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Variation in the biological properties of HIV-1 R5 envelopes: implications of envelope structure, transmission and pathogenesis

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Abstract

HIV-1 R5 viruses predominantly use CCR5 as a coreceptor to infect $CD4^+$ T cells and macrophages. While R5 viruses generally infect $CD4^+$ T cells, research over the past few years has demonstrated that they vary extensively in their capacity to infect macrophages. Thus, R5 variants that are highly macrophage tropic have been detected in late disease and are prominent in brain tissue of subjects with neurological complications. Other R5 variants that are less sensitive to CCR5 antagonists and use CCR5 differently have also been identified in late disease. These latter variants have faster replication kinetics and may contribute to CD4 T-cell depletion. In addition, R5 viruses are highly variable in many other properties, including sensitivity to neutralizing antibodies and inhibitors that block HIV-1 entry into cells. Here, we review what is currently known about how HIV-1 R5 viruses vary in cell tropism and other properties, and discuss the implications of this variation on transmission, pathogenesis, therapy and vaccines.

Keywords

CCR5; CD4; $CD4^+$ T cell; envelope; HIV-1; macrophage; macrophage tropism; neuroAIDS; R5; receptor; tropism

HIV-1 was first described in 1983 [1] and CD4 identified as its receptor in 1984 [2,3]. It was quickly apparent that CD4 alone was not sufficient for infection and that a putative second determinant was required for HIV-1 entry. The identification of CXCR4 in 1996 by Ed Berger's group as the coreceptor for T-tropic HIV-1 strains [4] thus heralded a new era for HIV-1 receptor and cell tropism research. Well before 1996, it was clear that there were two types of HIV-1 that infected primary T cells or macrophages with different efficiencies. The first type

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became known as T-tropic or syncytium-inducing, owing to their capacity to infect and induce syncytia in T-cell lines as well as in primary T-cell cultures. The second group was termed nonsyncytium-inducing, macrophage tropic or M tropic, owing to their inability to induce syncytia in T-cell lines or peripheral blood mononuclear cells (PBMCs) and their capacity to infect primary macrophage cultures [5–7]. While CXCR4 was the coreceptor for T-tropic, syncytium-inducing strains, CCR5 was identified as the coreceptor for M-tropic, nonsyncytium-inducing strains [8–10] and HIV-1 viruses are now termed R5, X4 and R5×4, depending on their use of the two coreceptors [11]. R5 viruses efficiently infect primary $CD4^+$ memory T cells that express CCR5. For many, so-called R5 tropism became synonymous with macrophage tropism or M tropism. However, extensive research from our group and others has shown that R5 envelopes confer widely divergent abilities to infect primary macrophages, along with diverse sensitivities to entry inhibitors and neutralizing antibodies (nAbs) [12–16]. Here, we review the current understanding of the cell tropisms of HIV-1 R5 viruses and discuss their implications for transmission, pathogenesis and therapy.

Variation in macrophage tropism of HIV-1 R5 viruses

In 1987, Koyanagi *et al*. described JR-FL and JR-CSF as macrophage-tropic and non-macrophage-tropic R5 isolates from brain tissue and cerebrospinal fluid (CSF) [17]. We also reported that primary R5 viral isolates varied in their capacity to infect primary macrophages by at least 1000-fold [18] and differed in their capacity to exploit low CCR5 levels for infection [19]. More recently, Gorry *et al*. described an R5 isolate from brain tissue that was highly tropic and fusigenic for primary macrophages [16]. We and others have confirmed Gorry's observations and demonstrated that R5 envelopes derived by PCR directly from brain tissue are frequently highly tropic for macrophages [12–14]. By contrast, R5 envelopes from blood and immune tissue frequently infected primary macrophages very inefficiently [14]. Overall, different studies have revealed a wide spectrum of macrophage infectivity conferred by R5 envelopes, which covered 3–4 orders of magnitude [12,14–16,20,21] (Figure 1). Similarly, others have reported that R5 virus isolates from the blood of adult and pediatric AIDS subjects conferred an enhanced macro-phage tropism compared with isolates from earlier stages of disease [22–24]. These reports suggest that HIV-1 variants with an increased capacity to infect macrophages may evolve or become more prevalent late in disease.

There are a number of points to make regarding the aforementioned studies. First, our recent work has focused on evaluating the phenotypes of envelopes as opposed to full-length clones or viral isolates. Although it is likely that the envelope predominantly controls macrophage infectivity it should be remembered that other HIV-1 genes or variation in long terminal repeat promoter sequences might also have affects. Second, while we have PCR-amplified macrophage-tropic envelopes from brain tissue and at a lower frequency from blood, semen and immune tissues, it is not known which cell type(s) supported their replication *in vivo*. It seems very likely that highly macrophage-tropic envelopes from brain tissue were derived from brain macrophages or microglial cells. However, the origin of macrophage-tropic R5 envelopes present in plasma, PBMCs, semen or immune tissue in these studies is less clear. When tested, highly macrophage-tropic R5 viruses have generally infected primary $CD4^+$ T cells with at least similar efficiencies as non-macrophage-tropic viruses [14]. Therefore, we do not know whether macrophage-tropic envs evolved by adapting for replication in macrophages or whether other selective pressures *in vivo* (e.g., nAbs) played a role. For example, low levels of nAbs in the brain may allow envelopes with a more open conformation, higher CD4 affinity and increased macrophage tropism to evolve. This subject will be discussed in more detail later.

Determinants of R5 macrophage tropism & effects on envelope structure

The capacity of R5 envelopes to confer macrophage infection correlated with their ability to exploit low levels of cell surface CD4 for infection [12,14,21]. In addition, we noted that macrophage infectivity correlated with sensitivity to reagents that blocked glycoprotein (gp) 120–CD4 interactions [13], including soluble CD4 and an anti-CD4 monoclonal antibody (mAb; Q4120), as well as BMS-378806, a small molecule that targets a hydrophobic cavity on gp120 close to the CD4 binding site (CD4bs) [25]. There was also a strong trend in our studies and a significant correlation in a study by Dunfee *et al*. between macrophage infectivity and increasing sensitivity to the CD4bs mAb, b12 [13,26], which was consistent with an increased exposure of the CD4bs and the b12 epitope. Together, these different studies point to changes in the affinity of the envelope for CD4 that result (at least in part) from enhanced exposure of the CD4bs and proximal epitopes. The increase in env–CD4 affinity, thus, explains the capacity of macrophage-tropic env proteins to exploit low levels of cell surface CD4 on macrophages for infection [27–29].

