

**SIV ESCAPE FROM IMMUNE RECOGNITION DUE TO SELECTION PRESSURE OF
IMMUNODOMINANT CD8+ EPITOPE GAG-CM9 AND TRIM5 RESTRICTION SITES**

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Kimberly Griffin, M.S.

University of Pittsburgh, 2013

ABSTRACT

HIV/AIDS has been a major public health focus for more than 40 years. By using the rhesus macaque/SIV model, we are able to learn more about immune response and host genetics than through human study alone.

There is a complex interplay between host and viral factors that determine the course of an HIV/SIV infection. The overall goal of this retrospective study is to analyze the dual evolution of both the immunodominant CD8+ GAG-CM9 epitope and the binding sites for restriction by the host protein TRIM5 α that are located on the highly conserved GAG region of the SIV genome. Early and late time points in an SIV infection will be analyzed to determine the favored drivers for viral escape.

In Specific Aim 1, a cohort of 15 Mamu A*01 rhesus macaques was characterized by viral load profile and TRIM5 haplotype. Six animals were “Controllers,” 5 animals maintained and “Intermediate Viral Load,” 3 animals maintained a “High Viral Load,” and 2 animals “Crashed and Burned.” Six animals had the TRIM5^{TFP/TFP} haplotype, 7 animals were classified as TRIM5^{TFP/Q}, and 2 animals were classified as TRIM5^{Q/Q}. No TRIM5^{CypA} allele was found in

the cohort. No statistical significance could be shown between TFP/TFP animals and TFP/Q animals, but there was significance when comparing TFP/TFP and Q/Q.

In Specific Aim 2, the sequence of both early and late time points from an immunotherapeutic trial were compared to the original inoculum. Early time points from the trial showed near complete homology with the inoculum at the residues of interest. Late time points showed that the R98S mutation was important for an animal's ability to control virus.

No significant mutation occurred at the GAG-CM9 epitope. Mutations at anchor residues did not contribute to an animal's ability to control virus and showed no correlation with TRIM5 haplotype. However, the polymorphisms in TRIM5 may be contributing to the variation in overall immune response as the avidity with which TRIM5 α binds to the viral capsid shows correlation with the strength of innate immune signaling.

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1.0 INTRODUCTION

There is a complex interplay between host and viral factors that determine the course of an HIV/SIV infection. Host genetics such as MHC class I variation has accounted for much of the difference seen in viral load during acute infection, set point, and overall disease progression. Apart from the innate and adaptive immune system, discoveries in the last decade have identified intrinsic immune factors, or restriction factors, that also contribute to virus control. HIV/SIV replication is highly error prone due to lack of accuracy by their respective reverse transcriptases resulting in frequent mutation. Successful and less successful infection is determined by selection pressure from the host, but the high mutation rate of the virus results in the failure of the immune system to clear the virus (25).

1.1 HIV/AIDS AND THE RHESUS MACAQUE MODEL

HIV/AIDS affects 34 million people worldwide (26). The development of a combination of drugs known as Highly Active Anti-Retroviral Therapy (HAART) has allowed those infected to live much longer lives than at the beginning of the epidemic. However, a preventative vaccine has yet to be developed and due to the virus' high mutation rate, new classes of drugs continually need to be found.

The challenge of studying this disease early on was finding an appropriate animal model. The SIV/macaque model has become the ideal due to similarity in disease progression and pathogenicity (4, 8, 10). This similarity is due to macaques and humans possessing similar genes which encode proteins important to immune system function (3). SIV in macaques originated from cross-species transmission of SIV_{sm} from sooty mangabeys in which the virus causes no immunodeficiency or disease; the resulting virus in macaques, however, is highly pathogenic (11, 18). The use of the non-human primate model also gives researchers the added benefit of being able to observe cross-species transmission of SIV strains amongst genetically diverse species (11).

1.2 CYTOTOXIC T LYMPHOCYTES (CTL)

1.2.1 The role of CTLs in acute and late infection

One of the most vital host defenses during an acute infection is the response of cytotoxic T Lymphocytes (CTL) or CD8⁺ T cells. Research has shown that the CTL response is important during the acute phase (first few weeks of infection) as well as containing the virus late in infection (3). CTL appearance in the peripheral blood coincides with decreased viremia during acute infection with CTL numbers inversely correlating to viral load (7). However, rhesus macaques still progress to end stage AIDS due to the emergence of viral escape mutants that prevent CTLs from recognizing infected cells (25).

1.2.2 Mamu A*01 status and CTL recognition of GAG-CM9

CTLs function by recognizing particular peptide fragment epitopes that have been processed intracellularly and presented on the surface of a cell by MHC (Mamu) class I. Just as in human MHC, Mamu class I genetic diversity in rhesus macaques can have varying effects on disease progression. CTL escape mutants are constantly being generated in both acute and chronic infections indicating that there is selective CD8⁺ T cell-mediated immune pressure on the virus population as well the realization that this is also an indicator of the failure of these cells to fully suppress virus replication (25).

The most well defined rhesus Class I molecule is Mamu A*01. CD8⁺ T cells of a Mamu A*01 positive macaque can recognize 32 different epitopes; one of the most immunodominant of these is CM9 (composed of the amino acid sequence CTPYDINQM) found on the highly conserved GAG region of the viral genome (GAG₁₈₁₋₁₈₉) (2, 3). This particular epitope was chosen for this study not only because it is immunodominant, but also that it is recognized by CTLs in both early and late stages of infection (6).

The original study that characterized GAG-CM9 used live cell binding assays to recognize that it requires three anchor residues for proper binding; these residues are at positions 2, 3, and at the C terminus (3). The most necessary residue for epitope binding is the proline at position 3 and the epitope will not bind without it (3). However, mutations at P2 that can occur including alanine (A), proline (P), and valine (V) will be accommodated (3).

1.3 TRIM5

The host cytoplasmic protein, Tripartite motif protein 5 α (TRIM5 α), is not part of either the innate or the adaptive immune response, but is referred to as an intrinsic cellular defense or restriction factor with the particular purpose of combating retroviral infection (9, 23). TRIM5 α acts by binding to and directly degrading the viral capsid, but it is also believed that TRIM5 proteins act as pattern-recognition receptors that signal the cellular innate immune response (1, 13, 21).

1.3.1 Discovery and Mechanism

The discovery of TRIM5 α was the result of researchers attempting to determine what blocked HIV-1 from replicating in old world monkey PBMC (23). In studies of rhesus macaque cell lines transduced to express human CD4 and CCR5, the viral receptors necessary for HIV-1 entry into cells, SIV replicated competently, but R5-tropic cytopathic HIV-1 did not (15). Researchers determined that HIV-1 was being targeted at the post-entry step and reverse transcription failed to be initiated (17). Heterokaryon analysis suggested the presence of inhibitory factor(s) and not the absence of a required factor was responsible for blocking HIV-1, but not its simian relative, SIV, in old world monkey cells (12, 15, 17). Upon viral entry of the cell, TRIM5 α targets the capsid and degrades it before reverse transcription can occur, thus halting viral replication before it starts (23). However, in SIV infections, due to differences in the viral capsid, TRIM5 α is less efficient and doesn't block viral replication entirely (20, 23).

Polymorphisms in the TRIM5 gene of rhesus macaques have been reported to evolve under balancing selection over millions of years (11, 14, 18, 19). The sole function of TRIM5

appears to be defense against retroviral infection; some would argue that it plays a key role in suppressing cross-species transmission (11). However, this is still up for debate.

1.3.2 TRIM5's RING domain-E3 ubiquitin ligase

A lesser known function of the TRIM5 gene is the RING domain-E3 ubiquitin ligase. TRIM5 has been shown to play a part in signal transduction. It promotes innate immune signaling with a strength that correlates to TRIM5's avidity for the retroviral capsid lattice (21). When TRIM5 proteins bind to the viral capsid, the ubiquitin ligase function of those proteins is also activated (1, 21). TRIM5 acts together with the UBC13-UEV1A enzyme complex to form ubiquitin chains that then activate the TAK1 kinase complex, which then stimulates AP-1 and NF- κ B signaling (Figure 1) (1, 21).

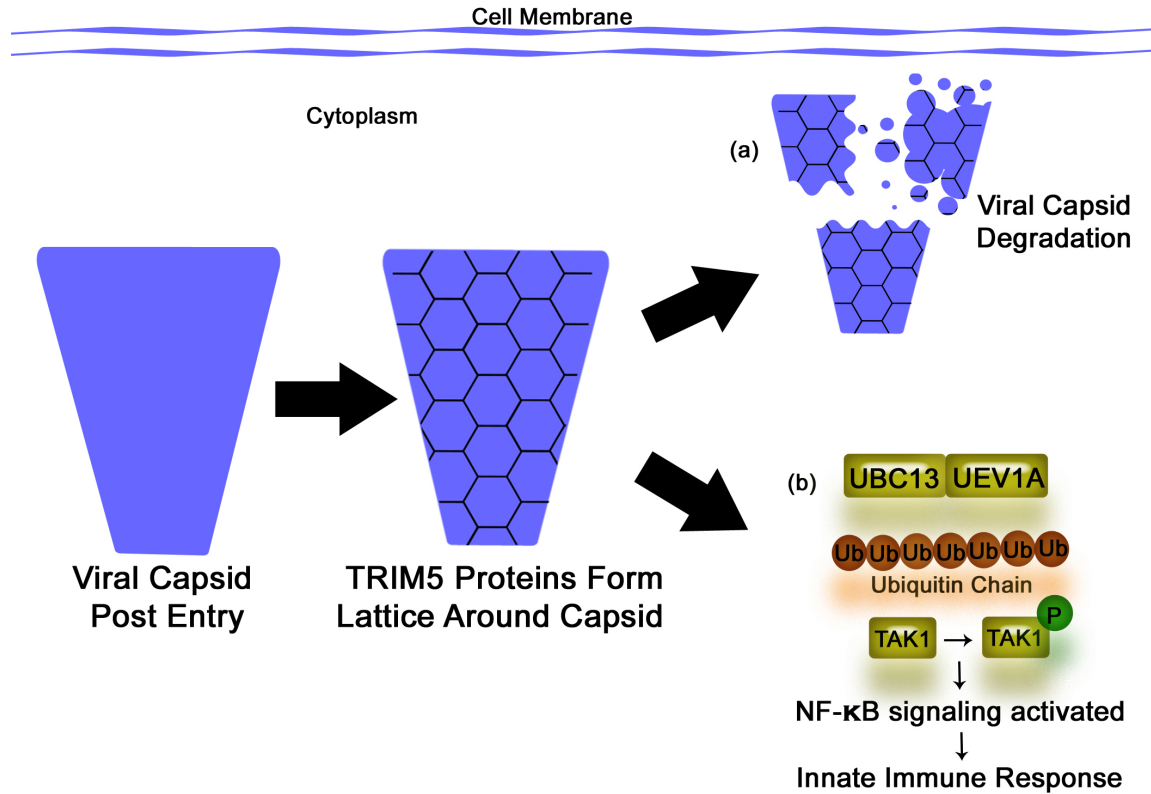


Figure 1. TRIM5's Methods of HIV/SIV Restriction

The two mechanisms of TRIM5 restriction are illustrated. (a) Capsid destruction and (b) Innate immune signaling.

1.3.3 Polymorphism

The variation in TRIM5 α -mediated restriction is determined by the sequence of the C-terminal B30.2/SPRY domain (11, 14). The polymorphisms in this domain result in three different alleles: TRIM5^{TFP}, TRIM5^Q, and TRIM5^{CypA} (Figure 2) (11). The TRIM5^{CypA} allele is the result of the SPRY domain being replaced entirely by an insertion of cyclophilin A (19).

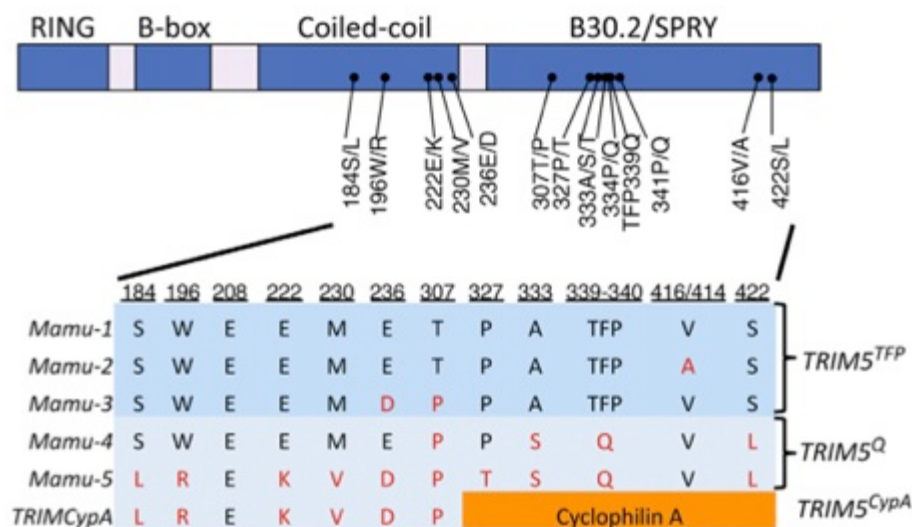


Figure 2. Illustration of the Rhesus Macaque TRIM5 Coding Sequence

Schematic of the TRIM5 gene domains with a detailed description of the B30.2/SPRY domain. The amino acid sequences for the alleles that distinguish the TRIM5 genotypes are in the chart on the bottom.

(Kirmaier, A et al. (2010) TRIM5 suppresses cross-species transmission of a primate immunodeficiency virus and selects for emergence of resistant variants in the new species. PLoS biology 8(8): doi:10.1371/journal.pbio.1000462.g001)

According to Kirmaier et al., the TRIM5 haplotypes that are the most efficient in controlling an SIVsmE543-3 infection are in order TRIM5^{TFP/CypA}, TRIM5^{TFP/TFP}, TRIM5^{Q/TFP}, TRIM5^{Q/CypA}, and TRIM5^{Q/Q}. Whether a virus is sensitive to the particular alleles of TRIM5 is determined by sequence variations in the viral capsid.

1.3.4 SIV TRIM5 α binding sites

The variation in the TRIM5 gene accounts for one aspect of viral degradation. The efficiency of TRIM5 α restriction is also determined by the viral sequence to which the protein binds. Several amino acid positions on the SIV capsid have been identified through previous studies as

important for TRIM5 α restriction. The residue at position 98 was the first identified due its striking switch from arginine (R) to serine (S) in rhesus macaques that had previously been seen in an experimental cohort of sooty mangabey SIV strains SIVsmE041 and SIVsmE543 (11). The second sequence identified was on the L 4/5 loop of the capsid at residues 89-91(Figure 3) (11).

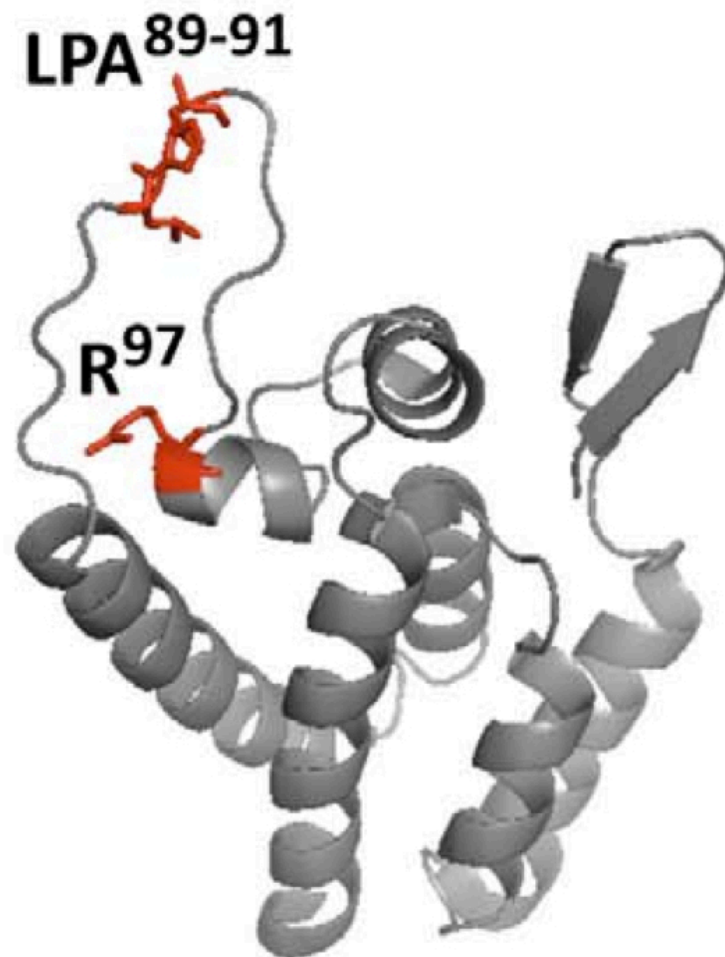


Figure 3. Crystal Structure of the HIV-2 Capsid

TRIM5 α binding sequences are highlighted in red.

(Kirmaier, A et al. (2010) TRIM5 suppresses cross-species transmission of a primate immunodeficiency virus and selects for emergence of resistant variants in the new species. PLoS biology 8(8): doi:10.1371/journal.pbio.1000462.g006)

1.4 SIV/DELTA B670

The virus used in this study was SIV/DeltaB670. It is a primary isolate comprised of a quasispecies containing at least 50 genotypes determined by V1 region diversity (Figure 4) (5). The strength of using a quasispecies as opposed to a single molecular clone is that it more accurately models how infection would occur in a non-lab setting as well as properly evaluating whatever vaccine or immunotherapy the researcher is testing against all of a virus' subclasses. The virus was propagated in rhesus monkey J943's (TRIM5^{CypA/CypA} haplotype) PBMCs *in vitro*.

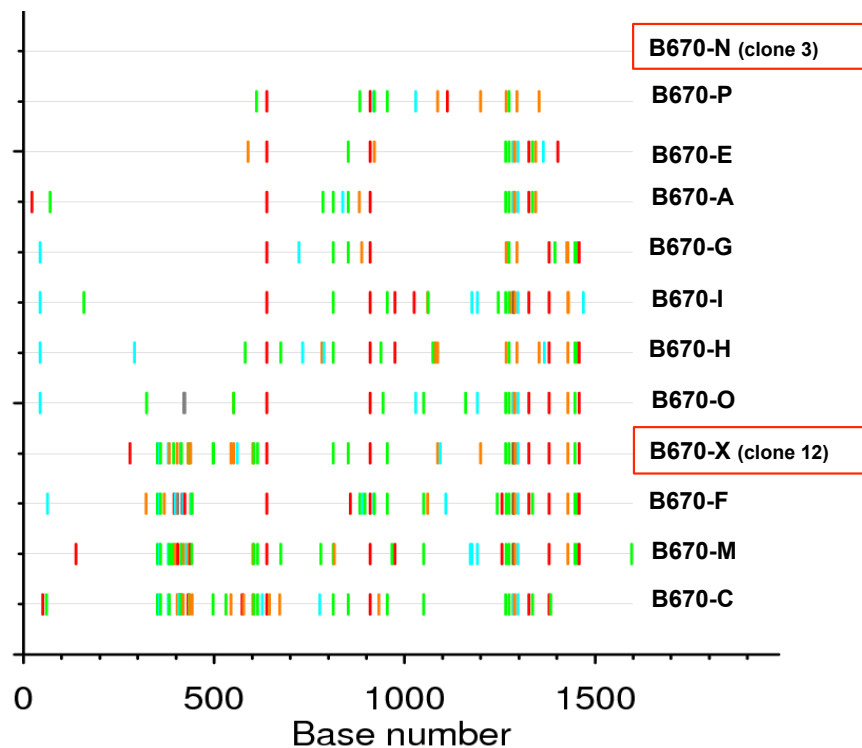


Figure 4. Highlighter Plot of SIV/DeltaB670 Stock Envelope Variants Obtained by SGA

Comparison to V1 region of Envelope of Clone 3 variant. Red boxes show most common clones.

