Identification and characterization of a macrophage-tropic SIV envelope glycoprotein variant in blood from early infection in SIVmac251-infected macaques

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Abstract

Macrophages play an important role in HIV/SIV pathogenesis by serving as a reservoir for viral persistence in brain and other tissues. Infected macrophages have been detected in brain early after infection, but macrophage-tropic viruses are rarely isolated until late-stage infection. Little is known about early variants that establish persistent infection in brain. Here, we characterize a unique macrophage-tropic SIV envelope glycoprotein (Env) variant from two weeks post-infection in blood of an SIVmac251-infected macaque that is closely related to sequences in brain from animals with neurological disease. SIVmac251 clones expressing this Env are highly fusogenic, and replicate efficiently in T cells and macrophages. N173 and N481 were identified as novel determinants of macrophage tropism and neutralization sensitivity. These results imply that macrophage-tropic SIV capable of establishing viral reservoirs in brain can be present in blood during early infection. Furthermore, these SIVmac251 clones will be useful for studies on pathogenesis, eradication, and vaccines.
Keywords

Human Immunodeficiency Virus (HIV); Simian Immunodeficiency Virus (SIV); Envelope glycoprotein (Env); Macrophage tropism; brain

Introduction

HIV and SIV infections are characterized by progressive CD4+ T cell depletion and chronic immune activation, eventually leading to immunodeficiency and AIDS. The main target cells infected by these viruses are CD4+ T cells and macrophages. While CD4+ T cells are progressively depleted by infection, macrophages are relatively resistant to cytopathic effects of these viruses (Ho et al., 1986) and serve as a reservoir for viral persistence in the central nervous system (CNS) and other tissues including bone marrow, lymph node, spleen, lung, and gut (Alexaki et al., 2008; Bissel et al., 2008; Clements et al., 2002; Gorry et al., 2005; Heise et al., 1994; Heise et al., 1993; Igarashi et al., 2001; Smith et al., 2003).

Infection of macrophages in the brain also causes neurological dysfunction and encephalitis, characterized by multinucleated giant cells, reactive astrocytes, and white matter abnormalities (Dunfee et al., 2006), and creates a sanctuary site for viral persistence and development of drug resistance due to poor CNS penetration of most anti-retroviral drugs (Gorry et al., 2005).

A major obstacle to studies on macrophage-related pathogenesis in animal models is the lack of a pathogenic SIV clone that reproducibly causes macrophage infection and CNS disease. The pathogenic clone SIVmac239 causes immunodeficiency and AIDS in non-human primate models (Kestler et al., 1990), but its tropism is restricted to CD4+ T cells (Mori et al., 1992) so it rarely causes neurological disease. SIVmac316, originally isolated from SIVmac239-infected animals at late-stage infection, replicates efficiently in monocyte-derived macrophages (MDM) in vitro (Mori et al., 1992) and induces AIDS in a subset of infected macaques (Johnson et al., 2009). However, infected macrophages and macrophage-associated pathology are rarely detected in SIVmac316-infected macaques (Borda et al., 2004; Johnson et al., 2009; Johnson et al., 2003a; Kodama et al., 1993). SIVmac251, a strain that replicates well in both CD4+ T cells and macrophages, is frequently used for HIV/AIDS pathogenesis studies in non-human primate models (Daniel et al., 1985; Kanki et al., 1985; Letvin et al., 1985; Miller et al., 1998). However, the composition of the swarm in this strain varies considerably after amplification in cell culture (Del Prete et al., 2013; Strickland et al., 2011), resulting in heterogeneous clinical outcomes and hindering studies of viral determinants important for pathogenesis and immune evasion. Viruses encoded by SIVmac251-derived molecular clones, including SIVmac251BK28 (Kornfeld et al., 1987), SIVmac251 clone (Choi et al., 1994; Naidu et al., 1988), SIVmac1A11 (Luciw et al., 1992), and SIVmac32H (Rud et al., 1994), have been inoculated into animals, but caused little or no disease in vivo. The uncloned SIVmac251 strain commonly used for pathogenesis and vaccine studies consists of more than 20 distinct env sequences; three env clones from this swarm mediated viral replication in alveolar macrophages in vitro (Bixby et al., 2010), but levels of replication were very low compared to SIVmac316 (Bixby et al., 2010). The availability of pathogenic molecular clones of SIVmac251 would facilitate development of
animal models to study macrophage-related pathogenesis and might also be useful as a challenge strain for vaccine studies.

HIV/SIV macrophage tropism is determined primarily by the viral envelope glycoproteins (Env). The Env gp120 external subunit is non-covalently linked to the gp41 transmembrane subunit, and organized as trimers on the viral membrane. gp120 binding to CD4 induces conformational changes that expose the CCR5 coreceptor binding site and enable gp120-CCR5 binding, which triggers additional conformational changes that lead to fusion and viral entry. The gp120 V1, V2 and V3 variable regions play important roles in mediating interactions with CD4 and CCR5. The V3 loop and bridging sheet region constitute the CCR5 binding site. The V1/V2 loop influences gp120 binding to CD4/CCR5 by partially occluding receptor binding sites in the unliganded structure (Johnson et al., 2003b; Pinter et al., 2004; Sullivan et al., 1998; Wyatt et al., 1995). Structural models of Env trimers suggest that the V1/V2 loop interacts with the V3 loop in the same or neighboring gp120 protomer (Chen et al., 2005b; Kwong et al., 2000; Liu et al., 2011; Rusert et al., 2011), an interaction that may influence CCR5 binding by affecting V3 loop orientation. Macrophage-tropic strains overcome the entry restriction imposed by low CD4 expression on macrophages via an enhanced capacity to mediate fusion and infection at low CD4 levels (Bannert et al., 2000; Gorry et al., 2002; Mori et al., 2000). However, structural changes that enhance gp120 interaction with CD4 often render macrophage-tropic viruses more susceptible to antibody recognition (Dunfee et al., 2009; Dunfee et al., 2007; Means et al., 2001; Musich et al., 2011; Puffer et al., 2002). Consistent with these findings, most macrophage-tropic SIV clones are highly neutralization sensitive. Together with previous studies suggesting that most transmitted/founder viruses replicate poorly in macrophages (Isaacman-Beck et al., 2009; King et al., 2013; Li et al., 2010; Ochsenbauer et al., 2012; Salazar-Gonzalez et al., 2009), these findings led to the prevailing view that macrophage-tropic HIV/SIV variants are rare or absent during early-stage infection.

