

***In vitro* and *in vivo* characterization of 239T1,
an R5X4 derivative of SIVmac239**

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A. Specific Aims.

Acquired Immunodeficiency Syndrome (AIDS) was responsible for over 3 million deaths in 2005, and over 40 million people are currently infected with the etiological agent of AIDS, human immunodeficiency virus (HIV). HIV shares a number of biological and structural features with simian immunodeficiency virus (SIV), making SIV a powerful tool for studying HIV infection. Both HIV and SIV typically enter permissive cells through sequential interactions of the viral envelope glycoprotein (Env) with two cellular receptors: CD4 and a chemokine coreceptor. CCR5 and CXCR4 are the most relevant of these chemokine coreceptors for HIV infection *in vivo*. Viruses that utilize CCR5 for entry (R5 viruses) normally predominate during the early stages of HIV infection, however viruses that utilize CXCR4 (R5X4 or X4 viruses) arise in ~50% of AIDS patients. The appearance of R5X4 or X4 viruses from an R5 population is known as coreceptor switching and is associated with advanced disease. Although nearly all SIV strains characterized to date utilize CCR5, SIVs that can efficiently utilize CXCR4 have been very rarely reported. Because SIV infection of non-human primates is the most commonly used model for *in vivo* HIV infection and pathogenesis, this paucity of well-characterized R5X4 or X4 SIVs limits our understanding of the acquisition of CXCR4-utilization and its pathogenic outcomes.

We have developed an R5X4 derivative of SIVmac239, a cloned virus that is pathogenic in rhesus macaques. This derivative of SIVmac239, termed 239T1, acquired the ability to utilize CXCR4 through *in vitro* adaptation to efficiently infect the CD4⁺ CXCR4⁺ SupT1 cell line. We hypothesize that specific amino acid changes in the Env glycoprotein of SIVmac239 enabled the virus to gain utilization of CXCR4 as a coreceptor for entry. We propose that these changes will alter the host range and increase the neutralization sensitivity of 239T1 compared with SIVmac239 and that *in vivo* replication of 239T1 as a CXCR4-utilizing virus will be restricted in rhesus macaques.

Specific Aim 1: Molecular, biological, and immunological characterization of 239T1. We have cloned and sequenced functional envelope genes (*env*) from 239T1. Using the sequences of the 239T1 *env* clones and the sequence of the parental SIVmac239 *env*, we have identified *env* mutations acquired by 239T1. We will use site-directed mutagenesis to mutate both the 239T1 and SIVmac239 *envs* to determine which of the 239T1 *env* mutations are necessary and

sufficient for CXCR4-utilization. We will create a recombinant SIVmac239 virus bearing the 239T1 envelope and evaluate how the 239T1 Env impacts viral host range, including infection of primary rhesus cells. Finally, we will evaluate the neutralization sensitivity of recombinant SIVmac239 bearing the 239T1 Env.

Specific Aim 2: Examination of *in vivo* infection of rhesus macaques with 239T1. We will infect rhesus macaques with wild type SIVmac239 or with recombinant SIVmac239 containing a cloned, R5X4 239T1 Env. The infected animals will be monitored for viral load and CD4⁺ T cell levels in the blood, lymph nodes, and gut-associated lymphoid tissue to characterize the pathogenic outcomes of infection with R5 (wild type) vs. R5X4 SIVmac. Over the course of infection, we will extract and sequence *env* clones from the infected animals to assess if selection pressures exist during *in vivo* infection that will drive mutations in the 239T1 *env*. We will utilize the cell-cell fusion assay to assess whether these mutations affect the coreceptor utilization of the Envs. We hypothesize that such pressures in infected rhesus macaques might select for mutations that lead to reversion of the viral population to an R5 phenotype.

B. Background and Significance

Since it was first recognized in the early 1980s, human immunodeficiency virus (HIV) has been responsible for over 25 million deaths, ranking it as one of the most deadly pandemics in recorded history (1). Despite efforts to increase HIV awareness and prevention, nearly 5 million people were newly infected with HIV in 2005, bringing the global total of living infected individuals to ~40 million. Although numerous antiretroviral drugs are currently in use and are becoming more readily available in many regions of the world, HIV disease still claimed the lives of over 3 million people this past year (1). The development of an HIV vaccine or new therapeutic anti-retroviral drugs is therefore critical and will require a more complete understanding of HIV infection, pathogenesis, and immunity. Because HIV does not productively infect old world monkeys and does not cause disease in infected apes (2), simian immunodeficiency virus (SIV) will continue to be an important tool for modeling HIV infection *in vivo*.

SIV as a model of HIV. There is strong genetic evidence to suggest that HIV-1 and HIV-2 arose as the result of zoonotic transmission of SIV into the human population in Africa, the region of the globe that has been hardest hit by HIV infection (3-6). While the study of SIV is relevant because it is the likely source of the HIV pandemic, there are also several characteristics of SIV infection of non-human primates that make SIV of great interest for HIV research. First, non-human primates naturally infected with SIV very rarely demonstrate any signs of disease despite ongoing viral replication (7-9). While the reasons that naturally infected animals can support high levels of viral replication without disease require further investigation, recent evidence suggests that natural SIV primate hosts do not suffer the deleterious chronic immune activation observed in HIV-infected individuals (10-12). Second, like HIV-infected humans, members of naïve primate species that become infected with SIV suffer CD4⁺ T cell depletion and an AIDS-like immunodeficiency (13, 14). This ability to induce an AIDS-like disease by infecting non-human primates with SIV, a virus genetically related to HIV, has presented researchers with a powerful tool for examining HIV infection and pathogenesis through controlled experiments in a manipulable *in vivo* model system. This model system allows researchers to examine the impact of defined viral properties by infecting animals with molecularly cloned viruses eliminating the potential confounding effects of a mixed viral population, or “quasi-species”, upon initial infection. The most widely studied and well-characterized SIV molecular clone is SIVmac239, a virus that is pathogenic in rhesus macaques (15). In our Preliminary Results, we describe the development and initial characterizations of 239T1, a virus derived from SIVmac239.