Individual nucleotide changes are shown: green=A, red=T, orange=G, blue=C, gray=gap.

(www.hiv.lanl.gov) (Figure designed by M. Murphey-Corb and A. Amedee)

SIV/DeltaB670 is closely related to the commonly used SIV E660. Both of these viruses originated from the same monkey E038, which was the sooty mangabey that was essential in the original discovery of SIV (16). The virus infecting E038 was used to infect two rhesus macaques, one of which was B670. The resulting virus was thusly named for monkey B670 as well as the then named Delta Primate Center.

As with many cross-species infections, SIV/DeltaB670 is highly pathogenic with an overall median time of survival post infection of 289 days (22, 24). There are several categories of disease progression. Animals that reach endstage AIDS less than one year post infection are classified as rapid progressors, animals that live between 1 and 3 years post infection are intermediate progressors, and animals that survive for more than 3 years post infection are labeled as slow/non-progressors (24). Virion RNA in plasma is usually detected in rapid-progressing animals within 3-5 days post infection followed by an extremely high peak viremia ($> 1.0 \times 10^7$ RNA copies/ml plasma) by 14 days post infection (24). Intermediate and slow/non-progressors follow a similar pattern, but with lower levels of virion RNA in plasma (24).

2.0 STUDY OVERVIEW

Both the immunodominant CD8+ GAG-CM9 epitope and the binding sites for TRIM5 α restriction are located on the highly conserved GAG region of the viral genome. The overall goal of this retrospective study is to analyze the dual evolution of these specific regions of GAG at multiple time points during the course of an SIV infection in an attempt to determine if either are the favored drivers for viral escape.

The data generated from this study together with published reports will provide an ideal environment in which to analyze viral evolution and determine the dependence or independence of these two systems. It could also make important contributions to future vaccine development and help to identify new immunotherapeutic targets.

2.1 SPECIFIC AIM 1: ANALYSIS AND CATEGORIZATION OF RHESUS MACAQUES BY TRIM5 HAPLOTYPE AND VIRAL LOAD PROFILE.

2.1.1 Hypothesis 1

Based on previous literature, there should be a noticeable difference in the viral load profile of rhesus macaques on the basis of TRIM5 haplotype.

2.1.2 Approach

Rhesus macaques were categorized by viral load profile after reaching viral setpoint (measured in RNA copies/ml of plasma) into one of four categories: “Maintained Low to Undetectable Viral Load (Controller),” “Maintained Intermediate Viral Load (<10e5 RNA copies/ml of plasma),” “Maintained High Viral Load (10e5-10e5 RNA copies/ml of plasma),” and “Crashed and Burned (set point >10e6 and >10e5 RNA copies/ml of plasma post set point).” TRIM5 haplotype of each macaque was determined by PCR, cloning, and sequencing as homozygous or heterozygous for the possible alleles TRIM5^{TFP}, TRIM5^Q, or TRIM5^{CypA}.

2.2 SPECIFIC AIM 2: ANALYSIS OF PERIPHERAL BLOOD PLASMA FOR IMMUNODOMINANT CD8+ GAG-CM9 ESCAPE MUTANTS TRIM5A BINDING SITE MUTATIONS.

2.2.1 Hypothesis 2

Trim5 α selection pressure will contribute to a stronger CD8+ response by slowing viral replication.

2.2.2 Approach

Virion RNA was isolated from peripheral blood plasma samples from the cohort of 15 rhesus macaques and converted to cDNA. To avoid amplifying a sequence of low copy number, three

independent nested PCRs were performed and then pooled. 10 ul of the pooled PCR product was electrophoresed in low-melting point agarose and gel purified. The purified PCR product was then cloned into a plasmid and 10 colonies/pool chosen for sequencing.

a. Analyze the sequences of GAG-CM9 and TRIM5 α restriction sites from a sample of the Day 10 stock SIV/B670 inoculum used to challenge the macaques in the immunotherapeutic trial. Identify any variation amongst these clones for comparison to trial samples. The inoculum sequence will also be compared with other closely related SIV strains.

b. Analyze the sequences of GAG-CM9 and TRIM5 α restriction sites of peripheral blood plasma sample clones from a time point during early acute infection and compare it with samples from later time points in the trial.

3.0 MATERIALS AND METHODS

3.1 SAMPLES

Samples for this retrospective study were taken from a cohort of 15 Mamu A*01 positive Indian-origin rhesus macaques in an immunotherapeutic trial that took place over a period of 672 days (Figure 5). Animals were challenged intravenously with SIV/DeltaB670. A Nucleotide Reverse Transcriptase Inhibitor (NRTI), PMPA, was administered for a period of 306 days (beginning on day 56 and ending on day 360). Animals that survived the entire length of the trial were sacrificed at the end of it.

Genomic DNA used for TRIM5 haplotyping was extracted from Peripheral Blood Lymphocyte (PBL) pellets using the QIAamp DNeasy Blood and Tissue Kit (QIAGEN).

Viral RNA was extracted from peripheral blood plasma using the Trizol (Life Technologies) method.

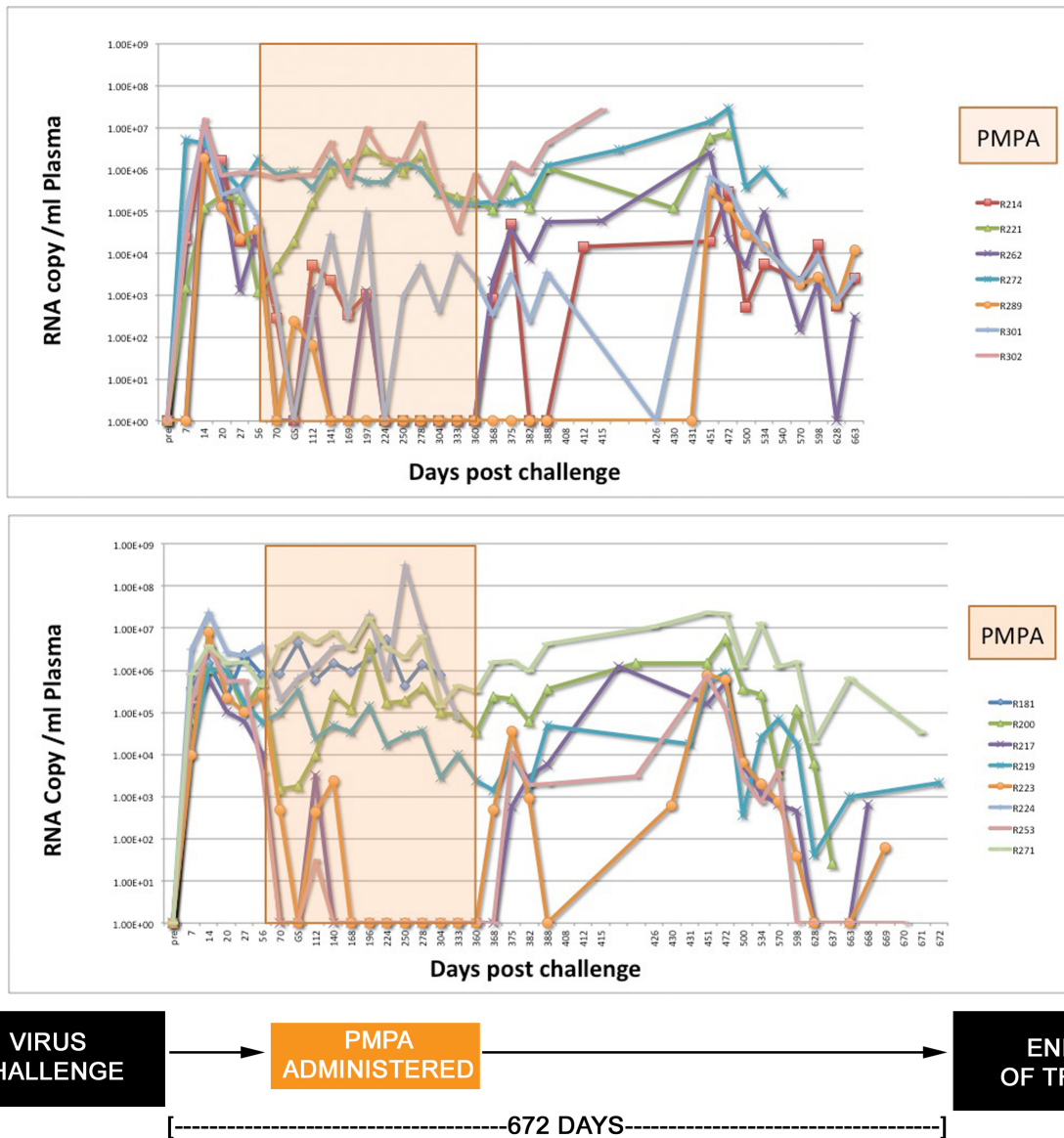


Figure 5. Immunotherapeutic Trial Schematic

Schematic of the trial from which samples for the current study were taken. Shaded orange box shows period of PMPA treatment.

3.2 RNA EXTRACTION

Plasma samples were gathered from the -80°C freezer and thawed on a heat block set at 37°C for 30 seconds. Once thawed, samples were centrifuged at 14000 RPM at 4°C for 60 minutes. After the 60 minutes, the samples were transferred from the centrifuge onto ice. Inside a Biological Safety Cabinet, the supernatant from each plasma tube was poured off into the bleach container with the desired pellet at the bottom of the tube remaining. 1 ml of Trizol (Life Technologies) was then added to each tube and allowed to sit at room temperature for 5-10 minutes to allow the pellet to dissolve. In order to shear any DNA, a 3mL syringe with a 23-gage needle was used to pull up and release the Trizol/pellet solution. 100 µl of 24:1 Chloroform/Isoamyl (Life Technologies) was then added and each sample was vortexed for 30 seconds. The tubes were then transferred to the centrifuge at 4°C and left to sit for 5 minutes. After the 5 minutes, the samples were centrifuged for 15 minutes at 14000 RPM. This last centrifugation separated the solution into 2 visible layers, the pink Trizol layer on the bottom and the clear layer containing the desired genetic material on top, with a membrane in between them. Being very careful not to disturb the bottom Trizol layer, the clear layer is pipetted out and added to a microcentrifuge tube containing 650 µl of Isopropanol (Life Technologies) and 20 µl of GlycoBlue (2mg/ml) (Life Technologies), which helps the RNA precipitate and the resulting pellet to be more visible. The tubes were then placed overnight in the -20°C freezer.

The next day, the tubes are removed from the -20°C freezer and centrifuged at 14000 RPM at 4°C for 15 minutes. After centrifugation, the supernatant is poured off and 1ml of 75% ethanol was added to the tube containing the now visible blue RNA pellet. The tubes were then spun again in the centrifuge at 14000 RPM at 4°C for 15 minutes. The supernatant was once again discarded and the tubes kept inverted on a kimwipe for 5 minutes. The tubes were then

placed upright on a rack to allow the remaining ethanol to evaporate. After the pellets were sufficiently dry, 20 µl water/1 ml plasma (the starting volume of the sample) was added and vortexed gently. The tubes were then placed on a heat block set at 65°C for 10 minutes. The tubes were then vortexed gently once again and spun in the centrifuge for a brief interval to make sure all the RNA was at the bottom of the tube. The RNA was then stored in the -80°C freezer.

3.3 POLYMERASE CHAIN REACTION (PCR)

3.3.1 TRIM5 Haplotype

To determine the TRIM5 haplotype, PCR was performed to amplify the C-terminal B30.2/SPRY domain of the TRIM5 gene using the primers and methods previously described in Kirmaier et al. and Newman et al (Tables 1-3). The PCR target sequence also included the 2 Single Nucleotide Polymorphisms (SNPs) in intron 6 used to determine if Cyclophilin A splicing has taken place.

Table 1. TRIMS Genotype PCR Primers

Forward primer	5'-CAGTGCTGACTCCTTTGCTTG-3'
Reverse primer	5'-GCTTCCCTGATGTGATAC-3'

Table 2. TRIM5 Genotype PCR Mixture

Water	17 μ l
Accuprime Supermix II (Invitrogen)	25 μ l
F primer	1.5 μ l
R primer	1.5 μ l
DNA (50 ng or undiluted)	5 μ l
Total Volume	50 μ l

Table 3. TRIM5 Genotype PCR Conditions

Initial denaturation	94°C	1 minute	These 3 steps are repeated for 30 cycles
Denaturation	94°C	15 seconds	
Annealing	55°C	30 seconds	
Extension	68°C	1 minute	
Final extension	68°C	10 minutes	
	4°C	∞	

3.3.2 Reverse Transcriptase Reaction

The RNA extracted from the plasma samples had to first be reverse transcribed into cDNA. Each individual sample yielded between 10-25 μ l of RNA depending on the original volume of the plasma. Each Reverse Transcription (RT) reaction utilized 2 μ l of RNA, so between 5-12 independent reactions were performed for each sample (Tables 4 and 5). Each independent reaction for an individual sample was then pooled into one tube. 100-240 μ l of cDNA was yielded per sample.

Table 4. Reverse Transcriptase Reaction Mixture

10x buffer 2	(Applied Biosystems)	2 μ l
MgCl ₂	(Applied Biosystems)	4 μ l
dNTPs (10 μ M)	(Applied Biosystems)	8 μ l
Reverse Transcriptase	(Applied Biosystems)	1 μ l
Rnase Inhibitor	(Applied Biosystems)	1 μ l
Random Hexamer	(Applied Biosystems)	1 μ l
Water		3 μ l
18 μ l of mixture added to each well		
RNA		2 μ l
Total Volume		20 μ l

Table 5. Reverse Transcription Reaction Conditions

25°	8 minutes
42°	30 minutes
99°	5 minutes
4°	∞

3.3.3 Nested PCR for CM9 epitope and TRIM5 α restriction sites for SIV/DeltaB670

It was necessary to design a nested PCR in order to amplify the sequence in SIV GAG containing the CM9 epitope and the TRIM5 α restriction sites (Tables 6-9). SIV/DeltaB670 does not have a fully sequenced genome available to access, so the PCR primers were designed by identifying areas of greatest homology amongst an alignment of various strains SIV and then by using the SIVmac239 sequence published on Genbank (AY588945.1). First and Second round primers were picked manually and evaluated with the NetPrimer tool available from PREMIER Biosoft online (<http://www.premierbiosoft.com/netprimer/index.html>) (Table 10). Primers were ordered from Invitrogen. The first round amplicon is 1052 bp and the second round amplicon is 546 bp.

In order to avoid amplification of sequence with low copy number, three independent PCRs were performed for each sample and pooled after the 2nd round reaction and before gel electrophoresis.

Table 6. CM9-TRIM5alpha Restriction Sites 1st Round PCR Mixture

10x Hotmaster buffer	(5 Prime)	5 μ l
1st Round Forward Primer (10uM)		0.5 μ l
1st Round Reverse Primer (10uM)		0.5 μ l
dNTPs	(Applied Biosystems)	4 μ l
Hotmaster Taq DNA Polymerase	(5 Prime)	0.3 μ l
Water		34.7 μ l
DNA (undiluted)		5 μ l
Total Volume		50 μ l

Table 7. CM9-TRIM5alpha Restriction Sites 1st Round PCR Conditions

Initial Denaturation	94°	2 minutes	These 3 steps are repeated for 35 cycles
Denaturation	94°	20 seconds	
Annealing	55°	20 seconds	
Extension	72°	30 seconds	
Final extension	72°	7 minutes	
	4°	∞	

Table 8. CM9-TRIM5alpha Restriction Sites 2nd Round PCR Mixture

10x Hotmaster buffer	(5 Prime)	5 µl
2nd Round Forward Primer (10uM)		0.5 µl
2nd Round Reverse Primer (10uM)		0.5 µl
dNTPs	(Applied Biosystems)	4 µl
Hotmaster Taq DNA Polymerase	(5 Prime)	0.3 µl
Water		34.7 µl
1st Round PCR Product		5 µl
Total Volume		50 µl

Table 9. CM9-TRIM5alpha Restriction Sites 2nd Round PCR Conditions

Initial Denaturation	94°	2 minutes	These 3 steps are repeated for 30 cycles
Denaturation	94°	20 seconds	
Annealing	55°	20 seconds	
Extension	72°	30 seconds	
Final extension	72°	7 minutes	
	4°	∞	

Table 10. CM9-TRIM5alpha Restriction Sites 1st and 2nd Round PCR Primers

1st Round Forward Primer	5' - ATGGGCGTGAGAAACTCCG - 3'
1st Round Reverse Primer	5' - CAAGCCGTCAGCATTCTTCTAG - 3'
2nd Round Forward Primer	5' - AAGCCCTAGAACATTAAATGC - 3'
2nd Round Reverse Primer	5' - GCAATCGTTAGCATTTTGAATCAG - 3'

3.4 GEL ELECTROPHORESIS AND PURIFICATION

3.4.1 Electrophoresis

After each of the three independent PCRs for each individual sample has been pooled, 10 µl of each pooled sample mixed with loading dye, along with 5 µl of a 100 bp DNA ladder (Promega), was run on a 1% low-melting point agarose gel. Each gel was composed of 1 g Invitrogen UltraPure Low-Melting Point Agarose, 100 ml 1x TAE buffer, and 10 µl ethidium bromide. This protocol, as well as the purification step, was used for both the TRIM5 genotype PCR as well as the CM9 and TRIM5α restriction sites nested PCR.

3.4.2 Purification

To ensure efficiency during cloning, each individual sample was gel purified using a vacuum manifold and the Wizard® PCR Preps DNA Purification System (Promega) protocol. The desired band was visualized using a UV light box and cut out of the gel using a scalpel. The yield of purified PCR product is 50 µl for each individual sample and was then used for the cloning step immediately following this step.

3.5 CLONING

The cloning process was used in order to observe representative examples of the variation present in each sample. The TOPO TA Cloning® Kit for Sequencing (Invitrogen) was used for both the TRIM5 genotype PCR as well as the CM9 and TRIM5 α restriction sites PCR. This is a protocol that utilizes the TOPO® vector which is ideal for the insertion of Taq polymerase-amplified PCR products.

Four µl of the purified PCR product was combined with 1 µl of salt solution and 1 µl of the cloning vector in a microcentrifuge tube and allowed to incubate at room temperature for 30 minutes. 2 µl of this cloning reaction was then gently added to a vial of One Shot® TOP10 Chemically Competent *E. coli* that had been allowed to thaw on ice and incubated for 15 minutes remaining on the ice. The vials of cells were then heat shocked for 30 seconds in a 42°C water bath and immediately put back on ice and allowed to sit for 2 minutes. 250 µl of room temperature S.O.C. media was then added to each vial of cells and incubated for 1 hour with the tubes placed horizontally in a shaking incubator at 37°C and 200 rpm.

