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Loss of the N-linked glycosylation site at position 386 in the HIV envelope V4 region enhances macrophage tropism and is associated with dementia

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Abstract

HIV infects macrophages and microglia in the central nervous system (CNS). Mechanisms that enhance HIV macrophage/microglial tropism are not well understood. Here, we identify an HIV Env variant in the V4 region of gp120, Asp 386 (D386), that eliminates an N-linked glycosylation site at position 386, enhances viral replication in macrophages, and is present at a higher frequency in AIDS patients with HIV-associated dementia (HAD) compared with non-HAD patients. D386 enhances HIV entry and replication in macrophages but not in microglia or peripheral blood mononuclear cells, possibly due to differential glycosylation in these cell types. A D386N mutation in the UK1br Env, which restores the N-linked glycan site, reduced neutralization sensitivity to the IgG1b12 (b12) monoclonal antibody, which recognizes a conserved neutralization epitope that overlaps the CD4 binding site. Molecular modeling suggested that loss of the glycan at position 386 increases exposure of the CD4 and b12 binding sites on gp120. Loss of a glycan at 386 was more frequent in Envs from HAD patients (26%; n=185) compared with non-HAD patients (7%; n=99; $p<0.001$). The most significant association of these Env variants with HAD was in blood or lymphoid tissue rather than brain. These findings suggest that increased exposure of the b12 epitope overlapping the CD4 binding site via elimination of a glycan at position 386 is associated with enhanced HIV macrophage tropism, and provide evidence that determinants of macrophage and microglia tropism are overlapping but distinct.

Keywords

Human immunodeficiency virus type 1 (HIV); envelope; CD4; macrophage tropism; neurotropism; neutralization; b12 antibody; neuropathogenesis; glycosylation

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Introduction

Human immunodeficiency virus type 1 (HIV) infects macrophages and microglia in the central nervous system (CNS) and causes HIV-associated dementia (HAD) or milder forms of neurocognitive impairment (minor cognitive motor disorder (MCMD)) in 10–20% of patients with AIDS (Gonzalez-Scarano and Martin-Garcia, 2005; Kaul et al., 2001; Kaul et al., 2005; McArthur et al., 2003; Sacktor et al., 2002). Highly active anti-retroviral therapy (HAART) has reduced the incidence of HAD (d'Arminio Monforte et al., 2004; Dore et al., 1999; Sacktor et al., 2001; Sacktor et al., 2002), but most current antiviral drugs have relatively poor CNS penetration and the prevalence of MCMD has increased as HIV-infected patients survive for longer times on anti-retroviral therapies (Dore et al., 2003; McArthur et al., 2003; Neuenburg et al., 2002; Valcour et al., 2004). Thus, neurological disease continues to be a significant complication of HIV infection.

HIV variants in brain are genetically distinct from those in lymphoid tissues and other organs, and sequences in the viral envelope glycoprotein (Env) have been associated with brain compartmentalization (Dunfee et al., 2006; Gartner et al., 1997; Korber et al., 1994; Morris et al., 1999; Ohagen et al., 2003; Power et al., 1995; Shapshak et al., 1999; Wang et al., 2001). Furthermore, brain-derived Envs from HAD and non-HAD AIDS patients are genetically and biologically distinct (Power et al., 1994; Power et al., 1998; Shah et al., 2006; Shapshak et al., 1999; Smit et al., 2001). The capacity of HIV or simian immunodeficiency virus (SIV) strains to replicate efficiently in macrophages has been correlated with increased neurotropism (defined here as the ability to replicate in microglia) (Ghorpade et al., 1998; Gorry et al., 2001; Li et al., 1999; Peters et al., 2004; Sharma et al., 1992; Wang et al., 2001), and may also be linked to progression of HIV/SIV disease (Desrosiers et al., 1991; Gray et al., 2005; Mori et al., 1992). However, mechanisms that enhance HIV replication in macrophages in the CNS and other macrophage-rich tissues such as lung, colon, and bone marrow are not well understood.

HIV Env, which is organized into trimers on virions, consists of the gp120 surface and gp41 transmembrane subunits. HIV entry into cells is initiated by interaction between gp120 and CD4, which induces a conformational change in gp120 that exposes the coreceptor binding site (Doms, 2000). The interaction of CD4-bound gp120 with the coreceptor triggers a conformational change in gp120, which leads to structural rearrangements in gp41 that enable fusion and virus entry. CCR5, the primary coreceptor used for infection of macrophages and microglia (Albright et al., 1999; Ghorpade et al., 1998; He et al., 1997; Li et al., 1999), is the coreceptor used by most viruses isolated from brain (Albright et al., 1999; Gorry et al., 2001; He et al., 1997; Li et al., 1999; Smit et al., 2001). However, CCR5 usage is neither necessary nor sufficient for neurotropism (Gorry et al., 2001; Yi et al., 2003). Tissue macrophages and brain microglia express lower cell surface levels of CD4 than CD4+ T cells in peripheral blood (Lewin et al., 1996; Wang et al., 2002). HIV Envs with enhanced tropism for macrophages/microglia have an increased capacity to mediate fusion with cells expressing low levels of CD4 and CCR5, suggesting that reduced dependence on CD4/CCR5 levels may enhance viral replication preferentially in the CNS (Gorry et al., 2002; Gray et al., 2005; Martin et al., 2001; Peters et al., 2004). We previously identified an HIV Env variant associated with brain infection and HAD, N283 in the C2 region of gp120, that increases gp120 affinity for CD4, enhancing the capacity of HIV Envs to use low levels of CD4 for virus entry and increasing viral tropism for macrophages and microglia (Dunfee et al., 2006). However, additional mechanisms by which HIV acquires an enhanced capacity to enter cells when receptor levels are low are unclear.

The gp120 surface subunit of HIV Env is heavily glycosylated (Leonard et al., 1990), resulting in occlusion of receptor binding sites and epitopes for neutralizing antibodies (Kwong et al.,

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1998; Wyatt et al., 1998). The HIV Clade B consensus gp120 amino acid sequence contains 26 motifs for attachment of N-linked glycans (NXS/T, where X is any amino acid except proline). Amino acid variants that result in the loss of N-linked glycosylation sites in the V1/V2 or V4 regions of HIV or SIV Envs can facilitate infection of cells expressing little or no CD4 (Edwards et al., 2001; Hoffman et al., 1999; Kolchinsky et al., 2001; Puffer et al., 2002). Furthermore, studies of pathogenic SIV and chimeric simian-human immunodeficiency virus (SHIV) infections in rhesus macaques demonstrated that macrophage tropism was associated with loss of conserved N-linked glycosylation sites in Env (Igarashi et al., 2003; Puffer et al., 2002). Whether loss of particular N-linked glycosylation sites in HIV/SIV Envs enhances viral replication in the brain *in vivo* remains unknown.

To investigate mechanisms by which HIV acquires enhanced tropism for macrophages and microglia, we analyzed sequences of brain-derived HIV Envs from 2 patients with HAD for changes in N-linked glycosylation sites and identified an amino acid variant, Asp 386 (D386), that eliminates an NXS/T motif at position 386 in the V4 variable region of HIV gp120. D386 preferentially enhances viral entry and replication in macrophages but not microglia or peripheral blood mononuclear cells (PBMC), possibly due to differential N-linked glycosylation in these cell types. Restoring the glycan at 386 increases resistance to neutralizing antibody IgG1b12 (b12), which recognizes a conserved epitope that overlaps the CD4 binding site. Molecular modeling suggested that loss of the glycan at position 386 increases exposure of the CD4 and b12 binding sites on gp120. Loss of a glycan at 386 was significantly more frequent in Envs from HAD patients compared with non-HAD patients. These findings suggest that increased exposure of the b12 epitope overlapping the CD4 binding site via elimination of a glycan at position 386 enhances HIV macrophage tropism, and provide evidence that determinants of macrophage and microglia tropism are overlapping but distinct.

