# Role of Lentiviral Restriction Factors in Resistance to SIV Infection *in vivo*

by

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**ROLE OF LENTIVIRAL RESTRICTION FACTORS IN RESISTANCE TO SIV INFECTION *IN VIVO***

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Lentiviral restriction factors (RFs) are cellular proteins potently capable of blocking virus replication *in vitro* and delaying disease progression *in vivo*. Currently, five RFs (APOBEC3G, TRIM5α, Tetherin, SAMHD1, and Schlafen 11) are considered part of the intrinsic immune response to viral infection for their ability to block virus replication prior to activation of the innate immune system. Although RFs are constitutively expressed, it is unknown whether basal and early induction levels of these RFs are capable of limiting virus dissemination following mucosal challenge. We hypothesize that higher basal RF levels can exert a protective effect by delaying systemic infection. Real-time PCR analysis was conducted to analyze basal and post-exposure RF expression in the blood, lymph nodes and duodenum. Eight Indian-origin rhesus macaques received repetitive low dose rectal exposures of SIV/DeltaB670: 3 macaques became infected after 2 challenges (susceptible), 2 became infected after 3 challenges (intermediate), while two became infected after 6 challenges (resistant). One animal remained persistently uninfected despite 7 challenges (elite controller). Analysis of basal RF and Mx1 expression in the blood revealed animals resistant to infection had significantly higher TRIM5α and Mx1 expression than susceptible animals. Longitudinal analysis of RF and Mx1 expression in the blood revealed no RF induction in the resistant group, while the susceptible and intermediate

groups experienced a 2 to 7-fold induction after virus appearance in the blood. Despite the lack of RF induction in the elite controller, a transient induction of Mx1 was evident after 2 of the 7 challenges, which may have provided further protection. Examination of RF induction in the gut revealed a similar pattern with expression mirroring that seen in the blood. Our results suggest that RFs are coordinately expressed in the blood and gut in response to infection and that levels remain unchanged, despite repeated exposure, until the animals become viremic. As such, induction of these RFs after virus exposure appears to be insufficient in containing virus dissemination. These results further suggest that basal RF levels in the blood may be used as a predictor for susceptibility to infection in macaques and possibly humans.

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# PREFACE

There are many people I wish to thank who continuously supported me with their advice and guidance. My parents and sister have played an essential role during my career. They were the ears that listened to my worries and the voice that encouraged me to chase my dreams. My parents have been my inspiration and the shoulder I leaned on during my tough times. I cannot thank my sister, Ameena, enough for the countless times she has sat with me like a loving sister, encouraging me to remain optimistic. She is the wonder woman I could not live without. She cooked for me during my long days at lab, drove me in the middle of the night for those late-night experiments, and accompanied me during my weekends at the lab.

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# 1.0 INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) epidemic has devastated the lives of millions of people worldwide with no reachable cure in sight. Despite the advent of antiretroviral therapy to limit virus replication and disease pathogenesis, the disease is still incurable. The human immunodeficiency virus (HIV), the causative agent of AIDS, was discovered more 30 years ago and found to primarily spread among individuals by sexual transmission [1]. While many sexually transmitted diseases have garnered notable attention from the medical community, HIV has been in the limelight due to the virus's inherent ability to infect the immune cells responsible for host defense and evading the subsequent immune response through mutational escape [2]. HIV is characterized with an extremely error prone reverse transcriptase (RT). The remarkable ability of HIV to constantly mutate allows it to evade neutralizing antibodies and the cytotoxic T cell response therefore rendering the host incapable of fully attenuating the virus [3]. Not only does its high error-prone RT devastate the mounted host immune response, but HIV, and retroviruses in general, can permanently integrate their genome into the host making them one of the most successful parasites in nature [4].

## 1.1 HIV VIROLOGY

HIV is a member of the retroviridae family and carries two (+) ssRNA strands encoding three essential genes *gag* (Glycoproteins), *pol* (Polymerases), and *env* (Envelope), and their mRNAs are further spliced to encode proteins necessary for the structure of the virus. In addition to these essential genes are

accessory genes (Vif, Vpu, Vpr, Nef) generally known to be dispensable for the survival of the virus *in vitro* but necessary for its pathogenesis *in vivo* [5,6]. HIV has a spherical morphology with a lipid bilayer membrane derived from the host cell and encapsidates a cone-shaped core called the nucelocapsid [7]. Within the nucleocapsid are the two strands of genomic RNA and proteins necessary for virus replication: protease, RT, integrase, Vpu, Nef, Vif, and Vpr. Tat and Rev are known as regulatory genes for their ability to control transcription and RNA export, respectively [8]. HIV's glycoprotein (gp120) mediates binding to the host cell through the CD4 receptor and the CCR5/CXCR4 coreceptors (**Figure 1**) [7]. Thereafter, the viral and host membranes fuse and the viral core is released into the cytoplasm. At this point, uncoating occurs and the viral RNA undergoes reverse transcription and the resulting double stranded cDNA can be transported into the nucleus for integration into the host genome. The integrated cDNA serves as the genetic template for the viral proteins to be encapsidated into new virions and also serves as the viral genomic RNA. Assembly and packaging of new virions occurs at the plasma membrane and newly made virions bud from the host lipid bilayer, acquiring a portion of the bilayer as a coat for the virus.

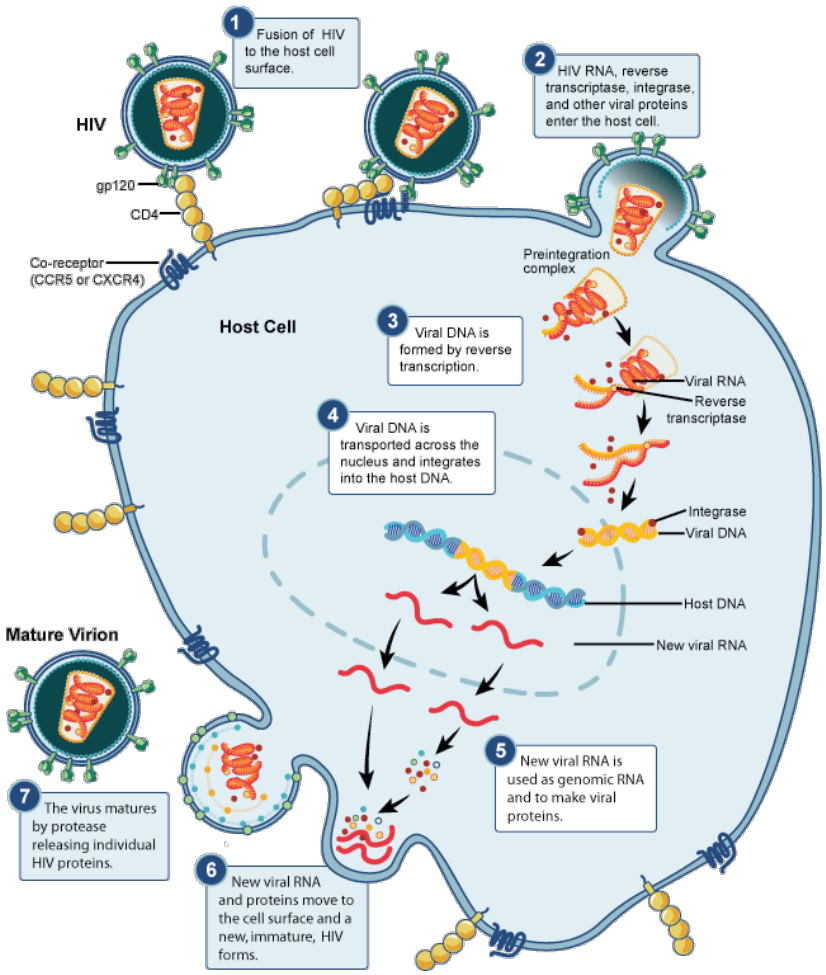



**Figure 1. Schematic of the HIV replication cycle in a host cell.**
Numbered are the different steps in the life cycle: 1,2) fusion and entry 3) reverse transcription 4) genome integration 5) translation 6) assembly 7) budding. Figure adapted from [9] with permission.

## 1.2 ORIGIN OF HIV

While it is not fully clear how HIV emerged into the human population, scientists attempted to approach this question by examining the genetic background of HIV. Two forms of HIV exist in the human population: HIV-1 and HIV-2. HIV-1 is the primary virus causing the global HIV epidemic while HIV-2

is less pathogenic and mostly localized to West Africa [10]. By analyzing the genetic sequence of HIV, scientists traced the origin of HIV-1 to a simian form of this virus found in chimpanzees (cpz). Specifically, HIV-1 group M was found to be the result of a chimpanzee to man zoonotic transmission of simian immunodeficiency virus (SIVcpz), a lentivirus commonly found in chimpanzees with high genetic similarity to HIV-1 [10]. Group M is the HIV-1 group that gave rise to the AIDS pandemic and is found throughout the world [10]. HIV-2, on the other hand, was derived from sooty mangabeys and is more genetically similar to SIV strains than HIV-1 [10]. Genetic analysis between HIV and SIV viruses shows high homology with differences in the presence/absence of accessory genes (**Figure 2**).

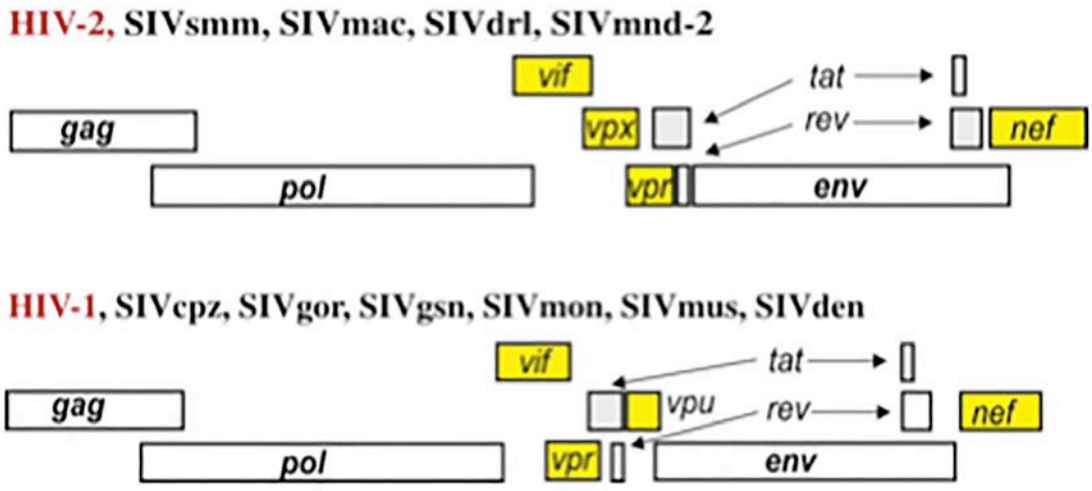



**Figure 2. Schematic of the SIV and HIV genomes**.
Accessory genes are shown in yellow. Figure adapted from [3] with permission.

## 1.3 SIV DIVERSITY

Both HIV-1 and HIV-2 were derived from a zoonotic transmission from an SIV isolate found in chimpanzees and sooty mangabeys, respectively [11]. Although SIV is genetically distinct from HIV-1 and HIV-2, it has allowed scientists to better explain the nature of HIV-1 pathogenesis by analyzing SIV pathogenesis in the nonhuman primates that harbor SIV. More than 40 different lentiviruses are naturally found in old world monkeys in sub-Saharan Africa [10]. Some of these lentiviruses underwent cross-species transmission to humans and great apes leading to the rise SIVcpz, SIVgor, HIV-1, HIV-2, and SIVmac. The most commonly studied SIV strains are 1) SIVcpz, found in chimpanzees 2) SIVsm, found in sooty mangabeys 3) SIVgor, found in gorillas 4) SIVagm, found in African green monkeys 5) SIVptm, found in pig-tailed macaques, and 6) SIVmac, found in rhesus macaques. Of particular interest is SIVmac, which was inadvertently transmitted from sooty mangabeys infected with SIVsm and became highly adapted in macaques after several passages of the original SIVsm virus in rhesus macaques [12]. SIVmac strains are the most commonly studied strains in SIV research for their ability to cause similar infection and disease pathogenesis as seen with HIV-1 infected humans.

## 1.4 HIV/SIV PATHOGENESIS

The cellular target of HIV/SIV is the primary reason no potent cure has been discovered. After HIV/SIV pass the mucosal epithelial layer, they specifically target and replicate in CD4+ T cells, the working horse of the host immune system. CD4+ T cells delegate tasks to other immune cells and are a stimulant of the adaptive immune system [13]. Both HIV and SIV infections are categorized by three phases: acute infection phase, asymptomatic phase, and the immunodeficiency onset phase (AIDs) [14]. The first phase is characterized by virus dissemination to lymphoid tissue and rapid virus replication [15]. The second phase involves low-level virus replication, an attribute of the lentivirus subfamily of retroviruses,

which can take several years. The third and final stage of infection is characterized by a compromised immune system as a result of significant gut CD4+ T cell depletion, immune activation, and microbial translocation [16]. Within 7-10 years, an HIV-infected individual begins the chronic stage of virus replication, immunodeficiency, and opportunistic infections [17]. At this point, the host's immune system has been severely compromised. Consequently, the acute phase of infection is the most critical phase for effective inhibition of further pathogenesis to the host.

The unique ability of RT to cause mutations in the viral genome and HIV's ability to integrate into the genome of host immune cells has contributed to the success of this virus in creating an incurable epidemic thus far. Overall, several factors contribute to the lack of effective drugs/vaccines: 1) HIV infects the immune cells (CD4+ T) that are responsible for defending the host; 2) HIV RT introduces numerous mutations in the viral genome; 3) the constantly evolving genetic sequence of HIV causes evasion from neutralizing antibodies and the cytotoxic T lymphocyte (CTL) response; 4) HIV becomes integrated into the host genome and capable of virus production indefinitely; and 5) HIV establishes reservoirs of latent virus in several tissues that cannot be easily accessed by drugs/immune system.

## 1.5 RHESUS MACAQUE MODEL

To understand the *in vivo* nature of the host immune response to HIV and to develop methods to intervene with virus replication, it was imperative to design an animal model that mimicked the HIV scenario seen with humans. Understanding HIV-host interactions at the site of transmission is imperative to design preventative drugs/vaccines that can effectively eliminate HIV in the mucosa (rectum, vaginal and intestinal tract). Due to ethical and practical reasons, it is impossible to sample humans immediately after virus transmission. However, with the development of SIV-infected rhesus macaque models, it is now possible for scientists to understand the course of virus dissemination from the genital tract to the periphery. SIV-infected rhesus macaques are the most successful model to study HIV transmission not

only for the ability to control tissue sampling and route/dose of exposure, but also because SIV is the most closely related virus to HIV both with regards to structure and host pathogenic effect [18,19]. Even more important are the similarities in the anatomy, physiology, and immunology of the reproductive tract of rhesus macaques and humans [20]. Essentially, the rhesus macaque model enables scientists to analyze early and late host immune responses to virus in many different tissue compartments.

## 1.6 SEXUAL TRANSMISSION

Sexual transmission of HIV is the world-leading cause of the AIDS epidemic. Despite the significant amount of effort and resources devoted to study mechanisms of HIV transmission, millions of humans become newly infected every year due to the lack of potent therapeutics that can eliminate the virus at the site of transmission. After sexual transmission, HIV can cross the female cervicovaginal tract by: 1) epithelial breaches; 2) uptake by dendritic cells (DCs); 3) transcytosis (**Figure 3)** [20]. Within hours, a founder population of infected resting CD4+ T cells is established. Local expansion of the founder population through the recruitment of CD4+ T cells occurs within days from virus entry and is necessary to allow for sufficient virus production and peripheral dissemination [20]. Within one week, the virus can disseminate to the draining lymph nodes and reach the peripheral lymphoid tissue and blood. At that point, the virus has taken hold of the host immune system and has established a lymphatic tissue reservoir. These reservoirs serve as virus-producing compartments, whereby the viral genome becomes integrated into the host genome and capable of producing virus indefinitely.

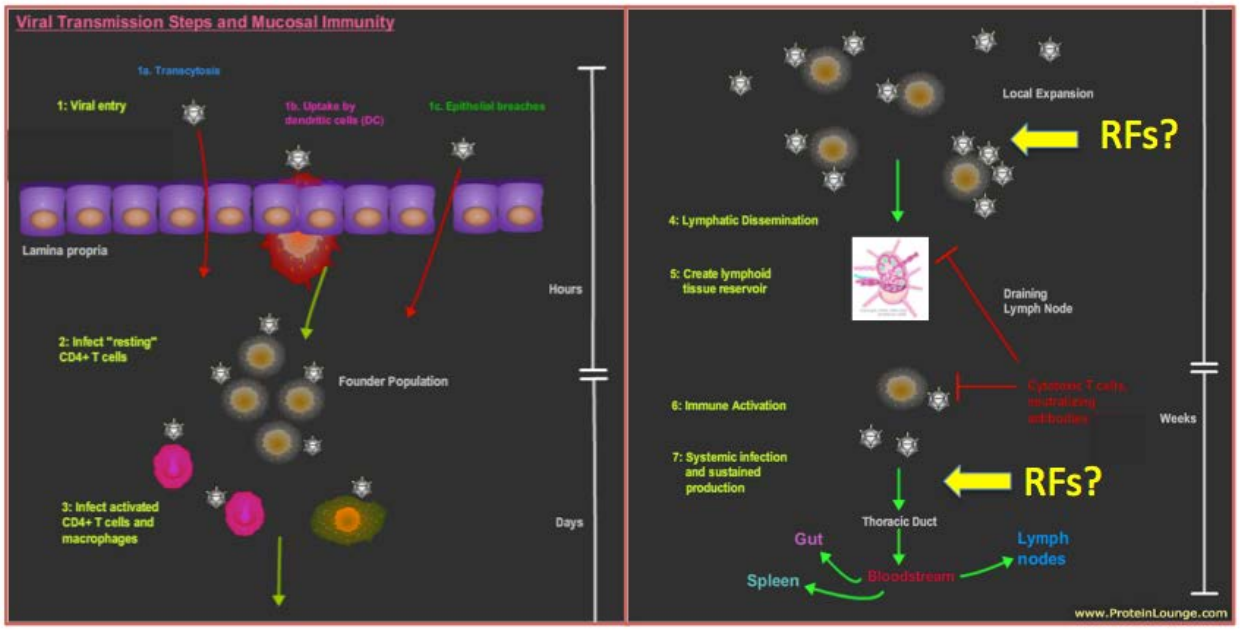



**Figure 3. Mucosal transmission of HIV/SIV.**
Virus can cross the epithelial barrier by: 1a) transcytosis; 1b) uptake by DCs; 1c) epithelial breaches. Virus then establishes a founder population and expands locally. Thereafter, virus establishes a lymphoid tissue reservoir and is disseminated systemically. To the right, is a timeline for each step during viral transmission and dissemination. Large yellow errors indicate possible time points when the restriction factors (RFs) may have a potent impact on virus replication and dissemination. Figure designed using www.proteinlounge.com and content was obtained from [20].

Studies have shown that the optimal time to prevent virus dissemination and further damage to the host is during the first week of exposure, when the virus is most vulnerable [21]. After SIV enters a host, DNA is not detectable in the blood or lymphatic tissue during the first seven days and this time period is regarded as the eclipse period. During these 7-10 days, the virus is attempting to establish a local infection and this timeframe is therefore regarded as the "window of opportunity" for potent intervention of virus replication and further damage to the host [21].

## 1.7 SIV RECTAL CHALLENGES

Sexual transmission of HIV can occur through the vaginal, rectal, or oral route [19,22]. While the majority of HIV transmissions occur through the vaginal route and thus necessitate female macaques for these studies, female macaques are often saved for breeding purposes. Consequently, the majority of SIV studies have focused on utilizing the rectal route of exposure to study HIV/SIV pathogenesis.

While many SIV animal studies have examined SIV transmission by the intravenous route, a mucosal model of SIV exposure is more relevant to studying HIV transmission in humans. This will be extremely beneficial in the long run in the development of therapeutics and drugs to curb HIV transmission. Several studies have delineated the pathway of SIV transmission from the rectum to the peripheral organs and blood. These studies have examined rectal infection through various methods of exposure. Among the possible routes of experimental SIV rectal exposure in macaques is: 1) a single high dose challenge; and 2) a repetitive, low dose challenge. Repetitive, low dose rectal SIV challenges have been shown to recapitulate HIV-1 mucosal infection in humans [23]. Similarly, studies show that this type of challenge may enable the host immune system to curb the virus during the early stages of replication due to the low number transmitted variants that pass the rectal barrier [24]. In my case, low-dose challenges can enable us to monitor the innate immune response to examine, in real-time, the effect of specific proteins before and after systemic infection. Not all challenges with repetitive low-doses of virus result in 100% infection and can thus be exploited by scientists to understand the reasons causing this effect. More importantly, repetitive, low dose rectal challenges may initiate a localized mucosal immune response that can block further virus replication and dissemination [25].

## 1.8 INNATE IMMUNE RESPONSE

For years, scientists have struggled to understand virus-host interactions of HIV by examining the virus's behavior in different tissue compartments. Only recently have researchers begun to focus closely on the mucosal portal of entry for HIV and the potential of innate immune responses in limiting viral dissemination from the vaginal tract/rectum. In order to avoid the deleterious effects of virus replication, it is imperative to halt the virus from disseminating beyond the mucosa. Several factors can contribute to blocking virus replication and include both intrinsic host factors and man-made interventions such as microbicides, antiretroviral therapy, and vaccines [21]. However, all these factors culminate in the engagement of the host innate immune response.

After a foreign particle enters the host, two forms of defense are capable of eliminating that particle: the innate immune response and the adaptive immune response. The innate immune system is considered the first line of defense against viral infection [26]. It is specifically characterized as an immediate immune response to any foreign antigen through the recruitment and activation of immune cells capable of killing the pathogen. These innate immune cells include macrophages, natural killer cells (NK), complement, and DCs, and are conveniently located at the portal of entry for HIV [21]. An extremely notable feature of the innate immune response is its ability to react within minutes to hours from stimulation by a foreign antigen.

Although many cytokines are involved in a mounted innate immune response, of particular importance is type I interferons. Interferons are crucial in the defense against viral infections [27]. With respect to HIV/SIV infection, the host's innate immune system responds to virus infection by upregulation of type I interferons that, in turn, induce a myriad of interferon-stimulated genes (ISGs) to create an "antiviral" environment in nearby cells (**Figure 4)** [28,29]. Well-known ISGs effective against a range of viruses include the Mx proteins. These proteins are considered among the most potent antiviral genes and are only induced by type I interferons. They are found as oligomers within the cell and bind to critical viral components to degrade them [27]. Studies have shown that the lack of Mx proteins in mice

contribute to decreased resistance to viral infection [27]. However, among the ISGs that have garnered significant attention for their potent ability to inhibit HIV/SIV replication are a group of cellular proteins termed restriction factors (RFs). These RFs, (TRIM5α, APOBEC3G, tetherin, SAMHD1, and schlafen 11), are both constitutively expressed and inducible by type I interferons, thereby making them members of the ISG family that defines innate immunity [28,30,31,32,33,34]. Their strength is further validated in their ability to restrict virus as single genes without the aid of cellular cofactors [30].

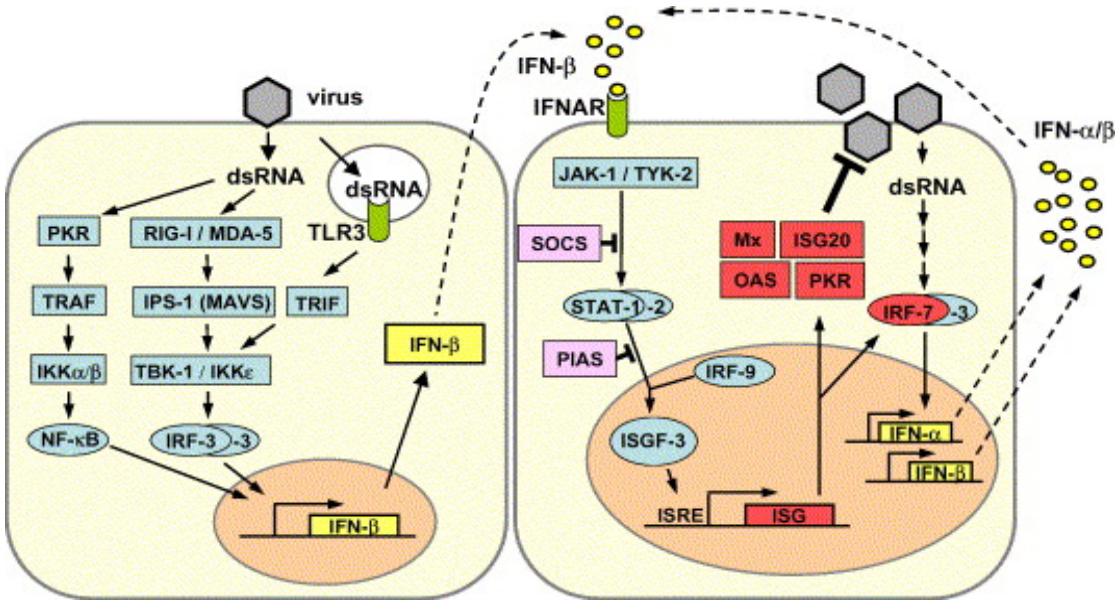



**Figure 4. Virus activation of type I interferons.**
After virus entry and detection in a cell by TLR3, a signaling cascade is initiated to allow IFNβ transcription and secretion. Secreted IFNβ binds to the IFNAR receptor in nearby cells and initiates another signaling cascade to stimulate transcription of interferon-stimulated genes (ISGs) such as Mx proteins. Virus can also directly activate IFNα and IFNβ transcription, which can then act in an autocrine fashion to further amplify the production of ISGs needed for defense against the pathogen. Figure adapted from [35] with permission.

## 1.9 RESTRICTION FACTORS (RFS)

Lentiviral RFs are specifically classified as part of the intracellular ***intrinsic*** immune response, in that they cause early resistance to virus infection without previous signaling/virus infection and are capable of acting autonomously without the aid of cellular cofactors [3,30,36,37,38,39,40,41,42]. RFs act as a frontline defense system due to their constitutive expression and capability of counteracting retroviruses before activation of the innate and adaptive immune system [43,44]. In fact, one study suggested that host factors may dominantly contribute to virus inhibition prior to the onset of immune responses and may be critical in establishing the early and post-acute viral replication patterns *in vivo* [18]. In other words, RFs may delay productive infection and dissemination. By definition, lentiviral RFs are dominantly-acting, germline-encoded cellular proteins that are constitutively expressed and capable of being induced by IFN [30]. They are species-specific and display signs of positive selection indicating coevolution between the virus and the RF [30]. In general, these RFs are potent cellular proteins but are inactive against the wildtype virus that is present in the natural host. This phenomenon is due to the presence of virally-encoded antagonists that counteract the restrictive function of these RFs through the ubiquitin-proteasome pathway. RFs have been in the spotlight of lentiviral research because of a discovery claiming the inhibition of HIV replication in rhesus macaque cells due to the rhesus RF, TRIM5α. In essence, rhesus macaque TRIM5α is capable of creating an outright block of HIV-1 replication in rhesus macaque cells but not in human cells; thus explaining the current inability of creating an HIV-rhesus macaque model [45]. This groundbreaking discovery proved that RFs alone have the power to completely block HIV entry in these cells. Such results spurred numerous studies to identify other RFs that can mimic the restrictive ability of TRIM5α.