To ensure efficient growth of colonies, the transformed cells are spread on plates that have been pre-warmed to 37°C. The plates used were LB plates containing 50 µg/ml ampicillin. An efficient cloning reaction produced numerous colonies, so the transformed cells were plated in amounts of both 25 µl and 50 µl per sample. The plates were incubated overnight at 37°C. The next day, 10 colonies per sample were chosen with autoclaved wooden sticks for analysis. Each colony was added to a 15-ml sterile plastic culture tube (Fisherbrand) containing 3 ml of a LB broth/ampicillin mixture (100 µg/ml). The chosen colonies were incubated overnight at 37°C and shaken at 250 rpm. After this last incubation, the plasmid DNA was isolated and purified for sequencing.

3.6 PLASMID ISOLATION, PURIFICATION, AND SEQUENCING

Plasmid DNA was isolated and purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega). This protocol yielded 100 µl of purified plasmid DNA per sample that can be stored at -20°C or immediately sent for sequencing.

Ten µl of each sample was sent off for Sanger sequencing at Genewiz, Inc. (South Plainfield, NJ).

3.7 SEQUENCE ANALYSIS

Sequence analysis was primarily carried out with Geneious Pro software (Biomatters, Ltd.), but many online tools were used as well, such as Nucleotide Blast (NCBI), ClustalW2 (EBI,

European Molecular Biology Laboratory), READSEQ (Center for Information Technology, National Institutes of Health), and the various online tools and HIV/SIV sequence database of the Los Alamos National Laboratory (NIH).

TRIM5 haplotype was determined by comparing the TRIM5 PCR clones with sequences published in Genbank (Mamu-1 through Mamu-5 EF113914–EF113918 and TRIM5CypA EU359036).

4.0 RESULTS

4.1 AIM 1: ANALYSIS AND CATEGORIZATION OF RHESUS MACAQUES BY VIRAL LOAD PROFILE AND TRIM5 HAPLOTYPE

4.1.1 Viral load profile

The cohort was separated into four groups on the basis of their viral load profile (measured by RT-PCR in RNA copies/ml plasma): Controllers (Figure 6), Maintained Intermediate Viral Load (Figure 7), Maintained High Viral Load (Figure 8), and Crashed and Burned (Figure 9).

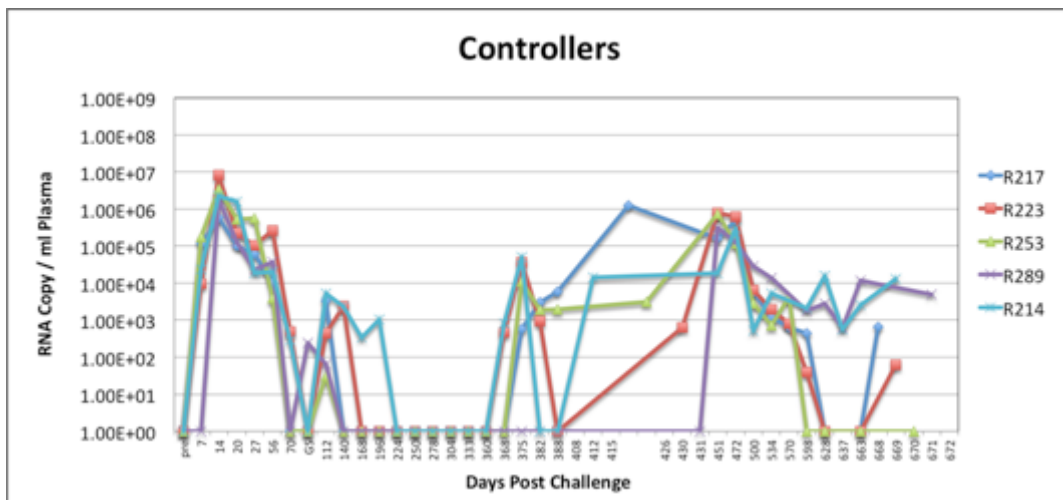


Figure 6. Rhesus Macaques Classified by Viral Load Profile: Controllers

Animals designated as “controlling their infection” survived the entire trial and had a lengthy period of undetectable virus after the acute infection stage. 5 out of the 15 animals in the cohort were in this group.

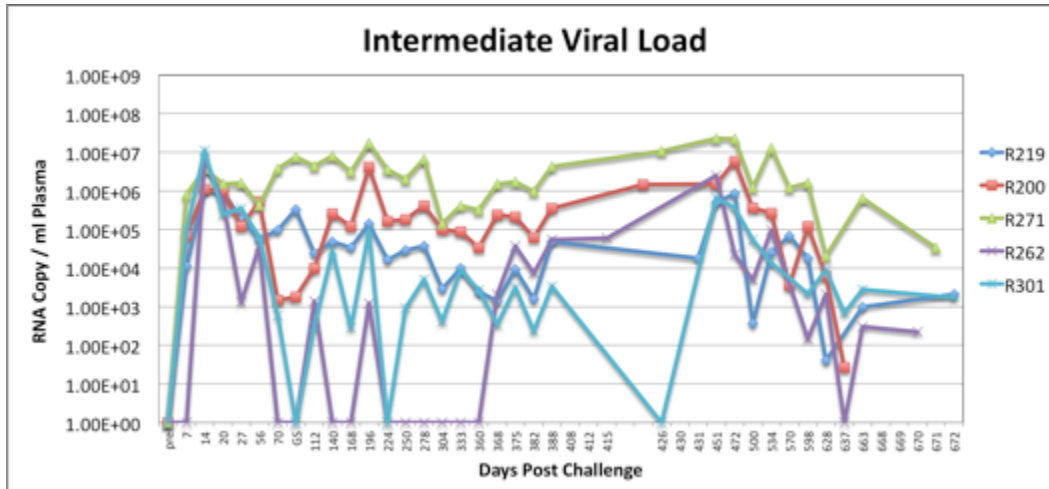


Figure 7. Rhesus Macaques Classified by Viral Load Profile: Maintained Intermediate Viral Load

Animals in this group survived the entire trial maintaining an intermediate viral load (<10⁵ RNA copies/ml of plasma) throughout. They did not have lengthy periods of undetectable virus like the controllers. 5 out of 15 animals in the cohort were in this group.

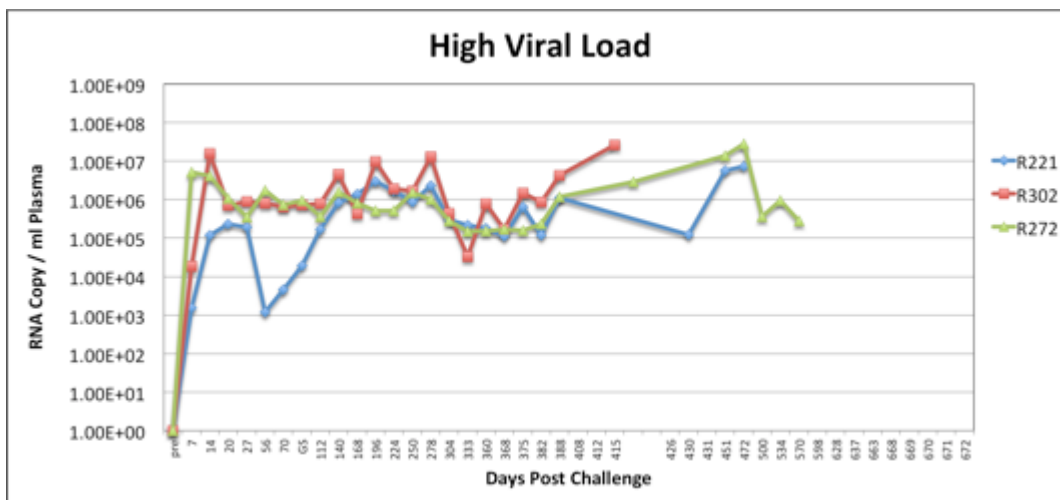


Figure 8. Rhesus Macaques Classified by Viral Load Profile: Maintained High Viral Load

Animals in this group did not survive the entire trial, had no time points with virus at undetectable levels, and maintained a high viral load (10⁵-10⁶ RNA copies/ml of plasma) until death. 3 out of 15 animals in the cohort were in this group.

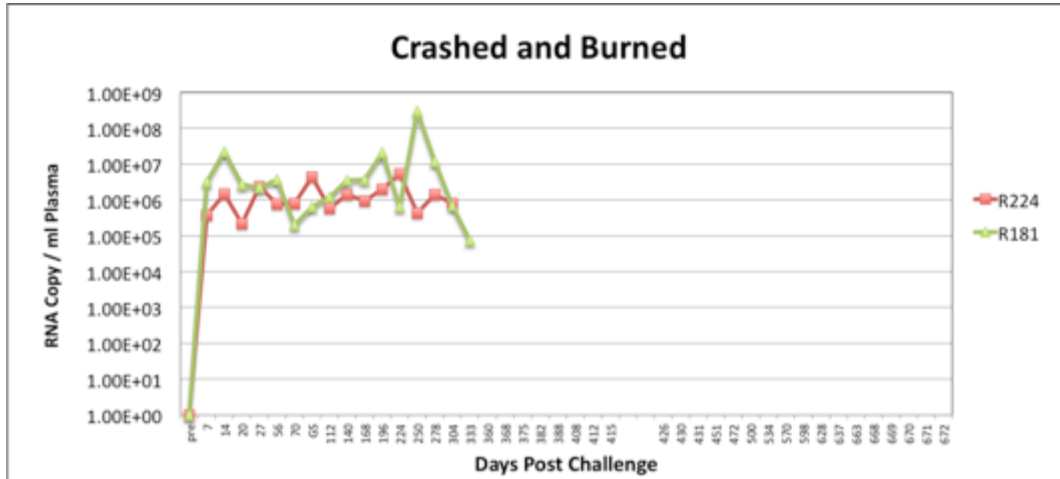


Figure 9. Rhesus Macaques Classified by Viral Load Profile: Crashed and Burned

Animals in this group died very early in the trial and maintained a viral load at acute infection levels ($>10^6$ RNA copies/ml of plasma). 2 out of 15 animals in the cohort were in this group.

4.1.2 TRIM5 haplotype

Six out of 15 animals were TRIM5^{TFP/TFP}, 7 out of 15 animals were TRIM5^{TFP/Q}, and 2 out of 15 animals were TRIM5^{Q/Q} (Table 11). The TRIM5 haplotypes were evenly distributed amongst the different viral load profile groups with the exception of the two TRIM5^{Q/Q} animals that were exclusively in the “Crashed and Burned” viral load group. This would suggest that my first hypothesis was only partially correct. Animals of TRIM5^{TFP/TFP} and TRIM5^{TFP/Q} could belong to any of the first three groups.

Unfortunately, the original immunotherapeutic trial from which samples were taken for this study was not designed to look at TRIM5 and no TRIM5^{CypA} alleles were found in the cohort.

Table 11. Rhesus Macaques by Viral Load Profile and TRIM5 Haplotype

Animal	Viral Load Profile	TRIM5 Haplotype
R217	Controller	TFP/TFP
R223	Controller	TFP/TFP
R253	Controller	TFP/Q
R289	Controller	TFP/TFP
R214	Controller	TFP/Q
R219	High Viral Load	TFP/TFP
R200	High Viral Load	TFP/Q
R271	High Viral Load	TFP/Q
R262	High Viral Load	TFP/TFP
R301	High Viral Load	TFP/Q
R221	Very High Viral Load	TFP/Q
R302	Very High Viral Load	TFP/TFP
R272	Very High Viral Load	TFP/Q
R224	Crashed and Burned	Q/Q
R181	Crashed and Burned	Q/Q

*No CypA allele found in cohort

4.1.3 Statistical analysis

To determine if there was a statistical significance between TRIM5 haplotypes by viral load for a specific time point, a Wilcoxon Rank Sum Test was performed comparing TFP/TFP animals and TFP/Q animals on Days 7, 14, 20, 27, and 56 (Table 12 and Figure 10). The sample size for Q/Q homozygotes was too small to determine significance. There was a significant difference in viral

loads between TFP/TFP and TFP/Q animals on Days 7 (P=0.0175), and 20 (P=0.0367), but not on Days 14, 27, and 56. On Days 7 and 20, TFP/TFP homozygotes at significantly lower viral loads compared to TFP/Q.

Table 12. Viral Load by TRIM5 Haplotype

		Day 7	Day 14	Day 20	Day 27	Day 56
TFP/TFP	R289	1.00E+00	1.84E+06	1.27E+05	2.30E+04	3.75E+04
	R262	1.00E+00	6.75E+06	5.30E+05	1.32E+03	3.80E+04
	R223	9.65E+03	8.25E+06	2.25E+05	1.05E+05	2.65E+05
	R217	1.15E+05	5.95E+05	1.01E+05	6.20E+04	1.02E+04
	R219	1.13E+04	1.09E+06	9.60E+05	1.80E+05	5.70E+04
	R302	1.85E+04	1.56E+07	7.05E+05	8.80E+05	8.10E+05
TFP/Q	R221	1.55E+03	1.19E+05	2.35E+05	1.90E+05	1.20E+03
	R214	2.40E+04	2.30E+06	1.60E+06	1.90E+04	2.00E+04
	R200	6.80E+04	1.09E+06	9.95E+05	1.20E+05	5.30E+05
	R301	9.60E+04	1.10E+07	2.50E+05	3.50E+05	6.50E+04
	R253	1.80E+05	3.55E+06	5.30E+05	5.60E+05	3.90E+03
	R271	8.00E+05	3.80E+06	1.50E+06	1.60E+06	4.30E+05
	R272	5.20E+06	4.16E+06	1.08E+06	3.50E+05	1.75E+06
Q/Q	R181	3.70E+05	1.45E+06	2.20E+05	2.35E+06	7.65E+05
	R224	3.20E+06	2.30E+07	2.70E+06	2.23E+06	3.70E+06

Virus loads are listed for Day 7, 14, 20, 27, and 56. Animals are listed by TRIM5 Haplotype:

TRIM5TFP/TFP (Blue), TRIM5TFP/Q (Yellow), TRIM5Q/Q (Red). Units are in RNA Copies/ml of Plasma.

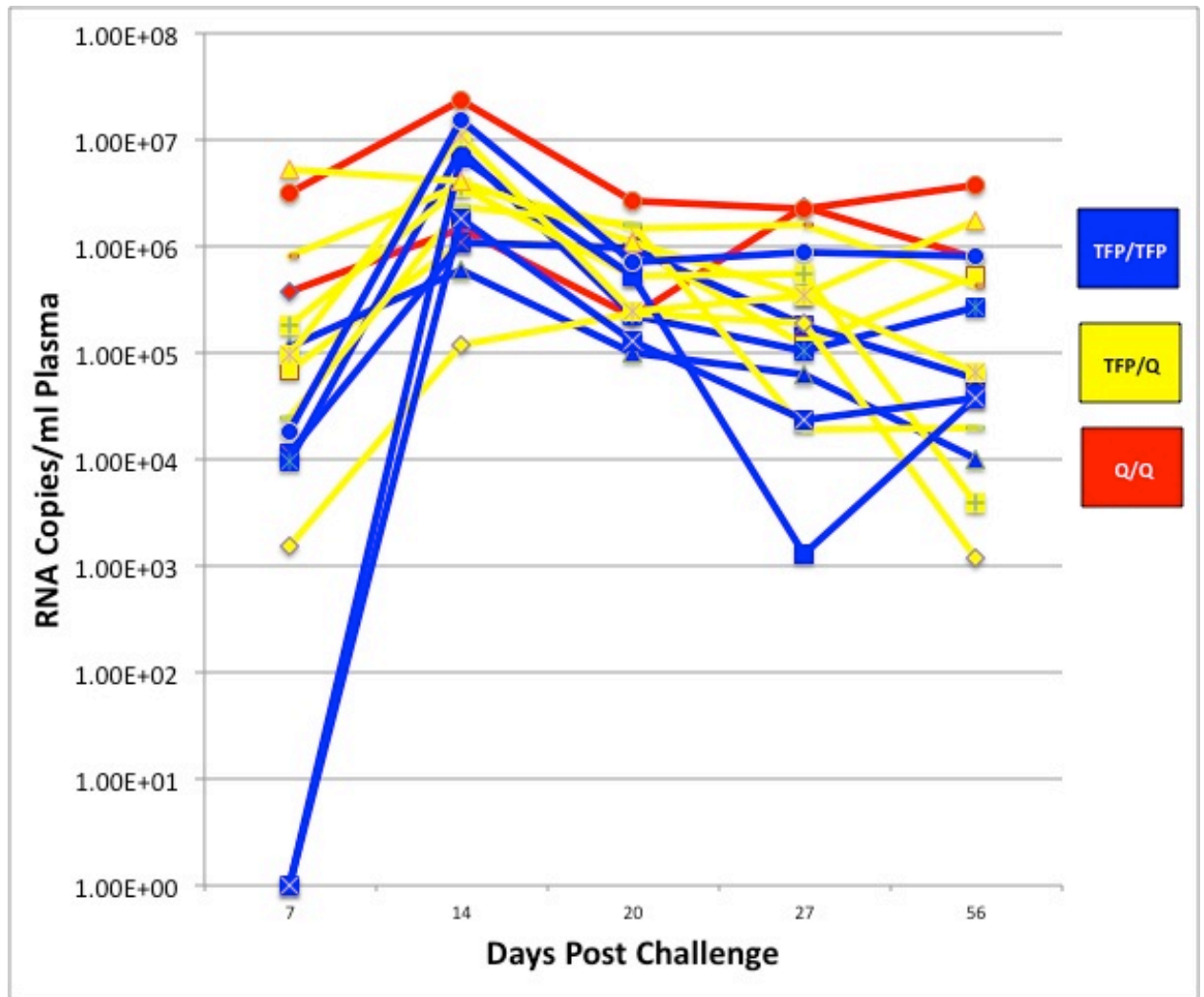


Figure 10. Viral Load by TRIM5 Haplotype

Viral loads are plotted for each animal by TRIM5 haplotype for Day 7, 14, 20, 27, and 56. TRIM5^{TFP/TFP} (Blue), TRIM5^{TFP/Q} (Yellow), and TRIM5^{Q/Q} (Red). Units are in RNA Copies/ml of Plasma.

4.2 AIM 2: ANALYSIS OF PERIPHERAL BLOOD PLASMA FOR IMMUNODOMINANT CD8+ GAG-CM9 ESCAPE MUTANTS AND TRIM5A BINDING SITE MUTATIONS

4.2.1 Analysis of Day 10 stock SIV/DeltaB670 inoculum

The first part of Specific Aim 2 was to analyze the sequences of GAG-CM9 and TRIM5 α restriction sites from a sample of the Day 10 stock SIV/B670 inoculum used to challenge the macaques in the immunotherapeutic trial. The sequence of all 10 clones showed complete homology at the sites of interest (Figure 11 and 12). This is not unexpected considering that GAG is highly conserved. It is also helpful for the analysis of the immunotherapeutic trial time points in the respect that any deviation from the inoculum sequence resulted from host selection pressure.