Results

Loss of a potential N-linked glycosylation site at position 386 in the V4 region in brain Envs from patients with HAD

Recently, we cloned and characterized HIV Envs from AIDS patients with HAD and showed that Envs with reduced dependence on CD4 and CCR5 levels are more frequent in brain compared to lymphoid tissues (Thomas et al., 2007), suggesting that viral adaptation for replication in the CNS selects for variants with an enhanced capacity to enter cells expressing low levels of receptors. To investigate mechanisms by which neurotropic Envs acquire the ability to use low CD4, we analyzed 23 full-length Env amino acid sequences from HAD patients MACS2 and UK1 (6 Envs cloned from viral isolates and 17 Envs cloned directly from brain and lymphoid tissues (Gorry et al., 2002; Thomas et al., 2007) for variability in N-linked glycosylation motifs (NXS/T, where X is any amino acid except proline) that might affect exposure of the CD4 binding site. 10/17 brain-derived Envs had an Asp at position 386 in the V4 region, which resulted in elimination of a potential N-linked glycosylation site relative to the Clade B consensus. In contrast, 6/6 lymphoid-derived Envs had an intact N-linked glycosylation site at this position (Fig. 1A). The frequency of D386 was similar in Envs with low versus high CD4 dependence (6/13 and 4/10, respectively). These results suggest that D386 in the V4 region, which results in loss of a potential N-linked glycosylation site, is more frequent in brain compared to lymphoid Envs from two HAD patients.

D386 makes a minor contribution to reduced CD4 dependence

To investigate whether D386 influences the capacity of Envs to use low CD4, we constructed a D386N mutant in UK1br15, a neurotropic Env that efficiently uses low levels of CD4 to mediate fusion and entry (Dunfee et al., 2006; Gorry et al., 2002). Hereafter, this Env is referred to as UK1br. To determine if the D386N mutation, which restores a NXS/T motif, resulted in

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a functional glycosylation site, UK1br wild-type and D386N mutant Envs were analyzed by partial tryptic digestion of 293T cell lysates. Trypsin-digested fragments of UK1br D386N Env migrated more slowly in SDS-PAGE gels compared to wild-type Env fragments, consistent with an additional ~2 kD N-linked glycan relative to wild-type Env (Fig. 1B). The D386N mutation in UK1br Env resulted in a minor reduction in virus entry at all levels of CD4 (Fig. 1C). The magnitude of this effect was less than that of the N283T mutation in UK1br Env, which significantly decreased virus entry into cells expressing low levels of CD4 (Dunfee et al., 2006). These data suggest that loss of a glycan at position 386 of UK1br results in a minor enhancement of virus entry at all CD4 concentrations.

D386 preferentially enhances macrophage tropism

To determine if D386 influences macrophage and/or microglia tropism, we cloned UK1br wild-type or D386N mutant Envs into pNL4-3 and infected PBMC, monocyte-derived macrophages (MDM), or microglia in primary human brain cultures. Viruses expressing wild-type or mutant Envs had similar replication kinetics and cytopathic effects in PBMC (n=4 donors; Fig. 2A) and microglia in primary brain cultures (n=4 of 5 donors; Fig. 2C and data not shown). In contrast, the D386N mutant virus had delayed replication kinetics and reduced syncytia formation in MDM (n=4 donors; Fig. 2B), similar to the phenotype of the UK1br N283T mutant virus that we described previously (Dunfee et al., 2006). These results suggest that D386, or the loss of a glycan at position 386, preferentially enhances UK1br tropism for macrophages.

To investigate whether D386 enhances macrophage tropism in other brain-derived Envs, we used site-directed mutagenesis to introduce N386D mutations in JRFL and YU2, well-characterized, macrophage-tropic Envs from brain tissues of HAD patients (Li et al., 1991; O'Brien et al., 1990), and in UK1fl2-15 and UK1bg4-13, Envs cloned directly from the brain of patient UK1 that enter MDM at high and intermediate levels, respectively, compared with other UK1 Envs (Thomas et al., 2007). We also introduced a D386N mutation in MACS2br13, a brain-derived Env from patient MACS2 with high CD4 dependence (Gorry et al., 2002) (Fig. 3A). Wild-type and mutant Envs were expressed and processed to gp120 at similar levels, and the loss or gain of an N-linked glycan was suggested by slight differences in mobility in SDS-PAGE gels compared to wild-type Envs (Fig. 3B). Consistent with our previous results (Thomas et al., 2007), UK1 Envs had less efficient processing of gp160 to gp120 compared to other Envs. MDM (from donor A in Fig. 2) and Cf2th cells expressing different levels of CD4 and CCR5 were infected with luciferase reporter viruses pseudotyped with wild-type and mutant Envs. In JRFL Env, a N386D change increased virus entry to a similar extent in MDM and Cf2th cells (50% and 58%, respectively). A N386D mutation in UK1fl2-15 Env decreased the ability to enter MDM by 37%, but had no effect on entry in Cf2th cells (Fig. 3C). Thus, D386 does not preferentially enhance macrophage tropism in these highly macrophage-tropic Envs (O'Brien et al., 1990; Thomas et al., 2007). In contrast to these results, in YU2 and UK1bg4-13 Envs, a N386D change increased virus entry in MDM by 200% and 450%, respectively, compared to wild-type (p=0.008 and 0.049, respectively, Student's t test), but did not increase entry in Cf2th cells (p=0.14 and 0.27, respectively) (Fig. 3C). A reciprocal D386N change in MACS2br13 Env resulted in a 65% decrease in the ability to infect MDM compared to the wild-type Env (p=0.027), but did not have a significant effect on entry in Cf2th cells (p=0.79) (Fig. 3D). Collectively, Envs with D386 had an increased capacity to enter macrophages and Cf2th cells compared to Envs with N386 (p=0.014, Student's t test, n=10 (5 Envs, 2 cell types)). These results suggest that loss of an N-linked glycan at position 386 enhances HIV entry in macrophages and transfected Cf2th cells in 5/6 Envs from 4 HAD patients, and that D386 preferentially enhances macrophage tropism in 4/6 Envs from 3 HAD patients (YU2, UK1br, UK1bg4-13, and MACS2br13).

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Increased glycosylation of UK1br Envs produced in MDM compared to microglia or PBMC

HIV Envs expressed in infected macrophage and PBMC cultures are differentially glycosylated (Lin et al., 2003; Willey et al., 1996). Thus, one possible explanation for the different effects of the UK1br D386N mutation on replication in PBMC, MDM, and microglia is differential N-linked glycosylation of Envs produced in these cell types. To investigate glycosylation of UK1br Env produced in primary cells, we purified virions from supernatants of infected PBMC, MDM, and primary brain cultures and analyzed virion-associated gp120 by Western blotting. Consistent with previous studies (Lin et al., 2003; Willey et al., 1996), UK1br Envs produced in MDM had decreased mobility and a more diffuse migration pattern in SDS-PAGE gels compared to Envs produced in PBMC (Fig. 4). Unexpectedly, Envs produced in MDM migrated more slowly and had a more diffuse pattern in SDS-PAGE gels than Envs produced in microglia (Fig. 4). Together with previous studies demonstrating that differences in mobility of MDM- and PBMC-derived Envs were due to glycosylation (Lin et al., 2003; Willey et al., 1996), these results suggest that UK1br Envs are more highly glycosylated when expressed in MDM compared to PBMC or microglia. These findings are consistent with the possibility that differences in replication of the UK1br wild-type and D386N mutant viruses in these primary cell types are due to differential N-linked glycosylation.

D386 influences sensitivity of UK1br to neutralization by the CD4 binding site monoclonal antibody b12

Env glycosylation is important for maintaining structure and shielding conserved structural epitopes from neutralizing antibodies (Burton et al., 2004). To investigate whether the loss of a glycan at position 386 influences sensitivity to neutralizing antibodies, we analyzed neutralization of viruses containing wild-type and D386N mutant UK1br Envs by a panel of monoclonal antibodies (mAbs) (summarized in Table 1). We previously demonstrated that the UK1-br primary isolate is sensitive to neutralizing antibody b12, which maps to an epitope that overlaps the CD4 binding site, and CD4-IgG2 (Gorry et al., 2002). A D386N change resulted in an 8-fold increase in resistance to neutralization by b12 and a 2-fold increase in resistance to CD4-IgG2 (Table 1 and Fig. 5). In contrast, there was no difference in sensitivity of the wild-type or D386N mutant UK1br viruses to neutralization by mAbs directed against other epitopes in gp120 and gp41 (Table 1). Together, these results suggest that D386 influences sensitivity of UK1br Env to neutralization by antibody b12, and raise the possibility that changes in amino acid sequence at this position may affect the conformation and/or exposure of the CD4 and mAb b12 binding sites.