Over the years, more lentiviral RFs have been discovered. Each of the currently known RFs has a different inhibitory target within the viral life cycle: APOBEC3G, TRIM5α, and SAMHD1 pose an early (post-entry) block on the first stages of reverse transcription while SCHL11 and tetherin inhibit translation and virion budding, respectively (**Figure 5**) [30,31,39,45,46,47]. Together, these RFs

act as independent barriers the virus must overcome to productively infect other cells. In addition to their positive selection and coevolution with HIV/SIV, the synergistic function of these RFs makes them powerful antagonists of lentiviral cross-species transmission [10,12,30,40].

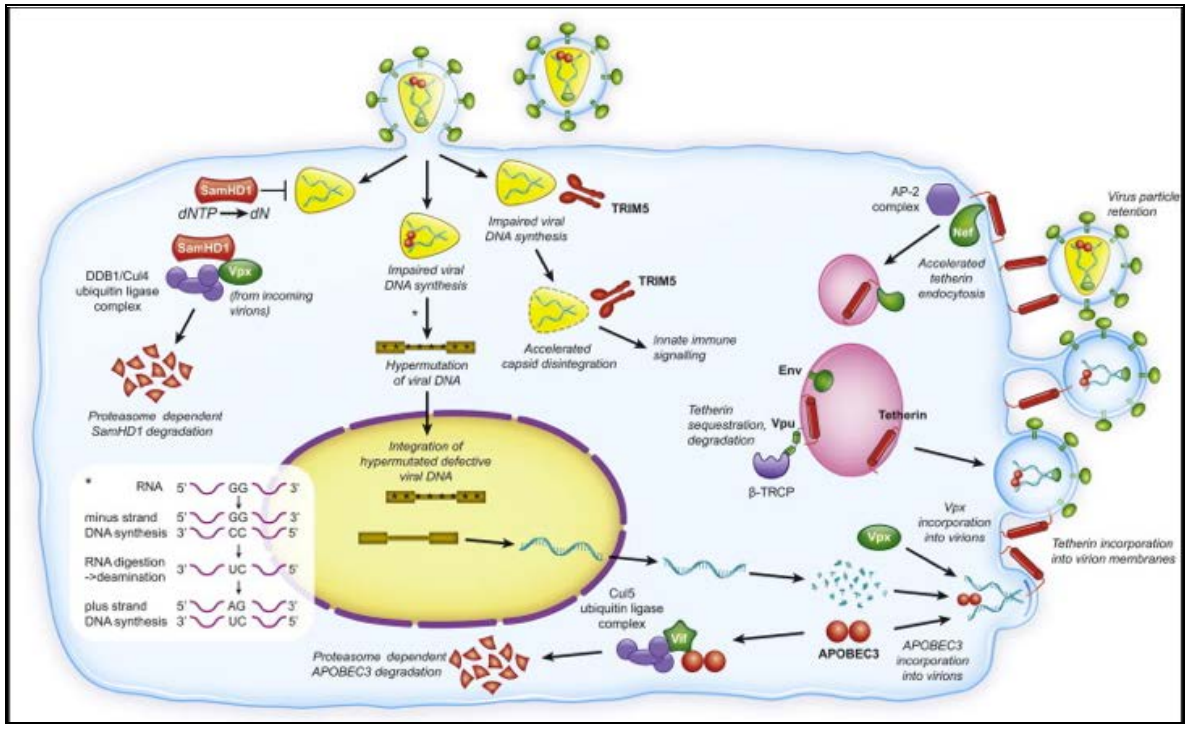



**Figure 5. Schematic of the direct role of RFs in the host cell.**
Shown are the RFs in red: TRIM5α, SAMHD1 and A3G pose an early block on reverse transcription and integration. Tetherin poses a late effect by blocking virion budding. This figure does not show the function of Schlafen 11. Corresponding antagonists for SAMHD1 (Vpx), A3G (Vif), and tetherin (Env, Nef, Vpu) are shown in green. Each antagonist recruits cellular proteins involved with the endocytosis or direct ubiqutination of the RF. Lower left box gives a detailed view of A3G hypermutation in the viral cDNA. Figure adapted from [39] with permission.

Both humans and rhesus macaques express all five RFs and they are fully ineffective in their natural hosts due to the virally-encoded antagonists. However, rhesus RFs are capable of strongly inhibiting HIV replication but are weakly capable of blocking SIV replication. The fact that lentiviruses have evolved specific proteins to combat these RFs reinforces the inherent ability of these RFs in potently blocking virus replication. In other words, HIV/SIV cannot simply escape RF restriction by mutating, but it has to specifically encode proteins within its genome to combat them individually [3]. As previously discussed, Ashley Haase proposed that two windows of opportunity exist for successful intervention against HIV replication and disease pathogenesis. The first window is during the first seven days after exposure and before systemic infection; and the second is before lymphatic dissemination. Since these RFs are constitutively expressed, I believe they may have a potent inhibitory effect during these two "windows of opportunity," before virus dissemination to the periphery. My study will specifically analyze this aspect.

### 1.9.1 APOBEC3G

Apolipoprotein B mRNA-editing, catalytic polypeptide-like 3G (A3G) is a member of a family of proteins that function as cytidine deaminases [48]. These proteins serve to protect the host from genomic instability caused by endogenous retroelements, especially in the germline [6,48,49]. A3G consists of an N-terminal deaminase inactive domain and a C-terminal deaminase active domain. The C-terminal domain functions as the cytosine deaminase while the N-terminal domain aids in A3G packaging into the virion and viral RNA binding through the nucleocapsid [30]. A3G is the only RF packaged within virions for immediate action post virus entry during reverse transcription. The restrictive function of A3G is manifested in both deaminse-dependent and deaminase-independent pathways (**Figure 6).** After packaging into virions from the producer cell, A3G functions in the target cell as: 1) **a cytidine deaminase** causing G to A hypermutations in the (-) strand of viral cDNA further inhibiting cDNA integration into the host genome; and 2) **an inhibitor** of the accumulation of HIV-1 reverse transcripts

(cDNA) [50,51,52]. Post viral entry into the cell, A3G begins deaminating cytosines to uracils during reverse transcription, causing deamination in up to 10% of the cytosines [30]. A3G, however, has a particular preference for the underlined cytosine 5'-CCCA [51]. These deaminations cause G to A hypermutations that can create frequent stop codons and compromise the integrity of the viral genome as a result of the introduction of an unnatural base into the DNA sequence [30,53]. In general, hypermutations have been associated with slower disease progression [54]. As a consequence of the aberrant DNA ends caused by hypermutations, the viral cDNA's ability to integrate into the host genome is compromised [55]. As part of its second function, A3G inhibits the translocation of reverse transcriptase during reverse transcription thereby hampering cDNA elongation and contributing to a lower viral cDNA level [50]. Specifically, A3G decreases tRNA specificity and processivity of translation by deaminating cytosines in the primer-binding site [56,57].

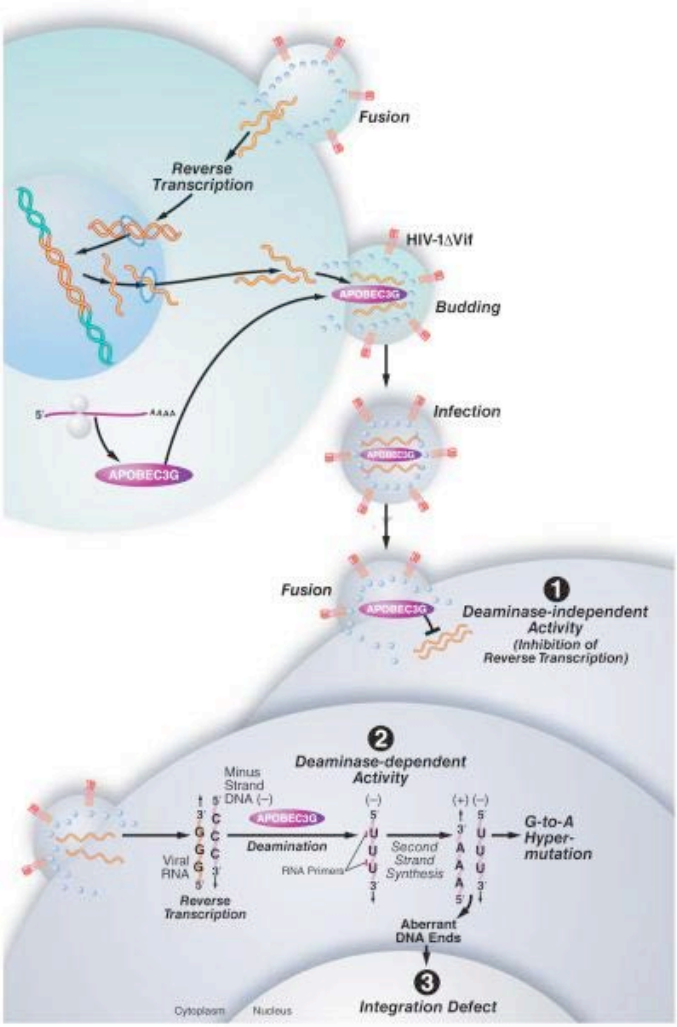



**Figure 6. A3G's diverse inhibitory effects on HIV-1.**
A3G is packaged within the budding virion and blocks virus replication in the target cell by: 1) deaminase-independent activity; 2) deaminase-dependent activity and 3) blocking integration as a result of aberrant ends. Figure adapted from [5] with permission.

Several studies have shown the inhibitory effect of A3G on HIV-1 and its restrictive ability on HIV-2, murine leukemia virus (MLV), and foamy viruses [48,58]. While A3G is effective in its restriction, HIV/SIV has evolved a protein, Vif (virion infectivity factor), that can block A3G's restrictive ability in three ways. First, Vif recruits the E3 ubiquitin ligase complex to ubiquitinate A3G and allow its proteasomal degradation prior to packaging in newly budding virions [55,59]. Second, Vif inhibits A3G

encapsidation into virions by competing with A3G for viral RNA binding by forming a complex with A3G [52,53,59]. Third, HIV-1 Vif binds to huA3G mRNA and inhibits its translation [60]. Vif is very efficient in its antagonistic ability regardless of the level of A3G present in a cell [30]. However, A3G neutralization by Vif is not absolute and studies show IFNα induction of A3G allows evasion from Vif's neutralizing effect [49,51,61].

Human and rhesus A3G are 75% similar but are species-specific [53]. A3G can have variable inhibitory effects in species depending on the genetic interaction between A3G and Vif [53,62]. For example, humans encode an L128 in A3G that allows A3G to be antagonized by HIV Vif while African green monkey A3G has an aspartic acid in that location that allows its specific inhibition by SIVagm Vif [30]. Residues D128 and D130 in A3G are critical residues for Vif binding [56]. A3G proteins in humans, macaques, African green monkeys, and chimpanzees can be combated by the natural lentivirus infecting that species (ex: SIVmac Vif inhibits rhesus (rh) A3G), but are unaffected by the Vif in other virus strains (ex: rhA3G unaffected by HIV-1 Vif) [49]. Studies have shown coevolution between A3G and Vif and it is believed that Vif's ability to counteract the A3G of new hosts has played a role in the zoonotic transmission of SIV to humans [30,63].

### 1.9.2 TRIM5α

TRIM5α is a member of the tripartite motif family known for their ability to self-associate and multimerize [64]. All TRIM proteins contain a RING, B-Box, and coiled coil domain [65]. Specifically, the RING domain functions as an E3 ubiquitin ligase, while the B-box and coiled coil are both involved in self association [64]. TRIM5α is an isoform of TRIM5 that is distinguishable from other TRIM proteins by the presence of the C-terminal B30.2/PRYSPRY domain. This domain is the most crucial component of the TRIM5α protein because it acts as the determinant for viral capsid binding and efficient restriction [65,66]. Its inhibitory effect is manifested both post-viral entry, pre-integration of the viral genome, and late during the viral life cycle. In other words, TRIM5α appears to have several routes of

restriction: 1) it binds to the capsid and mediates fragmentation, thereby inhibiting reverse transcription; 2) it blocks viral cDNA entry into the nucleus for integration; 3) it degrades Gag polyproteins during the late phase of replication; and 4) it triggers an alarm by activating the NfκB pathway to alert neighboring cells of an ensuing infection [67,68,69,70]. Therefore, TRIM5α activity is coupled with stimulation of the innate immune response.

Upon virus entry, TRIM5α binds to the capsid through the SPRY domain and allows multimerization in the form of a hexameric protein lattice [71]. Thereafter, the autocatalytic function of TRIM5α allows it to ubiquitinate itself, which consequently allows for degradation by the proteasome (**Figure 7)**. The movement of the capsid-bound TRIM5α to the proteasome disrupts the capsid, causing fragmentation [72,73].

Although several studies elucidated the current restrictive function of TRIM5α, the natural function of TRIM5α in cells was not clear. However, after the discovery of TRIM5α's capacity to activate the Nfκb pathway and promote cytokine production, it is now clear that it acts as a pattern recognition receptor that is specific for the retrovirus capsid [68].

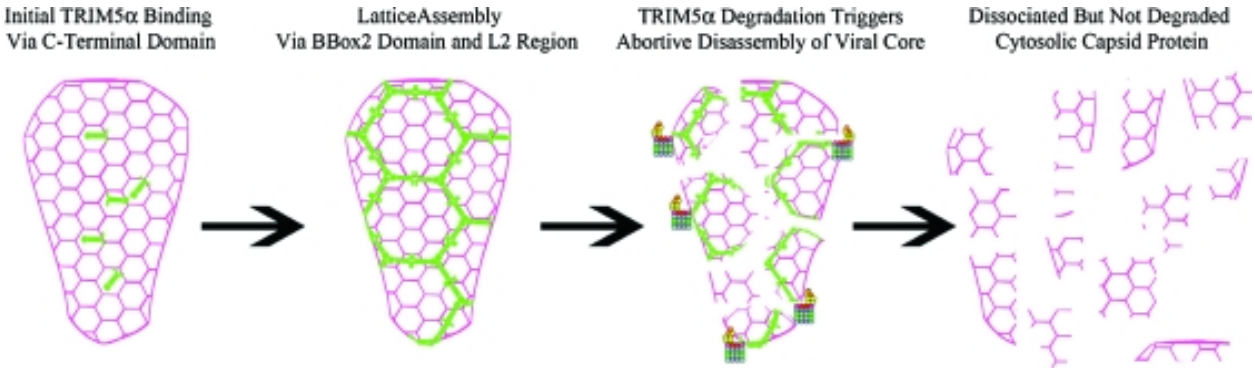



**Figure 7. TRIM5α-mediated uncoating of the viral capsid.**
TRIM5α binds to the capsid after viral entry and multimerizes on the capsid surface forming a hexamer lattice. TRIM5α's autoubiquitinating activity facilitates disassembly of the viral core by moving toward the proteasome. Figure adapted from [74] with permission.

Again, due to the species-specific nature of RFs, TRIM5α is generally ineffective against the retroviruses naturally found in a host species but is potent against retroviruses in other species [30]. Although human and rhesus TRIM5α are 87% similar, rhTRIM5α can block HIV-1 replication but not SIVmac, while huTRIM5α can potently block N-MLV but not HIV-1 [75,76]. The level of inhibition on N-MLV replication induced by huTRIM5α is similar to the level induced against HIV-1 by rhTRIM5α [66]. Differences in huTRIM5α's ability to potently combat N-MLV and not HIV-1 is due to the nature of the restriction site and the shape of the capsid [74,77]. The specific residues in the SPRY domain contribute to these differences among the two retroviruses [71]. Studies have revealed the importance of an amino acid mutation (R332P) in the huTRIM5α SPRY domain that can lead to efficient HIV-1 restriction [66]. Both rhesus macaque and African green monkey TRIM5α express the proline residue and therefore both are effective inhibitors of HIV-1 [66].

Rhesus TRIM5α is highly polymorphic in the C-terminal B30.2/PRYSPRY domain with a specific polymorphism at amino acids 339-341 highly linked to susceptibility to infection and disease progression induced by specific SIV isolates [12]. These polymorphisms reside in the regions that bind to the SIV capsid and affect the strength of the TRIM5α-capsid interaction and therefore restriction [78,79,80]. Three different allelic forms in the 339-341 region of TRIM5 exist: $TRIM5^{TFP}$, $TRIM5^{Q}$, and $TRIM^{CYPA}$. $TRIM^{CYPA}$ contains a complete substitution of the B30.2/PRYSPRY domain with the cyclophilin A gene as a result of retrotransposition and is restrictive against retroviruses whose capsids typically bind cyclophilin A such as HIV-1 and FIV [30,68,81,82].

These TRIM5α alleles may exhibit various restriction capabilities depending on the type of virus, route of exposure, viral dose, and prior vaccination [83]. Transmission and acquisition of SIV can be severely impaired if the recipient host expresses the restrictive TRIM5 alleles (TFP or CypA) and the effect may be more pronounced with low-dose challenges because it may contribute to the genetic bottleneck [12,84]. In fact, one group has shown that the restrictive TRIM5 alleles are capable of affecting mucosal acquisition of SIVsmE660 even without prior vaccination [85]. In other words, these alleles act as a standalone barrier that are potent in crippling the early stage of transmission. Macaques

with restrictive alleles show attenuated replication after SIV exposure by the IV route whereas those exposed mucosally show an outright block to SIVsmE660 [86]. Similarly, studies show that animals homozygous for the TFP allele are more resistant to SIV infection and display lower peak VLs and slower disease progression (lower viral set-point and less CD4+ T cell depletion) [12,79]. Thus, animals with restrictive alleles display significantly higher survival rates versus animals bearing at least one permissive allele [79]. In fact, these polymorphisms account for a 1.3-3 $\log_{10}$ decrease in SIV replication [84]. Animals homozygous for the Q allele show the opposite pattern and are known to be more susceptible to infection [12]. The CypA allele exerts restriction directly on SIVsmE041, SIVstm, and SIVsmE543-3 [12,79]. SIVmac251, on the other hand, is known to be unaffected by the TRIM5 polymorphisms regardless of the route of exposure, previous vaccination, viral dose, and protective MHC class I genotypes [83]. With respect to humans, these polymorphisms are not found in huTRIM5α; and while huTRIM5α is polymorphic, the polymorphisms are generally not associated with clinical disease, although other studies suggest otherwise [75,87,88,89].

The effect of TRIM5 polymorphisms applies to specific SIV strains (an outline for the derivation of each SIV strain is found in **Appendix A)**. For example, studies have shown that SIV strains not adapted to rhesus macaques (SIVsmE660, SIVsmE041, and SIVsmE543) are easily restricted by $TRIM5^{TFP}$ whereas the highly adapted SIVmac251 and SIVmac239 are generally unaffected by TRIM5α restriction regardless of the TRIM5 allele [12,80]. The primary difference lies in the capsid sequence of the TRIM5α binding site. Unlike the other RFs, a lentiviral-encoded antagonist for TRIM5α does not exist. The virus has evolved to escape TRIM5α restriction by mutating the TRIM5α binding site in the capsid to decrease the affinity of the interaction [3,12]. Several studies have pinpointed two critical regions for TRIM5α binding: amino acids 89-LPA-91 and R97 (according to the SIVmac239 capsid sequence) [12,84,90]. TRIM5α binds to these regions of the capsid and any mutation in these sites can hinder TRIM5α binding and restriction. The SIVsm strains are easily restricted by TRIM5α because they encode 89-LPA-91 and R97. However, both SIVmacs evolved a capsid sequence that expresses 89-QQ-90 instead of LPA and S97 instead of R [12]. These changes are potent enough to abrogate TRIM5α

binding to the viral capsid. In fact, studies show that TRIM5α alleles have no significant effect on SIVmac251 replication *in vivo* and disease progression despite challenge dose and prior vaccination [83]. It was suggested that the SIVmac capsid became adapted to counter TRIM5 restriction in comparison to the original SIVsm strain [12]. In fact, it is this adaptation that led to the emergence of the highly pathogenic SIVmac strain [12]. TRIM5α was therefore posited to be an important RF that can block cross-species transmission, limiting the early stage of virus replication.

### 1.9.3 Tetherin

Tetherin is a unique single-pass type II membrane protein with three domains: a cytoplasmic tail, a transmembrane domain (TM), and an extracellular domain [91]. Its uniqueness stems from its integration within the cellular membrane by both the N-terminal TM domains and the C-terminal glycophosphatidylinositol (GPI) anchor [92]. Tetherin is localized in the plasma membrane lipid rafts, trans-Golgi network, and endocytic compartments [93]. Its localization on plasma membrane lipids rafts is naturally advantageous since virions bud from lipid rafts [6]. Specifically synthesized in the endoplasmic reticulum (ER), tetherin shuttles between the ER, trans golgi network (TGN), and the surface membrane and is continuously recycled [94]. Interestingly, tetherin recognizes one highly conserved and immutable structure, the virion membrane, and causes retention of budding virions [92]. Consequently, tetherin is non-specific and is able to restrict all retroviruses in addition to others such as filoviruses and paramyxoviruses [91]. It exists as a parallel dimer linked through disulfide bonds [94]. This dimerization facilitates efficient restriction by allowing one tetherin to remain bound to the cell membrane and the second tetherin to embed in the budding virion membrane. During virion budding, tetherin is inserted into the virion envelope and bridging between the host and viral lipid bilayers occurs, causing retention of the virion [95]. After tethering, the virions can be endocytosed for eventual degradation [91]. The unique topology of tetherin distinguishes it from other RFs. Studies show that

proteins with similar topology to tetherin but with completely different genetic sequences, “artificial tetherin,” still allow virion retention, indicating the importance of tetherin’s overall structure [30,92,95].

Naturally, tetherin functions in a cell as a ligand for the pDC ILT7 receptor to downregulate type I IFN production and thereby regulate immune activation [96]. While tetherin is potent solely as a tetherer of virions, it can also augment the host immune response. Virion retention by tetherin is believed to enhance antibody opsonization, complement deposition, and recognition by phagocytes and NK cells [91]. Tetherin can specifically allow better antigen presentation on MHC class II molecules after virion endocytosis and degradation. However, newer studies have also revealed tetherin may function as a viral sensor by recognizing virions and inducing a signaling pathway to enhance NFκb activation and inflammatory cytokine secretion to alert the immune system [97].

While tetherin is effective against a wide range of viruses, it is highly species-specific with respect to antagonism by viral proteins. Unlike the other RFs, tetherin can be combated by several viral proteins, depending on the viral strain. Specifically, tetherin is restrictive against HIV-1 and SIV but is efficiently antagonized by Vpu and Nef, respectively. These differences lie in the genetic makeup of each virus: HIV-1 encodes Vpu whereas SIV does not. Additionally, a five amino acid deletion in the cytoplasmic tail of human tetherin important for binding to Nef abrogates its ability to be antagonized by SIV Nef [98,99]. Rhesus tetherin, on other hand, is not affected by HIV-1 Vpu due to differences in the sequence of the TM domain [98]. However, both human and rhesus tetherin block viral release in the same fashion [100]. The variability in tetherin antagonism emphasizes the remarkable plasticity of the lentiviral genomes in combating RFs [100].

The presence of the antagonists strongly cripple tetherin’s restrictive function. Vpu can decrease steady-state levels of cell-surface tetherin but can also mediate its degradation in endosomal compartments [94]. Through the recruitment of the E3 ligase complex, Vpu ubiquitinates the TM domain of tetherin and removes it from the cell surface. Since Vpu is localized primarily in the TGN, it can also contribute to the sequestration of tetherin that is being shuttled from the TGN to the plasma membrane [94]. In contrast, SIV Nef is localized at the cytosolic face of the plasma membrane and can downregulate

surface tetherin levels but does not reduce the overall protein level in the cell [98,101]. Specifically, Nef binds to the cytoplasmic tail of tetherin and recruits the AP-2 clathrin adaptor complex for tetherin downregulation.

### 1.9.4 SAMHD1

Sterile alpha motif and HD domain 1 (SAMHD1) is a cellular protein with dNTPase activity and is particularly found in myeloid cells [102]. The SAM domain is specifically involved in protein-protein/nucleic acid interaction while the HD domain functions as the nucleotidase [103]. The discovery of SAMHD1 came about after the observation that monocyte-derived macrophages (MDMs) and DCs are inefficiently infected by HIV-1. Specifically, SAMHD1 was discovered to hydrolyze intracellular dNTPs needed for retroviral reverse transcription [102]. It is particularly effective in that it depletes the available dNTP pool to concentrations lower than the threshold necessary for viral cDNA synthesis. Natuarlly, SAMHD1 functions as a negative regulator of the innate immune response by preventing the accumulation of retrotransposon DNA in order to inhibit activation of the innate immune response [102]. Mutations in SAMHD1 are associated with Aicardi-Goutières syndrome, a rare disease characterized with overproduction of IFNα and immune activation [102]. A recent study revealed that the restrictive ability of SAMHD1 is governed by a phosphorylation site and not by its dNTPase activity [104]. They specifically demonstrated that the enzymatic activity of SAMHD1 is not sufficient to prevent HIV-1 replication and that phosphorylation at residue T592 may allow interaction with an unknown cofactor to effectively restrict HIV-1.

Unlike the other RFs, SAMHD1 does not have a particular viral target. In essence, SAMHD1 functions to non-specifically lower cellular dNTPs levels to create an antiviral environment against a range of retroviruses [102]. This raises the question of how the cell is not adversely affected by lower dNTP levels needed for basic cellular functions. The key is in the expression of SAMHD1 in non-dividing or terminally differentiated cells, which do not require high levels of dNTPs [102]. While

lymphoid cells do express SAMHD1, levels are much higher in DCs followed by MDMs [102]. However, a recent report revealed high SAMHD1 expression in resting CD4+T cells, a cell type highly resistant to HIV-1 infection [105] .

SAMHD1 is antagonized by Vpx, a protein found only in the HIV-2-SIVsm lineage [102]. Vpx binds to SAMHD1 and mediates its ubiquitination and degradation. Conveniently, Vpx is pre-packaged in a virion and is ready to reverse SAMHD1's activity in the target cell [102]. Although HIV-1 does not encode Vpx, studies propose that SAMHD1 may actually serve to benefit HIV-1 by allowing the avoidance of activation of the cytoplasmic sensors and inflammatory cytokine production [102]. HIV-1 is able to withstand SAMHD1's effect despite the lack of Vpx by encoding a reverse transcriptase with a low Michaelis constant ($K_m$) [103]. In other words, HIV-1 RT has a high affinity for dNTPs and is able to locate them even when dNTP levels become limited [103]. Without Vpx, studies show SAMHD1 causes reduced peak virus loads [103]. Additionally, infection with SIV lacking the Vpx gene causes impaired viral dissemination and disease progression in macaques [103].