		GAG-CM9 epitope													
1_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
5_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
3_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
4_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
2_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
6_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
10_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
9_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
8_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												
7_Day10 Stock	TCAGAAAGGC	TGCACCCCTATGACATCAATCAAATG	CTAAATTGTGTAGGAGAG	CA											
Frame 2	S E G	C T P Y D I N Q M	L N C V G E H												

Figure 11. SIV/DeltaB670 Day 10 Virus Stock GAG-CM9 Epitope Sequences

Ten clones were sequenced from a sample of the Day 10 virus stock used to inoculate the animals for this study. The GAG-CM9 epitope is highlighted in blue.

[illegible]

The sequences of the same 10 clones of the Day 10 stock virus from Figure 11 were analyzed at the TRIM5alpha binding sites, which are highlighted in blue.

The TRIM5 α binding sites sequence of SIV/DeltaB670 aligned with other closely related SIV strains show that B670 is more closely related to the ancestral SIVsm sequences with an arginine (R) in position 97 rather than to those of the SIVmac strains. The unique aspect of B670 is the proline (P) in position 91 (Figure 13).

1. SIVsmE041	Q	P	G	P	I	P	A	G	Q	L	R	D	P	R	G	S	D
2. SIVE543	Q	P	G	P	L	P	A	G	Q	L	R	E	P	R	G	S	D
3. SIVB670	Q	P	G	P	I	P	P	G	Q	L	R	E	P	R	G	S	D
4. SIVMM251	Q	P	A	P	-	Q	Q	G	Q	L	R	E	P	S	G	S	D
5. SIVmac239	Q	P	A	P	-	Q	Q	G	Q	L	R	E	P	S	G	S	D

Figure 13. Alignment of the TRIM5alpha Restriction Sites of Different SIV Strains

Four SIV strains are aligned with SIV/DeltaB670 at the TRIM5alpha restriction sites. SIVsmE041 and SIVE543 or sooty mangabey viruses. SIVB670, SIVMM251, and SIVmac239 are rhesus macaque viruses. The restriction sites are highlighted in blue.

4.2.2 Analysis of peripheral blood plasma from the immunotherapeutic trial

The second part of Specific Aim 2 was to analyze the sequences of GAG-CM9 and TRIM5 α restriction sites of peripheral blood plasma sample clones from a time point during early acute infection and compare it with samples from later time points in the trial. The results are summarized in Table 13, and individual time points are listed for Controllers (Figures 14-23), Intermediate Viral Load (Figures 24-33), High Viral Load (Figures 34-39), and Crashed and Burned (Figures 40-43).

The sequences obtained for the early acute infection time points were nearly all identical to the consensus sequence of the SIV/DeltaB670 inoculum.

Table 13. Summary Table of Immunotherapeutic Trial Early and Late Time Points

Animal	Viral Load Profile	TRIM5 Haplotype	CM9 -- CTPYDINQM (WT)		Trim5 Binding Sites 89-91 -- IPP (WT)		Trim5 Binding Position 97 -- R (WT)	
			Early	Late	Early	Late	Early	Late
R217	Controller	TFP/TFP	CTPYDINQM (10)	CTPYDINQM (8), RTPYDINQM (2)	IPP (10)	IPP (10)	R (10)	S (10)
R223	Controller	TFP/TFP	CTPYDINQM (10)	CTPYDINQM (10)	IPP (9), TPP (1)	IPP (8), IPQ (2)	R (9), S (1)	S (8), T (2)
R253	Controller	TFP/Q	CTPYDINQM (10)	CTPYDINQM (10)	IPP (10)	IPP (10)	R (10)	G (10)
R289	Controller	TFP/TFP	CTPYDINQM (10)	CTSYDINQM (10)	IPP (10)	IPP (10)	R (10)	S (10)
R214	Controller	TFP/Q	CTPYDINQM (9), CAPYDINQM (1)	CIPYDINQM (9), CAPYDINQM (1)	IPP (10)	IPP (1), IPQ (9)	R (10)	S (10)
R219	Intermediate Viral Load	TFP/TFP	CTPYDINQM (8), RTPYDINQM (2)	CTPYDINQM (10)	IPP (9), VPP (1)	IPA (10)	R (10)	S (10)
R200	Intermediate Viral Load	TFP/Q	CTPYDINQM (9), CTPYGINQM (1)	CTPYDINQM (10)	IPP (10)	IPP (1), IPQ (8), LPQ (1)	R (9), G (1)	S (9), G (1)
R271	Intermediate Viral Load	TFP/Q	CTPYDINQM (10)	CTPYDINQM (10)	IPP (10)	IPP (9), VPP (1)	R (10)	S (10)
R262	Intermediate Viral Load	TFP/TFP	CTPYDINQM (10)	CTPYDINQM (10)	IPP (10)	IPP (10)	R (10)	S (10)
R301	Intermediate Viral Load	TFP/Q	CTPYDINQM (10)	CTPYDINQM (3), GTPYDINQM (7)	IPP (10)	IPP (7), IPQ (3)	R (10)	S (10)
R221	High Viral Load	TFP/Q	CTPYDINQM (9), CTPYDISQM (1)	CTPYDINQM (1), CAPYDINQM (9)	IPP (10)	IPP (10)	R (10)	R (10)
R302	High Viral Load	TFP/TFP	CTPYDINQM (9), CTPYDTNQM (1)	CAPYDINQM (4), CTPYDVNQM (4), CTPYDVNQI (2)	IPP (10)	IPP (10)	R (9), G (1)	S (10)
R272	High Viral Load	TFP/Q	CTPYDINQM (8), CAPYDINQM (2)	CTPYDINQM (10)	IPP (10)	IPA (10)	R (10)	R (10)
R181	Crashed and Burned	Q/Q	CTPYDINQM (10)	CAPYDINQM (10)	IPP (10)	IPP (10)	R (10)	R (10)
R224	Crashed and Burned	Q/Q	CTPYDINQM (10)	CTPYDINQM (9), CTPHINQM (1)	IPP (10)	IPP (10)	R (10)	R (10)

4.2.2.1 Statistical analysis

Viral mutations at TRIM5 α binding sites were evaluated using Fisher's Exact Test. Mutations were seen at residues 91 and 98. These mutations were evaluated on the basis of both TRIM5 haplotype as well as viral load profile. When looking at the difference between TRIM5 haplotypes, no statistical significance was found between TFP/TFP and TFP/Q animals and TFP/Q and Q/Q animals at either residue. However, when TFP/TFP and Q/Q are compared, there is a statistical significance shown at residue 98 (P=0.0357). Viral sequences obtained from

TFP/TFP homozygotes all show a mutation at this residue whereas ones obtained from Q/Q homozygotes do not.

For viral load profiles, statistical significance can only be shown when the 4 groups are combined into 2 groups: Controllers and Intermediate Viral Load, and High Viral Load and Crashed and Burned. Every animal in the first group had a mutation at residue 98 compared to only 1 that did in the second group and 4 that did not have the mutation ($P=0.0037$). This would indicate that a mutation at residue 98 is key to an animal's ability to control virus.

4.2.2.2 Viral load at a single time point as an indicator for mutation

In Table 13, it's indicated that 3 out of the 5 animals that were the best controllers of virus showed no mutation at residue 91 and mutation at residue 98. This is not statistically significant, but is worth noting for future consideration. If mutation at these residues later in infection is regarded in respect to the viral load at a single time point, we notice that on Days 7, 14, 27, and 56, those animals with the lowest viral load on each day also show no mutation at residue 91 and mutation at residue 98 (red boxes on Table 14). Once again, this cannot be stated with statistical certainty, but very well may show stronger significance with a larger sample size.

Table 14. Mutation as Determined by Viral Load at a Single Time Point

Viral Load in RNA copies/ml Plasma	Day 7		Day 14		Day 20		Day 27		Day 56	
	Mutation at position 91	Mutation at position 98	Mutation at position 91	Mutation at position 98	Mutation at position 91	Mutation at position 98	Mutation at position 91	Mutation at position 98	Mutation at position 91	Mutation at position 98
1.00E+00	no	yes								
1.00E+03	yes	yes					no	yes	no	yes
1.00E+04	yes	yes					yes	yes	yes	yes
1.00E+05	no	yes	no	yes	yes	yes	yes	yes	yes	yes
1.00E+06	yes	no	yes	yes	yes	yes	no	yes	yes	no
1.00E+07			yes	yes						

5.0 DISCUSSION

SIV/DeltaB670 behaves differently than other strains of SIV. Mamu A*01 status has been shown in the past to be a benefit to macaques infected with other strains such as SIVmac239. However, previous studies with B670 have not shown the same benefit. The drastic differences in virus loads from this study suggest that some other host factor is responsible for the variation that is seen. What we have learned about rhesus TRIM5 in the past decade suggests that polymorphisms in this gene could explain that variation.

In Aim 1, I sought to categorize the cohort of 15 Mamu A*01 rhesus macaques on the basis of viral load and TRIM5 haplotype. Hypothesis 1 stated that based on previous literature there should be a noticeable difference in viral load profile on the basis of TRIM5 haplotype. Homozygous TRIM5^Q animals (R181 and R224) were classified exclusively in the “Crashed and Burned” category as both maintained a viral load above 10e6 RNA copies/ml of plasma and died early on in the immunotherapeutic trial. However, both TRIM5^{TFP/TFP} and TRIM5^{TFP/Q} haplotypes were equally distributed amongst the remaining viral load profile categories (Figure 5: 5 Controllers, 5 Intermediate Viral Load, and 3 High Viral Load). This does not conflict with previous findings, but given that this study utilizes SIV/DeltaB670 instead of the strains from previous studies, the distribution of viral load by TRIM5 haplotype may not be as distinct requiring a much larger sample size. The TRIM5^{CypA} allele was not found at all in this cohort of animals. However, as was previously mentioned, the virus stock with which the study animals

were inoculated with was grown in PBMCs from J943, a macaque with a TRIM5^{CypA/CypA} haplotype. The reason this macaque's PBMCs were, and continue to be used is because the virus replicates efficiently in them. Another TRIM5^{CypA/CypA} macaque that was not included in this study and was also infected with B670 (data not shown) had an acute virus load of 10e7 that did not decrease at all throughout infection. This would suggest that the TRIM5^{CypA} allele does not restrict B670 at all.

In Aim 2, the SIV/DeltaB670 inoculum sequence was characterized. At residues 89-91, B670 (IPP) was distinctly different from closely related strains SIVsmE543 (LPA) and SIVE041 (IPA) as well as other macaque strains SIVmac239 and SIVmac251 (-QQ) (Figure 6). B670 also retained the ancestral arginine (R) at residue 98. According to Kirmaier et al., animals infected with SIVsmE543-3 (LPA89-91, R98) that were homozygous for the TRIM5^{TFP} allele and the TRIM5^Q allele were the most and least efficient controllers of virus respectively (11). The R98S mutation was also seen in their experimental cohort. By using site-directed mutagenesis of SIVmac239 and SIVsmE041, they determined that an R (from the ancestral SIVsm strain) at residue 98 and an LPA (also from the ancestral SIVsm strain) at residues 89-91 conferred sensitivity to the rhesus TRIM5^{TFP} allele, while the rhesus TRIM5^Q allele remained a poor controller (11). The sequence data from the 10 clones of the B670 inoculum showed no variation. As previously stated, TRIM5^{CypA} does not restrict B670 at all and this lack of restriction could be why we don't see any mutation in the sequence. As with the TRIM5^{Q/Q} homozygotes, poor restriction from TRIM5 puts no selection pressure on the virus to mutate. If no restriction is applied to the virus, it has no reason to mutate.

According to these data, there appears to be no statistically provable correlation between TRIM5 haplotypes and mutations at TRIM5 α binding sites late in infection with the exception of

TFP/TFP homozygotes and Q/Q homozygotes at residue 98. The same distinction is noted between viral load profile types and mutations at this site. Mutation or lack thereof at residue 98 seemed to be indicative of the animal's ability to control virus. The first two groups ("Controllers" and "Intermediate Viral Load") were the best controllers of virus and every animal in these groups notably showed mutation at residue 98. The last two groups ("High Viral Load" and "Crashed and Burned") had only one animal that showed mutation at this residue. This would indicate that mutation at this site is important for an animal's ability to control virus. This mutation is favorable to the host by allowing TRIM5 to restrict virus loads to a controllable level. This would suggest that a balancing selection by both the host and the virus for mutual survival is taking place and is a step in the direction of a chronic retroviral infection that does not cause pathogenicity much like its sooty mangabey ancestral strain.

The viral mutation that appears to be most favorable to the host is the mutation at residue 98 only. However, according to Kirmaier et al. (11), reversion of the sequence from a serine (S) to the ancestral sooty mangabey arginine (R), which is present in wild type SIV/DeltaB670, resulted in greater sensitivity of the virus to the TRIM5^{TFP} allele. In the current study, the R97S mutation was present in animals that were more efficient in controlling virus. It is clear that SIV/DeltaB670 behaves differently than its ancestral SIV strains in regards to TRIM5 activity. Going strictly on the basis of TRIM5 α binding site sequence, the one major difference between B670 and its ancestors as well as other SIVmac strains is the proline (P) in position 91. Proline is a unique amino acid in regards to its structure in that its side chain forms a cyclical structure with its amine nitrogen giving it extreme conformational rigidity. Also, considering that the proline in position 91 follows yet another highly conserved proline in position 90, this could create a major kink in the structure of L4/5 of the viral capsid and make the arginine in position

98 the less efficient residue and the mutant serine the more favorable residue when it comes to TRIM5 α restriction.

As mentioned before, I cannot make a significant statement that the best controllers of virus had a mutation at residue 98, but not at residue 91. However, it seems to be a pattern worth noting for future consideration.

Analysis of the GAG-CM9 sequence at both early and late time points showed no significant changes. Mutations that occurred at anchor residues had no impact on the individual animal's ability to control virus and showed no correlation to TRIM5 haplotype or subsequent mutations at the TRIM5 α binding sites. However, my second hypothesis relating TRIM5's contribution to a strong CD8⁺ T-cell response can neither be proven or disproven with these data. GAG-CM9 is a single CD8⁺ epitope that may or may not play an important role in this cohort of animals' ability to control SIV infection. What can be suggested here is that TRIM5 could be contributing to the immune response through innate immune signal transduction. The secondary action of TRIM5 correlates to the avidity that TRIM5 α has for binding to the viral capsid. The TRIM5 alleles that show poor restriction, Q and CypA, had no mutation at the TRIM5 α binding sites and therefore had a very weak overall immune response. No restriction by TRIM5 could cripple any T cell response. The answer to why Mamu A*01 shows varying ability to control infection of SIV/DeltaB670 is most likely associated with the unique sequence B670 possesses at the sites on the viral capsid that TRIM5 α binds to in order to degrade the virus.

5.1 FUTURE CONSIDERATIONS

As previously stated, the immunotherapeutic trial from which the samples were taken was not originally designed to study TRIM5 polymorphisms and no Cyclophilin A (TRIM5^{CypA}) allele was found in the cohort. To completely understand the mutations observed in this study, all alleles found in nature should be included. Unfortunately, time and resources prohibit such data from being included in this study.

To properly assess the impact of comparing differences in TRIM5 data with the presence of GAG-CM9 escape mutants, sequence data obtained from this study must be compared to previously performed T cell binding assays. This analysis is beyond the scope of this project and cannot be included in this thesis for time constraints.

The questions asked in this study could not be answered completely due to a small sample size. If data from more animals in the future were combined with the data from this study, there would be a more accurate distribution of significant differences.

5.2 PUBLIC HEALTH IMPLICATIONS

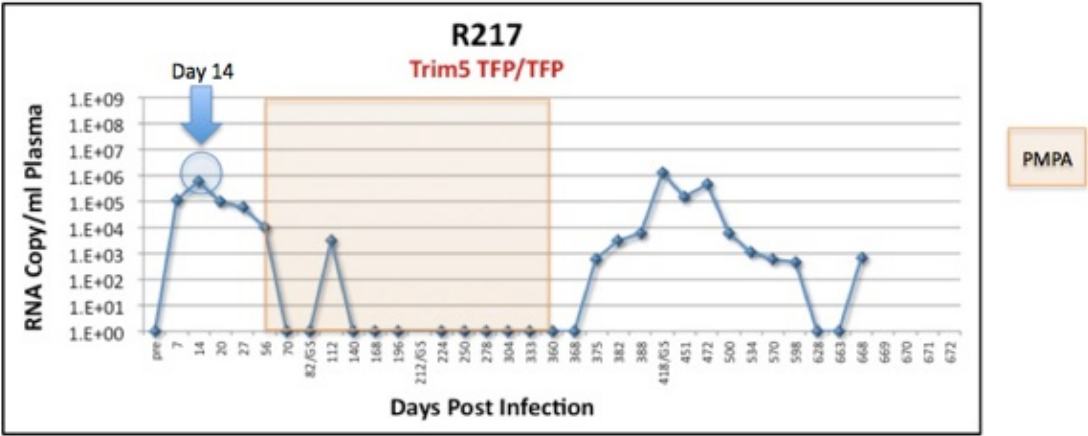
HIV/AIDS has been a major public health concern for more than 40 years. Rhesus macaques and SIV have been proven to be a valuable animal model for the study of this disease. New

chemotherapeutic targets are always needed due to the high mutation rate of the virus and the study of TRIM5 could provide this. The study of TRIM5 has also contributed to understanding the role that host genetics plays in the control of an HIV/SIV infection.