Consistent with this interpretation, determinants of R5 macrophage tropism were mapped to gp120 residues within or proximal to the CD4bs. Thus, Dunfee *et al*. described a polymorphism in the C2 part of the CD4bs that contained an asparagine at residue 283 (N283) (Figure 2). N283 was highly represented in R5 envelopes from brain tissue of subjects with HIV-associated dementia (HAD) and conferred enhanced macrophage infectivity in *env* mutants [30]. Non-HAD subjects predominantly carried I283 or T283. In Dunfee's study, N283 was structurally modeled as conferring a tighter gp120–CD4 interaction by facilitating the formation of a hydrogen bond with Q40 on CD4. We also demonstrated a profound influence of N283 on macrophage infectivity [31]. However, we identified many env proteins where the presence or absence of N283 did not correlate with macrophage infectivity [14,31]. In our studies, we identified further determinants on the variable flanks of the CD4 binding loop (Figure 2) that influenced macrophage infectivity [31]. Residues on the N-terminal flank of the loop were adjacent to CD4 contact residues and probably affect the exposure of this site on the trimeric envelope (Figure 2). In addition, Sterjovski reported that a potential glycosylation site (N362) on the same flank increased the fusigenicity of envelopes but did not examine macrophage infectivity [32]. Consistent with these observations, a recent study by Wu *et al*. indicated that the variable N-terminal flank of the CD4-binding loop plays a major role in resistance to neutralization by the CD4bs mAb, b12 [33].

In our study, determinants in the V3 loop (residues 308 and 332) also contributed to full macrophage tropism and to modulate sensitivity to entry inhibitors that target CD4–gp120 interactions. Substitutions at these sites were previously found in the brain of HAD subjects [34] and in CSF-derived env [35,36]. The location of the V3 loop on the ligand-free trimer is not known but presumably must sit close enough to the CD4bs to influence its exposure as suggested in an early study by Wyatt *et al*. [37].

Finally, the N-glycan at N386 at the N-terminal end of the V4 loop is also proximal to the CD4-binding loop (Figure 2). The presence or absence of this glycan has been demonstrated to affect macrophage tropism [31,38] and sensitivity to CD4bs mAb b12 [26,39,40], indicating that it acts to protect CD4 contact residues (most likely those in the proximal CD4 binding loop). Our recent work indicates that determinants on the N-terminal flank of the CD4-binding loop act by shifting the orientation of proximal glycans potentially protecting or exposing loop residues that contact CD4 [DUENAS-DECAMP M, UNPUBLISHED OBSERVATION.].

The effect of R5 macrophage tropism on other envelope properties

As discussed, variation in macrophage tropism of R5 envelopes results from changes in residues that alter gp120–CD4 interactions. This directly affects the sensitivity of envelopes

to reagents that block gp120–CD4 interactions, including soluble CD4 and an anti-CD4 mAb (Q4120) [13]. Variation in macrophage tropism also directly impacts on the sensitivity to nAbs that target the CD4bs, including b12, consistent with an increased exposure of this site and over-lapping neutralization epitopes [13,26]. In addition, we also noted a trend toward decreased sensitivity to the glycan-specific mAb, 2G12 [13], probably caused by (at least in part) the loss of critical glycan sites that may contribute to the exposure of the CD4bs in macrophage-tropic env proteins. Nevertheless, the focus of these determinants on env–CD4 interactions was further highlighted by the lack of correlation between macrophage tropism and sensitivity to reagents that targeted other envelope sites or functions and blocked other stages of entry. These included inhibitors of env–CCR5 interactions and gp41 conformational changes [13]. Finally, there was no evidence for increased exposure of CD4i epitopes, as indicated by the lack of sensitivity to 17b, a mAb that recognizes the conserved part of the coreceptor binding site [13]. Together, these observations point to a focused re-architecturing of the residues proximal to the CD4bs that affect the exposure of CD4 contact residues without dramatically affecting other envelopes sites or properties.

Whether macrophage tropism affects sensitivity to nAbs in HIV-1^+ human sera is under study. If modulation of macrophage tropism is relatively restricted to the CD4bs, there may only be limited effects on sensitivity to nAbs that target other sites on the envelope. It might be expected that the more macrophage-tropic envelopes will have enhanced sensitivity to CD4bs nAbs in human sera. However, such nAbs are relatively infrequent among HIV-1^+ sera, while the presence of nAbs directed to other conserved neutralization targets may obscure their effect [41–44].

Other envelope properties that affect receptor use & tropism

While our group has focused on macrophage tropism and CD4 use of R5 envelopes, others have investigated variation in the use of CCR5. Several studies have indicated that CCR5 use varied amongst different HIV-1 strains and evolved during the course of infection [45,46]. Altered use of CCR5 was revealed by changes in the capacity of R5 viral isolates to infect indicator cells expressing chimeric CCR5/CXCR4 receptors, with late disease isolates able to use a broader range of chimeras compared with early isolates [47–49]. These changes were observed in the absence of a switch to CXCR4 use. Repits *et al*. also described equivalent R5 variants from late-stage disease with an increased positive charge on the surface of gp120 [45,46] and fewer potential *N*-linked glycosylation sites [50]. The increased charge was focused in the variable loops (but not V3) and, again, was not associated with a switch to CXCR4 use. Such variants carried an enhanced replicative capacity and were more resistant to entry inhibitors including RANTES/CCL5 and CCR5 antagonists compared with viruses from earlier disease stages [45,46,51,52]. The increased positive charge of gp120 enhances the attachment of virions to cell surfaces that are negatively charged [46]. However, the precise mechanisms that affect CCR5 use are less clear, although changes in how gp120 interacts with the second extracellular loop were proposed [48].