Loss of a glycan at 386 increases exposure of the CD4 and mAb b12 binding sites

To investigate the mechanism by which changes at position 386 might affect the CD4 binding site, we used Swiss PDB Viewer (http://ca.expasy.org/spdbv) to model the amino acid at this position on the YU2 gp120-CD4-17b crystal structure (1G9N) (Kwong et al., 1998). The HIV Clade B consensus amino acid at this position is Asn (Fig. 6A). N386 is located on the highly glycosylated outer domain of gp120 (Kwong et al., 1998; Wyatt et al., 1998), proximal to the base of the V3 loop and the CCR5 binding site, and ~12 Å from the CD4 binding site. Introducing an N386D change in the structural model did not alter predicted interactions with amino acids that contact CD4 (data not shown). Previous studies demonstrated that N386 in monomeric gp120 produced in CHO cells is glycosylated with a high mannose group (Leonard et al., 1990). To better understand how a glycan at this position might affect the CD4 binding site, we aligned the N-acetylglucosamine (NAG) attached to N386 in the YU2 crystal structure with the first NAG of a high mannose group from the crystal structure of HIV mAb 2G12 bound to its $Man_9GlcNAc_2$ epitope (1OP5) (Calarese et al., 2003). The addition of the mannose group resulted in a modified N386 in which the carbohydrate side chains were only ~7 Å from CD4, partially occluding the CD4 binding site (Fig. 6B). Together with previous studies

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demonstrating that removal of the glycan at position 386 affects the conformation of surrounding carbohydrate chains (Scanlan et al., 2002), the structural modeling data suggest that removal of this N-linked glycosylation site may directly or indirectly increase exposure of the CD4 binding site.

To investigate the mechanism by which D386 might enhance sensitivity to mAb b12, we modeled the N386D change on the recently described HXB2 gp120-b12 crystal structure (Zhou et al., 2007). N386 and the attached NAG contact Trp 100 (W100) of the heavy chain of b12, with the amino acid side chains stacking together (Fig. 6C) and a hydrogen bond between the backbone of W100 and the NAG (data not shown) (Zhou et al., 2007). Modeling a D386 change in the structure eliminated the hydrogen bond between W100 and the NAG of N386, but D386 and W100 of b12 side chains had a similar stacking conformation as the wild-type N386 (Fig. 6D). Thus, a D386N change would be predicted to enhance b12 binding and neutralization by restoring the hydrogen bond between the NAG on N386 and the mAb heavy chain. However, a D386N mutation in UK1br Env had the opposite effect and increased resistance to neutralization by b12. These data suggest that a mechanism other than direct binding of b12 to D386, such as increased exposure of the b12 epitope, underlies the increase in b12 neutralization sensitivity mediated by D386.

Relationship between neutralization sensitivity to mAb b12 and enhanced macrophage tropism

To investigate whether increased neutralization sensitivity to mAb b12 is associated with enhanced macrophage tropism in a larger data set, we tested b12 neutralization of viruses expressing UK7br, ADA, or MACS2br13 Envs, and performed a meta-analysis of these results together with b12 neutralization data from 4 published studies (Binley et al., 2004; Gray et al., 2005; Thomas et al., 2007; Wei et al., 2003) (Table 2). For this analysis, we compiled results for HIV Envs or isolates that have been well-characterized for their macrophage tropism (Collman et al., 1989; Dunfee et al., 2006; Gray et al., 2005; Li et al., 1991; O'Brien et al., 1990; Thomas et al., 2007). Highly macrophage-tropic viruses had increased sensitivity to b12 neutralization (median 50% neutralization (IC_{50}) 0.16 μg/ml, n=10) compared to Envs with low or intermediate macrophage tropism (median IC_{50} 4.21 μg/ml, n=8) (p=0.0044, Mann-Whitney test). Furthermore, 9 of 11 (82%) of Envs with b12 $IC_{50} \leq 2$ μg/ml were highly macrophage-tropic, compared with only 1 of 7 (14%) of Envs with b12 $IC_{50} > 2$ μg/ml (p=0.013, Fisher's exact test). Thus, there is a significant association between increased sensitivity to b12 neutralization and enhanced macrophage tropism. In contrast, there was no correlation between sensitivity to other gp120 mAbs and enhanced macrophage tropism in this data set (Binley et al., 2004; Gray et al., 2005; Thomas et al., 2007; Wei et al., 2003) (Table 1). Viruses with Env variant D386 were sensitive to b12 neutralization (n=4, IC_{50} 0.03 to 2.8 μg/ml), whereas viruses with N386 had variable sensitivity to b12 neutralization (n=14, IC_{50} 0.03 to 25 μg/ml) (Table 2). Together, these findings suggest that increased exposure of the b12 epitope is associated with enhanced tropism of HIV for macrophages. The influence of the N-linked glycosylation site at position 386 on b12 neutralization sensitivity, however, is strain-dependent.

Loss of a glycan at position 386 is associated with dementia *in vivo*

To determine if the loss of an N-linked glycan at position 386 is associated with brain infection or dementia *in vivo*, we examined 284 matched brain- and blood- or lymphoid-derived Envs from 22 patients in 10 published studies (Dunfee et al., 2006; Gartner et al., 1997; Gorry et al., 2002; Korber et al., 1994; Liu et al., 2000; Martin-Garcia et al., 2006; Ohagen et al., 2003; Pang et al., 1991; Shapshak et al., 1995; Thomas et al., 2007). D386 or another amino acid variant that results in loss of the NXS/T motif at position 386 appeared in 18% of brain-derived sequences (n=152) and 20% of blood or lymphoid-derived sequences (n=132) (Table 3). Thus,

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loss of an N-linked glycan at position 386 is not associated with brain compartmentalization. To determine if loss of a glycan at this site is associated with HAD in this data set, we compared sequences from HAD and non-HAD patients. Amino acid variants that resulted in the loss of the glycosylation motif appeared in 26% of sequences from 13 HAD patients (n=185), compared to only 7% of sequences from 9 non-HAD patients (n=99) (Table 3). In contrast, variants that eliminated any of the 4 other NXS/T motifs in the Clade B consensus V4 region appeared at similar or lower frequencies in HAD compared to non-HAD patients (data not shown). Thus, the loss of an N-linked glycan at position 386 was significantly associated with dementia ($p<0.001$, Fisher's exact test, with Bonferroni correction for multiple comparisons). Although amino acid variants resulting in elimination of the NXS/T motif appeared in 22% of brain-derived sequences from HAD patients (n=103) compared to 10% from non-HAD patients (n=49), the most significant association of these variants with HAD was in the blood or lymphoid compartment. Amino acid variants resulting in the loss of a glycosylation site at position 386 appeared in blood or lymphoid-derived Envs in 30% of sequences from HAD patients (n=82) compared to only 4% of sequences from non-HAD patients (n=50; $p<0.001$) (Table 3). These results suggest that D386 and other variants that result in elimination of an N-linked glycosylation site at position 386 are associated with dementia *in vivo*.

Discussion

In this study, we demonstrated that D386 and other amino acids that result in the loss of an N-linked glycosylation site in the V4 region of HIV gp120 are more frequent in brain and lymphoid Envs from HAD patients (26%, n=185) compared to non-HAD patients (7%, n=99; $p<0.001$). D386 preferentially enhances HIV tropism for macrophages but not microglia or PBMC, possibly as a result of differential modification of N-linked glycosylation motifs in these primary cell types. A D386N mutation in UK1br Env increased resistance to b12 neutralization by 8-fold compared to wild-type. These results, together with molecular modeling studies, suggest that loss of a glycan at position 386 increases exposure of the CD4 binding site. These findings also suggest an association between exposure of the b12 epitope, which overlaps the CD4 binding site, and enhanced HIV tropism for macrophages. Consistent with this idea, increased b12 neutralization sensitivity is associated with enhanced macrophage tropism of other Clade B Envs as well ($p=0.0044$, Mann-Whitney test).

