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An altered and more efficient mechanism of CCR5 engagement contributes to macrophage tropism of CCR5-using HIV-1 envelopes

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ABSTRACT

While CCR5 is the principal coreceptor used by macrophage (M)-tropic HIV-1, not all primary CCR5-using (R5) viruses enter macrophages efficiently. Here, we used functionally-diverse R5 envelope (Env) clones to characterize virus-cell interactions important for efficient CCR5-mediated macrophage entry. The magnitude of macrophage entry by Env-pseudotyped reporter viruses correlated with increased immunoreactivity of CD4-induced gp120 epitopes, increased ability to scavenge low levels of cell-surface CCR5, reduced sensitivity to the CCR5 inhibitor maraviroc, and increased dependence on specific residues in the CCR5 ECL2 region. These results are consistent with an altered and more efficient mechanism of CCR5 engagement. Structural studies revealed potential alterations within the gp120 V3 loop, the gp41 interaction sites at the gp120 C- and N-termini, and within the gp120 CD4 binding site which may directly or indirectly lead to more efficient CCR5-usage. Thus, enhanced gp120-CCR5 interactions may contribute to M-tropism of R5 HIV-1 strains through different structural mechanisms.

Introduction

Human immunodeficiency virus type 1 (HIV-1) enters cells via interaction of the viral envelope glycoproteins (Env) with CD4 and a coreceptor. The Env is organized into trimers on virions and consists of the gp120 surface and gp41 transmembrane subunits. HIV-1 entry into cells is initiated by a high affinity interaction between gp120 and CD4, which induces a conformational change in gp120 that exposes the binding site for a chemokine coreceptor, either CCR5 or CXCR4 [reviewed in (Doms, 2000; Doms and Trono, 2000)]. Current models of gp120 binding to coreceptor suggest the gp120 V3 loop interacts principally with the coreceptor second extracellular loop (ECL2) region, while the gp120 bridging sheet formed between the C1, C2 and C4 domains of gp120 after CD4 binding interacts with the coreceptor N-terminus (Brelot et al., 1999; Cormier and Dragic, 2002; Farzan et al., 1999; Huang et al., 2005). While the coreceptor N-terminus and ECL2 region appear to be important for gp120-coreceptor binding, the ECL1 and ECL3 regions may also influence coreceptor function (Doranz et al., 1997, 1999; Farzan et al., 1998). The interaction of CD4-bound gp120 with coreceptor induces additional conformational changes in gp120, which leads to a structural rearrangement in gp41 that enables fusion and virus entry.

The tropism of HIV-1 for particular target cell populations in different tissue compartments is influenced by the coreceptor used by HIV-1 Env for virus entry [reviewed in (Gorry et al., 2004)]. Macrophage (M)-tropic HIV-1 viruses primarily uses CCR5 (R5) as a coreceptor (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996), whereas T-cell tropic viruses use CXCR4 (X4) (Feng et al., 1996). Dual-tropic viruses can use both coreceptors (R5X4) (Collman et al., 1992; Yi et al., 1999). Thus, the coreceptor specificity of primary HIV-1 isolates is frequently used to define cellular tropism; for example, R5 viruses are often collectively grouped as M-tropic viral strains (Gorry et al., 2004). However, CCR5

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usage by HIV-1 is neither necessary nor sufficient for M-tropism. Several studies have demonstrated the presence of non-M-tropic R5 viruses, which were replication competent in primary CD4+ T-cells but which could not productively infect monocyte-derived macrophages (MDM) (Gorry et al., 2001; Gray et al., 2005; Li et al., 1999; Peters et al., 2004, 2006, 2008). Thus, while CCR5 may be the main coreceptor for HIV-1 entry into MDM, not all R5 viruses are M-tropic. In addition, some highly M-tropic primary HIV-1 strains use CXCR4 for entry into MDM (Gorry et al., 2001; Gray et al., 2009). Therefore, the viral determinants that underlie HIV-1 tropism for macrophages are significantly more complex than the coreceptor specificity of the virus.

In addition to variations in the level of M-tropism, primary R5 HIV-1 strains have tremendous diversity in a number of other pathophysiological phenotypes including cytopathicity for CD4+ T-cells both *in vitro* (Kwa et al., 2003; Wade et al., 2010) and *in vivo* (Berkowitz et al., 1998; Fais et al., 1999; Scoggins et al., 2000), fusogenicity (Sterjovski et al., 2007), viral fitness (Borggren et al., 2008; Repits et al., 2005, 2008), and sensitivity to virus inhibition by β-chemokines (Borggren et al., 2008; Jansson et al., 1996, 1999; Koning et al., 2003; Repits et al., 2005, 2008) and HIV-1 fusion/entry inhibitors (Gorry et al., 2001, 2002; Gray et al., 2005; Repits et al., 2005; Sterjovski et al., 2007; Sterjovski et al., 2006). In addition, primary R5 HIV-1 strains have diversity in the exposure of the CD4 binding site (CD4bs) in gp120 which has been shown to influence the level of M-tropism (Duenas-Decamp et al., 2009; Dunfee et al., 2006, 2007, 2009; Martin et al., 2001; Martin-Garcia et al., 2005; Peters et al., 2004, 2008; Thomas et al., 2007), and suggested to influence the mechanism and efficiency of CCR5 usage (Dunfee et al., 2006).

Although the aforementioned studies indicate that exposure of the gp120 CD4bs and subsequent enhanced CD4 binding contributes to M-tropism of R5 Envs, other studies suggest that an augmented gp120-CCR5 interaction may also be important for efficient macrophage entry (Gorry et al., 2001, 2002; Gray et al., 2005). Augmented gp120-CCR5 interactions may occur in tandem with- or independent from enhanced CD4 binding (Gorry et al., 2002). In this study, we characterized alterations in the efficiency and mechanism of CCR5 engagement that contribute to efficient macrophage entry of R5 Envs derived from primary HIV-1 isolates.

Results and Discussion

Diversity in the ability of primary R5 Envs to enter macrophages

The R5 Envs selected for this study were derived from primary HIV-1 isolates and exhibit diversity in a number of phenotypes including cell-cell fusion activity, entry kinetics, sensitivity to the fusion inhibitor T-20, and sensitivity to neutralizing antibodies (Sterjovski et al., 2007), indicating a broad spectrum of Env function. Since variation in the ability of primary R5 viruses to enter macrophages occurs principally at the virus entry level (Duenas-Decamp et al., 2009; Gorry et al., 2001; Gray et al., 2005; Peters et al., 2006, 2008), we reasoned that these Envs may have diversity in their ability to enter macrophages efficiently and thus, be valuable reagents to probe the mechanisms contributing to CCR5-mediated M-tropism.

