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SIV envelope glycoprotein determinants of macrophage tropism and their relationship to

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A dissertation presented

by

Po-Jen Yen

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Dissertation Advisor: Dr. Dana Gabuzda   Po-Jen Yen

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Abstract

Macrophages are target cells for human immunodeficiency virus (HIV) and simian

immunodeficiency virus (SIV) infection that serve as viral reservoirs in brain, lung, gut, and other

tissues, and play important roles in disease pathogenesis, particularly HIV/SIV-associated

neurological disease. Macrophages express low levels of the HIV/SIV receptor CD4, but

mechanisms by which macrophage-tropic viruses use low CD4 to mediate spreading infections

are poorly understood. One mechanism involves enhanced envelope glycoprotein (Env)

interaction with CD4 or CCR5, but this phenotype is frequently associated with increased

neutralization sensitivity to antibodies targeting CD4/CCR5 binding sites. Moreover, this

mechanism does not explain how these neutralization-sensitive viruses evade immune

responses while establishing spreading infections. In this dissertation, we sought to identify SIV

Env determinants for macrophage tropism and characterize mechanisms by which they

enhance virus replication in macrophages. To identify viral variants capable of inducing

macrophage-associated pathogenesis, we cloned Env sequences from SIV-infected macaques

at early and late stage infection, and identified an early variant in blood that shares >98%

sequence identity with the consensus sequence of late variants in brain from macaques with

neurological disease. SIV viruses encoding this Env variant mediated high levels of fusion,

replicated efficiently in rhesus PBMC and macrophages, and induced multinucleated giant cell

formation upon infection of macrophage cultures. We identified an N-linked glycosylation site,

N173 in the V2 region, as a determinant of macrophage tropism. Loss of N173 enhanced

SIVmac239 macrophage tropism, while restoration of N173 in SIVmac251 reduced macrophage

tropism, but enhanced neutralization resistance to CD4/CCR5 binding site antibodies. SIVmac239 N173Q, which lacks the N173 glycosylation site, mediated CD4-independent fusion and cell-to-cell transmission with CCR5-expressing cells, but could not infect CD4-negative cells in single-round infections. Thus, CD4-independent phenotypes were detected only in the context of cell-cell contact. The N173Q mutation had no effect on SIVmac239 gp120 binding to CD4 in BIACORE and co-immunoprecipitation assays. These findings suggest that loss of the N173 glycosylation site increases SIVmac239 replication in macrophages by enhancing CD4-independent cell-to-cell transmission through CCR5-mediated fusion. This mechanism may facilitate escape of macrophage-tropic viruses from neutralizing antibodies, while promoting spreading infections by these viruses *in vivo.*

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CHAPTER 1: INTRODUCTION

The HIV/AIDS pandemic

More than 30 years since the first cases reported in 1981 (1, 2), acquired immune deficiency syndrome (AIDS) has been a global pandemic, resulting in more than 30 million deaths (UNAIDS 2012). Human immunodeficiency virus (HIV) was first isolated from AIDS patients in 1983 (3) and identified as the causative pathogen in 1984 (4-7). More than 60 million people have been infected, with 2.5 million new infections in 2011 alone, 2.2 million of which were adults and 0.3 million were children (UNAIDS 2012). As of 2011, 34 million people are living with HIV, the majority (69%) of which live in Sub-Saharan Africa, where 1 out of every 20 adults are infected (UNAIDS 2012). With antiretroviral therapy (ART) becoming more readily accessible, global AIDS-related deaths have been decreasing steadily from a peak of 2.3 million in 2005 to 1.7 million in 2011, especially in Sub-Saharan Africa, Caribbean, and Oceania regions, where the declines are 32%, 48%, and 41%, respectively. Furthermore, new HIV infections have been reduced by more than 50% since 2001 in 25 countries (UNAIDS 2012). However, current antiretroviral therapy does not cure HIV infection, and there is no effective vaccine available. Therefore, AIDS remains a serious worldwide healthcare issue.

HIV is a member of the genus *Lentivrus* that belongs to the family *Retroviridae*. As a retrovirus, HIV reverse transcribes its RNA genome into DNA, which is then incorporated into host genome for encoding viral proteins. The error-prone, lack of proof-reading mechanism of reverse transcription, together with high replication rate, high recombination rate, and APOBEC3G-
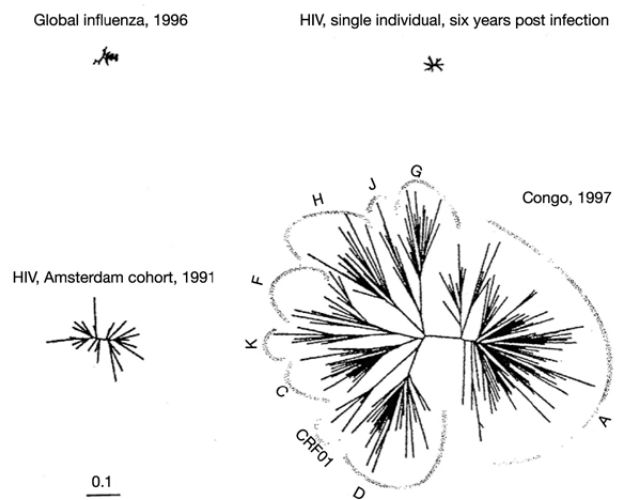
Figure 1.1 HIV variation. Adapted from Weiss et al, 2001 (8).

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induced hypermutation, make HIV one of the most diverse viruses ever identified (9). The variety of HIV population in one patient after 6 years of infection is comparable to that of global influenza in one year (Figure 1.1) (8, 10, 11). This enormous genetic variability has made the development of HIV vaccine an unprecedented challenge.

HIV is divided into two genetically distinct types, HIV-1 and HIV-2. HIV-1 is the predominant circulating virus of the current global pandemic, and is further divided into three subgroups: M (major), O (outlier), and N (Not-M, Not-O). The M group is the majority of global viral isolates, responsible for more than 90% of AIDS cases, and consists of 12 clades, A1, A2, B, C, D, F1, F2, G, H, J, and K. Clade B viruses are dominant in Europe and North America, and are most extensively studied. Clade C is dominant in Africa and India, and is the most prevalent subtype globally. HIV-2 is less transmitted, and is mostly confined in Africa. HIV-1 and HIV-2 are genetically distinct from each other, and are derived from simian immunodeficiency virus (SIV) of different primate sources (Figure 1.2) (12). HIV-1 is genetically close to SIV found in chimpanzees, and is believed to derive from chimpanzee-to-human infection, whereas HIV-2 is more close to SIV from sooty mangabey. SIV strains from different primates are also divergent from each other. SIV infection of primates resembles many features of HIV infection in human, and has been used as animal models to study AIDS pathogenesis.
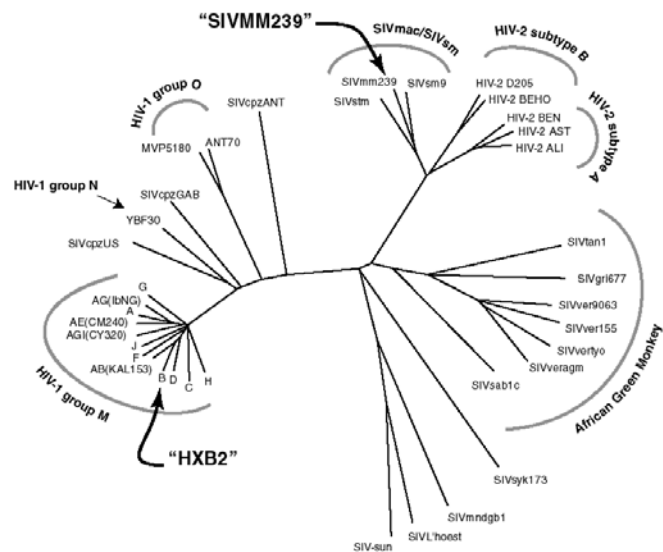
Figure 1.2 Phylogenetic tree of HIV and SIV. Adapted from Calef et al, 2001(12).

HIV/SIV infection

Clinical course and pathogenesis of HIV infection

HIV can be transmitted by specific body fluids, including blood, semen, vaginal secretions, and breast milk, through direct injection into the blood-stream or contact with mucosal membranes. Most HIV transmission occurs at mucosal sites through unprotected sexual behaviors (14). ~80% of infections transmitted through mucosal surfaces originate from one single transmitted / founder virus (T/F virus) (15-18). Gut mucosa and gut-associated lymphoid tissue (GALT) are the largest mucosal surface and lymphoid tissue in human body, respectively (19, 20), containing the largest pool of $CD4^+$ T cells and macrophages, the main target cells of HIV. A rapid and massive depletion of $CD4^+$ T cells from gut mucosa is induced in both HIV-infected humans (21-23) and SIV-infected macaques (24-28). 70-90% of $CD4^+$ T cells in the intestinal lamina propria are lost in the first two to three weeks of infection (Figure 1.3) (13). $CD4^+$ T cell counts in peripheral blood are also reduced in this period, although not as dramatically as the depletion in gut.

This difference may be explained in part by the availability of CCR5-expressing target cells (13). CCR5 is the main coreceptor used by HIV/SIV for entry into cells. In contrast to gut mucosa, where the majority of $CD4^+$ T cells are activated effector memory T cells expressing CCR5 (29), only 15% of blood T cells express CCR5 (24, 28, 29). In fact, only 0.01-1% of $CD4^+$ T cells

Figure 1.3 Clinical course of HIV infection. Adapted from Grossman et al, 2006 (13).

are infected in blood (30). During this acute phase, viruses replicate exponentially, reaching a

peak of more than 10^6 copies viral RNA per ml of peripheral blood. The high levels of viremia induce host adaptive cellular and humoral responses, which specifically target the viruses, but only partially control the infection and reduce viral load by one or two logs to a stable level (around 10^4 viral RNA copies per ml of blood) (Figure 1.3) (31, 32).

The reduction of peak viral load to a lower "set point" marks the onset of a clinically asymptomatic chronic phase, which can last for 10 years or longer in untreated patients. Peripheral blood CD4$^+$ T cell counts are partially restored and then gradually decline over the course of chronic infection (Figure 1.3).This phase is characterized by systemic chronic immune activation, a hallmark of HIV infection and the strongest predictor of disease progression (33). One cause of this chronic immune activation is microbial translocation (34), which may be due to the breakdown of mucosal barrier during acute infection as a result of the cytolytic viral infection and massive depletion of effector cells at the gut mucosal site. Chronic immune activation contributes to CD4$^+$ T cell depletion by providing more activated effector cells available for infection, by increasing turnover rate that leads to consumption of the naïve and resting cell pools, and by activation-induced apoptosis (32), all of which contribute to immune exhaustion and AIDS.

The symptomatic phase, AIDS, starts when the blood CD4$^+$ T cell count is <200 cells per µl, and is characterized by a breakdown of the immune system, uncontrolled HIV viremia, and opportunistic infections (35). 20-30% of patients also develop HIV-associated neurological disease. As the disease progresses, the immune system in patients is compromised, rendering them more susceptible for opportunistic infections and cancer, which are the main causes of AIDS-associated death.

Host immune responses to HIV/SIV infection

One of the first waves of detectable host immune responses is increased levels of cytokines and chemokines in plasma, including IL-15, type I interferons, CXCL10, IL-18, TNF,

IFN-γ, IL-22, and IL-10 (36). These cytokines may inhibit viral replication, while enhancing innate and adaptive immune responses. First, CD8$^+$ cytotoxic T lymphocyte (CTL) responses are induced as viremia approaches its peak. CTL responses partially control viral replication, reduce viral load, and are important for maintaining viral set point. However, they also exert immune selection pressure, and quickly lead to the emergence of viral escape mutants. CD8$^+$ cells expressing MHC-I alleles that recognize relatively conserved epitopes control virus more effectively (36). Patients with these protective alleles (HLA-B27, HLA-B57) progress to AIDS more slowly (37). Neutralizing antibody responses appear slowly at ~12 weeks after infection. Antibodies against autologous viruses usually are delayed by several months, and therefore are not able to control viral infection effectively. However, broadly neutralizing antibodies develop over time in 10-30% infected individuals (38-40). These broadly neutralizing antibodies can prevent infection when injected into animals (41-46). Further understanding of how these broadly neutralizing antibodies can be elicited will provide insights helpful to vaccine design.

Treatment

Current treatment of HIV infection, known as highly active antiretroviral therapy (HAART), combines multiple drugs targeting different stages of the virus replication cycle to prevent resistance by emergence of escape mutants. Classes of drugs used in current HAART include entry inhibitors, nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), integrase inhibitors, and protease inhibitors. Targets of these drugs will be described in further detail later. Although not able to cure HIV infection yet, HAART effectively controls viral load to low or undetectable levels, maintains immune system function, and prevents opportunistic infection. However, these drugs have poor penetration into the brain, and about 30-50% of patients develop HIV-associated neurological disorders even under treatment. Recently, a baby, whose mother has HIV, received HAART at 30 hours after birth, has shown no signs of infection for one year without drugs. The effectiveness of this treatment

is consistent with the idea that it is possible to clear the viruses if treated early, before they have the chance to establish the reservoir, which is the main hurdle to cure for current therapy.

Three cases of HIV-infected patients receiving bone-marrow transplant have been reported virus-free without HAART. The first "Berlin patient" had leukemia and was infected with HIV. He received a transplanted bone marrow with the CCR5Δ32 mutation, a deficient form of the coreceptor CCR5 that mediates HIV infection, and has no detectable virus without drugs for five years now. Recently, two HIV-infected Boston patients with lymphoma received similar procedures with bone marrow transplants without the CCR5Δ32 mutation, and showed no detectable viral levels after they stopped taking antiretroviral drugs. This procedure is too risky to be applied to most patients not at risk of dying from cancer already. However, studies of these patients may offer new insights into viral reservoirs and immune control of the viruses.

SIV animal model

SIV infection in non-natural hosts causes AIDS-like pathogenesis that in many ways resembles HIV infection in humans, with an early peak of high viral load, CD4$^+$ T cell depletion, chronic and systemic immune activation, microbial translocation, opportunistic infections, and finally progression to immunodeficiency and death. Utilization of SIV animal models has provided valuable insights into AIDS pathogenesis. Infection of SIV derived from rhesus macaques (SIVmac) or sooty mangabeys (SIVsm) in rhesus macaques is the non-human primate model most commonly used for AIDS pathogenesis studies. On the other hand, studies of non-pathogenic SIV infection of their natural hosts also provide valuable information understanding pathogenic infections. Similar to pathogenic HIV and SIV infections, non-pathogenic SIV infections of their natural hosts show high levels of viral replication, CD4$^+$ T cell depletion in blood and intestine during acute infection. However, non-pathogenic SIV infection lacks chronic immune activation, and CD4$^+$ T cells are restored during the chronic phase of infection. Therefore, the natural hosts of SIV co-evolved and are able to co-exist with the virus,

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not by controlling viral replication, but by mitigating immune response and preventing

exhaustion of the immune system.

HIV/SIV virus

Viral structure and genome organization

HIV and SIV contain two copies of linear, positive sense, single-stranded RNA genome that binds to nucleocapsid, and, together with reverse transcriptase, integrase, and protease, is enclosed in a conical capsid made of approximately 2,000 copies of viral protein p24 or p27, for HIV or SIV, respectively (Figure 1.4). The conical viral core is surrounded by a spherical layer of matrix protein p17, which is covered with a lipid bilayer

Figure 1.4 Structure of HIV virion. Adapted from NIAID.

envelope derived from the cell membrane when viruses bud from the cell. Embedded in the envelope are membrane proteins acquired together with the host cell membrane, and the only viral protein on the virion surface, envelope glycoprotein, which is responsible for viral entry by mediating fusion between viral and cell membranes.

The single-stranded RNA genome is about 10 kb. It is generated by utilizing the host transcription machinery, and therefore contains 5' cap and 3' poly(A) tail as cellular RNAs. The 5' and 3' ends of the genome are long terminal repeats (LTR), which contains important regulatory sequences for transcription initiation and polyadenylation (Figure
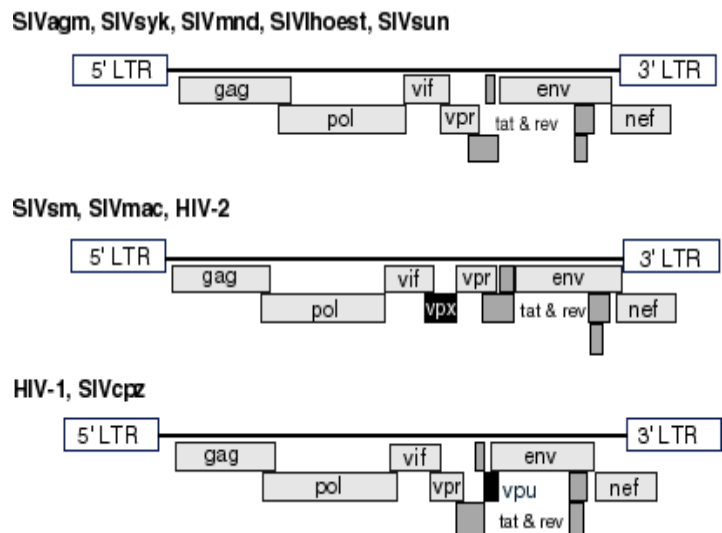
Figure 1.5 Genome organizations of HIV and SIV. Adapted from Beer et al, 1999 (47).

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1.5). As in other retroviruses, HIV/SIV contains three structural genes, gag, pol, and env, which encode major structural proteins and essential enzymes for forming viral particles. The gag gene expresses a myristylated protein precursor that associates with the plasma membrane for virus assembly. The Gag precursor is then processed to nucleocapsid, capsid, and matrix. The pol gene encodes protease, reverse transcriptase, integrase, and RNAse. Protease is essential for processing the viral polyprotein precursors. Reverse transcriptase and integrase mediate reverse transcription of the RNA genome into DNA and integration of the viral DNA into the host genome, respectively. The env gene encodes the Env polyprotein precursor gp160, which is cleaved to gp120 and gp41 subunits. gp120 and gp41 are non-covalently associated as a heterodimer, which is then associated with another two gp120-gp41 heterodimers to form trimer Env spikes on the virion surface.

In addition to the structural proteins, the HIV genome encodes six regulatory proteins important for viral function and infectivity, including Tat, Rev, Vif, Vpr, Vpu, and Nef (Figure 1.5). Tat (transactivator of HIV gene expression) binds to the TAR element at 5' LTR to activate transcription initiation and elongation. Rev (regulator of expression of viral proteins) binds to the RRE element to stabilize and transport unspliced viral mRNAs out of the nucleus. Vif (viral infectivity factor) is important for infection of T cells and macrophages (48, 49), and counteracts the host restriction factor APOBEC3G by targeting its degradation through the ubiquitin-proteosome pathway (50-52). Vpr plays a role in nuclear import of the preintegration complexes and cell growth arrest. Vpu is unique to HIV-1 and SIVcpz, and is involved in downregulation of CD4. Vpu also facilitates virion release from host cell by targeting the cell restriction factor tetherin for degradation (53, 54). Nef is a multifunctional protein that downregulates CD4 and MHC-I molecules, and interacts with cellular proteins in signaling pathways to promote target cell activation (55) and intercellular nanotube formation (56). Deletion of Nef attenuates viral infection and AIDS induction in rhesus macaques (57).The genomes of SIV from sooty mangabeys (SIVsm) and macaques (SIVmac) encode most of the same genes as HIV-1, except

that SIVsm and SIVmac do not have the Vpu gene. SIVsm, SIVmac and HIV-2 also express

another gene, Vpx, which is homologous to Vpr, and counteracts the host restriction factor

SAMHD-1 (58).

Virus replication cycle

The first steps of HIV/SIV replication cycle are attachment and viral entry into target

cells, mainly CD4$^+$ T cells and macrophages (Figure 1.6). The entry process can be mediated

by cell-free viral particles or cell-associated viruses. Transmission through cell-to-cell contact is

more efficient than by cell-free viral infection (60, 61). Both processes involve binding of gp120

of the viral envelope glycoprotein (Env) to the cellular receptor CD4 and a chemokine co-

receptor: CCR5 or CXCR4 for HIV, mainly CCR5 for SIV. These interactions then expose the

gp41 fusion peptide, which can insert into target cell membrane and mediate fusion between the

viral and cellular membranes. The fusion and entry mechanism will be described in further detail

later.
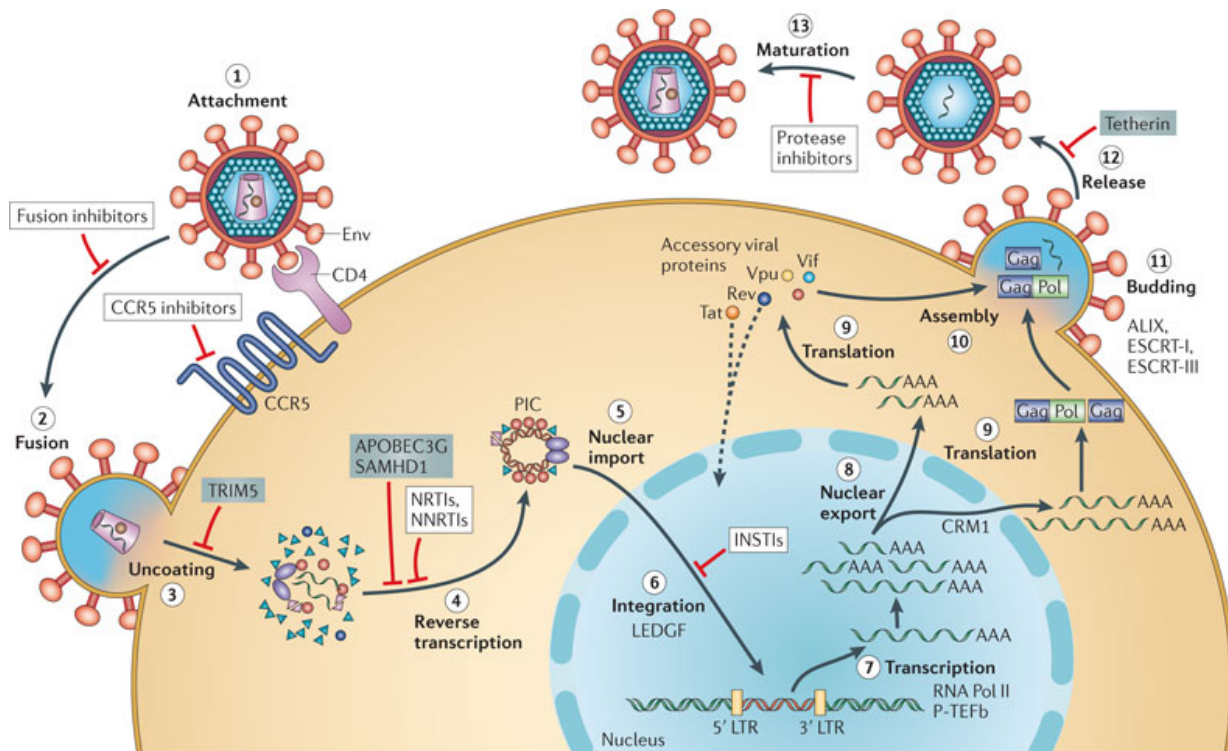
Figure 1.6 HIV replication cycle. Adapted from Engelman et al, 2012 (59).

Upon entry, the viral core is released into the cytoplasm and undergoes a tightly controlled uncoating process to form the reverse transcription complex, which consists of the viral RNA genome, $tRNA^{Lys}$ primer, reverse transcriptase, integrase, matrix, nucleocapsid, Vpr, and host proteins (62). Reverse transcriptase then copies the RNA genome, to generate double-stranded viral DNA and, together with integrase, matrix, and Vpr, forms the preintegration complex (PIC). In non-dividing cells, PIC is transported into the nucleus through nuclear pores, where integrase cleaves and inserts the newly formed double-stranded viral DNA into the host genome. Initial ineffective expression of the viral genes is driven by the 5' LTR promoter in the absence of Tat. Once expressed, Tat can bind to the TAR sequence element and recruit the positive transcription elongation factor b (P-TEFb) complex. Cdk9 of the P-TEFb complex can then phosphorylate RNA polymerase II for effective elongation (59, 62). The viral transcripts and viral RNA genome are transported to the cytoplasm for protein translation and viral assembly. Rev stabilizes and facilitates nuclear export of the transcripts by binding to the RRE element on viral RNA (59, 62).

The main viral structural protein Gag is synthesized in the cytoplasm as a polyprotein. Myristylation at the N-terminus enables Gag to associate with the inner surface of the cell membrane, where viral proteins and two copies of the RNA genome are recruited and assembled. For viral budding, Gag p6 protein recruits the cellular ESCRT complex and utilizes it to mediate membrane budding and membrane scission to release the immature virus. During or after the budding, proteases incorporated in the virion particle cleave the Gag precursor to matrix, capsid, and nucleocapsid. The cleaved proteins are re-assembled to form mature virions, which are capable of starting a new infection.