For the envelopes that we studied, overall gp120 charge also correlated with sensitivity to CCR5 antagonists, but not with sensitivity to other entry inhibitors (e.g., soluble CD4 and T20). However, neither gp120 charge nor sensitivity to CCR5 antagonists correlated with macrophage infectivity or with sensitivity to inhibitors of gp120–CD4 interactions. Thus, the evolution of gp120 charge in R5 isolates is independent and apparently unrelated to the variation in macrophage tropism described here.

There are also viral isolates and envelopes that confer use of low CCR5 levels for infection. We and others have reported that a subset of highly macrophage-tropic R5 isolates or envelopes from the brain that can exploit low CD4 levels for infection, can also infect cells via low levels

of CCR5 [12,16]. Nonetheless, we have also noted that a fraction of non-macrophage-tropic env proteins can also use low CCR5 levels for infection [PETERS PJ, UNPUBLISHED OBSERVATION]. The mechanisms and env determinants that confer low CCR5 use are not currently known. Low CCR5 use may enable such variants to target cells that express low amounts of CCR5, including dendritic cell (DC) lineage cells and particular CD4 T-cell populations (e.g., central memory cells) [53].

The possible implications of these different R5 envelope properties for transmission and pathogenesis are discussed later.

Environmental pressures *in vivo* that select for different R5 envelope tropisms

The selective pressures that modulate the properties of R5 envelopes *in vivo* are poorly understood. The simple view would be that macrophage-tropic variants have adapted for replication in macrophages while non-macrophage-tropic variants have been selected for T-cell replication. However, R5 viruses do not readily segregate into macrophage-tropic and non-macrophage-tropic groups. Instead there is a spectrum in the extent that different R5 viruses or envelopes confer macrophage infection (Figure 1). Moreover, all R5 envelopes that we tested conferred infection of primary phytohemagglutinin/IL-2 stimulated $CD4^+$ T cells or PBMCs [14]. Nevertheless, highly macrophage-tropic variants in the brain have probably adapted for efficient infection of macrophages and microglial cells present there. However, if all R5 variants can infect T cells anyway, what then selects for non-macrophage-tropic variants that interact less efficiently with CD4? It is likely that nAbs select for envelopes that have evolved to protect critical functional sites (e.g., the CD4bs). Such variants may be compromised in their capacity to bind CD4 but will not be as severely affected during infection of $CD4^+$ T cells that express high levels of CD4. By contrast, the brain is protected by the blood–brain barrier, which usually excludes antibodies [54–56]. Replication in this environment may select for envelopes with a more open conformation that can interact efficiently with CD4 and infect macrophages or microglia that carry low levels of CD4. This scenario is supported by the increased sensitivity of highly macrophage-tropic brain-derived env proteins to neutralization by the CD4bs mAb, b12 [13,26]. On the other hand, non-macrophage-tropic env proteins have been detected early in infection when nAbs are likely to be low or absent [57,58]. Thus, during this early stage of replication there would not be a selection pressure imposed by nAbs to prevent virus env proteins from evolving a more open conformation and allowing an efficient interaction with CD4. Thus, the selective pressures that prevent these early variants from evolving a more open envelope consistent with a macrophage-tropic phenotype are not understood.

Do different HIV-1 clades confer distinct or unique R5 envelope properties?

HIV-1 is highly variable and has been categorized into different groups and subtypes or clades. HIV-1 groups M, N and O represent three separate zoonotic transfers from chimpanzees or gorillas [59,60]. Group M has spread pandemically and has been further divided into subtypes or clades along with several circulating recombinant forms. The vast majority of research on HIV receptor use and tropism has been performed using clade B viral isolates or molecularly cloned viruses or *env* genes. Although it is clear that R5 envelopes from other clades or circulating recombinant forms confer similar properties to clade B, some important distinctions are emerging. It is well established that variants using CXCR4 evolve less frequently in clade C infections compared with clade B [61–65], while in clade D, such variants may be even more frequent [66]. Clade C envelopes also carry a relatively conserved variable V3 loop (compared with clade B) that frequently lacks the conserved *N*-linked glycosylation site at the N-terminus, and which is usually present in other clades [67]. Clade C R5 isolates were also reported to infect $CD34^+$ progenitor cells much more efficiently than clade B viruses, and were associated

with anemia in Africa [68]. The extent to which HIV-1 infects $CD34^+$ progenitors has become controversial, with some suggesting limited infection for clade B viruses [68,69], despite detection of both CD4 and CCR5 [68,70]. However, a recent study has demonstrated infection of $CD34^+$ progenitor cells *in vitro* using a reporter pseudovirus carrying an R5×4 clade B envelope. The same study also showed infection *in vivo* in (presumably) clade B subjects and the establishment of a latent viral reservoir in such cells [71]. Thus, the extent that clade B, C and other non-B clades infect $CD34^+$ progenitor cells requires further evaluation. Nonetheless, the distinct properties of clade C envelopes that are emerging may have been factors that contributed to the rapid spread of this clade, which is the most prevalent worldwide [72].

Whether different HIV-1 clades differ in their replicative fitness is currently unclear. Such differences (if they exist) could potentially have a profound effect on their pathogenic potential and rate of spread within a population. Thus, a recent study indicated that African clade C isolates were less fit for replication in PBMCs compared with other group M clades (including A, B and D), but competed well in cervical, penile and rectal explant cultures, consistent with efficient transmission [73]. However, another study reported that Indian clade C viruses conferred a greater replicative capacity in PBMCs compared with clade A [74]. It was also reported that heterosexually transmitted clade A [75] and C viruses [76] carried gp120s with a shorter sequence length, fewer glycosylation sites and increased neutralization sensitivity. Such envelope characteristics may confer an advantage for transmission and seemed to contrast with heterosexual [75] or male-to-male [77] clade B transmission, where such env proteins were not detected. These observations imply that the *env* genes of different clades selected at transmission may have distinct properties or that the 'transmission efficiency' of *env* genes can be conferred by different gp120 modifications in different clades. However, more recent data regarding mainly male-to-male transmission of clade B viruses indicate a weak relationship between transmitted viruses and shorter gp120s with fewer glycosylation sites [78].