We therefore determined the ability of luciferase reporter viruses pseudotyped with an alternative R5 Env to enter MDM (Fig. 1). As controls, we used luciferase reporter viruses pseudotyped with the highly M-tropic R5 ADA, YU-2 or JR-FL Envs, the non-M-tropic X4 HXB2 Env, or the non-M-tropic R5 JR-CSF Env. As expected (Peters et al., 2006), ADA, YU-2 and JR-FL Envs showed high levels of entry into MDM, whereas the HXB2 and JR-CSF Envs entered MDM very inefficiently (Fig. 1A). The primary R5 Envs showed considerable diversity in their ability to enter MDM, and comprise Env variants ranging from those that have very inefficient MDM entry, similar to the non-M-tropic JR-CSF Env, to one variant that enters MDM as efficiently as the highly M-tropic ADA and JR-FL Envs (NB23-C3) (Fig. 1B). The non-normalized luciferase values are summarized in Supplementary Table 1. Thus, we established a panel of primary virus-derived R5 Envs that have diversity in ability to enter MDM. Consistent with previous studies (Peters et al., 2006), many of the R5 Envs displayed very inefficient entry into MDM compared to controls, confirming that CCR5-usage of HIV-1 does not predict M-tropism.

The magnitude of macrophage entry correlates with increased ability to utilize low levels of CCR5

Although previous studies have shown that an increased ability to interact with CD4 contributes to M-tropism of R5 Envs (Duenas-Decamp et al., 2009; Dunfee et al., 2006, 2007, 2009; Peters et al., 2004, 2008), other studies suggest that an augmented interaction between gp120 and CCR5 may also be important for efficient macrophage entry (Gorry et al., 2001, 2002; Gray et al., 2005). Therefore, we conducted experiments using 293-Affinofile cells to determine whether efficient macrophage entry is associated with an increased ability of Env to scavenge low levels of cell surface CCR5. Alternative populations of cells expressing a moderate level of CD4 together with low ($CCR5^{low}$) or high ($CCR5^{high}$) levels of CCR5 were generated (Fig. 2A), and inoculated with equivalent infectious units of Env-pseudotyped reporter virus. Although CD4 and CCR5 expression levels on primary

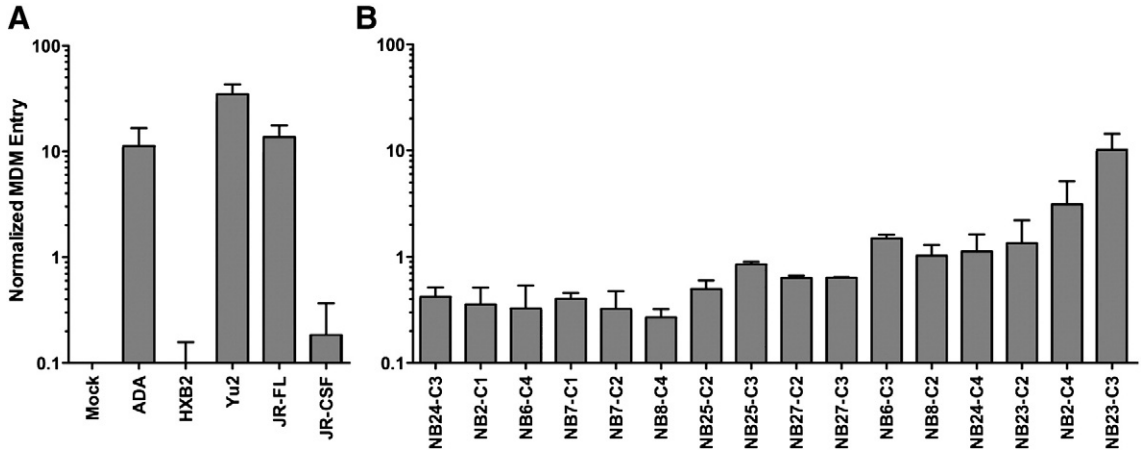

Fig. 1. Primary R5 Envs vary in their ability to enter macrophages. Monocyte-derived macrophages were inoculated with equivalent amounts of luciferase reporter virus pseudotyped with an alternative control Env (A) or primary R5 Env (B) as described in Materials and Methods. Macrophage entry levels were expressed relative to that attained by the same preparations of reporter virus in JC53 cells, as described previously (Peters et al., 2004, 2006, 2008). Values represent means from triplicate wells, and are representative of 5 independent experiments using MDM derived from 5 different donors. Error bars represent standard error of the mean.

macrophages are highly variable between donors, the CD4 and CCR5 expression levels in CCR5^{low} cells are within the range of what is typically observed in primary macrophages (Lee et al., 1999). The ability of viruses pseudotyped with the primary Envs to enter CCR5^{low} cells varied considerably (Fig. 2B). When the level of entry in CCR5^{low} cells was plotted against the level of entry in MDM achieved using the same preparations of reporter viruses, we observed a highly significant positive correlation between the magnitude of macrophage entry and the ability of Env to scavenge low levels of CCR5 (Fig. 2C). Similar results were obtained using Cf2th-CD4 cells expressing either low or high levels of CCR5 (data not shown). These results suggest a more efficient interaction between gp120 and CCR5 by M-tropic Envs.

M-tropic R5 Envs have reduced sensitivity to the CCR5 inhibitor maraviroc

To further investigate whether the efficiency of the gp120-CCR5 interaction influences M-tropism of R5 Envs, we determined the sensitivity of Env-pseudotyped luciferase reporter viruses to inhibition by the CCR5 inhibitor maraviroc (MVC). The IC_{50} values for MVC in JC53 cells were determined from virus inhibition curves, and plotted against the level of MDM entry achieved using the same preparations of reporter viruses (Fig. 3). We observed a positive correlation between the ability of Env to enter macrophages and reduced sensitivity to inhibition by MVC. Consistent with these results, the M-tropic R5 ADA Env was approximately 5-fold less sensitive to inhibition by MVC than the non-M-tropic R5 JR-CSF Env (data not shown). These results suggest greater competition between M-tropic Envs and MVC for CCR5 occupancy. Moreover, they indicate broad variation in the baseline sensitivity of primary R5 Envs to MVC, which may be clinically important. Together, the results shown in Figs. 2 and 3 are consistent with a more efficient CCR5 engagement by M-tropic R5 Envs.

M-tropic Envs have an altered mechanism of CCR5 engagement

More efficient CCR5 usage by M-tropic Envs raises the possibility that M-tropic Envs have an altered mechanism of coreceptor engagement. In particular, if the reduced sensitivity of M-tropic Envs to inhibition by MVC (Fig. 3) is indeed due to greater competition for CCR5, M-tropic R5 Envs would be expected to have a more favored- or more critical interaction with the CCR5 ECL2 region.