Coreceptor Utilization. HIV and SIV share many structural and biological features, including mode of viral entry. Both HIV and SIV enter target cells through interactions of the viral envelope glycoprotein (Env) with CD4 and a coreceptor on the surface of target cells. Although numerous G protein-coupled receptors (GPCR) have been shown to function as coreceptors for HIV infection *in vitro*, only two, the CCR5 and CXCR4 chemokine receptors, appear to be important for HIV infection *in vivo* (16). HIV isolates are therefore grouped into 3 categories: those that use CCR5 for entry are termed R5, those that use CXCR4 are termed X4, and viruses that can use both are termed R5X4. Normally, R5 viruses predominate during the early asymptomatic phase of infection, suggesting that they play a key role in HIV transmission (17, 18). The importance of R5 viruses for transmission is underscored by the fact that individuals

homozygous for a 32 base pair deletion in the CCR5 gene ($\Delta 32$), which renders the coreceptor nonfunctional, are highly resistant to HIV infection (19, 20). While R5 viruses often persist into the late stages of infection and disease, R5X4 and/or X4 viruses appear in ~50% of AIDS patients (17, 21). The appearance of R5X4 or X4 viruses has been associated with advanced disease and more rapid destruction of CD4⁺ T cells in peripheral blood. However, it is unclear if CXCR4-using viruses actually cause more severe disease or if they simply flourish in the presence of an exhausted immune system.

As is the case for many HIV strains, CCR5 is the main entry coreceptor for the vast majority of SIV strains characterized to date (22, 23). In contrast to HIV, however, R5X4 or X4 SIV strains have been very rarely reported. It is not known why R5X4 or X4 SIV appears so infrequently, however rhesus CXCR4 is functional for utilization by X4 HIV indicating that there should be no block against CXCR4 use *per se* in infected macaques (24). This suggests that the changes required for SIV to gain CXCR4 utilization may lead to a virus that is simply less fit or more exposed to immune system control *in vivo*. In Specific Aim 1, we will examine what changes in the SIVmac239 envelope glycoprotein permit the virus to gain the ability to utilize CXCR4 and how these changes affect sensitivity to neutralizing antibodies. Elucidating why R5X4 or X4 SIVs are so rare might improve our understanding of why CXCR4-using viruses do arise in many HIV-infected humans.

Because R5X4 or X4 SIV strains have been so rare and because those that have been described have been primarily non-pathogenic isolates from naturally infected primate hosts (25-27), *in vivo* studies of pathogenic X4 viruses have relied on the use of chimeric simian human immunodeficiency viruses (SHIV). These chimeric viruses usually comprise the HIV *tat*, *rev*, and *env* genes in an SIV backbone. While SHIVs are a useful tool for pathogenicity studies and for testing vaccine design strategies *in vivo*, they may not be useful for elucidating why SIVs that can utilize CXCR4 are so uncommon. Furthermore, it is unclear if the fulminant disease and reproducibly high replication rates of X4 SHIVs (28, 29) accurately models infection with X4 HIV, which does not always demonstrate high levels of viral replication or rapid disease progression in infected CCR5- $\Delta 32$ homozygous individuals (30-33). In Specific Aim 2, we will examine *in vivo* infection of rhesus macaques with an R5X4 SIV derived from the pathogenic SIVmac239 molecular clone and how pressures *in vivo* drive envelope evolution and coreceptor utilization.

C. Preliminary Results.

We have developed an R5X4 derivative of SIVmac239, termed 239T1. Here, we describe how 239T1 was derived by adapting SIVmac239 to productively infect SupT1 cells. We show preliminary data demonstrating the ability of 239T1 to infect SupT1 cells in a CXCR4-dependent manner and the identification and sequence analysis of a 239T1 envelope clone that induces fusion with cells expressing CD4 and CCR5 or CD4 and CXCR4.

Deriving 239T1 from SIVmac239. R5 SIVmac239 does not productively infect the CD4⁺ CXCR4⁺ CCR5⁻ SupT1 cell line (Appendix, Fig. 1A, closed symbols). However, SIVmac239 can infect SupT1 cells that also stably express CCR5 and the DC-SIGNR attachment factor (SupR5R cells) (data not shown). To adapt SIVmac239 to productively infect SupT1 cells, cocultures containing SupR5R and SupT1 cells in a 1:10 ratio were infected with SIVmac239. We passaged virus into the same mix of SupR5R and SupT1 cells until we observed infection spreading to greater than 10% of the culture by immunofluorescence, which suggested infection of the CCR5⁻ SupT1 cells (data not shown). Once infection of SupT1 cells was observed, virus was then passaged into a culture of pure SupT1 cells. After 17 cell-free passages in pure SupT1 cells, we collected stocks of the SupT1-adapted SIVmac239, now designated 239T1. Unlike parental SIVmac239, 239T1 productively infects SupT1 cell with kinetics similar to parental SIVmac239 infection of CEMx174 cells (Appendix, Fig. 1B, closed symbols; Fig. 1A, open symbols).

239T1 infection of SupT1 cells is CXCR4 dependent. To determine if 239T1 uses CXCR4 as a coreceptor for entry into SupT1 cells, we performed infection experiments in the presence of the CXCR4-specific antagonist AMD3100. First, we demonstrated that 100 nM concentrations of AMD3100 significantly inhibited the infection of SupT1 cells by the X4 HIV-1 strain HxB (Appendix, Fig. 2). This inhibition was specific for X4 viruses, as AMD3100 did not inhibit the infection of SupR5R cells by the R5 SIVmac239. Like infection with the X4 HxB, infection of SupT1 cells by the SIVmac239 derivative 239T1 was significantly inhibited by 100 nM AMD3100. Although we observed an increase in infection at 1 nM AMD3100 for 239T1 infection, a similar increase was observed for HxB infection at low concentrations of inhibitor. These data indicate that, unlike SIVmac239, 239T1 utilizes CXCR4 to infect SupT1 cells.

239T1 Envs can induce fusion with cells expressing CD4 and CXCR4. To verify that 239T1 Envs are indeed capable of utilizing CXCR4 to mediate fusion with target cells, we used primers

flanking *env* to PCR amplify *env* clones from the cellular DNA of 239T1 infected SupT1 cells. We screened 17 cloned 239T1 Envs in the cell-cell fusion assay for the ability to induce fusion with target cells expressing CD4 alone, CD4 and CXCR4, or CD4 and CCR5. One of the 239T1 Env clones, 2-32, was selected for sequencing and further study because of its high fusogenicity, comparable to that of the R5X4 HIV-2 VCP, on cells that express CD4 and CXCR4 (Appendix, Fig. 3). The ability of the 239T1 Env clone to induce fusion with cells expressing CD4 and CXCR4 was in contrast to the parental SIVmac239 Env, which could not utilize CXCR4 to induce fusion. Interestingly, clone 2-32 and the other CXCR4-utilizing 239T1 Env clones maintained the ability to utilize CCR5 after 17 passages on the CCR5⁻ SupT1 cell line (Appendix, Fig. 3 and data not shown). Sequence analysis of clone 2-32 revealed 7 amino acid changes between the R5X4 239T1 Env and the R5 SIVmac239 Env (Appendix, Fig. 4). These amino acid mutations included 1 change in the N-terminal region of gp120 (K47E), 1 change in the V2 loop (T202I), 3 changes in the V3 loop (N318K, I326M, L330W), 1 extracellular gp41 mutation (T558P), and 1 gp41 mutation that caused a truncation of the gp41 cytoplasmic tail (Q726Stop). Two of these mutations resulted in the loss of N-linked glycosylation sites (T202I, N318K).