HIV and SIV are genetically compartmentalized in the CNS due to founder effects and independent viral evolution, reflecting differences in target cells (i.e., macrophages) and immune selection pressures. Although viruses enter the brain within weeks after primary infection, infection usually remains latent until late-stage disease. Here, we identify a macrophage-tropic SIVmac251 variant in blood at two weeks post-infection that shares high sequence identity with gp120 sequences in the brain of animals with rapid disease progression and SIV encephalitis (SIVE). Infectious molecular clones encoding gp120 sequences from this early variant showed high fusion activity, and mediated high levels of viral replication and multinucleated giant cell formation in macrophages. Two N-linked glycosylation sites in the V2 and C5 regions were identified as determinants of macrophage tropism and neutralization sensitivity. These results suggest that macrophage-tropic SIV variants capable of establishing persistent viral reservoirs in macrophages in the CNS and other tissues can be present in blood during early infection. These SIVmac251 replication-competent molecular clones will be useful for future studies on macrophage-related pathogenesis and approaches to eradicate viral reservoirs in brain and other tissues.
Materials and Methods

Isolation of SIV sequence variants from SIVmac251-infected rhesus macaques

SIV gp120 sequence variants were cloned and sequenced from archived samples stored at −80°C, available from previous studies of SIVmac251-infected rhesus macaques (Orandle et al., 2002; Williams et al., 2002; Williams et al., 2001) (Table 1). Four macaques (Group I) were infected with SIVmac251 stock from the Desrosiers lab; two were inoculated intravenously and two intravaginally. The gp120 region was amplified by bulk PCR from genomic DNA of PBMC at two weeks post-infection (p.i.). Four macaques (Group II) were inoculated intravenously with 20 ng p27 of SIVmac251; two with stock provided by the Desrosiers lab (the same virus stock used to infect Group I) and two with stock provided by the Letvin lab. Frozen brain tissue, lymph node, and bone marrow samples were obtained at autopsy after these four animals died with AIDS, and the gp120 region was amplified by bulk PCR from genomic DNA isolated from these tissue samples. The amplified viral DNA was cloned into a TOPO vector for sequencing, and the gp120 amino acid sequences were aligned and analyzed using BioEdit and Clustal W.

Construction of recombinant Envs and viruses

Amino acid variants in the V1, V2, V3 and C3 regions with shared identity between the early variant in blood, desiv147#4, and the gp120 consensus sequence of late variants in brain, were introduced individually or in combination into SIVmac251 Env (SIVmac251_BK28 clone) by site-directed mutagenesis. gp120 and the N-terminus of gp41 (residues 1 to 213) of all recombinant Envs were then sub-cloned into pSIVΔgpv (provided by Joseph Sodroski) (Marcon and Sodroski, 1997) using restriction sites AfeI and NheI to generate Env clones, or into 239-FL plasmid (Bixby et al., 2010) using restriction sites BstBI and NheI to generate full-length replication-competent proviruses. The gene fragment containing desiv147c#4 Env was synthesized by GenScript Inc. and cloned into pSIVΔgpv or 293-FL provirus as described above.

Env expression and cell-cell fusion assays

Recombinant Envs were expressed by transfecting 293T cells with pSIVΔgpv, and Env expression examined by western blotting of cell lysates. For cell-cell fusion assays, 293T cells co-transfected with pSIVΔgpv and pLTR-Tat, a Tat expressing plasmid, were incubated with Cf2-luc cells (Etemad-Moghadam et al., 2000) transiently expressing rhesus CD4 and CCR5. Expression of the luciferase reporter gene in Cf2-luc cells is under the control of HIV-1 LTR. After 8 hours of co-incubation, cells were lysed and luciferase activity was quantified as an indication of cell-cell fusion.

Virus production and infectivity

Replication-competent viruses were generated by transfecting 293T cells with full-length provirus. For infection of TZM-BL cells, replication-competent viruses were incubated with cells in the presence of 15 μg/ml of DEAE-dextran. Two days p.i., luciferase activity in cell lysates was measured. Viruses used for infection were normalized by reverse transcriptase activity or p27 antigen concentration (ELISA from Advanced Bioscience Laboratories, Inc., Yen et al. Page 4
Kensington, MD). Assays were performed in duplicate wells in 96-well plates. For PBMC-derived viruses, viruses produced in 293T cells were passaged in rhesus macaque PBMC for 5 days. After five days of incubation, residual 293T-derived viruses were removed by washing, and viruses generated in PBMC were harvested on Day 7, 9, and 11 post-infection.

Neutralization assays
Viruses were pre-incubated with serial dilutions of heat-inactivated SIVmac251 antiserum (NIH AIDS Research and Reference Reagent Program), SIVmac239 antiserum, or mouse ascites containing SIV monoclonal antibodies (Edinger et al., 2000) at 37°C for one hour. After pre-incubation, TZM-BL cells were added with DEAE-dextran (final concentration 15 μg/ml). Two days later, cells were lysed and luciferase activity was measured. Assays were performed in duplicate wells in 96-well plates. Results are presented as IC<sub>50</sub> and IC<sub>90</sub>, the reciprocal dilution of antiserum or ascites required for achieving 50% and 90% inhibition of infection, respectively, relative to no serum or no ascites controls.

Viral replication in PBMC and MDM
PBMC were isolated from rhesus macaque peripheral blood (New England Primate Research Center) by Histopaque (Sigma) density centrifugation and activated in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 20 U/ml IL-2 and 1 ug/ml PHA-P for 3 days. Activated PBMC were then maintained in RPMI supplemented with 10% FBS, 1% P/S and 20 U/ml IL-2 and infected with replication-competent viruses. Three hours p.i., viruses were removed by washing cells three times with RPMI. To obtain MDM, PBMC were cultured in RPMI containing 15% FBS, 10% human serum type AB, 1% P/S and 20 ng/ml M-CSF for five days. Non-adherent cells were then removed by washing three times with RPMI. Adherent cells were cultured in RPMI supplemented with 15% FBS, 5% human serum type AB, 1% P/S and 20 ng/ml M-CSF for two additional days before infection. For infection, virus stocks were cultured with MDM for 24 hours and then removed by washing once with RPMI. Culture supernatant was collected twice a week and p27 concentration was quantified by ELISA (Advanced Bioscience Laboratories, Inc., Kensington, MD). Assays were performed in duplicate wells in 96-well plates.

Sequences
All gp120 sequences were deposited in GenBank (accession numbers JN376087 to JN376124).