To determine whether the more efficient CCR5 usage by M-tropic R5 Envs is linked to an altered mechanism of CCR5 engagement, we conducted fusion assays using target cells expressing CD4 and either WT CCR5 or a mutant coreceptor containing amino acid alterations in the N-terminus, ECL1, ECL2 or ECL3 regions of CCR5 which attenuate coreceptor activity (Doranz et al., 1997; Farzan et al., 1998), and effector cells expressing gp120 (Fig. 4). Cell surface staining by flow cytometry showed similar levels of gp120 expressed on the surface of effector cells (data not shown), and similar levels of WT or mutant CCR5 expressed on the surface of target cells (Fig. 4B). We observed a

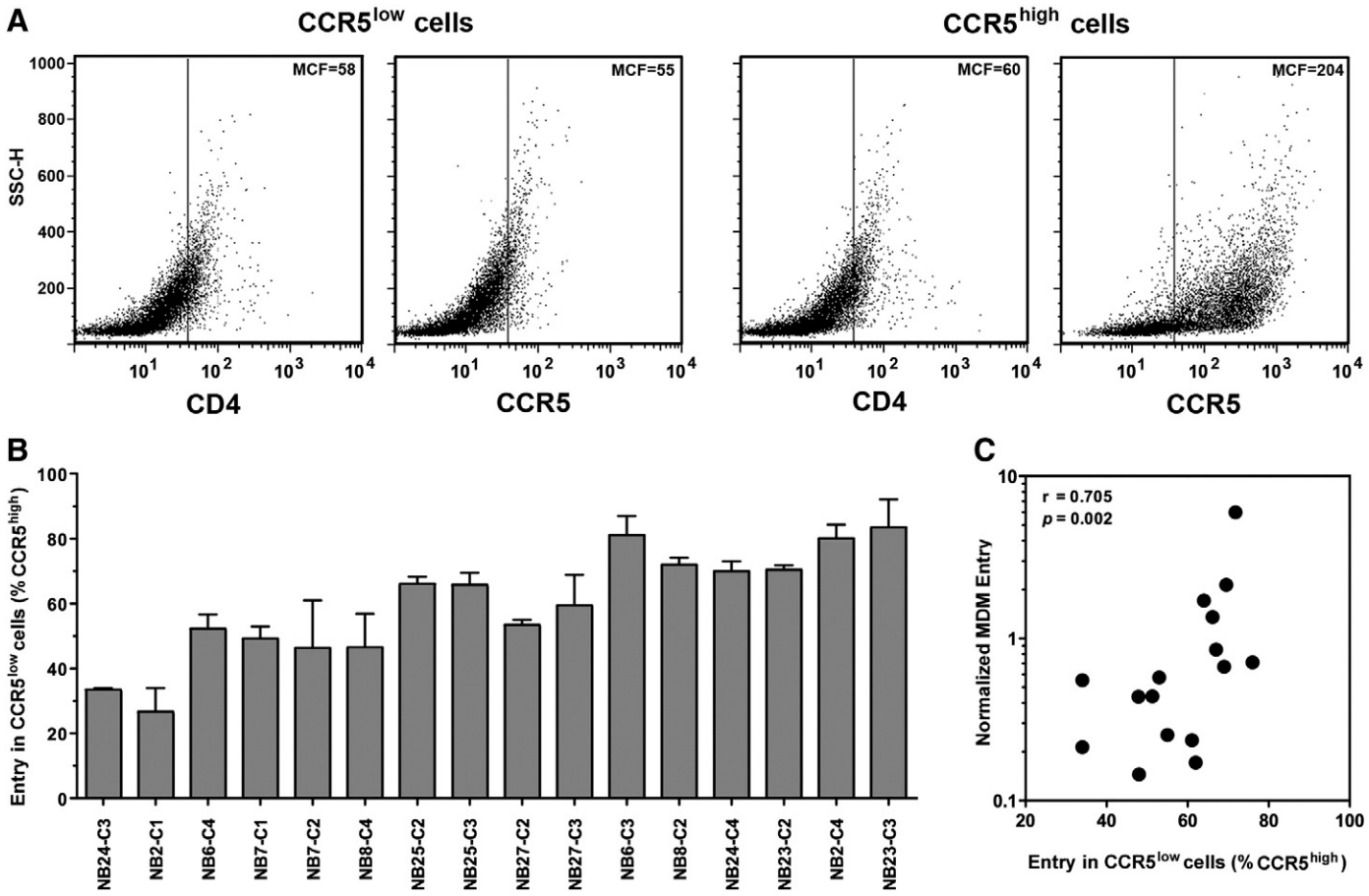

Fig. 2. Efficient macrophage entry is associated with more efficient use of CCR5. CCR5^{low} and CCR5^{high} cells expressing moderate levels of CD4 together with low or high levels of CCR5, respectively (A), were produced from 293-Affinofile cells (Johnston et al., 2009) and were inoculated with equivalent amounts of Env-pseudotyped luciferase reporter virus, as described in Materials and Methods. The entry levels in CCR5^{low} cells were expressed as a percentage of that attained in CCR5^{high} cells (B). The values shown are means from triplicate wells, and are representative of 3 independent experiments. Error bars represent standard deviation. The relative ability of Envs to enter CCR5^{low} cells was plotted against the level of entry attained by the same preparations of reporter virus in MDM, using Prism version 4.0c (GraphPad Software) (C). The Spearman correlation coefficient (r) and P values are shown. P values <0.05 were considered statistically significant. Similar correlations were made using data from 5 independent MDM donors. MCF, mean cell fluorescence.

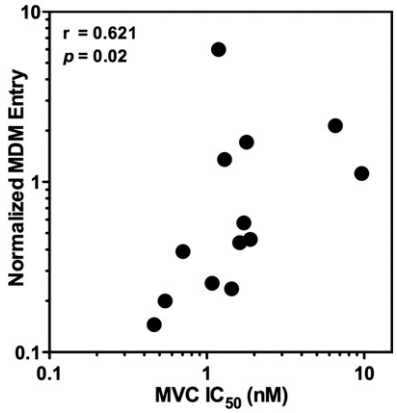

Fig. 3. Efficient macrophage entry is associated with reduced baseline sensitivity of Envs to the CCR5 antagonist maraviroc. The IC_{50} values for MVC were determined as described in Materials and Methods, and plotted against the level of entry attained by the same preparations of reporter virus in MDM, using Prism version 4.0c (GraphPad Software). The Spearman correlation coefficient (r) and P values are shown. P values <0.05 were considered statistically significant. Similar correlations were made using data from 5 independent MDM donors.

near-significant positive association between the magnitude of MDM entry and the ability of Env to tolerate mutation of Y15 in the CCR5 N-terminus (Figs. 4A,C). However, we found significant inverse correlations between the magnitude of MDM entry and the ability of Env to tolerate mutation of H181 or Y184 in the CCR5 ECL2 region (Figs. 4A,C). These results suggest increased reliance on H181 and Y184 by M-tropic R5 Envs, and a likely reduced reliance on Y15. Further studies with greater numbers of R5 Envs are required to determine more precisely whether Y15 in the CCR5 N-terminus influences M-tropism of HIV-1. Curiously, several Envs with inefficient MDM entry could not only tolerate ECL2 mutations well, they tended to fuse with cells expressing the ECL2 mutants more efficiently than cells expressing WT CCR5, particularly the H181A mutant. Whether this observation is significant and/or biologically relevant is uncertain. Mutation of Y14 and D11 in the CCR5 N-terminus abolished coreceptor activity against all Envs, while no correlations or trends were observed between MDM entry and the ability of Env to tolerate mutations at Y89 and H88 in the CCR5 ECL1 region, Q188 and K197 in the CCR5 ECL2 region, and E262 and F264 in the CCR5 ECL3 region (data not shown). Thus, the more efficient CCR5 usage by M-tropic R5 Envs is linked to an altered mechanism of CCR5 engagement.