Having isolated and sequenced an SIVmac239-derived Env clone, termed 239T1, that is capable of using CXCR4 in addition to CCR5 in cell-cell fusion assays, we propose to further characterize 239T1 *in vitro* and *in vivo*. We wish to identify what amino acid changes in the 239T1 Env permit its CXCR4 use and examine the impact of these changes on viral host range and neutralization sensitivity *in vitro* and infectivity and evolution *in vivo*.

D. Experimental Design and Analysis

D.1. Specific Aim 1: Molecular, biological, and immunological characterization of 239T1.

D.1.1. Rationale. The pathogenic, molecularly-cloned SIVmac239 is the most widely studied and commonly used SIV for modeling HIV infection. Like most SIV strains, SIVmac239 utilizes CCR5 as its major entry coreceptor (22, 23, 34). While R5X4 or X4 SIV strains have been reported, albeit infrequently, the vast majority of these viruses were non-pathogenic isolates found in natural SIV hosts (25-27). One X4 derivative of SIVmac239, dubbed SIVmac155T3, has been described (35), however SIVmac155T3 contains 22 amino acid mutations in Env when

compared with parental SIVmac239. Therefore SIVmac155T3 may not be amenable for careful molecular analysis of the Env determinants responsible for CXCR4 utilization. Although the HIV Env features associated with CXCR4 utilization have been investigated extensively, we are not aware of any studies that characterize the molecular determinants of an R5X4 or X4 SIV.

We have derived an R5X4 derivative of SIVmac239, termed 239T1, which contains only 7 amino acid mutations in envelope. We propose to examine the molecular, biological, and immunological characteristics of 239T1 *in vitro* to elucidate what changes permit SIVmac239 to gain CXCR4 utilization and what potential impact these changes have on host range and neutralization sensitivity. We hypothesize that specific amino acid changes in the Env glycoprotein of SIVmac239 enabled the virus to gain utilization of CXCR4 as a coreceptor for entry and that these changes will alter the host range and increase the neutralization sensitivity of 239T1 when compared with SIVmac239.

D.1.2. Identifying Envelope Mutations Necessary and Sufficient for CXCR4 Utilization. We

have isolated and sequenced an R5X4 Env clone from 239T1 infected SupT1 cells (Preliminary Results, Fig. 3). Comparison of the 239T1 *env* clone sequence with the parental SIVmac239 *env* sequence revealed 7 amino acid mutations in 239T1 (Preliminary Results, Fig. 4). Because R5X4 239T1 is derived from R5 SIVmac239, some or all of the 7 identified mutations in the 239T1 Env should be sufficient to confer CXCR4 utilization. First, we will determine which individual 239T1 mutations are necessary for efficient use of CXCR4. We will use site-directed mutagenesis to do “take away” mutations in the 239T1 *env* clone, singly changing each amino acid residue unique to 239T1 back to the corresponding residue in SIVmac239. Each altered 239T1 Env will then be functionally assessed in a cell-cell fusion assay for the ability to induce fusion with target cells expressing CD4 and CCR5 or CD4 and CXCR4 (36). Effector QT6 cells will be infected with recombinant vaccinia virus carrying a T7 RNA polymerase gene (VTF1.1) and then transfected with plasmids bearing *envs* under the control of a T7-dependent promoter. Target QT6 cells will be transfected with expression constructs bearing the genes for CD4 and either CCR5 or CXCR4 as well as a plasmid bearing a luciferase gene under the control of a T7-dependent promoter. We will also transfect target cells with CD4 alone or empty plasmids in addition to T7-luciferase to control for background fusion and luciferase expression levels. Env-mediated fusion of cocultured effector and target cells will result in the transcriptional activation

of the target cell luciferase gene by the T7 polymerase expressed in the effector cell. We will assess fusion levels by measuring the luciferase activity in the coculture cell lysates. It is possible that the combination of those mutations individually deemed necessary for CXCR4 use will not be sufficient to confer CXCR4 utilization by SIVmac239. We will therefore engineer 239T1 mutations into the SIVmac239 *env* and utilize the cell-cell fusion assay to determine what mutations are sufficient to confer CXCR4 utilization. We will first use cloning strategies and/or mutagenesis to add only those mutations individually deemed necessary for CXCR4 utilization by “take away” mutagenesis to the SIVmac239 *env*. If this set of mutations proves to be insufficient for the altered SIVmac239 Env to mediate fusion with target cells expressing CXCR4, we will then add in the remaining mutations alone and in combination to determine what minimal set of mutations are sufficient for SIVmac239 to gain CXCR4 utilization.

Variations in Env glycosylation sites have been associated with altered neutralization sensitivity for both SIVmac239 (37, 38) and HIV (39-41). Env glycosylation patterns have also been shown to be important determinants in HIV-1 coreceptor utilization and cell tropism (42, 43). Two of the mutations in the 239T1 *env* (T202I, N319K) eliminate 2 putative N-linked glycosylation sites (g9 in the V1/V2 region and g18 in V3, respectively). Because of the potential importance of altered N-linked glycosylation on viral tropism and neutralization sensitivity, we will determine if the effects of these mutations on coreceptor specificity are simply due to the loss of glycosylation or if they require the specific observed amino acid changes. To do this, we will mutate N200 and T321 to a glutamine and an isoleucine residue, respectively, while leaving T202 and N319 intact. Each of these two alternative mutations will also abolish g9 and g18, allowing us to evaluate if loss of N-linked glycosylation *per se* is responsible for any effects imposed by T202I and N319K on coreceptor usage. We will again implement the cell-cell fusion assay to assess these alternative glycosylation site mutants for fusion induction on cells expressing CD4 and CCR5 or CXCR4.