Results
Identification of a macrophage-tropic SIV variant in blood during early infection
To identify macrophage-tropic SIV viruses in blood during early infection of rhesus macaques, we analyzed unpublished SIV gp120 sequences generated in previous studies (Orandle et al., 2002; Williams et al., 2002; Williams et al., 2001). We reasoned that these viruses could be found using a sequence-based approach to search for gp120 sequences in blood at two weeks post-infection that share high sequence identity with late-stage variants in brain from macaques with SIVE. Early variants were amplified from PBMC genomic
DNA at two weeks post-infection from four SIVmac251-infected rhesus macaques (Group I in Table 1). Late variants were amplified from autopsy brain, lymph node, and bone marrow from a second group of four SIVmac251-infected rhesus macaques that progressed rapidly to AIDS, developed SIVE, and died within 8 months (Group II in Table 1). Among early variants, desiv147c#4 had the closest relationship to the gp120 consensus sequence in brain based on genetic distance (Fig. 1A). desiv147c#4 shared >98% sequence identity with the gp120 consensus sequence in brain and the brain variant most similar to this consensus sequence (A92-620BR2). Furthermore, desiv147c#4 was one of only two early variants in blood that clustered with late variants in brain in phylogenetic analysis (Fig. 1B). Based on its high sequence identity with gp120 sequences in brain, we predicted that desiv147c#4 is a macrophage-tropic Env.

Compared to published SIV sequences, desiv147c#4 is genetically unique based on BLAST searches of sequences in the NCBI database. desiv147c#4, A92-620BR2, and the gp120 consensus sequence in brain were genetically distinct from a lab-adapted non-pathogenic SIVmac251 clone (Edmonson et al., 1998; Hoxie et al., 1988; Kornfeld et al., 1987), with most differences mapping to the V1, V2, V3 and C3 regions (Fig. 2). Sequence variation in these regions can influence Env interactions with CD4/CCR5 (Chen et al., 2005b; Huang et al., 2005; Johnson et al., 2002; Means et al., 2001; Rizzuto et al., 1998) and thereby influence macrophage-tropism (Means et al., 2001; Mori et al., 1992; Musich et al., 2011). The G384R change in C3 (corresponding to G382R in other well-characterized SIV strains), at the N-terminus of the conserved GGPDE domain in the CD4 binding loop (Chen et al., 2005b), has been associated with macrophage-tropism and decreased CD4 dependence in macrophage-tropic strains SIVmac316 and SIV/17E-Fr (Flaherty et al., 1997; Mori et al., 1992; Mori et al., 2000; Otto et al., 2003). V1, V2, V3, and C3 sequences of desiv147c#4 were predominant in brain and bone marrow, and frequent in lymph node (Table 2). This tissue distribution suggests these gp120 sequences can support viral replication in tissues where macrophages (i.e., brain and bone marrow) as well as T cells (i.e., lymph node) are the predominant target cells.

**Viruses expressing V2 and C3 sequences from desiv147c#4 have enhanced fusion activity and viral infectivity**

To characterize phenotypes of SIV expressing V1, V2, V3 and C3 variants from desiv147c#4 (Table 2), we made recombinant Envs and infectious molecular clones (Fig. 3). The recombinant Envs were expressed at similar levels in 293T cells (Fig. 4A). In fusion assays, the 239/251 V1, V2, V3, and C3 Envs mediated higher levels of cell-cell fusion with cells expressing low or high rhesus CD4 compared to the parental Env (Fig. 4B). Next, we tested full-length replication-competent viruses expressing these Envs in infection assays using TZM-BL cells, which express high levels of human CD4 and CCR5. In these assays, 239/251 V2 and 239/251 C3 showed enhanced viral infectivity compared to the parental 239/251 virus (p<0.05) (Fig. 4C), whereas introducing the V1 and V3 variants reduced viral infectivity. The infectivity of 239/251 V1 was greatly impaired (p<0.01) (Fig. 4C). These results show that recombinant SIV 239/251 clones expressing V1, V2, V3, and C3 sequences from desiv147c#4 have high fusion activity, but different levels of viral infectivity in TZM-BL cell assays.
N173 and N481 decrease fusion activity but enhance viral infectivity

Next, we constructed a panel of infectious molecular clones expressing the V1, V2, V3, and C3 variant sequences in different combinations. In addition to these variants, we examined two additional amino acid changes, T173N and S481N, which map to the V2 and C5 regions, respectively. Although these potential N-linked glycosylation sites (NXS/T) are highly conserved among SIV strains, they are absent in the parental SIVmac251 clone used for experiments described above (Fig. 2). When mapped to the unliganded SIV gp120 structure (Chen et al., 2005a), N173 is located in the V1/V2 loop and N481 is located near the CD4 binding loop (Fig. 5). Introducing N173 and N481 into 239/251 Envs did not have a significant effect on Env expression or gp160 processing to gp120 and gp41; a mobility shift was observed for the gp160 and gp120 bands, consistent with addition of glycans at these sites (Fig. 6A).

Next, we tested desiv147c#4 and 239/251 Envs and replication-competent viruses in fusion and infectivity assays. The desiv147c#4 Env mediated high levels of cell-cell fusion and showed similar viral infectivity as SIVmac316, a prototype macrophage-tropic clone used as a positive control (Fig. 6B and C). 239/251 V2C3 and V2V3C3 Envs had increased fusion activity compared to the parental 239/251 (Fig. 6B). The high levels of fusion mediated by 239/251 V2V3C3 Env were similar to those of desiv147c#4 and SIVmac316 Envs, and were observed with cells expressing either low or high levels of rhesus CD4. Introduction of N173 and N481 reduced fusion activity of 239/251 Envs (Fig. 6B), but enhanced viral infectivity in TZM-BL cells by 2.5- to 10-fold (Fig. 6C). These results indicate that desiv147c#4 and 239/251 V2V3C3 Envs are highly fusogenic, similar to the macrophage-tropic SIVmac316 Env, and that introducing N173 and N481 into 239/251 Envs reduces fusion activity, but enhances viral infectivity.

N173 and N481 decrease SIV replication in macrophages

Next, we examined the ability of viruses produced from recombinant infectious molecular clones to replicate in primary rhesus macaque PBMC and MDM. SIVmac239 and SIVmac316 were included as controls. SIVmac239 replicates well in PBMC, but poorly in MDM, whereas SIVmac316 replicates well in both cell types. The 239/251 viruses replicated efficiently in PBMC, with similar replication curves and p27 peak titers (Fig. 7A). desiv147c#4 also replicated in PBMC, but with delayed kinetics and slightly lower peak levels compared to the other viruses (Fig. 7A). The same batch of virus stocks exhibited a different pattern when tested for viral replication in MDM (Fig. 7B). desiv147c#4, 239/251 WT, V2C3, and V1V2V3C3 replicated efficiently with similar kinetics, whereas 239/251 V2V3C3 replicated poorly. Adding N173 and N481 to the 239/251 recombinant viruses reduced replication of these viruses in MDM (Fig. 7B). Multinucleated giant cells (MNGC) were observed in macrophage cultures infected with SIVmac316, desiv147c#4, 239/251 WT, V2C3, and V1V2V3C3 replicated efficiently with similar kinetics, whereas 239/251 V2V3C3 replicated poorly. Adding N173 and N481 to the 239/251 recombinant viruses reduced replication of these viruses in MDM (Fig. 7B). Multinucleated giant cells (MNGC) were observed in macrophage cultures infected with SIVmac316, desiv147c#4, 239/251 WT, and 239/251 V2C3 (Fig. 8). MNGC induced by 239/251 V2C3 were larger than those induced by 239/251 WT, but smaller than those induced by SIVmac316 and desiv147c#4. These results suggest that desiv147c#4 is a macrophage-tropic Env that can mediate high levels of replication and MNGC formation in primary macrophages. Furthermore, these phenotypes can be enhanced by desiv147c#4 amino acid variants mapping to the V2 and C3 regions, but are inhibited by N173 and N481.
N173 and N481 enhance neutralization resistance