### 1.9.5 Schlafen 11

Schlafen 11 (SCHL11) is a member of the SLFN gene family commonly expressed in lymphoid tissue and known to regulate T cell development and cell growth [32,106,107]. This family of proteins is among the common ISGs induced after pathogen exposure [31]. Discovered in 2012, SCHL11 is the newest addition to the RF repertoire and was linked to a potent block in the late stage of HIV-1 replication. Specifically, it was found to bind to tRNAs and downregulate the expression of viral proteins by eliminating rare tRNA codons needed for HIV-1 protein translation [31]. In other words, SCHL11 has a late inhibitory effect by hindering viral protein translation and therefore virion production. Lentiviruses have high levels of the "A" nucleotide in their genome and SCHL11 exploits the viral codon bias by specifically sequestering the rare viral tRNA codons with an A/U in the third position [31]. Specifically, the GTA codon is the least used codon in human protein translation while it is the most used codon in HIV-1 protein translation [108]. Therefore, SCHL11 acts during the rate limiting step of HIV-1 protein translation by eliminating already rare tRNA codons needed for HIV-1 translation [31].

Compared to the other RFs, little is known about the behavior of SCHL11 in different immune cell subsets and in response to infection. Specifically, it is completely unknown how SCHL11 behaves in SIV-infected rhesus macaques. To date, no one has examined the role of SCHL11 in the context of SIV infections and this project is specifically geared to provide insight on this aspect.

**Table 1. Overview of RF function.**

| Restriction Factor | Time point of inhibition | Target | Mechanism of Action | Viral Antagonist |
|---|---|---|---|---|
| **TRIM5α** | Post-entry, integration | Viral capsid | Early capsid uncoating, inhibition of cDNA entry for integration | Capsid mutation |
| **APOBEC3G** | Reverse transcription | Viral (-) cDNA | Cytidine deaminations cause G to A hypermutations, aberrant DNA ends, and an integration defect | HIV/SIV Vif |
| **SAMHD1** | Reverse transcription | Cellular dNTPs | Deplete cellular dNTP pool needed for reverse transcription | HIV-2/SIV Vpx |
| **Schlafen 11** | Translation | Rare tRNAs | Sequester rare tRNA codons necessary for viral translation | ? |
| **Tetherin** | Budding | Virion membrane | Tether budding virions to cell membrane for endocytosis | HIV-1 Vpu HIV-2 Env SIV Nef |

## 1.10 SIV/DELTAB670

SIV/DeltaB670, the virus strain used in this study, was discovered in 1986 by Michael Murphey-Corb in a rhesus macaque displaying signs of lymphadenopathy [109]. Specifically, SIV/DeltaB670 was derived from a sooty mangabey with naturally-acquired leprosy and was passaged through two rhesus macaques (**Figure 8)** [110]. This isolate is highly pathogenic and can cause death in rhesus macaques within a median range of 289 days [22,111]. Additionally, the SIV/DeltaB670 stock consists of a swarm of genetically distinct viral sequences similar to HIV-1 [112]. Evaluation of the hypervariable V1 region in the Env gene of SIV/DeltaB670 revealed the presence of >23 genotypes [111].

In my study, the rhesus macaques were challenged intrarectally with repetitive, low doses of SIV/DeltaB670. The virus stock used for the challenge was obtained by co-culturing a lymph node homogenate from rhesus macaque B670 and primary human PBMC (huPBMC) and further expanded in the PBMC of two rhesus macaques with the CypA/CypA TRIM5 genotype.

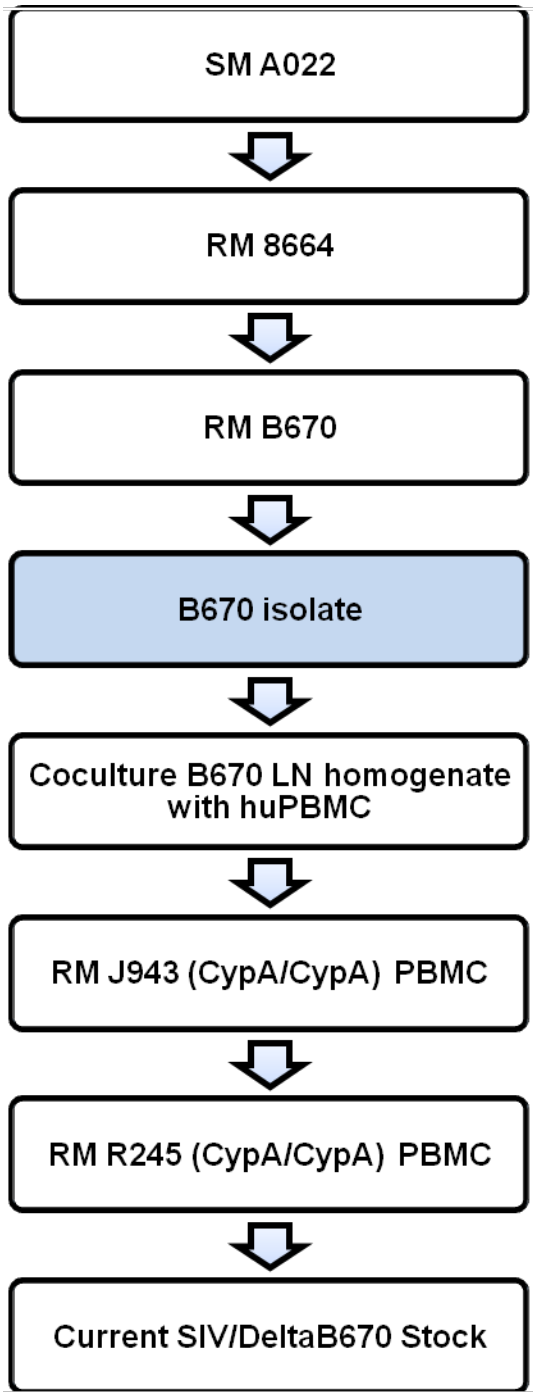



**Figure 8. Derivation of the SIV/DeltaB670 challenge stock virus.**
Each arrow indicates virus passage *in vivo* in a monkey or *in vitro* in PBMC. Animal numbers are noted. SM: sooty mangabey, RM: rhesus macaque, LN: lymph node. Blue box indicates isolation of the first SIV/DeltaB670 virus.

# 2.0 MATERIALS AND METHODS

## 2.1 ANIMAL CARE

Eight Indian-origin rhesus macaques ranging in age from 3-7 years were obtained from an approved vendor (Three Springs Scientific, Perkapsie, PA) and housed at the University of Pittsburgh primate facility (RIDC, Pittsburgh, PA). The animals were housed in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and with the approval of the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC) standards/regulations. Animals were cared for by competent veterinary and animal caretaker staff. Ketamine anesthesia was used throughout experimental procedures and any discomfort or pain was alleviated by appropriate use of analgesic agents at the discretion of the attending veterinarian. Animals were monitored monthly for signs of disease by examining CD4+ T cell counts, weight, and evidence of opportunistic infections. Humane euthanasia was performed in accordance with guidelines as established by the 2007 American Veterinary Medical Association Guidelines when clinical AIDS or signs of fatal disease were observed.

## 2.2 ANIMAL CHALLENGES

Each animal was rectally challenged on a weekly basis with 1mL cell free SIV/DeltaB670 ($10^6$ $TCID_{50}$). Briefly, sedated animals (10mg/kg Ketamine i.m.) were positioned in sternal recumbence and inoculated via atraumatic insertion of a 3-ml syringe (lubricated with Surgilube®) approximately 5 cm into the rectum. The inoculum was contained in 1.0 ml of saline. The inoculation was slowly pushed in for a full minute; the syringe left in place for five minutes and the animal left in sternal recumbence for a total of 15 minutes. Animals were initially challenged with $10^3$ $TCID_{50}$ of the SIV/DeltaB670 stock but were challenged with 10-fold more virus ($10^4$ $TCID_{50}$) for the remaining five challenges. Challenges were terminated when virus was detectable in the plasma. Blood (3-10mls) was collected twice a week from each animal until they reached a viral set point.

## 2.3 SAMPLE COLLECTION

Duodenal biopsies were obtained by an endoscope (4 days prior to each of the first three rectal challenges and 3 days later) and collected in 1ml TRIzol and stored at -80°C. Inguinal lymph nodes (ILNs) were surgically removed (4 days prior to each of the first three rectal challenges and 3 days later) and immediately processed for mononuclear cell purification. Fifteen to eighteen pinch biopsies of the rectal vault were obtained four days prior to the $6^{th}$ rectal challenge and 3 days later. The duodenum was specifically sampled due to accessibility by an oral endoscope, ease of sampling without causing harm to the animal, and relevance to understanding mucosal response to SIV infection. The ILNs were specifically obtained due to the location and ease of repetitively removing peripheral LNs without the animal undergoing surgery at each time point of sampling.

## 2.4 PLASMA VIRUS LOADS

Viral RNA was extracted from plasma using TRIzol (Ambion) and RT-PCR was performed as previously described using primers specific for the viral long terminal repeat [113]. RT-PCR was performed on a Prism 7700 (Applied Biosystems) using external standards with an 8-log range. Final values were extrapolated from the standard curve and were expressed as RNA copies/ml plasma. Sensitivity threshold for this quantitative assay was ~50 copies.

## 2.5 MONONUCLEAR CELL (MNC) PURIFICATION

PBMC were isolated by Ficoll-Paque (Pharmacia) density barrier centrifugation from rhesus macaque blood samples treated with ACD and were either viably frozen in liquid nitrogen or pelleted and frozen at -80°C. Mononuclear cells (MNC) were isolated from inguinal lymph nodes by teasing and soaking in 10% RPMI 1640 [supplemented with 15% fetal bovine serum, penicillin-streptomycin (100U/ml), L-glutamine (2mM), HEPES buffer solution (10mM)] followed by straining to obtain purified mononuclear cells. After several washes with 10% RPMI, purified MNCs were viably frozen in liquid nitrogen.

## 2.6 CELLULAR RNA EXTRACTION

Total RNA was isolated from viably frozen PBMC/ILN or frozen PBMC pellets using TRIzol (Ambion). RNA was isolated from the duodenal biopsies by first grinding the tissue with disposable tissue grinders and adding 1ml TRIzol. The Turbo DNA-free kit (Ambion) was used to purify the RNA and remove genomic DNA. RNA concentration was measured on the 2000c Nanodrop spectrophotometer (Thermo Scientific) and RNA purity and integrity was verified using the RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Biotechnologies).

## 2.7 V1 ENVELOPE SEQUENCING

Plasma viral RNA during acute infection was extracted with TRIzol (Ambion) and the V1 hypervariable region in the envelope was reverse transcribed. The RNA from the viral inoculum was also extracted using TRIzol (Ambion) and reverse transcribed. Nested PCR amplification of the V1 region of the cDNA was performed as described **(Table 2)** [112]. Similar PCR conditions were used for both rounds of PCR (**Table 3)**. PCR products from 2-4 individual amplifications of the viral inoculum were pooled prior to cloning. The ~200bp amplified DNA sequence was gel-purified (Wizard PCR Preps DNA Purification System-Promega). PCR products were cloned into a vector (pCR 4-TOPO) using the TOPO TA Cloning Kit (Invitrogen). Approximately 10-26 clones were isolated and purified using the Wizard Plus SV Minipreps (Promega). Cloning and purification was performed as described in the kits' manuals and sent for sequencing at Genewiz Inc., NJ. Geneious Pro Software (version 5.5.2) was used to align the V1 sequences and SeqPublish (Los Alamos National Laboratory) was used to prepare the formatted alignment. Each V1 nucleotide sequence was BLASTED® (NIH) to determine the identity of the clone as previously published [112].

**Table 2. Protocol for V1 Env PCR amplification.**

| Reagent | Volume (uL) |
|---|---|
| **10X Hotmaster Buffer (5 Prime)** | **10** |
| **Forward Primer (20pmol)** | **1** |
| **Reverse Primer (20pmol)** | **1** |
| **dNTPs (Applied Biosystems)** | **8** |
| **Hotmaster Taq DNA polymerase (5 Prime)** | **0.6** |
| **Nuclease Free Water** | **75.4** |
| **DNA** | **2** |
| **Total Volume** | **100** |

**Table 3. PCR conditions for V1 Env amplification.**

| Step | Temperature (°C) | Time |
|---|---|---|
| **1. Initial denaturation** | **94** | **2 min** |
| **2. Denaturation** | **94** | **15 sec** |
| **3. Annealing** | **55** | **15 sec** |
| **4. Extension** | **72** | **30 sec** |
| **5. Final extension** | **72** | **10 min** |
| **Hold** | **4** | ∞ |
| **Steps 2-4 repeated 35 cycles** | | |

## 2.8 RF EXPRESSION ASSAYS

A maximum of 2ug total cellular RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). A total of 25ng cDNA was used for RT-PCR amplification of each gene and reactions were performed in duplicate. Positive controls and no template controls were included in each assay. The assay IDs for the pre-developed Taqman assays (Applied Biosystems) are

displayed in **Table 4.** Each assay was specific for rhesus macaques and amplified exon-exon regions and did not detect genomic DNA. RT-PCR was performed on the 7900 HT Fast Real-time PCR system (Applied Biosystems) using the recommended reagents (Taqman Fast Universal PCR Mastermix 2X, no AmpErase UNG-Applied Biosystems) and conditions supplied with the Taqman assays. Reaction protocol is shown in **Table 5**. The TRIM5α Taqman assay listed in table 4 was used throughout the entire study. However, since this TRIM5α assay detects exons 7-8, which is absent in the TRIMCypA allele in one macaque (R701), we used another TRIM5α Taqman Gene Expression assay (Rh02788626_m1) that detects exons 1-2, a non-polymorphic site in both TRIM5α and TRIMCypA. Relative TRIM5 expression levels from both TRIM5 Taqman assays showed minor differences and therefore only the TRIM5α Taqman assay that detected exon 7-8 was used throughout the study.

**Table 4. Applied Biosystems Taqman Assay IDs.**

| Gene | Assay ID |
|---|---|
| **APOBEC3G** | **Rh02788475_m1** |
| **TRIM5α** | **Rh02788631_m1** |
| **Tetherin** | **Rh02848328_m1** |
| **SAMHD1** | **Rh01122752_m1** |
| **Schlafen 11** | **Rh02885088_m1** |
| **Mx1** | **Rh02842279_m1** |
| **IFNγ** | **Rh02788577_m1** |
| **TBP** | **Rh00427620_m1** |
| **HPRT** | **Rh02827360_m1** |
| **β2M** | **Rh02847367_m1** |
| **βGus** | **Rh02788764_m1** |

**Table 5. RF assay RT-PCR protocol.**

| Reagent | Volume (uL) |
|---|---|
| **20X Taqman Gene Expression Assay** | **1** |
| **2X Universal PCR Mastermix (no UNG)** | **10** |
| **Nuclease Free Water** | **8** |
| **25ng cDNA** | **1** |
| **Total Volume** | **20** |

All genes were validated for efficiency prior to the start of the assay by creating serial dilutions of a control cDNA sample (**Appendix B**). Linear regression analysis was performed to evaluate assay linearity ($R^2$) and efficiency ($1\text{-}10^{-1/slope}$). All lines had $R^2$ values >0.99 indicating linearity. Slopes were between -3.3 and -3.6 and therefore efficiency was >90%. A total of four endogenous controls were used at the start of the assay (TBP, HPRT, β2M, βGus) to determine the two most stable genes. Raw RT-PCR data were analyzed first by SDS RQ manager (version 2.3, Applied Biosystems) to obtain the Ct values. DataAssist Software (Applied Biosystems, version 3.01) was used to determine the top two most stable genes according to the scores calculated (the lower the score, the more stable the endogenous control). DataAssist also calculated $2^{-\Delta Ct}$ values. Raw $2^{-\Delta Ct}$ values were then multiplied by 1000 to obtain relative expression for each gene after normalization to TBP and HPRT. Fold change in expression was calculated by dividing the relative expression at each time point by the basal expression (four days prior to the first challenge).

## 2.9 IDENTIFICATION OF TRIM5 POLYMORPHISMS

Genomic DNA was extracted from PBMC using the DNeasy Blood and Tissue kit (Qiagen). TRIM5α was PCR amplified with primers and PCR conditions as described [12]. The PCR protocol is shown in **table 6**. Gel-purified DNA of ~500bp (Wizard PCR Preps DNA Purification System-Promega) was cloned into the pCR 4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) and four clones were isolated and purified using the Wizard Plus SV Minipreps (Promega). Cloning and purification was performed as described in the kits' manuals. The clones were sent for sequencing at Genewiz Inc., NJ. Geneious Pro Software (version 5.5.2) was used to align sequences and identify polymorphisms in the 339-341 amino acid region. Animals were either homo/heterozygous for TFP, Q, or CypA. The TRIMCypA allele was identified according to the presence of a published SNP (G to T substitution) in intron 6 [114].

**Table 6. TRIM5α PCR amplification protocol.**

| Reagent | Volume (uL) |
|---|---|
| **Accuprime Supermix II (Invitrogen)** | **25** |
| **Forward Primer (10uM)** | **1.5** |
| **Reverse Primer (10uM)** | **1.5** |
| **Nuclease Free Water** | **17** |
| **Undiluted DNA** | **5** |
| **Total Volume** | **50** |

## 2.10 SEQUENCING OF TRIM5A CAPSID BINDING REGION

Viral RNA from plasma was isolated by TRIzol (Ambion) and reverse transcribed using random hexamers (Applied Biosystems). Viral RNA was directly isolated (TRIzol-Ambion) from the inoculum stocks and reverse transcribed. Two rounds of PCR were performed to amplify the 546bp N-terminal region of the TRIM5α binding site in the capsid. The following primers were used for round 1 PCR: Forward-5'-ATG GGC GTG AGA AAC TCC G-3', Reverse-5'-CAA GCC GTC AGC ATT TCT TCT AG-3'. The following were the primers for round 2 PCR: Forward-5' AAG CCC TAG AAC ATT AAA TGC-3', Reverse-5'-GCA ATC GTT AGC ATT TTG AAT CAG-3'. The reaction protocol and conditions are shown in **Tables 7 and 8**, respectively. The amplicon was excised, gel-purified, and cloned into the TOPO TA cloning vector (Invitrogen). Two to four clones were selected, plasmid-purified, and sent for sequencing (Genewiz, NJ). Geneious Pro Software (version 5.5.2) was used to align capsid sequences.

**Table 7. Protocol for PCR amplification of the TRIM5α capsid-binding region.**

| Reagent | Volume (uL) |
|---|---|
| **10X Hotmaster Buffer (5 Prime)** | **5** |
| **Forward Primer (10uM)** | **0.5** |
| **Reverse Primer (10uM)** | **0.5** |
| **dNTPs (Applied Biosystems)** | **4** |
| **Hotmaster Taq DNA polymerase (5 Prime)** | **0.3** |
| **Nuclease Free Water** | **34.7** |
| **Undiluted DNA** | **5** |
| **Total Volume** | **50** |

**Table 8. PCR conditions for amplification of the TRIM5α capsid-binding region.**

| Step | Temperature (°C) | Time |
|---|---|---|
| **1. Initial denaturation** | **94** | **2 min** |
| **2. Denaturation** | **94** | **20 sec** |
| **3. Annealing** | **55** | **20 sec** |
| **4. Extension** | **72** | **30 sec** |
| **5. Final extension** | **72** | **7 min** |
| **Hold** | **4** | ∞ |
| **1st Round of PCR: steps 2-4 repeated 35 cycles** | | |
| **2nd Round of PCR: steps 2-4 repeated 30 cycles** | | |

## 2.11 MHC CLASS I GENOTYPING

Total RNA from PBMC of the eight animals was extracted and sent to the Wisconsin National Primate Research Center Genetics Service Unit (University of Wisconsin-Madison, USA) for MHC class I genotyping.

## 2.12 STATISTICAL ANALYSIS

Statistical analysis was performed using Prism 6.0b for Mac OS X and the appropriate statistical tests are listed under each figure. $P<0.05$ was considered significant.

# 3.0 STUDY OVERVIEW

## 3.1 CURRENT KNOWLEDGE

An association between expression of RFs and delayed disease progression has been shown in HIV+ humans. HIV-exposed seronegative individuals have significantly higher huA3G levels in PBMC and cervical tissue when compared to healthy controls, and PBMC of long-term nonprogressors (LTNPs) have significantly higher A3G levels than those of non-controllers [54,115,116,117]. Additionally, higher pre-infection A3G levels inversely correlate with the HIV viral set point [118]. With respect to tetherin, an association between *higher* tetherin levels and HIV-1 disease progression is observed [119], while in another, induction of tetherin expression during patient treatment with IFNα correlates with a reduction in HIV-1 virus burden [120]. Others show tetherin overexpression inhibiting particle release despite the presence of a viral antagonist [40]. These findings are further supported in rhesus macaques where higher A3G expression correlates with lower virus loads, increased survival, and protection from subsequent mucosal challenge [121,122,123]. While studies show higher TRIM5α expression associated with significant antiviral activity, the longitudinal expression of TRIM5α has not been examined, particularly in relation to viremia [124]. More importantly, less is known about SAMHD1 and SCHL11 expression in response to infection, especially with respect to the SIV macaque model.

## 3.2 GAPS OF KNOWLEDGE

While a significant contribution has been made in the field regarding the *in vitro* and *in vivo* role of these RFs, several questions remain to be answered. It is unclear if one or all RFs work in concert to provide optimal control early after viral exposure. Similarly, it is unknown whether animals with inherently lower basal RF levels are at a higher risk of infection. While it is established that RF (A3G) expression is inversely correlated with HIV/SIV viremia [54,121], analyzing the *in vivo* behavior of these RFs over a day to day time course during exposure can allow us to decipher how the host responds to virus early after exposure and when the expression threshold is reached to allow a host to succumb to disease. Whether these RFs are differentially induced in the mucosa in comparison to the periphery of infected macaques and whether induction fluctuates after each mucosal challenge is not known. Better understanding of how these RFs behave pre- and post-viral exposure is necessary to harness their innate ability to block the virus through gene therapy or vaccines that specifically upregulate these genes to arm the immune system prior to exposure. In other words, RFs may have a significant, understudied ability to hinder virus replication if expressed at higher levels prior to exposure. This speculation may be more evident with repetitive, low dose rectal challenges, which has not been examined previously.

To address these gaps of knowledge regarding the *in vivo* behavior of the five RFs, a thorough time course analysis of their expression in the mucosa and the periphery was performed by analyzing basal and induction levels and correlating these levels to susceptibility to infection in a cohort of eight Indian-origin rhesus macaques. I **hypothesized** that higher basal RF expression may exert a protective effect by delaying systemic infection. The two overarching questions that framed my work were: 1) why do some hosts remain persistently uninfected despite repeated exposure; and 2) could RF expression contribute to that variability. Therefore, I set out to understand whether inherent animal differences such as tissue-specific basal RF levels, early RF induction, maximum RF induction, and TRIM5α genotype all play a role in determining susceptibility to infection. I also wanted to determine if the changes seen in the blood mirror those seen in the mucosa (gut), the primary replication site for HIV/SIV [125].

## 3.3 PROJECT OVERVIEW

A cohort of eight Indian origin rhesus macaques were subjected to weekly low dose rectal challenges with the primary pathogenic isolate, SIV/DeltaB670 and evaluated for systemic infection. Pre and post-exposure mRNA levels of the five known lentiviral RFs (TRIM5α, A3G, Tetherin, SAMHD1, SCHL11) in addition to Mx1 and IFNγ were measured in each animal at 3-day intervals. Animals were divided into groups based on their susceptibility to infection and RF levels were monitored over time to analyze how expression of these RFs affects infection. Since TRIM5 polymorphisms impart a significant inhibitory effect on the replication of several SIV strains, I analyzed the TRIM5 polymorphisms in the cohort and its impact on an animal's susceptibility to infection with SIV/DeltaB670. Specifically, I examined whether the TRIM5α-capsid binding region in SIV/DeltaB670 exhibited signs of resistance to TRIM5α-mediated restriction as other SIV strains have shown.

## 3.4 SPECIFIC AIMS

The intrinsic ability of RFs to block lentiviral replication immediately post viral entry has refocused the attention of the field. Although some studies have examined specific expression of some RFs and linked their expression with lower viremia and slower disease progression, the vast majority of RF studies have examined the molecular behavior of each RF *in vitro* using specific cell lines or immune cell subsets. Very few studies have conducted a longitudinal analysis of RF expression *in vivo* to understand how these levels can impact susceptibility to infection. More importantly, no studies have examined basal RF expression in rhesus macaques and its potential in delaying systemic infection. By evaluating expression changes of these RFs *in vivo*, we are getting a closer look at the host's response to infection and understanding whether basal and early induction of the RFs serve to protect the host from the harmful consequences of an established infection. While prevention of HIV/SIV acquisition is of prime importance, prevention of an established infection can be more relevant and achievable by further understanding the behavior of these RFs prior to exposure.

**Aim 1: Compare basal RF expression in the mucosa and periphery of rhesus macaques exhibiting differences in susceptibility to infection.**

**Hypothesis**: Basal RF expression positively correlate with resistance to systemic infection.

**A)** Quantify basal levels of A3G, TRIM5α, tetherin, SAMHD1, and schlafen 11 in PBMC, duodenum, and ILNs to evaluate tissue-specific expression. **Approach**: Basal cDNAs from rhesus macaque PBMC, duodenal biopsies, and ILN purified cells were subjected to RT-PCR to determine relative expression levels of each gene using pre-developed Taqman assays.

**B)** Evaluate the relationship between basal RF expression and an animal's susceptibility to infection. **Approach:** Tissue-specific relative RF levels from Aim1a were compared to the number of challenges required for systemic infection using statistical tests and linear regression analysis.

**Aim 2: Examine the impact of RF induction on a host's susceptibility to infection.**

**Hypothesis:** Early RF induction delays systemic infection and controls virus replication.

**A)** Measure longitudinal RF induction to determine the relationship between early, maximum, and late RF induction and an animal's resistance to infection. **Approach:** Longitudinal RF induction in PBMC post first viral challenge were determined by calculating the fold change in RF expression relative to basal levels.

**B)** Determine if RF induction seen in the blood mimics that seen in the mucosa. **Approach:** PBMC RF induction as determined in Aim 2a was compared to RF induction in the duodenum.

**Aim 3: Investigate the in vivo role of TRIM5 polymorphisms.**

**Hypothesis:** TRIM5 polymorphisms exert no protective effect on rhesus macaques rectally challenged with SIV/DeltaB670.

**A)** Determine the TRIM5α genotype of each animal and its impact on susceptibility to infection and disease progression with SIV/DeltaB670. **Approach:** Sequenced the C-terminal B30.2/PRYSPRY domain of the eight rhesus macaques and identify the polymorphism present in amino acid region 339-341. To identify the presence of the CypA allele, a SNP in intron 6 was analyzed for the G>T substitution. The number of challenges required for systemic infection were compared to each animal's TRIM5 genotype to determine if TRIM5 polymorphisms impact susceptibility to infection. The impact of TRIM5 polymorphisms on disease progression were analyzed by correlating to the viral set point for each animal.