Interestingly, the vast majority of clade B R5 envelopes that have been amplified directly from patient material without culture can use CCR3 as well as CCR5 on $CD4^+$ indicator cells *in vitro* [12,79]. The use of CCR3 is intriguing and it is possible that there are important $CCR3^+$ cell types *in vivo* that are targeted by HIV-1. For example, $CD4^+$ Th2 T cells express CCR3 [80], while there is evidence that CCR3 may be an important coreceptor for the infection of microglial cells in the brain [81,82]. Nonetheless, infection of PBMCs *in vitro* by R5R3 envelopes is usually blocked entirely by CCR5 antagonists and a role for CCR3 *in vivo* has not yet been established [83]. Interestingly, not all R5 envelopes from different clades exhibit the R5R3 phenotype. Thus, clades A and C R5 envelopes frequently use formyl peptide receptor-like (FPRL)1 as a coreceptor in addition to CCR5, while clade D R5 envelopes use neither CCR3 nor FPRL1 [84]. Similar to CCR3, the significance of FPRL1 use *in vivo* remains to be proven. If CCR3 and FPRL1 are not important coreceptors *in vivo*, why do some R5 envelopes carry the capacity to use them? One possible explanation is that these coreceptors share some structural determinant with CCR5, which is critical for coreceptor function [85].

As more research is performed on non-clade B envelopes, other differences in properties are likely to be identified. Nonetheless, it is likely that the majority of data obtained for clade B R5 envelopes will be highly relevant for other clades.

Does variation in R5 envelope properties affect transmission?

The discovery of a polymorphism in the CCR5 coding region revealed that HIV-1 R5 strains are predominantly transmitted. Thus, individuals homozygous for a mutant form of CCR5 with a 32 bp deletion (Δ32 CCR5) do not express a functional CCR5 receptor and are substantially protected from infection via sexual transmission [86,87]. Other studies on homozygous Δ32/Δ32 CCR5 subjects have also indicated protection via other routes of infection (e.g., via blood

contact [88] or mother-to-child transmission [89]). The protection from HIV-1 infection conferred by the Δ32/Δ32 CCR5 genotype indicates that viruses using CXCR4 or other coreceptors rarely transmit. CXCR4-using strains have less opportunity for transmission since R5 strains predominate in most infected individuals until late in disease. Several studies also indicate that R5 viruses predominate in semen, despite the fact that CXCR4-using variants can sometimes be detected [90–93]. Extensive stromal cell-derived factor-1 expression by mucosal epithelia may also act as a barrier to X4 viruses [94]. Interestingly, all CXCR4-using viruses, including R5×4 viruses that can use CCR5, are selected against at transmission. However, these dual-tropic R5×4 strainsare frequently sensitive to inhibition by CCR5 chemokines when CXCR4 is absent. Such sensitivity may be sufficient for β-chemokines to prevent R5×4 transmitting via a CCR5 route. These, along with and a series of other potential advantages for R5 viruses [95], may combine to restrict transmission of CXCR4-using viruses.

Whether the different R5 env tropisms described previously affect HIV transmission has not yet been fully addressed. Variants that can exploit low CD4 or CCR5 for infection may have an advantage if cellular targets expressing low amounts of these receptors are critical for transmission. This paradigm will apply for any of the routes of transmissions. Several reviews have already extensively covered mechanisms of HIV-1 transmission [96–99]. This article will mainly focus on heterosexual male-to-female transmission. Sexual transmission of R5 viruses is very inefficient, with a transmission rate of one in 1000 exposures frequently cited for both male-to-female and female-to-male transmission [100–104]. High viral loads in the acute phase and in late disease, the presence of sexually transmitted infections and lack of male circumcision are factors that increase transmission to rates as high as one in 10–100 exposures [104]. The presence of a tight bottleneck at some stage in trans mission is supported by the fact that usually only a single variant is transmitted sexually. For example, Abrahams *et al*. recently reported that in 171 clade B and C transmission events, a single variant was transmitted on 78% of occasions [105]. However, recent studies have shown more frequent infection by more than one, and sometimes by several, strains in men who have sex with men and intravenous drug users [106,107].

The bottleneck is almost certainly in the recipient rather than the donor since viral RNA or DNA is readily detected in semen [90,108] and vaginal fluids of many infected male and female subjects, respectively [108–115]. In addition, we have observed a variety of R5 phenotypes in semen [14], while this has not been extensively investigated for vaginal fluids. Therefore, it seems probable that the very different properties of R5 envelopes that impact on the use of CD4 and CCR5 and affect replicative capacity will have an impact on the ability of HIV-1 to overcome the bottleneck and establish infection.

Cell types important for transmission

The cell types that are first targeted by HIV-1 following transmission have not been unambiguously defined (reviewed in [98,99]). For sexual transmission, HIV must penetrate either the stratified epithelia of the vagina, ectocervix or the penis, or, alternatively, the single columnar epithelial cell layers of the endocervix or the rectum. For transmission across mucosa that are protected by a stratified epithelium, there is evidence for infection of Langerhan's cells (LCs), $CD4^+$ T cells and macrophages, as well as capture and transfer of virions by LCs to T cells (see reviews [96,98]). LCs penetrate the cell layers of the stratified epithelium and extend dendrites through the extracellular spaces. They are the closest potentially susceptible cells to the surface of the vagina or penis [98,116,117]. LCs could become infected or capture virions via cell surface langerin (a C-type lectin) or other mechanisms [118]. Following maturation and migration into the stroma or to the regional lymph nodes, LCs may then transfer virions to susceptible T cells while presenting antigen via immunological synapses [96]. Although this is a compelling hypothesis, recent studies have suggested that capture of virions by langerin

on immature LCs results in their degradation rather than infection or transinfection of T cells [119]. Nonetheless, activated LCs were reported to be sensitive to infection or able to confer transinfection [96,120], indicating that the LC activation state may be critical in determining whether transmission occurs or fails. In addition, it has been difficult to clearly show productive infection of cervico-vaginal LCs following infection of ectocervical explant cultures [118]. By contrast, LCs purified from skin or present in abraded skin explants are permissive for HIV-1 R5 viruses *in vitro* [121], and LCs in the skin of infected subjects are clearly infected with HIV-1 [122,123]. Together, these observations indicate that at least some LC populations are permissive to HIV-1. In the simian immunodeficiency virus macaque (SIVmac) model, LCs in the genital mucosa of macaques were reported to be infected within 24 h of exposure to SIVmac [124]. Overall, the role of LCs in transmission remains unclear and requires further evaluation.