SIV macrophage tropism has been associated with high neutralization sensitivity, which renders macrophage-tropic SIV clones more susceptible to humoral immune responses. The V1/V2 and V3 loops are targets for neutralizing antibodies, and mutations in these regions can alter neutralization sensitivity (Johnson et al., 2002; Means et al., 2001; Musich et al., 2011; Pantophlet and Burton, 2006). To investigate whether the Env sequences characterized in the preceding experiments alter neutralization sensitivity, we tested recombinant viruses in a neutralization assay. SIVmac239, a highly neutralization resistant clone, and SIVmac316, a highly neutralization sensitive clone, were included as controls. desiv147c#4 was highly sensitive to SIVmac251 and SIVmac239 antiserum (Table 3). Introducing amino acid variants from the V2, V3 and C3 regions of desiv147c#4 into SIV239/251 had no significant effects on neutralization sensitivity. In contrast, adding N173 and N481 strongly enhanced neutralization resistance of the recombinant viruses to SIVmac251 and SIVmac239 antiserum (Table 3). 239/251 WT NN and 239/251 V2C3 NN were highly resistant to both antisera. Next, we used monoclonal antibodies targeting specific epitopes to probe structural changes. Antibodies 5B11, 7D3, and 36D5 target the CD4 binding site, CCR5 binding site, and V3 loop, respectively (Edinger et al., 2000). Viruses expressing N173 and N481 exhibited enhanced resistance to all three antibodies compared to those lacking these N-glycosylation sites (Table 3). Thus, N173 and N481 enhance neutralization resistance of SIV239/251 recombinant viruses, possibly through modulating accessibility of gp120 epitopes that overlap the CD4 and CCR5 binding sites.

To investigate relationships between neutralization sensitivity and macrophage tropism, we performed Spearman correlation analysis. Replication of SIV recombinant viruses in MDM was associated with higher neutralization sensitivity to SIV251 antiserum ($r = 0.7246, p = 0.0117$), SIV239 antiserum ($r = 0.7356, p = 0.0099$), 36D5 (anti-V3) ($r = 0.6416, p = 0.0333$), and 5B11 (anti-CD4 binding site) ($r = 0.5703, p = 0.0669$) (Fig. 9). Together, these results suggest that the ability of these recombinant SIV clones to replicate in macrophages is associated with higher overall neutralization sensitivity and increased exposure of epitopes that overlap the CD4 binding site and V3 region.

239/251 viruses with N173 and N481 are more neutralization resistant when generated in PBMC compared to 293T cells

HIV/SIV gp160 processing and N-linked glycosylation are cell-type dependent, which in part explains differences viral infectivity and neutralization sensitivity when viruses are produced in different cell types (Lin et al., 2003; Louder et al., 2005; Sawyer et al., 1994; Tremblay et al., 1998; Willey et al., 1996). The replication-competent viruses used in the experiments described above were generated in 293T cells transfected with full-length proviral DNA. To examine phenotypes of viruses generated in a more relevant cell type, we tested viruses generated in PBMC. PBMC-generated viruses used for these experiments were more infectious than the corresponding 293T-generated viruses based on infection of TZM-BL cells (0.1 ng of PBMC-derived virus stock induced comparable levels of infection as those induced by 10 ng of 293T-derived virus stock in TZM-BL cell assays), and induced more severe cytopathic effects in TZM-BL cells at lower doses of virus (data not shown). PBMC-generated viruses replicated as efficiently as 293T cell-derived viruses in PBMC.
Similar to experiments described above (Fig. 7), PBMC-generated desiv147c#4 replicated at moderate levels in MDM, while 239/251 viruses with N173 and N481 replicated at low levels in MDM (Fig. 10B). PBMC-generated 239/251 viruses without N173 and N481 (WT and V2C3) were highly neutralization sensitive, similar to those generated in 293T cells. However, 239/251 viruses containing N173 and N481 (WT NN and V2C3 NN) were more neutralization resistant when generated in PBMC, with one to three logs difference in IC$_{50}$ compared to the corresponding 293T-derived viruses (Table 4). These results suggest that viruses generated in PBMC were more infectious and neutralization resistant compared to those generated in cell lines, but their cell tropism was not changed.

Discussion

In this study, we identified a unique macrophage-tropic SIVmac251 early variant (desiv147c#4) in blood that shares high sequence homology with the gp120 consensus sequence in brain of animals that developed neurological disease. SIV251 clones expressing desiv147c#4 gp120, or V2 and C3 variants from this early variant, mediated high levels of fusion, replicated efficiently in rhesus PBMC and macrophages, and induced MNGC formation during infection of macrophages. The N-linked glycosylation sites N173 in the V2 region and N481 in the C5 region were identified as novel determinants of macrophage tropism and neutralization sensitivity. These N-glycosylation sites reduced fusion activity and macrophage tropism, but enhanced viral infectivity and neutralization resistance when introduced into SIVmac251. Together, these results suggest that desiv147c#4 may be a prototype for SIV strains that can establish early infection of macrophages in brain and other macrophage-rich tissues such as bone marrow, lung, and gut, and induce macrophage-associated pathogenesis in vivo. desiv147c#4 includes sequences similar to those in bone marrow and lymph node, suggesting this variant is not brain-specific. Compared to the well-studied macrophage-tropic SIVmac316 Env, unique features of this novel SIVmac251 variant include its isolation from early stage infection, close genetic relationship to sequences in brain from animals with encephalitis, and lower neutralization sensitivity. Together, these findings suggest that the infectious SIV clones expressing desiv147c#4 and other Envs described in the present study will be useful for future studies on HIV/SIV pathogenesis.