HIV/SIV envelope glycoprotein

Synthesis and processing

      The HIV/SIV envelope glycoprotein (Env) is the viral protein that mediates entry by inducing fusion between viral and cellular membranes. It is translated from a bicistronic vpu/env mRNA, and synthesized as a gp160 polyprotein precursor, which contains a signal sequence at the N-terminus that directs Env to the endoplasmic reticulum (ER) membrane, as well as a hydrophobic sequence at the C-terminus that keeps Env associated with the ER membrane (63, 64). The newly synthesized gp160 is then glycosylated with N-linked oligosaccharide side chains. The first step of N-linked glycosylation is addition of a carbohydrate core oligosaccharide to the asparagine of a glycosylation site (Asn-X-Ser or Asn-X-Thr, where X can be any amino acid except proline). The oligosaccharide consists of two N-acetylglucosamine, nine mannose, and three glucose residues, and undergoes multiple modifications after being added to the protein. All three of the glucose and one mannose are removed in the ER, resulting in carbohydrate chains ending in mannose (65). This high mannose structure is then further processed as Env is transported to the trans-Golgi network (66). More mannose residues are removed, and many additional sugars, including N-acetylglucosamine, galactose, fucose, and sialic acid, can be added to form various structurally different glycans. Based on the end of the two carbohydrate chains, fully processed N-linked glycans can be characterized as one of the three types: high mannose, hybrid, or complex. Both chains of the high mannose type end in mannose. Neither chains of the complex type end in mannose, but in galactose, N-acetylglucosamine, sialic acid, or glucose instead. Hybrid, as its name suggests, containing one chain ends in mannose, the other chain ends in the other sugar types as the complex type (Figure 1.7).

In addition to N-linked glycosylation, O-linked glycosylation has also been detected on SIV Envs in variable region 1 (V1) of gp120. The specific sequence of O-linked glycosylation site is not clearly defined, but it is suggested that stretches of serine and threonine can be potential O-linked glycosylation sites (65, 67, 68) O-linked glycosylation initiates by adding one N-acetylgalactosamine molecule to the hydroxyl group of Ser and/or Thr. Further modification by adding galactose, N-acetylglucosamine, and N-acetylneuraminic acid (sialic acid) forms different types of glycans, including Tn antigen, core 1, immature core 2, and core 2, with or without sialic acids (Figure 1.8). O-linked glycans may shield neutralization epitopes on Env and enhance neutralization resistance of SIV to sera from infected rhesus macaques (68).
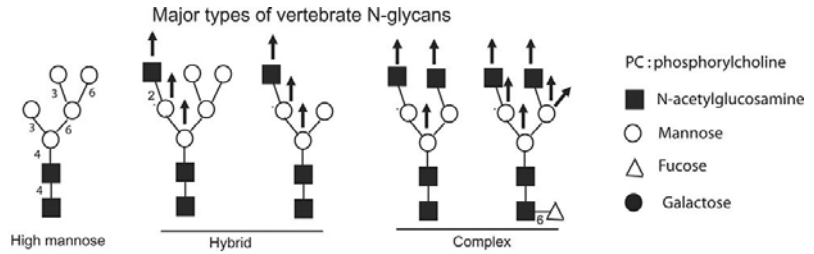
Figure 1.7 N-linked glycosylation Adapted from Berninsone et al, 2006 (69).
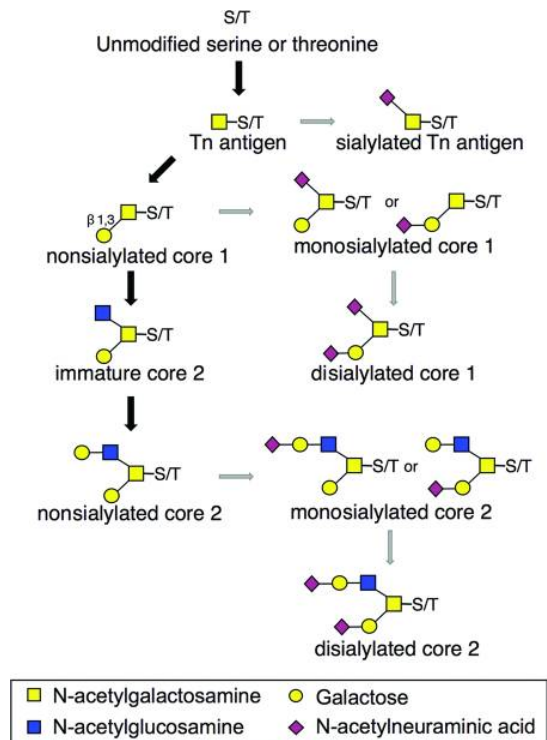
Figure 1.8 O-linked glycosylation. Adapted from Stansell et al, 2011 (67).

In order to form a functional fusogenic protein, the gp160 precursor needs to be cleaved to generate the surface subunit gp120 and the transmembrane subunit gp41. This cleavage occurs in Golgi, and is mediated by a cellular protease, furin, which specifically targets sequence (Lys / Arg)-X-(Lys / Arg)-Arg. The cleaved gp120 and gp41 remain associated

through noncovalent interaction. Three of the gp120-gp41 heterodimers then trimerize to form the Env spikes that are transported to the cell membrane. As Env reaches the cell membrane, it is rapidly recycled through endocytosis. In addition, gp120 sheds from the cell surface due to its relatively weak association with gp41. These two mechanisms, Env internalization and gp120 shedding, reduce Env levels from the cell surface, and therefore, on the virion surface, which helps viruses to minimize the host immune response. In fact, it is estimated that only eight to ten Env trimers are incorporated into each virion (70).

Sequence organization and structure of HIV/SIV Env

The external subumit HIV/SIV gp120 interacts with the cellular receptor CD4 and a chemokine coreceptor, mainly CCR5, to mediate viral entry. Approximately 511 amino acids in length for HIV and 525 amino acids for SIV, the gp120 sequence consists of five constant regions (C1-C5) interspersed between five variable regions (V1-V5). C1-C5 regions form the core structure of gp120, whereas the variable regions V1 -V4 form loop structures anchored by intramolecular disulfide bonds between cysteine residues. Based on the putative orientation in the trimer structure, a monomeric gp120 is divided into inner and outer domains, which are connected by a four β-strand bridging sheet. Two of the β-strands (β2 and β3) connect to the inner domain, and the other two (β20 and β21) connect to the outer domain. The CD4 binding site is mapped to several discontinuous epitopes in the interface between the inner domain, the outer domain, and bridging sheet, surrounding a hydrophobic cavity that provides a critical contact with Phe43 of CD4 (71, 72). The coreceptor binding site consists of part of the bridging sheet and the base of the V3 loop, and is not fully formed until CD4 binding.

The first crystal structure of monomeric gp120 in its native unliganded state is for a SIV gp120 core, with deletions of variable loops V1 to V3 and 43 and 22 amino acids from the N and C-termini, respectively (73). Unlike most of the CD4-bound HIV gp120 core structures, the β2 and β3 strands are separated from the β20 and β21 strands, and both the CD4 and the

coreceptor binding sites are not properly formed (Figure 1.9) (73). It was suggested that CD4

binding induces structural changes (mostly in the inner domain) that lead to formation of the

bridging sheet, which, together with the base of the V3 loop, constitutes the coreceptor binding

site (73). Recent studies show that structures of unliganded gp120 cores from HIV-1 clades B,

C, and E assume the CD4-bound conformation (74), suggesting that the CD4-bound

conformation represents the "ground-state" of the gp120 core (Figure 1.9). Quaternary

interactions and variable loops restrain the full length gp120 from spontaneous induction of the

CD4-bound state and allow it to sample a variety of potential conformations at the unliganded

state. Compared to the unliganded SIV gp120 core described above, these HIV-1 gp120 cores

retains longer stems of the variable loops and intact N terminus. Therefore, structural

differences observed between the unliganded SIV and HIV gp120s could be due to the

evolutionary changes between SIV and HIV, or differences between the "minimum core" and the

"extended core" of gp120 (74).

SIV mac32H core$_{min}$ HIV-1 Clade B$_{YU2}$ core$_e$
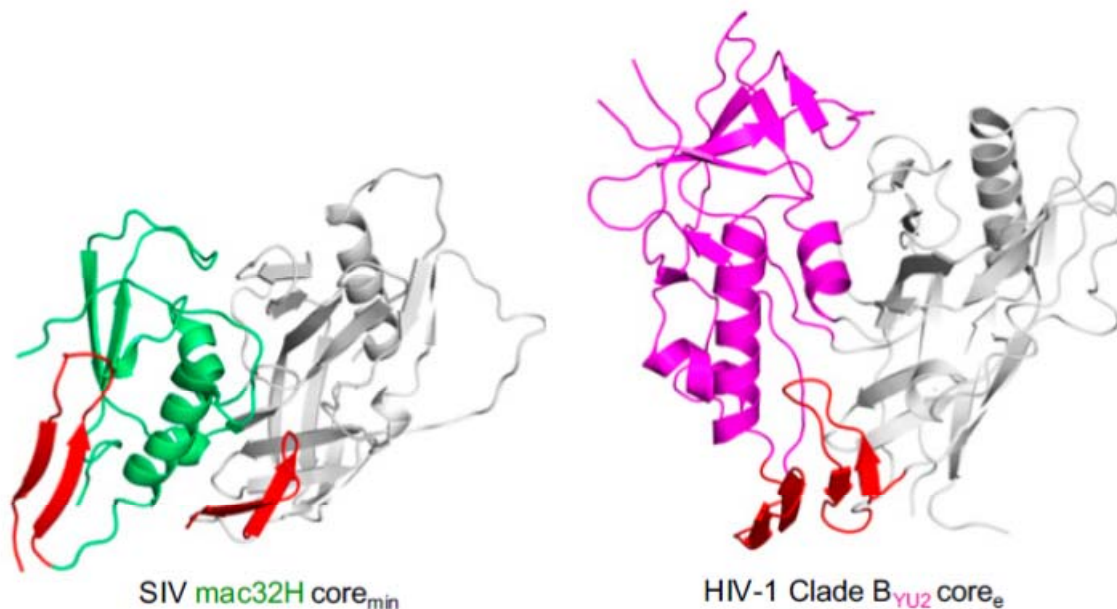
Figure 1.9 Unliganded structure of SIV and HIV gp120 core. Adapted from Chen et al, 2005
(73), and Kwon et al, 2012 (74).

The transmembrane subunit of Env, gp41, mediates fusion between viral and host membranes. HIV and SIV gp41 contain around 345 and 354 amino acids, respectively, and consist of three major domains: extracellular domain (or ectodomain), transmembrane domain, and cytoplasmic tail domain. The N-terminus of the extracellular domain is the fusion peptide with hydrophobic residues, followed by two hydrophobic α-helical repeat regions, HR1 and HR2 (or N-helix and C-helix, respectively). These domains provide the main driving force of the membrane fusion process, which will be described in more detail later. The C-terminus of the extracellular domain is referred to as membrane proximal external region, or MPER, whose role in membrane fusion is not clearly understood, but has been identified as targets of several neutralizing antibodies, 2F5, 4E10, and Z13.

The transmembrane domain of gp41 is a single membrane spanning α-helix consisting of 25 highly conserved amino acids. The cytoplasmic tail domain contains at least two motifs important for mediating the surface levels of Env expression. A membrane proximal motif, GYxxØ (where x can be amino acid, Ø represents bulky hydrophobic amino acids), is a sorting signal that interacts with the AP-2 clathrin adaptor protein to mediate clathrin-dependent endocytosis of Env. This mechanism may be important for reducing Env levels on the cell surface and thereby minimizing the host immune response. A second sorting motif is a dileucine at the C-terminus of the gp41 cytoplasmic tail. This dileucine motif serves redundant but imdependent functions of the GYxxØ through interacting with the AP-1 clathrin adaptor protein. Mutations of both the GYxxØ and the dileucine motifs abolish endocytosis of Env, result in high levels of Env expression on the cell membrane.

Variable regions

As the only viral protein on the virion surface, Env is the main target of the host humoral immune responses. As a result, Env evolves to be highly variable in sequences, particularly in the variable regions, V1 to V5. The structure of the V1/V2 variable loops is not well understood,

since they are removed from all of the gp120 molecules used for the purpose of crystallization. The only solved structure of the V1/V2 is an engineered V1/V2 domain placed onto a scaffold and crystalized in complex with PG9, a broadly neutralizing antibody specifically recognizes a conserved N-linked glycan on V1/V2 (N160) (75). Instead of a loop-like structure, the V1/V2 domain folds as four anti-parallel β-strands. The conformation of a CD4-liganded gp120 shows that the V3 loop protrudes from the gp120 core toward the target cell membrane (76). V1/V2 and V3 regions play an important role in determining the neutralization sensitivity of HIV and SIV strains (77, 78). As the major targets of elicited antibodies (79), the V1/V2 and V3 regions protect virions from detection by the immune system by forming a glycosylated surface, allowing high sequence variation, and shielding relatively conserved regions, such as the receptor and coreceptor binding sites. Removal of the V1/V2 loop from SIV results in extremely high sensitivity to antibody-mediated neutralization (80). V4 is a disordered loop structure exposed on the surface of the gp120 outer domain and is also a target for neutralizing antibodies (73). V5 is shorter than the other variable regions, and no definitive roles have been ascribed (81).

In addition to affecting neutralization sensitivity, the gp120 V1, V2 and V3 variable regions play important roles in mediating interactions with CD4 and CCR5. The V3 loop, together with the bridging sheet region, constitutes the CCR5 binding site. The V1/V2 loop does not interact directly with CD4 or CCR5, but may partially occlude the CD4 and CCR5 binding sites (82-85), thereby influencing gp120 interaction with these receptors. Structural models of Env trimers suggest that the V1/V2 loop may interact with the V3 loop on the same or neighboring gp120 protomer (72, 73, 86, 87), an interaction that may influence the orientation of the V3 loop and thereby modulate the CCR5 binding site. Recently, Clapham and colleagues showed that a determinant in the V1 loop modulates exposure of the V3 loop and CD4 binding site, resulting in effects on macrophage tropism together with altered sensitivity to monoclonal antibodies targeting these epitopes (88).

N-linked glycosylation

HIV/SIV Env is one of the most heavily glycosylated proteins, containing about 29 glycosylation sites, 25 in gp120 (ranging from 18 to 33) (89), 4 in gp41 (ranging from 3 to 5) (66). In fact, more than 50% molecular weight of gp120 is comprised of carbohydrate (65). The glycans cover the surface of viral protein, especially the outer domain, forming a glycan shield known as the "silent surface" that prevents targeting of neutralizing antibodies to Env. Therefore, the N-linked glycosylation sites have been associated with enhanced neutralization resistance to antiserum, soluble CD4 (sCD4), and antibodies targeting a broad spectrum of epitopes (82), suggesting that changes in glycosylation sites may not only affect local neutralizing epitopes, but also affect the global structure of Env. Exposure of the CD4 binding site is also modulated by N-linked glycans. Loss of the N386 glycosylation site is associated with enhanced neutralization sensitivity to b12, an antibody recognizes the CD4 binding site, and with enhanced replication in macrophages, which express low levels of CD4 on the cell surface. Substitutions in glycosylation sites in the V1/V2 region have also been identified as determinants of CD4-independence (90, 91). These findings suggest that glycans can influence Env interaction with the receptor, and thereby affect cell tropism. Instead of a static shield, viruses can also shift the position of the N-linked glycosylation sites in response to the immune selection pressure *in vivo*. Recent studies of transmitted/founder viruses showed that they contain fewer N-linked glycosylation sites and are more neutralization sensitive compared to viruses derived from chronic infection, suggesting that N-linked glycans may play a role in mucosal transmission.

In addition to its role in neutralization resistance, N-linked glycosylation is also important for normal folding, processing, and intracellular transport of Env, as it is for cellular proteins in general. A subset of N-linked glycans is necessary for correct forming of the CD4 binding site (92). Mutant Envs with missing glycosylation sites exhibit defects in gp160 processing to gp120 as well as defects in intracellular transport of Env (93, 94). These defects may lead to impaired

viral infectivity, as viruses generated in cells treated with glucosidase show lower infectivity, which is associated with altered glycosylation pattern, reduced surface expression of Env, and decreased gp160 processing to gp120 and gp41 (95-97). Finally, glycans may also facilitate viral interaction with target cells. Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a lectin containing a C-terminal carbohydrate recognition domain that can bind to glycans on gp120, promoting attachment of viruses to dendritic cells, which may not be infected by the virus, but can carry the virus to lymph nodes and infect other target cells through cell-to-cell transmission. These findings suggest that N-linked glycosylation sites play important roles in forming functional Env.

Env trimer structures

Recent studies using cryo-electron tomography have provided important insights into Env trimer structures in complex with sCD4 and antibodies at different states (Figure 1.10) (98-107). In its native unliganded state, HIV Env trimer adopts a "closed" conformation, with the V1V2 and V3 regions located at the center of the trimer apex (100, 106, 107). CD4 binding induces structural rearrangement that leads to transition to an "open" conformation. Interestingly, a CD4 independent
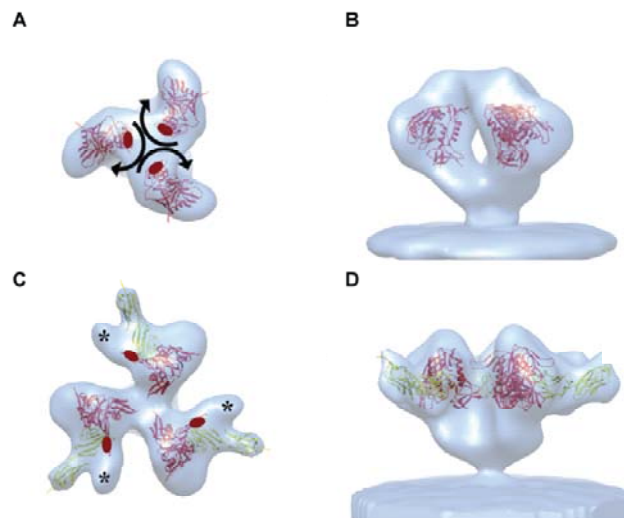
Figure 1.10 Density map and molecular architecture of the native (A, top view; B, side view) and CD4-bound (C, top view; D, side view) trimeric Env from HIV-1 BaL. The locations of the stems and the loops of the V1V2 are shown as red ovals and asterisks, respectively. The arrows in A depict the rotation directions of each monomer induced by CD4 binding that transform the Env trimer from a close conformation (A, B) to an open conformation (C, D). The density maps were fitted with subsets of the X-ray crystal structure of the gp120-sCD4/17b complex (PDB ID: 1GC1). Adapted from Tran et al, 2012 (98).

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strain, SIV CP-MAC is at an "open" conformation at its native state, which suggests that it may adopt a CD4-prebound structure that enables it to interact with the coreceptors in the absence of CD4 (Figure 1.11). It is also suggested that V1V2 and V3 regions interact with each other at the center and are involved in maintaining association of the trimer. Therefore, changes in the V1V2 and V3 regions may induce global effects that influence the trimer structure and expose epitopes originally buried within the trimer, consistent with the idea that the V1V2 regions are global modulators of neutralization sensitivity.
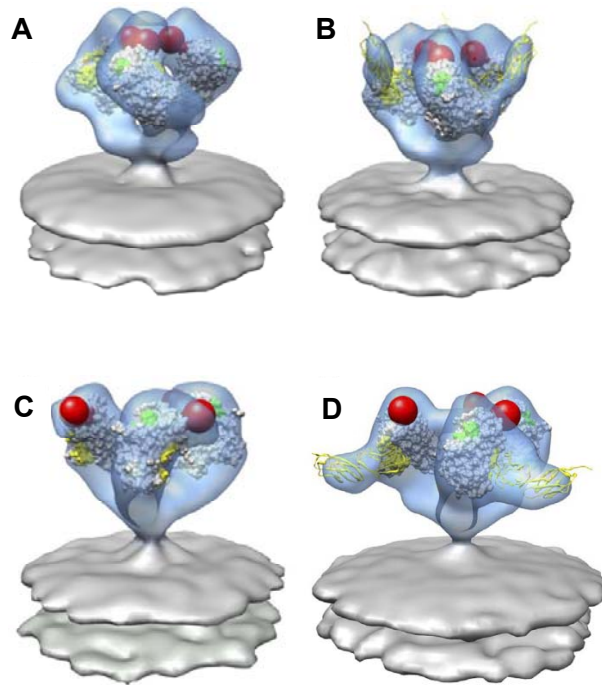
Figure 1.11 Density map and molecular architecture of the native (A, C) and CD4-bound (B, D) trimeric Env from SIVmac239 (A, B) and SIV CP-MAC (C, D). The red spheres indicate the locations of the V1V2 loop. The density maps were fitted with subsets of the X-ray crystal structure of the gp120-sCD4/17b complex (PDB ID: 1GC1). Adapted from White et al, 2011 (99).

Molecular mechanisms of fusion and entry
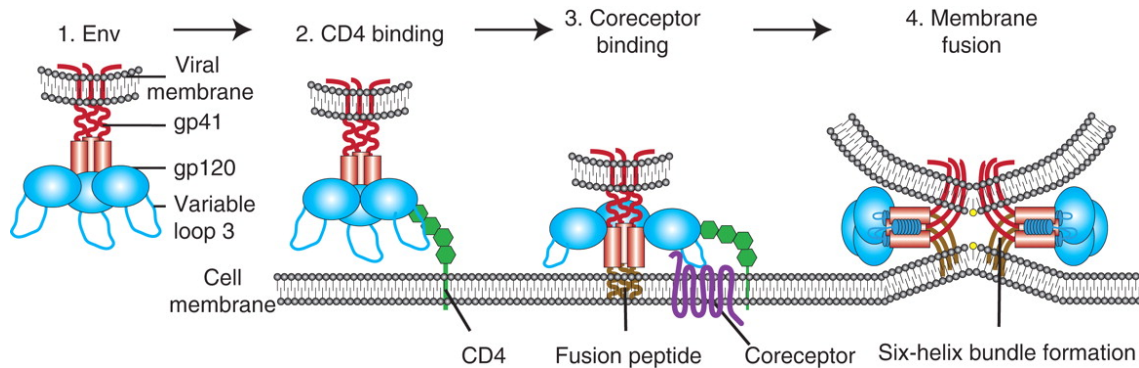
Figure 1.12 HIV/SIV entry. Adatped from Wilen et al, 2012 (108).

Attachment

The first step of viral entry involves attachment of the virus to the cell surface. Several

molecules have been identified as attachment factors for HIV infection. DC-SIGN is specifically

expressed on dendritic cells. It contains a C-terminal carbohydrate recognition domain that

binds to glycans on gp120. Dendritic cells themselves do not support productive infection by the

viruses. However, through this gp120-DC-SIGN interaction, viruses can attach to the surface of

dendritic cells, which migrate to lymph nodes and transmit the viruses to CD4$^+$ T cells. Heparan

sulfate proteoglycans bind to the V3 loop and the CD4-induced surface on gp120. The major

determinant for heparin sulfate proteoglycan interaction appears to be the charge on the V3

loop. The relevance of this interaction in infection of primary T cells is unclear. However, mature

macrophages express the proteoglycan syndecan-II, which promotes attachment to and

infection of these cells. Integrin α4β7 has also been shown to bind some HIV Envs, possibly

through a LDV peptide motif in the V2 region that resembles binding motifs on the natural

ligands of α4β7. Replication of some HIV strains in PBMC can be blocked by anti-α4β7

antibodies (109-111). However, not all HIV strains interact with α4β7. Parrish et al has recently

shown that replication of transmitted/founder viruses is not blocked by anti-α4β7 antibodies. Our

unpublished results also show that SIV replication in PBMC is not significantly affected by anti-

α4β7 antibodies. These results suggest that α4β7 may only used by certain strains of HIV as an attachment factor.

Receptor / coreceptor interaction

The primary receptor for HIV/SIV infection is CD4, which is a member of the immunoglobulin family that contains four immunoglobulin domains. It is expressed on T helper cells, monocytes, macrophages, and dendritic cells. Interaction of Env with CD4 induces rearrangement of V1/V2 and V3, as well as formation of the bridging sheet. These structural changes bring V3 and the bridging sheet into proximity, which constitutes the coreceptor binding site (Figure 1.12). The main coreceptor for HIV/SIV is CCR5, which is a C-C chemokine receptor. CCR5 is a transmembrane G protein-coupled receptor. The natural ligands of CCR5 include RANTES, MIP-1α and MIP-1β. CCR5 interacts with gp120 at two sites. The first site is the tyrosine-sulfated N-terminus, which reaches up to interact with the base of the V3 loop, and the second site is the second extracellular loop (ECL2), which interacts with the tip of the V3 (76, 108). CCR5Δ32 is a mutation of 32 bp deletion that results in a nonfunctional receptor, and therefore prevents HIV entry. People who carry homozygous CCR5Δ32 mutation are resistant to HIV. Maraviroc is an approved entry inhibitor used in anti-retroviral therapy. It binds and alters the structure of CCR5 to block Env interaction with CCR5. Interestingly, viruses resistant to maraviroc bind and use the maraviroc-bound CCR5 for entry, and therefore become dependent on the drug. Most transmitted viruses are CCR5-tropic. At late stages of infection, some HIV strains acquire the ability to use CXCR4 as the coreceptor. The emergence of CXCR4-tropic HIV-1 variants is associated with accelerated disease progression. In contrast, SIV mainly uses CCR5 as the coreceptor, and does not switch to CXCR4. SIV may also use other chemokine receptors, such as Bob/GPR15 and Bonzo/STRL33, as coreceptors for entry (112). The relevance of these alternate coreceptors *in vivo*, however, is less well understood.