Consistent with our results, previous studies have shown that gp120 proteins that become less reliant on the CCR5 N-terminus show greater dependence on the ECL2 region, which in turn promotes greater affinity for CCR5 (Platt et al., 2001). Moreover, studies with CCR5 chimeras have shown that primary R5 HIV-1 isolates obtained from patients with late stage HIV-1 infection, which typically have reduced sensitivity to inhibition by β-chemokines (Jansson et al., 1999; Karlsson et al., 2004; Koning et al., 2003; Scarlatti et al., 1997)

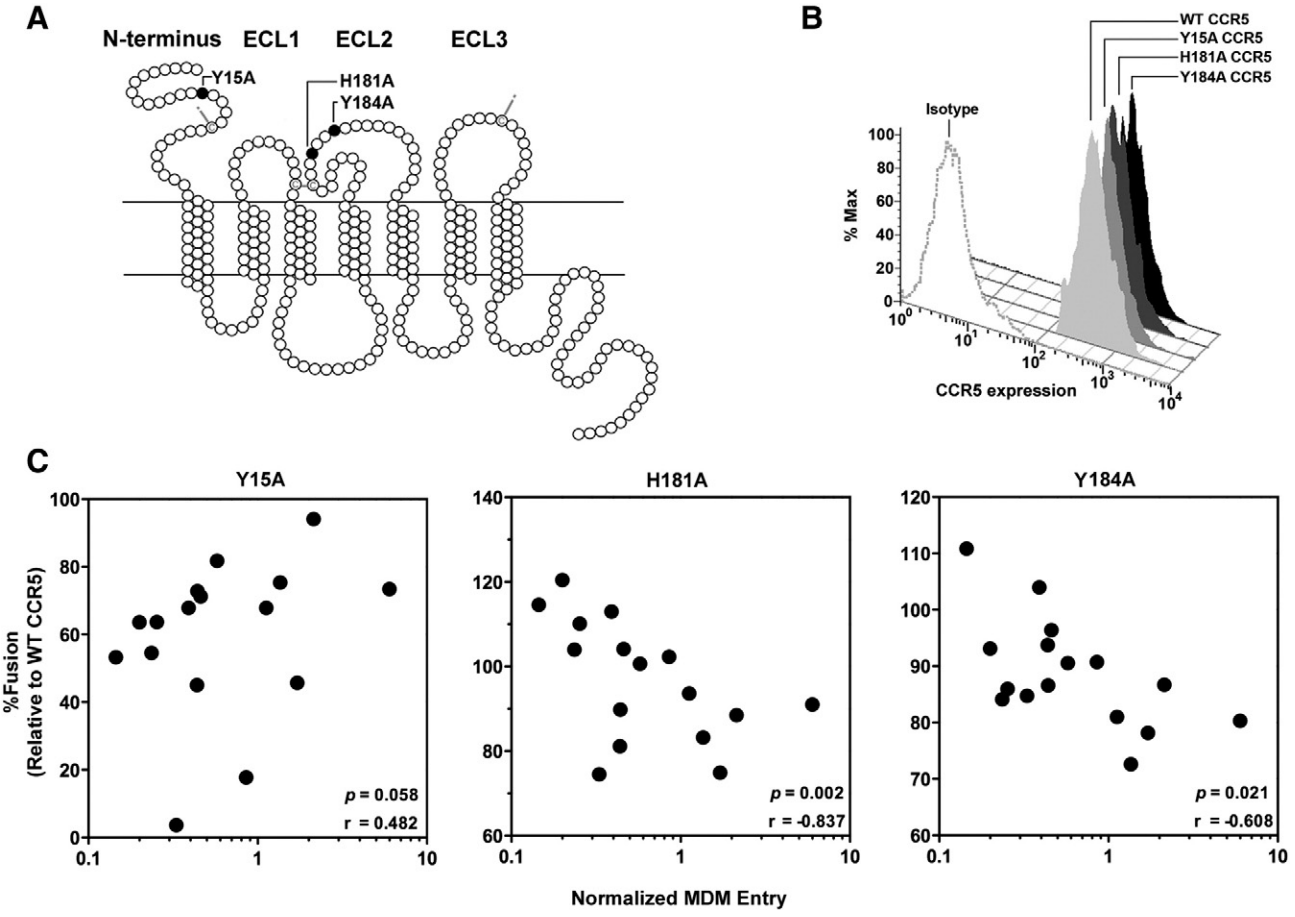

Fig. 4. Envs with efficient macrophage entry have an altered mechanism of CCR5 engagement. A schematic depicting the location of CCR5 residues that were shown to influence the efficiency of MDM entry is shown in (A). The level of WT and mutant CCR5 expression was measured by flow cytometry (B). Cells expressing CD4 together with equivalent levels of WT CCR5 or CCR5 with mutations at Y15, H181 or Y184 were used in cell-cell fusion assays with effector cells expressing equivalent levels of gp120, as described in Materials and Methods. The level of fusion in experiments using a particular CCR5 mutant was expressed as a percentage of that attained using WT CCR5, and plotted against MDM entry levels, using Prism version 4.0c (GraphPad Software) (C). The values shown are means from triplicate wells, and are representative of 3 independent experiments. The Spearman correlation coefficient (r) and P values are shown. P values <0.05 were considered statistically significant. Similar correlations were made using data from 5 independent MDM donors.

and reduced CCR5 dependence (Gray et al., 2005), have more efficient CCR5 usage (Karlsson et al., 2004). Together, our data and those from previous studies suggest that R5 Envs with efficient MDM entry have an altered- and more efficient mechanism of CCR5 engagement, characterized by a more critical interaction with the CCR5 ECL2 region, and a likely less critical interaction with the CCR5 N-terminus.