Based on sequence analysis, desiv147c#4 represents a minor variant in blood from SIVmac251-infected rhesus macaques at two weeks post-infection. The low prevalence of macrophage-tropic SIV variants in blood at early infection may in part be due to the low frequency of these variants in the original SIVmac251 inoculum. Consistent with this idea, only three of twenty Envs in a SIVmac251 virus stock mediated significant levels of viral replication in macrophages in a previous study (Bixby et al., 2010). gp120 sequences in the present study were generated by bulk PCR from previous animal studies using different virus stocks and inoculation routes. Two macaques were infected with SIVmac251 stock from one lab, and six were infected with SIVmac251 stock from another lab (Table 1). Despite the limitation of bulk PCR due to potential template switching and recombination during amplification, as well as differences in virus stock origin, dose, and inoculation route, we found high sequence identity between desiv147c#4 and late variants in brain from four...
animals. However, we have not proven that desiv147c#4 is infecting the brain and inducing SIVE. Furthermore, there are many variables in animal models that cannot be predicted by in vitro studies. Therefore, our conclusions must be restricted to those that can be reached based on the present in vitro studies. Similar to previously reported brain-derived SIV clones, including the macrophage-tropic neurovirulent clone SIV/17E-Fr (Flaherty et al., 1997; Gaskill et al., 2005; Puffer et al., 2002), SIV expressing desiv147c#4 replicates well in both rhesus T cells and macrophages, suggesting it is replication-competent in relevant target cells. Based on high similarity between desiv147c#4 and brain-derived sequences together with functional studies demonstrating that desiv147c#4 mediates high levels of fusion and spreading infection in rhesus macrophages in vitro, we predict that desiv147c#4 will be able to infect macrophages in brain and other tissues in vivo. Future in vivo studies in an animal model will be important to determine if this prediction is valid.

The monkey from which desiv147c#4 was isolated developed AIDS and died by 70 days post-infection, a relatively rapid disease course. As such, we cannot rule out the possibility that desiv147c#4 was selected because the animal was immunocompromised due to rapid disease. Consistent with this possibility, another animal model uses a macrophage-tropic clone, SIV/17E-Fr, together with an immunosuppressive virus, SIV/DeltaB670, to induce SIV-associated neurological disease (Zink et al., 1999), suggesting that an immunocompromised environment may help to allow macrophage-tropic SIV to induce pathogenesis in vivo. These findings are consistent with the observation that macrophage-tropic variants are present and can be detected at early infection in some HIV-infected patients (Fiore et al., 1994; Schuitemaker et al., 1992; Schuitemaker et al., 1991; Zhu et al., 1993), but usually do not emerge until late infection, when the host immune system is compromised.

desiv147c#4 is genetically unique compared to published sequences in the NCBI database. The V1, V2, and V3 sequences (Table 2) are present in some transmitted/founder viruses or SIVmac251 swarm-derived molecular clones described by others (Del Prete et al., 2013; Keele et al., 2009; Strickland et al., 2011), but these sequences have not been previously associated with macrophage tropism. Among published sequences, the one most closely related to desiv147c#4 is a sequence isolated from a SIVmac251 stock, 251-PM, by Del Prete et al (Del Prete et al., 2013). The sequence, AGG38406.1, shares 98% sequence identity (484 of 492 amino acids in gp120) with desiv147c#4, and contains the V1, V2, V3 and C3 sequences characterized in the present study. The 8 amino acid differences between desiv147c#4 and AGG38406.1 are E50D, D79E, I215L, G327E, V416L, N418T, G426R, and a P142 insertion in the V1 region. 251-PM was generated by infecting CEMx174 cells, and is genetically distinct from other SIVmac251 stocks, with 1.5% maximum sequence diversity within the swarm. No other features of this SIVmac251 stock or clone were characterized in the study (Del Prete et al., 2013). Most transmitted/founder HIV/SIV viruses have been derived from individuals or animals infected through mucosal routes, whereas desiv147c#4 was derived after intravenous (i.v.) infection. Viruses transmitted via the i.v. route undergo less stringent strain selection compared to mucosal transmission, but viruses still encounter substantial selection pressure. While most cases of mucosal transmission have only one transmitted/founder virus, a prior study found that three of five
animals infected with uncloned SIVmac251 or SIVsmE660 quasispecies through the i.v. route had four or fewer transmitted/founder viruses (Keel et al., 2009). Accordingly, viruses transmitted through the i.v. route may exhibit different phenotypes compared to those through the mucosal route. desiv147c#4 replicates efficiently in CD4+ T cells, the first cell type infected after viruses cross the mucosal barrier (Keel and Estes, 2011). As such, future in vivo studies will be of interest to test whether desiv147c#4 can mediate mucosal transmission.

Introducing desiv147c#4 gp120, or its V2 and C3 sequences, into SIV 239/251 viruses had little or no effect on viral replication in MDM, but enhanced fusion and MNGC formation in macrophages, an important feature of SIV encephalitis. 239/251 Envs with these V2 and C3 sequences were able to mediate high levels of fusion with cells expressing low or high CD4. V2 plays a role in gp120 interactions with CCR5, and may thereby modulate fusion activity (Rizzuto et al., 1998; Sullivan et al., 1998; Wyatt et al., 1995). Enhanced fusion activity of 239/251 V2 Envs might reflect structural changes involving the V2 region that lower CD4 dependence. G384R in the C3 region, flanking the conserved CD4 binding motif, GGDPE at the N-terminus, was previously identified as a determinant of macrophage-tropism and low CD4 dependence (Mori et al., 1992; Mori et al., 2000; Otto et al., 2003; Ryzhova et al., 2009). In these studies, G to R mutation of the first glycine in the GGDPE domain also showed CD4 independence, and was associated with clones from brain, but not spleen (Ryzhova et al., 2002b). Unexpectedly, this G to R mutation impaired gp120 binding to CD4 by increasing the dissociation rate constant (k\textsubscript{off}) (Ryzhova et al., 2002a), raising the possibility that mutations in this region may facilitate Env-mediated fusion with low CD4 expressing cells by facilitating interaction with CCR5 rather than CD4. These findings also suggest a close association between cell-cell fusion activity with target cells expressing low CD4 and MNGC induction in macrophages, phenotypes likely to be important for cell-cell transmission and viral pathogenicity in vivo.

Unexpectedly, the N-linked glycosylation sites N173 and N481 were identified as determinants of viral infectivity and macrophage tropism. These two N-glycosylation sites are highly conserved among SIV strains, but missing in the SIVmac251\textsubscript{BK28} clone, possibly due to prolonged passage in tissue culture. Notably, they were restored when SIVmac251\textsubscript{BK28} was introduced into animals (Edmonson et al., 1998), suggesting their importance for in vivo infection. Removal of glycans at positions analogous to N173 or N481 in HIV or SIV reduced infectivity in prior studies (Lee et al., 1992; Pikora et al., 2005). Consistent with these findings, removal of N173 and N481 reduced infectivity of 239/251 viruses in TZM-BL cells, but enhanced viral replication in macrophages in the present study. Viruses lacking these two N-glycosylation sites may be less efficient for cell-free infection, but may be more efficient at mediating fusion and cell-to-cell transmission with low CD4 cells, thereby enhancing replication in macrophages (Yen et al., 2014). These phenotypes might be strain-dependent, since the macrophage-tropic SIVmac316 clone expresses N173 and N481.