**B)** Determine SIV/DeltaB670's susceptibility to TRIM5α-mediated restriction by examining the TRIM5 binding site in the viral capsid. **Approach:** Sequenced the N-terminal region of the TRIM5α binding site in the capsid of the challenge virus and aligned to several well-studied SIV strains and various HIV-1 subtypes.

## 3.5 SIGNIFICANCE

Why certain individuals succumb to HIV infection, while others remain persistently uninfected despite repeated exposure has been a puzzling observation for scientists. The same scenario is seen with non-human primates that remain persistently uninfected after repetitive mucosal challenges. Additionally, natural hosts of SIV virus (ex: African green monkeys infected with SIVagm) are able to prevent disease progression while non-natural hosts (ex: rhesus macaques infected with SIVmac) develop an AIDS-like disease [126,127]. Many speculations have been made proposing differences in the mounted immune response or host genetics as the cause. During infection, the host has three forms of defense: intrinsic immunity (host restriction factors), innate immunity, and adaptive immunity [3,128]. Previous HIV/SIV studies have focused predominately on host adaptive immune responses to the virus in the peripheral organs/blood. While significant progress has been made in these aspects, more focus needs to be directed toward the intrinsic/innate immune system to elucidate possible mechanisms of virus inhibition in the mucosa prior to dissemination and further CD4+ T cell depletion.

An intense amount of research has linked the power of host RFs in the inhibition of HIV/SIV replication both *in vitro* and *in vivo*. Again, the primary reason HIV-1 cannot replicate in rhesus macaques is due to the restrictive function of rhesus TRIM5α and A3G [53,62,64,81,124]. Specific mutations in these RFs can influence the corresponding viral antagonists' function and change the virus's ability to adapt to a new host [5,63,129]. Such powerful conclusions should prompt rigorous research regarding the role of RFs early and late during an infection. It is also unknown whether these RFs are differentially expressed and induced in the mucosa in comparison to the periphery of infected macaques. Some studies in macaques have looked at RF expression changes in PBMC, but over a longer timeframe (weeks), while I believe it is critically important to analyze RF levels prior to exposure to understand their potential protective influence on the host. The significance of my study is threefold: 1) the ability to simultaneously measure the basal and induction levels of all five RFs throughout multiple exposures; 2) the ability to measure these changes directly in the mucosal and peripheral compartments; and 3) the

ability to monitor these changes *in vivo*. Additionally, studies have been limited to the *in vitro* antiviral ability of huSCHL11, but evidence examining its role *in vivo,* especially with respect to SIV, is lacking. Therefore, my study is the first to analyze SCHL11 expression *in vivo* after SIV infection and the first to specifically characterize the relationship of all five RFs both pre and post-exposure and their impact on an animal's susceptibility to infection.

# 4.0 RESULTS

## 4.1 OUTCOME OF REPETITIVE LOW DOSE RECTAL EXPOSURES

Eight Indian-origin rhesus macaques received up to six low-dose rectal challenges at weekly intervals with an initial dose of $10^3$ $TCID_{50}$ of SIV/DeltaB670 and then $10^4$ $TCID_{50}$ during the last five challenges. Challenges were terminated for each animal after it became systemically infected. Three macaques had detectable viremia after two challenges (R700, R701, R702; susceptible to infection), two macaques had detectable viremia after three challenges (R703, R704; intermediately susceptible), while three macaques required 6-7 challenges before virus was detected in the plasma (R705, R697, R698; resistant to infection) (**Figure 9**). All three susceptible animals behaved similarly, with a peak in viremia of $10^6$-$10^7$ RNA copies 10-14 days post their second rectal exposure, and a viral set point of $10^4$-$10^6$ reached within 31-37 days after the first viral exposure. These susceptible animals were sacrificed 37 days post first viral challenge for tissue collection. Viremia was detected in the intermediate animals after the third challenge. Virus levels gradually increased to a maximum of $10^5$ (R704) and $10^6$ (R703) copies/ml plasma and decreased thereafter to a set point of $10^3$ and $10^5$ copies/ml plasma, respectively. Two of the resistant animals (R697 and R698) required 6 challenges to become systemically infected. Monkey R697 had the highest peak viremia of all 8 animals ($10^8$), while R698 displayed better control of virus replication. However, the viral set point in both R697 and R698 was indistinguishable from those achieved in the susceptible group.

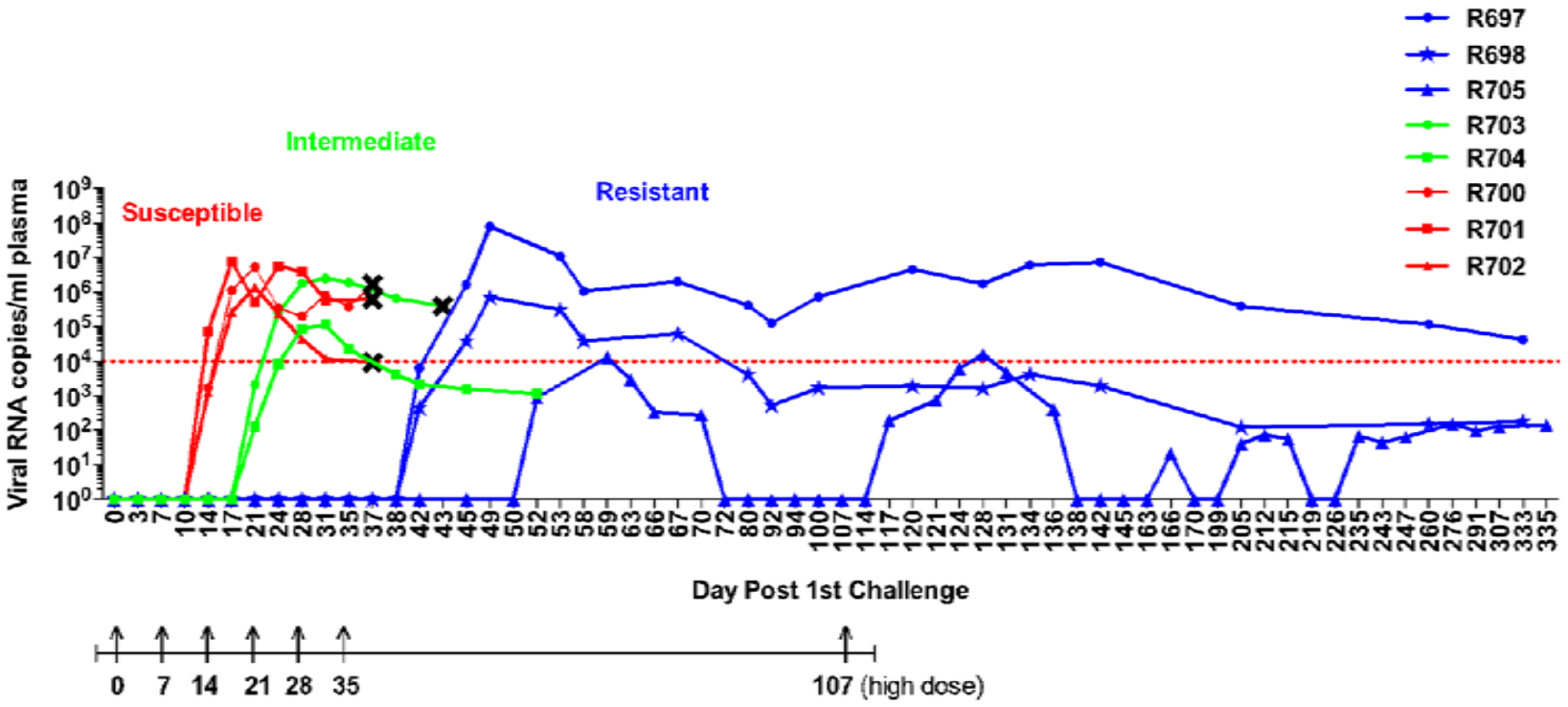

R697
R698
R705
R703
R704
R700
R701
R702
Intermediate
Susceptible
Resistant
Viral RNA copies/ml plasma
Day Post 1st Challenge
0 7 14 21 28 35
107 (high dose)

**Figure 9. Outcome of repeated, low-dose rectal challenges with SIV/DeltaB670.**
Eight Indian-origin rhesus macaques received weekly low dose rectal challenges. Animals were initially challenged with 1cc cell-free SIV/DeltaB670 ($TCID_{50}=10^3$) and then 10-fold more virus ($TCID_{50}=10^4$) for the remaining five challenges. Blood was collected twice a week and copies of viral RNA were quantified in plasma by qRT-PCR using external standards. Animals were divided into three groups based on the number of challenges required for systemic infection: susceptible (red); 2 challenges (R700, R701, R702), intermediate (green); 3 challenges (R703, R704), and resistant (blue); ≥6 challenges (R697, R698, R705). Red dotted line at $10^4$ viral RNA copies indicates the threshold virus load (VL) associated with clinical disease in rhesus macaques. Arrows indicate time points of challenge. **X** = sacrifice for tissue collection.

Of particular interest is R705, which developed a delayed, low-level transient viremia that was detectable 17 days after the 6th challenge. However, viremia was effectively controlled and virus was undetectable by day 72. R705 was exposed to a 100-fold higher dose of virus on day 107. Despite the high dose of virus, a low level of viremia was again detected that was transient; blips in viremia recurred thereafter without further challenge, with no more than 100 copies/ml of plasma persistently detected 235 days into the study. This animal was an elite controller, characterized by minimal levels of virus detected in the plasma and no signs of infection or disease. *Altogether, these eight animals depicted the full spectrum of infection and disease previously described for SIV/DeltaB670, with 3 susceptible, 2 intermediately susceptible, and 3 animals resistant to infection. These results demonstrated that the low dose rectal exposures in a cohort of 8 animals generated noticeable variability in macaque susceptibility to infection which will prove to be crucial in the upcoming experiments.*

## 4.2 COMPARISON OF TISSUE-SPECIFIC BASAL RF EXPRESSION

Following sexual exposure, the gut and/or vaginal mucosa serve as the initial sites for virus replication, followed by further expansion and dissemination of the virus via the draining lymph nodes and blood [21,130]. Since lentiviral RFs are part of the intracellular intrinsic defense system, in that they cause

resistance to virus infection without previous signaling [3,30,36,37,38,39,40], it was important to analyze *in vivo* expression of these genes in the mucosal and peripheral compartments prior to viral exposure. To address this issue, we began by evaluating basal mRNA levels of Mx1, A3G, TRIM5α, SAMHD1, tetherin, and SCHL11 in each animal four days prior to the first rectal challenge. Three compartments were examined: PBMC, ILNs, and the duodenum. IFNγ mRNA levels were also measured to examine the relationship between RF expression over time and induction of adaptive immunity [34]. Expression of Mx1, a common ISG, was measured to serve as a positive control in my study since RFs are also ISGs [28,30,31,39,131]. Mx1 was particularly chosen because it is among the most potent, long-lasting antiviral genes solely induced by type I interferons [26,132]. Previous studies have examined Mx1 mRNA levels in rhesus macaque PBMC and levels correlated with IFNα expression [121,132].

In general, RFs displayed higher mean basal expression in PBMC than in the ILNs and duodenum (**Figure 10).** Basal RF levels were more variable in PBMC compared to the ILNs and duodenum, with the latter two compartments displaying a tighter clustering of RF expression. Tetherin and SAMHD1 were more highly expressed than the other RFs in the three compartments (**Figure 10C,E)**. SCHL11 expression was especially low in the three tissues compared to the rest of the RFs with levels ranging from 30-900 copies (**Figure 10F**). As expected for naive animals, baseline IFNγ levels were lower in all three tissues compared to the RFs and Mx1 (**Figure 10G)**. *Together, these data illustrate that RFs are constitutively expressed and exhibit tissue-specific expression, with higher levels primarily found in the blood versus the duodenum and ILNs.*

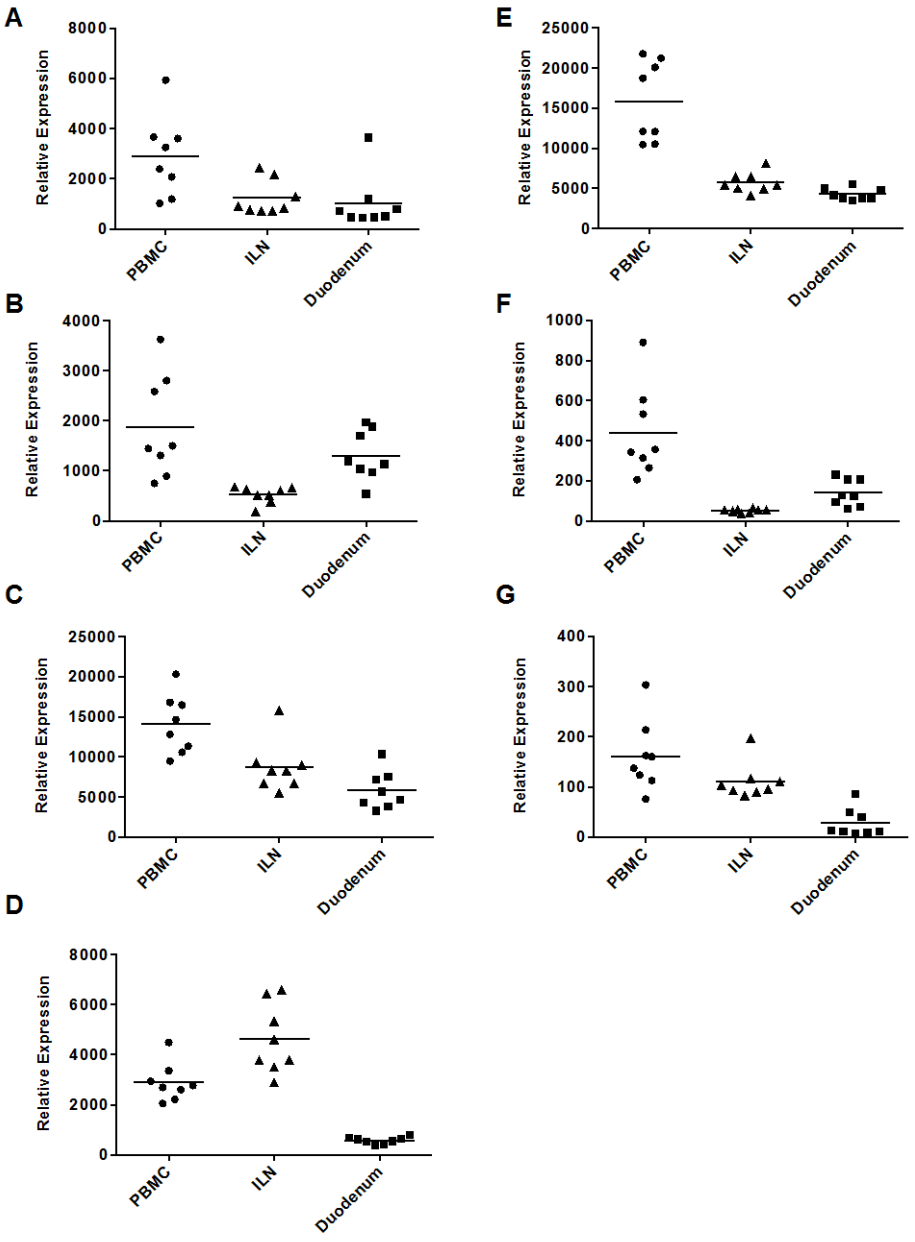



**Figure 10. Basal RF expression in PBMC, ILNs, and duodenum.**

Relative mRNA levels of **(A)** Mx1, **(B)** TRIM5α, **(C)** Tetherin, **(D)** A3G, **(E)** SAMHD1, **(F)** SCHL11 and **(G)** IFNγ was determined in PBMC, inguinal lymph nodes (ILNs), and duodenal biopsies in the eight animals. Samples were obtained 4 days prior to the first exposure (basal levels) on all animals except monkey R700 from which PBMC was obtained on day 0 (day of first exposure) while duodenal and ILN samples were obtained 4 days prior to first exposure. Relative expression was determined using the ΔΔCt comparative method and values were normalized to endogenous controls TBP and HPRT. Horizontal lines denote means values. Each symbol indicates the mean value from one animal.

## 4.3 BASAL RF EXPRESSION AND SUSCEPTIBILITY TO MUCOSAL INFECTION AND DISEASE PROGRESSION

To understand whether intrinsic differences in basal ISG (A3G, TRIM5α, tetherin, SAMHD1, SCHL11, and Mx1) expression related to the observed variability in the cohort's susceptibility to infection, RF expression in the susceptible, intermediate, and resistant groups were compared in the three compartments (**Figure 11**). In PBMC, there was a noticeable difference in basal Mx1 levels among the three groups with resistant animals exhibiting significantly higher levels of Mx1 (P=0.025) compared to the susceptible (**Figure 11A**). Remarkably, rhesus macaques resistant to infection also had significantly higher (P=0.01) basal TRIM5α levels in the blood versus the susceptible animals (**Figure 11B**). RF mRNA levels in ILNs displayed a similar pattern, with a significant difference in Mx1 expression among the three groups (P=0.025) and TRIM5α showing a trend toward significance (P=0.06). A similar trend was also observed for the remaining RFs in the blood (tetherin, A3G, SAMHD1, and SCHL11), although these differences were not as pronounced as that observed for Mx1 and TRIM5α (**Figure 11C-F)**. After TRIM5α and Mx1, the greatest trend was seen in PBMC tetherin and A3G expression (P=0.06, P=0.09, respectively), where resistant animals expressed higher basal levels of these two RFs than the susceptible (**Figure 11C,D**). Notably, the patterns seen in the PBMC and ILNs were not observed in the duodenum.

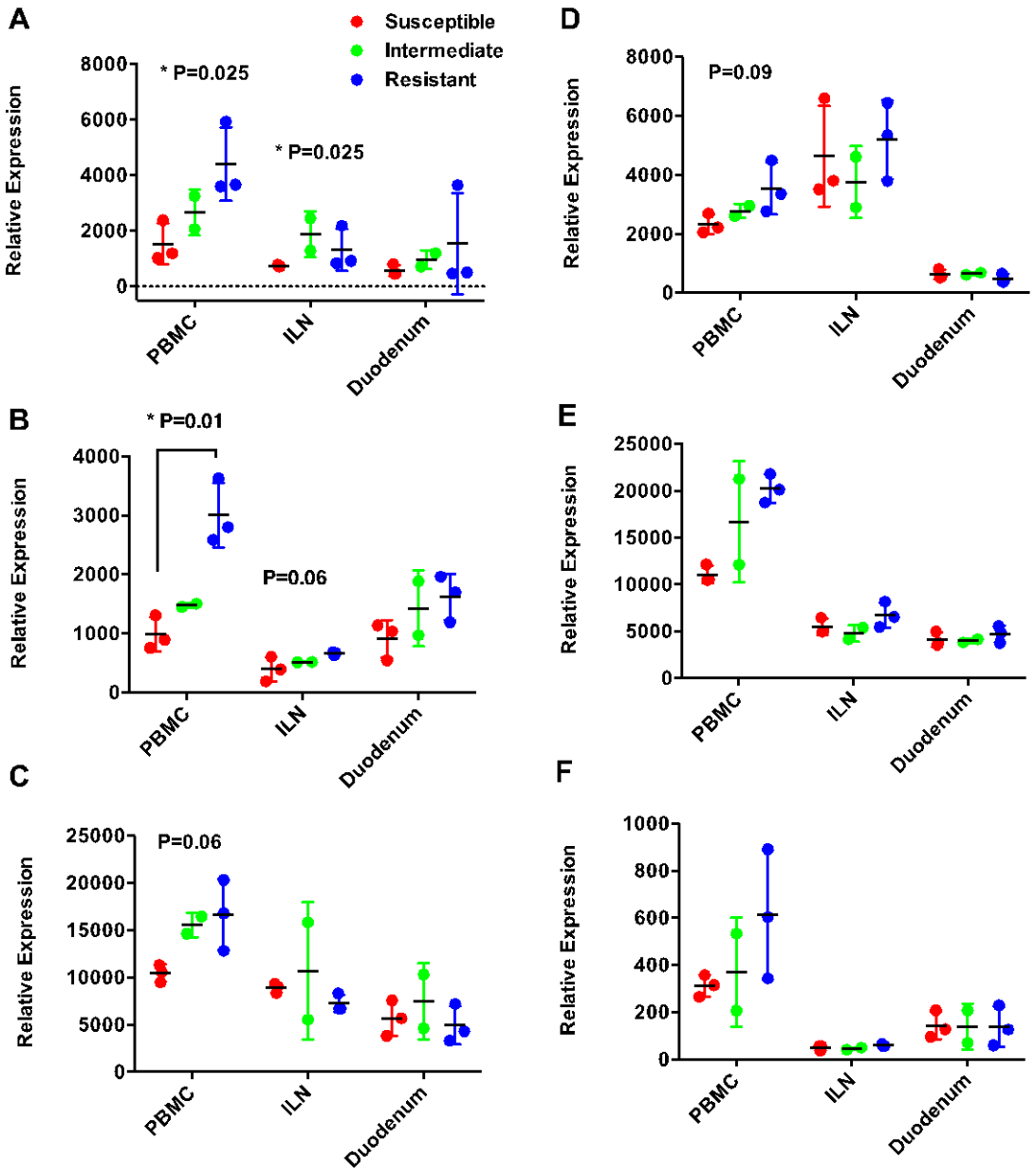



**Figure 11. Tissue-specific basal RF expression and susceptibility to infection.**
Basal expression of **(A)** Mx1, **(B)** TRIM5α, **(C)** Tetherin, **(D)** A3G, **(E)** SAMHD1, **(F)** SCHL11 was measured as in figure 10. Samples were obtained 4 days prior to the first exposure (basal levels) on all animals except monkey R700 from which PBMC was obtained on day 0 (day of first exposure) while duodenal and ILN samples were obtained 4 days prior to first exposure. Animals were divided into susceptible (red), intermediate (green), and resistant (blue) groups according to figure 9. Mean +/- SD of basal mRNA values in each tissue are shown for each group. Significance was calculated using the Kruskal-Wallis test with Dunn's multiple comparison test. Asterisks indicate significance at $P<0.05$. P values indicating trends toward significance are shown. Each symbol indicates one animal.

A significant association between higher basal RF expression and resistance to mucosal infection was further confirmed by linear regression analysis. Tissue-specific basal ISG expression for each animal was plotted against the number of exposures required for systemic infection **(Figure 12)**. A significant positive correlation was observed between basal A3G, TRIM5α, SAMHD1, and Mx1 levels in PBMC and resistance to infection (**Figure 12A)**. This correlation was especially pronounced for TRIM5α ($r^2$=0.85, P=0.001). Basal TRIM5α levels in the duodenum and ILNs were also positively correlated with the number of exposures required for systemic infection but were not significant (**Figure 12B,C**). Although higher tetherin and SCHL11 expression in PBMC did not significantly correlate with resistance to infection, a positive trend was observed (P=0.06, P=0.09, respectively) which was also seen for SCHL11 in the ILNs (P=0.08). No significant correlation between basal ISG expression and resistance to infection was observed in the duodenum.

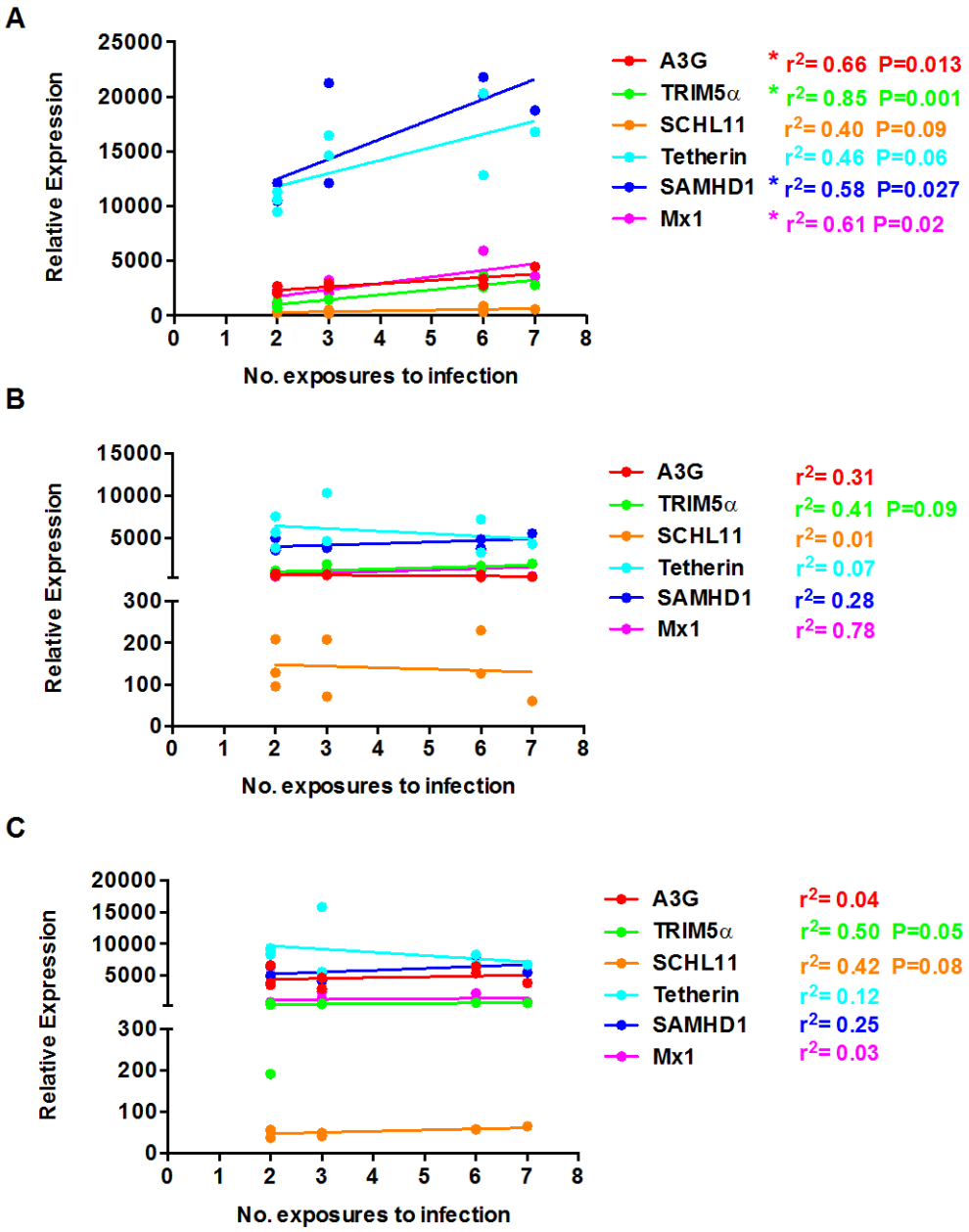



**Figure 12. Relationship between tissue-specific basal RF expression and resistance to infection.** Basal ISG levels in **(A)** PBMC, **(B)** Duodenum, **(C)** ILNs were compared to the number of exposures an animal required for systemic infection. Samples were obtained 4 days prior to the first exposure (basal levels) on all animals except monkey R700 from which PBMC was obtained on day 0 (day of first exposure) while duodenal and ILN samples were obtained 4 days prior to first exposure. Each dot indicates one animal. Lines depict linear regression analysis with $r^2$ and p values indicated next to each gene. Asterisks indicate significance with $p<0.05$. Genes without a p value were not significant or trending toward significance ($p<0.09$).