D.1.3. Examining the Host Range of Recombinant SIVmac239 Bearing the 239T1

Envelope. Because 239T1 was passaged 17 times on SupT1 cells, it is possible that viral genes other than *env* have accumulated mutations for improved viral replication in these specific host cells. Additionally, the 239T1 swarm is not a clonal population, which makes it difficult to draw definitive conclusions about the impact of a specific set of *env* mutations on the virus phenotype.

Therefore, we will create recombinant SIVmac239 bearing the cloned 239T1 envelope (SIVmac239_{239T1}) to examine the impact of altered coreceptor utilization on cell tropism in the context of a clonally-derived virus lacking other potential confounding viral mutations. To create SIVmac239_{239T1} we will use the pVP-1 and pVP-2(Nef+) SIVmac239 hemigenome plasmids (38). pVP-2(Nef+) contains the 3' half of the SIVmac239 genome, including the 3' long terminal repeat (LTR) and the *env*, *tat*, *rev*, *nef*, *vpx*, and *vpu* genes. Using molecular cloning strategies, we will exchange the SIVmac239 *env* in pVP-2 for the 239T1 *env* clone. We will then linearize pVP-2(Nef+) and pVP-1 (which contains the 5' LTR and the *gag*, *pol*, and *vif* genes) and ligate them together to form a whole recombinant provirus. SupT1 cells will be electroporated with the recombinant proviral DNA and cultured to produce a stock of clonally-derived SIVmac239_{239T1} to be used in further infection and neutralization assays. Collected SIVmac239_{239T1} viral titers will be quantified by measurements of p27 antigen as well as by determining the 50% tissue culture infectious dose (TCID₅₀).

We will assess the infectivity of SIVmac239_{239T1} in U87 cells expressing CD4 and CCR5 or CD4 and CXCR4 and in a range of CD4⁺ CXCR4⁺ cell lines in the lab including: SupT1, MT-2, CEMx174, Molt 4 clone 8, HUT-78, and CEM. To assess infection levels in these cell lines, we will use a radioactive assay to measure viral reverse transcriptase (RT) activity in the cell supernatants over time. We will compare SIVmac239_{239T1} infection in these cell lines to that of SIVmac239 to evaluate how Env differences that alter coreceptor utilization affect viral host range. To verify our cell-cell fusion assay data, which indicates the efficient utilization of CXCR4 by 239T1 *env* clones (Preliminary Results, Fig. 3), the CXCR4 specific antagonist AMD3100 will be used to verify that SIVmac239_{239T1} is indeed utilizing CXCR4 to infect CXCR4⁺ cell lines. We will test the ability of various concentrations of AMD3100 to inhibit SIVmac239_{239T1} infection of CD4⁺ CXCR4⁺ cell lines. We will use the X4 HIV-1 HxB as a positive control for infection of CXCR4⁺ cell lines as well as inhibition by AMD3100. AMD3100 treatment of SIVmac239 infection of SupR5R cells will serve as a negative control for AMD3100 inhibition.

We will perform infection experiments using peripheral blood mononuclear cells (PBMC) isolated from rhesus macaques to determine if SIVmac239 bearing the 239T1 Env maintains the ability to infect primary cells and if it can utilize CXCR4 to do so. Ficoll gradient centrifugation will be used to isolate PBMC from rhesus blood (44). We will stimulate extracted

PBMC with phytohemagglutinin (PHA) and IL-2 for 72 hours prior to infection. Because SIVmac239_{239T1} bears the 239T1 Env, which we have shown induces fusion with cells expressing either CCR5 or CXCR4 (Preliminary Results, Fig. 3), we will first determine if SIVmac239_{239T1} can infect rhesus PBMC using CCR5 and/or CXCR4. We will infect PBMC with SIVmac239_{239T1} or SIVmac239, which can productively infect PBMC in culture, and monitor infection by measuring the RT activity in the culture supernatant over time. If we find that SIVmac239_{239T1} can productively infect PBMC, we will then determine if it can do so in a CCR5-independent, CXCR4-dependent manner by infecting PBMC in the presence of the CCR5 antagonist CMPD 167 (45); to ensure full inhibition of CCR5 usage, we will use CMPD 167 concentrations high enough to completely inhibit SIVmac239 infection of PBMC. If SIVmac239_{239T1} infects in the presence of CMPD 167, we will verify that infection is CXCR4-dependent by infecting in the presence of high concentrations of both CMPD 167 and AMD3100. Again, infection will be monitored by measuring RT activity in the culture supernatant.

D.1.4. Determining if Env Changes Associated with CXCR4-utilization Alter the SIVmac239 Neutralization Sensitivity. SIVmac239 is highly resistant to neutralization by sera from SIVmac239-infected macaques (46), however amino acid changes in the SIVmac239 Env have been associated with increased neutralization sensitivity (47). In particular, certain Env mutations resulting in deglycosylation have been shown to increase the neutralization sensitivity of SIVmac239 (38). Env deglycosylation has also been associated with increased neutralization sensitivity for HIV-1 (39, 40, 48-50). For these reasons, we wish to examine the neutralization sensitivity of SIVmac239 bearing the 239T1 Env, which has several amino acid changes and the loss of 2 N-linked glycosylation sites when compared with the SIVmac239 Env. To examine neutralization, we will use the CEMx174SIV-SEAP reporter cell line (46). CEMx174SIV-SEAP cells contain a secreted alkaline phosphatase (SEAP) gene under the transcriptional control of the SIV LTR. Thus, when CEMx174SIV-SEAP cells are infected by SIVmac, the viral Tat protein will induce transcription of the SEAP gene. Infection can therefore be measured as SEAP activity in the culture.

We will infect CEMx174SIV-SEAP cells with recombinant SIVmac239_{239T1}, SIVmac239, and SIVmac239 Δ V1V2, a replication competent, neutralization sensitive derivative

of SIVmac239 that lacks the Env V1/V2 loop (38, 51). SIVmac239 will serve as our neutralization resistant control and SIVmac239 Δ V1V2 will serve as our neutralization sensitive control. Infections will be carried out in the presence of serial dilutions of sera from SIVmac239-infected rhesus macaques as well as in the presence of uninfected control macaque sera to determine baseline SEAP levels induced by infection with these 3 virus strains. We will determine the 50% neutralization titer for each virus to determine if, like SIVmac239 Δ V1V2, SIVmac239_{239T1} demonstrates increased neutralization sensitivity when compared with parental SIVmac239.