Molecular modeling with the D386 variant in the YU2 crystal structure (1G9N) (Kwong et al., 1998) suggests that removal of the high mannose group attached to position 386, which is located on the heavily glycosylated outer domain of gp120, increases exposure of the CD4 binding site. Restoring the N-glycan at 386 increased UK1br resistance to neutralization by sCD4-Ig by only 2-fold, consistent with our finding that a D386N mutation had only a minor effect on the capacity of UK1br Envs to use low CD4. In contrast, the N283T mutation, which influences gp120 affinity for CD4 but has no significant effect on b12 neutralization (R. Dunfee and D. Gabuzda, unpublished data), has a significant effect on virus entry into cells expressing low CD4 (Dunfee et al., 2006). The observation that UK1br D386N mutant virus was 8-fold more resistant to neutralization by b12 than the wild-type virus was unexpected, since molecular modeling with the HXB2 gp120-b12 crystal predicted that a D386N mutation would enhance b12 binding by restoring interactions between the glycan at N386 and the mAb heavy chain. The influence of this glycan on b12 sensitivity is strain-dependent, however (Table 2). D386 may work cooperatively with changes in the V1/V2, C2, V3, and/or C3 regions, since these regions also influence sensitivity to b12 neutralization (Koch et al., 2003; Mo et al., 1997; Pugach et al., 2004). Alternatively, changes in glycan structure at position 386 could affect the conformation and packing of other carbohydrates on the outer domain of gp120 to modulate exposure of the CD4 and mAb b12 binding sites. The finding that loss of an N-linked glycan at position 386 in an HXB2 Env adapted to use only CXCR4 for entry enhanced sensitivity to neutralization by HIV-infected patient sera (Edwards et al., 2001) provides further

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evidence that changes at position 386 influence exposure of the CD4 binding site and conserved neutralization epitopes on gp120. Position 386 is located at the base of the V3 loop and proximal to the coreceptor binding site. Thus, changes at this position may influence Env interactions with CCR5 as well.

Brain macrophages and microglia are both derived from the mononuclear phagocyte lineage, yet they differ in some phenotypic and functional characteristics (Gonzalez-Scarano and Martin-Garcia, 2005). Whether HIV tropism for macrophages and microglia is similar (Ghorpade et al., 1998; Gorry et al., 2001), or overlapping but distinct (Strizki et al., 1996), has been debated. The D386N mutation in UK1br decreased viral entry and replication in macrophages but had no effect on viral replication in microglia. In contrast, the N283 variant, which increases gp120 affinity for CD4, enhances viral tropism for both macrophages and microglia (Dunfee et al., 2006). MDM-derived Envs migrated more slowly on SDS-PAGE gels than microglia- or PBMC-derived Envs, consistent with increased gp120 glycosylation. Thus, D386 may preferentially enhance HIV replication in macrophages as a consequence of differential N-linked glycosylation in these primary cell types.

The mechanism by which loss of an N-linked glycan at position 386 contributes to enhanced tropism for macrophages, but not PBMC or microglia, is unknown. Restoring the glycosylation site with a D386N mutation in UK1br Envs had a minor effect on virus entry in Cf2th cells at all levels of CD4, raising the possibility that D386 enhances Env interactions with CD4, and/or CCR5, albeit to a minor extent. One possible explanation for preferential enhancement of macrophage tropism by D386 is differential glycosylation of the NXS/T motif at 386 in PBMC, macrophages, and microglia. An alternative possibility is that changes in glycosylation at position 386 influence Env interactions with molecules important for initial attachment to macrophages, such as DC-SIGN, macrophage mannose receptor, syndecans, or other attachment factors (Binley et al., 2006; Nguyen and Hildreth, 2003; Saphire et al., 2001). Further studies are needed to investigate these possibilities.

The CNS has lower concentrations of neutralizing antibodies compared to peripheral tissues (Goudsmit et al., 1987; Kaul et al., 2001), and may therefore be a favorable environment for replication and persistence of HIV variants with exposed receptor binding sites. Most HIV-infected individuals do not develop neurological dysfunction until after development of immunosuppression and progression to AIDS. Loss of immune control associated with disease progression is one possible reason why HAD typically occurs only in the late stages of HIV infection (Gonzalez-Scarano and Martin-Garcia, 2005). van Marle *et al* (Van Marle et al., 2002) demonstrated that sera from HAD patients was less efficient at neutralizing HIV than sera from non-HAD patients, suggesting that patients with HAD may have a decreased ability to generate neutralizing antibodies. The significantly higher frequency of D386, a viral variant that enhances b12 neutralization sensitivity, in blood- and lymphoid-derived Envs from HAD patients compared to non-HAD patients, is consistent with the possibility that loss of a glycan at 386 may have evolved more readily in these individuals because they have lower levels of neutralizing antibodies that target the b12 epitope. In a longitudinal study of neutralizing antibody responses, changes at positions 400–406 in the V4 region were indicative of adaptive mutations selected by immune responses (Wei et al., 2003). In our Env sequence data set, position 406 was the only N-linked glycosylation motif in the V4 region that was eliminated at a significantly higher frequency in non-HAD patients (49%, n=99) compared to HAD patients (26%, n=185; $p<0.001$, Fisher's exact test, with Bonferroni correction for multiple comparisons) (data not shown). These observations and the association between enhanced macrophage tropism and increased exposure of the b12 epitope demonstrated here and by others (Gray et al., 2005) raise the possibility that macrophage-tropic strains may evolve more readily in individuals with low levels of neutralizing antibodies directed against outer domain regions of Env.

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The identification and characterization of the loss of an N-linked glycan at position 386 in the V4 region of gp120 suggests that increased exposure of the b12 epitope overlapping the CD4 binding site enhances HIV macrophage tropism, and provide evidence that determinants of macrophage and microglia tropism are overlapping but distinct. These findings also provide insights that may facilitate development of immunogens that elicit broadly neutralizing antibodies as well as therapeutics to prevent CNS infection and neurologic injury in HIV-infected patients.

Materials and methods

Cells

293T cells and canine thymocyte cell line Cf2th (Choe et al., 1996) were cultured in DMEM medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 100 μg/ml penicillin and streptomycin. Cf2-Luc cells, which are derived from Cf2th cells and stably express luciferase under control of HIV-1 LTR (Etemad-Moghadam et al., 2000), were cultured in medium supplemented with 0.7 mg/ml G418 (Mediatech, Herndon, VA). Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy HIV-1-negative donors as described (Gorry et al., 2001), stimulated with 2 μg/ml phytohemagglutinin (PHA; Sigma, St. Louis, MO), and cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 100 μg/ml penicillin and streptomycin, and 20 U/ml interleukin-2 (Boehringer Mannheim, Germany). CD8+ T cells were depleted with anti-CD8-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA). Monocyte-derived macrophages (MDM) were isolated from PBMC by plastic adherence and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μg/ml penicillin and streptomycin, and 10 ng/ml macrophage colony stimulating factor (M-CSF; R&D Systems, Minneapolis, MN) (Gorry et al., 2001). Primary human fetal brain cultures containing a mixture of astrocytes, neurons, and microglia were prepared and maintained in 48-well tissue culture plates as described (Gorry et al., 2001; Gorry et al., 2002) and cultured in DMEM medium supplemented with 10% bovine calf serum (HyClone, Logan, UT), 100 μg/ml penicillin and streptomycin, 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA), and 10 ng/ml M-CSF.

Virus isolates, HIV Env cloning, and sequence analysis

Primary viruses MACS2-br and UK1-br were previously isolated from autopsy brain tissues from AIDS patients with HAD (Gorry et al., 2001). Env genes amplified from genomic DNA extracted from PBMC on day 7 post-infection with these primary isolates or from autopsy brain tissue and cloned into pCR3.1 were described previously (Dunfee et al., 2006; Gorry et al., 2002; Thomas et al., 2007). Env protein expression was verified by Western blot with goat anti-gp120 (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, MD) (Gorry et al., 2002). Sequences were aligned using Clustal X. D386N and N386D mutant Env plasmids were created by PCR-based mutagenesis and changes were verified by DNA sequencing. Differences in migration of UK1br wild-type and D386N mutant Envs on SDS-PAGE gels, consistent with changes in glycosylation, were verified by Western blot with goat anti-gp120 of 293T lysates incubated with 0.01 μg/ml trypsin (Sigma, St. Louis, MO) for 2 h at 37°C, run on a 4–20% SDS-PAGE gradient gel, and analyzed by Western blot with goat anti-gp120.