These data clearly revealed an association between higher basal RF levels and an animal's resistance to infection. However, it was not clear whether higher basal RF levels could contribute to a lack in disease progression after infection. It is already established in the field that viral set point determines the extent of host disease progression [133]. As such, basal RF, Mx1, and IFNγ levels were examined in the three tissues and compared to each animal's viral set point. Correlation analysis revealed no significant relationship between basal RF levels in the blood and ILNs and an animal's viral set point and therefore disease progression (**Figure 13).** Interestingly, a negative correlation was observed between expression of all genes in the blood (except IFNγ) and viral set point, indicating the impact of higher RF levels in slowing disease progression (**Figure 13A)**. A significant trend was observed in the duodenum, however, for TRIM5α (spearman r=-0.74, P=0.046) and less so for A3G (spearman r=0.71, P=0.057) (**Figure 13B)**. It is important to note that TRIM5α expression in all three tissues was consistently negatively correlated with viral set point. This observation was not evident for the other RFs in all three tissues.

*In summary, animals resistant to mucosal infection expressed higher basal ISG levels than those more susceptible to infection, with differences in the levels of Mx1 and TRIM5α significant with expression of the remaining RFs trending toward significance. These differences were strongest in the blood and followed by the ILNs, with expression in the duodenum varying only marginally among the three groups. No significant association between basal ISG expression and disease progression was observed in either of the tissues. Only basal TRIM5α levels in the duodenum showed a significant negative correlation with viral set point, which may indicate its potential protective effect in the gut.*

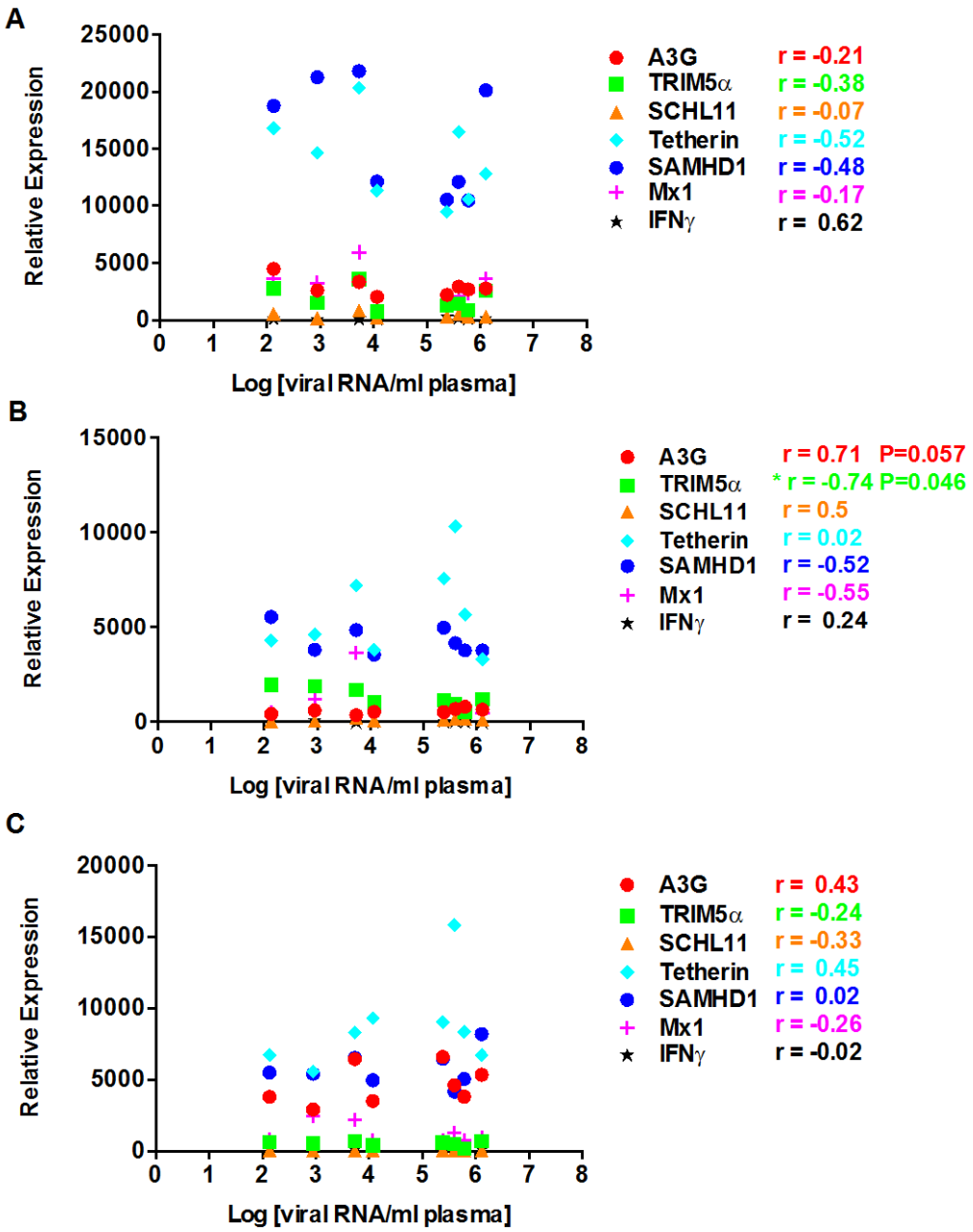



**Figure 13. Relationship between tissue-specific basal RF expression and viral set point.**
Basal ISG and IFNγ levels in **(A)** PBMC **(B)** Duodenum **(C)** ILNs were plotted against an animal's corresponding viral set point. Samples were obtained 4 days prior to the first exposure (basal levels) on all animals except monkey R700 from which PBMC was obtained on day 0 (day of first exposure) while duodenal and ILN samples were obtained 4 days prior to first exposure. Spearman correlation analysis was performed to evaluate the relationship between the two variables. Spearman r with two-tailed p values are indicated next to each gene for the three tissues. Asterisk indicate significance at $p<0.05$. P values tending toward significance were noted. Each symbol indicates one animal.

## 4.4 LONGITUDINAL ANALYSIS OF ISG INDUCTION IN PBMC

After examining pre-exposure RF levels in the blood, it was important to determine whether repetitive challenge induced RFs and whether induction in the blood correlated with resistance. A time course analysis examining RF induction in the blood post virus exposure was conducted for each animal over the series of repetitive rectal challenges. Longitudinal analysis was performed only in the blood and not the duodenum or ILNs due to the availability of blood samples throughout the time course. Induction levels are displayed as fold-change which was determined by dividing relative mRNA copy values at each time point with those at baseline (day -4 of the challenge series), with a 2 fold change serving as the minimum threshold. These results are plotted in relation to virus loads.

### 4.4.1 ISG time course in the susceptible animals

Transient induction of the six ISGs was observed in all susceptible animals during the acute viremic episode associated with systemic infection (**Figure 14)**. As expected for genes induced by IFNα, high expression was observed at the first time point virus was detectable in the blood, but declined to basal levels 3-7 days later. A 5-fold induction of TRIM5α and a 7-fold induction of tetherin was observed for R700 on day 17 (**Figure 14A**). Induction of these two RFs was particularly higher than A3G, SAMHD1, and SCHL11. R701 had a 5-6 fold induction of tetherin and TRIM5α on day 14 while the other RFs were induced to a lower extent, about 3-5 fold (**Figure 14B)**. RF expression remained high for R701 three days later with TRIM5α and tetherin showing persistently high induction among the rest of the RFs. R702 also had a 4-5 fold induction of TRIM5α, tetherin, and A3G on day 17, three days after the appearance of virus in the blood (**Figure 14C)**. RF induction, however, quickly subsided after the initial viremia remaining below the 2-fold threshold for the three animals. As expected, Mx1 induction corresponded with RF induction in the susceptible animals. R700 had a 20-fold induction of Mx1 at the first viremic time point and levels increased to 50 fold three days later (**Figure 14A)**. R701 and R702, on the other

hand, had a 40-fold induction of Mx1 during initial viremia that decreased to 30- and 5-fold, respectively (**Figure 14B,C)**. Similar to the RFs, Mx1 levels were downregulated after initial viremia in the susceptible group, remaining below the 2-fold mark (**Figure 14)**. IFNγ expression, on the other hand, remained well below the 2-fold threshold in the susceptible animals with no induction observed throughout the time course. Although the ISGs were induced during initial viremia, their induction levels did not appear to affect virus replication later in the time course.

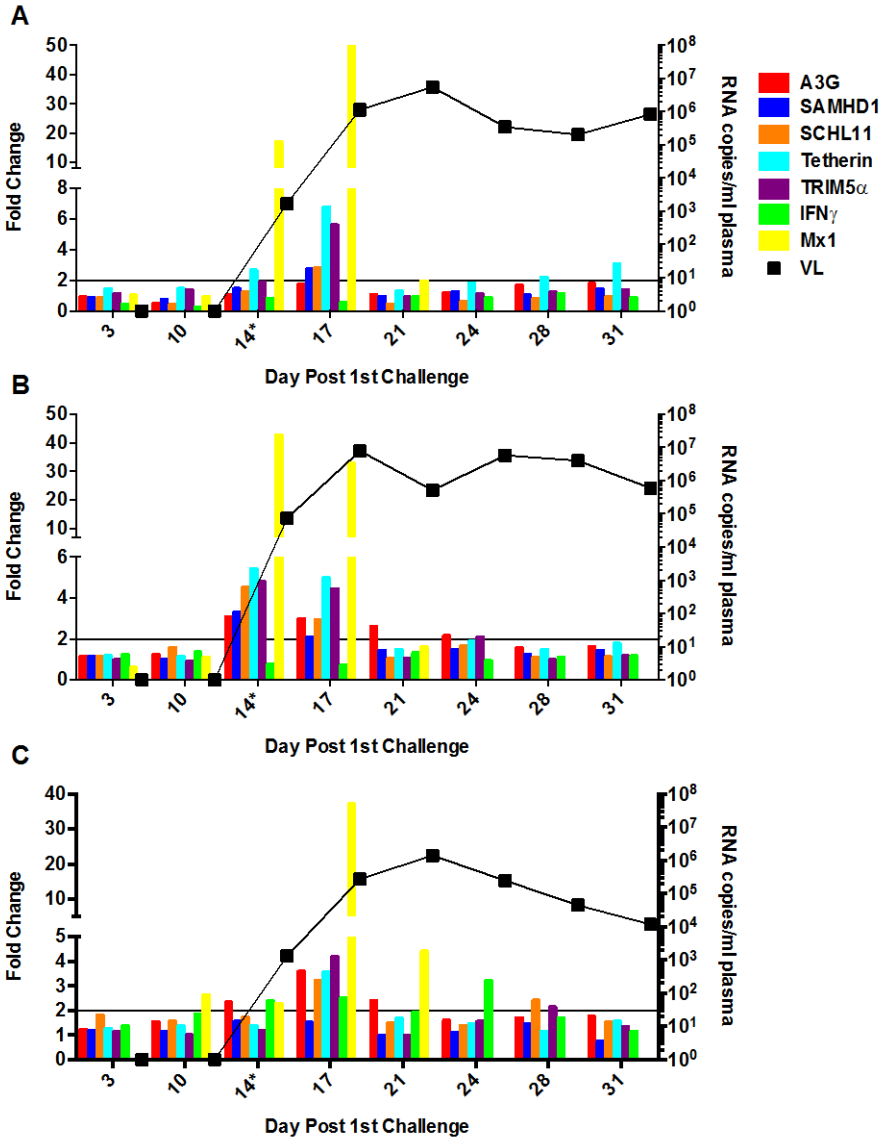


**Figure 14. Longitudinal analysis of RF and IFNγ induction in susceptible animals.**
The fold change in the relative expression of each RF, Mx1, and IFNγ in PBMC are plotted (**A)** R700, **(B)** R701**, (C)** R702**.** Samples were obtained 4 days prior to the first exposure (basal levels) on all animals except monkey R700 from which PBMC was obtained on day 0 (day of first exposure). Each gene is depicted by a different colored bar as indicated in the figure legend; plasma virus loads are shown by the black line and the right Y-axis. Fold change was calculated by dividing the relative mRNA level at each time point by the constitutive level observed at baseline. Mx1 was not measured at all time points (R702 day 3, all animals days 24-31). The black solid line indicates the threshold for the observed induction to be true. Asterisks next to day numbers indicate days of rectal challenge.

### 4.4.2 ISG time course in the intermediate animals

Intermediate animals displayed transient RF induction similar to the susceptible group. Expression during the earlier time points (days 3-10) was below the 2-fold threshold (data not shown). RF induction for R703 and R704 occurred primarily on day 24 (10 days after the third rectal challenge) and after virus detection in the blood (**Figure 15)**. Levels were downregulated thereafter. RF induction levels in the intermediate animals were much lower than the susceptible. A 4-fold induction of Mx1 was observed for R703 on day 21 which increased to 40 fold just three days later, corresponding with the 2-3 fold RF induction (**Figure 15A)**. After day 24 for R703, levels remained below the 2-fold mark for all genes examined. R704's time course was different from R703 in that IFNγ was persistently induced at 2-5 fold from days 17-31 (**Figure 15B)**. RF induction was not observed in R704 and remained below the 2-fold mark throughout the time course. In contrast to R703, Mx1 induction in R704 was much lower, where a 7-fold increase was observed on day 24 with a slight increase four days later.

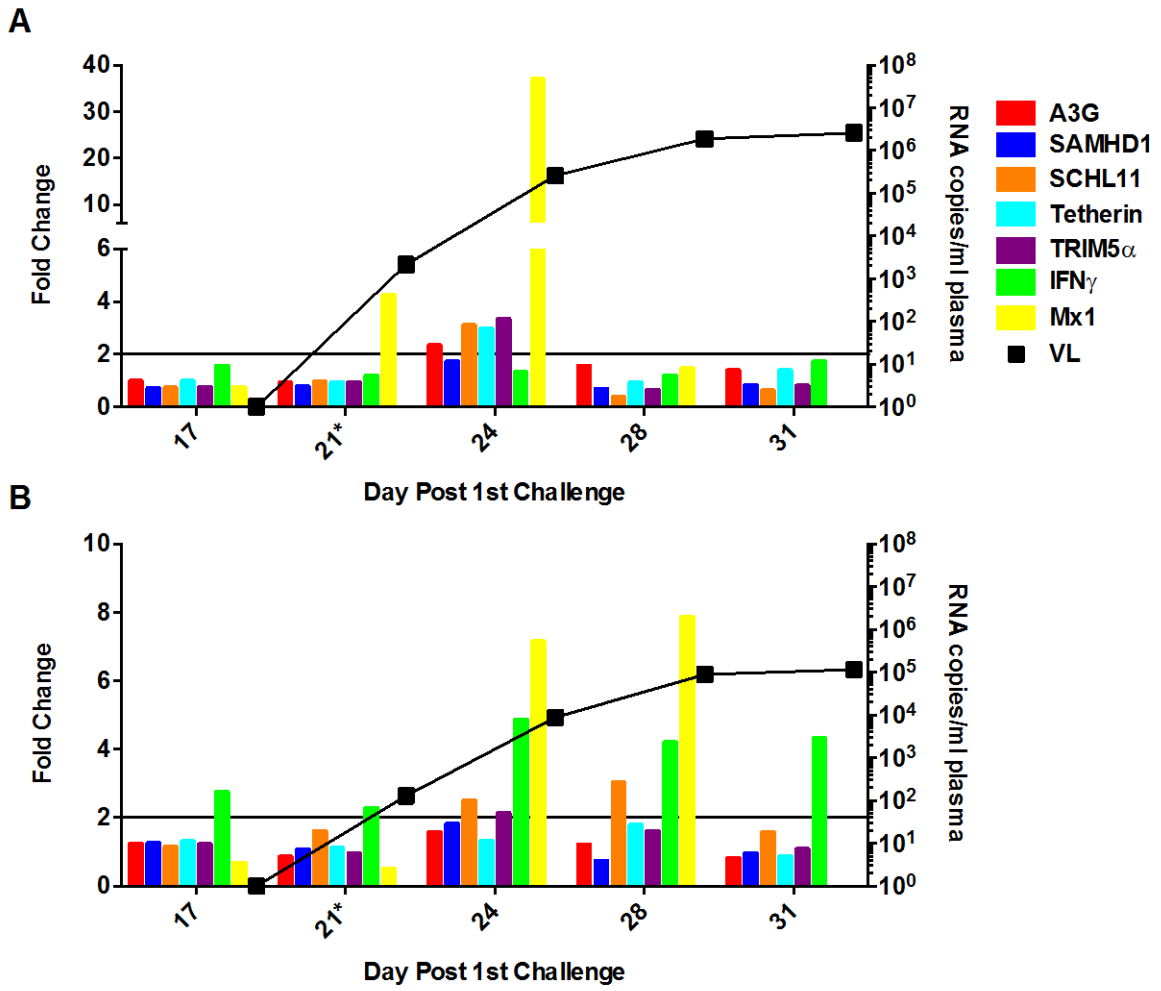



**Figure 15. Longitudinal analysis of RF and IFNγ induction in intermediate animals.**
The fold change in the relative expression of each RF, Mx1, and IFNγ in PBMC are plotted (**A)** R703, **(B)** R704**.** Each gene is depicted by a different colored bar as indicated in the figure legend; plasma virus loads are shown by the black line and the right Y-axis. Fold change was calculated by dividing the relative mRNA level at each time point by the constitutive level observed at baseline. Mx1 was not measured on day 31 for both animals. Black solid line indicates the threshold for the observed induction to be true. Asterisks next to day numbers indicate days of rectal challenge.

### 4.4.3 ISG time course in the resistant animals

A different scenario was observed with the PBMC time courses of the resistant animals. Both R697 and R698 displayed a lack of ISG or IFNγ induction, despite six repetitive challenges, until they became viremic (**Figure 16)**. Induction levels in both macaques did not pass the 2-fold threshold mark until 10 days after the 6$^{th}$ challenge (day 45). However, R697 displayed a 4-fold increase in Mx1 levels by day 42, the first time point of detectable viremia. Due to lack of available sample, it is unknown if R698 also induced Mx1 to similar levels on day 42. Ten days after the 6$^{th}$ challenge (day 45), both animals showed a 10-14 fold increase in Mx1 which was maintained over time in R697 but not in R698, where Mx1 levels were downregulated 25 days after initial viremia (day 67). RF levels were slightly induced, about 2-3 fold, in R697 on day 67 and 80 but remained below the 2-fold mark thereafter (**Figure 16A)**. Interestingly, tetherin was the most highly induced RF in both R697 and R698. On day 100 (58 days after viremia) for R697, an 8-fold increase in tetherin expression was observed and was maintained to a lesser extent on days 142 and 205. R698 also had a 3-fold increase in tetherin on day 142 (**Figure 16B)**. It is interesting to note that despite five challenges, little to no RF induction was observed over time for both animals until the 6$^{th}$ challenge that resulted in a systemic infection. Unlike the RFs, IFNγ seemed to be slightly more induced over the time course of infection in R697, with a 4-fold induction on day 205 (163 days after viremia) which corresponded with the log decrease in virus load. Interestingly, persistent Mx1 induction in R697 corresponded with persistently high virus loads ($10^5$-$10^7$) whereas transient Mx1 induction in R698 corresponded with decreasing virus loads ($10^2$-$10^5$). Earlier time points are shown for the resistant animals, as opposed to the other two groups, to better understand how repeated challenges affect longitudinal RF levels in animals persistently resistant to infection. *In general, these two resistant animals did not show notable RF induction over time despite repeated challenges. However Mx1, IFNγ, tetherin, and to a lesser extent A3G, were induced in this group, but only after virus appearance in the blood.*

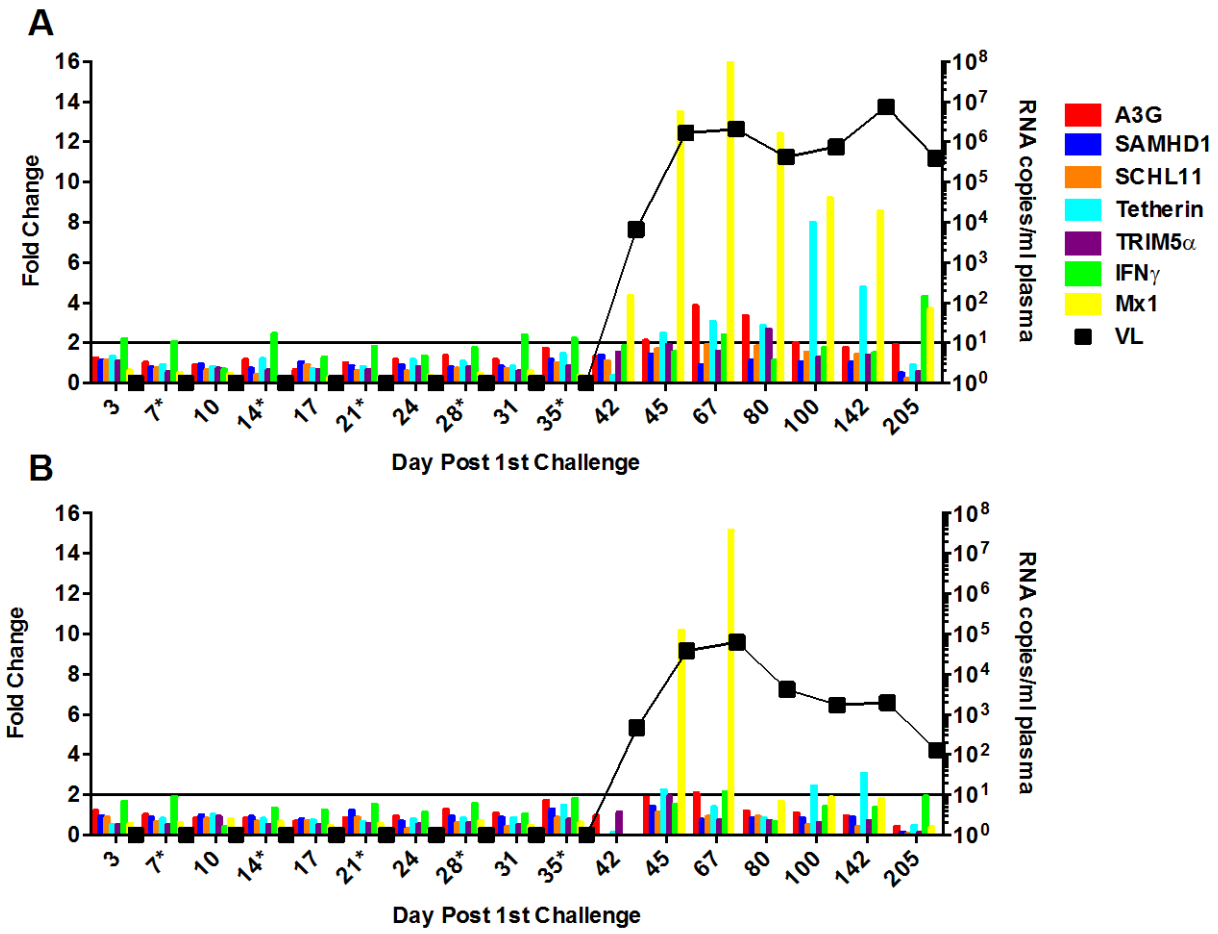



**Figure 16. Longitudinal analysis of RF and IFNγ induction in resistant animals.**
The fold change in the relative expression of each RF, Mx1, and IFNγ in PBMC are plotted (**A)** R697, **(B)** R698**.** Each gene is depicted by a different colored bar as indicated in the figure legend; plasma virus loads are shown by the black line and the right Y-axis. Fold change was calculated by dividing the relative mRNA level at each time point by the constitutive level observed at baseline. Mx1 was not measured at all time points. The black solid line indicates the threshold for the observed induction to be true. Asterisks next to day numbers indicate days of rectal challenge.

R705 is also considered among the animals in the resistant group; however, it is specifically classified as an elite controller. This animal was challenged six times with escalating doses of SIV/DeltaB670. No sign of virus was evident until 17 days from the $6^{th}$ challenge. Levels of virus reached $10^4$ copies/ml plasma and was maintained at that level for 18 days before virus was no longer detectable in the blood for 42 days. After the $7^{th}$ single high dose challenge (100 fold higher than previous challenge) on day 107, R705 became systemically infected once more while maintaining a lower VL in comparison to the other animals. R705 blipped a total of three times before it became systemically infected on day 235. Throughout the time course, no RF induction was observed despite the repeated challenges and the single high dose exposure (**Figure 17)**. Induction remained below the 2-fold mark for all examined genes with the exception of Mx1 and IFNγ. Interestingly, there was a 4-5 fold induction of Mx1 very early during the time course on days 14-28. This induction was observed despite the lack of detectable virus, which is opposite to what was observed with the rest of the cohort. The highest level of Mx1 induction was observed on day 59 during the first viremic blip but levels remained below the 2-fold mark thereafter. It is noteworthy to emphasize that Mx1 was not induced later in the time course despite several viremic blips. In contrast to early Mx1 induction, IFNγ was induced later and to a higher extent than the RFs. A 2-3 fold increase was observed on days 215, 219, 226, and 247. *From these time courses, we were able to conclude that RF induction occurred after detectable virus in the blood (except for R705), RFs were coordinately and transiently induced during initial viremia, Mx1, TRIM5α and tetherin were the most highly induced ISGs, repeated challenges had no apparent effect on RF induction, and RF induction did not appear to correlate with protection.*

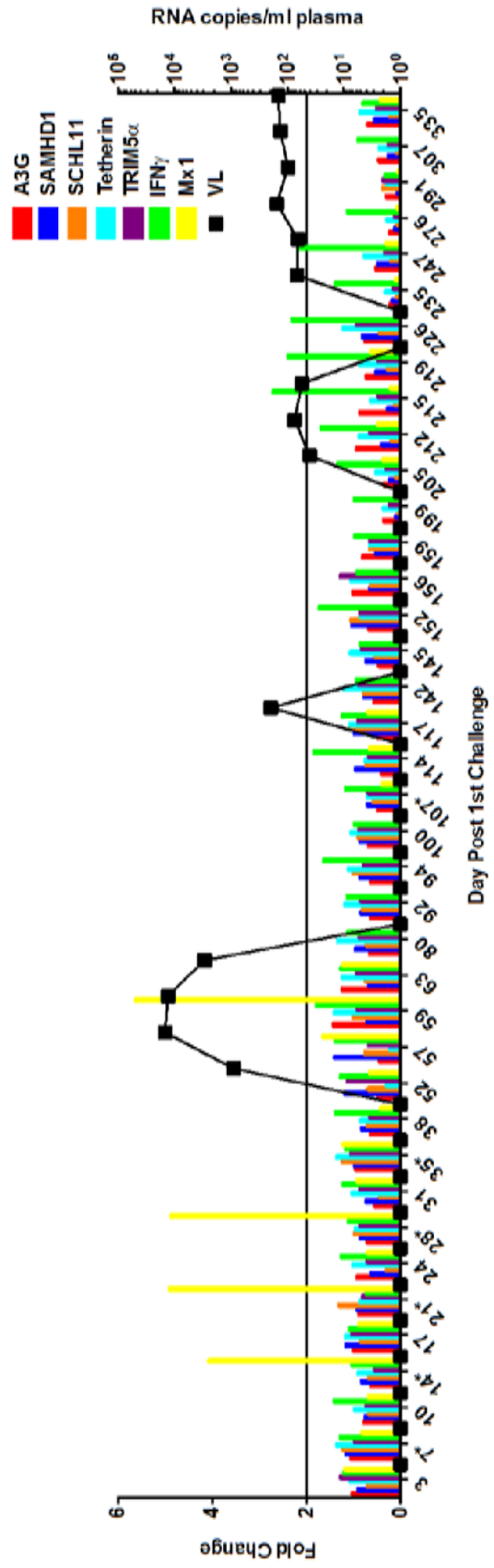

RNA copies/ml plasma
A3G
SAMHD1
SCHL11
Tetherin
TRIM5α
IFNγ
Mx1
VL
Fold Change
Day Post 1st Challenge

**Figure 17. Longitudinal analysis of RF and IFNγ induction in the elite controller.**
The fold change in the relative expression of each RF, Mx1, and IFNγ in PBMC are plotted for R705. Each gene is depicted by a different colored bar as indicated in the figure legend; plasma virus loads are shown by the black line and the right Y-axis. Fold change was calculated by dividing the relative mRNA level at each time point by the constitutive level observed at baseline. Mx1 was not measured at all time points. The black solid line indicates the threshold for the observed induction to be true. Asterisks next to day numbers indicate days of rectal challenge.