APPENDIX

IMMUNOTHERAPEUTIC TRIAL EARLY AND LATE TIME POINTS

A



B

CM9

SIVB670 Inoculum consensus	G	C	T	P	Y	D	I	N	Q	M	L
1_R217 (Day14)
3_R217 (Day14)
5_R217 (Day14)
2_R217 (Day14)
4_R217 (Day14)
9_R217 (Day14)
10_R217 (Day14)
7_R217 (Day14)
8_R217 (Day14)
6_R217 (Day14)

C

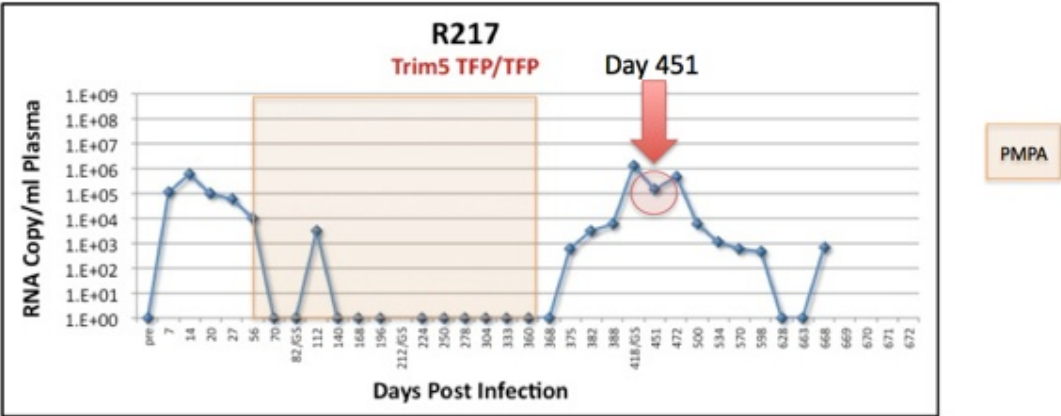
Trim5α Binding

SIVB670 Inoculum consensus	P	I	P	P	G	Q	L	R	E	P	R	G
1_R217 (Day14)
3_R217 (Day14)
5_R217 (Day14)
2_R217 (Day14)
4_R217 (Day14)
9_R217 (Day14)
10_R217 (Day14)
7_R217 (Day14)
8_R217 (Day14)
6_R217 (Day14)

Figure 14. R217 Early Time Point

(A) R217 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B

CM9

SIV8670 Inoculum consensus	G	C	T	P	Y	D	I	N	Q	M	L	N
6_R217 (Day451)	.	R
8_R217 (Day451)
7_R217 (Day451)
9_R217 (Day451)
10_R217 (Day451)	.	R
3_R217 (Day451)
4_R217 (Day451)
5_R217 (Day451)
2_R217 (Day451)
1_R217 (Day451)

C

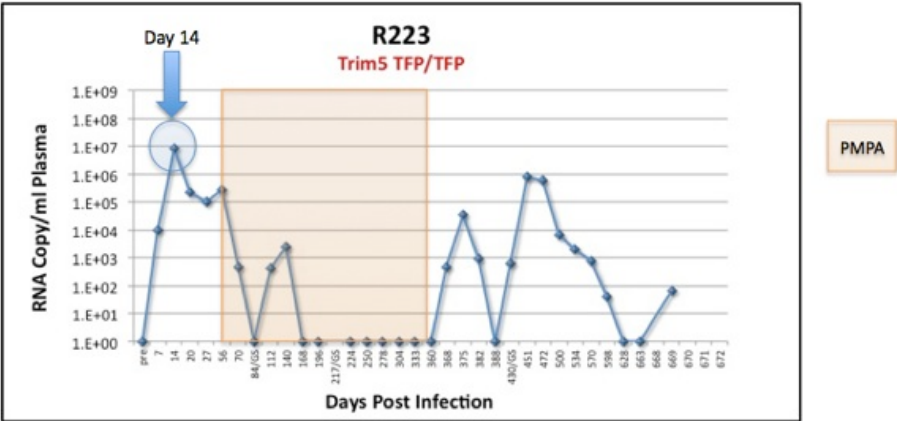
Trim5α Binding

SIV8670 Inoculum consensus	P	I	P	P	G	Q	L	R	E	P	R	G
6_R217 (Day451)	S	.
8_R217 (Day451)	S	.
7_R217 (Day451)	S	.
9_R217 (Day451)	S	.
10_R217 (Day451)	S	.
3_R217 (Day451)	S	.
4_R217 (Day451)	S	.
5_R217 (Day451)	S	.
2_R217 (Day451)	S	.
1_R217 (Day451)	S	.

Figure 15. R217 Late Time Point

(A) R217 Individual virus loads with Day 451 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 451. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B

CM9

SIVB670 Inoculum Consensus	G	C	T	P	Y	D	I	N	Q	M	L	N
4_R223 (Day14)
1_R223 (Day14)
3_R223 (Day14)
6_R223 (Day14)
9_R223 (Day14)
8_R223 (Day14)
7_R223 (Day14)
10_R223 (Day14)
5_R223 (Day14)
2_R223 (Day14)

C

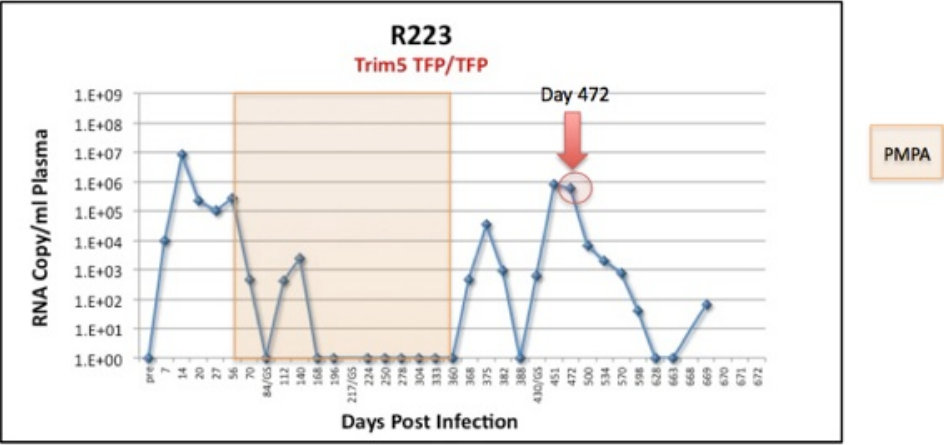
Trim5α Binding

SIVB670 Inoculum Consensus	P	I	P	P	G	Q	L	R	E	P	R	G
4_R223 (Day14)
1_R223 (Day14)
3_R223 (Day14)
6_R223 (Day14)
9_R223 (Day14)
8_R223 (Day14)
7_R223 (Day14)
10_R223 (Day14)	S	.
5_R223 (Day14)
2_R223 (Day14)	.	T

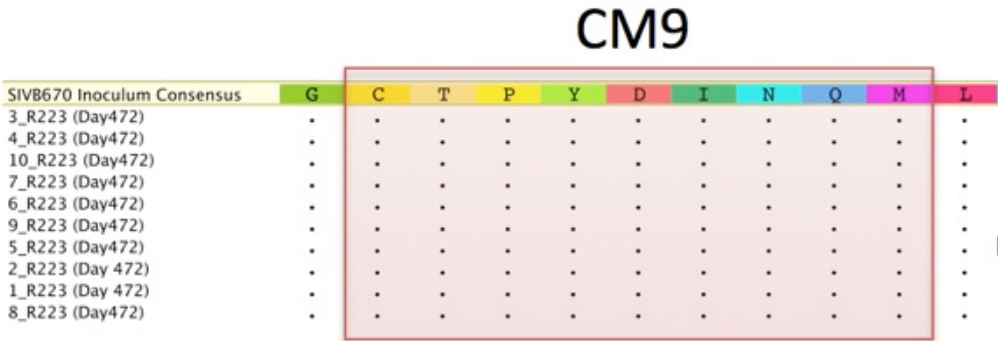
Figure 16. R223 Early Time Point

(A) R223 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



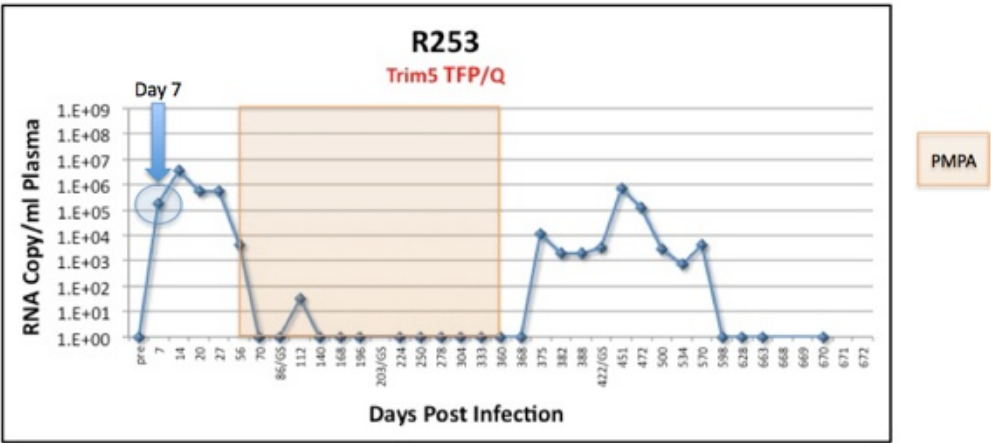
C



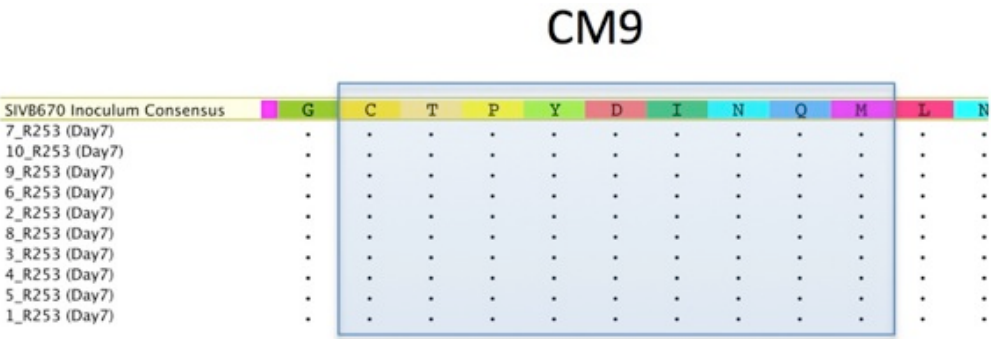
Figure 17. R223 Late Time Point

(A) R223 Individual virus loads with Day 472 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 472. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



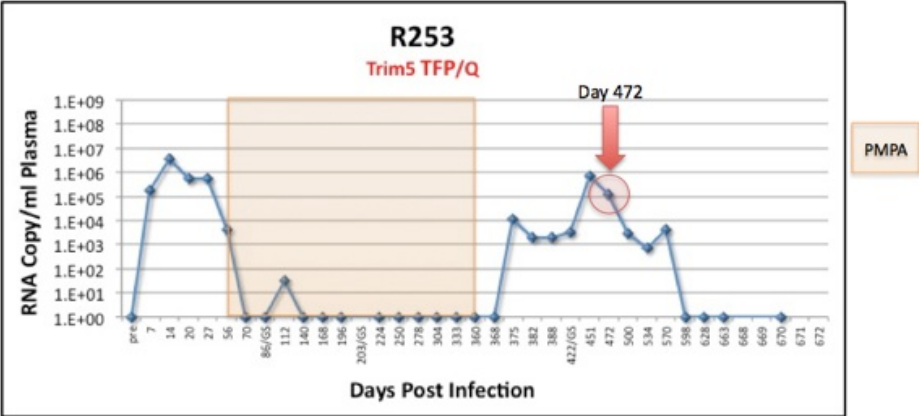
C



Figure 18. R253 Early Time Point

(A) R253 Individual virus loads with Day 7 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 7. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B

CM9

SIV8670 Inoculum Consensus	G	C	T	P	Y	D	I	N	Q	M	L	N
1_R253 (Day472)
2_R253 (Day472)
5_R253 (Day472)
4_R253 (Day472)
3_R253 (Day472)
7_R253 (Day472)
10_R253 (Day472)
8_R253 (Day472)
9_R253 (Day472)
6_R253 (Day472)

C

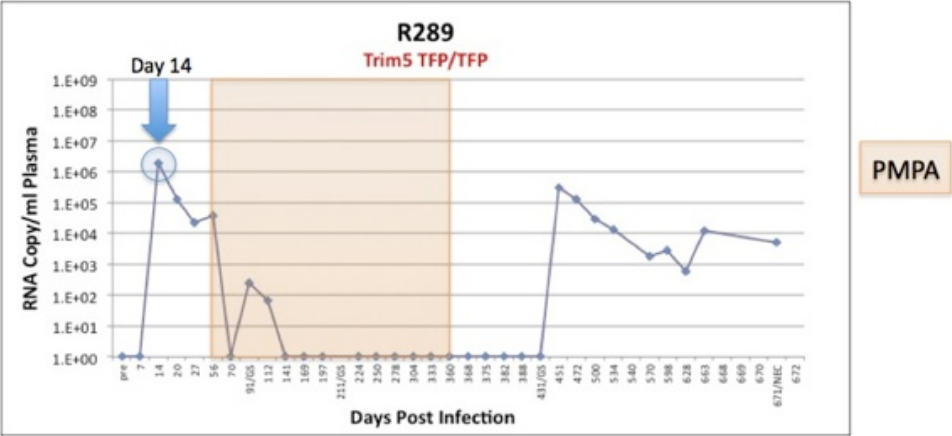
Trim5α Binding

SIV8670 Inoculum Consensus	P	I	P	P	G	Q	L	R	E	P	R	G
1_R253 (Day472)	G	.
2_R253 (Day472)	G	.
5_R253 (Day472)	G	.
4_R253 (Day472)	G	.
3_R253 (Day472)	G	.
7_R253 (Day472)	G	.
10_R253 (Day472)	G	.
8_R253 (Day472)	G	.
9_R253 (Day472)	G	.
6_R253 (Day472)	?	.	.	G	.

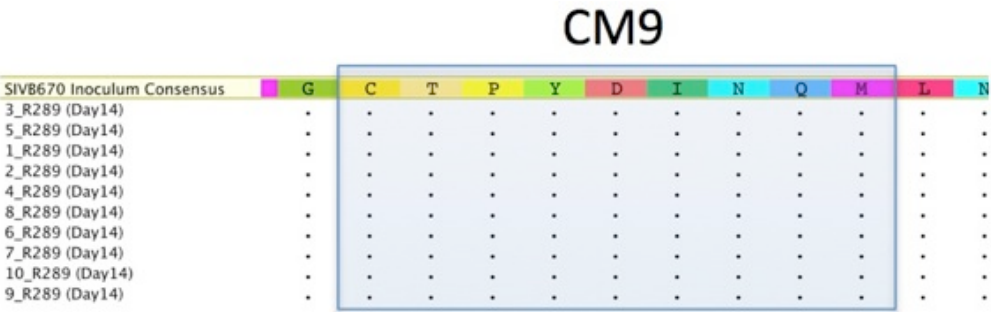
Figure 19. R253 Late Time Point

(A) R253 Individual virus loads with Day 472 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 472. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



C

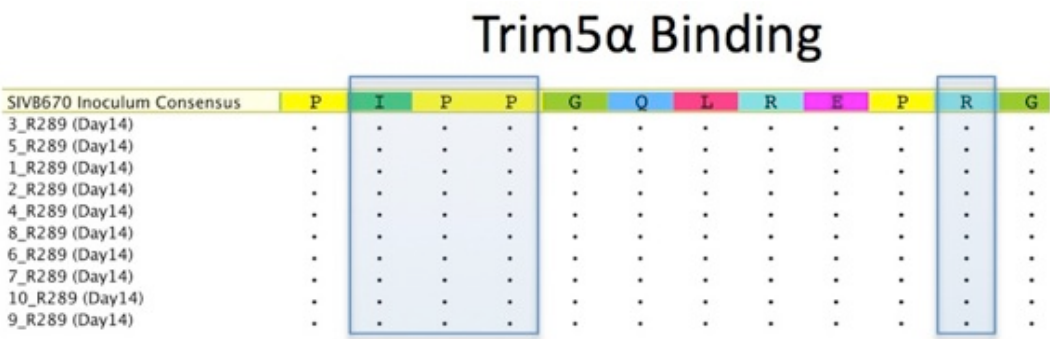
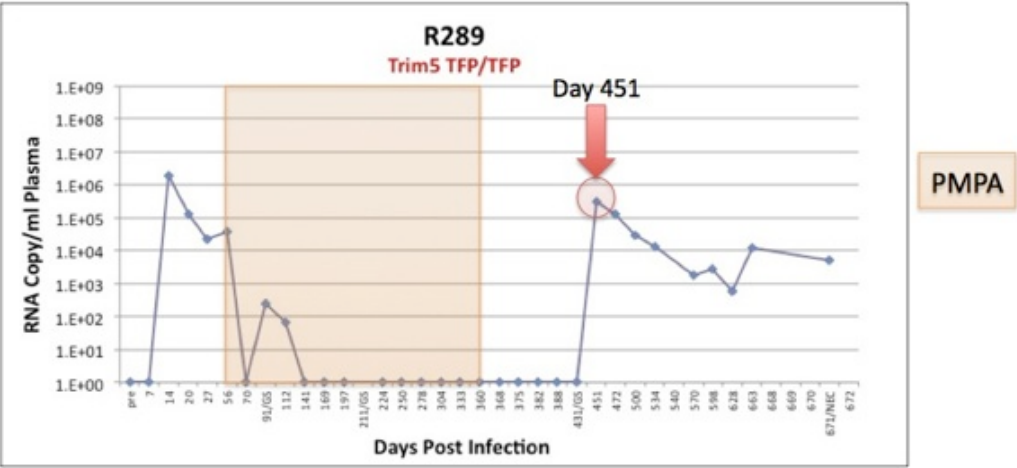


Figure 20. R289 Early Time Point

(A) R289 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B

CM9

SIVB670 Inoculum Consensus	G	C	T	P	Y	D	I	N	Q	M	L	N
6_R289 (Day451)	.	.	.	S
9_R289 (Day451)	.	.	.	S
7_R289 (Day451)	.	.	.	S
8_R289 (Day451)	.	.	.	S
10_R289 (Day451)	.	.	.	S
1_R289 (Day451)	.	.	.	S
2_R289 (Day451)	.	.	.	S
5_R289 (Day451)	.	.	.	S
3_R289 (Day451)	.	.	.	S
4_R289 (Day451)	.	.	.	S

C

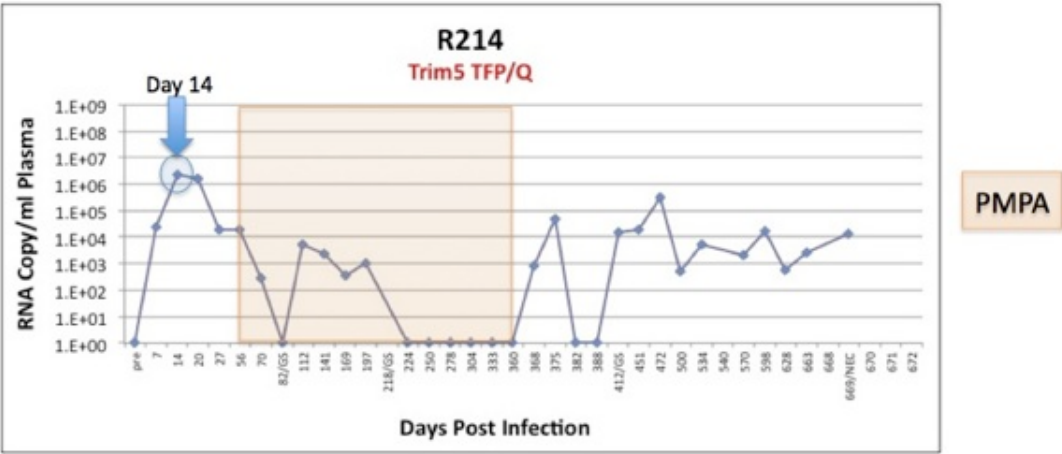
Trim5α Binding

SIVB670 Inoculum Consensus	P	I	P	P	G	Q	L	R	E	P	R	G
6_R289 (Day451)	S	.
9_R289 (Day451)	S	.
7_R289 (Day451)	S	.
8_R289 (Day451)	S	.
10_R289 (Day451)	S	.
1_R289 (Day451)	S	.
2_R289 (Day451)	S	.
5_R289 (Day451)	S	.
3_R289 (Day451)	S	.
4_R289 (Day451)	S	.