D.1.5. Expected Results. The Env V3 loop is a critical determinant for HIV-1 tropism (52) and changes in V3 amino acids have also been associated with altered SIVmac239 tropism (53, 54). In particular, residue 324, which is one of three altered amino acid positions in the 239T1 V3 loop, has been shown to be a critical determinant in SIVmac239 coreceptor interactions (55). Although no molecular studies have yet examined an X4 SIV, we expect that the three amino acid changes in the V3 loop of the 239T1 Env will be critical for CXCR4 usage. It is possible, however, that the rules for CXCR4 usage by SIV will be quite different from those of HIV and that the changes in the 239T1 V3 loop will not be the important mutations for conferring CXCR4 utilization. Indeed, the addition of positively charged residues into the V3 loop, which is strongly associated with HIV-1 CXCR4 use (56-59), does not confer CXCR4 utilization on SIVmac239 (54). Thus our molecular studies may reveal an unexpected mechanism for the gain of CXCR4 utilization demonstrated by 239T1; identifying what is required for SIVmac239 to acquire CXCR4 utilization is a first important step for addressing why X4 SIV strains are apparently so rare and assessing further biological, immunological, and pathological characteristics of 239T1.

We anticipate that SIVmac239_{239T1} will be able to infect a range of CD4⁺ CXCR4⁺ cell lines and rhesus PBMC in a CXCR4-dependent manner; if so, our cell-cell fusion assay results would be verified and SIVmac239_{239T1} could be utilized for further studies as a bona fide R5X4 derivative of SIVmac239. It is possible, however, that SIVmac239_{239T1} will have a restricted phenotype and be unable to infect a broad range of CXCR4⁺ cells or primary cells in a CXCR4-dependent manner. Because SIVmac239_{239T1} contains the 239T1 Env but is otherwise identical

SIVmac239, this result would suggest that the ability of a virus to utilize a given coreceptor for infection may require more than just the presence of the appropriate receptor and coreceptor.

While we expect SIVmac239_{239T1} to be more neutralization sensitive than SIVmac239, primarily because of the loss of 2 N-linked glycosylation sites in the 239T1 Env, this may not be the case. Not all SIVmac239 glycosylation sites examined thus far have shown equal importance for neutralization resistance (38). Also, we will be examining neutralization by sera from SIVmac239-infected animals. Although SIVmac239-infected macaque sera has been shown to successfully neutralize several viruses derived from SIVmac239 (38, 51), this may not be the case for SIVmac239_{239T1}. Therefore, we may observe greater neutralization resistance for SIVmac239_{239T1} because the sera do not recognize this heterologous virus. Thus, if we observe that SIVmac239_{239T1} is equally or more neutralization resistant than SIVmac239, we cannot necessarily conclude that animals infected with SIVmac239_{239T1} would produce antibodies that are as unsuccessful at neutralization. If we observe increased neutralization sensitivity, however, it will strongly suggest that SIVmac239_{239T1} is more exposed than SIVmac239 for neutralization by host humoral immunity.

D.1.6. Limitations and Alternative Approaches. SupT1 cells electroporated with SIVmac239_{239T1} proviral DNA might not successfully produce a viral stock because of the harsh nature of electroporation. In this event, we will instead transfect 293T cells with SIVmac239_{239T1} proviral DNA to produce a clonally-derived SIVmac239_{239T1} viral stock. Additionally, our neutralization assays will implement the CEMx174SIV-SEAP reporter cell line, the use of which necessarily requires that the examined virus be capable of infecting CEMx174 cells. Although CEMx174 cells are CD4⁺ CXCR4⁺, it is still possible that SIVmac239_{239T1} will be unable to infect these cells. In this event, we will instead infect GHOST reporter cells expressing CD4, CCR5, and CXCR4 to evaluate neutralization sensitivity. Alternatively, we could examine neutralization by infecting SupR5R cells, which support both 239T1 and SIVmac239 infection, and measure RT activity in the supernatant at an appropriate time point post-infection. While we could alternatively measure p27 antigen release following infection, the presence of antibodies directed toward *gag* gene products in the rhesus sera could confound results; we therefore prefer an RT activity assay. As mentioned in Expected Results, it is possible that the CXCR4 utilization that we have demonstrated in the cell-cell fusion assay

(Preliminary Results, Fig. 3), which measures the fusion of large surfaces overexpressing envelope with large surfaces overexpressing CD4 and coreceptors, will not correlate with the infection of CXCR4⁺ cell lines or PBMC by actual virions. In this event, we will adapt SIVmac239 to infect an alternative CD4⁺ CXCR4⁺ cell line, using the same methodology used to derive 239T1, to obtain a more relevant virus for further *in vitro* and *in vivo* studies.

D.2. Specific Aim 2: Examination of *in vivo* infection of rhesus macaques with 239T1.

D.2.1. Rationale. Because X4 SIV strains have been identified so infrequently, *in vivo* animal infection studies examining viruses that can utilize CXCR4 have relied on the use of X4 SHIVs.

X4 SHIVs reproducibly replicate to high titers in infected animals and lead to the precipitous depletion of CD4⁺ cells in peripheral blood and rapid progression to AIDS (28, 29, 60).

Although the drastic depletion of CD4⁺ cells in the blood is observed in X4 HIV-1 infected humans homozygous for the CCR5-Δ32 allele (30-33), extremely high viral titers and rapid progression to disease is not as common as that observed in macaques infected with X4 SHIVs.

It is therefore unclear if infection of macaques with X4-SHIVs is the most relevant model system for examining the pathogenicity and immunogenicity of X4 HIV. Indeed, Picker *et al.* have shown that although the X4 SIVmac155T3 rapidly depletes peripheral CD4⁺ cells, it does not replicate to high titers in infected animals nor does it lead to rapid disease progression (35). An examination of how the SIVmac155T3 Env is affected by immune system pressure has not been reported; the complexity of the SIVmac155T3 envelope, which bears 22 amino acid differences when compared with SIVmac239, might preclude such a study. We therefore propose to work in collaboration with the Centers for AIDS Research (CFAR) primate research core at the Tulane Regional Primate Research Center to infect rhesus macaques with the R5X4 SIVmac239 derivative SIVmac239_{239T1}. We hypothesize that, like SIVmac155T3, SIVmac239_{239T1} will replicate to lower levels than SIVmac239 and that immune system pressure will drive the evolution of the SIVmac239_{239T1} back to an R5 phenotype.