## 4.5 RELATIONSHIP BETWEEN MAXIMUM RF INDUCTION AND SUSCEPTIBILITY TO INFECTION

To determine whether intrinsic differences in maximally induced ISG expression could impact susceptibility to infection, the maximum expression levels induced post systemic infection was compared among the three groups (**Figure 18**). All animals except R705 were included in this analysis. The maximum relative expression achieved for all RFs, Mx1 and IFNγ was fairly similar among the three groups, suggesting that the ability to mount a type I interferon response to viral infection was unaffected. When fold change in expression was measured at the maximum induction time point relative to basal levels, a maximum induction of 2-9 fold was observed in the examined genes (**Figure 19B-G)**. Mx1 induction at the maximum expression time point was the greatest among the other ISGs with levels increasing to 50-fold in the susceptible and resistant groups (**Figure 19A)**. It is important to remember that the fold-change in maximum induction for the resistant animals was not as high as the susceptible due to the resistant animals' intrinsically higher basal ISG expression. *Overall, these data suggest that the maximum induced levels of these genes did not vary among the three groups and further indicated that higher RF induction post exposure did not necessarily protect from SIV infection.*

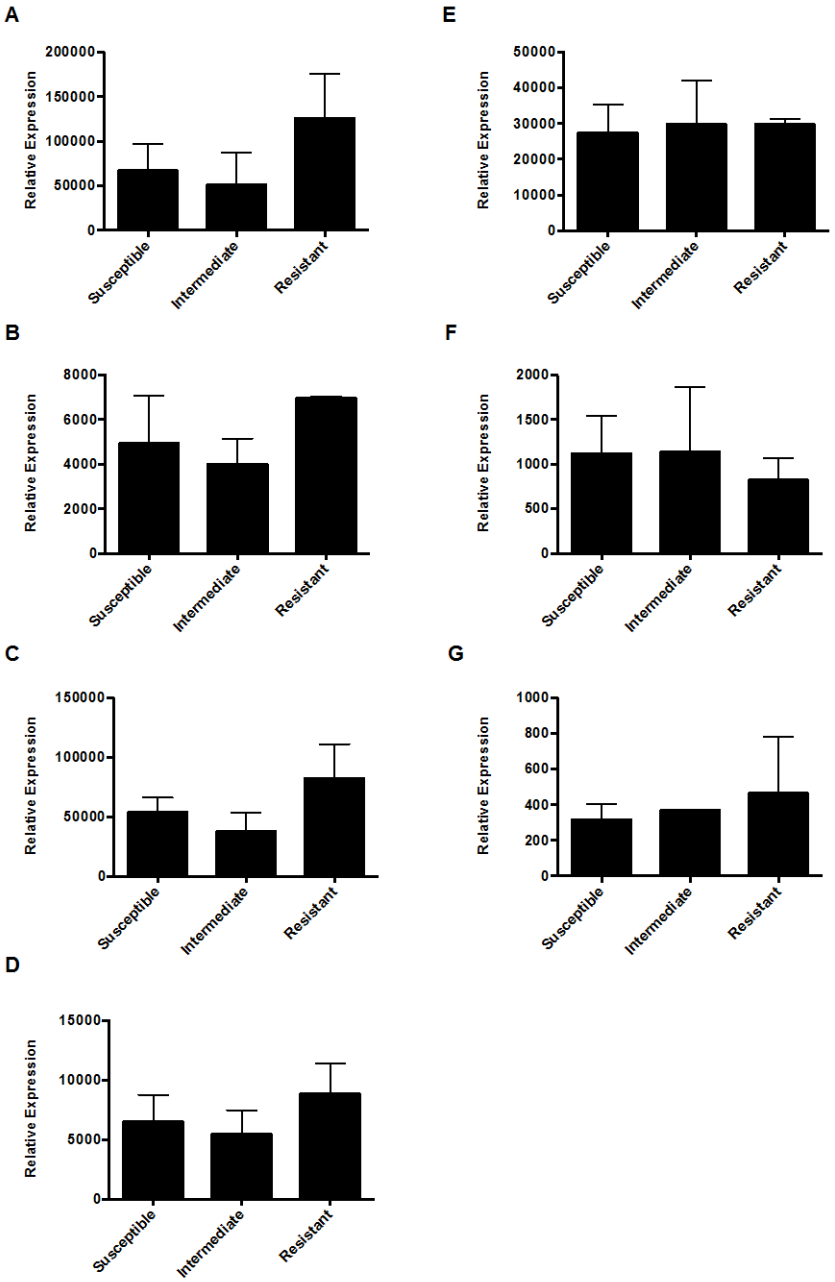



**Figure 18. Maximum ISG and IFNγ expression among the three groups.**

The maximum expression value for each gene throughout the entire PBMC time course was plotted for the three groups. **(A)** Mx1 **(B)** TRIM5α **(C)** Tetherin **(D)** A3G **(E)** SAMHD1 **(F)** SCHL11 **(G)** IFNγ. Maximum values were 3-24 days after the 2nd challenge for the susceptible animals, 10-17 days after the 3rd challenge for the intermediate animals, and 10-170 days after the 6th challenge for the resistant animals. Shown is the mean value with SD bars. R705 was not included in the resistant animals.

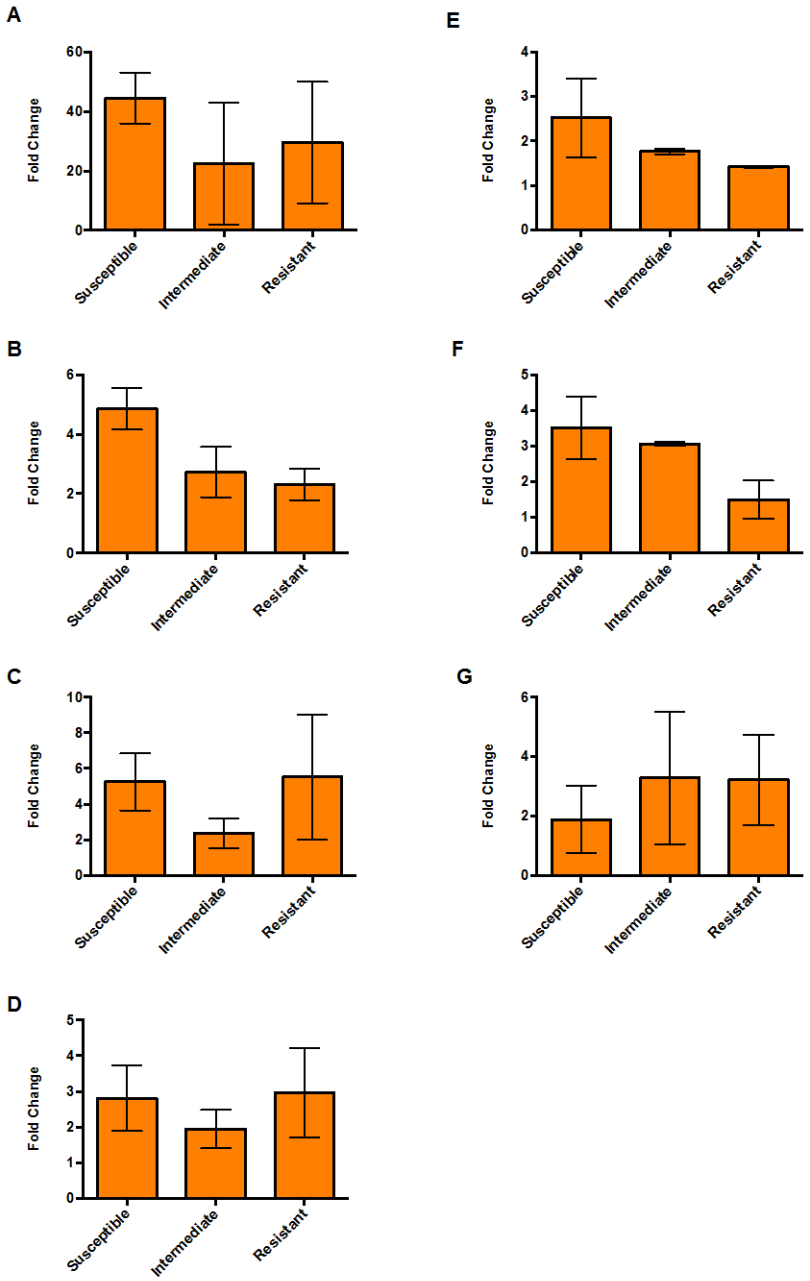



**Figure 19. Fold change in maximum ISG and IFNγ expression.**

Maximum PBMC expression values from figure 18 were divided over the relative basal values for each gene **(A)** Mx1 **(B)** TRIM5α **(C)** Tetherin **(D)** A3G **(E)** SAMHD1 **(F)** SCHL11 **(G)** IFNγ. Shown is the mean value with SD bars. R705 was not included in the resistant animals.

## 4.6 TISSUE-SPECIFIC ISG EXPRESSION IN THE ELITE CONTROLLER

Although the rectum would have been the optimal site to examine the impact of early RF expression on susceptibility to infection, the animal's natural resistance to infection could have been compromised during later rectal challenges. However, rectal biopsies were obtained four days prior to and three days after the 6$^{th}$ challenge for R705, the elite controller. Relative rectal ISG and IFNγ expression was therefore compared to levels in the blood and duodenum to evaluate tissue-specific differences that may contribute to resistance. Similar RF expression was observed in the 3 tissues (**Figure 20)**. More importantly, all five RFs exhibited coordinate induction in the three tissues. Even though R705 was exposed 6 times before this examined time point, IFNγ levels were extremely low in all three tissues, especially at the site of exposure (the rectum), indicating the lack of a mounted adaptive immune response. While minor expression variability was observed among the three tissues, these data suggest that RFs are coordinately expressed in the blood, gut, and rectum. *Collectively, these data suggest that ISGs are coordinately expressed in the PBMC, duodenum, and rectum and further reveal the ability of the blood to mimic mucosal RF expression. Thus, the blood may serve as a better compartment for examining RF behavior in response to pathogen exposure than other lymphoid and mucosal compartments.*

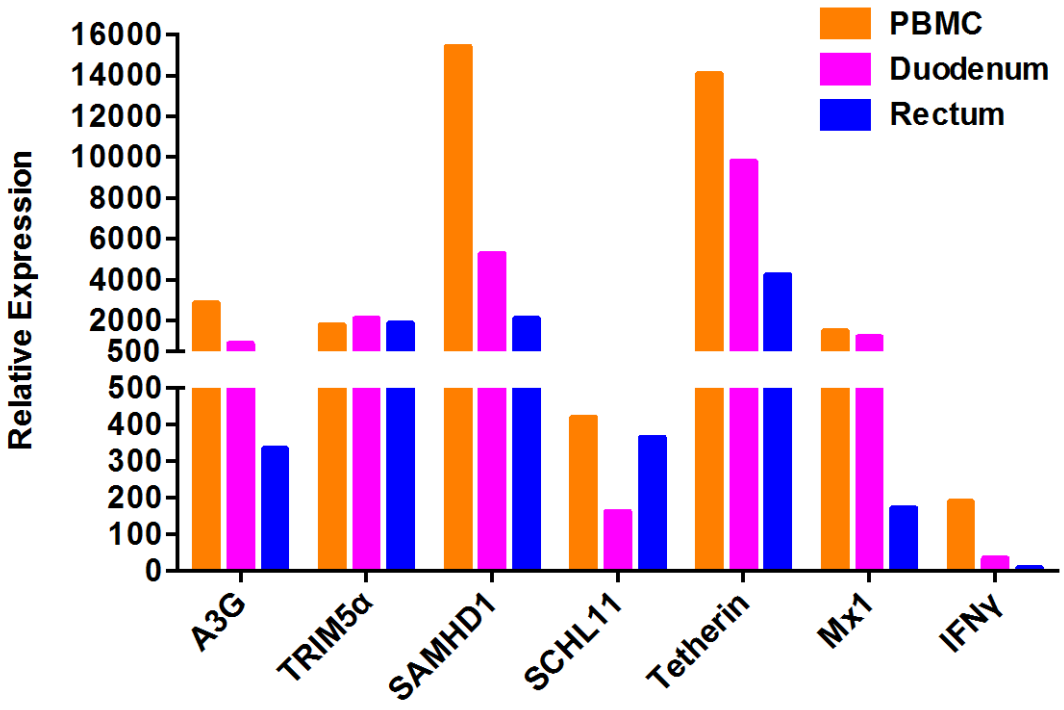



**Figure 20. Tissue-specific ISG and IFNγ expression in the elite controller.**
Relative expression values in the PBMC, duodenum, and rectum are plotted for each gene three days post the 6$^{th}$ rectal challenge in R705.

## 4.7 LONGITUDINAL ISG INDUCTION IN THE DUODENUM

In order to understand the significance of the RF induction observed in the blood, it was important to correlate longitudinal PBMC RF expression with the longitudinal RF expression seen in the duodenum. By evaluating the duodenum, I can specifically understand the interplay of ISG induction in the mucosa and its response to rectal exposure. Comparison of the two possible bottlenecks, "windows of opportunity," for systemic infection, as described in the sexual transmission section, may also reveal whether one or both barriers contribute to protection. A time course analysis was conducted by examining fold-change in expression relative to basal levels of the five RFs, Mx1, and IFNγ in duodenal biopsies

from the susceptible animals (**Figures 21)**. The intermediate and resistant animals were not examined due to lack of duodenal biopsies at their first viremic time points. Only days 3, 10, and 17 in the duodenum of the susceptible animals were examined. While the susceptible animals became viremic by day 14, a duodenal biopsy was not available at this time point. Three days after the first challenge, a noticeable induction (5-12 fold) in Mx1 was observed in the duodenum of R701 and R702 (**Figure 21B,C)**. Three days after virus appearance in the blood (day 17), all three animals displayed pronounced Mx1 and RF induction. Specifically, tetherin and TRIM5α were more highly induced (2-7 fold) in all three animals compared to the other RFs. This again mimics what was seen in the blood for selective induction of TRIM5α and tetherin in these animals. Interestingly, Mx1 induction was the greatest (150 fold) during the examined viremic time points in the duodenum of R700 and R701. These induction levels were 3-fold greater than Mx1 induction levels at the corresponding time point in the blood of R700 and R701. R702, on the other hand, upregulated Mx1 on day 17 to levels that were 30-fold high and these levels closely matched that seen in the blood on day 17 **(Figure 21C)**. *Taken together, these data suggest that Mx1 induction as early as day 3 in the susceptible animals can be detected in the duodenum much earlier than in the blood, which may suggest earlier and more sensitive virus detection in the duodenum. Additionally, Mx1 induction during the first viremic time point was much greater in the duodenum than in the blood indicating that both compartments responded differently to the viral challenges but remained relatively similar with respect to RF induction over time.*

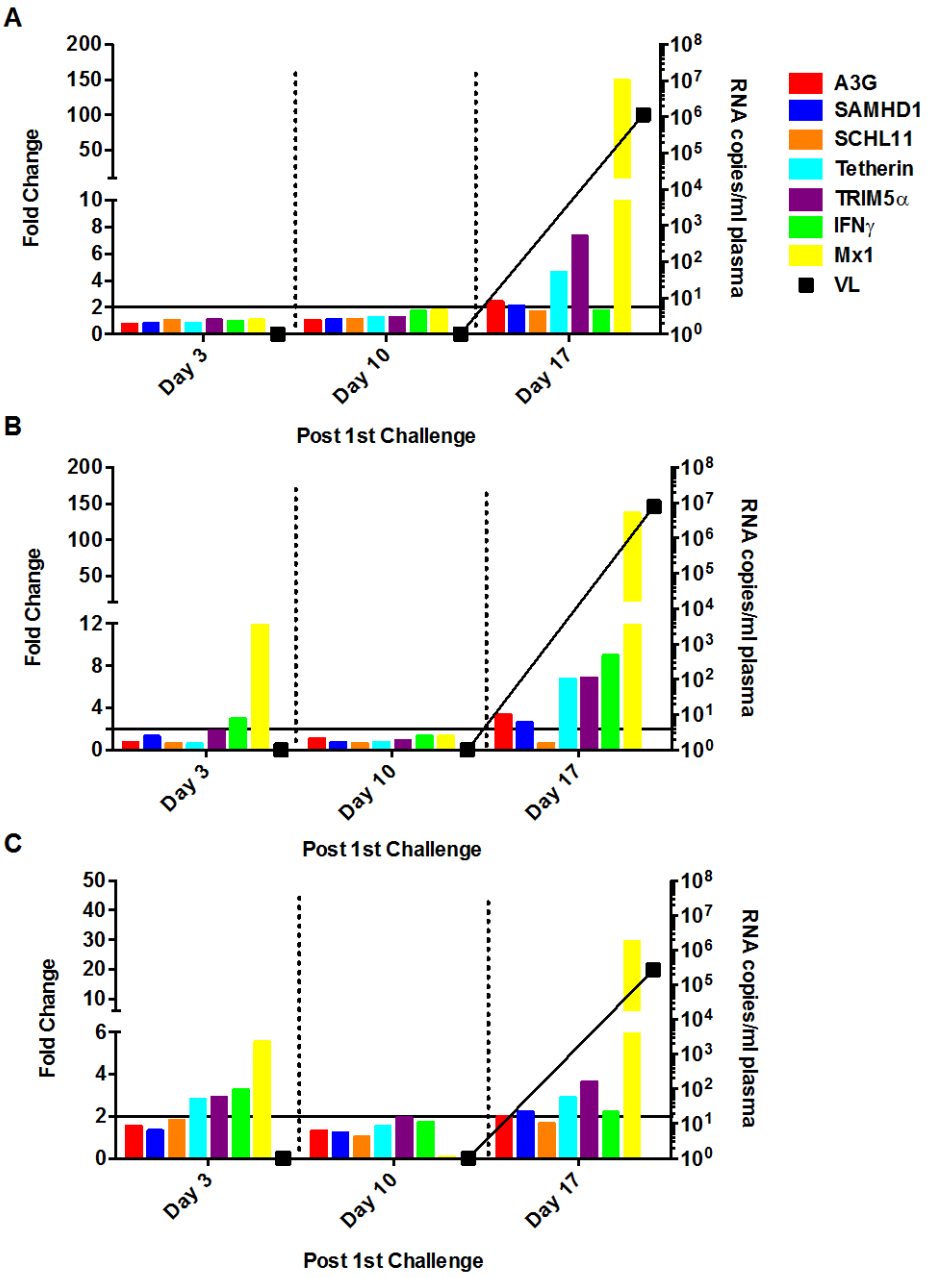



**Figure 21. Longitudinal analysis of ISG and IFNγ expression in the duodenum.**
The fold change in the relative expression of each gene is plotted for the duodenum of the susceptible animals (**A)** R700, **(B)** R701 **(C)** R702. Samples were obtained 4 days prior to the first exposure (basal levels). Each gene is depicted by a different colored bar as indicated in the figure legend; plasma virus loads are shown by the black line and the right Y-axis. Fold change was calculated by dividing the relative mRNA level at each time point in the duodenum over the basal level observed four days prior to the first challenge. The black solid line indicates the threshold for the observed induction to be true. Vertical dotted lines are for clarity and separate the RF expression for each day. Asterisks next to day numbers indicate days of rectal challenge.

## 4.8 IMPACT OF TRIM5 POLYMORPHISMS ON A HOST'S SUSCEPTIBILITY TO INFECTION AND DISEASE PROGRESSION

Studies suggest that repetitive low-dose rectal challenges may allow better manifestation of TRIM5α's restrictive ability due to the ability of specific TRIM5 polymorphisms in limiting productive infection [134]. Since TRIM5α is the first RF to respond to virus entry, it is plausible that low dose rectal challenges may block the few viruses that penetrate the mucosal barrier [134]. Polymorphisms in the TRIM5 SPRY domain that encode the binding region of the SIV capsid affect the strength of the TRIM5α-capsid interaction and therefore efficient restriction of virus replication [78,79,80]. As previously mentioned, three different allelic forms in the 339-341 region of the capsid exist: $TRIM5^{TFP}$, $TRIM5^{Q}$, and $TRIM^{CYPA}$. To determine whether these TRIM5 polymorphisms were associated with the observed differences in susceptibility to infection with SIV/DeltaB670 in the cohort, the TRIM5α genotype was determined for each animal (**Table 9 and Appendix C)**.

Six of eight macaques expressed the TFP/Q genotype, while R703 and R701 expressed the Q/Q and Q/CypA genotype, respectively. The G to T nucleotide substitution in intron 6 was observed for two of the TRIM5 clones from R701 indicating presence of the CypA allele (**Appendix C, panel B)**. Since the TFP allele is a dominantly restrictive allele, it is reasonable to assume that all macaques (except R701) would restrict virus replication and exhibit lower viral set points as previously shown for several SIV strains. However, a widespread variability was observed in the cohort's susceptibility to infection despite 6 out of 8 macaques encoding the TFP/Q genotype. When these TRIM5 genotypes were also compared to viral set point levels, no correlation was observed to suggest that animals with a restrictive allele have lower viral set points and therefore slower disease progression as others have seen [79]. *Therefore, despite these common alleles that would render the animals resistant to infection with other SIV isolates, the full spectrum of susceptibility to infection and disease was observed indicating that TRIM5 polymorphisms may not have a restrictive effect on SIV/DeltaB670 replication and host susceptibility to infection.*

**Table 9. Relationship between host genetics and susceptibility to infection and disease progression.**

| **Infection Phenotype** | **Animal #** | **Genotype** | | | ***Viral Set Point** |
|---|---|---|---|---|---|
| | | **TRIM5α** | **Mamu A** | **Mamu B** | |
| Susceptible | R700 | TFP/Q | A002a/A004 | B017a/B048 | 2.40E+05 |
| | R701 | Q/CYPA | A002a/A011 | B008/B055 | 6.00E+05 |
| | R702 | TFP/Q | A004/A011 | B002/B008 | 1.18E+04 |
| Intermediate | R703 | Q/Q | A004/A011 | B008/B028 | 3.95E+05 |
| | R704 | TFP/Q | A004/A007 | B002/B024a | 9.00E+02 |
| Resistant | R697 | TFP/Q | A002a/A051 | B017a/B017a | 1.30E+06 |
| | R698 | TFP/Q | A011/A011 | B012b/B043a | 5.35E+03 |
| | R705 | TFP/Q | A002a/A003 | B043b/B093 | 1.35E+02 |

* RNA copies/ml plasma

As such, MHC Class I genotyping was conducted for the eight animals to determine whether the Mamu A and Mamu B haplotypes contributed to the variability in animal disease progression. Two dominant alleles Mamu A*01 and Mamu B*017 are known to be associated with protection and delayed disease in macaques infected with SIVmac239/251 [135]. These specific alleles are critical for the induction of an effective T cell response to lower the viral burden. Genotyping results revealed the absence of the Mamu A*01 allele in the cohort but the presence of the Mamu B*017 allele in R700 and R697, a susceptible and resistant macaque, respectively. Although R697 was homozygous for the B017a allele, this animal had the highest viral set point at $1.3x10^6$ mRNA copies/ml plasma. Similarly, R700 carried the Mamu B*017A allele and had a high viral set point of $2.4x10^5$ mRNA copies/ml plasma. These results therefore revealed a lack of a relationship between the animals' Mamu A or B genotype and disease progression.

*Collectively, these data suggest the lack of an association between: 1) TRIM5 polymorphisms and susceptibility to infection with SIV/DeltaB670; and 2) Mamu A or Mamu B haplotype and disease progression. Although a larger rhesus macaque cohort will better solidify these findings, the data suggest that each SIV isolate has independently evolved with respect to TRIM5α restriction and that SIV/DeltaB670 did not appear to be affected by the MHC class I genotypes and TRIM5 polymorphisms, in contrast to what others have seen with other SIV isolates.*

## 4.9 EXAMINATION OF THE TRIM5A CAPSID BINDING REGION OF SIV/DELTAB670 AND DIVERSE SIV AND HIV-1 ISOLATES

The negative effect observed on SIV replication from the TRIM5 polymorphisms is only apparent with specific SIV strains as a result of variability in the TRIM5α-capsid binding sequence. Although only eight macaques were examined in this study, the apparent independence of infection and disease on the

host TRIM5 haplotype in SIV/DeltaB670 infection was puzzling, particularly since SIV/DeltaB670 originated from one of the first sooty mangabeys found to harbor SIV asymptomatically and since it is closely related to another well-described sooty mangabey isolate (SIVsmE660), whose infection and disease is highly influenced by TRIM5 polymorphisms [84,86,109,136,137].

To determine whether the apparent lack of association between TRIM5 haplotype and control of SIV/DeltaB670 replication was due to sequence differences in the TRIM5α binding region of the viral capsid, the inoculum used for the challenges was sequenced. As expected, this site was highly conserved and all clones had identical protein sequences. To understand how SIV/DeltaB670 compared to other SIV and HIV strains, a portion of the SIV/DeltaB670 capsid sequence was aligned to analogous sequences from multiple SIV, HIV-2, and HIV-1 sequences obtained from the Los Alamos National Laboratory database and the Kirmaier et al., study [12]. This is a relevant comparison because unlike the other RFs, no virally encoded antagonist exists for TRIM5α and thus each virus has escaped TRIM5α restriction by specific mutations in the TRIM5α capsid-binding region.