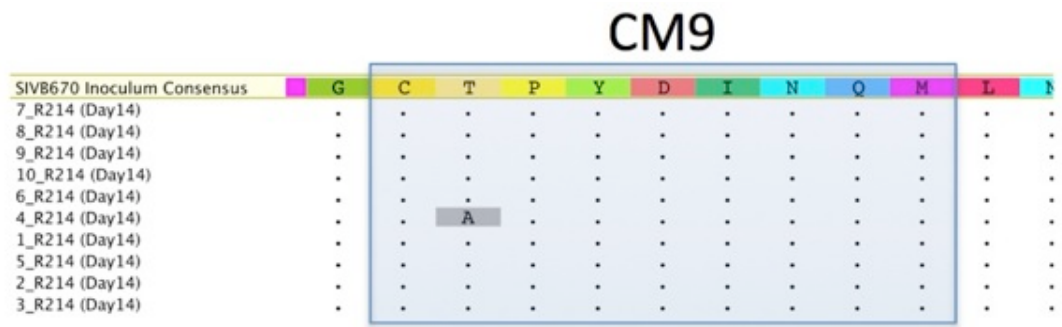
Figure 21. R289 Late Time Point

(A) R289 Individual virus loads with Day 451 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 451. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



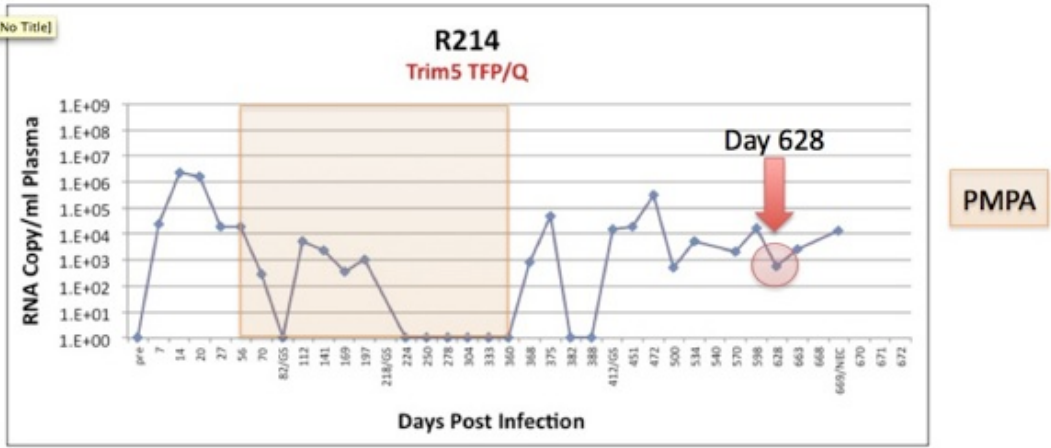
C



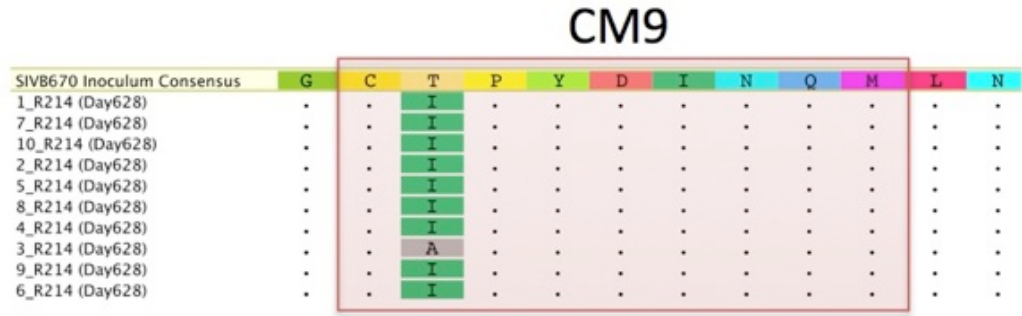
Figure 22. R214 Early Time Point

(A) R214 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C

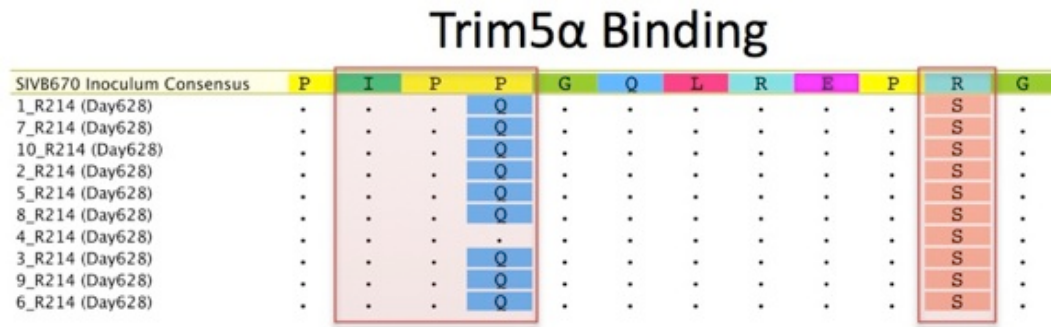
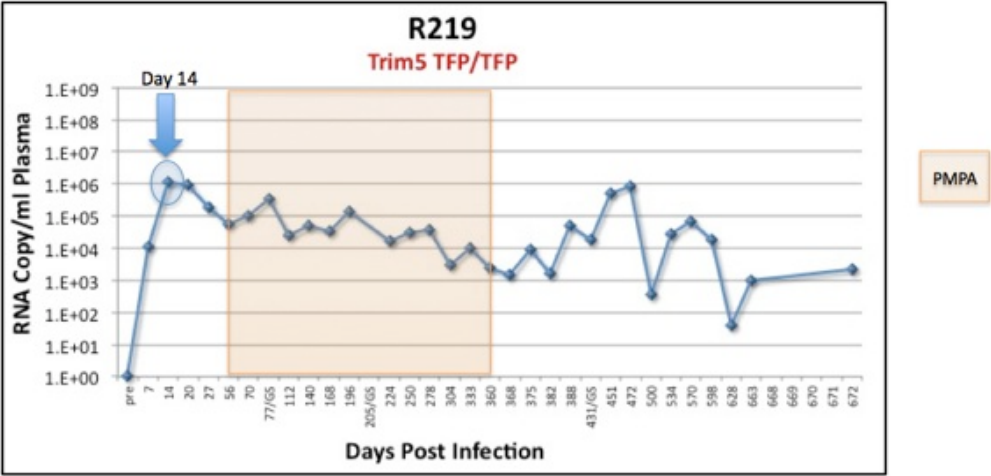


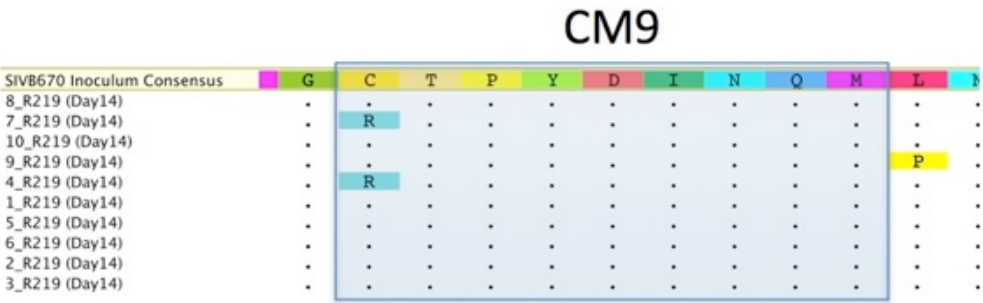
Figure 23. R214 Late Time Point

(A) R214 Individual virus loads with Day 628 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 628. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



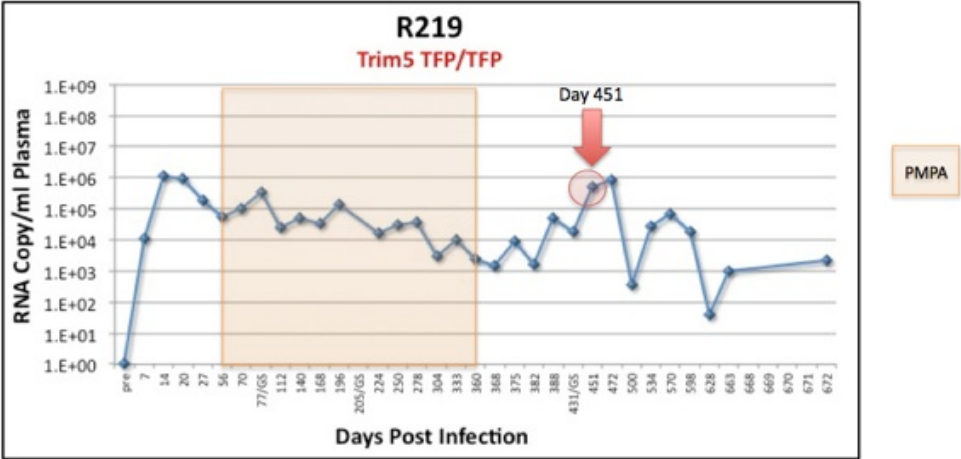
C



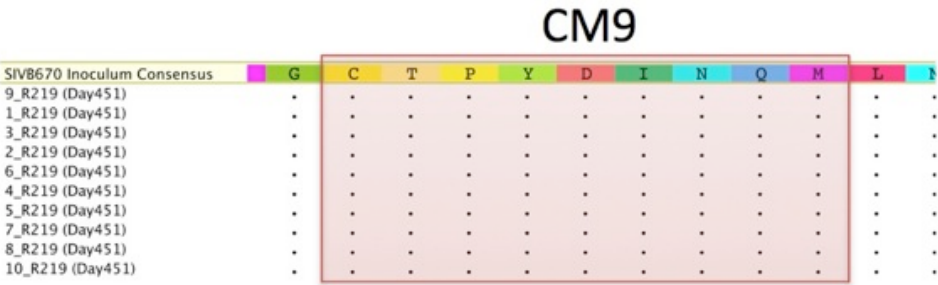
Figure 24. R219 Early Time Point

(A) R219 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C

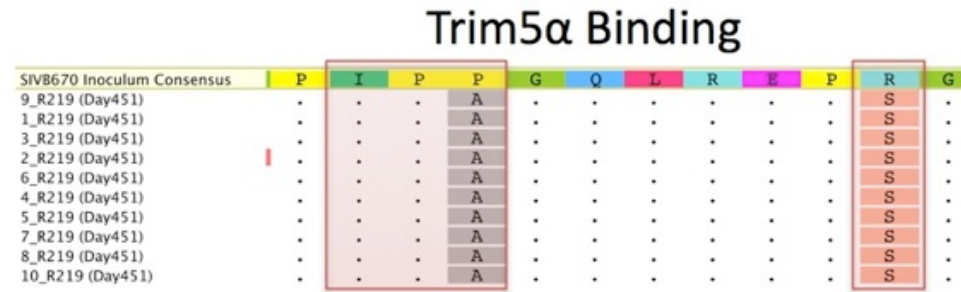
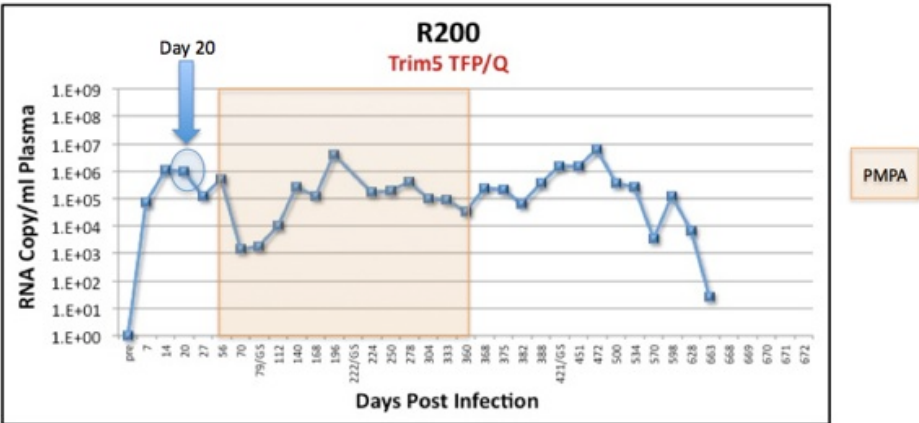


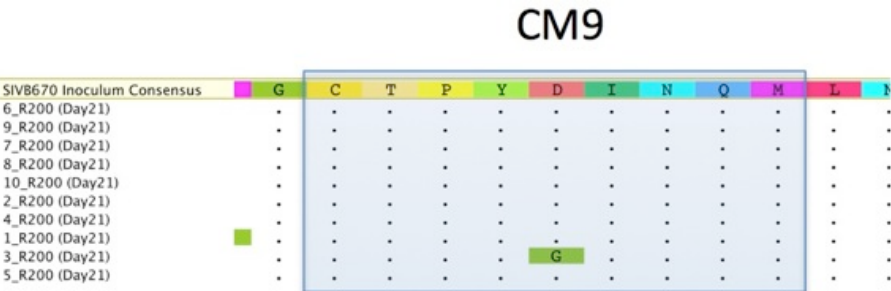
Figure 25. R219 Late Time Point

(A) R219 Individual virus loads with Day 451 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 451. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



C

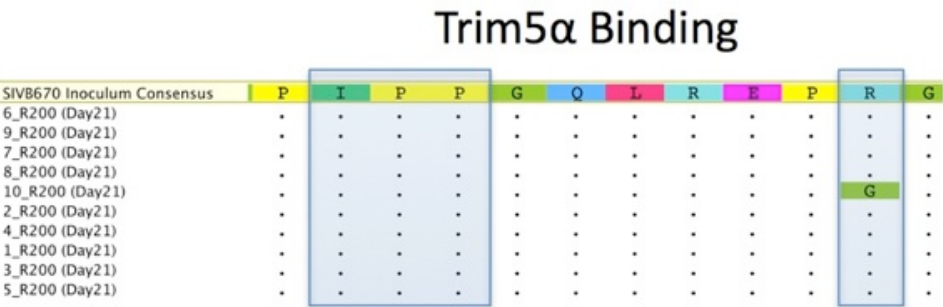
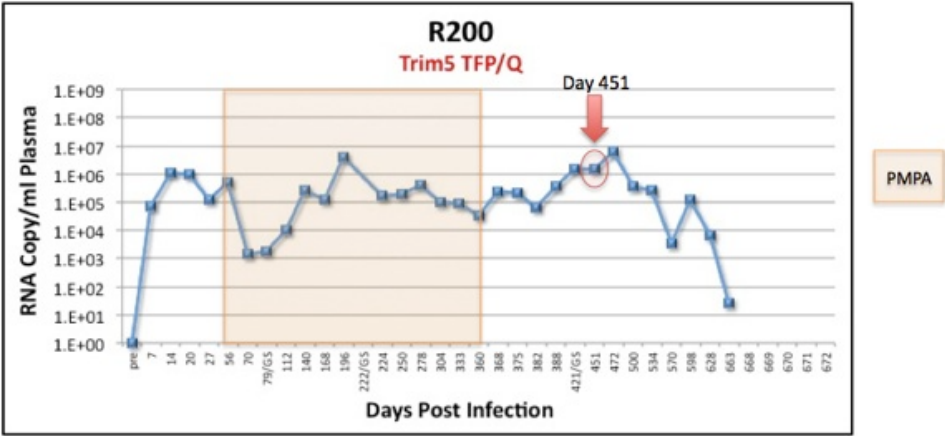


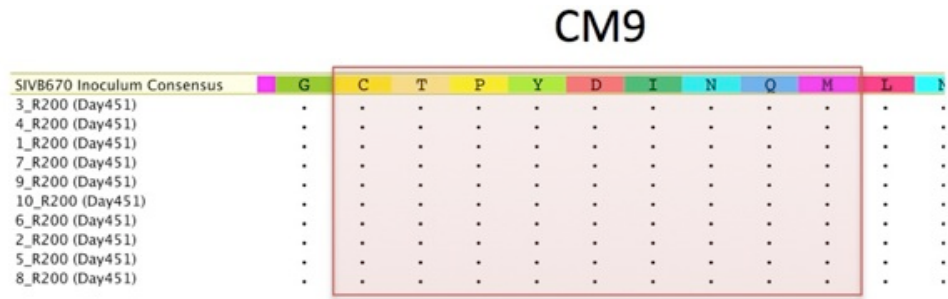
Figure 26. R200 Early Time Point

(A) R200 Individual virus loads with Day 21 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 21. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C

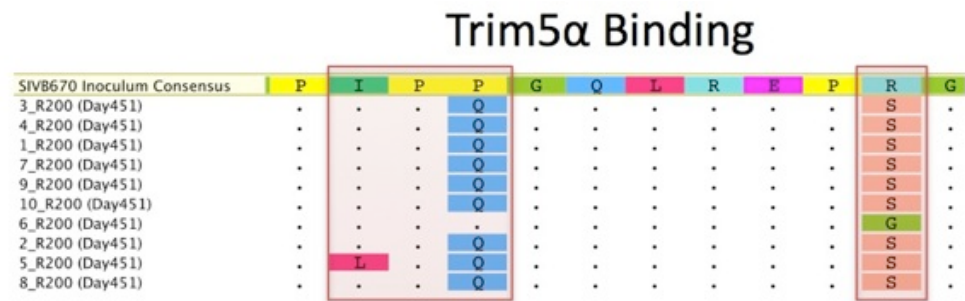
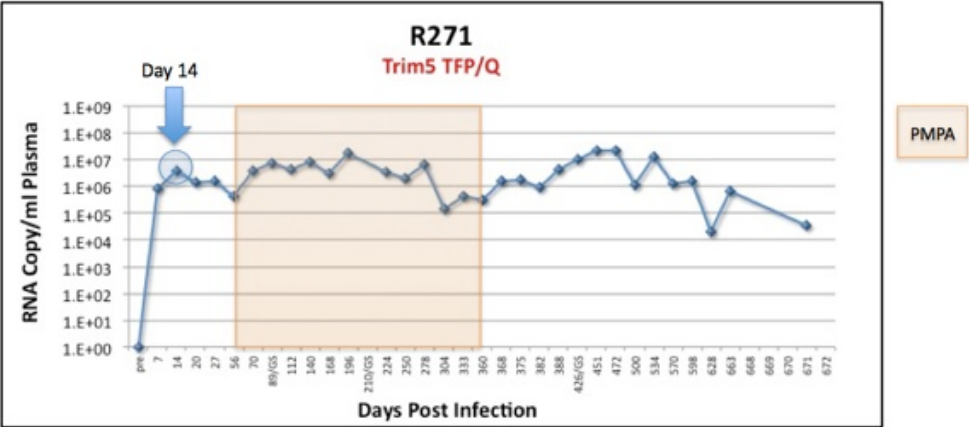


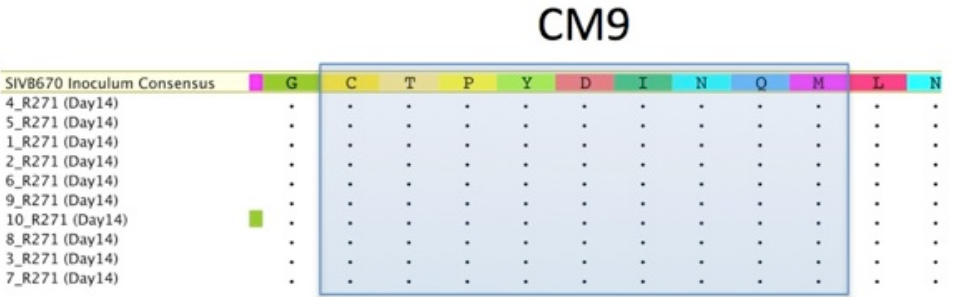
Figure 27. R200 Late Time Point

(A) R200 Individual virus loads with Day 451 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 451. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