D.2.2. Infection of Rhesus Macaques. We will infect rhesus macaques (*Macaca mulatta*) intravenously with viruses produced in rhesus PBMC. We will infect 6-8 animals in total; 4-5 monkeys will be infected with SIVmac239_{239T1} and another 2-3 monkeys will be infected with

SIVmac239. We will infect fewer animals with SIVmac239 because infection with this virus has already been examined and described extensively.

D.2.3. Monitoring Viral Load. We will examine the replication of SIVmac239_{239T1} in rhesus macaque hosts, both to characterize *in vivo* infection with R5X4 SIVmac239_{239T1} and to determine if the results of Picker and colleagues, which demonstrate low replication levels for X4 SIVmac155T3 (35), are typical for SIVs that can utilize CXCR4. To quantify viral replication in rhesus macaque hosts, we will measure SIV RNA copies in plasma taken at multiple time points following infection with SIVmac239_{239T1} or SIVmac239. We will reverse transcribe viral RNA in plasma and then use real-time PCR with primers derived from the SIVmac239 *gag* gene to quantify viral cDNA from multiple time points taken throughout infection (61).

D.2.4. Evaluating CD4⁺ Cell Depletion in Various Anatomical Compartments. It has been shown that SIVmac239 infection leads to the rapid and profound destruction of CD4⁺ cells in the gut-associated lymphoid tissue (GALT) (62). This pathogenic consequence of infection has also been observed in humans infected with HIV (63). Evidence from SHIV-infected rhesus macaques suggests that this depletion of CD4⁺ cells in the GALT is caused by R5 viruses, but not X4 viruses (28). Instead, an X4 SHIV was found to induce the massive depletion of CD4⁺ lymphocytes in peripheral blood and lymph nodes (LN), whereas an R5 SHIV showed a more gradual loss of CD4⁺ cell in these compartments. Therefore, we will use flow cytometric analysis to compare CD4⁺ lymphocyte destruction in peripheral blood, lymph nodes, and the GALT in monkeys infected with R5X4 SIVmac239_{239T1} or R5 SIVmac239. We will extract cells from blood and from peripheral lymph node and intestinal lamina propria biopsies (64, 65) taken early and late in infection. Extracted lymphocytes will be stained with conjugated monoclonal antibodies against CD3, CD4, and CD8 β as well as with isotype matched negative control antibodies to control for non-specific antibody binding to cells. We will use flow cytometry to examine the change in CD4⁺ cells within each compartment by determining the percent of CD3⁺ T cells that are CD4⁺ at each post-infection time point. To ensure that any observed decrease in CD4⁺ T cells is due to cell depletion and not the downregulation of CD4 on cell surfaces, we will determine if there is an increase in the number of CD3⁺ CD4⁻ CD8⁻ cells in each compartment.

All analyses will also be performed on lymphocytes taken from uninfected control animals for comparative purposes.

D.2.5. Examining *env* Evolution *in vivo*. We hypothesize that immune system pressure in the macaque host will drive mutations in the SIVmac239_{239T1} *env* such that the virus will lose the ability to utilize CXCR4 and revert back to an R5 phenotype. To address our hypothesis, we will evaluate the sequence and function of Envs extracted from the infected animals before, during, and after peak viral load. By examining Envs from these 3 time points, we can track how envelope evolves from the input virus before, during, and after the initiation of the host adaptive immune response. RT-PCR will be used to create cDNA from viral RNA in the plasma of the infected animals. We will use primers flanking the SIVmac239 *env* gene to PCR amplify *env* clones from cDNA extracted at each of the 3 time points from infected macaques. We will screen the extracted Envs for functionality in the cell-cell fusion assay (described above) (36) to ensure that we have 10 functional envelope clones from each of the SIVmac239_{239T1} and SIVmac239-infected animals for each time point. We will assess the ability of each of the extracted *env* clones to induce fusion with target cells expressing CD4 and CCR5 or CD4 and CXCR4. We will include the R5 SIVmac239 Env and the R5X4 239T1 Env as controls in each of our cell-cell fusion assays. We will sequence the functional *env* clones and examine consensus *env* mutations over the course of infection and how these changes correlate with the Env functionality. Because the 239T1 Env may revert back to an R5 phenotype without reverting back to the SIVmac239 Env sequence, we will sequence *env* clones from the SIVmac239-infected animals to discern what mutations are common to SIVmac239 *envs* and what mutations are unique to the R5X4 239T1 *env* during *in vivo* infection.

D.2.6. Expected Results. SIV strains that can utilize CXCR4 as an entry coreceptor have been very rarely reported, perhaps because the mutations required for SIV to utilize CXCR4 result in a virus that is more easily controlled by the host immune system. We therefore expect that R5X4 SIVmac239_{239T1} will replicate with a lower viral set point than SIVmac239 in rhesus macaque hosts. As has been observed with X4 SHIV infection (28), we anticipate that SIVmac239_{239T1} will cause a greater depletion of CD4⁺ T cells in peripheral blood and lymph nodes and a lesser depletion of CD4⁺ T cells in the GALT when compared with SIVmac239. We believe that

pressures during *in vivo* infection will drive SIVmac239_{239T1} to revert back to an R5 phenotype. The capability of X4 SHIV to replicate with set points similar to R5 SHIV in rhesus macaques, implies that there is no obvious block against the use of CXCR4 in the macaque host *per se*. Therefore, if the 239T1 *env* does, indeed, revert back to an R5 phenotype, it would suggest that the host immune system is better equipped to control SIV that utilizes CXCR4.

Alternatively, SIVmac239_{239T1} might maintain its R5X4 phenotype into the chronic phase of infection. If this is the case, it would suggest that CXCR4-utilizing SIV strains are not necessarily selected against by the host immune system, but instead perhaps appear so infrequently due to a competitive disadvantage with their R5 counterparts. SIVmac239_{239T1} may still replicate to lower set points than SIVmac239, even if the host immune system does not select against CXCR4-utilizing viruses, as other aspects of the SIVmac239_{239T1} biology, including productively infected CD4⁺ T cell subsets, could impact replication levels.

It is of course possible that SIVmac239_{239T1} replication and evolution will vary between infected animals. Because the initial virus inoculum will be a molecularly cloned virus, any differences in disease outcome and viral evolution will be reflective of differential host factors, including immune system pressure.