$CD4^+$ memory T cells that localize in the lamina propria at sites of transmission are likely to play a major role at some stage during transmission. Under conditions of immune activation (e.g., sexually transmitted infections), $CD4^+$ memory T cells can penetrate the epithelial layers close to the surface and become targets for infection in cultures of intact vaginal epithelial tissue [97,118,125]. In this situation, such cells could be the first infected, resulting in a localized focus of replication and dispersal of virus to sites in the underlying stroma. Gupta *et al*. provided evidence that $CD4^+$ T cells were initial targets for infection in cervical explant cultures when the barrier was maintained [126], while others have found infected T cells within 24 h of infection of nonbarrier cervical explant cultures [127,128].

Macrophages and DC-$SIGN^+$ DC cells in the submucosa below the stratified epithelium are probably not close enough to the surface of the stratified epithelium to be the first cells infected during transmission via the vagina/ectocervix or penis. Nonetheless, these cell types may play other roles in establishing and spreading the infection in a new host. Since macrophages are long lived, they could help establish a sustained infection. Greenhead used ectocervical or vaginal explants that were infected with-out a barrier and reported that the majority of infected cells were $CD68^+$ macrophages [127]. Macrophages also became infected in a similar study of ectocervical explant infection by Cummins *et al*. [129].

The DC-$SIGN^+$ DCs may act to spread an initial focus of HIV infection from sites of transmission to regional lymph nodes [99]. DCs support transinfection of $CD4^+$ T cells by virions captured via glycosaminoglycans or other mechanisms (e.g., DC-SIGN) [130–133]. Myeloid DCs appear to carry an early postentry restriction to HIV but may still support a low-level productive infection [133]. Regardless, HIV-1 envelopes that confer more efficient entry into such cells will have a better chance of overcoming this restriction to set-up a productive infection.

Langerhan’s cells are present in mucosa that have stratified epithelia on their surfaces, but are largely absent at mucosal sites with a single-cell barrier [134]. Such mucosa include the endocervix and rectum, which are protected by a single layer of columnar epithelial cells [134]. Submucosal macrophages and DCs may play a more significant role in transmission across mucosa at these sites, although macrophages in the intestine were reported to be resistant to HIV-1 infection [135,136].

Receptor levels & transmission

The amount of CD4 and CCR5 on the surface of cells that are potential targets during transmission is likely to have a profound effect on whether they can be infected or not. While macrophages express low levels of CD4, LCs carry low amounts of both CD4 and CCR5. If infection of these cell types is required for transmission, then virus strains that can exploit these low receptor levels will be required. However, examination of the tropism properties of HIV-1

viruses and their envelopes amplified from the plasma of acutely infected individuals has not yet indicated a role for macrophages. Thus, so-called 'founder' viruses did not efficiently infect macro phages [137]. These viruses were derived from consensus sequences of entire genomes amplified by PCR shortly after transmission and are predicted to represent those of the transmitted founder virus [137]. Similarly, clade B envelopes from the acute or early stages of infection were demonstrated to confer variable and, at best, modest levels of infectivity for macrophages [57]. These latter env proteins were not 'corrected' to consensus founder sequences and it is not known whether they accurately represent the viral strains that were transmitted. Nonetheless, these studies strongly indicate that macrophage tropism is not important for HIV-1 transmission. Since DCs, including LCs, express low levels of CD4 and CCR5, it is likely that these observations also rule out both cell types as important targets for infection during transmission, although expression of surface molecules (e.g., DC-SIGN) that efficiently capture virions could potentially compensate for low receptor levels. Nonetheless, these observations point towards $CD4^+$ T cells as the critical cell type targeted during transmission. The bottleneck could then be explained by the requirement of the transmitting virus (or infected donor cell) to find a susceptible $CD4^+$ T cell for infection. However, whether such T cells are the first cell contacted by the transmitting virus or LCs (that are not infected) play a role in transferring virus to T cells remains to be determined. It is also possible that transmitting viruses carry other unique properties (e.g., enhanced CCR5 use or an increase in replicative capacity) that may increase the likelihood of transmission.

Influence of R5 envelope variation on pathogenesis

While R5 viruses are predominantly transmitted, CXCR4-using (X4 and R5×4) variants can be isolated from at least 50% of AIDS patients in clade B infections and confer a more rapid loss of $CD4^+$ T cells and faster disease progression [5,51,138–140]. CXCR4 is more widely expressed on different $CD4^+$ T cell populations (compared with CCR5) and CXCR4-using viruses have a broader T-cell tropism [141,142]. Nevertheless, CD4 depletion and AIDS occur in patients from whom only CCR5-using viruses can be isolated [65,143]. This is particularly apparent in clade C infections, where CXCR4-using variants are detected in far fewer individuals [61–65] and AIDS presumably occurs in the absence of CXCR4-using variants, caused directly by CCR5-using R5 viruses. The role of the switch from CCR5 to CXCR4 use for disease progression has been covered by previous reviews [144–147] and will not be addressed in detail here.

HIV-1 infection and destruction of the $CD4^+$ T-helper population is the primary cause of the resulting immunodeficiency. In the first few weeks of infection, HIV-1 R5 viruses decimate the CCR5 $CD4^+$ memory cell population present at the mucosa, including in the intestine [148–151]. Whether R5 viruses with distinct envelope properties impact on this early loss of the memory cell population or on subsequent systemic pathogenic outcomes is unclear.

R5 variants with an enhanced macrophage tropism [22–24] or alternatively with altered CCR5 use [45,46,51,52] have been detected in late disease and may instigate the late decline of $CD4^+$ T cells in the absence of CXCR4-using variants or represent a stage in R5 envelope evolution prior to the emergence of CXCR4-using variants. Enhanced R5 macrophage tropism is associated with an increased affinity of gp120 for CD4, which may increase the efficiency of entry for all $CD4^+$ cell types, including T cells. R5 variants with an increased gp120 charge and altered CCR5 use are associated with faster replication kinetics [45,46] and it is easy to envisage how the emergence of such variants could precipitate a faster decline in $CD4^+$ T cells during late disease. However, this is not proven.