C

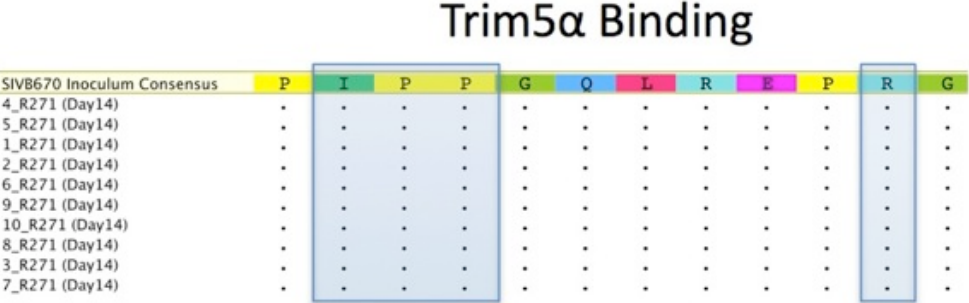
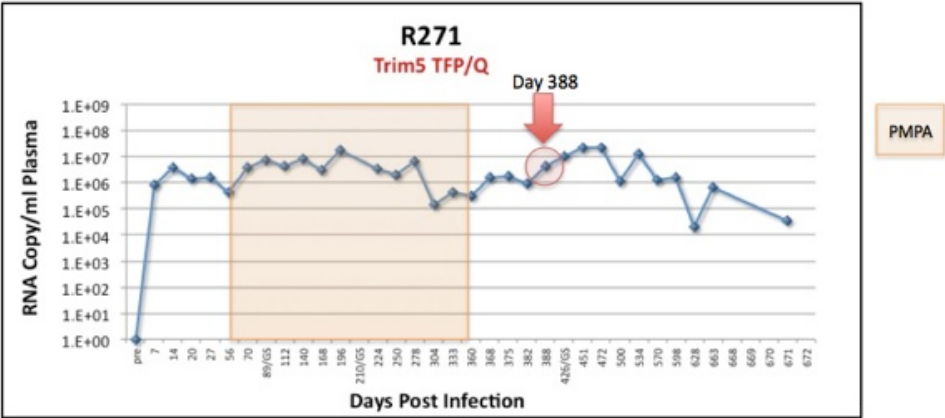


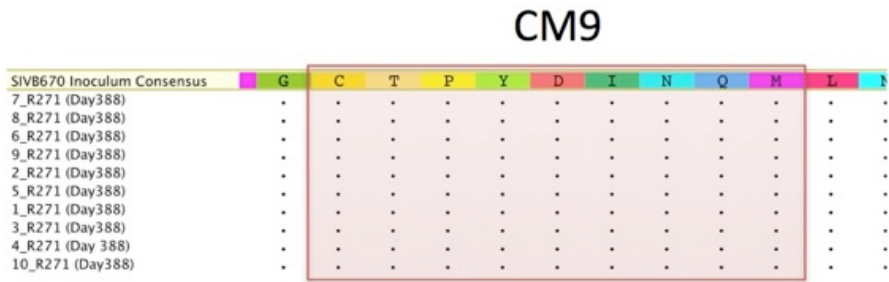
Figure 28. R271 Early Time Point

(A) R271 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



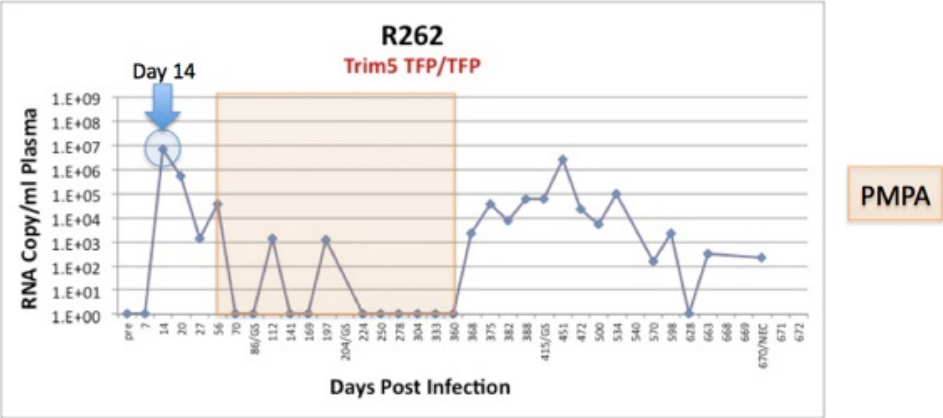
C



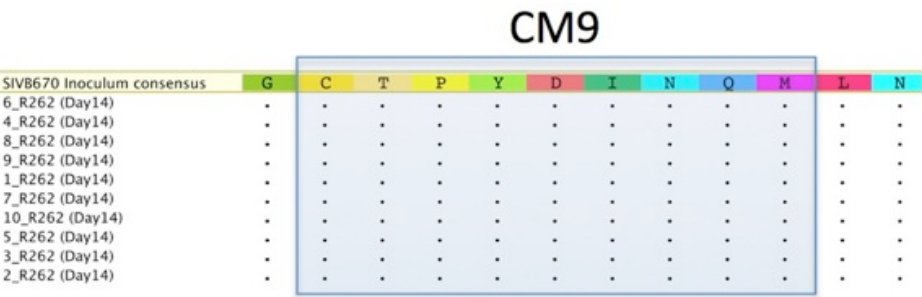
Figure 29. R271 Late Time Point

(A) R271 Individual virus loads with Day 388 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 388. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



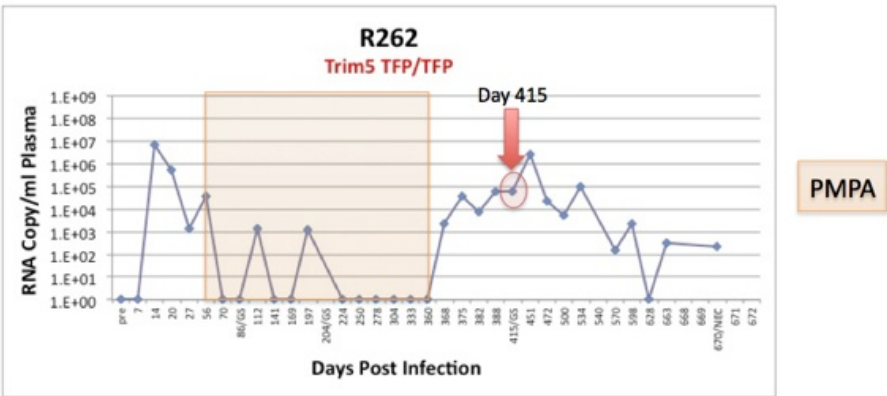
C



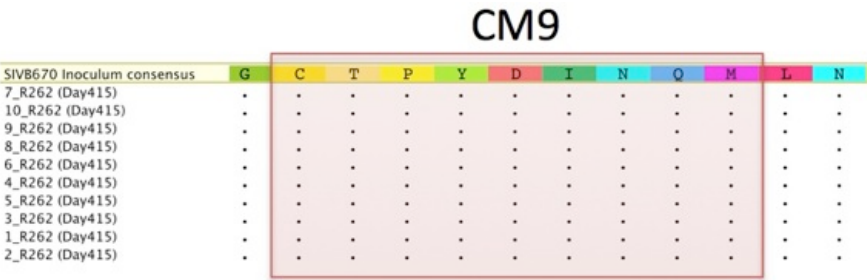
Figure 30. R262 Early Time Point

(A) R262 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



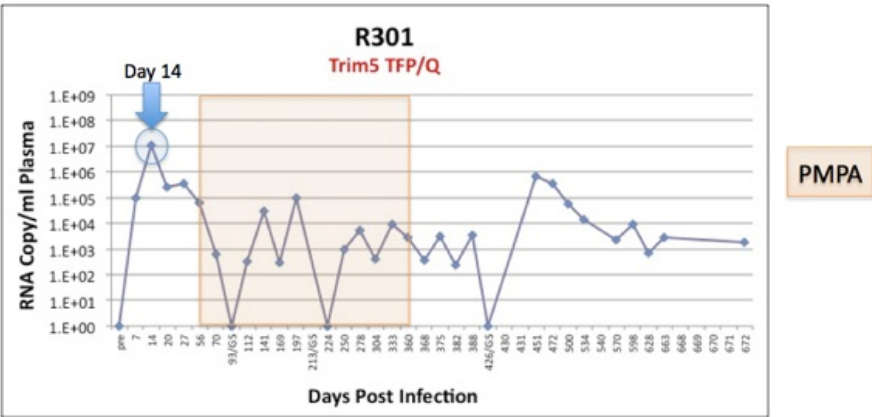
C



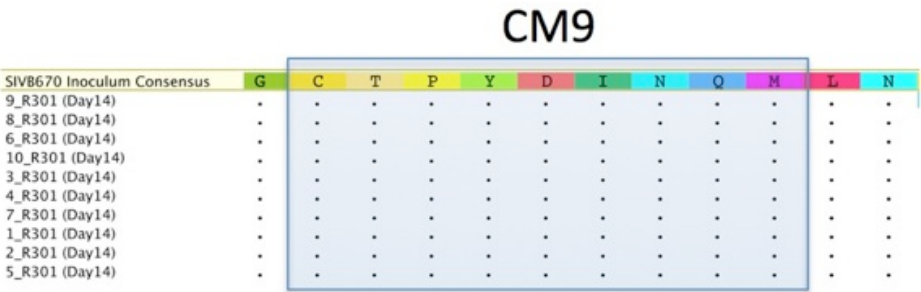
Figure 31. R262 Late Time Point

(A) R262 Individual virus loads with Day 415 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 415. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



C

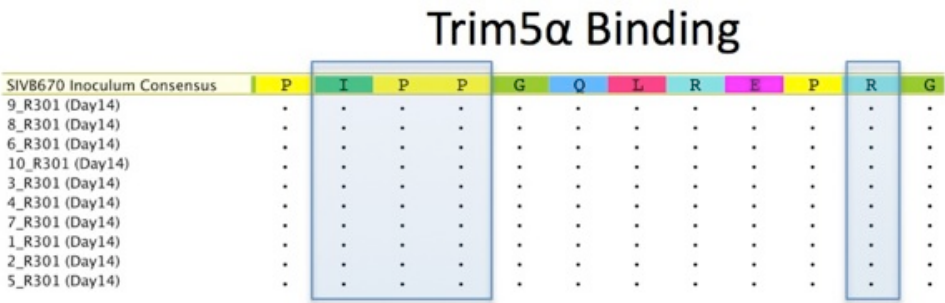
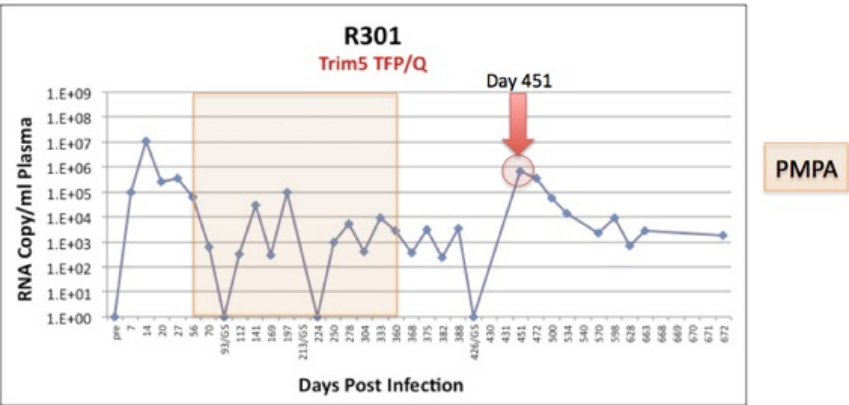


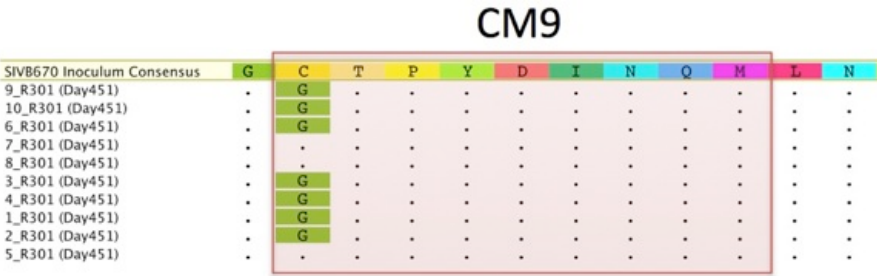
Figure 32. R301 Early Time Point

(A) R301 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C

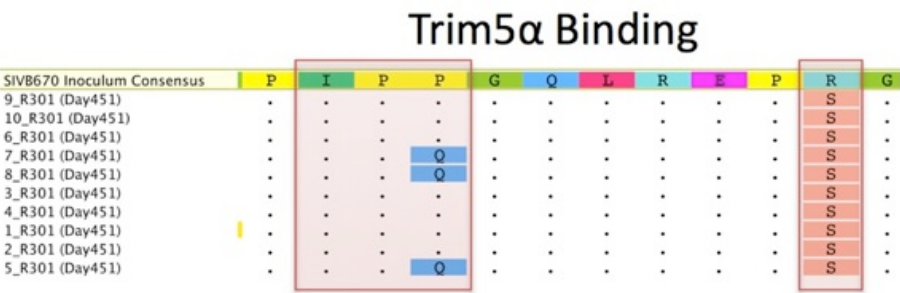
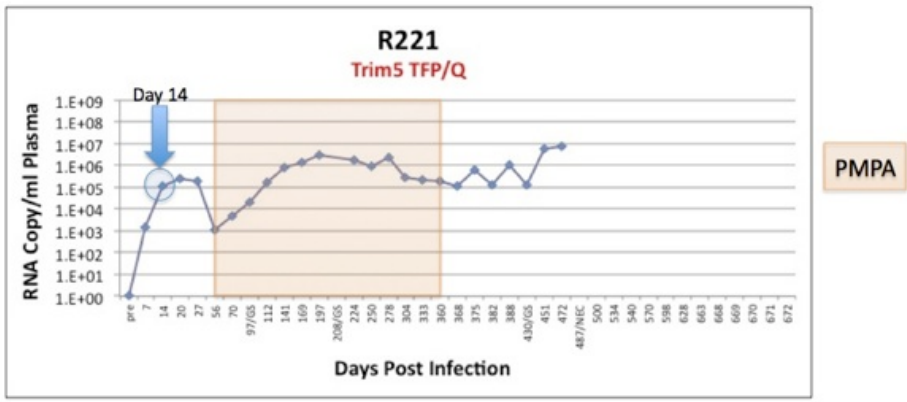


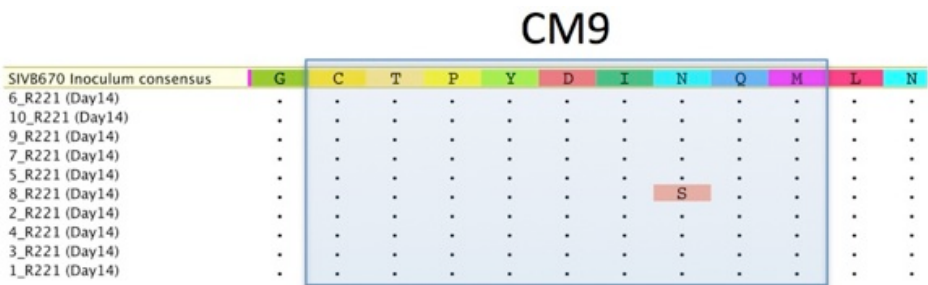
Figure 33. R301 Late Time Point

(A) R301 Individual virus loads with Day 451 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 451. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



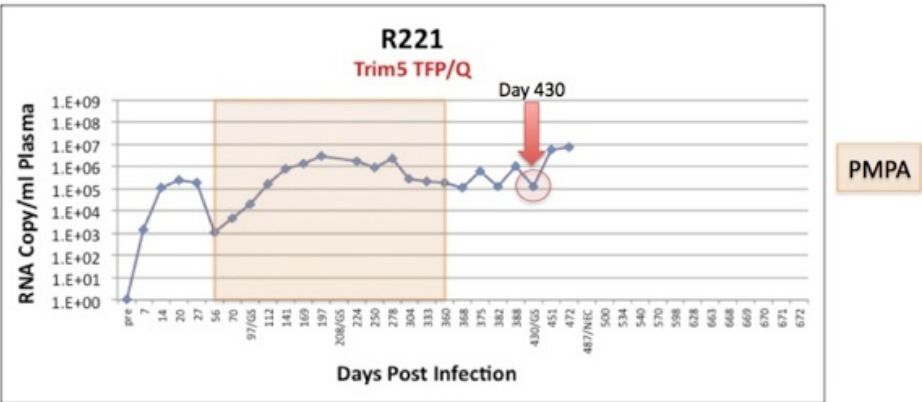
C



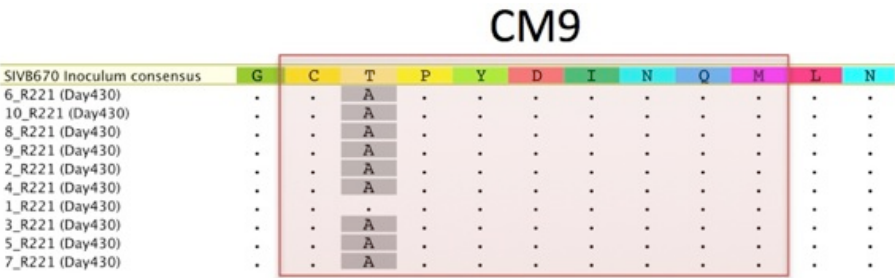
Figure 34. R221 Early Time Point

(A) R221 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C

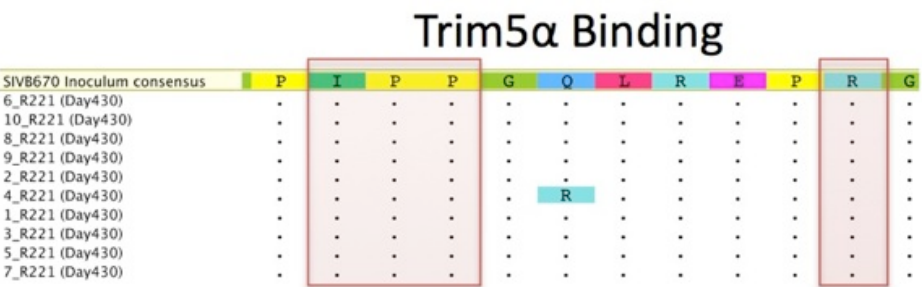
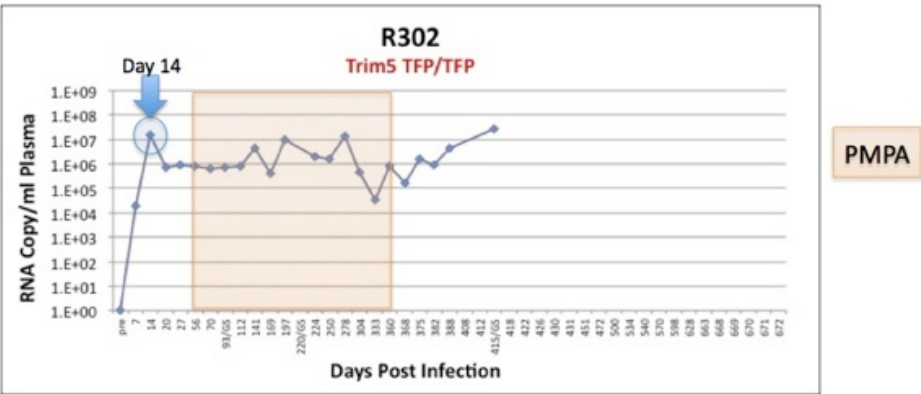


