Short Communication:
HIV-1 Variants That Use Mouse CCR5 Reveal Critical Interactions of gp120's V3 Crown with CCR5 Extracellular Loop 1

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Abstract

The CCR5 coreceptor amino terminus and extracellular (ECL) loops 1 and 2 have been implicated in HIV-1 infections, with species differences in these regions inhibiting zoonoses. Interactions of gp120 with CD4 and CCR5 reduce constraints on metastable envelope subunit gp41, enabling gp41 conformational changes needed for infection. We previously selected HIV-1$_{JRCSF}$ variants that efficiently use CCR5(Δ18) with a deleted amino terminus or CCR5(HHMH) with ECL2 from an NIH/Swiss mouse. Unexpectedly, the adaptive gp120 mutations were nearly identical, suggesting that they function by weakening gp120's grip on gp41 and/or by increasing interactions with ECL1. To analyze this and further wean HIV-1 from human CCR5, we selected variants using CCR5(HMMH) with murine ECL1 and 2 sequences. HIV-1$_{JRCSF}$ mutations adaptive for CCR5(Δ18) and CCR5(HHMH) were generally maladaptive for CCR5(HMMH), whereas the converse was true for CCR5(HMMH) adaptations. The HIV-1$_{JRCSF}$ variant adapted to CCR5(HMMH) also weakly used intact NIH/Swiss mouse CCR5. Our results strongly suggest that HIV-1$_{JRCSF}$ makes functionally critical contacts with human ECL1 and that adaptation to murine ECL1 requires multiple mutations in the crown of gp120's V3 loop.

HIV-1 ENTRY REQUIRES interactions of gp120-gp41 envelope glycoproteins with cell surface CD4 and coreceptors that normally function as G-protein-coupled chemokine receptors.[1–6] Transmitted viruses use CCR5 as coreceptor, whereas variants employing CXCR4 often form during disease progression.[7–9] Coreceptor shifts require mutations in the V3 loop of gp120, and V3 mutations also adapt HIV-1 to other factors that limit entry including coreceptor antagonists and suboptimal concentrations of CD4 or coreceptors.[9,19]

CCR5's amino terminus (Nt) and extracellular loop (ECL) 1 and 2 regions contribute to coreceptor activity.[20–31] Affinities of sCD4-gp120 complexes for CCR5 are weakened by Nt and ECL2 mutations.[22,24–26,32–37] Tyrosine sulfates in Nt enhance infection and sCD4-gp120 binding,[26,34,35,38] and tyrosine sulfated Nt peptide binds to the base of gp120 V3.[33,37] Additionally, antibodies to ECL2 block entry.[36,39–41] Studies of chimeric human CCR5s with substitutions from murine CCR5 or other chemokine receptors also suggest involvement of Nt and ECL1 and 2.[21,23,24,27,30,42] African green monkeys (AGMs) have been endemically infected by

SIV$_{AGM}$ at high prevalence for millennia and their CCR5s contain many polymorphisms at functionally important sites in Nt, ECL1, and ECL2.[27,43,44]

Damaging mutations in CCR5 can be overcome by adaptive mutations in HIV-1$_{JRCSF}$ gp120 centered in V3.[14–16,44,45] Surprisingly, as described previously and summarized below, mutations adaptive for CCR5(Δ18) with a deleted Nt or CCR5(HHMH) with ECL2 from NIH/Swiss mice were overlapping, with S298N and F313L in V3 and elimination of an N-glycan at N403 (by substitutions N403K,S or T405N,A) in V4 being common.[45] These common mutations increased syncytia formation and susceptibilities to sCD4 inactivation and reduced the activation energy barrier that restricts gp41 refolding, thereby enabling weak coreceptors to function efficiently.[45] Conceivably, these common mutations might strengthen gp120 interactions with ECL1, thereby compensating for reduced reliance on Nt and ECL2.