M-tropic R5 Envs have greater exposure of CD4-induced epitopes in gp120

Binding of gp120 to CD4 induces exposure of the CCR5 binding site. To determine whether M-tropic R5 Envs with altered and more efficient CCR5 usage have greater exposure of CD4-induced (CD4i) epitopes, we next determined the association between the magnitude of MDM entry and immunoreactivity of CD4-bound gp120 to the monoclonal antibodies 17b and 48d, which recognize distinct CD4i epitopes in the gp120 core overlapping the CCR5 binding site (Kwong et al., 1998; Moore et al., 1993; Thali et al., 1993). We observed positive correlations between the magnitude of MDM entry and the ability of CD4-bound gp120 to recognize 17b (Fig. 5A) and 48d (Fig. 5B). None of the Envs recognized 17b or 48d in the absence of soluble CD4 (data not shown), suggesting that M-tropic R5 Envs do not have CD4-independent exposure of the coreceptor binding site in gp120. Equivalent reactivity to the polyclonal HIV-1 sera BB10, which strongly recognises gp120 (Gorry et al., 1999), indicated equivalent levels of cell-surface Env expression (data not shown). In addition, we saw no correlation between MDM entry and the ability of gp120 to bind the V3-specific monoclonal antibody 39F (Kwong et al., 2002; Pantophlet et al., 2004) (data not shown), which recognizes a linear epitope on the ascending (N-terminal) strand of the V3 loop. Together, these results provide evidence that greater exposure of the CD4-induced coreceptor binding site in gp120 contributes to the more efficient CCR5 usage by M-tropic R5 Envs.

The coreceptor binding site in gp120 is formed by dramatic conformational changes that are triggered by CD4 binding (Chen et al., 2005; Kwong et al., 1998; Myszka et al., 2000). The 17b mAb binds to a small (~500 Å^2) basic surface area of gp120 via the highly protruding acidic complementarity determining region (CDR) H3 loop, as well as a hydrophobic interaction between a CDR H2 loop of 17b and the gp120 bridging sheet (Huang et al., 2004; Kwong et al., 1998, 2000). The relatively small interface area and protruding CDR H3 loop paratope of 17b is likely to be the result of limited accessibility of the coreceptor binding site. Enhanced binding of 17b by M-tropic Envs may be due to electrostatic interactions with 17b CDR loops or protection of hydrophobic residues to result in a higher affinity interaction. Thus, structural alterations in gp120 that result in greater exposure of CD4i epitopes within the coreceptor binding site may contribute to enhanced MDM entry and more efficient CCR5 usage by R5 Envs.

A more efficient gp120-CCR5 interaction may occur through a variety of structural mechanisms

To better understand the structural basis of efficient CCR5 usage and MDM entry by M-tropic R5 Envs, we compared three paired Env sequence sets that had consistent differences in levels of M-tropism when tested in five independent MDM donors, despite being cloned from the same primary virus isolates (Table 1). Thus, we took advantage of the fact that a similar genetic background would help pinpoint Env amino acid alterations that may contribute to efficient MDM entry.

Molecular models of the Env pairs were produced to examine sequence changes in the context of predicted three-dimensional gp120 structures. Sequence analysis together with models based on the 2B4C crystal structure of the V3 loop-containing, CD4-liganded gp120 showed that, in NB24 clones, alterations associated with more efficient MDM entry were located predominantly within- or immediately adjacent to the V3 loop (Fig. 6A). Interestingly, NB24-C3 Env which enters MDM very inefficiently, was more sensitive to mutation of Y15 in the CCR5 N-terminus compared to NB24-C4 Env (data not shown), consistent with the overall trend observed between increased M-tropism and reduced dependence on Y15 (Fig. 4C). In NB24-C3, changes in the G-P-G-R/K motif at the tip of the V3 loop, which is highly conserved among clade B Envs (Plantier et al., 1999), may contribute to the reduced tolerance of mutation in the N-terminus of CCR5 compared to NB24-C4 by abrogating the interaction with negatively charged sulfate groups of sulfotyrosine residues.

Using the recently-described 3JWO crystal structure of gp120 containing the gp41 interactive domain (Pancera et al., 2010) to model amino acid substitutions, we showed that an R5 Env pair that displayed significant disparity in M-tropism contained alterations in the gp41 interface region within gp120 (Fig. 6B). All but one amino acid substitution between NB2-C1 and NB2-C4 Envs were located within or near sites in the C1 and C5 regions of gp120 that have been shown by mutagenesis to interact with the gp41 fusion protein (Helseth et al., 1991; Pancera et al., 2010; Sen et al., 2008; Yang et al., 2003). Transmission of an activation signal from the CD4/coreceptor-

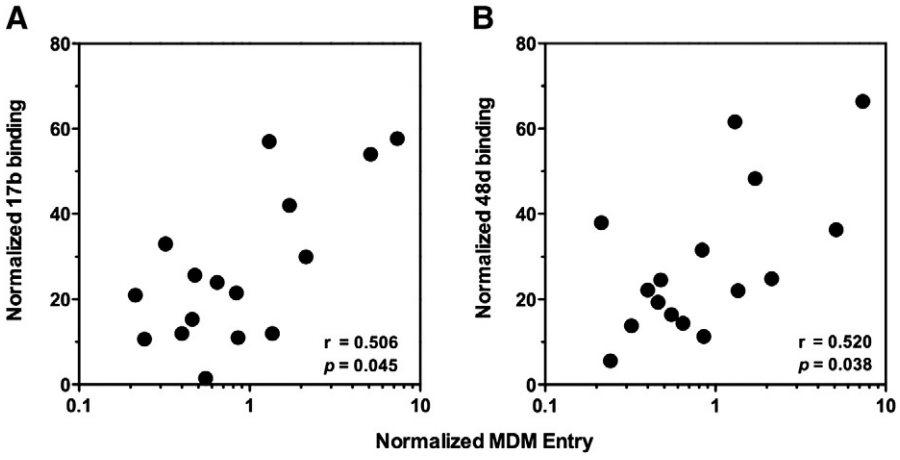

Fig. 5. Efficient macrophage entry is associated with greater exposure of CD4-induced epitopes in gp120. Immunoreactivity of CD4-bound gp120 to the monoclonal antibodies 17b (A) and 48d (B) was determined by flow cytometry, as described in Materials and Methods. The level of antibody binding was expressed as a percentage of that achieved for the control R5 Env ADA, and plotted against MDM entry levels using Prism version 4.0c (GraphPad Software). The values shown are means of triplicates, and are representative of 3 independent experiments. The Spearman correlation coefficient (r) and P values are shown. P values <0.05 were considered statistically significant. Similar correlations were made using data from 5 independent MDM donors.

Table 1
Paired Env clones with variable relative levels of M-tropism.