Membrane fusion

Coreceptor binding induces further conformational changes in gp120 that leads to the exposure of the gp41 hydrophobic fusion peptide. The fusion peptide inserts into the host cell membrane and then the HR1 and HR2 domains fold to form a six-helix bundle, which is the driving force that brings together the cell and viral membranes, resulting in the formation of a fusion pore, through which the viral core enters into the cell (Figure 1.13). T-20, a HIV fusion inhibitor, targets this step of entry. It mimics the HR2 domain, and binds to the HR1 domain, which prevents the formation of the six-helix bundle, and thereby blocks fusion and viral infection.
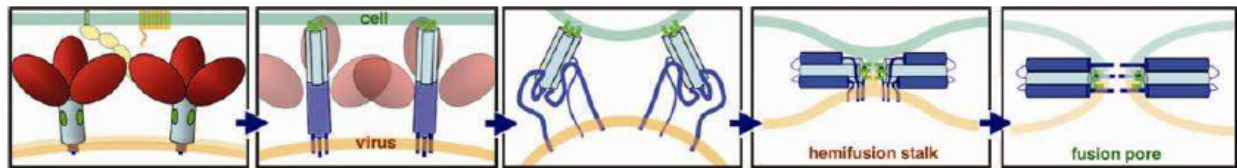
Figure 1.13 HIV/SIV gp41-mediated membrane fusion. Adapted from Frey et al, 2008 (113).

Neutralizing antibodies / epitopes

Although the variable sequences and glycan shield make Env a difficult target for the host humoral immune response, neutralizing antibodies are induced in some infected individuals. These antibodies recognize specific epitopes of Env and are able to neutralize viral infection *in vitro*. However, they are not able to control viral replication in individuals from whom they are derived, mainly because viral evolution outpaces the host antibody responses by months. Although the antibodies derived at a certain time point may show potent neutralization activity against viruses derived from the same individual at an earlier time point, they may not be effective against virus derived concurrently.

The Env epitope that has been identified to be targeted by most broadly neutralization antibodies is the CD4 binding site. b12 is the earliest broadly neutralizing antibody identified to

target this epitope. It neutralizes about half of the viruses within a test panel. More recent studies identify a series of broadly neutralizing antibodies, represented by VRC01, that also target the CD4 binding site, and are able to neutralize more than 90% of the virus strains. Although both antibodies resemble CD4 binding, structural studies show that, VRC01 approaches the epitope with less steric hindrance compared to b12, and even less than soluble CD4. This effectiveness of VRC01 targeting this epitope may explain its broad neutralizing activity against the virus.

Some antibodies, such as 17b, only bind the CD4-bound gp120. This type of antibody targets epitopes that are only induced and formed after CD4 binding (CD4i). Most residues constituting the CD4i epitopes are within the bridging sheet or nearby structures. These epitopes usually overlap the coreceptor binding site, and therefore binding of these antibodies may neutralize virus by interfering with coreceptor binding.

Dozens of newly identified broadly neutralizing antibodies (PG9, PG16, CH01-04, PGT140s, PGT120s, and PGT130s), as well as the previously identified 2G12, exhibit unusual target specificity and bind to unique patterns of glycans on the Env surface. These antibodies bind to glycans in the V2, V3/V4, or the outer domain of gp120. Activities of some of these antibodies depend on the quaternary structure of Env trimer. For example, PG9 exhibit an unusual asymmetric recognition. Structural analysis suggests that one PG9 fragment antigen-binding (Fab) makes two interactions with two gp120 protomers within an Env trimer; one with the N156 and N160 glycan in a V1/V2 domain, and a second one with N160 glycan from an adjacent gp120 protomer.

HIV/SIV macrophage tropism

Roles of mononuclear phagocytes in HIV/SIV infection

Cells of the mononuclear phagocyte lineage, including monocytes, dendritic cells, and macrophages, are involved in various aspects of HIV infection. Monocytes originate from myeloid precursors in the bone marrow and circulate in the peripheral blood, where they remain for only a few days. Upon migrating into tissues, they can differentiate into dendritic cells or different macrophage subsets, such as microglial cells and alveolar macrophages, and live from months to years, depending on the tissue type. While expressing the receptors and coreceptors required for HIV entry, monocytes are not productively infected by the virus. This may be due to restrictions at multiple steps of virus replication cycle. For example, it has been shown that, compared to $CD4^+$ T cells and macrophages, monocytes express lower levels of CycT1, a factor of the P-TEFb complex, which is required for transactivation mediated by Tat. A subset of monocytes, $CD14^+CD16^+$ cells (only 5% of the total monocyte population), appears to be more susceptible to infection than $CD4^{high}CD16^-$ monocytes. The low percentage of this susceptible subset may explain the infrequent recovery of viral DNA from monocytes in elite controllers, which suggest monocytes are not the major viral reservoir (114).

Dendritic cells contribute to HIV infection mainly by capturing and transporting virions from peripheral tissue to distal lymph nodes, where they transmit the virus to infect $CD4^+$ T cells. The detailed mechanism is not fully understood, but involves the formation of a "virological synapse", a tight junction similar to the "immunological synapse". Multiple cellular factors are involved in the formation of the virological synapse. Upon cell-cell contact, viral particles and cellular receptors/coreceptors, as well as the adhesion molecule (LFA-1) are recruited to the site of contact within minutes. This mechanism results in a highly concentrated environment facilitating viral transmission, which may explain the higher efficiency of cell-to-cell transmission, compared to cell-free virus transmission.

Macrophages in different tissues show extensive heterogeneity and plasticity. For example, alveolar macrophages express high levels of pattern recognition receptors and scavenger receptors for removing pathogens and environment particles, whereas osteoclasts express markers for bone tissue remodeling. Macrophages in the intestine and gut mucosa show high phagocytic and anti-bacterial activity, but produce low levels of inflammatory cytokines to prevent over stimulation by constant exposure to commensal microbes. These differences may play an important role in influencing their susceptibility to HIV infection and contribution to pathogenesis (discussed below).

Macrophage infection in HIV/SIV pathogenesis

HIV and SIV infections are characterized by progressive local and systemic CD4$^+$ T cell depletion and chronic immune activation, ultimately leading to immunodeficiency and AIDS. While CD4$^+$ T cells get depleted by infection, macrophages are relatively resistant to the cytopathic effects of these viruses and have a slower turnover rate than CD4$^+$ T cells (115). Therefore, they serve as a major reservoir for viral persistence in the central nervous system (CNS) and other tissues including bone marrow, lymph node, spleen, lung, and gut (116-123). These viral reservoirs are particularly important in the CNS. Infection of macrophages in the CNS causes neurological dysfunction and encephalitis, characterized by multinucleated giant cells, reactive astrocytes, and white matter abnormalities (124). Most current highly active antiretroviral therapy (HAART) drugs have poor penetration into the CNS (119), making it a sanctuary site for viral persistence and development of drug resistance.

In addition to serving as a major viral reservoir, macrophage infection also plays a significant role in pathogenesis in gastrointestinal tract mucosa, the largest lymphoid organ and macrophage reservoir in the body (19, 20), and the major site of CD4$^+$ T cell depletion (125). Identification of activated monocytes and macrophages in immunologic inductive sites and gastrointestinal tract of SIV-infected rhesus macaques (126, 127) suggests that these cells

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contribute to chronic immune activation, a hallmark of disease progression (128). Indeed, progression to advanced disease has been correlated with an enhanced ability of HIV-1 isolates to replicate in macrophages (129, 130).

Macrophage infection in CNS

HIV/SIV infection of CNS induces HIV/SIV-associated neurological diseases in 20-30% of untreated patients or animals. Viruses enter the brain early during acute infection, as infected macrophages can be detected in brain as early as two weeks post-infection. To enter the CNS, viruses need to cross the blood-brain barrier (BBB), which is a layer of brain microvascular endothelial cells connected with tight junctions. The mechanism by which viruses pass through BBB and enter brain is not clearly understood. A trojan horse model has been proposed, in which infected CD4$^+$ T cells and monocytes migrate through BBB and transmit viruses to perivascular macrophages and microglial cells. Alternatively, infected monocytes can differentiate in brain and give rise to infected perivascular macrophages and microglial cells. Viruses persist in brain, but usually remain latent until late stage of infection. While viral DNA can be detected in brain at a constant level throughout the course of infection, viral RNA is down-regulated after acute infection, and remains at background level until development of HIV/SIV Encephalitis and AIDS.

HIV/SIV infection of macrophages and microglia, the main target cells for infection in the CNS, results in neurological diseases such as HIV-associated dementia (HAD) and HIV encephalitis (HIVE). HIV infected macrophages and microglia can induce neuronal dysfunction or cell death directly by secreting neurotoxic viral proteins (such as gp120, Tat, and Vpr) (131-134), or, indirectly by inducing inflammation in the brain (135). The induction of inflammation results in further activation of macrophages and microglia, as well as astrocytes, enhanced production of cytokines (i.e. TNF-α, IL-1β, IL-6) (136) and chemokines (i.e. MIP-1α, -1β, RANTES, MCP-1) (137), disruption of the blood-brain barrier, and infiltration of other immune

cells, which ultimately leads to neuronal injury and dysfunction and cell injury. Astrocytes may also be infected by HIV/SIV, but infection of these cells may not result in productive viral replication (135).

The CNS is an isolated compartment, separated from other body fluids and tissues by the BBB. The unique microenvironment, target cells, and immune selection pressures in the CNS may lead to independent evolution of viral variants after entering the brain. Consistent with this prediction, phylogenetic studies show distinct compartmentalization of viral variants in brain compared to those in lymph nodes. Sequences derived from brain are more closely related to each other than they are to sequences from other tissues. Since macrophages are the main target cells for brain infection, viral isolates from brain usually show higher macrophage-tropism and higher efficiency of infecting low CD4/CCR5-expressing cells, compared to viral variants from lymph nodes. This genotypic compartmentalization of viral variants in brain suggests that adaptive evolution may occur in the CNS, independently of other tissues.

SIV strains for pathogenesis studies *in vivo*

A major obstacle to studies on macrophage-related pathogenesis in animal models is the lack of a pathogenic macrophage-tropic SIV molecular clone. Currently, there is no pathogenic SIV clone that reproducibly causes macrophage infection and CNS disease *in vivo*. The most frequently used SIV clone for *in vivo* pathogenesis studies is SIVmac239 (138), which is highly neutralization resistant and replicates in CD4$^+$ T cells, but not in macrophages (139). In some monkeys infected with SIVmac239, viruses evolve *in vivo* and adapt to replicate in macrophages and induce macrophage-associated pathogenesis, such as SIV encephalitis (SIVE) (140). Several macrophage tropic molecular clones have been isolated from these animals, including SIVmac316 (139) and SIV/17E-Fr (141, 142). Unlike SIVmac239, these viruses replicate well in both CD4$^+$ T cells and macrophages *in vitro*; however, as is true for most experimentally characterized SIVmac molecular clones, they are attenuated when

introduced into animals and do not induce pathogenesis *in vivo*. These viruses are isolated at the late stage of infection, when the immune system has been compromised, and thus might evolve in the absence of strong immune selection pressure. Indeed, these strains show significantly increased neutralization sensitivity compared to the highly neutralization resistant SIVmac239 (77, 90). Determinants that contribute to macrophage tropism and neutralization sensitivity are strain specific, but have been mapped to regions including the V1/V2 loops, V3 loop, and the CD4 binding site (77, 91, 139). Furthermore, these viruses were able to mediate fusion and enter cells with little or no surface CD4, the main receptor of HIV and SIV, an unusual phenotype rarely seen in HIV isolates (77, 90, 91, 143, 144). This CD4 independence is associated with macrophage tropism (77, 90), and may facilitate entry into macrophages, which have lower CD4/CCR5 surface expression than $CD4^+$ T cells (143, 145, 146). A neuropathogenic model was established by co-inoculation of SIV/17E-Fr with an immunosuppressive virus, SIV/DeltaB670, into pig-tailed macaques (147). This model reproducibly induces encephalitis in over 90% of the infected macaques, and has provided remarkable insights for SIV neuropathogenesis.

SIVmac251 (148-150) is an uncloned SIV strain that replicates well in both $CD4^+$ T cells and macrophages, and are commonly used for AIDS pathogenesis studies in non-human primate models (148-151). Compared to molecular clones, experimental infection with these uncloned strains has the advantages of more closely resembling the variety of variants encountered during natural infection. However, clinical outcomes using this strain are heterogeneous due to the various compositions of viral stocks generated by amplification in tissue culture (152). Furthermore, studies to identify viral determinants associated with pathogenesis or immune evasion are hampered by the undefined composition of the viral inoculum. The available molecular clones derived from SIVmac251 include SIVmac251$_{BK28}$ (153), SIVmac251 clone (154, 155), SIVmac1A11 (156), and SIVmac32H (157), but these clones are neutralization sensitive, and cause mild or no disease in vivo. The uncloned

SIVmac251 strain consists of more than 20 distinct env sequences but only three of these env clones were shown to mediate significant levels of viral replication in alveolar macrophages in vitro (158), and the replication levels were much lower than that of SIVmac316 (158). A pathogenic molecular clone of SIVmac251 would facilitate development of an animal model to study macrophage-associated pathogenesis and might also be useful as a challenge strain for vaccine studies.

SIVsmE660 (159, 160) is another uncloned SIV strain commonly used for *in vivo* pathogenesis studies. In contrast to SIVmac, viruses derived from the SIVsm lineage (e. g. SIVsmE543-3 (161), SIVsmPBj (162), SIVsmFGb (163)) often induce rapid disease in rhesus macaques, and progression to AIDS and death within months or even within two weeks in some cases (SIVsmPBj). These acute rapid progressions may represent a distinct disease course from that of human AIDS. Notably, SIVsmFGb-derived viruses caused nerupathogenic effects in 100% of infected pig-tailed macaques (usually 30-40% in infected macaques (140) and 10-20% in infected human without HAART (164)). Two interesting molecular clones, PGm5.3 and BPZm.12, were derived from SIVsmFGb. While PGm5.3 recapitulated many properties of the parental strain in vitro, it was low pathogenic in vivo. BPZm.12, on the other hand, acquired a unique macrophage-only tropism (165) not described in any other SIV strains.

Mechanisms of macrophage tropism

Due to the lower levels of CD4 on macrophages compared to CD4$^+$ T cells (143, 145, 146), HIV/SIV macrophage-tropism is associated with lower dependence on CD4 to mediate fusion and entry, in some cases due to increased exposure of the CD4 or CCR5 binding site and in others due to increased affinity between Env and CD4 (77, 90, 91, 124, 143-145, 166). In order to use the lower levels of CD4 on macrophages, macrophage-tropic Envs may adopt a more open conformation of gp120, including exposure of the CD4 binding site, which renders the Env more susceptible to antibody recognition, and thus increases neutralization sensitivity

(167, 168). Therefore, entry into macrophages is associated with enhanced CD4 and/or CCR5 interaction and neutralization sensitivity. This is consistent with association of macrophage-tropic strains with late stage infection, when the immune system is compromised and neutralizing antibody responses are diminished. Together with the recent studies of a small number of transmitted/founder viruses showing that these viruses are poorly macrophage-tropic, it is generally believed that macrophage-tropic viruses emerge in the later stage of disease after the immune system has been compromised.

Comparative studies of amino acid sequences between macrophage-tropic and non-macrophage-tropic viral clones have identified several molecular determinants in the V1V2, V3, V4, C1, C2, and C3 regions of Env for macrophage tropism. Studies of these determinants may help to explain potential mechanisms by which macrophage tropism is enhanced. For example, N283, in the CD4 binding site and the C2 region of HIV gp120, is associated with brain infection and dementia, and enhances viral replication in macrophages and entry in cells expressing low levels of CD4 (124). The enhanced macrophage tropism and usage of low levels of CD4 are due to enhanced gp120 affinity with CD4, through the hydrogen bond formed between N283 and Q40 of CD4. N386D mutation, which eliminates an N-linked glycosylation site in the V4 region of gp120, enhances viral entry and replication in macrophages, as well as neutralization sensitivity to the monoclonal antibody, b12, which targets an epitope that overlaps the CD4 binding site (167). These results suggest that this N-linked glycan may partially occlude the CD4 binding site. Loss of the N386 glycan may therefore increase exposure of CD4 binding site, facilitate gp120-CD4 binding, and promote usage of low levels of CD4 on macrophages. More recently, a mutation, E153G in the V1 region of HIV gp120 was identified to be associated with enhanced macrophage tropism and neutralization sensitivity to sCD4 and monoclonal antibodies targeting the V3 region (88). These findings suggest that macrophage tropism determinants in gp120 may increase the exposure of the CD4 binding site, enhance gp120-CD4 interaction, and thereby enhance viral entry and replication in macrophages.

Studies of macrophage-tropic SIV clones revealed a CD4-independent phenotype, which is less frequently seen in primary HIV isolates (91). Macrophage-tropic SIV, including SIVmac316, SIV/17E-Fr, and SIVmac1A11, are able to infect CD4-negative, CCR5-positive cells, whereas the non-macrophage-tropic SIVmac239 strictly depends on the presence of CD4 for infection (90, 91). These CD4-independent Envs are more neutralization sensitive to monoclonal antibodies to the V3 loop, and to the coreceptor binding site, suggesting that they may exist in a state similar to the partially triggered state after CD4 binding, with constitutively exposing coreceptor binding sites. Consistently, pre-triggering SIVmac239 with soluble CD4 makes it susceptible to these antibodies (90). Two residues, position 165 at the V1/V2 region and position 573 at gp41, have been mapped as determinants for CD4 independence. Introduction of M165I and K573T mutations into SIVmac239 Env enables it to mediate CD4-independent fusion, whereas I165M and T573K mutations have the opposite effect on SIVmac316 and abolish CD4-independent fusion (91). The ability to mediate CD4-independent fusion and infection may be relevant for using the low levels of CD4 on the surface of macrophages for infection and replication.

Scope of this dissertation

Macrophages are important target cells for human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infection that 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. They play important roles in disease pathogenesis, particularly for development of HIV/SIV-associated neurological disease. Macrophages are the predominant target cells for HIV/SIV infection in brain. Infection of macrophages in the CNS causes neurological dysfunction and encephalitis. Activated monocytes and macrophages in immunologic inductive sites and gastrointestinal tract contribute to chronic immune activation, a hallmark of HIV/SIV infection disease progression.

Macrophages express low levels of the HIV/SIV receptor CD4 compared to CD4$^+$ T cells, but mechanisms by which these viruses use low CD4 to mediate spreading infections are poorly understood. One mechanism involves enhanced envelope glycoprotein (Env) interaction with CD4 or CCR5, but this phenotype is frequently associated with increased sensitivity to antibody neutralization due to increased exposure of neutralizing epitopes overlapping the CD4/CCR5 binding sites. Moreover, this mechanism does not explain how these neutralization-sensitive viruses evade immune responses while establishing spreading infections. In this dissertation, we sought to identify SIV Env determinants for macrophage tropism and characterize mechanisms by which they enhance virus replication in macrophages.

In Chapter 2, we sought to identify viral variants capable of inducing macrophage-associated pathogenesis at early stage of infection. We cloned Env sequences from SIVmac251-infected macaques at early and late stage infection, and identified an early variant in blood that shares >98% sequence identity with the consensus sequence of late variants in brain from animals with neurological disease. Infectious SIV clones encoding this Env variant mediated high levels of cell-cell fusion, replicated efficiently in rhesus PBMC and macrophages, and induced multinucleated giant cell formation upon infection of macrophage cultures. Two N-

linked glycosylation sites (N173 and N481) in the gp120 V2 and C5 regions were identified as important determinants of macrophage tropism and neutralization sensitivity.

In Chapter 3, we identified the N-linked glycosylation site, at position 173 in the V2 region, but not N481 in the C5 region, as a determinant of macrophage tropism. Loss of N173 enhanced SIVmac239 replication in macrophages, while restoration of N173 in SIVmac251 reduced macrophage tropism but enhanced neutralization resistance to antibodies targeting the CD4 and CCR5 binding sites. SIVmac239 N173Q, which lacks the N173 glycosylation site, mediated CD4-independent fusion and cell-to-cell transmission with CCR5-expressing cells, but could not infect CD4-negative cells in single-round infection assays. Thus, CD4-independent phenotypes were detected only in the context of cell-cell contact. The N173Q mutation had no effect on binding of recombinant SIVmac239 gp120 to CD4 in BIACORE and co-immunoprecipitation assays. These findings suggest that loss of the N173 glycosylation site increases replication of SIVmac239 in macrophages by enhancing CD4-independent cell-to-cell transmission through CCR5-mediated fusion. This mechanism may promote replication and cell-to-cell spread of macrophage-tropic viruses *in vivo*, since these viruses are typically neutralization sensitive, but cell-to-cell transmission facilitates escape from neutralizing antibodies.

In summary, we identified macrophage-tropic viral variants from SIV-infected rhesus macaques, suggesting that macrophage-tropic variants are present at early stage of infection. This early variant replicates well in rhesus PBMC and macrophages, and mediated high levels of cell-cell fusion. Genetic analysis shows that this variant is closely related to variants from brain from late infection, suggesting that viruses containing this variant sequence may enter and establish infection in brain. Therefore, this newly identified molecular clone may be useful for development of an animal model to study macrophage-associated pathogenesis. Furthermore, we identified a novel mechanism by which macrophage-tropic viruses replicate and transmit in spreading infection. Enhanced CD4-independent cell-to-cell transmission may enable

macrophage-tropic viruses that are relatively neutralization sensitive to spread infection while evading host humoral response. These findings provide further understanding of mechanisms by which Env determinants modulate macrophage tropism, and may help to develop an animal model for future studies of macrophage-associated pathogenesis.

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CHAPTER 2: Identification and characterization of a macrophage-tropic SIV envelope glycoprotein variant in blood from early infection in SIVmac251-infected macaques

Po-Jen Yen,[1,2] Megan E Mefford,[1,2] James A. Hoxie,[3] Kenneth C. Williams,[4] Ronald C. Desrosiers,[5] and Dana Gabuzda[1,6]

Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts, USA[1]

Division of Medical Sciences Program in Virology, Harvard Medical School, Boston, Massachusetts, USA[2]

Department of Medicine, Hematology-Oncology Division, University of Pennsylvania, Philadelphia, Pennsylvania, USA[3]

Department of Biology, Boston College, Chestnut Hill, Massachusetts, USA[4]

New England Primate Research Center, Department of Microbiology and Immunobiology, Harvard Medical School, Southborough, Massachusetts, USA[5]

Department of Neurology, Harvard Medical School, Boston, Massachusetts, USA[6]

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 as early as two weeks post-infection, but macrophage-tropic viruses are rarely isolated until late-stage infection. Little is known about viruses in blood during early infection that may have the ability to establish persistent infection of macrophages in brain. Here, we identified an SIV envelope glycoprotein (Env) variant at two weeks post-infection in blood of an SIVmac251-infected macaque that shares >98% sequence identity with the gp120 consensus sequence of late variants in brain from macaques with neurological disease. SIVmac251 clones expressing gp120, or V2 and C3 sequences, from this early variant mediated high levels of fusion, replicated efficiently in rhesus peripheral blood mononuclear cells and macrophages, and induced multinucleated giant cell formation during infection of macrophages. The highly conserved N-linked glycosylation sites N173 and N481 in the V2 and C5 regions of these SIV Envs were identified as determinants of macrophage tropism and neutralization sensitivity. These results suggest that macrophage-tropic SIV viruses capable of establishing viral reservoirs in macrophages in brain and other tissues can be present in blood during early infection. Furthermore, these replication-competent SIVmac251 clones will be useful for non-human primate studies on macrophage-related pathogenesis, persistence and eradication of viral reservoirs, and vaccine development.

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 (1) 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 (2-9). Infection of macrophages in the brain also causes neurological dysfunction and encephalitis, characterized by multinucleated giant cells, reactive astrocytes, and white matter abnormalities (10), and creates a sanctuary site for viral persistence and development of drug resistance due to poor CNS penetration of most anti-retroviral drugs (3).

      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 (11), but its tropism is restricted to CD4$^+$ T cells (12) 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* (12) and induces AIDS in a subset of infected macaques (13). However, infected macrophages and macrophage-associated pathology are rarely detected in SIVmac316-infected macaques (13-16). 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 (17-20). However, the composition of the poorly defined swarm in this strain varies considerably after amplification in cell culture (21), resulting in heterogeneous clinical outcomes and hindering studies of viral determinants important for pathogenesis and immune evasion. Currently available molecular clones derived from SIVmac251 include SIVmac251$_{BK28}$ (22), SIVmac251 clone (23, 24), SIVmac1A11 (25), and SIVmac32H (26), but these clones cause 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* (27), but levels of replication were very low compared to SIVmac316 (27). 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 (28-31). Structural models of Env trimers suggest that the V1/V2 loop interacts with the V3 loop in the same or neighboring gp120 protomer (32-35), 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 (36-38). However, structural changes that enhance gp120 interaction with CD4 often render macrophage-tropic viruses more susceptible to antibody recognition (39-43). 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 (44-48), 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 over 98% 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 demonstrate 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 (49-51) (Table
2.1). 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
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 PCR
from genomic DNA isolated from these tissue samples. 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) were then sub-cloned into pSIVΔgpv (provided
by Joseph Sodroski) (52) to generate Env clones, or into 239-FL plasmid (provided by Ronald
Desrosiers) (27) to generate full-length replication-competent viruses. 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 (53) 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., 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 (provided by Ronald Desrosiers), or mouse ascites containing SIV monoclonal antibodies (54) 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 percentage infectivity 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 (49-51). 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 2.1). 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 2.1).