A major goal of our investigation has been to wean HIV-1$_{JRCSF}$ from dependency on human CCR5 by adapting it in incremental stages for utilization of NIH/Swiss mouse

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CCR5. In addition, this approach provides evidence concerning the interactions of specific gp120 amino acids with sites in CCR5. To investigate these issues, we used previous methods.[14–17,27,44–46] We made CCR5(HMMH) by substituting the *Msc*I–*Bsa*BI fragment from the cDNA of NIH/Swiss mouse CCR5 for the corresponding human sequence (Fig. 1A), and used the HI-J clone of HeLa-CD4 cells to derive subclones with distinct cell surface CCR5(HMMH) amounts measured using monoclonal antibody 3A9 to human Nt.[47]

Human and mouse CCR5 sequences differ in Nt, ECL1, and ECL2, but are identical in ECL3 except for conservative N-to-T and V-to-A substitutions at the TM6-ECL3 and ECL3-TM7 junctions (Fig. 1B). We initially assumed that HIV-1$_{JRCSF}$ variants adapted to CCR5(HHMH) with murine ECL2 and CCR5(G163R) that disrupts gp120 binding and occurs at the TM4/ECL2 junction[36] would be partially

adapted to CCR5(HMMH). However, these variants could not use CCR5(HMMH) at low or medium concentrations although they weakly used a high concentration (e.g., ~86,000/cell) (Fig. 1C), confirming that HIV-1s use coreceptors in a concentration-dependent manner.[16,44,45] JC.53 cells, which contain a supersaturating number (~130,000/cell) of human CCR5 and are infected efficiently by all HIV-1 variants, were used to normalize titers of our virus preparations. These results suggested that HIV-1$_{JRCSF}$ mutations adaptive for CCR5(G163R) and CCR5(HHMH) were not partially adapted to use CCR5(HMMH) and might be maladaptive for use of murine ECL1.

Isolation of adapted variants occurs optimally when the initial virus replicates weakly in the cells used for selection. Accordingly, we used the HeLa-CD4/CCR5(HMMH) clone with ~86,000 coreceptors/cell and the HIV-1$_{JRCSF}$(G163R)-
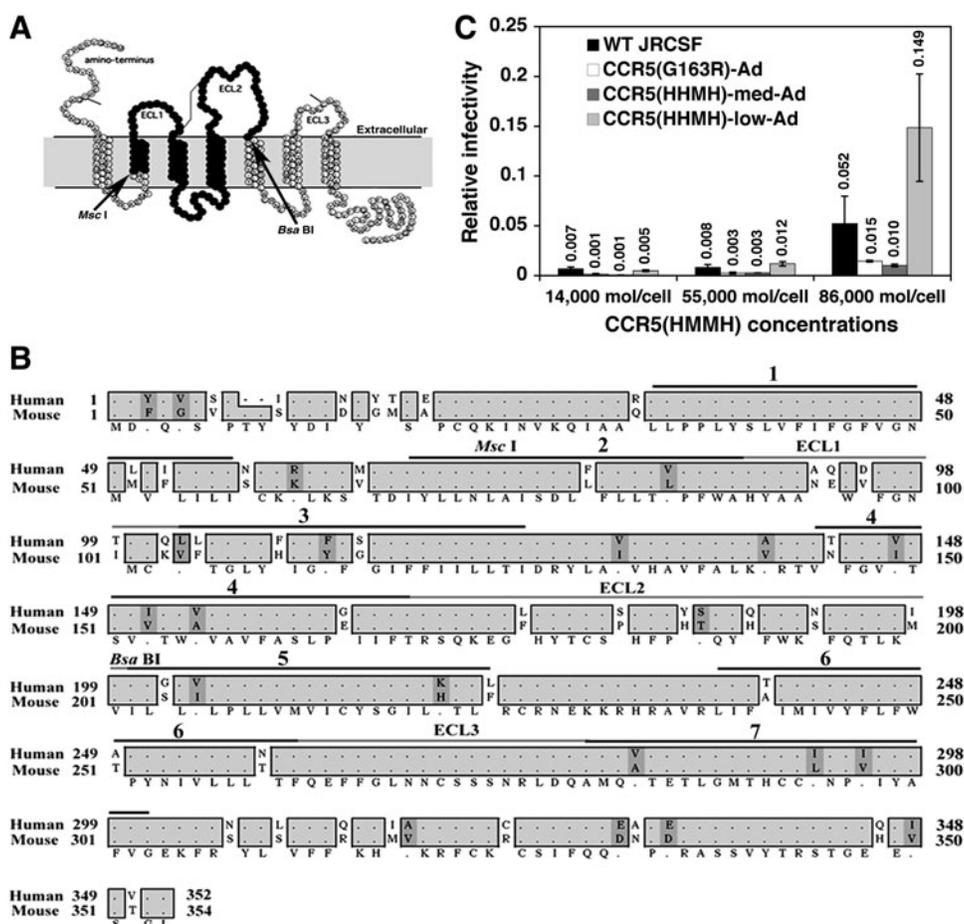