D.2.7. Limitations and Alternative Approaches. As discussed in Specific Aim 1, SIVmac239_{239T1} may not infect rhesus PBMC in a CXCR4-dependent manner, which would indicate that the virus will not be infectious in a rhesus host. In this event, as mentioned above, we will adapt SIVmac239 to infect alternative CD4⁺ CXCR4⁺ cell lines using the same techniques used to generate 239T1 to produce a virus more relevant for *in vivo* studies. Also, because it may require a high number of PCR cycles to amplify *env* clones from plasma RNA extracted from infected rhesus hosts, non-specific amplification could become problematic for isolating *env* genes. To increase the specificity and yield of our amplifications from cDNA, we may implement a nested PCR strategy, in which a second set of primers that fall within the region amplified by the first set of primers are additionally used to amplify *env* clones. Alternatively, we could amplify *env* clones from cellular DNA extracted from infected monkeys.

Appendix.

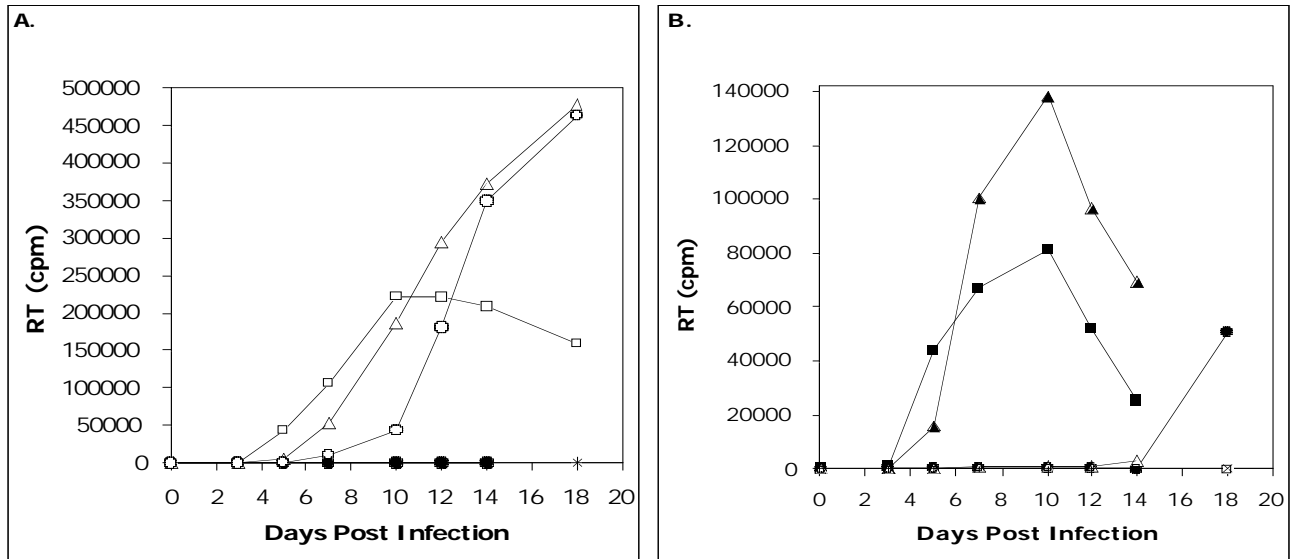


FIG. 1. Replication of SIVmac239 (A) and 239T1 (B). CEMx174 cells (open symbols) and SupT1 cells (closed symbols) were infected with virus containing 25 ng (squares), 2.5 ng (triangles), or 0.25 ng (circles) of p27 antigen. Virus replication was measured as reverse transcriptase (RT) activity in the cell supernatant.

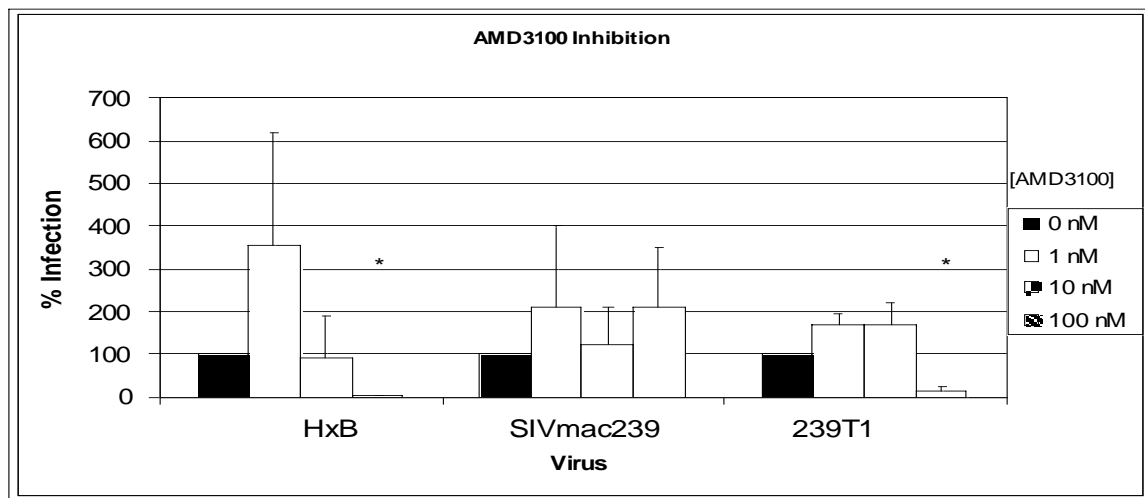


FIG. 2. Inhibition by AMD3100. SupT1 cells were infected with HxB or 239T1 and SupR5R cells were infected with SIVmac239 in the presence of 0 nM, 1 nM, 10 nM, or 100 nM concentrations of AMD3100. Culture supernatants were assayed for viral reverse transcriptase (RT) activity at 7 days post-infection to quantify infection. RT values from infections in the presence of inhibitor are represented as mean % infection of untreated controls from 3 experiments. Asterisks (*) indicate the RT values that are significantly lower than the untreated control ($P < 0.01$). Error bars represent standard deviations.