N173 and N481 enhanced neutralization resistance of 239/251 SIV viruses. This finding is consistent with previous studies showing that glycosylation sites, particularly those located in the V1/V2 regions, contribute to neutralization resistance against antiserum and multiple
antibodies targeting a broad range of epitopes (Chackerian et al., 1997; Johnson et al., 2003b; Pikora et al., 2005; Sagar et al., 2006). Increased neutralization resistance to monoclonal antibodies targeting CD4 binding site and V3 loop suggests that N-glycans at these positions may modulate the structure or accessibility of these epitopes. Indeed, previous studies showed that loss of the N160 N-glycosylation site in HIV Env (analogous to N173 in SIV) impaired CCR5 usage in a cell-cell fusion assay (Ogert et al., 2001). Furthermore, N160 is a critical residue in HIV gp120 for recognition and binding of a broadly neutralizing antibody, PG9 (McLellan et al., 2011). The recent finding that antibodies targeting the V2 region were the only variable correlating with protection in the RV144 vaccine trial highlight the importance of V2 as an immunogenic epitope (Haynes et al., 2012; Rolland et al., 2012). The N173 glycan may shield these epitopes from antibody recognition, and thereby enhance neutralization resistance. 239/251 NN viruses generated in PBMC were more neutralization resistant than those generated in 293T cells, consistent with previous studies indicating that gp120 glycosylation is dependent on the cellular source of viruses (Lin et al., 2003; Willey et al., 1996). These finding suggest that N173 may be an important determinant of neutralization sensitivity for HIV and SIV infections. Further studies are required to address this question and dissect individual roles of N173 and N481 in determining neutralization sensitivity.

The association between neutralization sensitivity and SIV macrophage tropism demonstrated in the present study and prior studies may have different consequences for pathogenesis in the periphery versus the CNS. In particular, strong neutralizing antibody responses in the periphery are expected to inhibit these strains, while lower levels of neutralizing antibodies in the CNS may allow them to replicate and persist in this compartment.

In conclusion, we characterized a unique macrophage-tropic SIV Env variant from early stage infection that is closely related to sequences in brain from several animals with neurological disease, and identified novel determinants of SIV macrophage tropism and neutralization sensitivity. The findings suggest that macrophage-tropic SIV viruses capable of establishing persistent viral reservoirs in macrophages in brain and other tissues can be present in blood during early infection. Furthermore, the replication-competent SIVmac251 clones described in this study replicate well in both T-cells and macrophages, and are closely related to sequences not only in brain, but also in non-brain tissues such as lymph node and bone marrow, suggesting they will be useful for non-human primate studies on HIV/SIV macrophage-related pathogenesis, persistence and eradication of viral reservoirs, and vaccine development.

Acknowledgments

We thank J. Sodroski, A. Engelman, R.P. Johnson, D. Barouch, and K. Reimann for helpful discussions and advice. We are also grateful to the NIH AIDS Research and Reference Reagent Program for providing SIVmac251 antiserum, J. Sodroski for pSIVΔgpv plasmid, N. Letvin for TZM-BL cells, and S. Westmoreland and W. Lauer for providing infection and pathology data from animal studies.

This work was supported by NIH grants MH83588 and MH97659 to D.G. M.E.M. was supported in part by NIH fellowship F31NS060611. Core facilities were supported by Harvard Medical School Center for AIDS Research (CFAR) and DFCI/Harvard Cancer Center grants.
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We characterize a macrophage-tropic SIVmac251 variant in blood from early infection.

This Env variant is similar to brain variants from animals with neurological disease.

These sequences are also related to those in non-brain tissues such as lymph nodes.

SIVmac251 clones expressing these variants replicate in both T-cells and macrophages.

These SIVmac251 clones can be used to study pathogenesis, eradication, and vaccines.
Figure 1.
Identification of an early SIV variant in blood closely related to late variants in brain from macaques with SIV encephalitis. (A) Genetic distance between early variants in blood from four macaques at two weeks post-infection and the gp120 consensus sequence of late variants in brain from four other macaques that developed rapid disease progression and SIV. Among seven early variants, desiv147c#4 had the lowest genetic distance to the gp120 consensus sequence in brain, indicating highest genetic similarity. (B) Phylogenetic analysis shows clustering of desiv147c#4 with sequences from brain (BR), bone marrow (BM), and lymph node (LN) tissues at autopsy from four macaques that died with AIDS. Clones from early infection are colored in cyan. Clones from late infection are colored based on the animal from which they were derived. Scale bar represents 0.01 nucleotide substitutions per base.
Figure 2.

gp120 sequence differences between desiv147c#4 and a lab-adapted SIVmac251 clone map primarily to the V1, V2, V3 and C3 regions. Alignment of the gp120 consensus sequence in brain tissue from four macaques obtained at autopsy (Table 1), desiv147c#4, and A92-620BR2 (the brain variant most similar to the brain consensus sequence) with the lab-adapted SIVmac251 gp120 sequence. Amino acid variants in the V1, V2, V3 and C3 regions are highlighted. N-linked glycosylation sites are marked with $\psi$. 