Env clone	Primary virus isolate	Relative level of MDM entry	Genbank accession number
NB24-C3	NB24	-	EU308561
NB24-C4		++	EU308562
NB2-C1	NB2	-	EU308541
NB2-C4		++	EU308543
NB23-C2	NB23	++	EU308557
NB23-C3		+++	EU308558

MDM entry of primary R5 Envs was scored as – (≤ 0.5-fold relative entry), + (0.5- to 2-fold relative entry), ++ (2- to 10-fold relative entry), and +++ (≥ 10-fold relative entry).

bound gp120 to the gp41 fusion protein is thought to occur via the gp120-gp41 interface (Chan et al., 1997; Melikyan et al., 2000; Tan et al., 1997; Weissenhorn et al., 1997). Mutations in these regions have been shown to modulate gp120-gp41 association (Helseth et al., 1991; Sen et al., 2008; Yang et al., 2003), including the valine residue at position 496 in C5 (Sen et al., 2008), which differs between NB2-C1 and NB2-C4 Envs. These observations suggest that some R5 Envs may become more efficient at utilising CCR5 by way of altered transmission of gp120 signalling events to the gp41 fusion peptide.

While coreceptor interaction or subsequent signal transduction pathways may contribute to efficient MDM entry by certain R5 Envs, others may indirectly have an enhanced interaction with CCR5 via a more efficient interaction with CD4, as suggested by previous studies using particular neurotropic R5 Envs (Dunfee et al., 2006). For example, the gp120-CD4 interaction is short-lived and weak compared to the interaction between CD4-bound gp120 and CCR5 (Chang et al., 2005). Since CCR5 is more mobile in the cell membrane than CD4 (Steffens and Hope, 2004), a more stable gp120-CD4 interaction by R5 Envs could potentially allow the Env-CD4 complex to more readily co-localize with CCR5, thus increasing the efficiency of CCR5 usage. This possibility is illustrated by comparing the predicted gp120 structures of NB23-C2 and NB23-C3 Envs. In addition to loss of a potential glycosylation site in V1, NB23-C2 Env contains a rare aspartic acid to glycine mutation at position 477 near the CD4bs when modeled on the 2B4C gp120 crystal structure (Fig. 6C). When mapped to the surface of gp120, Asp477 increases the local negative charge and may play an important role in maintaining electrostatic interactions between the predominantly negatively charged CD4bs on gp120 and the predominantly positively charged binding site on the CD4 molecule.

Together, these analyses provide evidence that more efficient CCR5-usage that contributes to M-tropism of R5 HIV-1 strains, may be gained through different structural mechanisms. Specifically, primary R5 Envs may acquire enhanced MDM entry by changing the way they interact with CCR5, by improving the efficiency by which signals are transmitted to the gp41 fusion peptide, or by augmenting the interaction with CD4 and thus reducing the reliance on high levels of CCR5 to mediate fusion and entry. Further mutagenesis studies are required to fully elucidate the biological role of Env amino acid alterations associated with more efficient CCR5 usage and MDM entry.

Conclusions

In summary, we showed that the efficiency of MDM entry by R5 Envs is linked to a more efficient and altered mechanism of CCR5 engagement characterized by increased dependence on specific amino acids in the CCR5 ECL2 region, at least among the panel of primary Env clones studied. Furthermore, greater exposure of CD4i gp120 epitopes overlapping the coreceptor binding site among M-tropic R5 Envs provided evidence that structural alterations in CD4-bound gp120 augment gp120-CCR5 interactions and contribute to efficient MDM entry. Finally, sequence analysis together with molecular modeling of gp120 suggests that enhanced gp120-CCR5 interactions may contribute to M-tropism of R5 HIV-1 strains through different structural mechanisms.

Materials and Methods

Plasmids

The HIV-1 Envs used in this study were cloned from primary R5 HIV-1 isolates which have been described in detail previously, including the clinical characteristics of the subjects from whom they were isolated (Gray et al., 2005; Li et al., 1999). The Env clones used were NB23-C2, NB23-C3, NB24-C3, NB24-C4, NB25-C2, NB25-C3, NB27-C2, NB27-C3, NB2-C1, NB2-C4, NB6-C3, NB6-C4, NB7-C1, NB7-C2, NB8-C1, NB8-C2 and NB8-C4, which have been described in detail previously (Sterjovski et al., 2007) (accession numbers EU308533 to EU308568). Briefly, the 2.1Kb KpnI-to-BamHI fragment of the HIV-1 *env* gene was amplified from virus isolates and cloned into the pSVIII-Env expression vector (Gao et al., 1996), as described previously (Gray et al., 2006, 2009; Sterjovski et al., 2007). The pcDNA3-CD4 and pcDNA3-CCR5 plasmids express human CD4 and CCR5, respectively (Gorry et al., 2001). pSVL-Tat expresses the HIV-1 Tat protein. The CCR5 mutants used in this study have been described previously (Doranz et al., 1997; Farzan et al., 1998).

Cells

Cf2-Luc cells (Etemad-Moghadam et al., 2000), derived from the Cf2th canine thymocyte cell line (Choe et al., 1996), stably express the luciferase gene under the control of the HIV-1 long terminal repeat and were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS), 100 µg of penicillin and streptomycin per ml, and 0.7 mg of G418 per ml. 293 T cells were cultured in DMEM supplemented with 10% (vol/vol) FCS, and 100 µg of penicillin and streptomycin per ml. JC53 cells are derived from the HeLa cell line and stably express high levels of CD4, CXCR4 and CCR5 on the cell surface (Platt et al., 1998), and were cultured in DMEM supplemented with 10% (vol/vol) FCS, and 100 µg of penicillin and streptomycin per ml. Peripheral blood mononuclear cells (PBMC) were purified from the blood of healthy HIV-1 negative donors by density gradient centrifugation. Monocyte-derived macrophages (MDM) were produced from elutriated monocytes (from PBMC) that were cultured for 5 days in RPMI 1640 medium supplemented with 10% (vol/vol) pooled human sera, 100 µg of penicillin and streptomycin per ml, and 12.5 ng of macrophage colony-stimulating factor per ml. The dually-inducible 293-Affinofile cell line (Johnston et al., 2009), in which expression of CD4 and CCR5 can be induced and regulated by the addition of minocycline or ponasterone A (ponA), respectively, was maintained in DMEM supplemented with 10% (vol/vol) FCS, 100 µg of penicillin and streptomycin per ml, and 50 µg blasticidin per ml. To produce cell populations expressing a moderate level of CD4 together with low ($CCR5^{low}$) or high ($CCR5^{high}$) levels of CCR5, 293-Affinofile cells were treated with 1.25 ng/ml minocycline together with 0 or 2.0 µM ponA, respectively. Note that in 293-Affinofile cells there is low level basal CCR5 expression without induction by ponA (Pugach et al., 2009), so cells were treated with minocycline alone to produce the $CCR5^{low}$ population.