Among early variants, desiv147c#4 had the closest relationship to the gp120 consensus

sequence in brain based on genetic distance (Figure 2.1A). 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

(Figure 2.1B). Based on >98% sequence identity with gp120 sequences in brain, we predicted

that desiv147c#4 is a macrophage-tropic Env.

Table 2.1 Clinical and pathological findings in SIVmac251-infected rhesus macaques

Group	Animal	Inoculum	Survival dpi[a]	AIDS	Pathology	Reference
I	147-94	SIVmac251 (0.032 ng p27, i.v.[b])	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.[c])	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[d] (MNGC[e]); CMV pneumonia; severe segmental purulent enteritis and peritonitis and lymphangiectasia	Williams et al, 2002 (49)
	A95-346	SIVmac251 (20 ng p27, i.v.)	190	Yes	SIVE (MNGC); giant cell pneumonia; giant cell enteritis	Williams et al, 2001 (51)
	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; Enterocytozoon bieneusi, bile duct duodenum; Proliferative and fibrinous peritonitis; Giant cell pneumonia	Williams et al, 2001 (51); Williams et al, 2002 (49)
	A97-251	SIVmac251 (20 ng p27, i.v.)	233	Yes	SIVE (MNGC); Pneumocystis carinii pneumonia (PCP); CMV[f] orchitis	Orandle et al, 2002 (50)

[a] dpi, days post-infection.

[b] i.v., intravenous inoculation.

[c] vag., vaginal inoculation.

[d] SIVE, SIV encephalitis.

[e] MNGC, Multinucleated giant cells.

[f] CMV, Cytomegalovirus.

Figure 2.1 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 high 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.
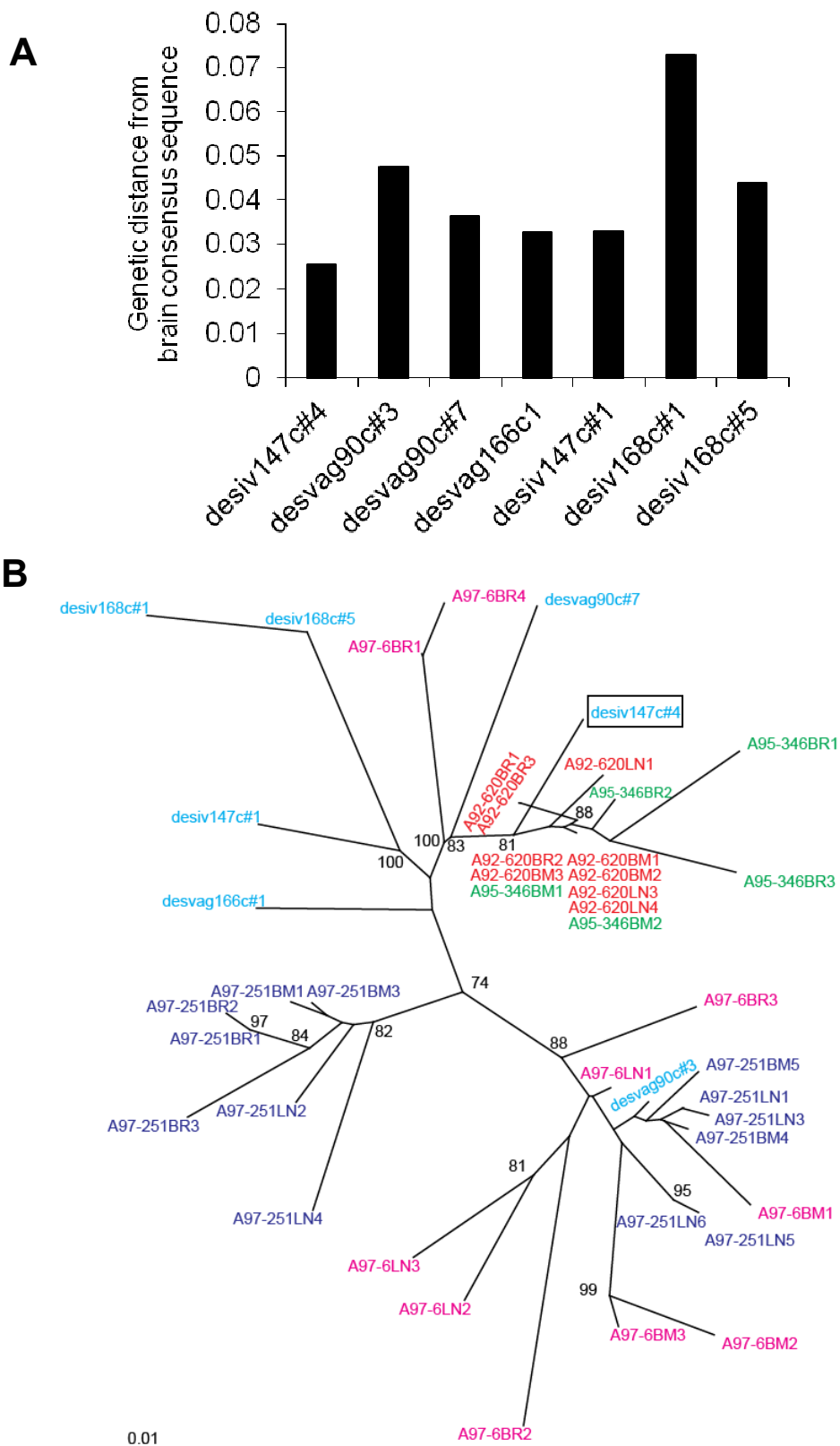
Figure 2.1 (continued)

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desiv147c#4, A92-620BR2, and the gp120 consequence sequence in brain were genetically distinct from a lab-adapted non-pathogenic SIVmac251 clone (22, 55, 56), with most differences mapping to the V1, V2, V3 and C3 regions (Figure 2.2). Sequence variation in these regions can influence Env interactions with CD4/CCR5 (33, 40, 57-59) and thereby influence macrophage-tropism (12, 40, 43). The G382R change in C3, at the N-terminus of the conserved GGPDE domain in the CD4 binding loop (33), has been associated with macrophage-tropism and decreased CD4 dependence in macrophage-tropic strains SIVmac316 and SIV/17E-Fr (12, 38, 60, 61). V1, V2, V3, and C3 sequences of desiv147c#4 were predominant in brain and bone marrow, and frequent in lymph node (Table 2.2). 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.

```
                10        20        30       Ψ  40        50        60        70        80        90        100
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SIVmac251  MGCLGNQLLIAILLLSVYGIYCTQYVTVFYGVPAWRNATIPLFCATKNRDTWGTTQCLPDNGDYSELALNVTESFDAWENTVTEQAIEDVWQLFETSIKP
Consensus  ....................................................................................................
desiv147c#4 ..................................................E.........................D......................
A92-620BR2 ....................................................................................................
```

V1 ⟷   V2

```
               110    Ψ   120       130     Ψ 140       Ψ150      Ψ160       170  Ψ    180     Ψ  190       200
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SIVmac251  CVKLSPLCITMRCNKSETDRWGLTKSSTTITTAAPTSAPVSEK-IDMVNETSSCIAQNNCTGLEQEQMISCKFTMTGLKRDKTKEYNETWYSTDLVCEQG
Consensus  .........................IT...PT.P-TT.T.S..........VHD..........G...N...............................
desiv147c#4 ...................K.......--LT...PT.PNTT.T.-..........VHD..........G...N..............................
A92-620BR2 ...................K........IT...PT.PNTT.T.S..........VHD..........G...N..............................
```

```
          Ψ   Ψ        210         220       230       240     Ψ 250       260       270       280   Ψ  290    Ψ  300
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SIVmac251  NSTDNESRCYMNHCNTSVIQESCDKHYWDTIRFRYCAPPGYALLRCNDTNYSGFMPKCSKVVVSSCTRMMETQTSTWFGFNGTRAENRTYIYWHGRDNRT
Consensus  ...................I...............................................................................
desiv147c#4 ...................I...............................................................................
A92-620BR2 ...................I...............................................................................
```

V3 ⟷

```
          Ψ310     Ψ320        330       340       350       360       370  Ψ    380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SIVmac251  IISLNKYYNLTMKCRRPGNKTVLPVTIMSGLVFHSQPINDRPKQAWCWFGGKWKDAIKEVKQTIVKHPRYTGTNNTDKINLTAPGGGDPEVTFMWTNCRG
Consensus  ..................................V.E..N........N..............................R................
desiv147c#4 ..................................V.E..N........N..............................R................
A92-620BR2 ..................................V.E..N........N..................I...........R................
```

```
                410       420       430       440       450       460 Ψ     470      Ψ480Ψ     490       500
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SIVmac251  EFLYCKMNWFLNWVEDRDVTTQRPKERHRRNYVPCHIRQIINTWHKVGKNVYLPPREGDLTCNSTVTSLIANIDWTDGNQTSITMSAEVAELYRLELGDY
Consensus  ...................L................................................................N...................
desiv147c#4 ...................N.......G........................................................N...........------
A92-620BR2 ..................................................................................N...................
```

```
                510       520
         ....|....|....|....|....|....|...
SIVmac251  KLVEITPIGLAPTDVKRYTTGGTSRNKR
Consensus  ............--------------
desiv147c#4 ----------------------------
A92-620BR2 ............--------------
```

Figure 2.2 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 2.1), 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 Ψ.

Table 2.2 desiv147c#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	brain	bone marrow	lymph node	total
			% (n=37)	% (n=13)	% (n=12)	% (n=12)	% (n=7)
V1	132-141	**PTTPNTTSTK**	**35**	**38**	**42**	**25**	**14**
		PTSAPVSEK	35	8	42	58	14
		PTAPTAASK	16	23	17	8	0
		STTPKTISTK	5	15	0	0	0
		PTTPSTTSTK	3	8	0	0	0
		PTAPTAAPK	3	0	0	8	0
		PPSAPASEP	3	8	0	0	0
V2	153-155	**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	0	8	0
		IHD	3	8	0	0	0
		AQD	3	0	0	8	0
V3	335	**V**	**100**	**100**	**100**	**100**	**100**
	337	**E**	**76**	**100**	**58**	**67**	**67**
		D	24	0	42	33	33
	340	**N**	**100**	**100**	**100**	**100**	**100**
C3	382	**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 2.1).

[b] Clones from early infection were derived from PBMC two weeks post-infection from four macaques infected with uncloned SIVmac251virus stock (Table 2.1).

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 2.2), we made recombinant Envs and infectious molecular clones (Figure 2.3). The recombinant Envs were expressed at similar levels in 293T cells (Figure 2.4A). In fusion assays, the 239/251 V1, V2, V3, and C3 Envs mediated higher levels of cell-cell fusion compared to the parental Env (Figure 2.4B). 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 ($p<0.05$) (Figure 2.4C), whereas introducing the V1 and V3 variants reduced viral infectivity. The infectivity of 239/251 V1 was greatly impaired ($p<0.01$) (Figure 2.4C). 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.
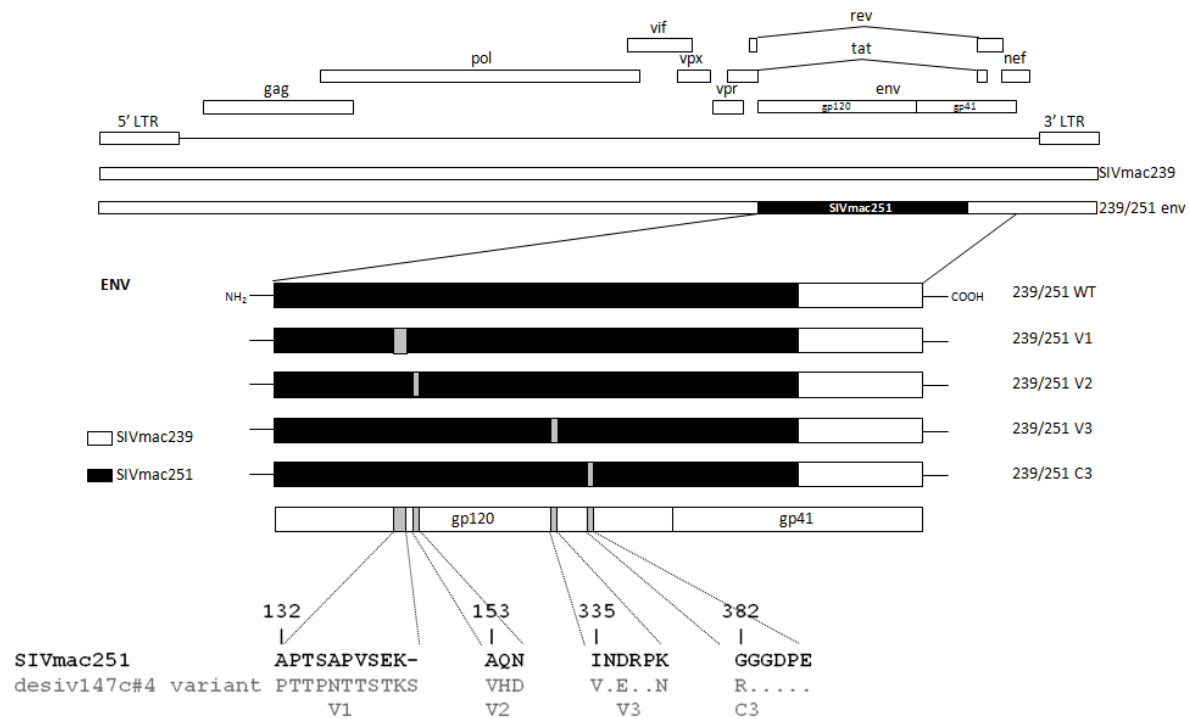

 Figure 2.3 Construction of full-length recombinant SIV 239/251 proviruses expressing gp120 sequences from the early variant desiv147c#4. The gp120 region and N-terminus of gp41 of SIVmac251 was cloned into the full-length SIVmac239 provirus. Sequences in the V1, V2, V3

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and C3 regions of desiv147c#4 were introduced into SIVmac251 gp120 to create recombinant viruses.
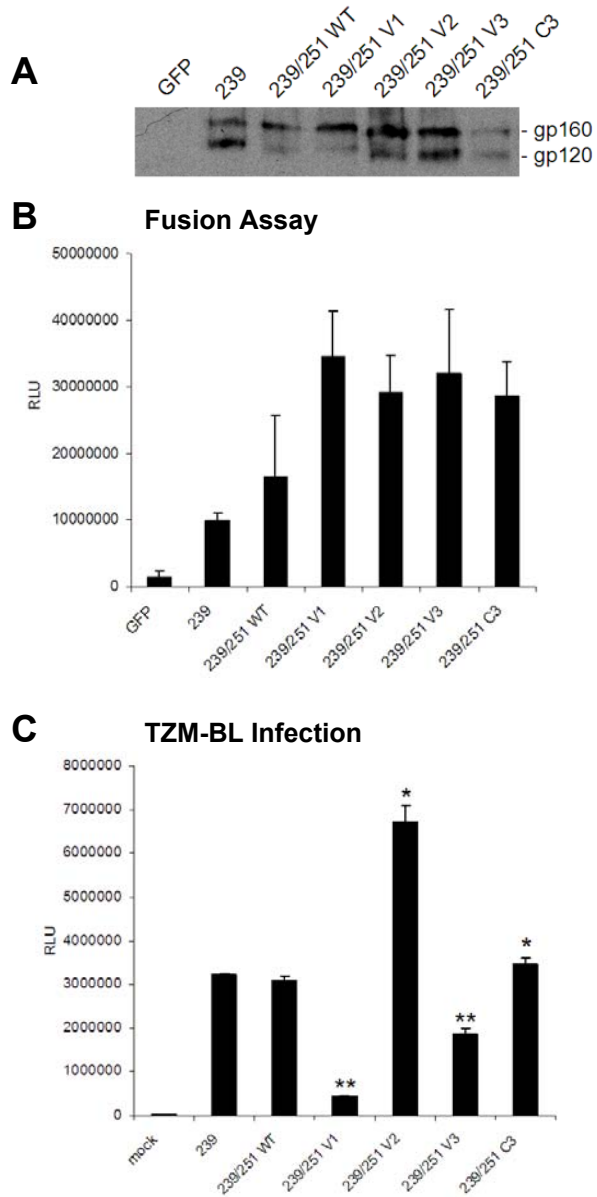

Figure 2.4 SIV Envs expressing sequences from desiv147c#4 mediate high levels of cell-cell fusion but variable infection of TZM-BL cells. (A) Expression of recombinant Envs in transfected 293T cells examined by western blot. (B) Cell-cell fusion assay. 293T cells transfected with GFP (negative control) or the indicated Env-expressing plasmids were mixed with Cf2-luc cells expressing rhesus CD4 and CCR5. 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.

N173 and N481 decrease fusion activity but enhance viral infectivity. Next, we constructed a panel of infectious molecular clones expressing the V2, V3, and C3 variant sequences in different combinations. Because viruses containing the V1 variant had poor infectivity, this variant was not included (Figure 2.4C). 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$_{BK28}$ clone used for experiments described above (Figure 2.2). When mapped to the unliganded SIV gp120 structure (62) , N173 is located in the V1/V2 loop and N481 is located near the CD4 binding loop (Figure 2.5). 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
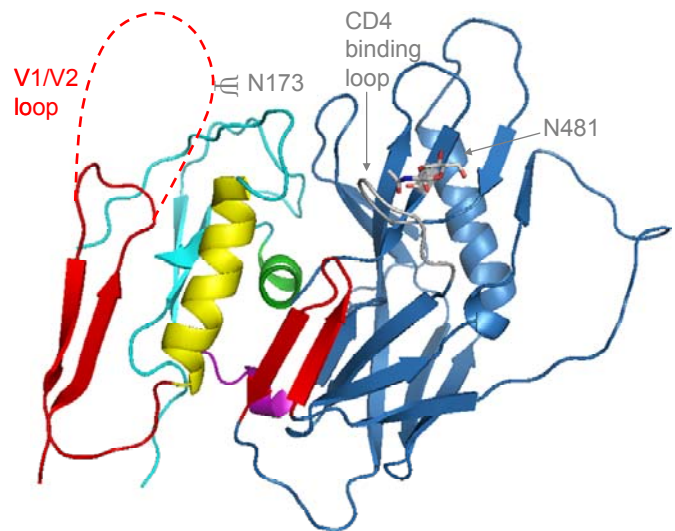

Figure 2.5 N173 and N481 mapped onto the unliganded SIV structure published by Chen et al, 2005 (33). N173 (shown as Ψ) is in the V1/V2 loop, which is absent in the structure, shown here as a dashed line. N481 and CD4 binding loop containing the conserved GGDPE domain are marked in gray. In this model, the N481 N-glycosylation site is located near the CD4 binding loop.

with addition of glycans at these sites (Figure 2.6A).

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 viral infectivity, similar to those of SIVmac316, a prototype macrophage-tropic clone used as a positive control (Figure 2.6B and C). 239/251 V2C3 and V2V3C3 Envs had increased fusion activity compared to the parental 239/251 (Figure 2.6B). The high levels of fusion mediated by 239/251 V2V3C3 Env were similar to those of desiv147c#4 and SIVmac316 Envs. Introduction

of N173 and N481 reduced fusion activity of 239/251 Envs (Figure 2.6B), but enhanced viral

infectivity in TZM-BL cells by 2.5- to 10-fold (Figure 2.6C). 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,
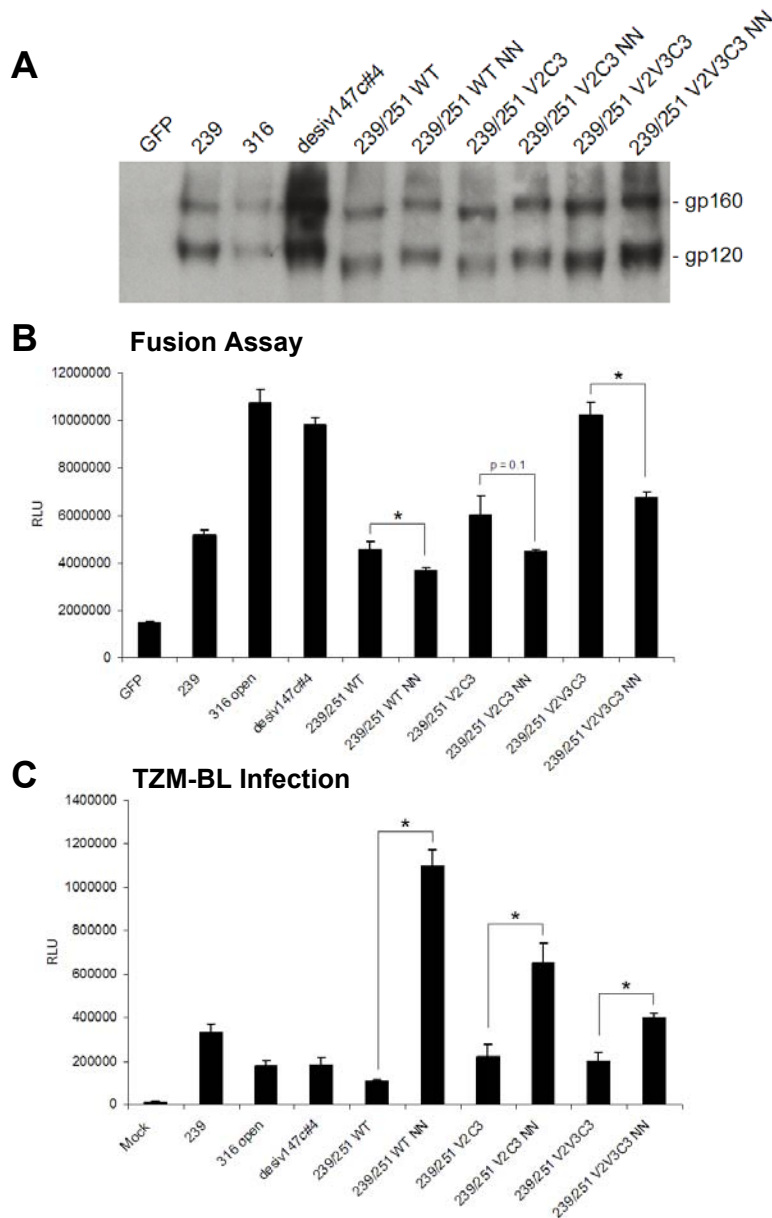
but enhances viral infectivity.

Figure 2.6 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

Figure 2.6 (continued)
(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.

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 (Figure 2.7A). desiv147c#4 also replicated in PBMC, but with

delayed kinetics and slightly lower peak levels compared to the other viruses (Figure 2.7A). The

same batch of virus stocks exhibited a different pattern when tested for viral replication in MDM

(Figure 2.7B). desiv147c#4, 239/251 WT and 239/251 V2C3 replicated efficiently with similar

kinetics, whereas 239/251 V2V3C3 replicated poorly. Adding N173 and N481 to the 239/251

recombinant viruses reduced viral replication in MDM (Figure 2.7B). Multinucleated giant cells

(MNGC) were observed in macrophage cultures infected with SIVmac316, desiv147#4, 239/251

WT, and 239/251 V2C3 (Figure 2.8). 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

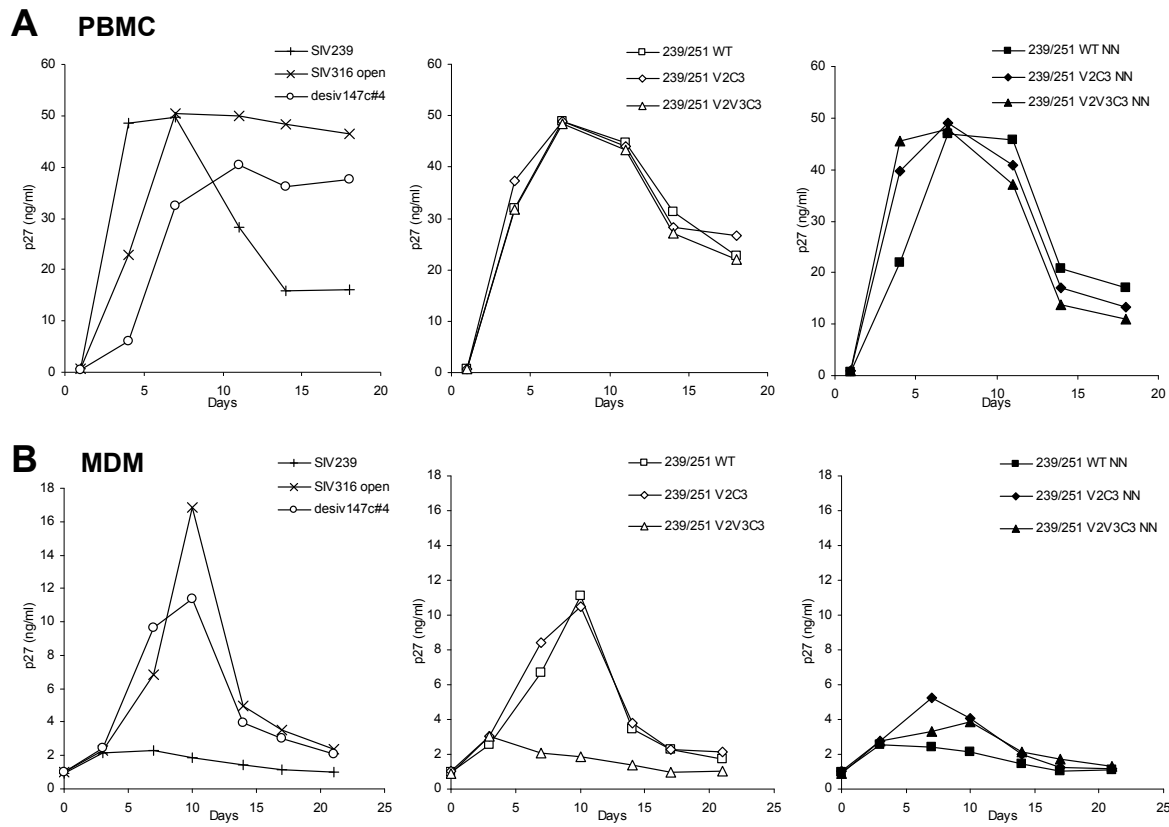regions, but are inhibited by N173 and N481.