Furthermore, it is not known whether R5 variants detected in the blood along with an enhanced macrophage tropism are predominantly replicating in $CD4^+$ T cells, or whether they have been

transported to the blood from tissues where macrophages are major reservoirs of viral infection (e.g., the brain). For example, as demonstrated for SIV, when $CD4^+$ T cells are depleted in late disease, macrophage-tropic R5 variants derived from macrophages in immune tissue may predominate in the blood [152].

It is also worth considering the $CD4^+$ central memory T cell (Tcm) population. Such cells express substantially lower levels of CCR5 compared with effector memory cells and survive the acute phase of infection [153,154]. It has been suggested that the preservation of this cell population allows for sufficient $CD4^+$ effector memory cells to be generated during the asymptomatic phase of disease following the decimation of effector memory cells at mucosal sites early in infection [53,153]. Nevertheless, disease progression is associated with the eventual loss of the Tcm population [53,153] and it is tempting to speculate that their loss is connected to the emergence of putative R5 variants capable of efficiently infecting cells via low levels of CCR5. Consistent with this possibility, Groot *et al.* reported that Tcms were less susceptible to R5 HIV-1 compared with effector memory cells [155], while Heeregrave *et al*. reported that HIV-1 sequences recovered from Tcms were compartmentalized compared with those from naive or effector cells [156].

Highly macrophage-tropic R5 viruses play a clearer role in neuropathogenesis (see reviews [157–159]). In the absence of therapy, up to approximately 30% of AIDS patients suffer neurological complications, including HAD [157]. The brain is colonized by HIV-1 early in infection [160,161], but it is then difficult to detect virus there during the asymptomatic phase of infection [162–165]. As disease progresses, the $CD16^+$ pool of monocytes detected in blood become increasingly activated and expanded [166,167], and this may predispose monocytes for traffic through the blood–brain barrier [168]. Chemokines (e.g., MCP-1), produced by perivascular macrophages in the brain, also act to attract blood monocytes, further exacerbating the situation [169]. Some of the $CD16^+$ monocytes recruited through the blood–brain barrier are likely to be infected and will take HIV-1 in with them [170,171]. This scenario is consistent with the highly macrophage-tropic viruses or envelopes detected in brain tissue of AIDS subjects [12,14,16,21]. As previously discussed, perivascular macrophages and microglia are the main targets for infection [157]. However, there is also evidence that HIV-1 infects astrocytes in the brain *in vivo* [172–183], although this has been controversial [184]. Churchill *et al*. used laser capture microscopy to isolate immunostained astrocytes and multinucleated giant cells (MNGCs; fused macrophages) before PCR-amplifying V3 loop sequences. Although only two subjects were analyzed, it was striking that V3 sequences from astrocytes were distinct from those derived from proximal MNGCs [175]. This observation is consistent with infection of astrocytes by distinct HIV-1 variants rather than their contamination with fragments of infected MNGCs during their preparation for laser capture microscopy [184]. Astrocyte infection is curious since these cells do not express CD4, although CCR5 expression has been detected *in situ* in rhesus macaques [185]. The mechanism of HIV entry into astrocytes is therefore unclear, although cell surface receptors, including the mannose receptor, galactocerebroside and undefined receptors, have been demonstrated to bind gp120 and virions [186–188]. Whether any of these receptors can explain astrocyte infection by substituting for CD4 so that CCR5 can act as a coreceptor is not known.

Highly macrophage-tropic R5 variants present in the brain probably play a major role in instigating the pathological processes that result in dementia and other neurological dysfunctions. Replication of HIV-1 R5 viruses in the brain could also result in the emergence of variants that are resistant to highly active antiretroviral therapy drugs that penetrate the blood–brain barrier inefficiently. The precise mechanisms that result in neuropathogenesis remain unclear. HIV-1 does not usually infect neurons, despite the fact that there is frequently substantial loss of such cells [157,189]. Neuropathology is closely associated with the accumulation of activated monocyte-derived macrophages in the perivascular regions and

generalized immune activation in the brain [157]. A proportion of the macrophages are infected with HIV-1 and some fuse with uninfected macrophages to form MNGCs, which are a hallmark of dementia [157]. Infected and uninfected macrophages that are activated produce an array of cellular and viral proteins. These proteins are believed to further activate bystander cells, including astrocytes, and thus contribute to general immune activation in the brain [157]. Such products may also cause apoptosis of neurons and astrocytes, which is associated with dementia [157].

Although it is clear that R5 viruses present in the brain are highly macrophage tropic, it is not known whether neurovirulent variants are associated with neuropathogenesis. In the SIV/rhesus macaque model, macrophage-tropic variants were described that differed drastically in neurovirulence outcomes and may be a precedent for HIV-1 in the brain [190,191]. How the enhanced macrophage tropism that we and others have described relates to neurovirulence is also unknown. This is because the viral determinants of neurovirulence are not known and may include nonenvelope genes (e.g., *nef*) [190]. Many studies have reported that the HIV-1 envelope has various toxic affects on different brain cells *in vitro* in many different assays [157]. However, it remains unclear how these *in vitro* assays of toxicity relate to the neuropathogenic events *in vivo*. Currently, there are no suitable animal models to ascertain whether a particular HIV-1 isolate or envelope can cause or even influence neuropathogenesis.

Does variation in R5 virus tropism & receptor use affect therapy with CCR5 antagonists?