Fusion assays

293T cells were cotransfected with 15 μg of Env-expressing plasmid and 2 μg of pLTR-Tat using calcium phosphate. Cf2-Luc cells (Gorry et al., 2002) were cotransfected with different amounts of pcDNA3-CD4 and pcDNA3-CCR5 as indicated using Lipofectamine 2000 (Invitrogen). The total amount of DNA in each transfection was normalized using pcDNA3. 2.5×10^4 293T cells and 2.5×10^5 Cf2-Luc cells were mixed in 48-well plates and incubated

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at 37°C for 8 to 12 h. Cells were lysed and assayed for luciferase activity. 293T cells cotransfected with a nonfunctional Env (pSVIII-ΔKSenv) and pLTR-Tat were used to determine background levels.

Entry assays

The KpnI to BamHI region of *env* was PCR amplified and cloned into pNL4-3 to generate replication-competent chimeric viruses (Ohagen et al., 2003). HIV luciferase reporter viruses were generated by cotransfection of 293T cells with pNL4-3env^{-}luc, an HIV provirus with *env* deleted and *nef* replaced by luciferase, and an Env-expressing plasmid as described (Gorry et al., 2002). Cf2th or Cf2-Luc cells were cotransfected with pcDNA3-CD4 and pcDNA3-CCR5 as above. Transfected cells were infected with 10^4 ^{3}H cpm reverse transcriptase (RT) units of virus stock. Cells were lysed 48 to 60 h post-infection and assayed for luciferase activity. MDM were prepared as above in 48-well plates and infected with 2×10^4 ^{3}H cpm RT units of virus stock. Cells were lysed 6 days post-infection and assayed for luciferase activity.

HIV replication kinetics

3×10^6 PBMC were prepared as described (Gorry et al., 2001) and incubated with 5×10^4 ^{3}H cpm RT units of virus stock for 3 h at 37°C. MDM were prepared by plastic adherence as described above in 24-well tissue culture plates, cultured in the presence of M-CSF for 7 days prior to infection, and incubated with 2×10^4 ^{3}H cpm RT units for 3 h at 37°C. Primary human fetal brain cultures containing a mixture of astrocytes, neurons, and microglia were prepared and maintained in 48-well tissue culture plates as described (Gorry et al., 2001; Gorry et al., 2002) and incubated with equivalent amounts of virus stock (10^4 ^{3}H cpm RT units) for 16 h at 37°C. 50% media changes were performed every 3 to 7 days for 28 to 35 days. Virus replication was monitored by p24 ELISA (Perkin Elmer, Boston, MA).

Virion preparation and Western blot

Virions were purified from cell culture supernatant of infected PBMC, MDM, or primary human fetal brain cultures by high-speed centrifugation on a 20% sucrose gradient and resuspended in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.05 M Tris hydrochloride buffer [pH 7.5], 0.15 M NaCl, 1 mM EDTA, Complete protease inhibitor cocktail (Roche, Indianapolis, IN)) at a concentration of 1 ng p24 antigen/μl. Equivalent amounts of virions were run on a 4–20% SDS-PAGE gradient gel, and analyzed by Western blotting with goat anti-gp120 as above or with rabbit anti-p24 (Abbott Laboratories, Abbott Park, IL).

Neutralization assays

Replication-competent chimeric virus stocks were incubated with a range of concentrations of human monoclonal Abs (mAbs), or CD4-IgG2 1 h prior to infection of Cf2-Luc cells transiently expressing CD4 and CCR5. Cells were harvested 48 h post infection and assayed for luciferase activity. mAbs b12 (Roben et al., 1994), 2G12 (Buchacher et al., 1994), and 2F5 (Buchacher et al., 1994; Purtscher et al., 1994) were obtained from D. Burton and P. Parren, and H. Katinger (2G12 and 2F5), respectively, through the AIDS Research and Reference Reagents Program. mAb 17b (Thali et al., 1993) was kindly provided by J. Robinson. mAb F105 was kindly provided by M. Posner and L. Cavacini. The plasmid expressing CD4-IgG2 was kindly provided by M. Farzan.

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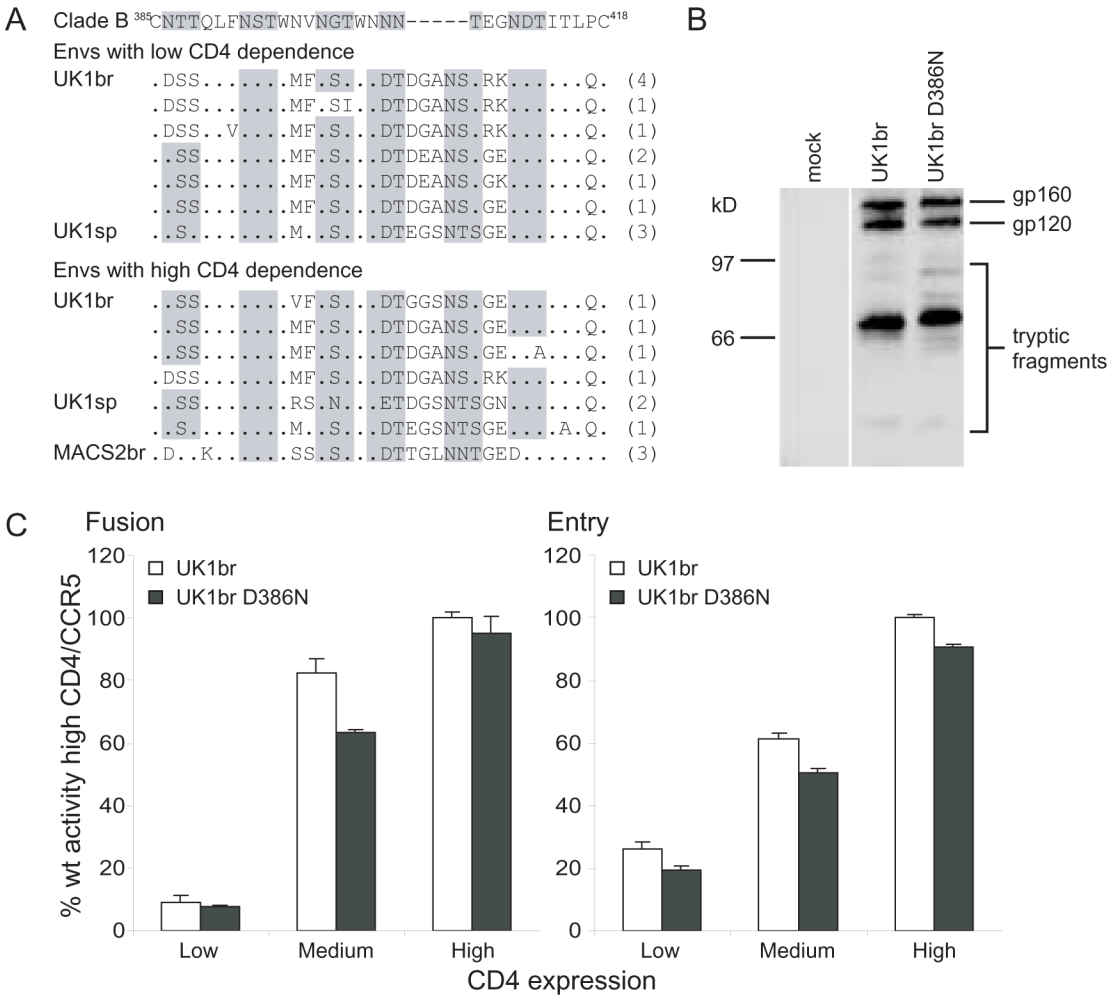

Fig. 1. D386 is associated with brain-derived Envs from 2 HAD patients and has a minor effect on reduced CD4 dependence

(A) Amino acid sequences from the V4 region of gp120 from 2 HAD patients were obtained from published studies (Gorry et al., 2002; Thomas et al., 2007) and aligned using Clustal X. Potential N-linked glycosylation sites are highlighted in gray. Sequences are numbered relative to the HXB2 reference sequence. Sequence names are notated as follows: br, brain-derived; sp, spleen-derived. The number in parentheses represents the number of Env clones with each sequence. Dots represent residues identical to the Clade B consensus sequence, and dashes represent gaps. (B) Lysates from 293T cells transfected with UK1br wild-type or D386N mutant Envs were incubated with 0.01 μg/ml trypsin for 2 h at 37°C and analyzed by Western blotting with anti-gp120. (C) Cf2-Luc cells expressing different levels of CD4 and high CCR5 were mixed with 293T cells expressing UK1br wild-type or D386N mutant Envs in cell-cell fusion assays (left) or infected with recombinant viruses expressing wild-type or mutant Envs in entry assays (right). Cells were lysed after 8–12 h incubation or 48 h post-infection, respectively, and analyzed for luciferase activity. Data were normalized to luciferase activity of the wild-type Env on cells expressing high CD4 and are expressed as means of duplicate wells from a single experiment. Error bars represent standard deviations. Data are representative of 3 independent experiments.