Figure 2.7 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. Shown are representative results of three independent experiments.
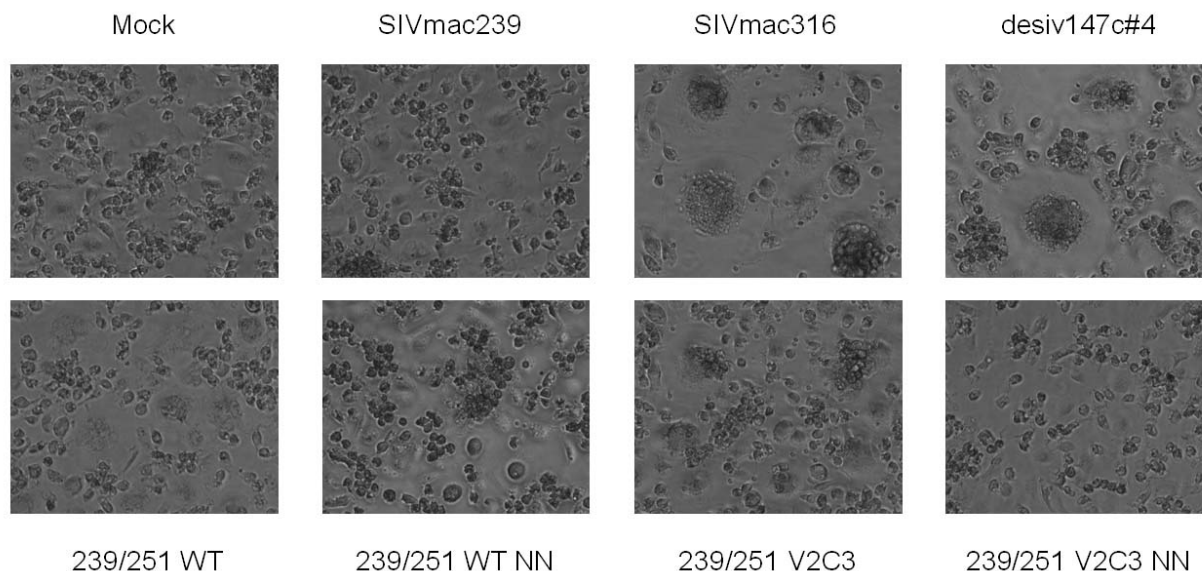
Figure 2.8 Induction of multinucleated giant cells in primary rhesus macrophages infected with recombinant SIV viruses. Shown are multinucleated giant cells on day 7 post-infection in rhesus MDM cultures infected with 10 ng of p27 of the indicated SIV viruses.

N173 and N481 enhance neutralization resistance. The V1/V2 and V3 loops are targets for neutralizing antibodies, and mutations in these regions can alter neutralization sensitivity (40, 43, 58, 63). 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 2.3). 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 2.3). 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 (54). Viruses expressing N173 and N481 exhibited enhanced resistance to all three antibodies

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compared to those lacking these N-glycosylation sites (Table 2.3). 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.

Table 2.3 Neutralization sensitivity profile of replication-competent recombinant SIV viruses generated in 293T cells

Viruses	SIV251 antiserum		SIV239 antiserum		5B11[c]		7D3[c]		36D5[c]	
	IC$_{50}$[a]	IC$_{90}$	IC$_{50}$	IC$_{90}$	IC$_{50}$	IC$_{90}$	IC$_{50}$	IC$_{90}$	IC$_{50}$	IC$_{90}$
SIVmac239	<100[b]	<100	<100	<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	7330	<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

[a] IC$_{50}$ and IC$_{90}$ were calculated as the reciprocal dilution of antiserum or ascites required for achieving 50% and 90% inhibition of infection with no serum control, respectively.

[b] IC$_{50}$ or IC$_{90}$ could not be achieved at 1:100 dilutions of antiserum or ascites containing the monoclonal antibodies.

[c] Epitopes of 5B11, 7D3, and 36D5 were previously mapped to the CD4 binding site, CCR5 binding site, and V3 loop, respectively (54).

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) (Figure 2.9). 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

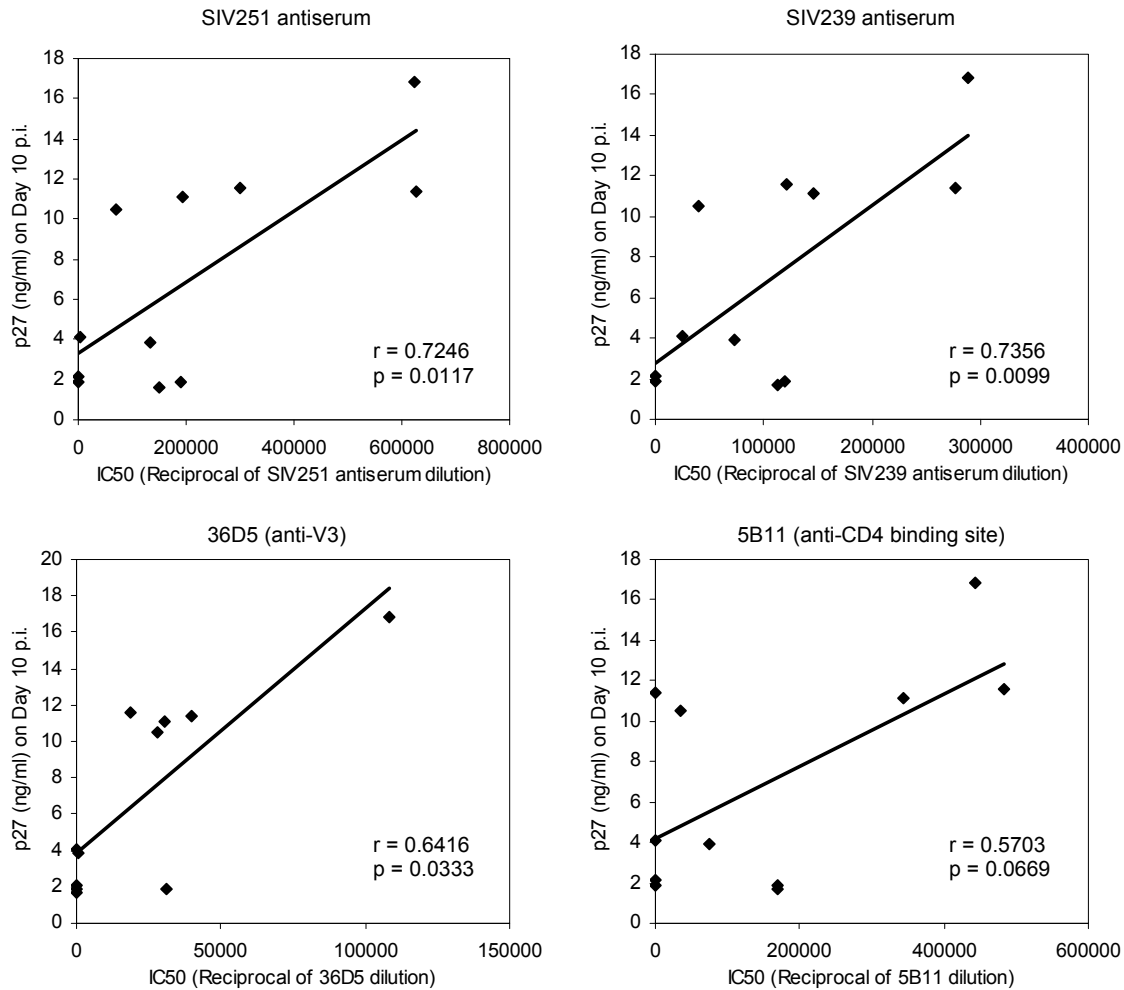overlap the CD4 binding site and V3 region.

Figure 2.9 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 (Figure 2.7B). Neutralization sensitivity was expressed as the reciprocal dilution required for achieving 50% inhibition of infection with the no serum control.

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 (64-68). 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 were more infectious than the corresponding 293T-generated viruses, 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 (Figure 2.10A). Similar to experiments described above (Figure 2.7), PBMC-generated desiv147c#4 replicated at moderate levels in MDM, while 239/251 viruses with N173 and N481 replicated at low levels in MDM (Figure 2.10B). 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 2.4). 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.
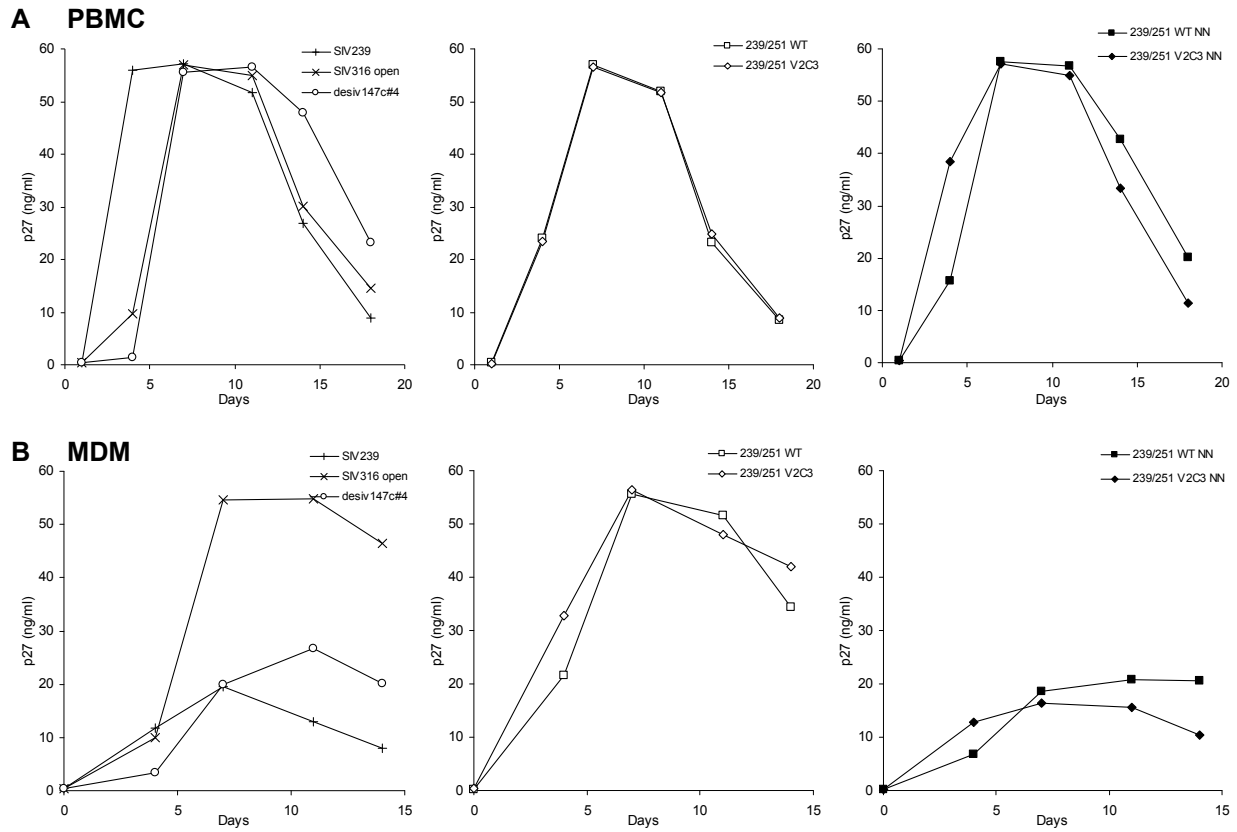
Figure 2.10 Replication of PBMC-derived recombinant SIV viruses in primary rhesus macaque PBMC (A) and MDM (B). Viral replication was quantified in rhesus PBMC and MDM infected with 2 ng p27 of the indicated PBMC-derived viruses by measuring SIV p27 antigen concentration in cell cultures supernatants by ELISA. Values shown are means of duplicate wells.

Table 2.4 Neutralization sensitivity profile of replication-competent recombinant SIV viruses generated in PBMC

Viruses	SIV251 antiserum		SIV239 antiserum		5B11[c]		7D3[c]		36D5[c]	
	IC_{50} [a]	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
SIVmac239	<100[b]	<100	<100	<100	<100	<100	<100	<100	158	<100
SIVmac316	629106	132455	609154	115496	129037	156	752972	418318	166164	7959
desiv147c#4	627934	134022	296499	103497	101	<100	7512	<100	131625	3159
239/251 WT	513696	<100	219312	<100	133481	<100	601386	<100	7708	118
239/251 WT NN	<100	<100	<100	<100	<100	<100	<100	<100	144	103
239/251 V2C3	110632	<100	35489	<100	1840	<100	470150	<100	9992	138
239/251 V2C3 NN	<100	<100	<100	<100	<100	<100	<100	<100	198	107

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

[a] IC_{50} and IC_{90} 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.

[b] IC_{50} or IC_{90} could not be achieved at 1:100 dilutions of antiserum or ascites containing the monoclonal antibodies.

[c] Epitopes of 5B11, 7D3, and 36D5 were mapped to the CD4 binding site, CCR5 binding site, and V3 loop, respectively (54).

Discussion

In this study, we identified a macrophage-tropic SIVmac251 early variant (desiv147c#4) in blood that shares over 98% sequence homology with the gp120 consensus sequence in brain of rhesus macaques that developed neurological disease. SIV251 clones expressing desiv147c#4 amino acid variants in the V2 and C3 regions 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 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*. As such, the infectious SIV clones expressing desiv147c#4 and other Envs described in the present study will be useful for future studies on these and other aspects of 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 (27). gp120 sequences in the present study were generated 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 SIVmac251stock from another lab (Table 2.1). Despite 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. This finding is consistent with the possibility that desiv147c#4 represents a founder

virus for macrophage infection in brain. V1, V2, V3 and C3 sequences in desiv147c#4 (Table 2.2) are also present in some transmitted/founder viruses described by others (21). Most transmitted/founder HIV/SIV viruses have been derived from individuals or animals infected through mucosal routes, whereas desiv147c#4 was derived from an animal inoculated intravenously. Since desiv147c#4 replicates efficiently in CD4$^+$ T cells, the first cell type infected after viruses cross the mucosal barrier (69), it will be interesting to examine whether desiv147c#4 can mediate mucosal transmission followed by establishment of infected macrophage reservoirs in brain during early infection.

Introducing desiv147c#4 gp120, or V2, V3, 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. Enhanced fusion activity of 239/251 V2 and V3 Envs might reflect structural changes involving the V2 and V3 regions. These regions play a role in gp120 interactions with CCR5, and may thereby modulate fusion activity (30, 31, 59). G382R 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 (12, 38, 61). These findings suggest a close association between cell-cell fusion activity 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$_{BK28}$ clone, possibly due to prolonged passage in tissue culture. Notably, they were restored when SIVmac251$_{BK28}$ was introduced into animals (56), 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 (70, 71). Consistent with these findings, addition of N173 and N481 enhanced infectivity of 239/251 viruses in TZM-BL cells, but reduced viral replication in macrophages in the present

study. However, 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. 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 (72). Furthermore, N160 is a critical residue in HIV gp120 for recognition and binding of a broadly neutralizing antibody, PG9 (73). 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 (74, 75). 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 (65, 68). 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.

In conclusion, our findings suggest that macrophage-tropic SIV capable of establishing viral reservoirs in macrophages in brain and other tissues can be present in blood during early infection. SIV infectious molecular clones expressing sequences from this variant replicate efficiently in PBMC and macrophages, and induce large MNGC in macrophages. As such, these SIV molecular clones will be useful for future animal model studies on macrophage-associated pathogenesis, persistence and eradication of viral reservoirs, and vaccine development.

Acknowledgments

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CHAPTER 3: Loss of a conserved N-linked glycosylation site in the SIV envelope glycoprotein V2 region enhances macrophage tropism by increasing CD4-independent cell-to-cell transmission

Po-Jen Yen,[1,2] Alon Herschhorn,[1] Christopher Gu,[1] Hillel Haim, [1] Ignacio Salas, [1] Joseph Sodroski,[1] and Dana Gabuzda[1,3]

Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts, USA[1]

Division of Medical Sciences Program in Virology, Harvard Medical School, Boston, Massachusetts, USA[2]

Department of Neurology, Harvard Medical School, Boston, Massachusetts, USA[3]

Abstract

HIV and SIV strains differ in their capacity to replicate in macrophages, but mechanisms underlying these differences are not fully understood. Here, we identify a highly conserved N-linked glycosylation site (N173) in the V2 region of the SIV envelope glycoprotein (Env) as a novel determinant of macrophage tropism and characterize mechanisms underlying this phenotype. Loss of N173 in the non-macrophage-tropic SIVmac239 virus enhanced viral replication and multinucleated giant cell formation in rhesus macrophages, while adding N173 to SIVmac251 had the opposite effect. Removal of N173 in SIVmac239 by introducing an N173Q mutation enhanced CD4-independent cell-to-cell transmission with CCR5-expressing cells. SIVmac239 with N173Q also mediated CD4-independent cell-cell fusion, but could not infect CD4-negative cells in single-round infections. Thus, CD4-independent phenotypes were detected only in the context of cell-to-cell contact. Similar results were obtained in SIVmac251 with and without N173. N173 decreased neutralization sensitivity of SIVmac251, but had no effect on neutralization sensitivity of SIVmac239. N173Q mutation had no effect on SIVmac239 binding to CD4 in BIACORE and co-immunoprecipitation assays. These findings suggest that loss of the N173 N-linked glycosylation site increases SIVmac239 replication in macrophages by enhancing CD4-independent cell-to-cell virus transmission through CCR5-mediated fusion. This mechanism may facilitate escape of macrophage-tropic viruses from neutralizing antibodies, while promoting spreading infections by these viruses *in vivo.*

Introduction

HIV and SIV strains differ in their ability to infect and replicate in macrophages. Macrophage tropism is determined primarily by the envelope glycoprotein (Env), which forms trimers composed of three non-covalently bound heterodimers of gp120 and gp41 subunits. The gp120 exterior subunit interacts with CD4 and a co-receptor, mainly CCR5 (1, 2) and the gp41 transmembrane subunit mediates virus-cell fusion. Env determinants of macrophage tropism have been shown to confer reduced CD4 dependence, a phenotype frequently associated with enhanced neutralization sensitivity to soluble CD4 (sCD4) and antibodies targeting the CD4 and CCR5 binding sites (3-7). These findings suggest that macrophage-tropic Envs might adopt structures with more exposed CD4 and/or CCR5 binding sites, a phenotype that allows these Envs to overcome the restriction to virus entry imposed by low CD4 expressed on macrophages by facilitating Env interaction with the receptors, and thereby enhance membrane fusion and entry in cells expressing low CD4 (8-10).

As the only viral protein on the virion surface, Env is the main target of humoral immune responses, and therefore the main determinant of neutralization sensitivity. Mechanisms that viruses evolve to prevent antibody recognition include high sequence variability and a heavily glycosylated Env (11-14). The variable regions of Env, in particular the V1V2 region, can shield other conserved epitopes on Env, including the CD4 and CCR5 binding sites (15-18). The glycans cover 50 % of the molecule and form a silent face protecting Env from binding of neutralizing antibodies (11, 19). Recent findings suggest more complex roles of the V1V2 regions and glycans in this region in determining Env structure and immunogenicity. In particular, studies of RV144 vaccine trial indicate the presence of V2-specific antibodies as the only variable correlating with protection, suggesting that V2 itself may be a key immunogenic epitope (20, 21). Series of broadly neutralizing antibodies, including 2G12, PG9, PG16, PGT120s and PGT130s, have been identified to recognize and target specific glycan patterns

on Env (22-27), suggesting glycans are also susceptible to neutralizing antibody binding. Together, these findings suggest that immune selection pressures can induce mutations in the V2 region, including glycosylation sites, which in turn may alter Env structure.

The V1V2 region and N-linked glycosylation sites in V4 have been previously identified as determinants of macrophage tropism (4, 28-38). Furthermore, changes in V1V2 sequences have been associated with emergence of macrophage-tropic Simian/Human Immunodeficiency Virus (SHIV) *in vivo* (37, 38). Studies using chimeric recombinant viruses have shown that the V1V2 region is a key determinant of macrophage-tropism (29, 30, 34, 36). The V1V2 loop may interact with other regions of Env, including the V3 loop, which constitutes part of the coreceptor binding site, and thereby modulate Env structure and interaction with CCR5 (39-42).

In a previous study, we identified two N-linked glycosylation sites in the V2 and C5 regions of SIV Env that modulate macrophage tropism and enhance neutralization resistance of SIVmac251 (P. J. Yen et al, unpublished data). The N-glycosylation site in V2, N173, is at a position analogous to HIV N160, a critical residue for PG9 binding (23). The N-glycosylation site in C5, N481, is located near a region of the CD4 binding site. Here, we examined the functional roles of these N-glycosylation sites in macrophage tropism in SIVmac251 and SIVmac239, and mechanisms by which they mediate effects on viral infection and replication in macrophages.

Materials and Methods

      Recombinant SIV Envs and viruses. N173 and N481 mutations were introduced into Env expressing plasmids in pSIVΔgpv (provided by Joseph Sodroski) by site-directed mutagenesis. The recombinant Envs were then sub-cloned into full-length SIVmac239 proviruses (293-FL, provided by Ronald Desrosiers) (43), which are used to transfect 293T cells for producing replication-competent viruses. Pseudotyped viruses were generated by co-transfecting 293T cells with pSIVΔgpv and a SIV-based Env⁻ luciferace vector (SIV-luc, provided by Joseph Sodroski) (44). Viruses used for infection were normalized by reverse transcriptase activity or SIV p27 antigen concentration (ELISA from Advanced Bioscience Laboratories, Inc., Kensington, MD).

      Viral replication in PBMC and monocyte-derived macrophage (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% FBS, 1% 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% penicillin/streptomycin (P/S) and 20 U/ml IL-2 prior to infection with replication-competent viruses (10 ng p27) in duplicate wells in 96-well plates. Three hours post-infection (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 in 96-well plates. 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, viruses (10 ng p27) 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 in supernatant was measured by ELISA (Advanced Bioscience Laboratories, Inc., Kensington, MD).

Cell-to-cell transmission. Cf2 canine thymocyte donor cells were infected with VSV-G

pseudotyped SIV generated in 293T cells co-transfected with plasmids expressing VSV-G

envelope and full-length replication-competent SIV proviruses. Two days after infection, donor

cells were washed and mixed with target Cf2-luc reporter cells (45) at a 1:1 ratio, directly or in

transwells (24-well plates). Cf2-luc target cells were transfected to express different levels of

rhesus CD4 and CCR5, and cell surface receptor levels were quantified by flow cytometry. Viral

transmission to target cells was quantified by measuring luciferase activity in cell lysates two

days after co-incubation. Cell-cell fusion assay was performed in a similar format by using Env-

expressing instead of SIV-infected donor cells.

Env expression and cell-cell fusion assays. Recombinant Envs were expressed in

293T cells transfected with pSIV∆gpv. Two days post-transfection, cells were lysed and Env

expression was examined by western blotting. Env levels on virions were analyzed by western

blotting of virions, which were normalized by p27. For cell-cell fusion assays, 293T cells co-

transfected with pSIV∆gpv and pLTR-Tat, Tat expressing plasmid, were incubated with Cf2-luc

cells transiently expressing rhesus CD4 and CCR5. Expression of the reporter luciferase gene

in Cf2-luc cells is under the control of HIV-1 LTR. The ratio of 293T cells to Cf2-luc cells is 1:10.

Ten hours after co-incubation, cells were lysed and luciferase activity was quantified as an

indication of cell-cell fusion. 293T cells transfected with GFP served as a negative control for

background luciferase activity.