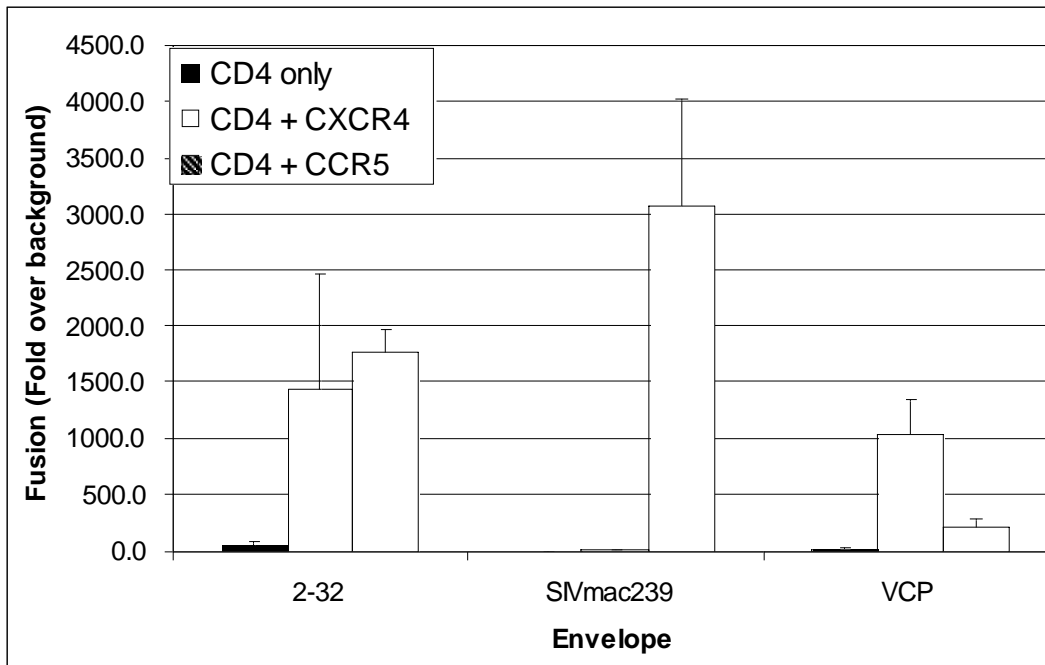
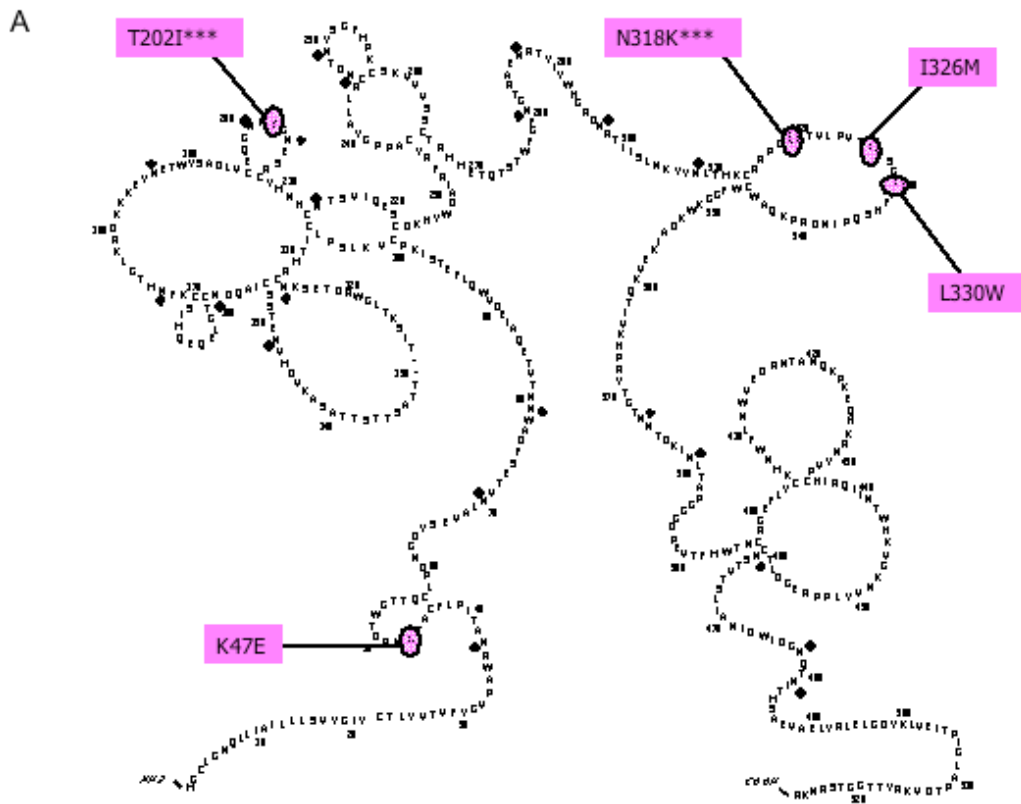


FIG. 3. Coreceptor use in cell-cell fusion. Effector cells expressing cloned Envs and T7 polymerase were mixed with target cells carrying a T7 promoter-driven luciferase gene and expressing CD4 alone or in combination with CXCR4 or CCR5. Env-induced fusion was measured as luciferase activity in cell lysates. Fusion levels with target cells lacking CD4 and coreceptors were set as background for each Env. Data shown are means of values from 3 experiments. Error bars represent standard deviations.



B

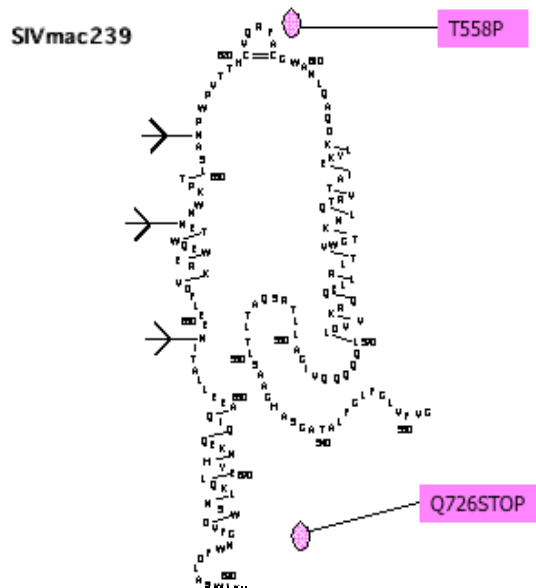


FIG. 4 Amino Acid Changes in the 239T1 envelope. Shown are the predicted disulfide bonding patterns of the gp120 (A) and gp41 (B) subunits of the SIVmac239 envelope glycoprotein (66). The residues highlighted in pink are altered as indicated in the 239T1 clone 2-32 envelope. Amino acid changes indicated with asterisks (***) eliminate an N-linked glycosylation site.

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