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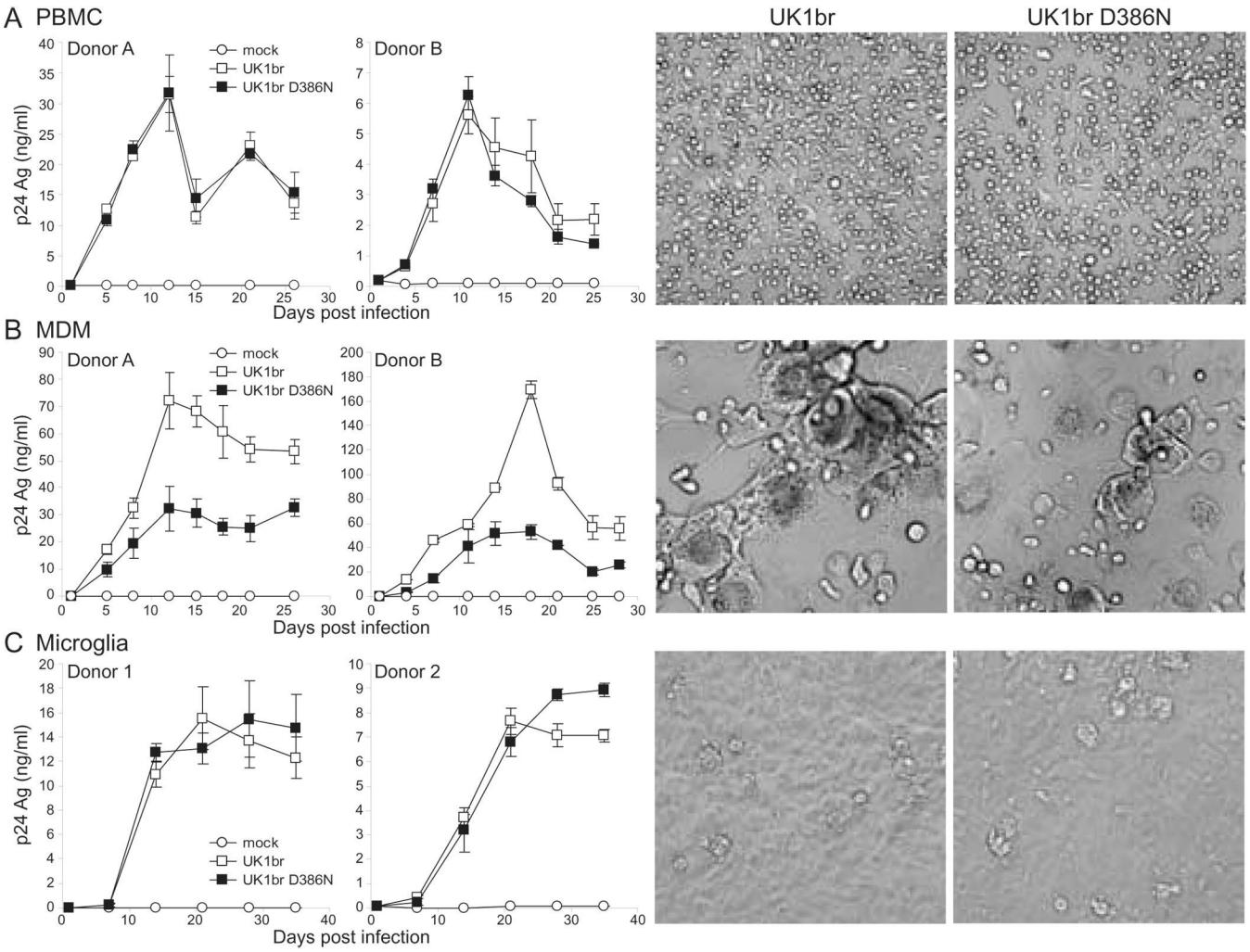

Fig. 2. Replication kinetics and cytopathic effects of viruses expressing UK1br wild-type and D386N mutant Envs in PBMC, MDM, and microglia

PBMC (A), MDM (B), and microglia in primary human brain cultures (C) were infected as described in Materials and Methods. Supernatants were collected every 3–7 days and replication was monitored by p24 ELISA. Results shown are from triplicate samples. Error bars represent standard deviations. Data are representative of independent experiments in 4 different donors per cell type. Photographs were taken at 14 (PBMC and MDM) or 28 (microglia) days post-infection at a 200X magnification.

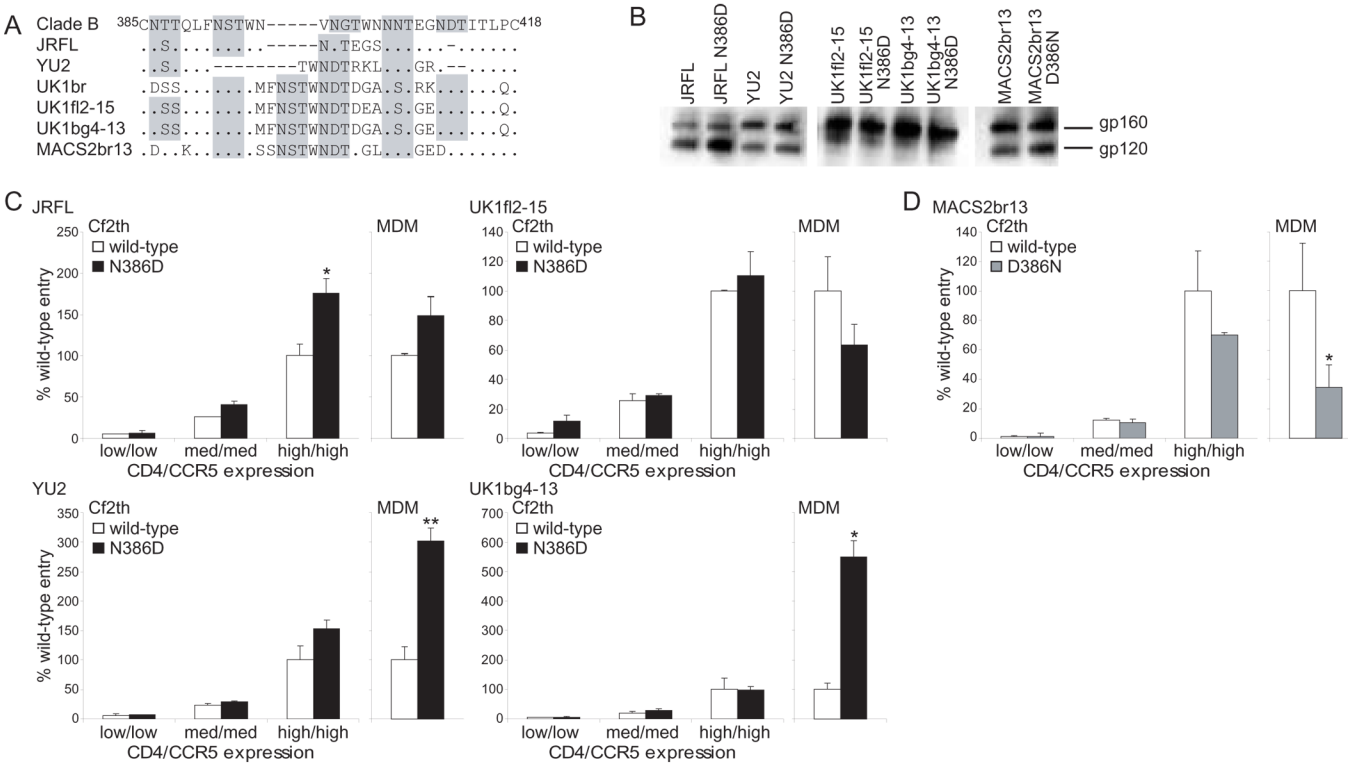

Fig. 3. D386 in Envs with low or intermediate macrophage-tropism increases virus entry into macrophages