FIG. 1. Construction and coreceptor activity of CCR5(HMMH). **(A)** Schematic diagram. The region *shaded black* includes ECL1 and 2 and contains CCR5 sequences from NIH/Swiss mice. The lines emanating from cysteine residues indicate disulfide bonds linking ECL1 to ECL2, and the amino terminus (Nt) to ECL3. **(B)** Clustal alignment of human and mouse CCR5 sequences. TM domains 1–7 and ECL1, 2, and 3 regions are indicated. Nonconservative sequence differences are shown in *white*, and conservative substitutions in *dark shading*. **(C)** CCR5(HMMH) is a severely disabled coreceptor for wild-type HIV-1$_{JRCSF}$ and variants that have been selected to employ human ECL1. HeLa-CD4 cell clones expressing distinct amounts of CCR5(HMMH) were tested for susceptibilities to infection by HIV-1$_{JRCSF}$ and variants previously selected for use of CCR5(G163R) or CCR5(HHMH), which contain intact human ECL1 but damaged ECL2s. None of these viruses used CCR5(HMMH) at low or moderate concentrations and only weakly employed it at high concentration. Infectivity values were normalized relative to titers in JC.53 cells that express a large excess of wild-type CCR5 (1.3×10^5/cell). The CCR5(G163R) and CCR5(HHMH) adapted viruses were generated by selection on HeLa-CD4 cells with the following mutant coreceptor expression levels: CCR5(G163R),1.9×10^4 /cell; CCR5(HHMH)-med, 1.0×10^5/cell; CCR5(HHMH)-low, 2.0×10^4/cell.[44,45] Error bars are SEMs.
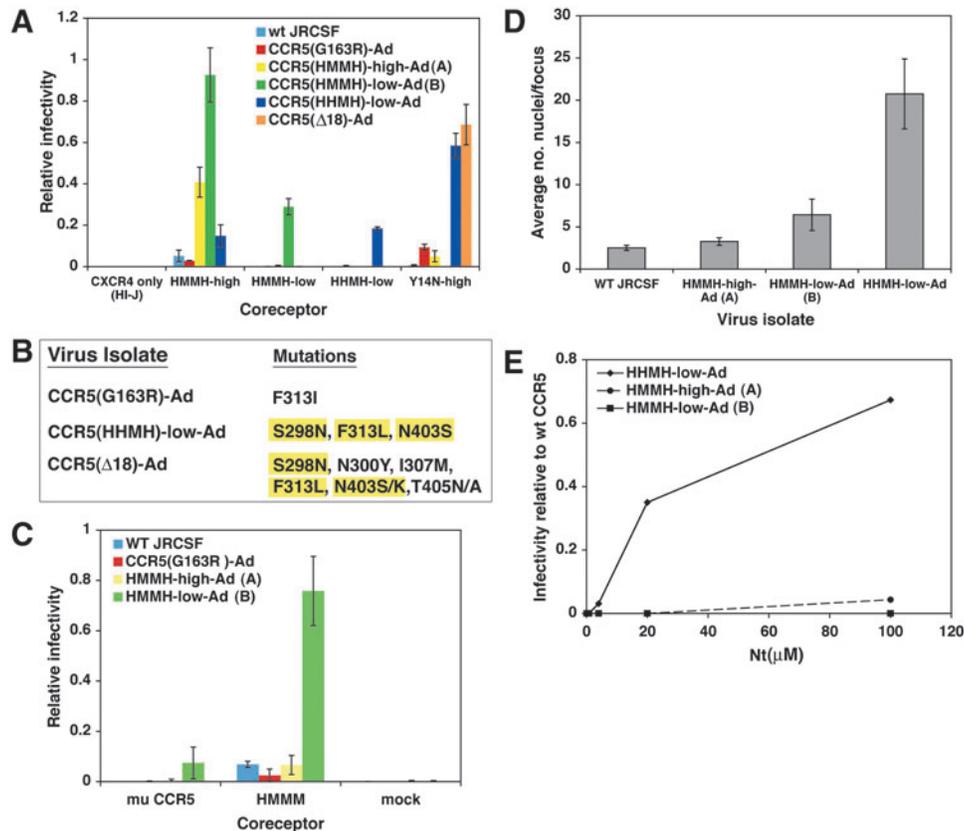
FIG. 2. Characterization of HIV-1$_{JRCSF}$ variants adapted for use of CCR5(HMMH). **(A)** Use of CCR5(HMMH) and other mutant coreceptors by diverse viral isolates. Wild-type HIV-1$_{JRCSF}$ and variants adapted to use high **(A)** or low concentrations of CCR5(HMMH) **(B)** were titered in cells expressing different coreceptors. The variants adapted to CCR5(HMMH) used this coreceptor efficiently, in contrast to all viruses adapted to CCR5s with intact human ECL1 [i.e., wild-type CCR5, CCR5(HHMH), CCR5(G163R), and CCR5(Y14N)]. Conversely, variants adapted to mouse ECL1 cannot use coreceptors with human