Affinofile cells, Cf2-luc, and TZM-BL cell single-round infection assays. Affinofile

cells (46) were seeded at 3×10^4 cells per well into 96-well plates the day prior to induction. Cells

were induced with 0, 0.6, 0.8, and 1 ng/ml doxycyclin (to induce CD4) and 0, 0.25, 0.5, and 1

µM ponasterone (to induce CCR5) in a 3×3 matrix format for 21 hours at 37°C. CD4 and CCR5

expression was analyzed by flow cytometry and quantified with QuantiBRITE PE (BD Biosciences). The induced cells were infected with the indicated pseudotyped viruses in the presence of 40 µg/ml DEAE-dextran for 16 hours. The viruses were then removed by replacing the medium, and luciferase activity was measured 48 hours later as an indication of infection.

For Cf2-luc and TZM-BL cell infections, cells were infected with replication-competent viruses (10 ng p27) in the presence of 15 µg/ml of DEAE-dextran. Two days p.i., luciferase activity in cell lysates was measured. Cf2-luc cells were transfected with CD4 and CCR5 expression plasmids 24 hours before infection.

Neutralization assays. Viruses were pre-incubated with serial dilutions of heat-inactivated SIVmac251 antiserum (NIH AIDS Research and Reference Reagent Program) or mouse ascites containing monoclonal antibodies (provided by James Hoxie) (47) at 37°C for one hour. After pre-incubation, TZM-BL cells (provided by Norman Letvin) were added with DEAE-dextran (final concentration 15 µg/ml). Two days later, cells were lysed and luciferase activity was measured.

Generation of soluble gp120. To generate soluble gp120 (sgp120), 293F cells were transfected with a His-tagged sgp120 expressing plasmid (provided by Joseph Sodroski) by 293 Fectin (Invitrogen). Three days post-transfection, supernatant of the transfected cells was harvested, filtered through 0.45 µm filter, mixed with equilibration buffer (500 mM NaCl, 500 mM NaH_2PO_4, pH7.9) and superflow Ni-NTA beads (Qiagen), and rotate overnight at 4°C. The solution was then loaded onto a Poly-Prep Chromatography Column (Bio-Rad). The column was washed with wash buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH7.9). After washing, sgp120 was eluted stepwise with elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH7.9, containing 10, 20, 50, 100, or 250 mM Imidazole). The eluted sgp120 was dialyzed, concentrated in PBS, and analyzed by SDS-PAGE.

Co-immunoprecipitation (Co-IP). sgp120 (2 ug) was mixed with human CD4-Ig (2 ug) (provided by Joseph Sodroski) and placed on a nutator at room temperature for one hour. Protein G PLUS-Agarose (Santa Cruz) was then added and incubated at room temperature for another hour. After the incubation, the protein-agarose complex was washed three times with PBS, and binding of sgp120 to human CD4-Ig was analyzed by western blot.

Biacore. Kinetic analysis was performed on a Biacore 3000 optical biosensor (General Electric) as previously described (48), with the following modifications. Soluble CD4 (sCD4) was immobilized onto flow cells 2, 3 and 4 on a CM5 sensor chip to surface densities of ~700, ~1400 and ~2100 response units, respectively. Flow cell 1 was activated and deactivated and used as a control for nonspecific binding and refractive index changes. Different concentrations of sgp120 were injected over all flow cells at a flow rate of 50 μl/min for 1.2 min. Each concentration was injected in triplicates, the order of the injections was randomized and dissociation was measured at the end of each injection for 10 min. The binding surfaces were regenerated after each injection by 2 sequential injections of 25 and 10 μl of 10 mM Glycine (pH 2.5). All procedures were done at 25°C using the standard HBS (Hepes buffer saline, GE) as running buffer. The response from the reference flow cell was subtracted from the responses from all active surfaces. The association and dissociation phase data of triplicate injections were fitted simultaneously with BIAevalution (version 3.2) software using a 1:1 Langmuir model of binding.

Cell-based ELISA. CD4 binding to Env trimers was examined by using a cell-based ELISA as previously described (49), in which we measured binding of human CD4-Ig and SIV251 antiserum (to normalize CD4-Ig binding relative to Env cell surface expression levels) to Env trimers expressed on the cell surface. Briefly, HOS cells cultured in a 96-well plate were

transfected with SIV Env expressing plasmids. Three days later the cells were incubated with human CD4-Ig or with SIV251 antiserum for 30 minutes at room temperature. The cells were then washed and HRP-conjugated secondary antibody was added. For samples incubated with human CD4-Ig, we used goat anti-human HRP-conjugated polyclonal antibody as the secondary antibody. For samples incubated with SIV251 antiserum, we used HRP-conjugated Protein G. Binding was quantitated after adding Western Lightning reagents by measuring luminescence.

Results

N173 and N481 N-linked glycosylation sites are highly conserved in SIV strains, but missing in SIVmac251$_{BK28}$. In a previous study, we identified two N-linked glycosylation sites in the V2 and C5 regions of gp120 that reduce macrophage tropism but enhance neutralization resistance of SIVmac251$_{BK28}$ (P. J. Yen et al, unpublished data). These N-linked glycosylation sites, N173 and N481, are present in other well-studied SIVmac clones and highly conserved in 7,119 SIV sequences in the Los Alamos HIV sequence database (99.79% and 99.83% for N173 and N481, respectively), but N173 and N481 are lost in SIVmac251$_{BK28}$ (Figure 3.1A). The analogous glycosylation site of N173 in HIV, N160, is also highly conserved (91.75%) in 3,710 representative HIV sequences from all the clades in the database.

Modeling glycans at N173 and N481 on the unliganded SIV gp120 crystal structure (40) and the V1/V2 domain structure (23) provides clues for possible roles in Env function (Figure 3.1B). N173 is located in the V2 region, which plays a role in modulating formation and exposure of the CD4 binding site, interacting with V3 loop and modulating CCR5 binding site, and Env trimer association (15-18, 39-42). N-linked glycans in the V1V2 region have also been identified as determinants of neutralization sensitivity and fusion activity (17, 50-55). N481 is located in the outer domain of gp120, which is on the surface of the Env trimer, in close proximity to the CD4 binding loop. Therefore, glycosylation at this site might play a role in shielding gp120 neutralizing epitopes from antibody recognition, and may also influence structure of the CD4 and CCR5 binding sites.

A

V1V2

C4V5C5

B

Figure 3.1 N-linked glycosylation sites N173 and N481 are highly conserved among SIV strains but missing in the SIVmac251$_{BK28}$ clone. (A) Alignment of SIV gp120 sequences of the V1/V2 and the C4-V5-C5 regions shows that the N173 and N481 glycosylation sites are conserved in other well-studied SIV clones but missing in SIVmac251$_{BK28}$. N-linked glycosylation sites are shown in red, with N173 and N481 highlighted in red rectangles. (B) The two N-linked glycosylation sites are mapped to the unliganded SIV gp120 structure published by Chen et al (40) and the V1/V2 domain structure by McLellan et al (23). N173 is in the V1/V2 loop; N481 is in the C5 region, in close proximity to the conserved GGDPE domain of the CD4 binding loop.

Loss of N173 in SIVmac239 enhances viral replication and syncytia formation in rhesus macrophages, while adding N173 to SIVmac251 has the opposite effect. We previously showed that introduction of both N173 and N481 decreased macrophage tropism of SIVmac251 (P. J. Yen et al, unpublished data). To dissect the roles of each N-glycosylation site, we introduced them individually into the SIVmac251 clone. We also generated N173Q and N481Q mutants of the T-cell-tropic clone SIVmac239 to test whether removal of these glycosylation sites enhanced macrophage tropism. Mutations at either N-glycosylation site did not significantly influence viral replication in rhesus PBMC (Figure 3.2A). In contrast, adding N173 to SIVmac251 reduced viral replication in MDM, whereas adding N481 had only a modest effect, resulting in delayed replication and lower peak levels (Figure 3.2B). Removing N173 from SIVmac239 enhanced viral replication in MDM, while removing N481 had no significant effect. Infection with SIV lacking N173 (SIVmac251 and SIVmac239 N173Q) induced cytopathic effects and multinucleated giant cell (MNGC) formation in MDM (Figure 3.2C). These results suggest that N173 was a more important determinant of macrophage tropism and MNGC formation than N481.
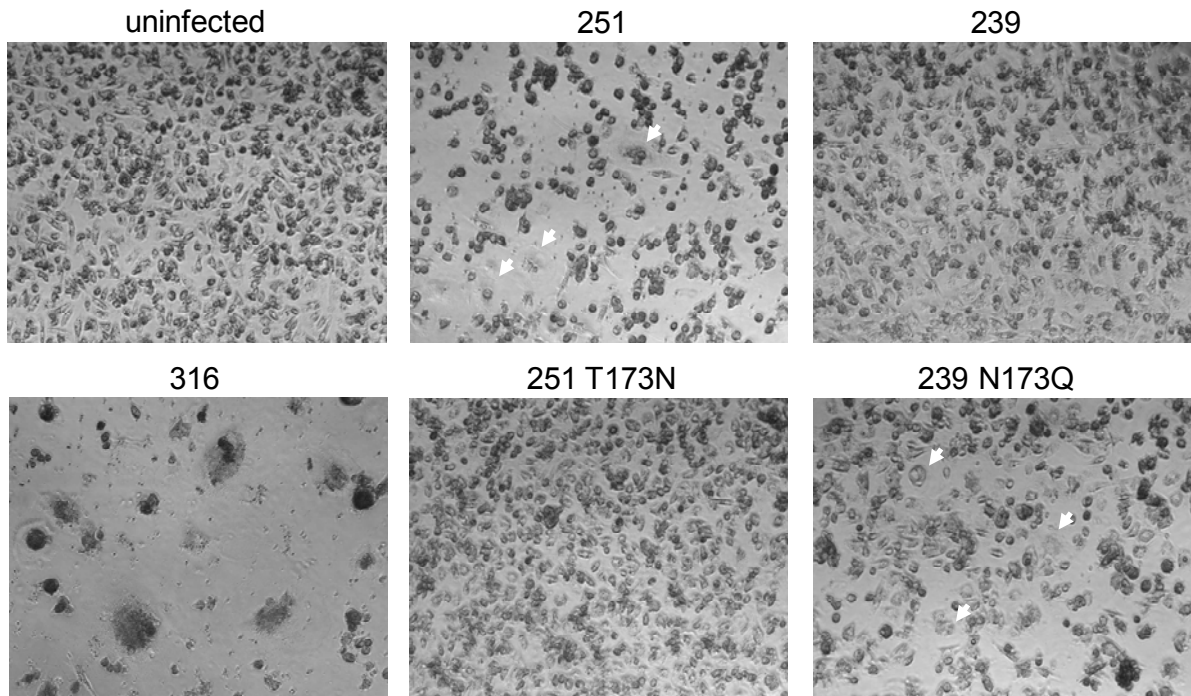
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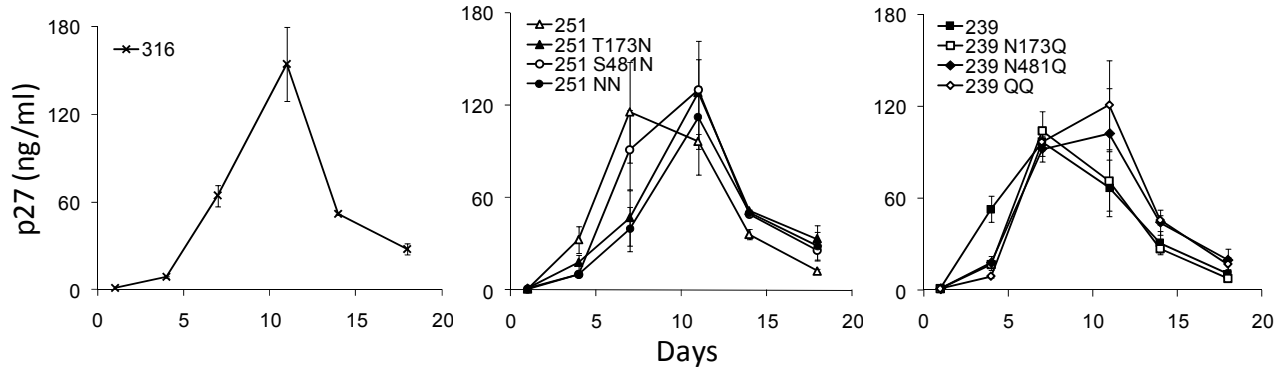
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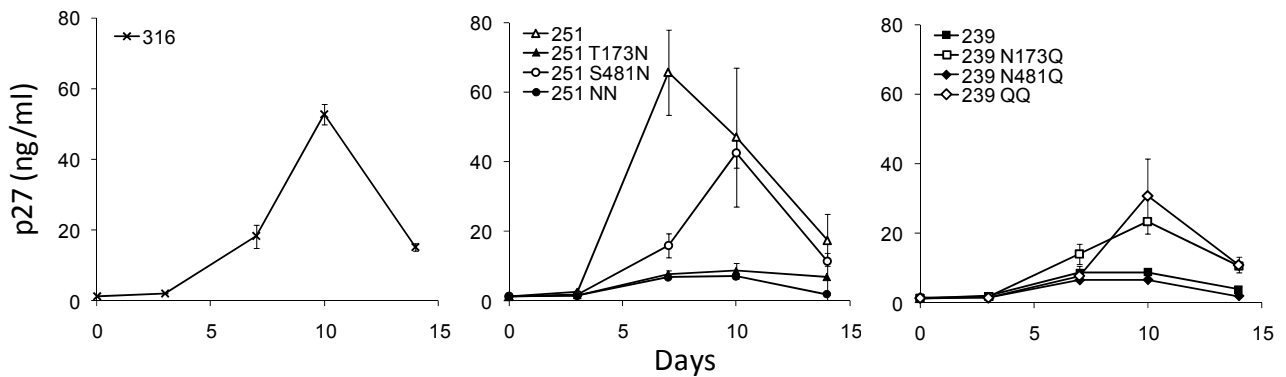
C

Figure 3.2 SIVmac251 and SIVmac239 viruses with N173 replicate well in rhesus macaque PBMC, but poorly in macrophages. (A) All viruses replicated at high levels in Rhesus PBMC. (B) Addition of N173 reduced SIVmac251 replication in macrophages, while loss of N173 enhanced
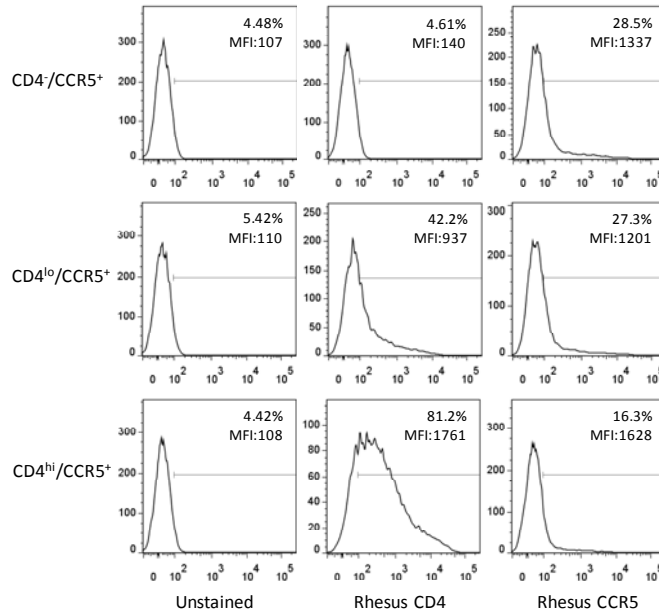
Figure 3.2 (continued)

SIVmac239 replication in macrophages. The macrophage-tropic SIVmac316 virus was included as control. (C) Infection of SIVmac251 and SIVmac239 N173Q induced MNGC (arrows) in MDM. Viruses used for infection were normalized by p27 concentration (10 ng p27). Shown are means and standard deviations of samples from duplicate wells.

Loss of N173 increases cell-to-cell transmission of SIV to CCR5+ cells expressing low or no rhesus CD4. SIV infection of rhesus macrophages with viruses lacking N173 (SIVmac251 and SIVmac239 N173Q) induced multinucleated giant cells (Figure 3.2C), suggesting these macrophage-tropic viruses mediate high levels of cell-cell fusion and may spread infection through cell-to-cell transmission. To determine whether the enhanced viral replication in rhesus macrophage is due to more efficient transmission between cells, we designed and optimized a cell-to-cell transmission assay. First-round infection of donor Cf2 cells was normalized by using VSV-G pseudotyped SIV. Cf2-target cells were transfected to express different levels of rhesus CD4/CCR5 (Figure 3.3A) When target cells expressed high levels of CD4 and CCR5, such as TZM-BL expressing high levels of human CD4/CCR5, and Cf2-luc transfected to express high levels of rhesus CD4/CCR5, viruses with or without N173 were transmitted at similar high levels (Figure 3.3B). In contrast, for target cells expressing CCR5 and low CD4 or no CD4, macrophage-tropic SIV viruses (239 N173Q and 251) mediated higher levels of cell-to-cell transmission compared to non-macrophage-tropic viruses (239 and 251 T173N) (Figure 3.3C). SIV239 N173Q mediated cell-to-cell transmission to $CD4^-CCR5^+$ target cells about three-fold more efficiently than the parental SIV239. Viral transmission was dependent on direct cell-cell contact, as it was abolished when donor and target cells were separated by transwells. Cell-cell transmission was also strictly CCR5-dependent, as these viruses were not transmitted to cells lacking both CD4/CCR5. These results suggest that these macrophage-tropic viruses mediate efficient CD4-independent cell-to-cell transmission to CCR5-expresing cells, and raise the possibility that the ability of viruses to mediate cell-to-cell transmission to target cells with low or no CD4 may be an important mechanism for promoting viral replication and spreading infection in macrophages.

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Figure 3.3 Macrophage-tropic SIVmac239 N173Q mediates CD4-independent cell-cell transmission and fusion more efficiently compared to non-macrophage-tropic SIVmac239. (A) Expression of rhesus CD4/CCR5 on Cf2-luc target cells used in both cell-cell transmission and cell-cell fusion assays. Percentage and MFI of $CD4^+$ or $CCR5^+$ cells are shown. (B) VSV-G pseudotyped SIVmac239 or SIVmac251 with or without N173 were transmitted at similar levels from infected Cf2 donor cells to TZM-BL target cells expressing human CD4/CCR5 or Cf2-luc target cells expressing high levels of rhesus CD4/CCR5. Loss of N173 in SIVmac239 enhances CD4-independent cell-cell transmission (C) and Env fusion activity (D). Results are representative of 2 to 3 independent experiments. Error bars represent standard deviation of two replicate samples. * indicates significant differences by student's t-test ($p < 0.05$).
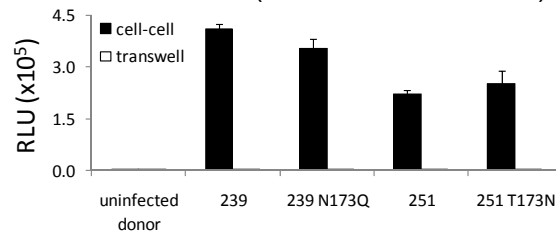
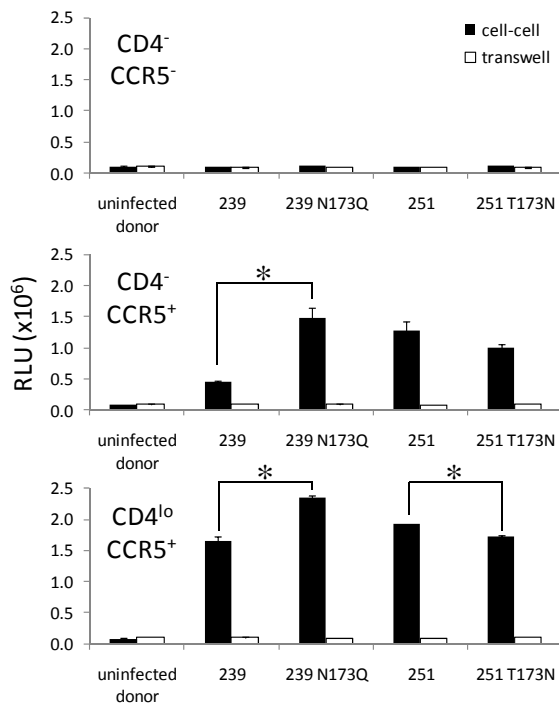**A** Rhesus CD4/CCR5 expression on transfected Cf2-luc cells

B
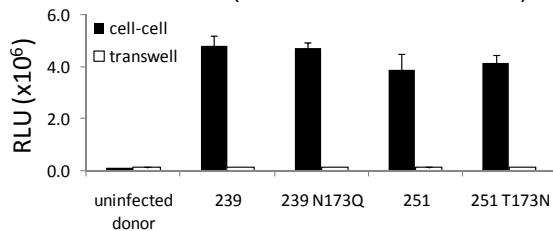
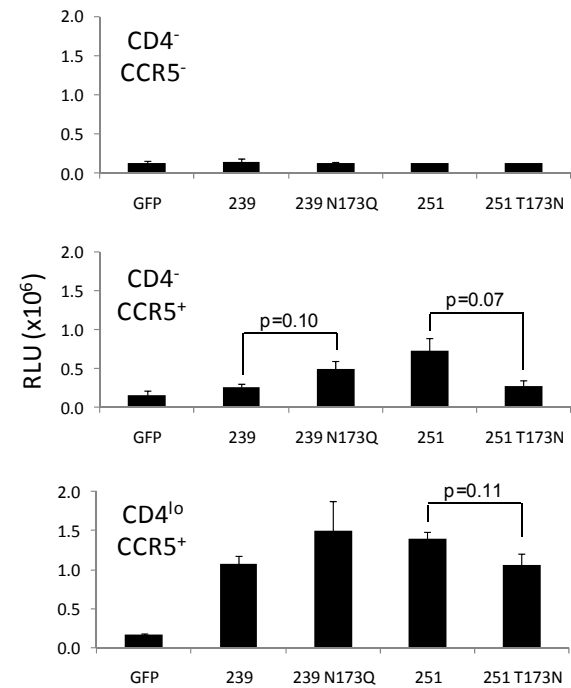**C** cell-cell transmission

D cell-cell fusion

Figure 3.3 (continued)

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Macrophage-tropic SIV Envs (239 N173Q and 251) mediate CD4-independent fusion with CCR5-expressing target cells. Next, we examined the relationship between Env-mediated fusion and cell-cell virus transmission. To address these questions, we performed a cell-cell fusion assay in a format similar to that used for the preceding cell-to-cell transmission experiments, but with Env-expressing instead of SIV-infected donor cells. Using this assay, we showed that cell-cell fusion activity corresponded to the ability of viruses to mediate cell-to-cell transmission, with the macrophage-tropic Envs (239 N173Q and 251) mediating CD4-independent fusion with CCR5-expressing target cells more efficiently than non-macrophage-tropic Envs (239 and 251 T173N) (Figure 3.3D). Similar to cell-to-cell transmission, CCR5 was required for cell-cell fusion, as fusion was not detected with target cells lacking both CD4/CCR5. Together, these findings suggest that loss of the N173 N-glycosylation site increases SIV239 macrophage-tropism by enhancing Env-CCR5 interactions and CD4-independent cell-to-cell virus transmission during spreading infections in macrophages, which express very low levels of CD4.

Loss of N173 enhances cell-cell fusion activity of SIVmac239 Env, but has no significant effect on Env expression, processing, or incorporation into virus particles. Glycosylation plays an important role in the correct folding of HIV Env (56, 57). To examine whether N173 and N481 have effects on Env expression and processing, we examined expression of the N173 and N481 mutants in 293T cells transfected with Env expression plasmids. Western blots showed similar expression levels for SIVmac239 and SIVmac251 recombinant Envs with and without the N-glycosylation sites (Figure 3.4A). gp120 and gp160 protein bands for Envs with N173 and N481 migrated more slowly than those without N173 and N481, consistent with addition of glycans at these sites (Figure 3.4A).
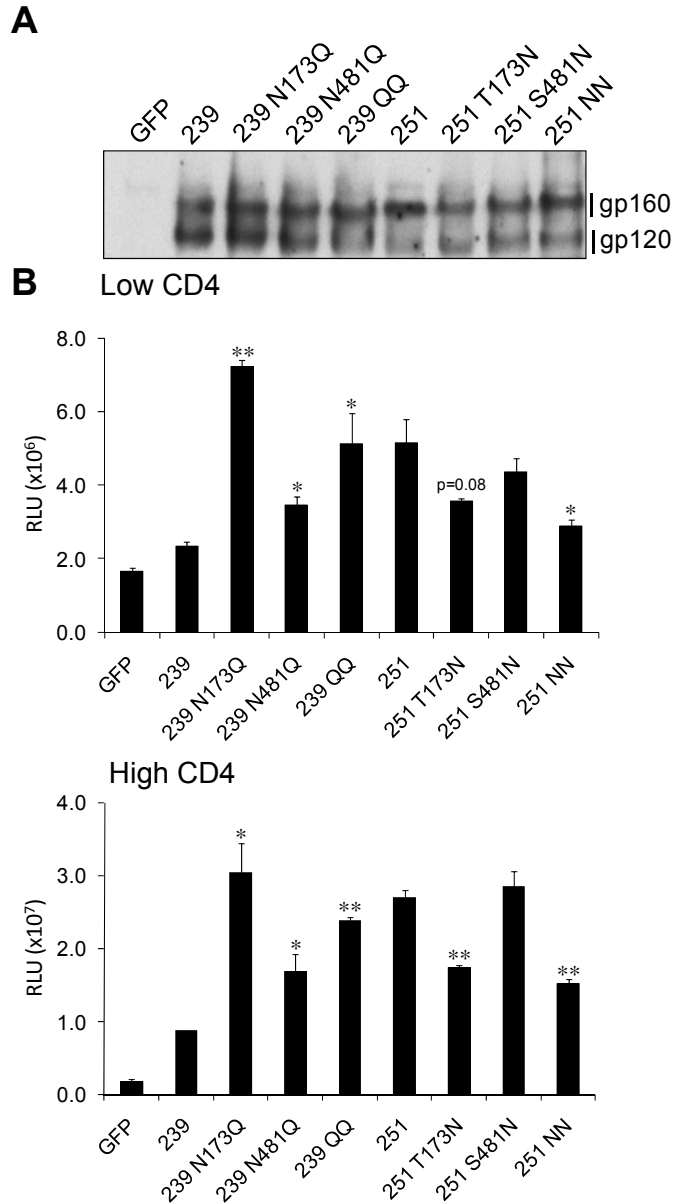
Figure 3.4 Loss of N173 enhances fusion activity but does not alter Env expression. (A) 293T cells transfected with Env expression plasmids were analyzed by western blot. N173 did not significantly affect Env expression and incorporation into virions. Shift in mobility of the gp120 and gp160 bands between the parental and N173/N481 mutants suggests addition of glycans at these two sites. (B) Env fusion activity was examined by a cell-cell fusion assay measuring fusion between 293T cells expressing SIV Envs and Cf2-luc cells expressing low or high levels of rhesus CD4 and CCR5. Loss of N173 enhanced cell-cell fusion mediated by SIVmac239 Env, whereas addition of N173 reduced cell-cell fusion mediated by SIVmac251 Env. * indicates significant difference vs. parental (student's t-test; $p<0.05$). ** indicates significant difference vs. parental ($p<0.01$).