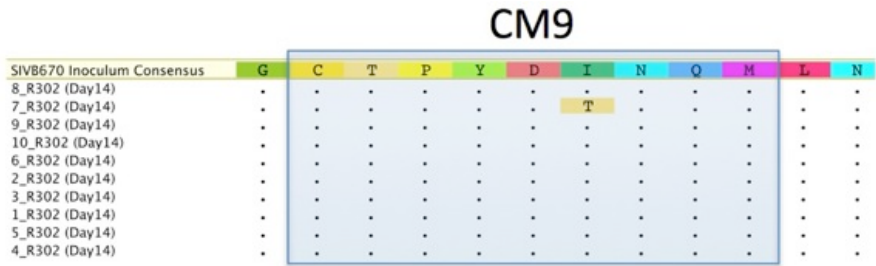
Figure 35. R221 Late Time Point

(A) R221 Individual virus loads with Day 430 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 430. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



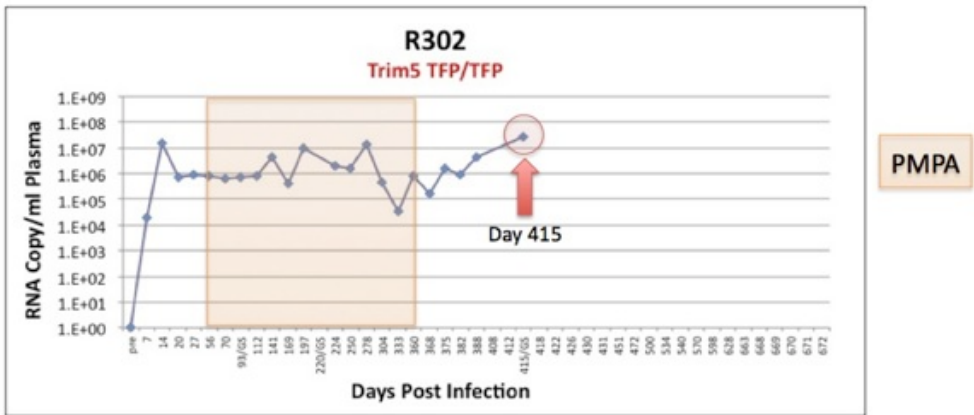
C



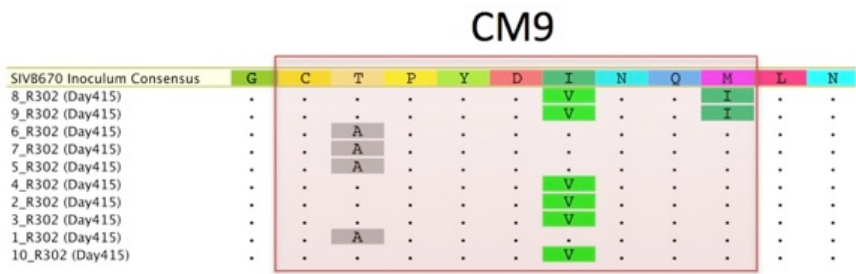
Figure 36. R302 Early Time Point

(A) R302 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C

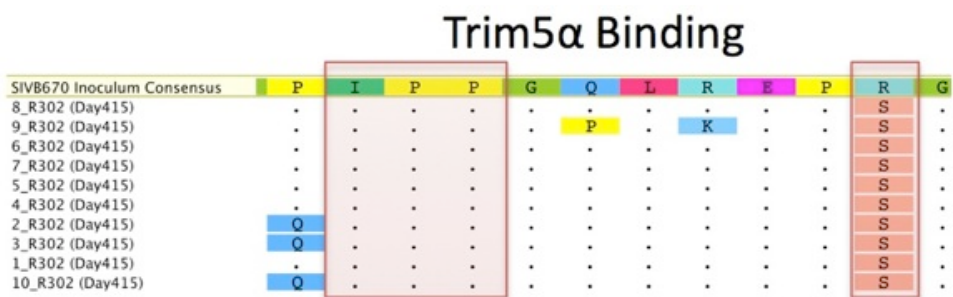
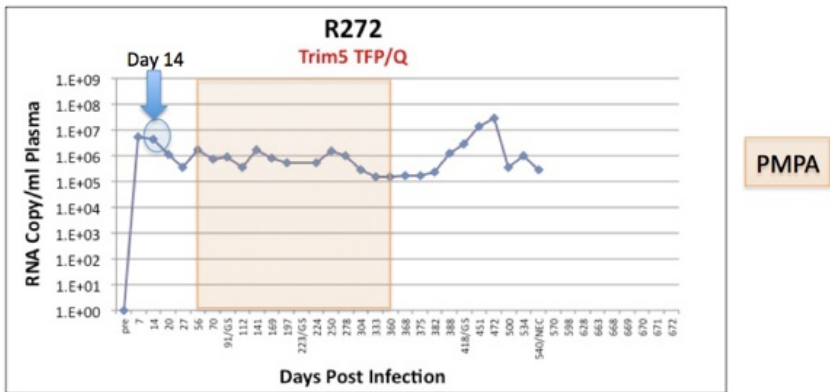


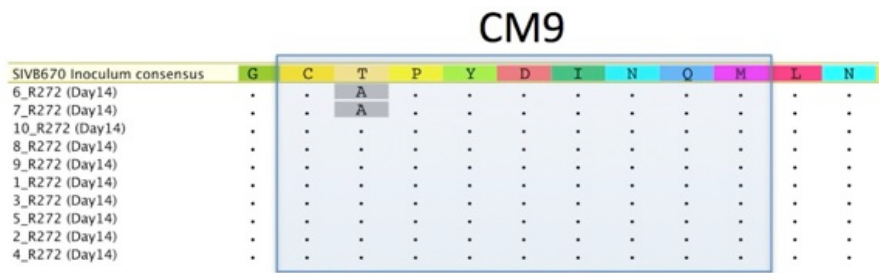
Figure 37. R302 Late Time Point

(A) R302 Individual virus loads with Day 415 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 415. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



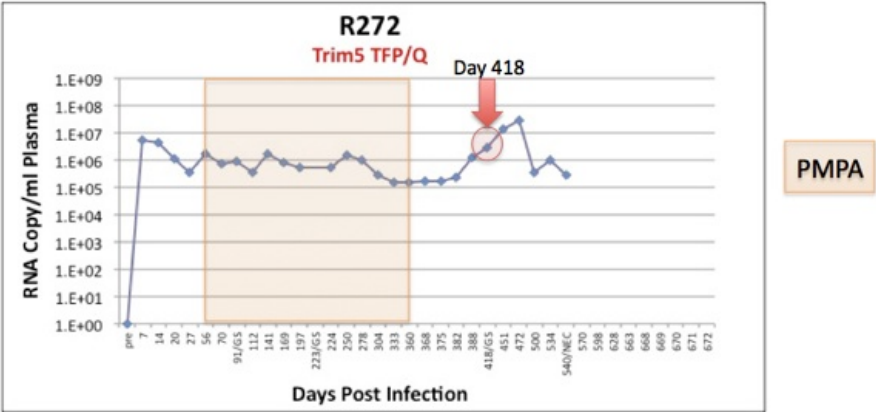
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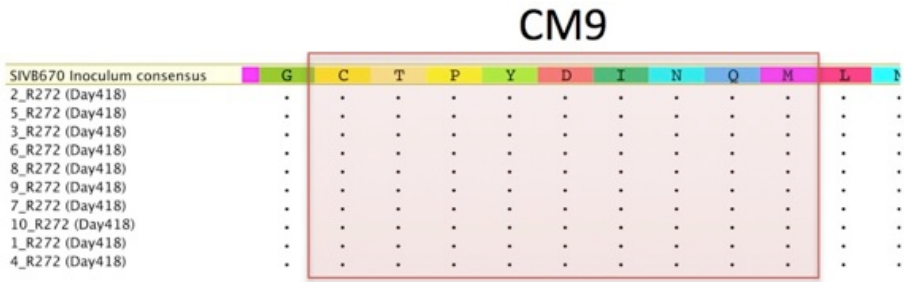
Figure 38. R272 Early Time Point

(A) R272 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C

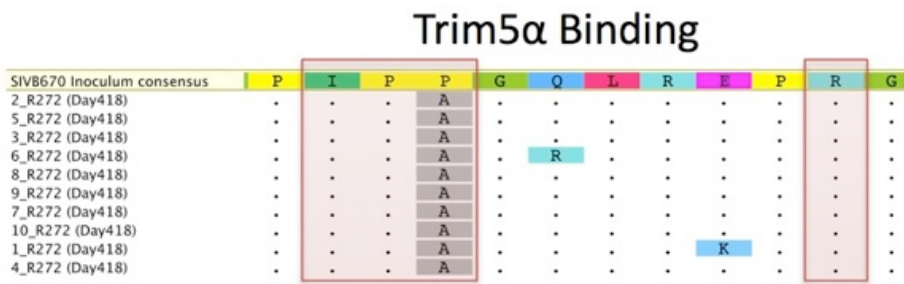
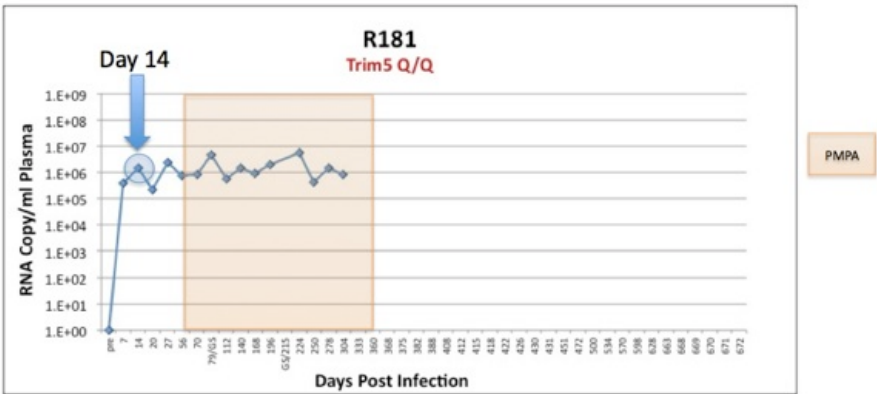


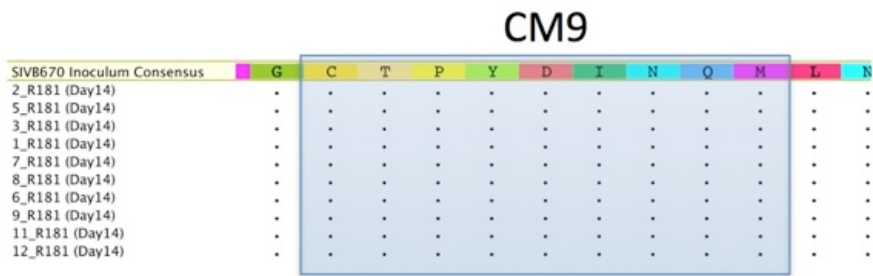
Figure 39. R272 Late Time Point

(A) R272 Individual virus loads with Day 418 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 418. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



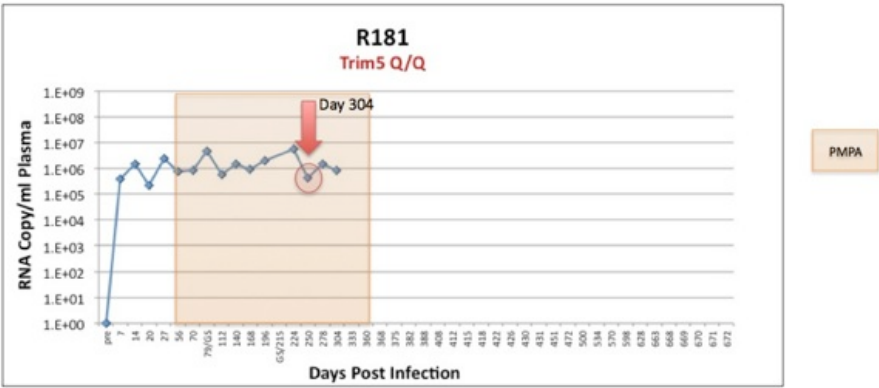
C



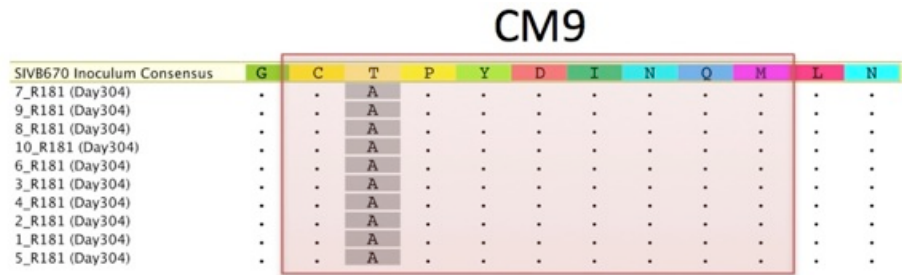
Figure 40. R181 Early Time Point

(A) R181 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C

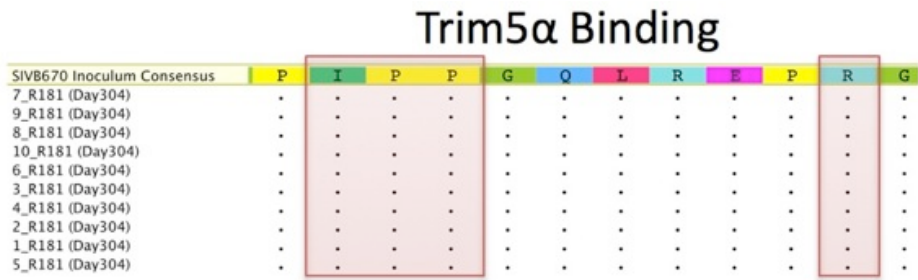
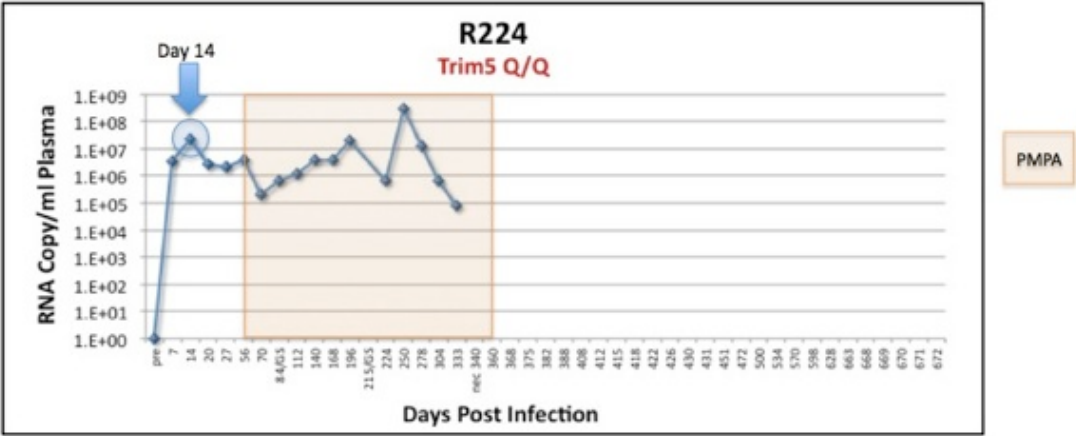


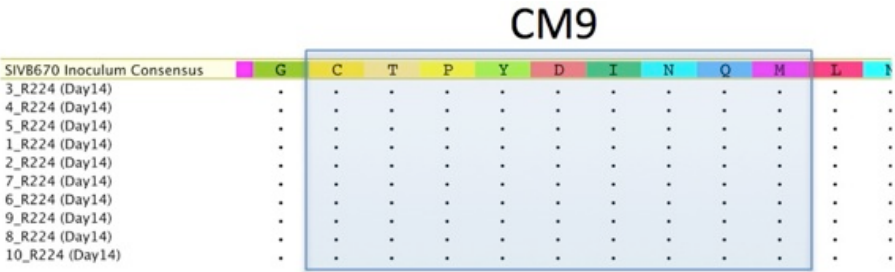
Figure 41. R181 Late Time Point

(A) R181 Individual virus loads with Day 304 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 304. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

A



B



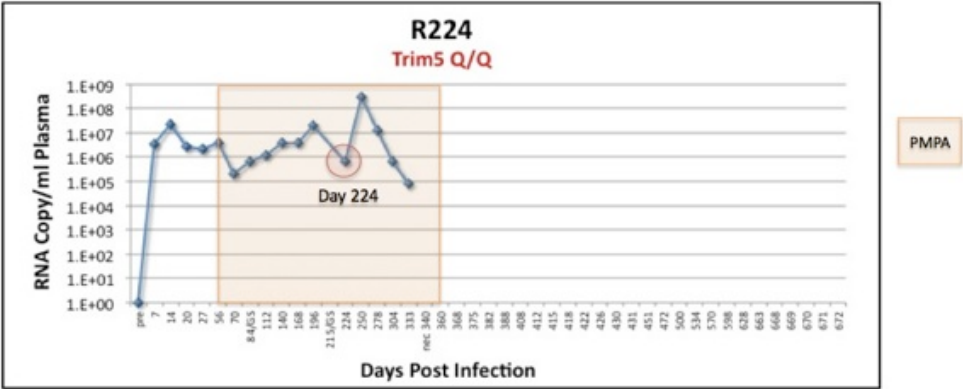
C



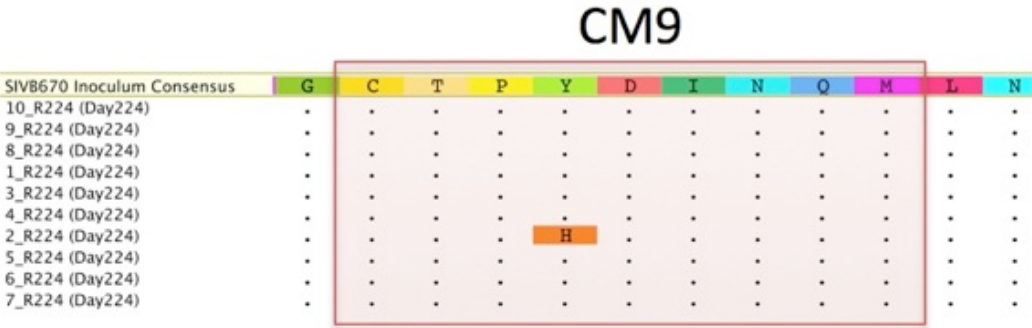
Figure 42. R224 Early Time Point

(A) R224 Individual virus loads with Day 14 highlighted in blue. (B) SIV GAG-CM9 sequences of 10 clones from Day 14. Epitope highlighted in blue. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in blue.

A



B



C



Figure 43. R224 Late Time Point

(A) R224 Individual virus loads with Day 224 highlighted in red. (B) SIV GAG-CM9 sequences of 10 clones from Day 224. Epitope highlighted in red. (C) SIV TRIM5α binding site sequences of same 10 clones from B. Restriction sites highlighted in red.

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