ECL1. None of the variants employs CXCR4. Titers normalized relative to JC.53 cells are averages of two to three experiments with error bars SEM. **(B)** Adaptive mutations in the previously isolated viruses used in **(A)**. The adaptive mutations for viruses able to use the CCR5(G163R), CCR5(HHMH)-low, and CCR5(Δ18) coreceptors are listed. The CCR5(HHMH)-low-Ad virus was generated by first passaging CCR5(G163R)-Ad virus on CCR5(HHMH)-med cells, and the variant virus (adaptive mutations: F313L, N403S, A428T) that emerged was then selected on CCR5(HHMH)-low cells.[45] Mutations shared between CCR5(HHMH)-low-Ad and CCR5(Δ18) adapted viruses are highlighted in *yellow*. The CCR5(Δ18)-Ad virus was created by passaging CCR5(Y14N)-Ad virus on CCR5(Δ18) cells [6.3×10^4 CCR5(Δ18)/cell]. Adaptive mutations in the CCR5(Y14N)-Ad virus are S298N, N300Y, and F313L.[15,44] The N300Y mutation enhances CCR5(Δ18) use specifically,[15,45] while mutations at residue I307 appear to enhance use of disparate mutant coreceptors [i.e., CCR5(Δ18) and CCR5(HMMH)]. **(C)** Mouse CCR5 use by adapted viruses. 293T cells were transiently cotransfected with expression vectors for CD4 and either human CCR5, mouse CCR5, or the previously described chimera CCR5(HMMM) containing only human Nt. The relative infectivities are averages of three experiments with error bars the SEMs. **(D)** Syncytia-forming abilities of variants adapted to use high **(A)** or low **(B)** concentrations of CCR5(HMMH) compared to wild-type HIV-1$_{JRCSF}$ (low syncytia formation) and the variant adapted to CCR5(HHMH) (high syncytia formation). Syncytia were scored by counting the number of nuclei in 100 infected foci in JC.53 cultures. Error bars are SEMs. **(E)** Ability of adapted viruses to infect HeLa-CD4((18) cells lacking Nt in the presence of different concentrations of the soluble tyrosine sulfated Nt peptide. Infectivity values normalized relative to JC.53 cells. Unlike the variant adapted to CCR5(HHMH), which uses Nt peptide efficiently, the variants adapted to CCR5(HMMH) cannot infect in these conditions.