Maraviroc, a CCR5 antagonist, is a small organic molecule (molecular weight: 513.67) that is licensed for use in therapy throughout the developed world. *In vitro* selection experiments have indicated that R5 variants found in the asymptomatic phase will be highly sensitive and unlikely to readily evolve escape variants [192]. Maraviroc-resistant viruses selected *in vitro* had adapted to use CCR5 bound by the drug rather than switching to use CXCR4 [192,193]. *In vitro* escape was not straightforward, requiring multiple passages in increasing concentration of maraviroc to generate the set of mutations required for resistance [192,194]. Currently, maraviroc is not being used as a first line of defense owing to the superior effectiveness and pharmacological profile of other drugs. Nevertheless, an important niche has been found for maraviroc as a salvage or change therapy for individuals who develop resistance variants or cannot tolerate first-line therapies [195]. However, this means maraviroc is used at later stages of disease compared with the first-line drugs and there are obvious issues with this strategy. CXCR4-using variants are more likely to emerge at later stages of infection and their presence would probably render treatment ineffective. It is also possible that CCR5 blockade may actually select for the more virulent CXCR4-using variants if they are present at low levels, as has been suggested in some of the maraviroc trials [196,197]. This possibility means that subjects who are candidates for maraviroc therapy need to be screened for the presence of CXCR4-using variants before treatment can begin [198,199]. This process is expensive and may further weigh therapeutic choices against maraviroc [196]. Finally, the detection of R5 variants with decreased sensitivity to CCR5 antagonists late in disease [45,46,51,192] is also a consideration for the use of maraviroc at this stage, as they may represent viral strains that have a head start in evolving resistance. Nevertheless, maraviroc and other candidate CCR5 antagonists are potent inhibitors of CCR5-mediated entry. Such reagents will be used in combination with other HIV inhibitors, which will contribute to the prevention of viral replication and greatly reduce the likelihood of the emergence of resistant variants that retain CCR5 use or have switched to CXCR4.

Conclusion

R5 viruses are predominantly transmitted and cause AIDS even in the absence of CXCR4-using variants. Over 25 years after the identification of HIV-1, we are still discovering the extent of variability in the biological properties conferred by the envelopes of R5 viruses. R5 envelopes confer variable extents of macrophage infectivity, vary in their use of receptors CD4 and CCR5 and in sensitivity to inhibitors, and antibodies that block viral entry and viral entry kinetics. Appreciating the extent of the variation in these different properties is crucial for fully understanding how HIV-1 transmits and for the optimal design of vaccines that will target transmissible HIV-1 variants. In addition, highly macrophage-tropic R5 variants predominate in the brain tissue of subjects with HAD, although it is unclear how these variants confer neurovirulence. Finally, HIV-1 replication in brain tissue may result in the emergence of variants that are resistant to highly active antiretroviral therapy drugs that only inefficiently penetrate the blood–brain barrier.

Future perspective

The effects of HIV-1 R5 variability on transmission and pathogenesis are still poorly understood and will be the focus of future studies. Better appreciation of the environmental pressures *in vivo* that select for or against R5 envelopes with distinct properties will provide insights into the phenotypes and envelope changes that are associated with AIDS and neuropathogenesis, and will aid the design of novel approaches for intervention. Understanding the full characteristics of transmitted non-macrophage-tropic R5 viruses will facilitate the generation of vaccines and microbicides that target the most vulnerable stages of viral entry and envelope sites.

Executive summary

Variation in macrophage tropism of HIV-1 R5 viruses

- Macrophage tropism of R5 viruses varies greatly and increases in later stages of disease.
- R5 macrophage tropism is associated with enhanced interactions with CD4 and variation in residues within or proximal to the CD4 binding site.
- Highly macrophage-tropic R5 viruses are present in the brain and associated with neuropathogenesis.

Evolution in CCR5 use

- As disease progresses, R5 envelopes also evolve altered use of CCR5, an increase in envelope positive charge and reduced sensitivity to CCR5 chemokines.
- Altered use of CCR5 appears unrelated to changes in macrophage tropism.

Cross-clade variation of R5 viruses

- Clade C R5 viruses may infect $CD34^+$ progenitor cells more efficiently than clade B.

Role of R5 virus variation in transmission

- Transmission is usually conferred by R5 viruses.
- Transmitted viruses appear non-macrophage tropic, indicating that $CD4^+$ T cells are probably the first targets for infection.

Role of R5 virus variation in pathogenesis

- R5 virus variants that confer enhanced interactions with CD4 and/or CCR5 become more prevalent late in disease and may cause the depletion of $CD4^+$ T cells in the absence of CXCR4-using variants.
- Macrophage-tropic R5 viruses cause neuropathogenesis via infection of brain macrophages and microglia.
- Replication of R5 viruses in the brain results in the upregulation of factors toxic to neurons and astrocytes, and leads to the activated state of astrocytic cells.

Considerations for therapy with CCR5 antagonists

- The use of CCR5 antagonists in therapy may select for CXCR4-using variants or resistant R5 variants that can exploit drug-occupied CCR5.
- However, the use of CCR5 antagonists with other highly active antiretroviral therapy drugs will greatly reduce the chances of resistance developing.

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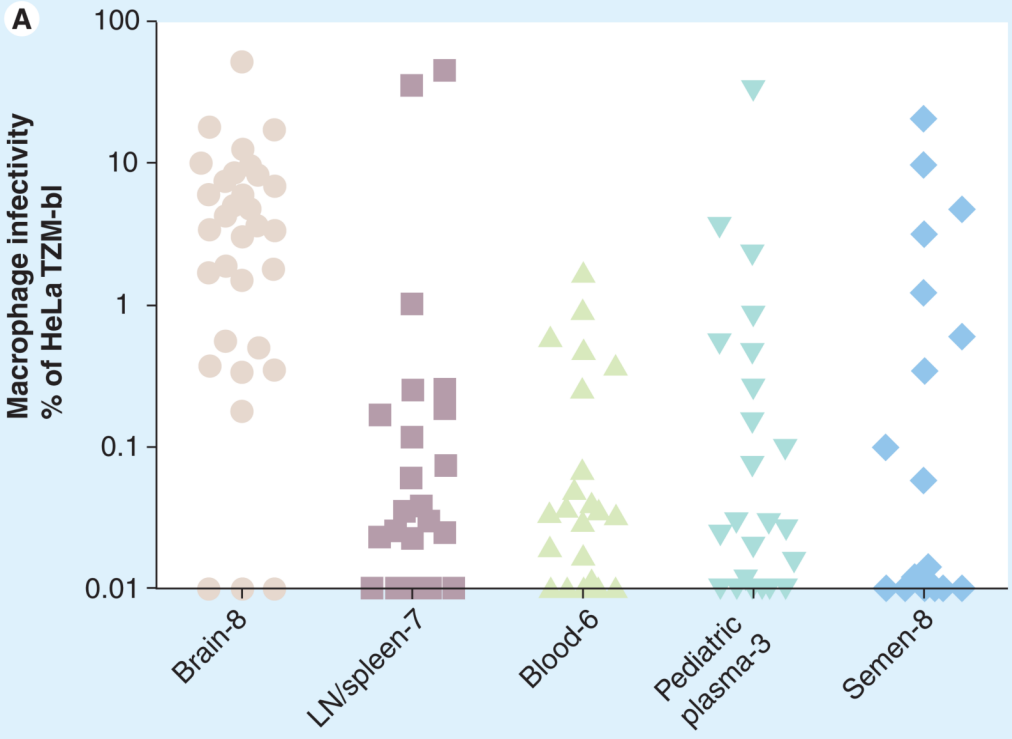
A