We then examined fusion activity of Envs expressing the N173 and N481 mutants, using a cell-cell fusion assay different from the one described above. In this alternative assay format, we used 293T cells as Env-expressing effector cells instead of Cf2 cells, the ratio of effector to target cells was 1:10 instead of 1:1, and co-incubation time was 10 hours rather than 2 days. N173 mutation induced a more significant difference in fusion activity under these conditions (Figure 3.4B compared to Figure 3.3D). Envs without N173 mediated higher levels of fusion than those with N173, with cells expressing either low or high levels of CD4. In contrast, N481 mutation had only minor effects, and did not significantly alter fusion activity when introduced into SIVmac251.

Viruses lacking N173 do not infect cells expressing low or no CD4 more efficiently. We examined viral proteins in virions with western blot and showed that Env levels on virions were similar between the parental and the N173 mutants (Figure 3.5A), suggesting that N173 does not influence Env incorporation into virus particles. Macrophages express lower surface levels of CD4 than $CD4^+$ T cells (8-10). To test whether enhanced replication of SIV clones in macrophages is due to better infectivity on low CD4 or CCR5 expressing cells versus better usage of low levels of these receptors, we used Affinofile cells, an inducible cell line that can be simultaneously and independently induced to express different levels of human CD4 and CCR5 (46) (Figure 3.5B). SIVmac239, SIVmac251, and the corresponding N173 mutants infected Affinofile cells with a similar pattern. Adding or removing N173 did not alter the infection pattern of these viruses on Affinofile cells expressing different levels of CD4 and CCR5 (Figure 3.5C). Unexpectedly, viruses expressing N173 showed higher infectivity than those lacking N173. Infection of Cf2-luc cells with rhesus CD4 showed similar results (Supplemental Figure 3.1A). SIVmac239 and SIVmac251 without N173 did not infect cells with or without CD4, and lack of N173 impaired infectivity of these SIV clones in cell-free infections. Furthermore, removing N173 impaired infectivity of SIVmac239 in TZM-BL cells, whereas adding N173 enhanced

SIVmac251 infectivity in TZM-BL cells (Supplemental Figure 1B). These results contrast with those of cell-cell transmission assays, in which viruses lacking N173 were transmitted to the same Cf2-luc target cells more efficiently than those with N173. In particular, these viruses mediated CD4-independent cell-cell transmission, but could not infect CD4-negative cells in cell-free virus infections. Together, these findings suggest that CD4-independent infection required cell-cell contact.
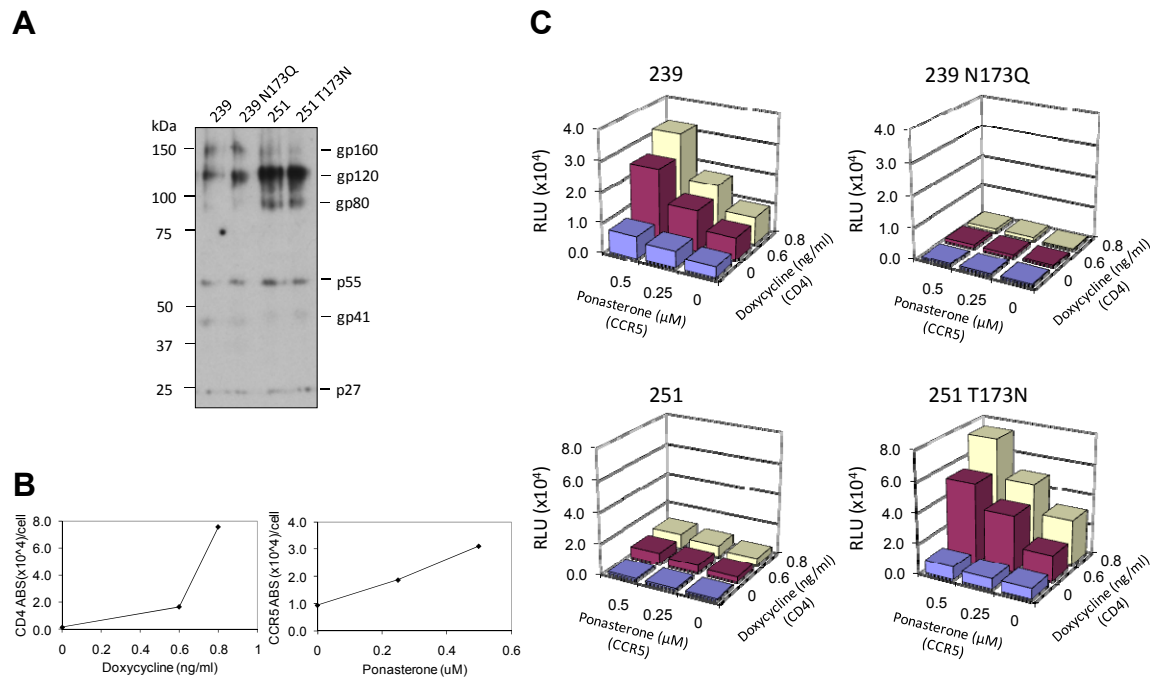

Figure 3.5 Loss of N173 impairs single-round infection of Affinofile cells. (A) Viral proteins in virions were analyzed by western blot. (B) Affinofile cells were induced with doxycyclin and ponasterone to express CD4 and CCR5. Levels of expression were analyzed by flow cytometry and quantified by using QuantiBRITE. (C) SIV lacking N173 showed impaired infectivity compared to those with N173, and did not mediate CD4-independent infection in Affinofile cells.

N173 mutation alters SIV neutralization sensitivity in a strain-dependent manner.

To probe structural changes induced by N173 and N481 mutations, we next tested the neutralization sensitivity of the recombinant viruses to SIVmac251 antiserum and monoclonal antibodies targeting specific epitopes, including CD4 and CCR5 binding sites. Previous studies showed that adding both of these N-glycosylation sites to SIVmac251 enhanced neutralization

resistance (P. J. Yen et al, unpublished data). Here we showed that this enhanced neutralization resistance was due to changes in N173, but not N481 (Table 3.1). N173 enhanced neutralization resistance of SIVmac251 to SIV251 antiserum, and monoclonal antibodies 5B11 and 7D3 (targeting CD4 and CCR5 binding sites, respectively) (47), resulting in more than 3-log differences in IC_{50}, whereas N481 did not alter neutralization sensitivity. In contrast to results for SIVmac251 clones, removal of N173 and N481 did not alter neutralization sensitivity of SIVmac239. SIVmac239 without N173 or N481 were still highly neutralization resistant, similar to the parental SIVmac239. These results suggest that N173 has different effects on neutralization sensitivity of SIVmac251 and SIVmac239. While N173 enhanced neutralization resistance of SIVmac251, removing N173 from SIVmac239 had no effect on neutralization resistance.

Table 3.1 Neutralization sensitivity of SIV clones to SIV251 antiserum and monoclonal antibodies

Viruses	SIV251 antiserum IC_{50}[a]	5B11[c] IC_{50}	7D3[c] IC_{50}
251	156192	432486	607806
251 T173N	158	<100[b]	<100
251 S481N	175437	86143	550114
251 NN	111	<100	<100
239	<100	<100	<100
239 N173Q	<100	<100	<100
239 N481Q	<100	<100	<100
239 QQ	<100	<100	<100

color
- <100
- 100 - 1000
- 1000 - 100000
- >100000

[a] IC_{50} was calculated as the reciprocal dilution of antiserum or ascites required for achieving 50% inhibition of infection with no serum control, respectively.

[b] IC_{50} could not be achieved at 1:100 dilutions of antiserum or ascites containing the antibodies.

[c] Epitopes of 5B11 and 7D3 were mapped to CD4 and CCR5 binding sites, respectively (47).

N173 does not significantly affect SIV gp120-CD4 binding. To directly test

interactions between the recombinant Envs and CD4, we purified His-tagged soluble gp120

(sgp120) from supernatants of transfected 293F cells (Figure 3.6A), and examined binding to

CD4 by co-IP (Figure 3.6B) and Biacore assays (Figure 3.6C). For the co-IP assay, similar

amounts of 239 and 239 N173Q sgp120 were pulled-down by human CD4-Ig (Figure 3.6B),

suggesting similar binding to CD4 by both sgp120s. The binding kinetics and affinities between

sgp120 and CD4 were analyzed by Biacore with human sCD4 immobilized on the chip surfaces

at three concentrations. Data from each surface were fit to a 1:1 binding model using

BIAevaluation software to derive k_{on}, k_{off}, and K_d, averages of which are shown in Figure 3.6C.

The results suggest that 239 and 239 N173Q gp120 bind to CD4 with similar kinetics and affinity.

Finally, to examine binding of Env trimers to CD4, we used a cell-based ELISA as described

(49). Consistent with results from the co-IP and Biacore assays, 239 and 239N173Q Env trimers

bound to CD4 at similar levels (Figure 3.6D). Thus, N173 does not appear to have significant

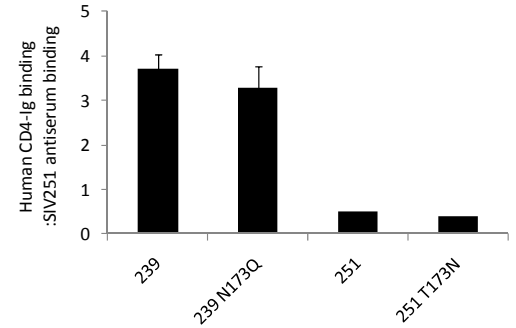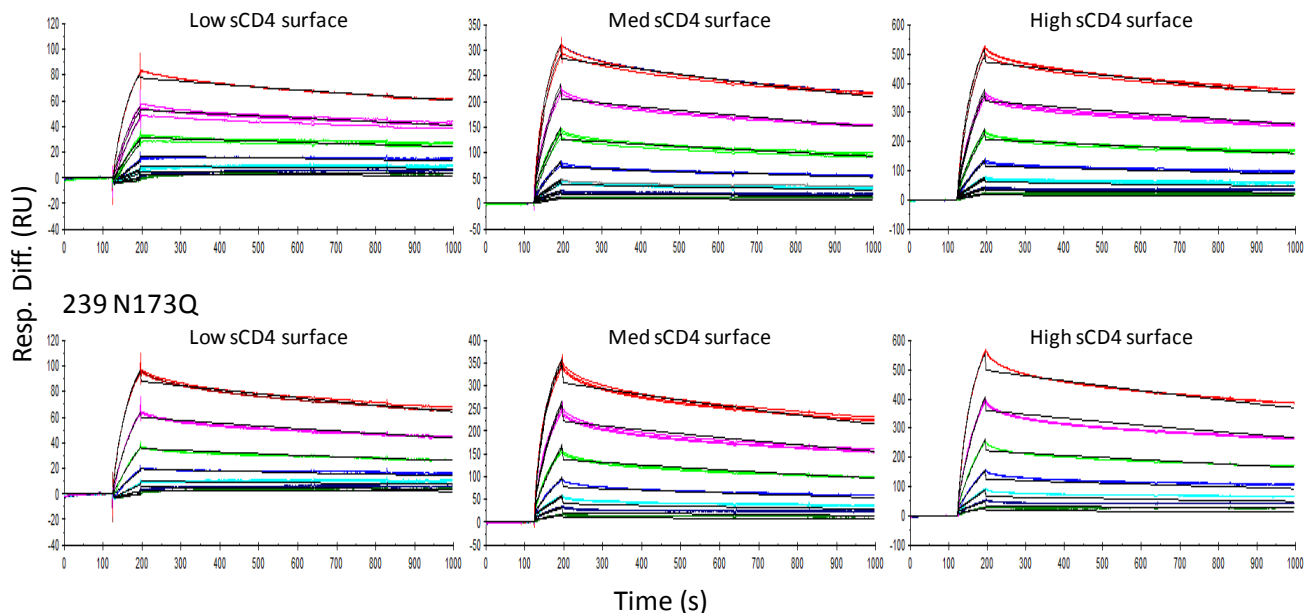effects on binding of gp120 monomers or Env trimers to CD4.

A purified sgp120
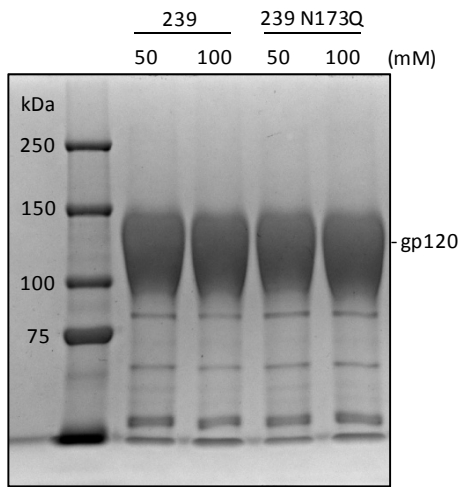
B co-IP
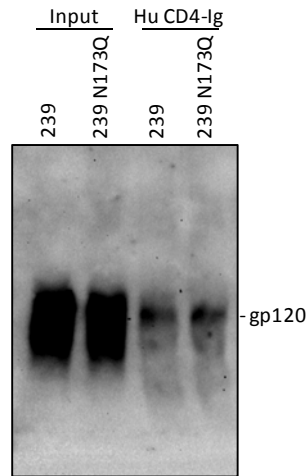
D cell-based ELISA

C Biacore

gp120	k_{on}, 10^3 M^{-1} s^{-1}	k_{off}, 10^{-4} s^{-1}	K_d, 10^{-7} M
239	3.30 ± 0.36	3.45 ± 0.34	1.05 ± 0.07
239 N173Q	2.68 ± 0.35	4.02 ± 0.33	1.52 ± 0.22

Figure 3.6 No significant differences between 239 and 239 N173Q sgp120 binding to human CD4. (A) Coomassie blue stained SDS-PAGE gel of 239 and 239 N173Q sgp120 generated and purified from transfected 293F. (B) co-IP of sgp120 with human CD4-Ig. (C) Kinetic constants for sgp120 and human sCD4 interactions analyzed by BIACORE. (D) N173 mutation does not significantly affect CD4-Ig binding to Env trimers in a cell-based ELISA.

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Discussion

In this study, we investigated functional roles of N173, a conserved N-linked glycosylation site in the V2 region of the SIV envelope glycoprotein in macrophage tropism and neutralization sensitivity. Removing N173 from SIVmac239 enhanced macrophage tropism and CD4-independent cell-to-cell transmission, but had no significant effect on neutralization sensitivity. Likewise, adding N173 to SIVmac251 reduced viral replication in macrophages and decreased cell-to-cell transmission, but in contrast to SIVmac239 neutralization resistance was enhanced. Infection of macrophages by SIV lacking N173 was associated with induction of MNGC formation, a phenotype that may be explained by the increased fusion activity of Envs of these macrophage-tropic viruses. These findings suggest that loss of the N173 glycosylation site increases SIVmac239 replication in macrophages by enhancing CD4-independent cell-to-cell transmission through CCR5-mediated fusion. This mechanism may be important for promoting spreading infections by these viruses in tissues such as brain *in vivo.*

CD4-independent cell-to-cell transmission represents a novel mechanism to explain enhanced macrophage tropism. Consistent with our results, previous studies showed that infected macrophages can transmit HIV to T cells (58-60), and that Env determinants of macrophage tropism in the V1V2 region influence entry and spread infections in macrophages (34, 36). Cell-to-cell transmission is more efficient than cell-free virus infection (59, 61, 62), and has been shown to protect viruses from inhibition by neutralizing antibodies, anti-retroviral drugs, and cellular restriction factors (62-67). Furthermore, macrophages can form conduits that connect to other target cells for transport of viral proteins, and thereby facilitate viral transmission and evasion of immune responses (68-70). This mechanism may be important to promote cell-to-cell spread and replication of macrophage-tropic SIV viruses *in vivo*, since these viruses are typically neutralization sensitive, but cell-cell transmission facilitates escape from neutralizing antibodies.

The CD4-independent SIV phenotypes were detected only in the context of cell-cell interaction, but not cell-free virus infection. Although macrophage-tropic viruses mediated enhanced CD4-independent cell-cell fusion and cell-to-cell transmission, they were not able to infect cell lines in the absence of CD4 in single-round infection assays. This may be due to the transient and short-lived intermediate state of Env required for CD4-independent fusion. In the SIV Envs lacking N173, the unliganded Env might have an increased propensity to sample a CD4-bound conformation that facilitates interaction with CCR5, and thereby allow CD4-independent fusion. This intermediate state may be short-lived before undergoing spontaneous and irreversible conformational changes, and may not be sustained long enough to achieve cell-free virus infection. Also, rapid kinetics of cell-to-cell transmission compared to cell-free virus infection (minutes versus hours) may be a factor allowing this transient form of Env to mediate CD4-independent cell-cell fusion. Within minutes of cell-cell contact, virus on the donor cell (71), and cellular receptors on the target cell (72, 73) cluster to the junction of cell-cell contact. This results in a high concentration of CCR5 locally at the cell-cell contact, another mechanism that may facilitate CD4-independent infection in the context of cell-to-cell transmission.

To our knowledge, this is the first study to identify an N-linked glycosylation site as a determinant for cell-to-cell transmission of HIV/SIV. In macrophages, HIV assembles in intracellular vesicles, late endosomes, or multivesicular bodies (74-78). HIV Env determines the site of viral release (79), and deletion of an N-linked glycosylation site in gp41 blocked gp160 cleavage and transport (80). Although no significant differences were detected in Env expression and incorporation into virions by western blot, we did not examine sites of virus assembly and release, which may be relevant for cell-to-cell transmission. Nonetheless, results from fusion assays suggest that loss of N173 enhances CCR5-mediated fusion activity, which in turn facilitates cell-to-cell transmission. Loss of N173 may affect structure and/or orientation of

the V1V2 loop, which in turn may affect V3 loop and CCR5 binding site exposure and/or orientation, thereby increasing gp120-CCR5 interaction and facilitating CD4-independent cell-cell fusion and transmission. Alternatively, unliganded SIV Env lacking N173 may have a higher propensity to sample a CD4-bound conformation, resulting in a more exposed CCR5 binding site that promotes CD4-independent fusion and cell-cell transmission.

The macrophage-tropic SIV clones (SIVmac251 and SIVmac239 N173Q) induced cell fusion and MNGC formation in macrophages and mediated higher levels of fusion in cell-cell fusion assays than their counterparts expressing N173. We originally hypothesized that removal of N173 would enhance gp120 interaction with CD4, and thereby increase fusion activity and usage of low levels of CD4 on macrophages. Unexpectedly, results from sCD4 neutralization assays showed that viruses with N173 were more sensitive to sCD4 neutralization. However, sensitivity of virus to sCD4 neutralization is not a direct indication of binding; other factors such as propensity to conformational changes after binding could also be involved (49). In co-IP and Biacore assays, removal of N173 had no effect on binding of purified SIV239 gp120 monomers to CD4. Likewise, results of cell-based ELISA showed no significant differences in CD4 binding between 239 and 239 N173Q Env trimers. Together with results of assays testing CD4-independent cell-to-cell transmission and cell-cell fusion, these finding suggests that loss of N173 increases SIV239 macrophage-tropism by enhancing Env-CCR5 interactions and CD4-independent cell-cell virus transmission to CCR5-expressing cells during spreading infections in macrophages, which express low levels of CD4.

The viruses examined in our study replicated at similar high levels in rhesus PBMC regardless of presence or absence of N173. In contrast, viruses without N173 replicated more efficiently in macrophages, but were impaired in single round infection of multiple cell lines including Affinofile, Cf2-luc, and TZM-BL cells. One possibility is that viruses without N173

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infect poorly in the first round of infection in macrophages, as suggested by the infectivity defect we found in single round infections in several cell lines, but the second round of infection via cell-to-cell transmission is enhanced in these viruses. N173 is highly conserved among HIV and SIV strains. Lacking this glycan may affect Env folding or post-translational modification, which could result in structural changes linked to the infectivity defect. An alternative explanation is that other cellular factors facilitate infection by the viruses lacking N173 are present on primary macrophages but absent on these cell lines. Previous studies suggested that some HIV Envs interact with integrin α4β7, which may serve as an attachment factor and facilitate viral infection (81-83). α4β7, however, is unlikely to be involved in facilitating viral replication in the present study. Although primary rhesus macrophages express α4β7 after seven days in culture with M-CSF and N173 is located near the putative α4β7 motif, we found that presence of anti-α4β7 antibody had no significant inhibitory effect on macrophage infection with viruses characterized in the present study (data not shown). Further studies are required to examine whether the infectivity defect in viruses lacking N173 is related to interaction with another attachment factor, effects on Env folding or structural changes, or other mechanisms.

In summary, we identified an N-linked glycosylation site, N173 in the V2 region, as an important determinant of SIV macrophage tropism. Loss of N173 enhanced SIVmac239 macrophage tropism, while restoration of N173 in SIVmac251 reduced macrophage tropism but enhanced neutralization resistance to CD4/CCR5 binding site antibodies. SIVmac239 lacking N173 mediated CD4-independent fusion and cell-to-cell transmission with CCR5-expressing cells, suggesting that loss of N173 increases SIVmac239 macrophage tropism by enhancing CD4-independent cell-to-cell transmission through CCR5-mediated fusion. This mechanism may facilitate escape of macrophage-tropic viruses from neutralizing antibodies, while promoting spreading infections by these viruses *in vivo.*

Acknowledgments

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CHAPTER 4: DISCUSSION

Summary

Macrophages are important target cells for HIV/SIV infection by serving as a long-lived reservoir for viral persistence in brain and other tissues, including bone marrow, lymph node, spleen, lung and gut. Macrophages play important roles in disease pathogenesis, particularly in the CNS, where macrophages are the predominant target cells and infection of macrophages causes neurological dysfunction. Macrophages become activated in mucosal and peripheral lymphoid tissues and the gastrointestinal tract of SIV-infected rhesus macaques, which contributes to systemic immune activation, a hallmark of HIV/SIV infection disease progression. HIV/SIV strains show different capacities to infect and replicate in macrophages. The main determinant for macrophage tropism is Env. To infect macrophages that express lower levels of CD4 on their cell surface, viruses frequently adopt Env conformations that increase exposure of CD4 and/or CCR5 binding sites, which can also increase neutralization sensitivity. To further understand mechanisms by which macrophage-tropic viruses spread infection while evading host neutralizing responses, we identified SIV Env determinants of macrophage tropism by isolating and characterizing Env clones from viruses in blood and brain *in vivo*, and a novel mechanism by which these Envs increase infection and replication in macrophages through enhancement of CD4-independent cell-to-cell transmission.

To isolate macrophage-tropic variants that may be responsible for establishing infection in macrophage-rich tissues and inducing pathogenesis in these tissues *in vivo*, we took advantage of existing studies and derived Env sequences in blood from early stage of infection from SIVmac251-infected rhesus macaques, as well as from brain, bone marrow, and lymph node from late stage of infection from SIVmac251-infected rhesus macaques that developed encephalitis. Sequence analysis identified a variant, desiv147c#4, from early infection that was closely related to the late variants and shared >98% sequence identity with the consensus sequence of variants from brain at late stage infection. desiv147c#4 mediated high levels of fusion, replicated efficiently in PBMC and macrophages, and was relatively neutralization

sensitive to serum from SIVmac251- or SIVmac239-infected animals. We identified an N-linked

glycosylation site, at position 173 in the V2 region, as a determinant of macrophage tropism.