adapted virus that infects these cells ∼1.5% as efficiently as JC.53 cells (see Fig. 1C). After five cell passages a virus emerged (variant A) that we used to select variant B that replicates efficiently in cells with ∼10,000 CCR5(HMMH)/cell. This selection was very difficult and required 15 cell passages. Figure 2A shows infectivity assays of the A and B variants and several other HIV-1$_{JRCSF}$ variants previously adapted to other mutant CCR5s. Interestingly, viruses adapted to CCR5s containing human ECL1 used a high concentration of CCR5(HMMH) inefficiently and were unable to

use low concentrations. Conversely, viruses adapted to CCR5(HMMH) cannot use CCR5(HHMH) or CCR5(Y14N) containing human ECL1. Importantly, none of the viruses uses CXCR4 (Fig. 2A). Figure 2B lists the previously isolated viruses that we employed in this investigation and identifies the specific adaptive *env* mutations that they contained.

Interestingly, variant B also efficiently used CCR5(HMMM), which differs from mouse CCR5 only in Nt,[27] and weakly used intact murine CCR5 in HEK293T cells that had been cotransfected with expression vectors for CD4 and CCR5s (Fig.

TABLE 1. ADAPTIVE ENVELOPE MUTATIONS IN HIV-1$_{JRCSF}$ ISOLATES GROWN ON HeLa-CD4 CELLS EXPRESSING CCR5(HMMH)

	Env clone[a,b]	A217T	I305V	I307L	R311G	G317E	N403D	A524T
Variant A[c]	1	−	−	+	−	−	+	−
	2	−	−	+	−	−	+	−
	3	−	−	+	−	+	−	−
	4	−	−	+	−	+	−	−
	5	−	−	+	−	−	+	−
	6	−	−	+	−	−	+	−
	7	−	−	+	−	+	−	−
	8	−	−	+	−	−	−	−
	9	−	−	+	−	−	+	−
Variant B[d]	10	−	+	+	−	+	−	−
	11	−	−	+	+	−	+	−
	12	+	+	+	−	+	−	+
	13	+	+	+	−	+	−	−

[a]Entire envelope genes, encoding gp120/gp41, were obtained by PCR of genomic DNA harvested from infected cells and individual clones were then sequenced.

[b]We have used HIV-1$_{JRCSF}$ numbering. The corresponding HIV-1$_{HXBc2}$ numbering for the adapted envelope mutations is 219T, 307V, 309L, 314G, 320E, 411D, and 532T.

[c]Envelope clones isolated from variant A virus-infected HeLa-CD4 cells expressing large amounts of CCR5(HMMH).

[d]Envelope clones isolated from variant B virus-infected HeLa-CD4 cells expressing low amounts of CCR5(HMMH).

2C). In contrast, other tested viruses lacked this capability. The A and B variants were also less syncytium inducing in JC.53 cells than the highly fusogenic variant with S298N, F313L, and N403S that was adapted for efficient use of CCR5(HHMH) (Fig. 2D). In contrast to the latter virus, the A and B variants were also unable to infect HeLa-CD4/CCR5(Δ18) cells in the presence of the tyrosine sulfated Nt peptide (Fig. 2E). This suggests that CCR5(HMMH) adaptations did not enhance viral reliance on human Nt. Although gp120 adaptations to damaged

CCR5 Nt and ECL2 substantially overlap (see Fig. 2B) and neither of these regions is essential, HIV-1 infection evidently requires specific interactions with ECL1.

The gp120-gp41 sequences of *env* cDNA clones from adapted viruses A and B are summarized in Table 1. Importantly, mutations adaptive for CCR5s with human ECL1 but damaged Nt or ECL2 were absent in CCR5(HMMH)-adapted variants except for the N-glycan loss mutation N403D in V4 in a proportion of A and B gp120s. The V3
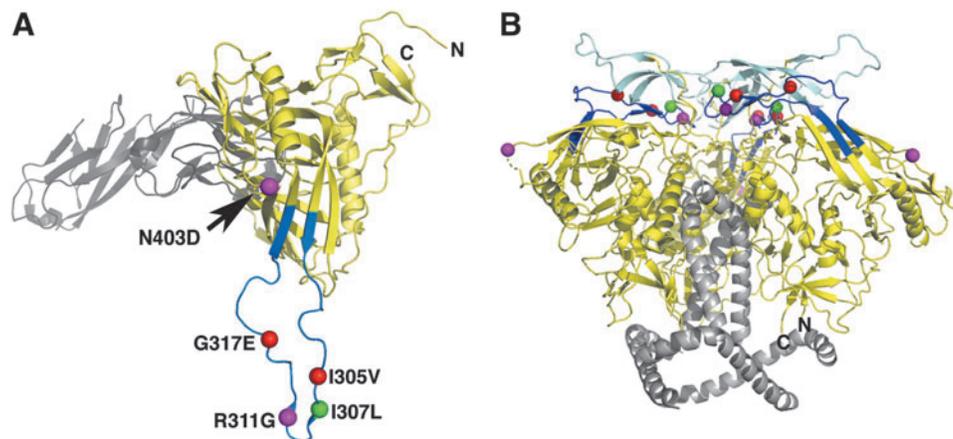