Virology. Author manuscript; available in PMC 2015 June 01.
Figure 3.
Construction of full-length recombinant SIV 239/251 proviruses expressing gp120 sequences from the early variant desiv147c#4. The gp120 region and N-terminus of gp41 of SIVmac251 was cloned into the full-length SIVmac239 provirus. Sequences in the V1, V2, V3 and C3 regions of desiv147c#4 were introduced into SIVmac251 gp120 to create recombinant viruses.
Figure 4.
SIV Envs expressing sequences from desiv147c#4 mediate high levels of cell-cell fusion but variable infection of TZM-BL cells. (A) Expression of recombinant Envs in transfected 293T cells examined by western blot. (B) Cell-cell fusion assay. 293T cells transfected with GFP (negative control) or the indicated Env-expressing plasmids were mixed with Cf2-luc cells expressing rhesus CCR5 and low or high levels of rhesus CD4 after transfection with CCR5 and either 0.5 or 10 μg CD4 plasmid DNA, respectively. Cell-cell fusion was quantified by measuring luciferase activity. (C) Infection of TZM-BL cells with the indicated replication-competent SIV viruses (10 ng of p27). Shown are means and standard deviation of samples from duplicate wells. * indicates significant difference vs. 239/251 WT (Student’s t-test; p<0.05). ** indicates significant difference vs. 239/251 WT (p<0.01). Shown are representative results of 3 to 6 independent experiments.
Figure 5.
N173 and N481 mapped onto the unliganded SIV structure published by Chen et al, 2005 (Chen et al., 2005b). N173 (shown as Ψ) is in the V1/V2 loop, which is absent in the structure, shown here as a dashed line. N481 and CD4 binding loop containing the conserved GGDPE domain are marked in gray. In this model, the N481 N-glycosylation site is located near the CD4 binding loop.
Figure 6.
N173 and N481 N-glycosylation sites influence cell-cell fusion and viral infectivity in TZM-BL cells. Env expression was examined by western blot (A). Fusion assays using Env clones (B) and TZM-BL infection assays using replication-competent viruses (C) were performed as described in Figure 4. Introduction of N173 and N481 into SIVmac251 gp120 (indicated as NN) reduces Env fusion activity, but enhances viral infectivity in TZM-BL assays. Shown are mean and standard deviation of samples from duplicate wells. * indicates significant differences by student’s t-test (p < 0.05). Shown are representative results of one to five independent experiments.
Replication of recombinant SIV viruses in primary rhesus macaque PBMC (A) and MDM (B). Viral replication was quantified in culture supernatants from PBMC and MDM cultures infected with the indicated virus stocks (10 ng of p27) by measuring the concentration of SIV p27 antigen in cell culture supernatants by ELISA at the indicated time points. Introduction of N173 and N481 into SIVmac251 gp120 (indicated as NN) reduced viral replication in macrophages, while not affecting viral replication in PBMC. Shown are means of samples from duplicate wells. Data in each panel are from a single experiment, with viral replication of the panel of mutants examined in cells from the same animal donor. Shown are representative results of three independent experiments.
Figure 8.
Induction of multinucleated giant cells in primary rhesus macrophages infected with recombinant SIV viruses. Shown are multinucleated giant cells on day 7 post-infection in rhesus MDM cultures infected with 10 ng of p27 of the indicated SIV viruses.
Figure 9.

Macrophage tropism of recombinant SIV is associated with neutralization sensitivity to SIV251 antiserum, SIV 239 antiserum, 36D5 (anti-V3), and 5B11 (anti-CD4 binding site). The relationship between SIV replication in macrophages and neutralization sensitivity was analyzed by Spearman correlation. Replication in macrophages was expressed as SIV p27 antigen concentration in cell culture supernatants at 10 days post-infection (Fig. 7B). Neutralization sensitivity was expressed as the reciprocal dilution required for achieving 50% inhibition of infection with the no serum control.

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Figure 10.
Replication of PBMC-derived recombinant SIV viruses in primary rhesus macaque PBMC (A) and MDM (B). Viral replication was quantified in rhesus PBMC and MDM infected with 2 ng p27 of the indicated PBMC-derived viruses by measuring SIV p27 antigen concentration in cell cultures supernatants by ELISA. Data in each panel are from a single experiment, with viral replication of the panel of mutants examined in cells from the same animal donor. Values shown are means of duplicate wells.
| Group | Animal | Inoculum | Survival dpi<sup>a</sup> | AIDS | Pathology | Reference |
|-------|--------|----------|--------------------------|------|-----------|-----------|
| I     | 147-94 | SIVmac251 (0.032 ng p27, i.v.<sup>b</sup>) | 70 | Yes | E. coli enteritis, E. bieneusi cholecystitis, lymphoid depletion | Current publication |
|       | 168-94 | SIVmac251 (0.032 ng p27, i.v.) | 856 | Yes | Severe intestinal mycobacteriosis, peritonitis, lymphoid depletion | Current publication |
|       | 90-95  | SIVmac251 (24 ng p27, vag.<sup>c</sup>) | 122 | Yes | SIV giant cell pneumonia, adenovirus, CMV, candidiasis | Current publication |
|       | 166-95 | SIVmac251 (24 ng p27, vag.) | 463 | Yes | SIV pulmonary arteriopathy, pericarditis, myocarditis, and sepsis due to S. aureus | Current publication |
| II    | A92–620| SIVmac251 (20 ng p27, i.v.) | 115 | Yes | SIVE<sup>d</sup> (MNGC<sup>e</sup>); CMV pneumonia; severe segmental purulent enteritis and peritonitis and lymphangiectasia | Williams et al., 2002 (Williams et al., 2002) |
|       | A95–346| SIVmac251 (20 ng p27, i.v.) | 190 | Yes | SIVE (MNGC); giant cell pneumonia; giant cell enteritis | Williams et al., 2001 (Williams et al., 2001) |
|       | A97-6  | SIVmac251 (20 ng p27, i.v.) | 243 | Yes | SIVE (MNGC); severe chronic active gastritis; Severe AIDS encephalopathy, brain & spinal cord; Severe diffuse glomerulonephritis; Cirrhosis; Enteroctozen boleus, bile duct duodenum; Proliferative and fibrinous peritonitis; Giant cell pneumonia | Williams et al., 2001 (Williams et al., 2001); Williams et al., 2002 (Williams et al., 2002) |
|       | A97–251| SIVmac251 (20 ng p27, i.v.) | 233 | Yes | SIVE (MNGC); Pneumocystis carinii pneumonia (PCP); CMV<sup>f</sup> orchitis | Orandle et al., 2002 (Orandle et al., 2002) |

<sup>a</sup> dpi, days post-infection.

<sup>b</sup> i.v., intravenous inoculation.

<sup>c</sup> vag., vaginal inoculation.

<sup>d</sup> SIVE, SIV encephalitis.

<sup>e</sup> MNGC, Multinucleated giant cells.

<sup>f</sup> CMV, Cytomegalovirus.
Table 2

Desivir147c#4 sequences in the V1, V2, V3, and C3 regions (shown in bold) match the predominant sequences in brain, bone marrow, and lymph node at late-stage infection.