Loss of N173 enhanced macrophage tropism of SIVmac239, while restoration of N173 in

SIVmac251 reduced macrophage tropism and enhanced neutralization resistance to

SIVmac251 or SIVmac239 antiserum. SIV Envs without the N173 glycosylation site mediated

enhanced CD4-independent cell-cell fusion and cell-to-cell transmission, compared to their

counterparts expressing N173. However, these CD4-independent phenotypes were only

detected in the context of cell-cell contact, as the viruses were not able to infect cells with no

CD4 as cell-free virus. Env interaction with CD4 was not significantly changed by the N173

mutation, suggesting that other mechanisms, potentially enhanced Env-CCR5 interaction, may

contribute to enhanced CD4-independent cell-cell fusion and cell-to-cell transmission. This

enhanced cell-to-cell transmission may facilitate spreading infection of macrophage-tropic

viruses *in vivo*, enabling these neutralization sensitive viruses to transmit while being protected

from the host humoral immune response.

Together, these results suggest that macrophage-tropic SIV variants can be present at

early stages of infection, and provide a novel mechanism that may help to explain spreading

infection of macrophage-tropic viruses *in vivo*, through enhanced CD4-independent cell-to-cell

transmission. These findings also provide mechanistic insights into the identity and roles of SIV

Env determinants in macrophage tropism, and may be helpful for establishing a new animal

model for studies on SIV pathogenesis.

Identification of macrophage-tropic SIV at early stage infection

In Chapter 2, we identified a SIV variant, desiv147c#4, in blood from early infection that

replicates at high levels in PBMC and macrophages. This is different from most SIV

macrophage-tropic clones, which were previously isolated from macrophage-rich tissues, such

as brain, lung, and spleen at late stage of infection (1-3), In HIV-infected individuals, isolates

from patients with advance disease stage show enhanced macrophage tropism compared to isolates from early stage (4, 5). Furthermore, previous studies show that most transmitted/founder viruses replicate poorly in macrophages (6-10). These previous findings resulted in the prevailing view that macrophage-tropic variants are rare or absent in early stage of infection and that HIV/SIV macrophage-tropism is generally associated with late stage infection (11). However, infected macrophages can be detected in brain as early as two weeks post-infection (12-14), suggesting that variants capable of infecting macrophages are present at early infection. In the present study, we found that desiv147c#4 shares over 98% sequence identity with the consensus sequence of viral variants from brain at late stage infection, suggesting that it may represent a macrophage-tropic variant at early stage of infection capable of entering and establishing infection in the brain tissue. Indeed, desiv147c#4 replicated at high levels in both rhesus PBMC and macrophages, and induced multinucleated giant cell formation in cultured macrophages. Together, these results suggest that macrophage-tropic variants are present during early stage infection and suggest that examining the infectivity and pathogenicity of desiv147c#4 in an animal model *in vivo* are important questions for future research.

New animal model for studying SIV pathogenesis

The high sequence homology between desiv147c#4 and gp120 variants in autopsy brain, bone marrow and lymph node, and the fact that these sequences were identified not only in clones from late stage of infection, but also in clones from acute infection, before the immune system is compromised, suggest that this unique clone is likely to be macrophage-tropic and pathogenic *in vivo*. It will therefore be interesting to test whether this macrophage-tropic clone, desiv147c#4, will be pathogenic when inoculated into rhesus macaques, and to determine whether it will infect macrophages in tissues including brain, lung, gut, etc. in vivo.. Our findings suggest this infectious molecular clone of SIV might be useful for establishing a new animal model for studies of macrophage-associated pathogenesis *in vivo*. An additional implication of

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our work is that due to its enhanced capacity to replicate in macrophages and spread infection via cell-cell transmission, desiv147c#4 may induce rapid disease progression associated with higher levels of immune activation (i.e. expression of T cell activation markers) and possibly preferential depletion of Th17 cells in gut mucosal tissues, which are two important indicators of HIV/SIV disease progression (15-17). Th17 cell differentiation is dependent on TGF-β and IL-6 in mice (18), or IL-23 and IL-1β in human (19). IL-6, IL-23 and IL-1β can all be secreted by macrophage; the mechanisms of Th17 cell differentiation in monkeys are not yet known. Therefore, an animal pathogenesis model in which macrophages are reproducibly infected *in vivo* will be useful for studying macrophage-associated disease phenotypes that cannot be studied in SIVmac239 infected animal models, such as whether macrophage infection affects Th17 cell differentiation and results in preferential depletion of Th17 cells. In addition, given the heterogeneity of most viral strains compared to viruses generated using a single infectious molecular clone, availability of a molecular clone for an *in vivo* animal model will be valuable for studying viral and host genetic determinants of interesting phenotypes and adaptive changes in the virus occurring during viral evolution over time and in response to immune selection.

CD4 and CCR5 interaction of macrophage-tropic SIV

In Chapter 3, viruses lack the N-linked glycosylation site N173 (SIVmac251 and SIVmac239 N173Q) induced high levels of cell fusion and multinucleated giant cell formation in cultured macrophages. Compared to CD4$^+$ T cells, macrophages express lower levels of CD4 on their cell surface (20-22). One mechanism for HIV/SIV to utilize the low levels of CD4 on macrophages for mediating fusion and entry involves increased affinity between Env and CD4 (20, 23-28). However, when we investigated the effect of N173 on Env interaction with CD4 by co-IP and BIACORE assays, purified SIVmac239 and SIVmac239 N173Q soluble gp120 did not show significant differences in binding to soluble CD4 (sCD4). In addition, the macrophage-tropic SIVmac251 and SIVmac239 N173Q were not more neutralization sensitive to sCD4,

compared to their counterparts expressing the N173 glycan (P.J. Yen and D. Gabuzda, unpublished data). These results suggest that mechanisms other than enhanced Env interaction with CD4 may contribute to the enhanced macrophage tropism.

These macrophage-tropic viruses were able to mediate cell fusion and cell-to-cell transmission with CD4-negative, CCR5-positive cells more efficiently than the non-macrophage-tropic viruses, suggesting that interaction with CCR5 may play a role in enhanced macrophage tropism. Increased Env-CCR5 interaction is one of the mechanisms used by HIV Env clones for mediating cell-cell fusion with cells expressing low CD4 or CCR5 (21, 29). This enhanced interaction between macrophage-tropic Env and CCR5 may facilitate cell-cell fusion, leading to efficient cell-to-cell transmission. This CD4-independent enhancement of Env-CCR5 interaction may be explained by a more exposed coreceptor binding site due to a higher propensity for macrophage-tropic Envs to sample the CD4-bound conformation. Unliganded Env is at a high energy state that may spontaneously sample Env conformations at different stages of fusion (30, 31). It is possible that macrophage-tropic Envs have a higher propensity to sample the CD4-bound conformation, and therefore facilitate interaction with CCR5 independent of CD4.

Roles of cell-to-cell transmission in macrophage tropism

Our results suggest that macrophage-tropic viruses can be present in early infection, when there is strong immune response. desiv147c#4 is relatively neutralization sensitive, which is one of the characters of transmitted/founder viruses (32, 33). No specific genetic sequence signatures have been identified for transmitted/founder viruses, and it is not clearly understood how these neutralization sensitive viruses spread and establish infection. Here we identified cell-to-cell transmission as a novel mechanism by which macrophage-tropic viruses can spread, while escaping from neutralizing antibodies *in vivo*. Cell-cell transmission involves the tight junction formed between the cell-cell contact, known as the virological synapse, a structure resembling that formed between antigen presenting cells (APC) and T cells, the immunological

synapse (34). Formation of the immunological synapse is dependent on the interaction between MHC molecules on the APC and its T cell receptor/CD3 complex on T cells, and also involves the interaction between intracellular adhesion molecule-1 (ICAM-1) on the APC and the lymphocyte function-associated adhesion molecule (LFA-1) on T cells. In the virological synapse, additional interaction is formed between Env on the donor cell and cellular receptors on the target cell. The macrophage-tropic clones characterized in this study were able to mediate CD4-independent cell-to-cell transmission, and therefore may be dependent more on Env interaction with the coreceptor, CCR5.

The CD4-independent SIV phenotypes were only detected in the context of cell-cell interaction, such as cell-cell fusion and cell-to-cell transmission, but not cell-free virus infection. Although macrophage-tropic viruses mediated enhanced cell-cell fusion and cell-to-cell transmission, they were not able to infect cell lines in the absence of CD4 in single-round infection assays. This may be reflect a transient and short-lived intermediate state of Env that is required for CD4-independent fusion. Unliganded Env might sample a CD4-bound conformation that facilitates interaction with CCR5 and CD4-independent fusion. This intermediate state of Env may be short-lived before it undergoes spontaneous and irreversible conformational changes. Therefore, it may not be sustained long enough to achieve cell-free virus infection. Cell-to-cell transmission is much more efficient than cell-free virus infection (35-37), ranging from 5 fold (37) to 18,000 fold (36), and viruses spread via cell-cell transmission are protected from inhibition of antibodies, anti-retroviral drugs, and cellular restriction factors (36, 38-42). Also, the kinetics of cell-to-cell transmission is much more rapid compared to those of cell-free virus infection (minutes versus hours), which may enable the transient state of Env to mediate CD4-independent cell-cell fusion. Furthermore, within minutes of cell-cell contact, virus on the donor cell (43), and cellular receptors on the target cell (44, 45) cluster to the junction of cell-cell contact. This results in a high concentration of CCR5 locally at the cell-cell contact, another

mechanism that may facilitate CD4-independent infection in the context of cell-to-cell transmission.

α4β7 does not appear to be involved in the N173 glycan dependent effects on macrophage tropism.

In addition to CD4 and CCR5 interaction, we also investigated the potential roles of other factors in enhanced macrophage tropism. Recent studies reported that some HIV Envs bind the integrin α4β7, and replication of some HIV strains in PBMC can be blocked by anti-α4β7 antibodies (46-48). Structural modeling of the V1V2 peptide on a scaffold and the natural ligand of α4β7, MAdCAM-1, shows similar β strands structure, with the critical binding residue of the putative α4β7 binding motif, D, locates at a similar orientation on the molecules (Figure 4.1). N173 is twenty amino acids away from D193. Therefore, we sought to investigate whether changes in N173 may affect the potential interaction between Env and α4β7. α4β7 expression was expressed on PBMC and MDM under our culture conditions (Figure. 4.2A). To test whether α4β7 is involved in N173-mediated effects on SIV macrophage tropism, we examined viral replication in PBMC and MDM in the presence of anti- α4β7 antibody. Anti-α4β7 did not block SIV replication in PBMC and MDM (Figure 4.2B and 4.2C). We also monitored α4β7 surface levels on infected PBMC. α4β7 was downregulated in SIV-infected PBMC on day 3 and day 4 post-infection (Figure 4.2D). The levels of α4β7 downregulation, however, were not associated with the presence or absence of N173. SIVmac251- and SIVmac251 T173N-infected PBMC showed similar levels of α4β7. Instead, α4β7 levels were inversely associated with SIV p27 levels in the supernatant of infected PBMC (Figure 4.2E), suggesting that SIV replication downregulated α4β7 expression in PBMC. Together, these results suggest that enhanced replication of SIV in macrophages does not depend on interaction with α4β7.
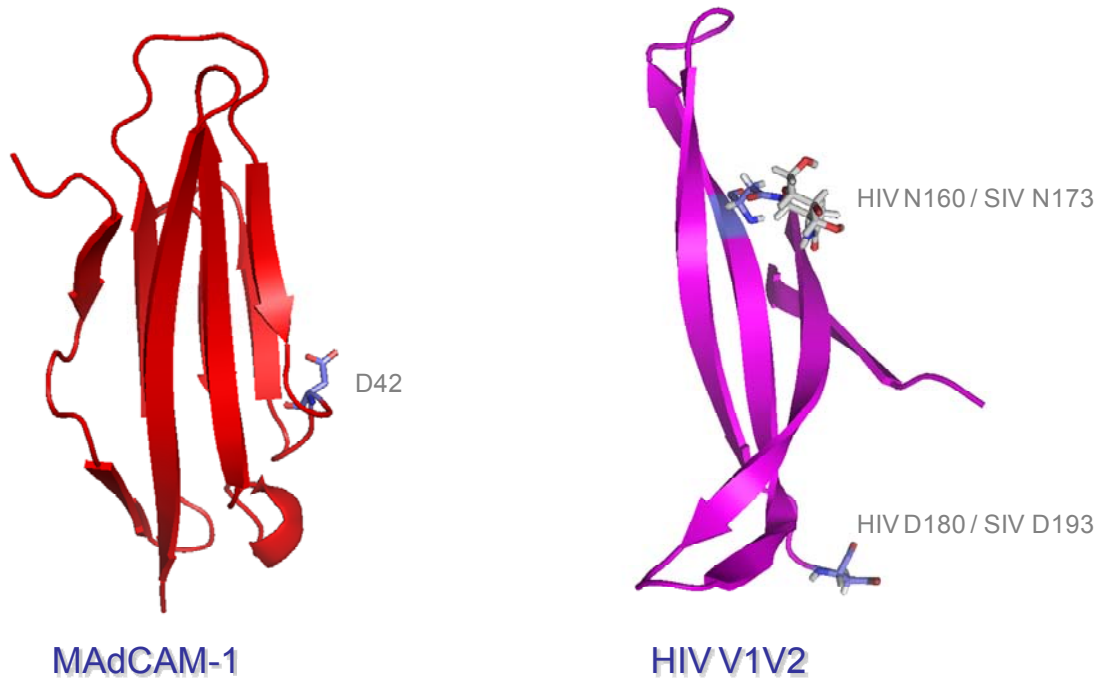
Figure 4.1 Asp residues critical for α4β7 interaction on MAdCAM-1 and HIV V1V2. MAdCAM-1, adapted from Tan et al, 1998 (49). HIV V1V2 peptide on a scaffold, adpapted from McLellan, 2011 (50).

Figure 4.2 α4β7 expression and SIV replication in PBMC and MDM. (A) α4β7 expression was increased in activated PBMC and MDM. α4β7 did not appear to be involved in the N173 glycan dependent effects on macrophage tropism. Anti-α4β7 antibody did not block SIV replication in PBMC (B) and MDM (C). SIV infection downregulated α4β7 expression in infected PBMC (D). Downregulation of α4β7 is associated with SIV replication in PBMC, but not with the presence or absence of the N173 glycan (E).

A
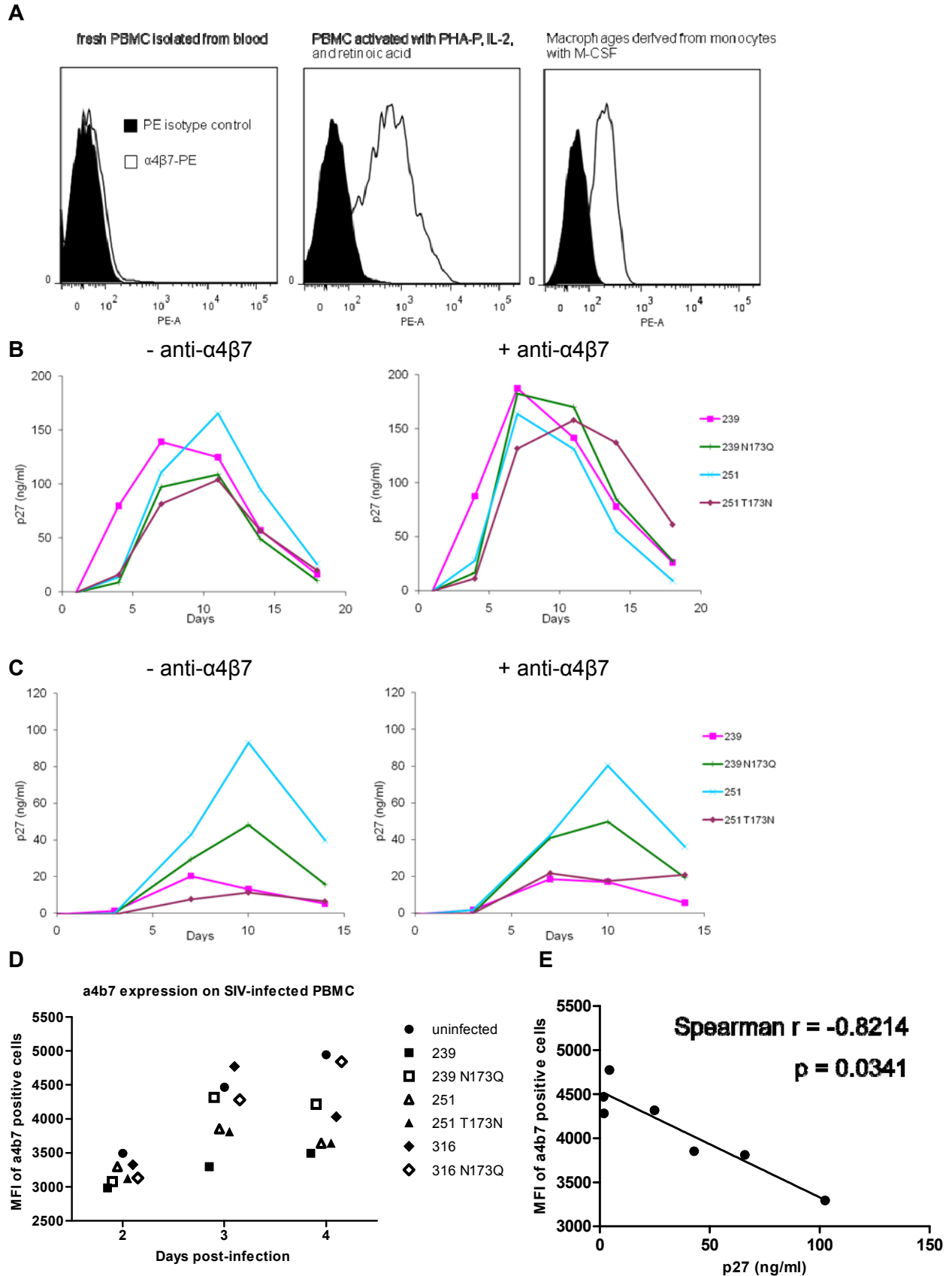

B

C

D

E

Figure 4.2 (continued)

N-linked glycosylation site as a novel determinant of cell-to-cell transmission

Our study identified an N-linked glycosylation site, at position 173 in the V2 region, as a novel determinant for cell-to-cell transmission of SIV. Only a few studies directly compared the effects of different Envs on cell-to-cell transmission of HIV (37, 38, 51), but no specific viral genetic determinants have been identified. Therefore, N173 represents a novel Env determinat of cell-to-cell transmission. N-linked glycosylation plays important roles in modulating Env function. The glycans cover a large surface of Env trimer, protecting the viral protein from recognition and neutralization of host antibody response. Glycans, as a post-translational modification, may also influence the processing and transport of Env (52). The transportation and localization of Env, in turn, may determine the location of viral assembly and release (53). It is particularly important for viruses generated in macrophages. In macrophages, HIV assembles in intracellular compartments. These compartments were initially characterized as late endosome or multivesicular body (54-57). However, recent studies found that they are specialized compartments connected with the plasma membrane (34, 58). The location of virus assembly and release might be critical in determining the efficiency of cell-to-cell transmission. Thus, it is possible that change in this glycosylation site may affect trafficking of the protein, which in turn affect sites for virion assembly and release, and as a result, affect cell-to-cell transmission. However, according to our results, loss of the N173 glycosylation site did not affect Env expression and processing. Together, these results imply that changes in the N173 glycosylation site do not significantly alter Env expression and processing, and suggest that examining virion distribution and localization are potential directions for future research.

Model for glycan modulates SIV macrophage tropism

N-linked glycosylation may also play a role in modulating the structure of Env. Changes in the N173 glycosylation site may have a direct impact on the orientation or structure of the V2

region. The V1/V2 regions themselves do not interact with CD4 directly, but may partially occlude the CD4 binding site, and modulate the interaction between Env and CD4 (59-62). Furthermore, the V1/V2 regions may interact with the V3 loop on the same or neighboring gp120 protomer (63-66), and thereby modulate the structure of V3 loop, and affect CCR5 binding. Our results of CD4 binding assays, including Biacore, co-Immunoprecipitation, and ELISA, showed no significant differences of binding to CD4 between SIVmac239 gp120 with or without the N173. Together with the CD4 independent phenotypes in cell-cell fusion and cell-to-cell transmission, we proposed a model in which glycan modulates SIV macrophage tropism by influencing CD4-independent cell-cell fusion and cell-cell transmission. Loss of N173 may affect structure and/or orientation of the V1V2 loop, which in turn may affect the V3 loop and the CCR5 binding site, thereby increase gp120 and CCR5 interaction and facilitate CD4-independent cell-cell fusion and transmission. Alternatively, unliganded SIV Env lacking N173 may have a higher propensity to sample a CD4-bound conformation, resulting in a more exposed CCR5 binding site that promotes CD4-independent fusion and cell-cell transmission.

Strain-specific effects of N173 on neutralization sensitivity

SIV macrophage tropism is generally associated with high neutralization sensitivity. Introduction of N173 into SIVmac251 reduced macrophage tropism and neutralization sensitivity. In contrast, loss of N173 on SIVmac239 enhanced macrophage tropism, but did not significantly alter neutralization sensitivity. Env trimers of different SIV strains exhibit distinct quaternary structures (67), which may provide clues to the strain-specific effects of N173 mutation on neutralization sensitivity. One major structural difference between SIVmac239 and SIV CP-MAC (another lab-adapted SIVmac251-derived strain) is the orientation of the V1V2 loop (Figure 4.3). The V1V2 loops of SIVmac239 are located at the center of the Env trimer, and the trimer is at a relatively "closed" state. On the other hand, SIV CP-MAC displays an open conformation, and the V1V2 loops are at an outward position of the trimer. It is possible that the N173 mutation

induces more conformational changes in the V1V2 structure/orientation in SIVmac251 than it does in SIVmac239; transforming SIVmac251 into a conformation that is more protected from antibody, while inducing modest changes in SIVmac239 that do not reduce neutralization resistance.
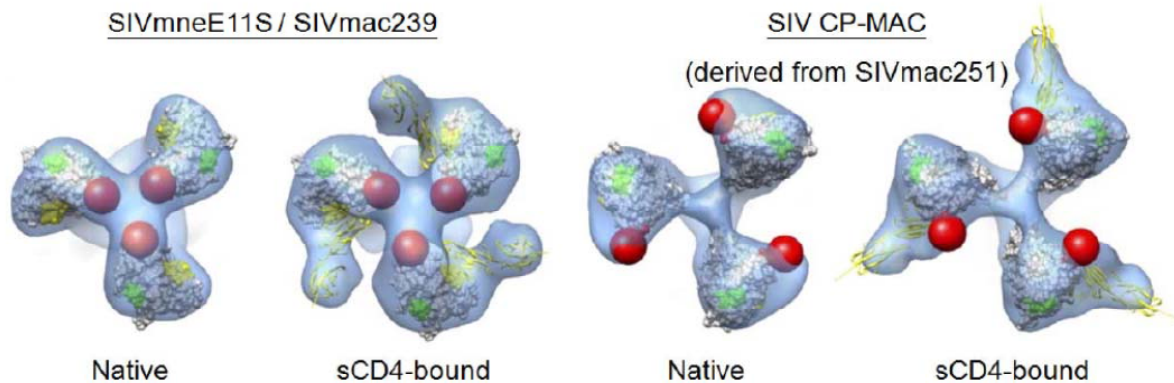

Figure 4.3 Strain-specific variation in Env trimer quaternary structure. CryoEM structures of Env trimers at native and sCD4-bound states (adapted from White et al, 2011 (67)). Structural variation between different SIV strains, including different orientation of the V1V2 loop (red sphere), may explain the strain-specific effects observed due to the N173 mutation.

Conclusion

HIV infection has been a global health issue that has resulted in more than 30 million deaths since its discovery more than 30 years ago (UNAIDS 2012). It is estimated that 34 million people were living with HIV as of 2011. Although ART is becoming more readily available, and is able to suppress viral load, restore and maintain immune system function, and prevent opportunistic infections, there is still no cure for the vast majority of patients. The major block to cure is the persistent viral reservoir. Compared to CD4$^+$ T cells, macrophages are less susceptible to cytopathic effect, and serve as long-lived viral reservoir after CD4$^+$ T cells get depleted. Furthermore, macrophages play important roles in other aspects of HIV/SIV pathogenesis, including neurological dysfunction. Understanding macrophage-associated pathogenesis would therefore provide insights into improving treatment strategies, and ultimately, facilitate control or cure of the disease.

In this thesis, we identified a new macrophage-tropic SIV variant that may be a prototype for strains that establish early infection and induce pathogenesis in macrophage-rich tissues such as brain and lung *in vivo*. This newly identified SIV molecular clone may be useful to establish a new animal model for studying macrophage-associated pathogenesis *in vivo*. We also identified and characterized SIV Env determinants of macrophage tropism. Studies of these determinants revealed novel mechanistic insights explaining how macrophage-tropic viruses may transmit and spread infection despite immune selection pressures. Overall, these studies provide further understanding of mechanisms by which HIV/SIV enhance macrophage tropism, and will promote future studies of macrophage-associated pathogenesis *in vivo*.

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