Using SIV/DeltaB670 as the reference sequence, a number of amino acid changes were noted in comparison to other SIV, HIV-2, and HIV-1 strains (**Figure 22**). With respect to the SIV strains, SIV/DeltaB670 encoded an 89-IPP-91 as opposed to the commonly seen 89-LPA-91 sequence, but still retained the R97 residue (**Figure 22A)**. SIVsmE041, SIVsmE660, and SIVsmE543 exhibited similar sequences to SIV/DeltaB670 differing only in one amino acid in the 89-91 region. The TRIM5α capsid-binding sequences of a sooty mangabey (PBJA) and a pig-tail macaque-specific SIV isolate (PT573) were identical to the SIV/DeltaB670 capsid sequence. Additionally, an SIV capsid sequence isolated from a chimpanzee in Cameroon had identical TRIM5α-binding sites as SIV/DeltaB670. The SIVmacs and SIVmne were unique among the other strains in the 89-QQ-91 and S97 residues causing evasion from TRIM5α restriction. Strikingly, SIV/DeltaB670, among other prominent SIV strains, displayed a TRIM5α-capsid binding sequence that was most similar to African HIV-1 subtype A isolates (**Figure 22B**). SIV/DeltaB670 differed from the other HIV-1 subtypes at residues 89 or 90. *These results emphasized that the TRIM5α capsid-binding region of SIV/DeltaB670 was unique among other well-*

*studied SIV strains and the most similar to African HIV-1 subtype A viruses further suggesting similarity in response to TRIM5 restriction. Such an association emphasizes the West African origin of this SIV/DeltaB670. Despite the presence of capsid residues known to allow TRIM5α restriction, variability in resistance to infection with SIV/DeltaB670 does not appear to be driven by the TRIM5 polymorphisms.*

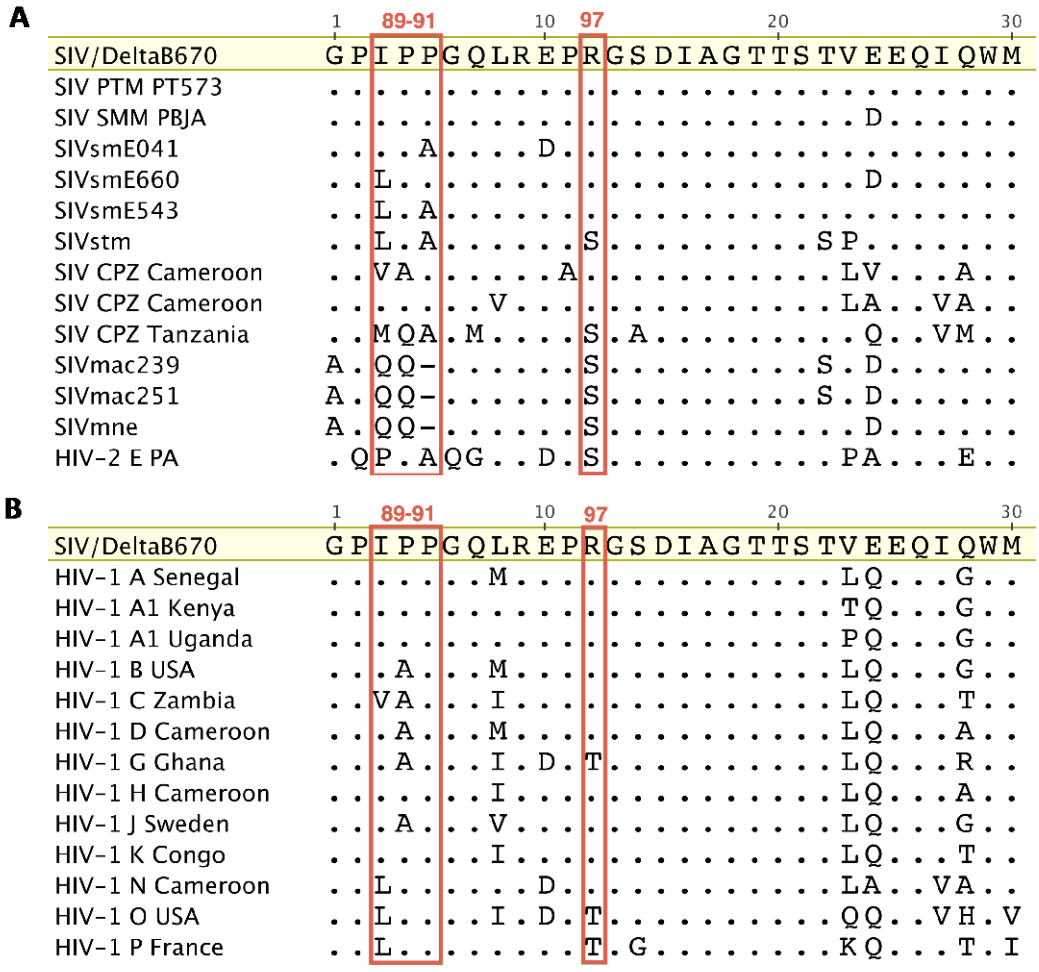



**Figure 22. SIV/DeltaB670 capsid alignment with SIV and HIV-1 strains.**
A portion of SIV/DeltaB670's capsid sequence was used as the reference sequence for alignment with various **(A)** SIV and **(B)** HIV-1 sequences. A sequence from each of the HIV-1 subtypes and its country of origin is shown. Red box indicates the TRIM5α binding region in the capsid. Red numbers identify the location of these two regions in SIVmac239. All sequences were obtained from the Los Alamos National Library Database and the Kirmaier et al. study [12]. Dots indicate residues identical to the reference sequence shown in yellow. GenBank accession numbers for each sequence are found in **Appendix D**.

## 4.10 GENETIC DIVERSITY OF THE VIRAL INOCULUM AND THE STRAINS CAUSING SYSTEMIC INFECTION

To further understand if the variability in infection observed in the cohort was due to selection of specific viral strains, envelope sequencing was performed on the inoculum and the viruses selected during early infection. The HIV/SIV capsid is a highly conserved gene but can mutate upon host pressure (ex: restriction by TRIM5α) [138,139]. In contrast, the Env gene, and the V1 region in particular, is hypervariable and known to be the defining region for the presence of specific viral strains in an inoculum [112]. Previous studies by our lab revealed that SIV/DeltaB670 is a quasispecies, harboring >10 viral variants/clones [112,140]. As such, the V1 region of the Env gene in the inoculum used for this study was sequenced to confirm the quasispecies nature of the inoculum. An inoculum that harbors more than one viral variant is important to fully recapitulate the genetic diversity of the HIV strains seen in humans. Sequence analysis revealed that the virus stock was indeed a quasispecies, harboring five different V1 clones (**Table 10, Appendix E)**.

To understand whether differences in susceptibility to infection observed in this study was influenced by the mucosal selection of a viral variant, viral sequences were analyzed from plasma samples during the acute viremic episode of each animal. In general, the susceptible animals selected for clones 2 or 3 (**Table 10)**. The intermediate animals selected for clones 2, 3, or 12. The resistant animal R697 selected clones 3 and 12 while R698 selected clone 12 only. The elite controller R705 only selected for clone 2 despite the sequencing of 23 viral clones. To further confirm the specific selection seen in R705, virus was sequenced from plasma 67 days later. Clone 2 was indeed solely selected for again in R705. *Together, these data indicate that the viral inoculum was indeed a quasispecies and that each animal exhibited variable selection in the viral strain that caused systemic infection. These findings indicate that no specific clone seems to be associated with the animal's susceptibility to infection. Further studies are warranted to understand the impact of these clones on the host immune response especially with respect to impact of clone 2 on the resistance of R705.*

**Table 10. V1 Env clone frequency in the cohort and challenge stock.**

| Infection Phenotype | Animal # | Clone 2 | Clone 3 | Clone 12 | Clone 14 | Clone 17 | Total |
|---|---|---|---|---|---|---|---|
| | **Virus Stock** | **17** | **2** | **2** | **1** | **4** | **26** |
| **Susceptible** | R700 | | 20 | | | | **20** |
| | R701 | | 13 | | | | **13** |
| | R702 | 13 | | | | | **13** |
| **Intermediate** | R703 | 16 | 2 | 2 | | | **20** |
| | R704 | | 10 | | | | **10** |
| **Resistant** | R697 | | 10 | 3 | | | **13** |
| | R698 | | | 16 | | | **16** |
| | R705 early | 23 | | | | | **23** |
| | R705 late | 17 | | | | | **17** |

# 5.0 DISCUSSION

RFs have been an intriguing area of lentiviral research for their potent ability to block virus replication. Specific adaptation of HIV/SIV to evade the restrictive function of RFs through virally encoded antagonists has contributed to their increased attention among other proteins [40]. The power of these RFs lies in their ability to be among the first weapons of defense against invading lentiviruses due to their constitutive expression in the immune cells affected by HIV/SIV. Additionally, the IFN-inducible nature of these RFs makes them an extremely powerful source of innate defense. The constitutive and IFN-inducible nature of these RFs makes them an integral component of the host intrinsic and innate defense system, respectively. The more the impact of these RFs is understood, the closer the field is to hindering this deadly virus from irreparable host damage.

Despite the current knowledge of RF expression in HIV/SIV infection, little is known about the potential role of basal expression of A3G, TRIM5α, tetherin, SAMHD1, and schlafen 11 in delaying systemic infection and whether their induction may impact the course of an infection. Overall, the data I presented has clarified the importance of host RF expression prior to SIV exposure and their ability to delay systemic infection. The first major observation of this study was that basal levels of A3G, TRIM5α, tetherin, and SAMHD1 in the blood significantly correlated with resistance to infection. Animals resistant to intrarectal repetitive low doses of SIV/DeltaB670 had significantly higher basal TRIM5α levels ($P=0.025$, Kruskal-Wallis with Dunn's multiple comparison test) compared to animals susceptible to infection. Second, analysis of RF induction and virus load over an extended time course revealed no relationship between RF induction and protection from infection, as seen with high virus

loads. Overall, my study has provided important insight into the role of these RFs in the blood, mucosa, and lymphoid tissue with the possibility of basal PBMC RF levels being used as a predictor for susceptibility to infection.

Three different forms of susceptibility to infection were observed in the cohort as a result of the repetitive low dose rectal challenges: susceptible, intermediately susceptible, and resistant. Most peculiar are the three resistant animals, especially the elite controller R705. The resistant animals, R697, 698, and R705, required 6-7 rectal challenges before systemic infection was detectable, while the rest of the cohort became infected after 2 or 3 challenges. Variability in infection has been seen in other studies involving repetitive low dose SIV exposure [83,84]. Many factors may explain this variability such as the lack of necessary target cells for virus expansion or a strong immune response [141]. Since the cohort underwent repetitive low dose rectal challenges, the resistant animals may have experienced a localized mucosal infection that was quickly overcome by either innate immune cells such as NK cells or macrophages, or the virus was eliminated by a T cell response in the mucosa induced by the early challenges. This was previously seen with rhesus macaques protected from SIV/DeltaB670 infection as a result of an SIV env-specific MHC-restricted CTL response induced in the lamina propria by a single low dose mucosal exposure [25].

The variability in susceptibility to infection was hypothesized to be due to higher basal RF levels. Examination of the basal mRNA levels of the RFs, Mx1, and IFNγ by RT-PCR revealed higher expression in the blood versus the gut. Such differences may rise from variability in the cell type population in each compartment. In general, all five RFs are expressed in hematopoietic cells and the population of these cells varies in the blood, ILNs, and the duodenum Approximately 40% of the total lymphocyte population resides in the lymph nodes whereas only 2% is found in the blood and 6% in the lamina propria of the gut [142]. Expression of A3G and SAMHD1 is particularly high in myeloid cells, whereas tetherin is constitutively expressed in mature B cells, plasma cells and pDCs [30,52,92,102].

Studies report that SLFN gene expression can also vary during a cell's response to infection and during macrophage and T-cell development [143]. Such observations may therefore account for the differences in basal levels seen in the three compartments. Tissue-specific variability may also result from differences in basal IFNα expression. Basal type I interferon expression can vary in different cell types and lymphoid tissues with higher expression seen in CD4+ T cells than in CD8+ T cells [144,145]. These discrepancies can be confirmed in future studies by single cell analysis to allow for a more accurate quantification of RF mRNA levels in various immune cell subsets that express these RFs.

Examination of basal RF expression in the three compartments revealed a positive correlation between basal RF levels and resistance to mucosal infection, indicating the potential impact of RFs in delaying systemic infection. Animals resistant to infection with SIV/DeltaB670 exhibited significantly higher basal levels of TRIM5α and Mx1 in the blood compared to susceptible animals. Similar trends were also seen with SAMHD1, tetherin and A3G in the blood. These findings are consistent with another study where higher basal huA3G levels in PBMC correlate with a decreased susceptibility to *in vitro* infection with HIV-1 [115]. Similarly, another group propose a positive correlation between basal human A3G expression and resistance to disease progression. Jin et al. show that highest A3G expression is found in long-term nonprogressors>HIV-uninfected>progressors [117]. They further hypothesize that basal A3G mRNA levels in PBMC is a constant that is determined by host genetics and individuals more resistant to disease progression will have higher basal A3G levels [117,146]. It is intuitive to believe that higher basal RF expression would render an animal more resistant to infection; however, I did not expect this trend to be more prominent in the blood versus the lymphoid and mucosal compartments, especially since virus predominately replicates in the latter two tissues due to higher target cell levels. The observed trend may be more prominent in the blood than in the ILNs or duodenum as a result of differences in the cell population as previously stated. Similarly, since the LNs and gut experience the greatest virus replication, basal RF levels may have no protective effect in these compartments due to saturation by virus that may hinder cellular activities including RF mRNA

transcription. In general, higher basal RF levels in the blood may serve to delay systemic infection during the second “window of opportunity” when virus is disseminating to lymphoid tissue and establishing a reservoir.

It is interesting that basal TRIM5α and Mx1 expression exhibited the strongest positive correlation with resistance to infection. Closer scrutiny of the data revealed that TRIM5α’s behavior in the blood and ILNs of the three groups mimicked the behavior seen for Mx1 expression. This suggests that, among the other RFs, TRIM5α may respond in a similar and early fashion as Mx1 after virus exposure and IFNα induction. The similar expression profile of Mx1 (a common ISG) and TRIM5α confirmed that the differences seen among the groups are not an artifact and are in fact a novel observation worthy of attention. These observations may be useful in future macaque studies allowing the use of basal TRIM5α levels in the blood as a screening tool to predict host susceptibility to repetitive low dose rectal challenges for a more accurate randomization of study groups.

In contrast to the correlation between basal RF levels and resistance to infection, no correlation was observed between basal RF expression in the PBMC/ILNs and resistance to disease progression. However, there was a significant negative correlation between basal TRIM5α levels in the duodenum and viral set point. In the blood, higher basal TRIM5α expression contributed to resistance to infection; and in the duodenum, higher basal TRIM5α expression contributed to a lower viral set point and slower disease progression. It is unclear why this effect on disease progression was only observed in the duodenum. One possible explanation is since the majority of HIV/SIV replication during chronic infection occurs in the gut, any form of protection in the gut (ex: higher basal RF expression) is likely to aid the host by limiting virus replication and dissemination. Additionally, since the gut includes a large number of HIV/SIV target cells [147], higher basal levels can inhibit further expansion of virus replication in the gut to allow for a more potent adaptive immune response to take place. The persistently negative correlation observed only in the blood may indicate RF levels in the blood operate as a potent barrier for the establishment of infection and disease.

After observing a correlation between basal RF levels and resistance to infection and disease, it was important to understand whether early RF induction had an impact on virus replication in the mucosa and therefore control of systemic infection. A coordinate but transient induction of the RFs and Mx1 was observed in the susceptible and intermediate animals only during viremia. The similarity in the expression profile of Mx1 and the RFs, which are also ISGs, further verifies the integrity of my results. My findings are consistent with another study observing a transient 2-3 fold induction of A3G in rhesus PBMC within 2 weeks of infection that corresponded with the 30 fold induction of Mx1 [121]. Transient RF induction may reflect the transient nature of the IFNα response during a viral infection [132]. RFs work in a synergistic fashion with type I interferons. Since IFN expression is transient and primarily effective during the initial stage of infection, it is understandable that the RFs were induced transiently during viremia [27]. Transient expression may also reflect cellular damage to the host cell by the incoming virus, which may disable cellular antiviral defense by the upregulation of these RFs through type I IFNs.

Although all five RFs are ISGs, the pattern of induction of Mx1 and the RFs differed in the animals. Mx1, TRIM5α, and tetherin were selectively induced in tandem. Selective induction of Mx1 among the other ISGs, may be due to the nonspecific induction of Mx1 during an infection by IFNβ prior to RF induction by IFNα as shown in figure 4. Mx1 induction was particularly high for the susceptible and intermediate animals (8-40 fold) while resistant animals only exhibited a 4-16 fold increase. These findings are in agreement with another study observing persistently higher Mx1 induction in progressor macaques while little to no Mx1 induction was observed in the nonprogressor [132]. Previous studies show that Mx mRNA levels are a reliable indicator of ongoing virus replication, where higher Mx levels in chronically-infected macaques strongly correlate with increased VL [132]. The persistent Mx1 induction in R697 and the corresponding high VL may confirm earlier studies that show persistent IFNα production causing a heightened level of immune activation and T cell apoptosis thereby facilitating further virus replication and disease progression [34].

The transient, selective induction of Mx1 during the early failed challenges in R705 distinguished this elite controller from the rest of the cohort. The 4-5 fold increase in Mx1 levels confirms R705 was exposed to the virus during the first or second challenge but was able to limit productive replication because it expressed significantly higher basal Mx1 levels than the susceptible animals. Indeed, type I interferon and Mx induction can occur through the presence of viral proteins alone without virus replication [132,144]. Since Mx1 was not induced once infection was established in the elite controller, this lack of Mx1 induction may indicate protection as others have seen [148]. For example, the nonpathogenic AGM model of SIV is known to show a decrease in Mx1 response during the chronic stage of infection [132]. Specifically, AGMs and SMs have the unique ability to control immune activation by rapidly downregulating acute phase type I IFN during the transition to chronic infection [148]. This feature is what differentiates them from the rhesus macaque pathogenic model.

TRIM5α and tetherin were the most highly induced RFs in the PBMC of the susceptible animals during initial viremia. Contrary to my hypothesis, RFs were not induced in the resistant animals, despite repeated viral challenges, until they became viremic. These findings are consistent with a previous study showing higher A3G, TRIM5α, and tetherin mRNA levels in HIV-1 infected untreated patients (UT) than patients on antiretroviral therapy [119]. Variability in the selective induction of these RFs during initial viremia in the susceptible animals could reflect differences in T cell activation. This may be true since in HIV-1 infected untreated patients, T cell activation positively correlates with TRIM5α and tetherin expression [119]. Additionally, selective RF induction may be due to stimulation by cofactors other than IFNα [39]. For example, tetherin can be induced through type I interferon-independent pathways through TLR 8 in myeloid cells [91].

It is known that HIV exposure can trigger A3G induction in PBMC even without signs of infection [116]. However, induction was not observed beyond the 2-fold threshold in the resistant macaques until they became viremic from the $6^{th}$ or $7^{th}$ challenge. More importantly, there was a lack of

RF induction in the elite controller which is in agreement with others who have shown no difference in A3G, TRIM5α, or tetherin expression in HESN and healthy controllers [119]. One group proposes that early PBMC type I IFN responses (example: RFs) do not control virus replication nor result in a nonprogressor monkey [132]. This is supportive of our findings because early RF induction in PBMC was associated with viremia and not protection.

A possible explanation for the lack of RF induction in the resistant macaques can be due to lack of virus that successfully penetrated the epithelial layer to induce the innate immune response. This may be especially true since RF induction only occurred during viremia. Another explanation is there may be a difference in RF function before and after infection. Essentially, higher **basal** RF levels in the blood can have a potent effect on resistance to infection by arming the host with necessary defense proteins. However, it may be "too late" for RF **induction** to play a role in the blood after infection if a host has lower basal levels to begin with. This may be especially true since at the point of virus detection in the blood, virus is establishing lymphoid reservoirs [20].

Collectively, these data suggest that RF induction does not correlate with protection, especially since susceptible animals showed the greatest RF induction [33]. Despite the 2-7 fold induction of the RFs and the 30-50 fold induction of Mx1, virus levels remained high. My observation is in agreement with another study observing higher tetherin levels directly associated with VLs and disease progression [119]. Further corroborating this hypothesis is the high VL of R697 in addition to the 8-fold induction of tetherin during chronic infection. Contrary to other reports where higher PBMC A3G levels in HIV-1 exposed seronegative individuals inversely correlated with disease progression, I noticed a lack of A3G induction among the three resistant animals to indicate they are protective [115,116]. Similarly, no inverse relationship between RF expression and virus load was observed as others have seen with rhA3G [121].

The lack of an association between induction and resistance to infection was also confirmed by examining the maximum RF levels achieved for each animal. Maximum levels attained for the animals in the three groups were highly similar indicating that the overall expression of RFs post-exposure had no impact on an animal's resistance to infection. RFs may become passive to repeated challenge, reaching a threshold of induction whereby they could no longer control virus replication and contribute to protection. Studies in HIV-1 infected humans show that with increasing VL, ISGs involved in antiretroviral defense also increase [27]. When antiretroviral therapy is given to these individuals with high levels of HIV-1, the ISG profile returned to levels similar to uninfected individuals indicating that the lack of ISG induction in macaques, especially in R705, may be causing protection. Since transient IFN expression is known to limit the first rounds of replication and since IFNα has a finite time to effectively prevent productive HIV replication as previously seen in macrophages, RF induction during the first 24-48 hours after exposure may be more informative to study and allow for a better understanding for the possible protective effect of these RFs [27,61].

Since RF induction was not observed after the first 5 exposures in the resistant animals, this raised the question of what was causing these animals to remain resistant. One of the common parameters for determination of resistance to HIV disease has been analysis of host genetic factors (HLA Class I alleles, CCR5 polymorphisms, and chemokine copy numbers) [149]. Many studies have shown an animal's MHC Class I genotype capable of affecting VL and disease progression. Specifically, alleles A*01 and B*17 are known to cause protection for SIVmac239/251-infected macaques [135]. I therefore determined the MHC Class I Mamu haplotypes of the cohort and found no association between the MHC class I Mamu haplotypes and virus loads and disease progression. This supports previous findings from our lab. Specifically, the protective Mamu A*01 allele was shown to have no impact on SIV/DeltaB670 replication [150]. My study, however, has added the lack of an impact for the Mamu B*17 allele as well. Therefore, other unknown factors may play a role in the persistently low viremia detected in R705.

Since the rectum and gut are exposed to virus prior to the blood, it was important to understand how RF expression varied in these compartments. Studies have shown SIV can be detected in the digestive mucosa within four hours of intrarectally-infected rhesus macaques [151]. RF expression was therefore examined in the duodenum and rectum of the elite controller and compared to RF expression in the blood. I hypothesized that RF expression would vary in the blood versus the mucosa with higher expression in the mucosa since that is the primary portal of entry for HIV. Examination of the RFs in the three tissues revealed similar expression levels. The slight variability can simply be due to immune signaling. After virus enters the mucosa, the innate immune response is triggered. At that time the rectum may have low basal levels of these RFs and becomes overwhelmed by the entering virus and unable to upregulate RF expression. However, the immune system has been alerted which may allow for upregulation of these RFs in the blood before the virus enters the periphery. Only rectal biopsies on day 38 for R705 were available and while that might have not been an optimal time point, the data suggest that future analysis of RF expression changes in the mucosa can be more easily monitored in the blood due to similarity in expression among the three tissues.

The coordinate expression of RFs in the blood in response to infection was further verified by longitudinal analysis of RF expression in the duodenum. RF induction was observed in the duodenum of animals susceptible to infection, especially during initial viremia. Of particular interest is the 5-10 fold induction of Mx1 on day 3 in the duodenum of R701 and R702, which was not observed in the blood. This induction confirms exposure in the mucosal compartment allowing a rapid response from the innate immune cells in the duodenum than the blood. However, this Mx1 induction did not appear to protect the host, as these two animals became systemically infected 11 days later. Therefore, selective induction of Mx1 alone is insufficient in protecting a host from subsequent mucosal exposure if they are intrinsically more susceptible to infection. While the duodenum and blood appeared to exhibit similar ISG expression in the susceptible animals, Mx1 was the exception as seen by earlier induction in the duodenum and a stronger induction by day 17. This observation is supported by another group that found

higher Mx levels in lymphoid tissues than in the PBMC of acutely infected monkeys [132]. Higher Mx1 induction in the duodenum can also be due to persistent virus replication since mucosal mononuclear cells experience a greater viral burden than PBMC [147].

Genetic polymorphisms in TRIM5 have also been shown to play a role in the susceptibility to infection and disease with some SIV isolates and not others. Previous studies have established that macaques with restrictive TRIM5 alleles (TFP or CypA) are more resistant to SIVsmE660 and SIVsmE543 infection than macaques expressing the susceptible (Q) allele. These polymorphisms inhibit virus replication regardless of the route of exposure but are dependent on the SIV strain used. However, I did not identify a relationship between the TRIM5 polymorphisms in the SPRY domain and susceptibility to infection with SIV/DeltaB670. This implied other cofactors imparting a role on host susceptibility that may or may not work in concert with the TRIM5 polymorphisms. Previous studies have established that animals expressing restrictive TRIM5 alleles require more rectal/penile exposures to become infected with SIVsmE660 [84,86]. Specifically, animals with the $TRIM5^{TFP/CYPA}$ genotype require more than 10 challenges to become systemically infected. However, I observed a widespread variability in animal susceptibility to infection, which did not seem to relate with the TRIM5 polymorphisms as others have seen with SIVsmE660 and SIVsmE543. Therefore, despite the origin similarity of SIVsmE660 and SIV/DeltaB670, the latter virus did not appear to be affected by the TRIM5 polymorphisms as the former virus.

The primary difference in variable TRIM5 restriction lies in the capsid sequence of the TRIM5α binding site. SIV/DeltaB670 appears to have behaved differently from other SIV strains in that it was not affected by the TRIM5 polymorphisms, but was susceptible to restriction. By analyzing the TRIM5α binding site in the SIV/DeltaB670 capsid, I was able to determine that SIV/DeltaB670 may be resistant to TRIM5 polymorphisms due to the genetic sequence of the capsid. The 89-91 and 97 amino acid regions in the TRIM5α binding site of SIV/DeltaB670 encoded a unique sequence not seen in any

other SIV strain with the exception of the SMM PBJA and PTM PT573 isolates. The similarity of SIV/DeltaB670 to the latter two isolates may indicate that all three respond to TRIM5 restriction in a similar fashion. Strikingly, the SIV/DeltaB670 TRIM5α binding site was most similar to several African HIV-1 subtype A isolates which confirms that SIV/DeltaB670 may have originated from West Africa. This similarly may further suggest that SIV/DeltaB670 is a virus more representative of HIV-1 than other SIV strains with respect to TRIM5 restriction. Further analysis of how TRIM5 polymorphisms may impact rhesus macaque disease progression will be immensely beneficial in creating a more HIV-1 tropic rhesus macaque model and perhaps pave the way for effective gene therapy against HIV-1 [152].