(A) Amino acid sequences from the V4 region of gp120 of brain-derived Envs used in this study were obtained from Genbank (JRFL and YU2, accession numbers U63632 and M93258, respectively) or published studies (Gorry et al., 2002; Thomas et al., 2007) and aligned using Clustal X. Potential N-linked glycosylation sites are highlighted in gray. Sequences are numbered relative to the HXB2 reference sequence. Dots represent residues identical to the Clade B consensus sequence, and dashes represent gaps. (B) 293T cells expressing wild-type or mutant Envs were lysed and analyzed by Western blotting with anti-gp120. The positions of gp160 and gp120 are indicated on the right. (C, D) Cf2th cells expressing different levels of CD4 and CCR5 or MDM were infected with luciferase-expressing reporter viruses pseudotyped with wild-type, N386D mutant (C), or D386N mutant (D) Envs. Cells were lysed 48 to 72 h postinfection and analyzed for luciferase activity. Data were normalized to the amount of luciferase activity of the wild-type Env on cells expressing high CD4/CCR5 or on MDM. Results shown are from duplicate (Cf2th) or triplicate (MDM) samples. Error bars represent standard deviations. *, $p<0.05$, **, $p<0.01$, Student's t test.

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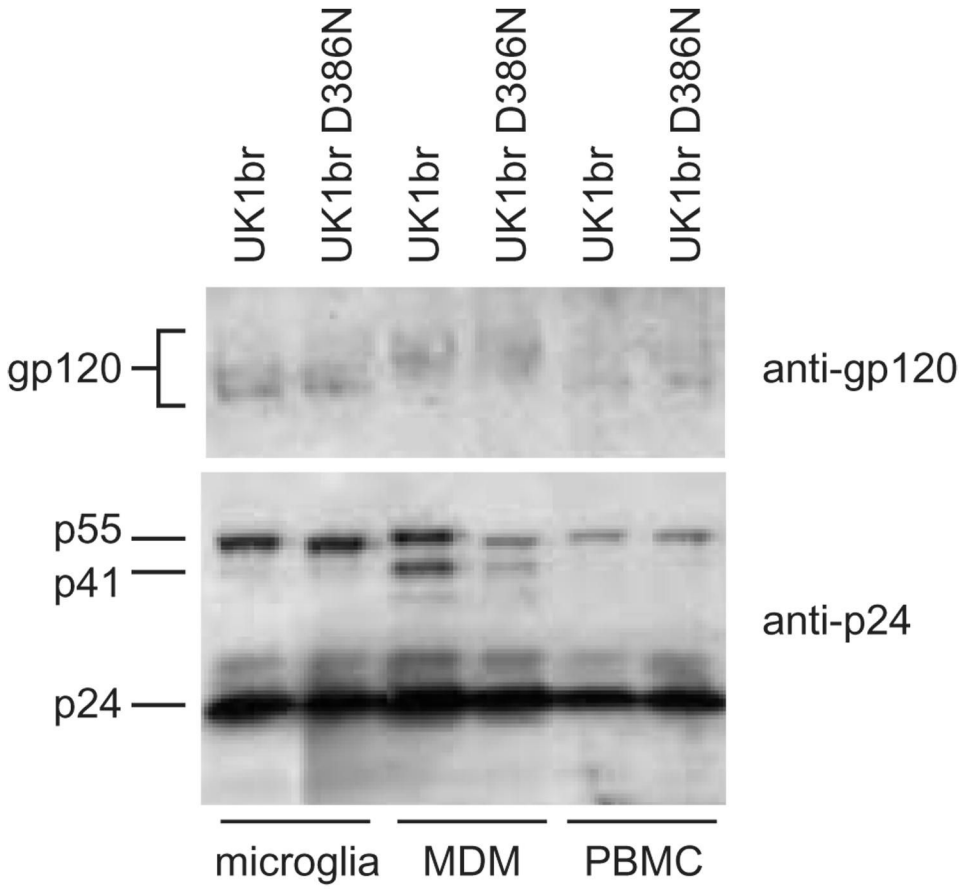

Fig. 4. Western blot of virions from infected microglia, MDM, and PBMC

Virions from infected microglia, MDM, or PBMC cultures were purified by high-speed centrifugation on a sucrose cushion as described in Materials and Methods, lysed, and analyzed by Western blotting with anti-gp120 or anti-p24. The positions of gp120, p24, and the Gag precursor proteins p55 and p41 are indicated on the left.

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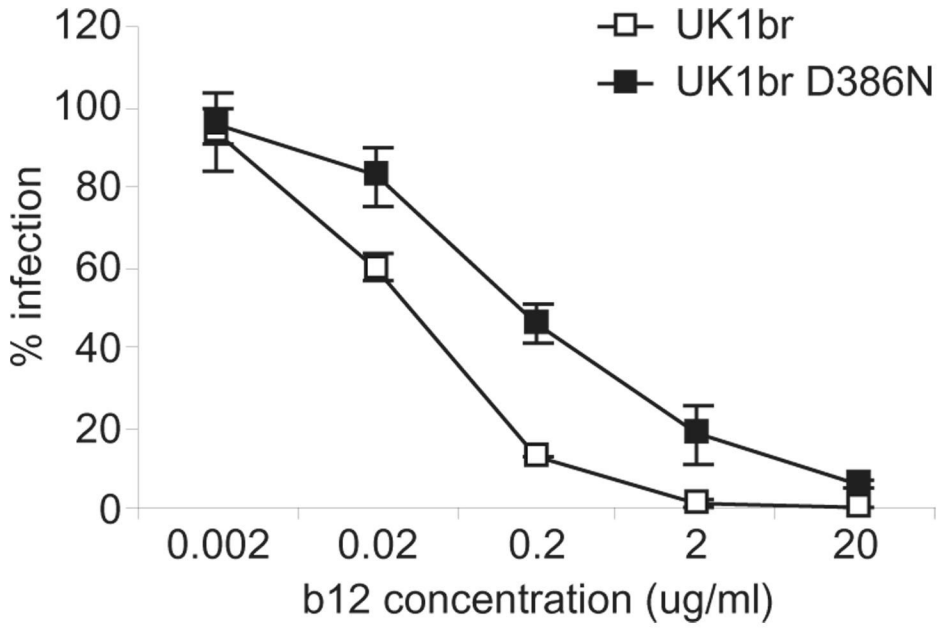

Fig. 5. Neutralization of UK1br wild-type and D386N mutant viruses by mAb b12

Recombinant viruses expressing UK1br wild-type or D386N mutant Envs were incubated with mAb b12 for 1 h and then used to infect Cf2-Luc cells expressing high levels of CD4 and CCR5. Cells were lysed 36 h postinfection and analyzed for luciferase activity. Data are normalized to the amount of luciferase activity in cells infected with virus in the absence of b12 and expressed as the mean of duplicate samples. Error bars represent standard deviations. Results are representative of 4 independent experiments.