B

	LN/spleen	Blood	Pediatric plasma	Semen
Brain	<0.0001	<0.0001	<0.0001	<0.0045
LN/spleen		0.4383	0.4695	0.338
Blood			0.99	0.5874
Pediatric plasma				0.7822

Figure 1. Macrophage infectivity of HIV-1 R5 envelopes amplified from different tissues

(A) The infectivity of HIV-1 env^{+} pseudovirions for primary macrophages was plotted as a percentage of infection for HeLa TZM-bl cells.

(B) Mann–Whitney ana lysis of the variation of macrophage infectivity for envelopes from different compartments (p values). Macrophage infectivity conferred by brain-derived envelopes is significantly higher than for envelopes from blood, immune tissue and semen. Numbers shown on tissue labels represent subjects studied.

LN: Lymph node.

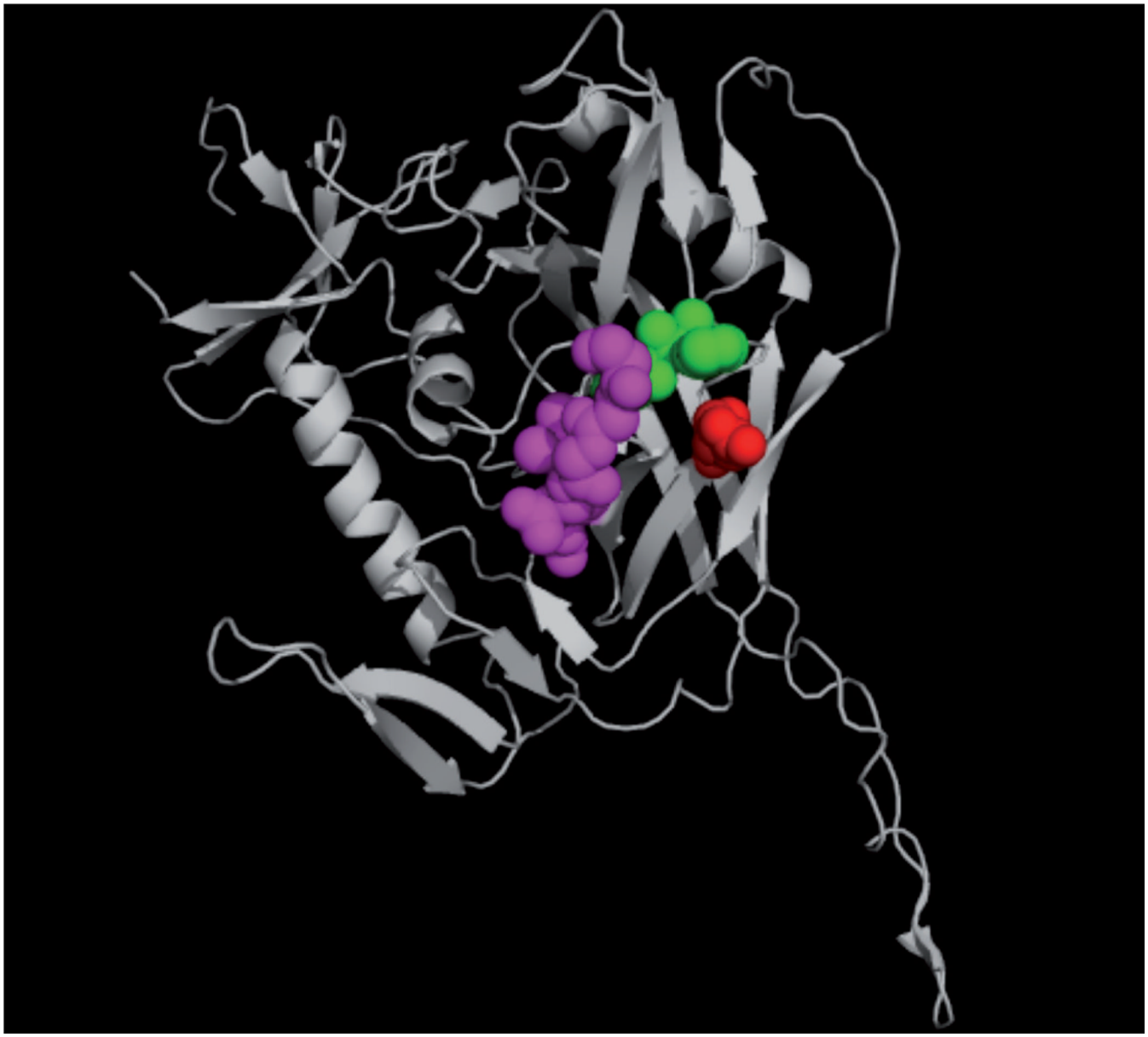


Figure 2. HIV-1 glycoprotein 120 residues involved in CD4-binding, exposure of the CD4 binding site and macrophage tropism

CD4 contact residues SGGD-PE on the CD4-binding loop are shown in magenta. Green spheres are immediately adjacent residues on the CD4-binding loop that affect macrophage tropism, presumably by modulating the exposure of the proximal CD4 contact residues. The *N*-linked glycan at N386 has also been demonstrated to affect exposure of the CD4 binding site and macrophage tropism. N386 is shown in red. A V3 loop containing gp120 in the CD4-bound form is shown (MMDB ID: 36137).