Binding assays

Detection of ligand binding to gp120 expressed on the surface of 293 T cells was carried out using flow cytometry. 293 T cells were transfected with Env expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocol, and stained for surface gp120 expression using pooled polyclonal HIV + sera BB10, as described previously (Sterjovski et al., 2007). Approximately 2 x 10^5 293 T cells transfected with each Env expression plasmid were used in

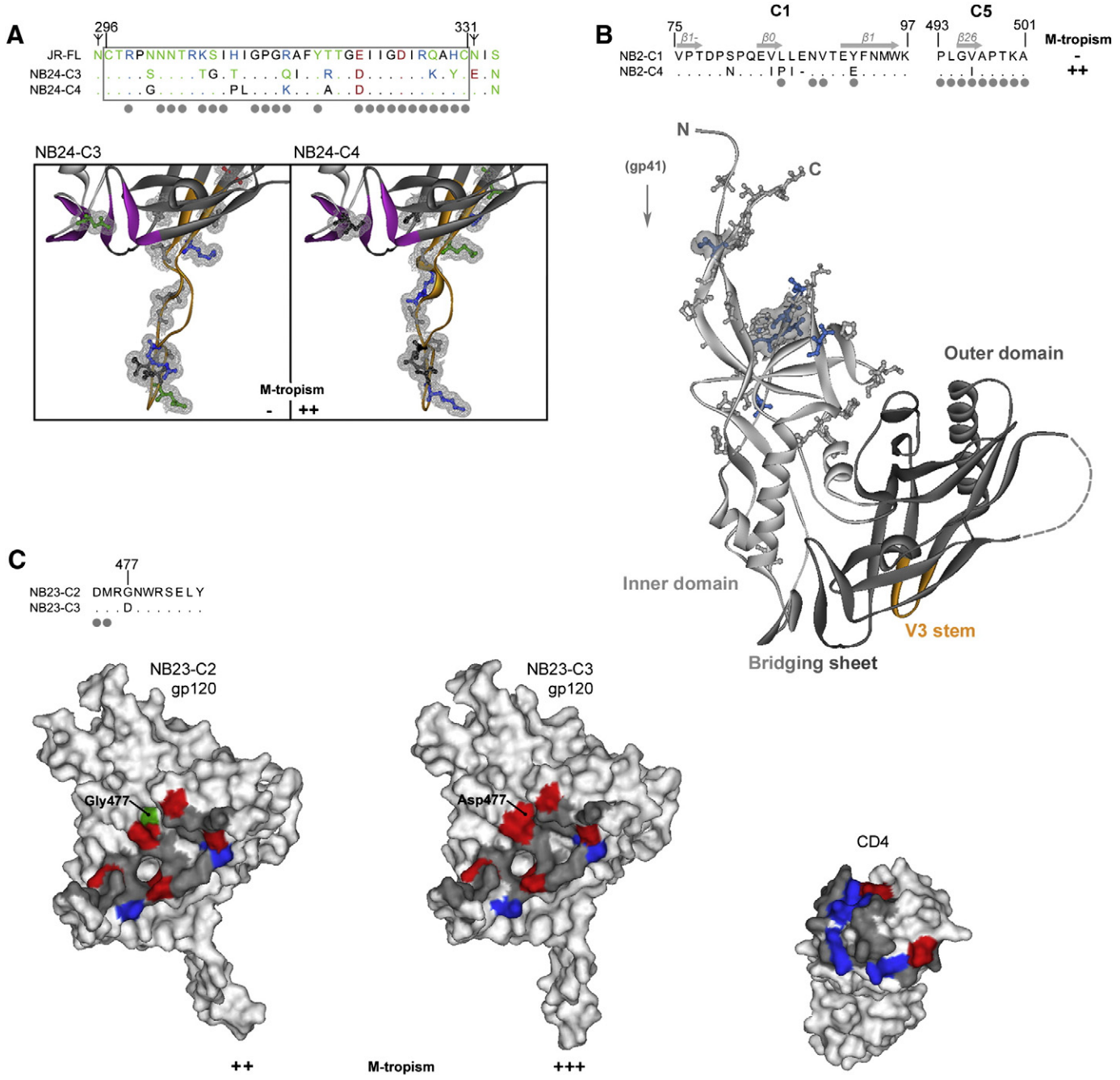

Fig. 6. Several structural mechanisms could potentially enhance the interaction between M-tropic Envs and CCR5. A subset of Envs contain changes in the V3 loop of gp120 which may contribute to M-tropism (A). The V3 region (boxed) of NB24-C3 and NB24-C4 Envs was aligned against the M-tropic R5 JR-FL Env (A, top panel). Amino acid sequence is colored according to polarity: Polar, green; Non-polar, black; Positively-charged, blue; Negatively charged, red. Potential N-linked glycosylation sites are marked by , and grey circles mark the position of residues that have previously been shown by mutagenesis to be important for coreceptor binding (Cormier and Dragic, 2002; Rizzuto et al., 1998; Suphaphiphat et al., 2007). Amino acid differences in the V3 loop of NB24 Envs were analyzed in the context of their predicted three-dimensional structures that were generated using the V3-containing JRFL crystal structure (2B4C) (Huang et al., 2005) (A). Residues that differed between NB24 Envs are shown in stick representation and colored as in the sequence alignment, with van der Waals surface in grey. The gp120 structures are shown in grey ribbon representation with the V3 loop in gold and the bridging sheet in magenta. In (B), the Envs derived from virus NB2, which displayed significant disparity in M-tropism, contained changes in the gp41-interractive region of gp120. Regions within the N-terminus (residues 75 to 97) and C-terminus (residues 493 to 501) that contained amino acid changes were aligned (B, top panel), grey circles mark the position of residues that have previously been shown by mutagenesis to be involved in gp41 interaction (Pancera et al., 2010). The gp120 structure containing the gp41 interactive domain (Protein Data Bank ID: 3JWO, (Pancera et al., 2010)) is shown in ribbon representation (B, bottom panel), with the inner and outer domains colored in light and dark grey, respectively. Residues involved in gp41 interaction are shown in grey stick representation, and residues that differ between NB2-C1 and NB2-C4 are shown in blue stick representation, with those that occur at positions involved in gp41 interaction highlighted with grey van der Waals surface. The stem of the V3 loop is colored gold. In (C), the molecular surface of the predicted structures of NB23-C2 (C, left) and NB23-C3 (C, center) Envs and of CD4 (C, right) are shown with the gp120-CD4 interface highlighted in grey. Basic, acidic and polar uncharged residues found at the interface are colored blue, red and green, respectively. The location of the G477D mutation is shown in the sequence alignment (C, top left); grey circles mark the position of residues previously shown to be involved in CD4 binding (Kwong et al., 1998; McCaffrey et al., 2004).