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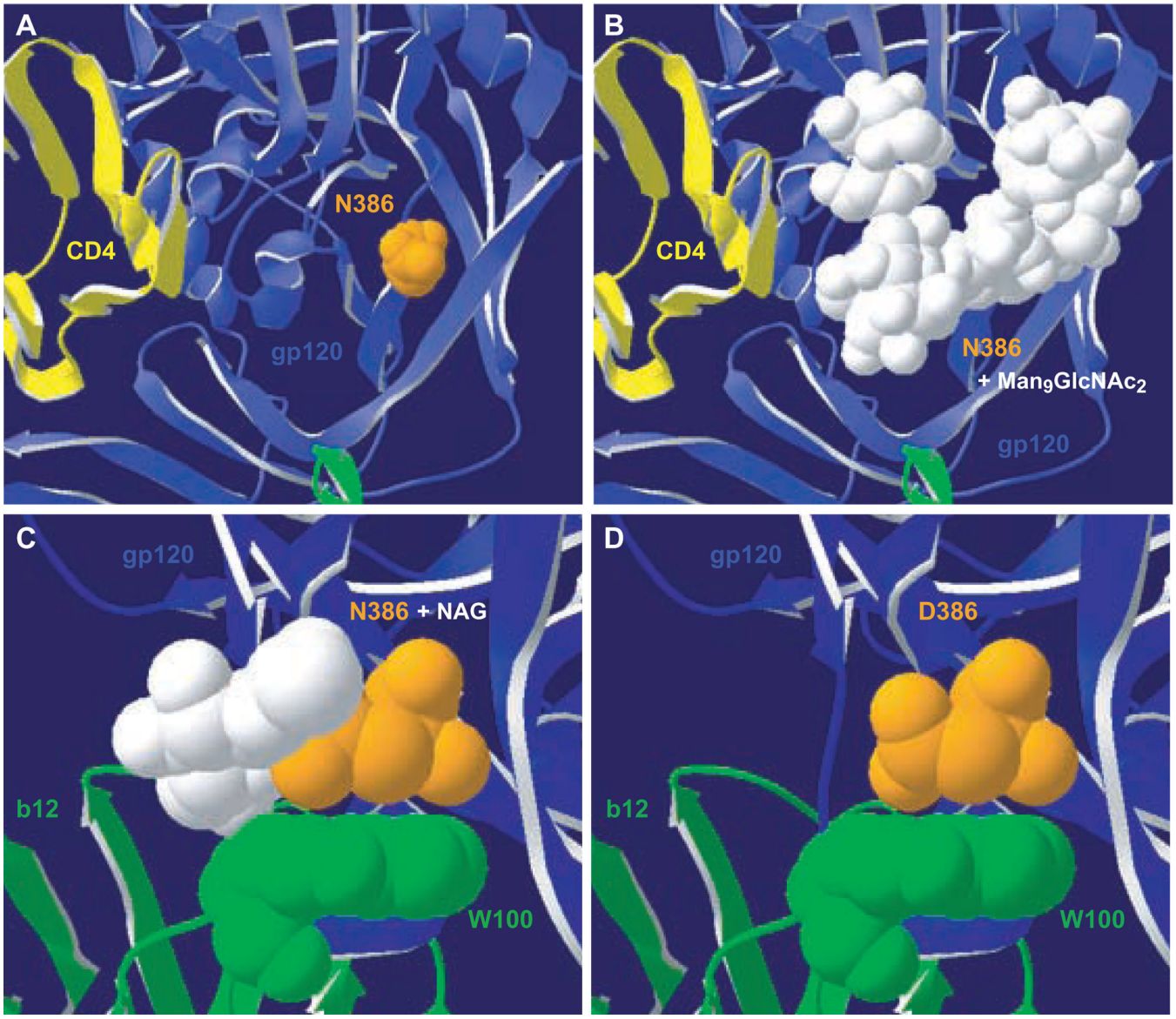

Fig. 6. Structural modeling of position 386 in Envs bound to CD4 or mAb b12

Swiss PDB Viewer was used to model changes at position 386 on the V4 region of the YU2 gp120-CD4-17b CD4i Ab crystal structure (1G9N) (Kwong et al., 1998) (A, B) or the HXB2 gp120-mAb b12 crystal structure (2NY7) (Zhou et al., 2007) (C, D). Blue, gp120. Yellow, CD4. Green, 17b CD4i Ab (A, B) or mAb b12 (C, D). Orange, position 386. White, carbohydrate moieties. (A) Location of N386 in the YU2 crystal structure. Position 386 is ~12Å from the CD4 binding site. (B) The N-acetylglucosamine (NAG) attached to N386 was aligned with NAG1 from a high mannose group ($Man_9GlcNAc_2$) derived from the crystal structure of mAb 2G12 bound to its carbohydrate epitope (1OP5) (Calarese et al., 2003) to produce a model of gp120 with intact glycosylation at position 386. (C) N386 and the attached NAG make contacts with W100 of mAb b12. (D) Swiss PDB Viewer was used to model an N386D change in HXB2, which results in elimination of the NAG but retains the conformation of the b12 W100 and Env amino acid 386 side chains.

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Table 1

Neutralization of UK1br wild-type and D386N mutant viruses by mAbs and CD4-IgG2

mAb	Epitope	UK1br	UK1br D386N
b12	CD4 binding site	0.04	0.31
F105	CD4 binding site	> 20	> 20
17b	CD4-induced	> 20	> 20
2G12	Outer domain glycans	> 20	> 20
2F5	gp41	2.87	3.11
CD4-IgG2	CD4 binding site	0.92	1.78

mAb or CD4-IgG2 concentration (μg/ml) at which luciferase expression was reduced by 50% compared to infection in the absence of mAb (IC_{50}).

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Table 2

Neutralization sensitivity of HIV-1 Envs and viral isolates with variable macrophage tropism to mAb b12

Env clone or isolate[a]	M-tropism	Amino acid at 386	b12 IC_{50}[b]	Reference
UK1br	high	D	0.03	
SF162	high	N	0.03	Binley et al., 2004
JRFL	high	N	0.09	Binley et al., 2004
NB6	high	D[c]	<0.1	Gray et al., 2005
UK7br	high	N	0.16	
NB2	high	N[c]	0.16	Gray et al., 2005
BaL	high	N	0.19	Binley et al., 2004
ADA	high	N	0.91	
NB7	high	N[c]	2	Gray et al., 2005
NB8	high	D[c]	2.8	Gray et al., 2005
MACS2br13	intermediate	D	0.48	
YU2	intermediate	N	2.3	Wei et al., 2003
MACS2fl8-12	intermediate	N	5.22	Thomas et al., 2007
JRCSF	low	N	0.16	Binley et al., 2004
NB25	low	N[c]	3.2	Gray et al., 2005
NB27	low	N[c]	7	Gray et al., 2005
NB24	low	N[c]	12	Gray et al., 2005
NB23	low	N[c]	25	Gray et al., 2005

[a] Data represents results of neutralization assays performed using cloned HIV Envs except for BaL (viral quasispecies) and the data from Gray et al. (primary isolates).

[b] μg/ml

[c] J. Sterjovski and P. R. Gorry, unpublished data

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Table 3

Loss of an N-linked glycosylation site at position 386 is associated with HAD[a]

		Amino acid at 386			Glycosylation site at 386	
Comparison	N[b]	D	K	S	Present	Absent
By tissue (22 patients)						
Brain (n=152)	0.83[c]	0.14	0.03	0	0.82	0.18
Lymphoid (n=132)	0.82	0.10	0.04	0.04	0.80	0.20
By disease						
HAD (13 patients)						
Total (n=185)	0.76	**0.16***	0.05	0.03	0.74	**0.26****
Brain (n=103)	0.79	0.16	0.04	0.01	0.78	0.22
Lymphoid (n=82)	0.73	**0.15***	0.06	0.06	0.70	**0.30****
non-HAD (9 patients)						
Total (n=99)	0.95	**0.05***	0	0	0.93	**0.07****
Brain (n=49)	0.92	0.08	0	0	0.90	0.10
Lymphoid (n=50)	0.98	**0.02***	0	0	0.96	**0.04****

[a] Sequences were obtained from published studies (Dunfee et al., 2006; Gartner et al., 1997; Gorry et al., 2002; Korber et al., 1994; Liu et al., 2000; Martin-Garcia et al., 2006; Ohagen et al., 2003; Pang et al., 1991; Shapshak et al., 1995; Thomas et al., 2007) and Genbank.

[b] N is the Clade B consensus residue.

[c] The frequency is calculated by dividing the number of sequences with each amino acid variant or with or without the NXS/T glycosylation motif (where X is any amino acid except proline) at position 386 by the total number of sequences. Sequences were limited to 2 to 10 per patient.

* $p < 0.05$.

** $p < 0.001$. p values calculated using Fisher's exact test.

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