In this report, I recapitulated the intrinsic role of RFs in defending the host from SIV infection. As stated earlier, RFs are specifically part of the intrinsic immune system in that they are constitutively expressed without aid from the host immune system [44]. RFs may serve as the initial trigger for stimulation of innate and adaptive immunity in an infected host. My report further supports this definition through the discovery of basal levels of TRIM5α potently delaying susceptibility to infection. While the size of the cohort is a limitation in this study, statistically significant data was obtained with clear differences among the groups (susceptible, intermediate, and resistant). Additionally, there was a notable variability in susceptibility to infection with the cohort that truly allowed me to validate the conclusions made.

Taken together, this study provided several novel observations that have the potential to significantly impact the field: 1) higher basal RF levels may serve as a predictor for susceptibility to infection; 2) early and late RF induction does not appear to protect from infection but may impact disease progression; 3) TRIM5 polymorphisms do not appear to impact SIV/DeltaB670 replication kinetics like they do for other SIV isolates; 4) SIV/DeltaB670's TRIM5α capsid binding site is unique and most similar to African HIV-1 isolates and may thus serve as a better strain for HIV macaque studies; and 5) blood may serve as a window for understanding RF behavior in the mucosa.

## 5.1 PUBLIC HEALTH IMPLICATIONS

Identification of the potential protective impact of basal RF levels on a host's susceptibility to infection and disease progression is a novel observation that may significantly impact the field. My study has shown higher basal RF levels can contribute to delayed mucosal infection which can be further translated in other macaque and human studies to allow for screening of blood samples for RFs as a predictor of susceptibility. The ability of basal mRNA levels of TRIM5α to predict susceptibility to infection and disease progression can have a significant impact on the field. These data suggest that future clinical studies that immunize macaques with an adjuvant to upregulate RF expression (especially TRIM5α) may allow prevention of infection or delayed disease progression. Similarly, perhaps therapeutics or microbicides in the form of RF gene therapy can allow a host to eliminate the virus early after exposure. Upregulation of basal RF expression in the form of microbicides will be especially beneficial in the mucosa (rectum or vaginal tract) to effectively block virus dissemination. The observation that RF induction in the blood mimics that seen in the duodenum also has a significant impact on the field by allowing scientists to easily assess and obtain macaque blood samples to evaluate the changes seen in the gut after mucosal exposure.

Thus, in order to fully harness the potency of these RFs in the context of HIV infection in humans, scientists must explore different avenues to enhance RF function or block antagonist function. Several groups have already experimented with various methodologies to maximize the potency of RFs in humans and nonhuman primates. First, small molecular inhibitors of viral antagonists can be designed to specifically allow RFs to practice their function unhindered [51]. This has already been performed against HIV-1 Vif and results reveal reduced viral infectivity and higher cytidine deamination of the viral genome [153]. Therefore, by specifically eliminating these antagonists through the use of drugs or siRNA, RFs will be more functional. Second, the inhibitory function of RFs can be coupled with vaccines to maximize the antiviral effect by usage of adjuvants to specifically upregulate RF expression [154]. One study observed that the TRIM5 haplotype coupled with vaccine-induced immune response may have a potent

protective impact on a host [85]. Finally and perhaps most importantly, RFs can be utilized as a form of gene therapy. For example, a form of gene therapy using huTRIM5α that carries the P332R mutation known to allow for HIV-1 restriction may contribute to a better block of HIV-1 in humans [26].

In essence, identifying the role of these RFs in virus inhibition may identify possible mechanisms to optimize their interplay with the innate immune response to further aid in the design of drugs/microbicides that could target virus prior to dissemination. By implementing these methods, humans may maximize the natural potency of these RFs and enable them to curtail virus replication at the onset of transmission.

## 5.2 FUTURE DIRECTIONS

While this work has provided valuable insight into the role of RFs during repetitive, low dose infections, more work is warranted to fully understand their cellular function. First, and of high importance, is single cell analysis of RF expression in various cell types to determine whether RFs are specifically induced in immune cell and whether the cell that is constitutively expressing the RF is the same one responsible for induction. By understanding the basal mRNA and protein levels of each RF in hematopoietic cells, powerful conclusions can be made regarding the specific immune cells responsible for protection after exposure. Second, the observation that resistant animals have significantly higher levels of TRIM5α will be further validated by screening rhesus macaque blood samples for basal levels of TRIM5α to predict an animal's susceptibility to infection with repetitive low dose rectal challenges. Third, since RFs are early responders to virus invasion, perhaps examining induction within hours from infectious exposure in other mucosal and lymphoid tissues including the jejunum and mesenteric lymph nodes would confirm our observation that the blood may in fact be a more valid source for understanding host susceptibility to infection. Fourth, immunological studies are necessary to understand the nature of the adaptive immune

response in all animals, especially in the elite controller. Fifth, siRNA knockdown studies examining the individual potency of each RF in infected human and rhesus cells may elucidate the power of a single RF or the necessary cooperative interaction among them. Future studies examining the ability of animals shown to be resistant to SIV infection *in vivo* to also resist infection *in vitro* will further confirm my findings [155].

# APPENDIX A

## DERIVATION OF SIV CHALLENGE STOCKS USED IN NONHUMAN PRIMATE STUDIES

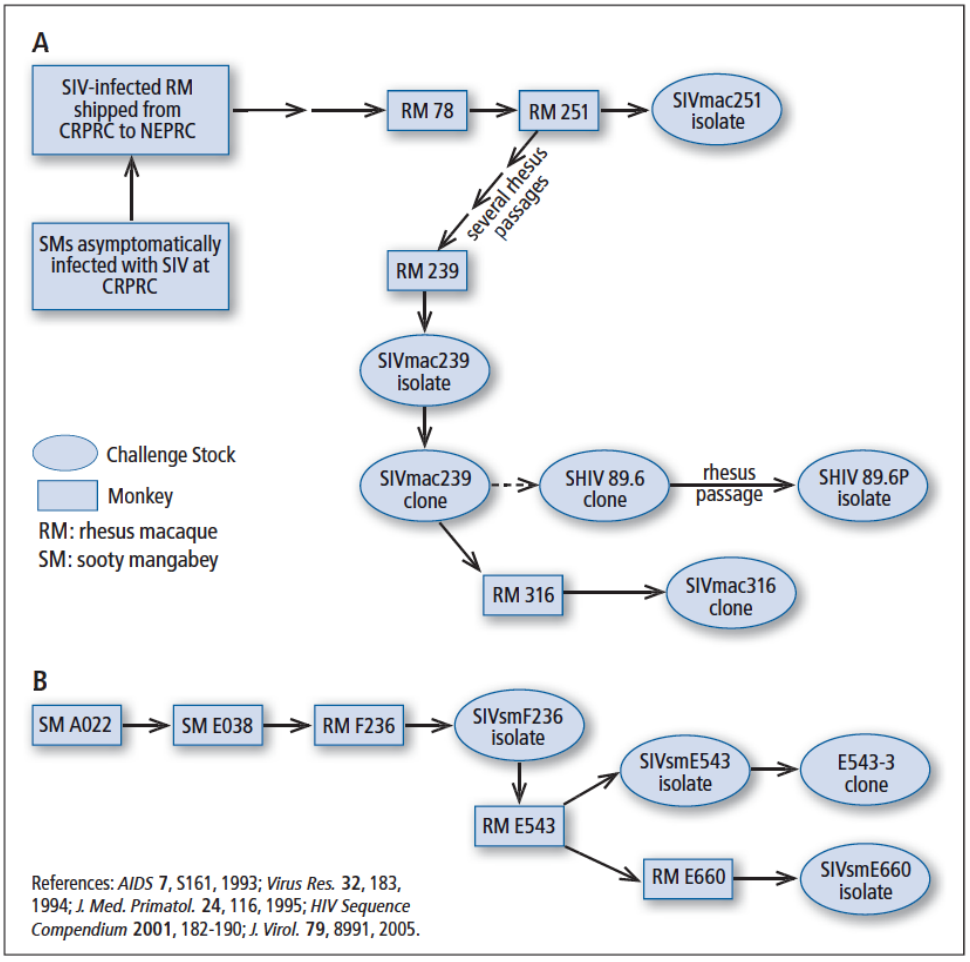
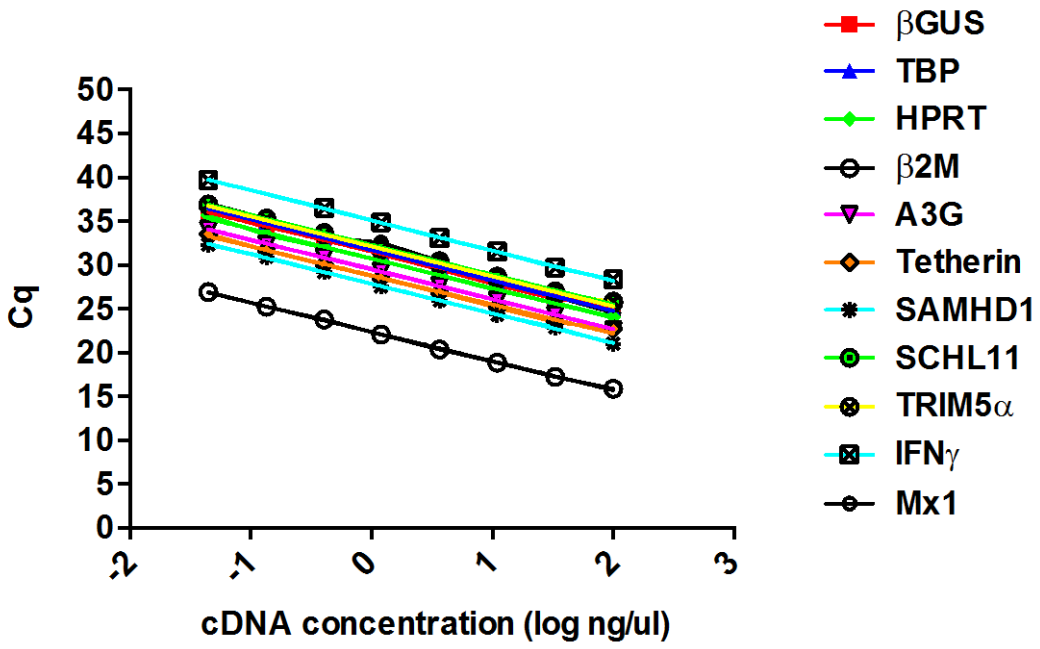



Figure adapted from [156].

## APPENDIX B

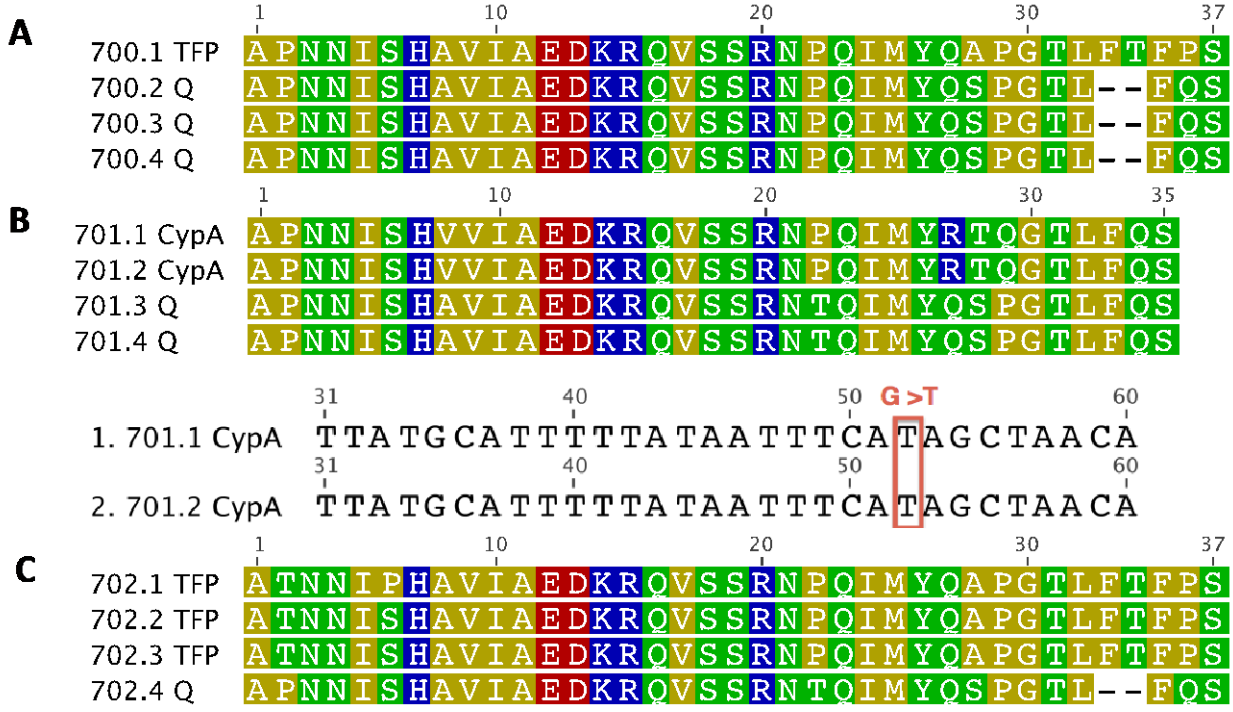
### LINEARITY AND EFFICIENCY OF THE TAQMAN ASSAYS

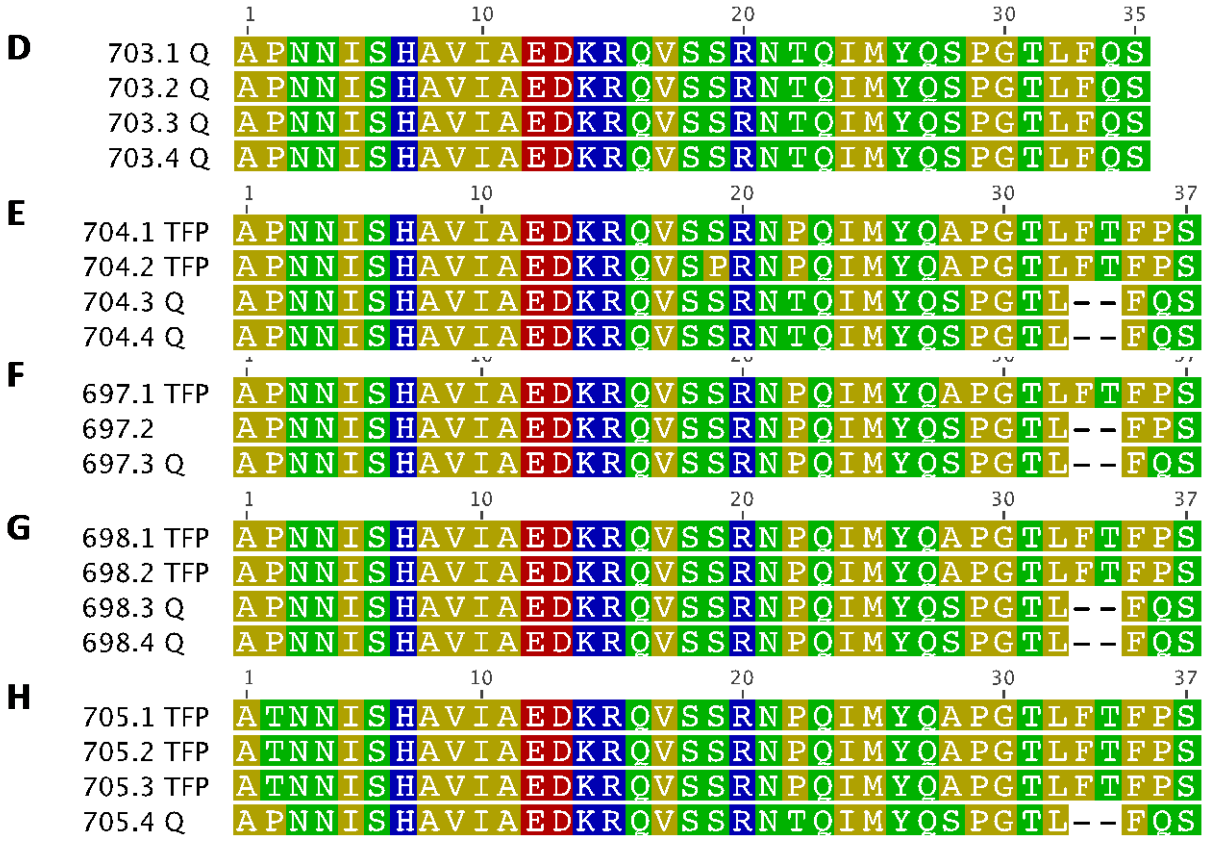



PBMC cDNA was serially diluted and amplified using each Taqman primer/probe set and the corresponding Cq (quantification cycle) was plotted at each cDNA concentration. Linear regression analysis was performed to evaluate assay linearity ($R^2$) and efficiency ($1\text{-}10^{-1/slope}$). All lines had $R^2$ values >0.99 indicating linearity. Slopes were between -3.3 and -3.6 and therefore efficiency was >90%.

## APPENDIX C

### SEQUENCE ALIGNMENTS OF THE TRIM5A B30.2/SPRY DOMAIN






A portion of the TRIM5α B30.2 domain was PCR amplified to determine the polymorphisms present in region 339-341. Four clones were chosen from each animal and sequences were aligned **(A)** R700 **(B)** R701 **(C)** R702 **(D)** R703 **(E)** R704 **(F)** R697 **(G)** R698 **(H)** R705. Noted next to each animal clone number is the polymorphism present. The TFP polymorphism corresponding to region 339-341 is found in region 34-36 above and the Q polymorphism (characterized by a deletion of residues 338 and 339) is found in residue 34 or 36. The residue numbers in the alignments do not correspond with the residue location in the TRIM5 gene. Below the TRIM5 sequence alignment for R701 (B) is a portion of the nucleotide region in TRIM5 for two clones showing the G to T nucleotide substitution indicating presence of the CypA allele.

## APPENDIX D

### ACCESSION NUMBERS FOR THE SIV AND HIV SEQUENCES

**A**

| Sequence Name | Genbank Accession # |
|---|---|
| SIV PTM PT573 | AY221515 |
| SIV SMM PBJA | M31325 |
| SIVsmE041 | Kirmaier et al. |
| SIVsmE660 | JQ864087.1 |
| SIVsmE543 | U72748.2 |
| SIVstm | Kirmaier et al. |
| SIV CPZ Cameroon | AF115393 |
| SIV CPZ Cameroon | JN835460 |
| SIV CPZ Tanzania | DQ374657 |
| SIVmac239 | AY588946.1 |
| SIVmac251 | M74931.1 |
| SIVmne | Kirmaier et al. |
| HIV-2 E PA | Kirmaier et al. |

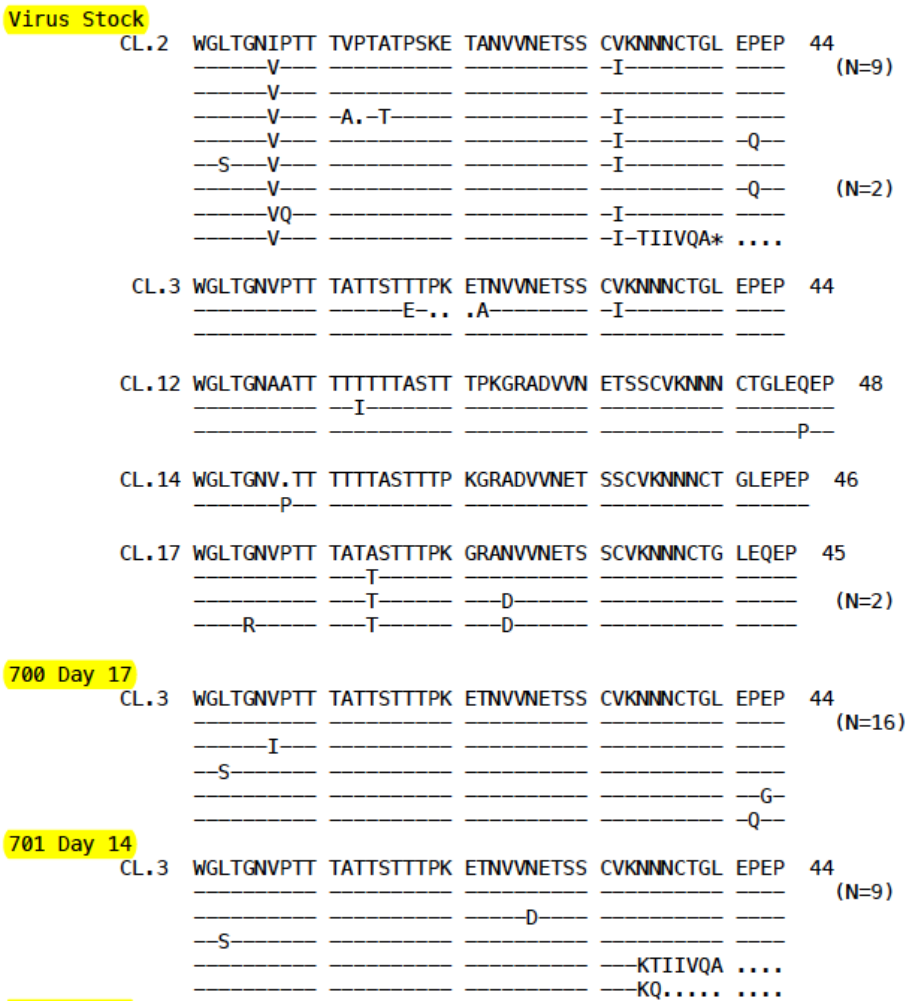
**B**

| Sequence Name | Genbank Accession # |
|---|---|
| HIV-1 A Senegal | AJ274582 |
| HIV-1 A1 Kenya | GQ429844 |
| HIV-1 A1 Uganda | AY803406 |
| HIV-1 B USA | AB078005 |
| HIV-1 C Zambia | AB254141 |
| HIV-1 D Cameroon | AJ555084 |
| HIV-1 G Ghana | AB231893 |
| HIV-1 H Cameroon | EU619439 |
| HIV-1 J Sweden | AF082394 |
| HIV-1 K Congo | AJ249235 |
| HIV-1 N Cameroon | AJ006022 |
| HIV-1 O USA | AB485668 |
| HIV-1 P France | GQ328744 |

All accession numbers were obtained from GenBank and Kirmaier et al. [12].

# APPENDIX E

## V1 ENV SEQUENCE ALIGNMENTS OF THE INOCULUM AND THE STRAINS SELECTED IN EACH ANIMAL DURING INITIAL VIREMIA



```
702 Day 17

        CL.2  WGLTGNIPTT TVPTATPSKE TANVVNETSS CVKNNNCTGL EPEP  44
              ------V--- ---------- ---------- -I-------- ----    (N=10)
              ------V--- ---------- ---------- RI-------- ----
              ------V--- ---------- ------K--- -I-------- ----
              ------V--- ---------- ---------- -I-------- Q---
703 Day 24
        CL.2  WGLTGNIPTT TVPTATPSKE TANVVNETSS CVKNNNCTGL EPEP  44
              ------V--- ---------- ---------- ---------- -Q--    (N=12)
              ------V--- ---------- ---------- ---------- -QV-
              ------V--- ---------- ---------- ---T------ -Q--
              ------A--- ---------- --I------- ---------- -Q--
              ------V--- ---------- -----D---- ---------- -Q--

        CL.3  WGLTGNVPTT TATTSTTTPK ETNVVNETSS CVKNNNCTGL EPEP  44
              ---------- -------A-- ---------- ---------- ----
              ---------- ---------- ---------- ---------- ----

        CL.12 WGLTGNAATT TTTTTTASTT TPKGRADVVN ETSSCVKNNN CTGLEQEP  48
              ---------- ---------- ---------- ---------- --------(N=2)

704 Day 24
         CL.3 WGLTGNVPTT TATTSTTTPK ETNVVNETSS CVKNNNCTGL EPEP  44
              ---------- ---------- ---------- ---------- ----    (N=10)


697 Day 45
        CL.3  WGLTGNVPTT TATTSTTTPK ETNVVNETSS CVKNNNCTGL EPEP  44
              ---------- ---------- ---------- ---------- ----    (N=7)
              ---------- ---------- ---------- ---------- -Q--    (N=3)

        CL.12 WGLTGNAATT TTTTTTASTT TPKGRADVVN ETSSCVKNNN CTGLEQEP  48
              ---------- ---------A- ------N--- ---------- --------
              ---------- ---------- ------N--- ---------- --------
              ----------. .-------S- ------N--- ---------- --------
698 Day 45
        CL.12 WGLTGNAATT TTTTTTASTT TPKGRADVVN ETSSCVKNNN CTGLEQEP  48
              ---------- ---------- ---------- ---------- --------(N=11)
              ---------- ---------- ---------- ---------- -----*--
              ---------- ---------- -----G---- ---------- --------
              ---------- ---------- ---------- -------KQ. ........(N=3)

705 Day 57
         CL.2 WGLTGNIPTT TVPTATPSKE TANVVNETSS CVKNNNCTGL EPEP  44
              ------V--- ---------- ---------- ---------- -Q--    (N=16)
              ------V--- ---------- ---------- --R------- -Q--
              ------V--- ---------- ---------- -I-------- ----
              ------V--- ---------- -----D---- ---------- -Q--
              *-----V--- ---------- ---------- ---------- -Q--    (N=2)
              ------V--- ---------- ---------- ---KQ..... ….     (N=2)

705 Day 124
         CL.2 WGLTGNIPTT TVPTATPSKE TANVVNETSS CVKNNNCTGL EPEP  44
              ------V--- ---------- ---------- -I-------- ----    (N=9)
              ------V--- ---------- ---I------ -I-------- ----    (N=3)
              ------V--- ---------- ---------- ---------- -Q--
              ------V--- ---------- ---------- -I-KQ..... ....    (N=4)
```

A portion of the V1 Env protein sequence is shown. Viral cDNA from animal plasma samples during acute viremia were sequenced and aligned using SeqPublish (Los Alamos). Clone reference sequences were obtained from GenBank and Amedee et al [112]. Highlighted in yellow are the animal numbers and the time point of the isolated cDNA. Parentheses in the far right indicate the number of clones sequenced with identical sequences. Dashes indicate residues identical to the reference sequence. Dots indicate incomplete sequences. The following are the accession numbers for the reference sequences: clone 2: AY118205.1, clone 3: AY118204.1, clone 12: AY118213.1, clone 14: AY118216.1, clone 17: AY118217.1

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