binding reactions. In 17b and 48d binding reactions, cells were pre-incubated in FACS buffer [phosphate buffered saline (PBS) containing 10% (vol/vol) FCS and 0.05% (wt/vol) sodium azide] with or without 1 μg of sCD4 per ml (Progenics Pharmaceuticals Inc., Tarrytown, NY) for 1 h at room temperature. Cells were then washed twice with 1 ml FACS buffer and resuspended in 50 μl of FACS buffer containing 20 μg of 17b (Kwong et al., 1998; Sullivan et al., 1998; Thali et al., 1993; Trkola et al., 1996; Wyatt et al., 1995, 1998) or 48d (Moore et al.,

1993; Thali et al., 1993) per ml. These concentrations were empirically determined to be within the linear range of binding (data not shown). Following incubation for 1 h at room temperature, cells were washed twice with 1 ml FACS buffer and resuspended in 50 µl of FACS buffer containing a 1:200 dilution of FITC-conjugated anti-human IgG $F(Ab)_2$ fragment (Millipore Corporation, Billerica, MA). Cells were incubated for 1 h at room temperature prior to being washed twice with 1 ml of FACS buffer and resuspended in 150 µl of PBS containing 4% (wt/vol) paraformaldehyde, and analysed by flow cytometry as described previously (Gorry et al., 1999).

Fusion assays

Cell-cell fusion assays were conducted as described previously (Sterjovski et al., 2007). Briefly, Cf2-Luc target cells seeded in 25 cm^2 tissue culture flasks were transfected with 1 µg of CD4 plasmid and or 3 µg of wild-type (WT) or mutant CCR5 plasmid. 293 T effector cells seeded in 6-well tissue culture plates were co-transfected with 3.4 µg of Env-expressing plasmid and 0.6 µg pSVL-Tat. Target and effector cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocol. Approximately 2.5×10^4 293 T effector cells were added to 2.5×10^4 Cf2-Luc target cells. After mixing, target and effector cells were incubated at 37 °C in replicate wells containing 200 µl of culture medium. Cells from replicate wells were harvested at 10 h post-mixing and assayed for luciferase activity (Promega) according to the manufacturers' protocol. Luminescence was measured using a FLUOStar microplate reader (BMG LABTECH, GmbH, Germany). 293 T cells transfected with pSVL-Tat alone were used as negative controls to determine the background level of luciferase activity.

Production and quantitation of Env-pseudotyped, luciferase reporter viruses

Env-pseudotyped, luciferase reporter viruses were produced by transfection of 293 T cells with pCMVΔP1ΔenvpA, pHIV-1Luc and pSVIII-Env plasmids using Lipofectamine 2000 (Invitrogen) at a ratio of 1:3:1, as described previously (Gray et al., 2006; Sterjovski et al., 2007; Yang et al., 2001). Supernatants were harvested 48 h later, filtered through 0.45 µm filters and stored at -80 °C. The $TCID_{50}$ of virus stocks was determined by titration in JC53 cells (Platt et al., 1998), as described previously (Gray et al., 2009; Sterjovski et al., 2007).

HIV-1 Entry assays

For entry assays using JC53 cells or 293-Affinofile cell populations, 2×10^4 cells were inoculated with 200 $TCID_{50}$ of Env-pseudotyped luciferase reporter virus (equating to a multiplicity of infection [MOI] of 0.01) in a volume of 100 µl for 12 h at 37 °C. The cells were washed twice with culture medium to remove residual inoculum and incubated for a further 60 h at 37 °C. For entry assays in MDM, 5×10^4 cells were inoculated with 500 $TCID_{50}$ of Env-pseudotyped luciferase reporter virus (equating to an MOI of 0.01) in a volume of 100 µl for 12 h at 37 °C. The cells were washed twice with culture medium to remove residual inoculum and incubated for a further 96 h at 37 °C. In all cell types, the level of HIV-1 entry was measured by luciferase activity in cell lysates (Promega), according to the manufacturers' protocol. Luminescence was measured using a FLUOStar microplate reader (BMG LABTECH, GmbH, Germany). Negative controls included mock-infected cells that were incubated with culture medium instead of virus, and cells inoculated with luciferase reporter virus pseudotyped with the non-functional ΔKS Env (Etemad-Moghadam et al., 2000). Macrophage entry levels were normalized against that attained by the same preparations of reporter virus in JC53 cells to more accurately control for virus infectivity, as described previously (Peters et al., 2004, 2006, 2008). In experiments measuring the inhibition of HIV-1 entry by maraviroc (MVC) in JC53 cells, the cells were left untreated or preincubated with serial 5-fold dilutions of drug for 30 min prior to inoculation with virus (0.064 to 1000 nM). The drug concentrations were maintained during the virus inoculation and the subsequent culture period. After the background activity was subtracted, the amount of luciferase activity in cells treated with MVC was expressed as a percentage of that in untreated cells. The percent inhibition was calculated by subtracting this number from 100. Data were fitted with a nonlinear function, and 50% inhibitory concentrations (IC_{50}) were calculated by least squares regression analysis of inhibition curves, as described previously (Gorry et al., 2002, 2007; Gray et al., 2005; Sterjovski et al., 2007).

Computer-aided structure prediction of gp120

Homology models of CD4-bound and primary gp120 sequences were prepared using the Build Model protocol of the Discovery Studio suite, version 1.6 (Accelrys, San Diego, CA, USA). This approach uses the Modeller algorithm to generate an atomic model of the target protein from a template molecule and a sequence alignment. The template-based models are optimised by iterative cycles of conjugate-gradient minimisation against a probability density function that includes spatial restraints derived from the template and residue specific properties (Sali and Blundell, 1993). The crystal structure of JRFL gp120 containing the V3 variable loop and bound to CD4 and the X5 Fab antibody fragment was used as the template for CD4-bound models (Protein Data Bank ID: 2B4C; (Huang et al., 2005)). The X5 antibody fragment was deleted from the CD4-bound template prior to modeling. The coordinates for gp120 and CD4 were extracted from the 2B4C crystal structure. Sequence alignments were generated between JRFL gp120 and the primary gp120 Env clones. The sequence for CD4 was included as a second polypeptide chain such that the models of gp120 were constructed as complexes with CD4. The V1/V2 variable loops were replaced with a GAG linker sequence and the N- and C- termini overhangs were cut using the modeling software. Similarities in three-dimensional structure were measured by the root mean square deviation (RMSD) of the distances between main-chain atoms (N, Cα, C and O atoms) from crystal and model structures after rigid body superposition, where an RMSD of <1 Å signifies a high level of homology of three-dimensional structure between overlayed proteins. The overall quality of the geometry of gp120 models generated was verified using PROCHECK (Laskowski et al., 1993). Additional energy minimisation using the Crystallography and Nuclear Magnetic Resonance System (CNS) program package (Brunger et al., 1998) did not further improve the geometry of model structures or lower the RMSD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.05.006.

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