FIG. 3. Locations of the CCR5(HMMH)-adaptive mutations in the monomeric and trimeric crystal structures of gp120. **(A)** Monomeric gp120. Adaptive mutations were modeled onto the crystal structure of monomeric gp120 bound to sCD4 [Protein Data Bank (PDB) accession number 2B4C].[11] In this sCD4 liganded structure, the V3 loop projects away from the virus toward the cell surface. *Gray*, CD4; *yellow*, gp120; *blue*, V3. All envelopes cloned from adapted viruses had the I307L mutation (*green*) and CCR5(HMMH)-low adapted viruses had additional mutations of G317E and I305V (*red spheres*) or R311G and N403D (*purple spheres*), while CCR5(HMMH)-high adapted viruses had I307L and G317E or N403D. The image is oriented with the target cell membrane below. **(B)** Trimeric gp120. The CCR5(HMMH) adaptive mutations were modeled onto the crystal structure of the soluble cleaved trimeric envelope in complex with the neutralizing antibody PGT122 (PDB accession number 4NCO).[50] In this structure, V3, along with V1/V2, overlays the portion of the virus that faces the cell. *Gray*, external portion of gp41; *yellow*, gp120; *light cyan*, V1/V2; *blue*, V3. Visible gaps in the image due to missing or ambiguous electron density are filled in with *dashed lines*. The color coding for the adaptive mutations is as in **(B)**, and the image is oriented with the target cell membrane above. The figure was generated using the PyMOL Molecular Graphics System (Version 1.2r1, Schrödinger).

mutation F313I in the initially employed CCR5(G163R)-adapted virus also reverted during adaptation to CCR5(HMMH). The A and B viruses both contain two viral populations, all having the I307L V3 substitution. The highly adapted B viruses also contain I305V plus G317E in V3 or R311G in V3 plus N403D in V4. These mutations suggest that loss of positive charge (R311G) or gain of negative charge (G317E) in V3 is adaptive for mouse ECL1 and maladaptive for human ECL1. Unlike our previously isolated N-glycan loss mutations (N403K,S or T405N,A), N403D also adds a negative charge. These charge differences may be important because murine ECL1 is positively charged (Fig. 1B) and because electrostatic interactions strongly influence coreceptor choice.[18,48,49]

These V3 mutations cluster in the structures of sCD4-gp120 monomers[11] and gp120-gp41 trimers[50] (Fig. 3). Importantly, the V3 substitutions controlling ECL1 usage occur in gp120's V3 crown. The conservative V3 substitutions I305V and I307L occur in a conserved hydrophobic cluster that influences the orientation and packing of V3. Mutation R311G alters the highly conserved GPGR consensus sequence in the crown tip of V3.[51] The N403D N-glycan loss mutation is situated near positive charges in the V3 base of the unliganded trimer structure. Although some B variant clones contained mutations in C2 (A217T) and in the gp41 fusion peptide (A524T), they occur in regions not known to interact with CCR5 and were not present in a coherent pattern in the B clones. Consequently, we presume they may have been caused by genetic drift during the prolonged selection process. In contrast, the other A and B virus mutations were located specifically in gp120 sites previously shown to interact with CCR5.[15,16,45]

These experiments suggest that the gp120 mutations adaptive for both CCR5(HHMH) and CCR5(Δ18) (i.e., S298N, F313L, and N-glycan loss at N403) (Fig. 2B) make the virus more reliant on human ECL1 and consequently less tolerant of mutations in this region. Previous mutagenic studies also strongly imply that CCR5 ECL1 plays an important role in HIV-1$_{JRCSF}$ entry.[27] Thus, the gp120 V3 loop mutations that were adaptive for CCR5(HHMH), CCR5(Δ18), and CCR5(G163R), which contain human ECL1, were maladaptive for use of murine ECL1. Conversely, the A and B CCR5(HMMH) HIV-1$_{JRCSF}$ variants adapted for murine ECL1 were maladapted for use of human ECL1.

Whereas gp120 mutations adaptive for CCR5s with damaged Nt or ECL2 are substantially identical, implying that gp120 interactions with these regions cooperate in a common process, we conclude that gp120 interactions with CCR5 ECL1 have a functionally different role that is essential for infection. We propose that binding steps involving Nt and ECL2 precede the ECL1-dependent entry process that involves its interactions with the gp120 V3 crown. This interpretation is concordant with previous evidence of Cormier et al.[37]

Acknowledgments

Author Disclosure Statement

No competing financial interests exist.

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