| regions | residue no. | sequence  | late infection\(^a\) | early infection\(^b\) |
|---------|-------------|-----------|----------------------|----------------------|
|         |             |           | total % (n=37)        |                      |
|         |             |           | brain % (n=13)        | bone marrow % (n=12) |
|         |             |           | lymph node % (n=12)   | total % (n=7)        |
| V1      | 134–143     | PTTPNTTSTK| 35                   |                      |
|         |             |           | 38                   | 42                   |
|         |             |           | 25                   | 14                   |
|         |             | PTSAPVSEK | 35                   |                      |
|         |             |           | 8                    | 42                   |
|         |             |           | 58                   | 14                   |
|         |             | PTAPTAASK | 16                   |                      |
|         |             |           | 23                   | 17                   |
|         |             |           | 8                    | 0                    |
|         |             | STTPKTSTK | 5                    |                      |
|         |             |           | 15                   | 0                    |
|         |             |           | 0                    | 0                    |
|         |             | PTTPSTTSTK| 3                    |                      |
|         |             |           | 8                    | 0                    |
|         |             |           | 0                    | 0                    |
|         |             | PTAPTAAPK | 3                    |                      |
|         |             |           | 0                    | 0                    |
|         |             |           | 8                    | 0                    |
|         |             | PPSAPASEP | 3                    |                      |
|         |             |           | 8                    | 0                    |
|         |             |           | 0                    | 0                    |
| V2      | 155–157     | VHD       | 41                   |                      |
|         |             |           | 54                   | 42                   |
|         |             |           | 25                   | 29                   |
|         |             | AQN       | 24                   |                      |
|         |             |           | 0                    | 42                   |
|         |             |           | 33                   | 14                   |
|         |             | THD       | 24                   |                      |
|         |             |           | 31                   | 17                   |
|         |             |           | 25                   | 57                   |
|         |             | AHN       | 3                    |                      |
|         |             |           | 8                    | 0                    |
|         |             |           | 0                    | 0                    |
|         |             | AHD       | 3                    |                      |
|         |             |           | 0                    | 8                    |
|         |             |           | 0                    | 0                    |
|         |             | IHD       | 3                    |                      |
|         |             |           | 8                    | 0                    |
|         |             |           | 0                    | 0                    |
|         |             | AQD       | 3                    |                      |
|         |             |           | 0                    | 8                    |
|         |             |           | 0                    | 0                    |
| V3      | 337         | V         | 100                  |                      |
|         |             |           | 100                  | 100                  |
|         |             |           | 100                  | 100                  |
|         | 339         | E         | 76                   |                      |
|         |             |           | 100                  | 58                   |
|         |             |           | 67                   | 67                   |
|         |             | D         | 24                   |                      |
|         |             |           | 0                    | 42                   |
|         |             |           | 33                   | 33                   |
|         | 342         | N         | 100                  |                      |
|         |             |           | 100                  | 100                  |
|         |             |           | 100                  | 100                  |
| C3      | 384         | R         | 100                  |                      |
|         |             |           | 100                  | 100                  |
|         |             |           | 100                  | 100                  |

\(^a\)Clones from late infection were derived from autopsy brain, bone marrow, or lymph node tissue samples from four macaques that died with AIDS (Table 1).

\(^b\)Clones from early infection were derived from PBMC two weeks post-infection from four macaques infected with uncloned SIVmac251 virus stock (Table 1).
### Table 3

Neutralization sensitivity profile of replication-competent recombinant SIV viruses generated in 293T cells

| Viruses         | SIV251 antiserum | SIV239 antiserum | 5B11<sup>c</sup> | 7D3<sup>c</sup> | 36D5<sup>c</sup> |
|-----------------|------------------|------------------|------------------|-----------------|-----------------|
|                 | IC<sub>50</sub><sup>a</sup> | IC<sub>90</sub> | IC<sub>50</sub> | IC<sub>90</sub> | IC<sub>50</sub> | IC<sub>90</sub> | IC<sub>50</sub> | IC<sub>90</sub> |
| SIVmac239       | <100<sup>b</sup>  | <100  | <100  | <100  | <100  | <100  | <100  | <100  |
| SIVmac316       | 622542           | 126456          | 288437          | 104103          | 444272          | 518  | 537622          | <100  | 107991          | 7401 |
| desiv147c#4     | 626748           | 131824          | 276853          | 62275           | 570  | <100  | 730  | <100  | 39535 | 1707 |
| 239/251 WT      | 192810           | <100            | 145862          | <100            | 343764          | <100  | 10294          | <100  | 30746 | <100  |
| 239/251 WT NN   | 562              | <100            | <100            | <100            | 447  | <100  | <100  | <100  | <100  | <100 |
| 239/251 V2C3    | 68476            | 9134            | 39471           | 6469            | 34679          | <100  | 477384         | <100  | 27878 | <100  |
| 239/251 V2C3 NN | 2753             | <100            | 24312           | <100            | 146  | <100  | 38502          | <100  | <100  | <100  |
| 239/251 V2V3C3  | 189907           | 44743           | 119171          | 26760           | 169195         | 654  | 713938         | 118419| 31203 | <100  |
| 239/251 V2V3C3 NN | 133147         | 101             | 72208           | <100            | 75038          | <100  | 502726         | <100  | 690  | <100  |

**Color code:**
- <100
- 100 – 1000
- 1000 – 10000
- >10000

<sup>a</sup> IC<sub>50</sub> and IC<sub>90</sub> were calculated as the reciprocal dilution of antiserum or ascites required for achieving 50% and 90% inhibition of infection with no serum control, respectively.

<sup>b</sup> IC<sub>50</sub> or IC<sub>90</sub> could not be achieved at 1:100 dilutions of antiserum or ascites containing the monoclonal antibodies.

<sup>c</sup> Epitopes of 5B11, 7D3, and 36D5 were previously mapped to the CD4 binding site, CCR5 binding site, and V3 loop, respectively (Edinger et al., 2000).
### Table 4

Neutralization sensitivity profile of replication-competent recombinant SIV viruses generated in PBMC

| Viruses          | SIV251 antiserum | SIV239 antiserum | 5B11<sup>c</sup> | 7D3<sup>c</sup> | 36D5<sup>c</sup> |
|------------------|------------------|------------------|------------------|------------------|------------------|
| SIVmac239        | <100<sup>b</sup> | <100<sup>b</sup> | <100<sup>b</sup> | <100<sup>b</sup> | 158<sup>a</sup>  |
| SIVmac316        | 629106           | 609154           | 115496           | 129037           | 752972          |
| desiv147c#4      | 627934           | 296499           | 103497           | 101<sup>c</sup>  | 512<sup>c</sup>  |
| 239/251 WT       | 513696           | 219312           | 133481           | 601386           | 7708<sup>a</sup> |
| 239/251 WT NN    | <100<sup>b</sup> | <100<sup>b</sup> | <100<sup>b</sup> | <100<sup>b</sup> | 144<sup>a</sup>  |
| 239/251 V2C3     | 110632           | 35489            | <100<sup>c</sup> | 1840<sup>c</sup> | 470150          |
| 239/251 V2C3 NN  | <100<sup>b</sup> | <100<sup>b</sup> | <100<sup>b</sup> | <100<sup>b</sup> | 198<sup>a</sup>  |

**color code:**
- **<100**
- **100 – 1000**
- **1000 – 10000**
- **>10000**

<sup>a</sup> IC<sub>50</sub> and IC<sub>90</sub> were calculated as the reciprocal dilution of antiserum or ascites required for achieving 50% and 90% inhibition of infection with the no serum control, respectively.

<sup>b</sup> IC<sub>50</sub> or IC<sub>90</sub> could not be achieved at 1:100 dilutions of antiserum or ascites containing the monoclonal antibodies.

<sup>c</sup> Epitopes of 5B11, 7D3, and 36D5 were mapped to the CD4 binding site, CCR5 binding site, and V3 loop, respectively (Edinger et al., 2000).