

# HIV-1 Envelope Determinants of CXCR4-Mediated Infection of Macrophages

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## INTRODUCTION

HIV-1 phenotype is defined by tropism and coreceptor utilization. Use of CXCR4 (X4) coreceptor provides a biomarker for HIV-1 pathogenesis in vivo. Env gp120 V3 amino acid composition predicts X4 usage on lymphocytes, but its value as a major determinant for phenotype diminishes with regard to X4 mediated entry into monocyte derived macrophages (MDM). We hypothesize that X4 entry into MDM requires discontinuous determinants in HIV-1 gp120, which differ from PBMC and T-cell lines.

## METHODS

**Sample population** Fifty HIV-1 infected individuals were prospectively enrolled to examine the relationship between viral phenotype and clinical outcomes. **Creations of expression plasmids** Plasmids containing envelope sequences derived from HIV-1<sub>LAI</sub> or HIV-1<sub>JRFL</sub> have been described previously [Tuttle et al, ARHR, vol 18: 353-362, 2002]. For production of chimeric Envelope Expression Vector (EEV), each plasmid was constructed into the gp120 of HIV-1<sub>LAI</sub> or HIV-1<sub>JRFL</sub> by sub-cloning cognate regions of D-X4 envelopes from primary viruses using unique natural or mutant restriction sites. The restriction sites used for sub-cloning V1-V5 regions are shown in figure 1.

**Pseudotyped virus preparation** Viral stocks were prepared by transfecting 293 cells. Transfection mixtures containing full-length pNL4-3 proviral DNA with an envelope frame shift mutation that prevents the expression of gp160, a functional vpr, a luciferase gene replacing nef (NL4-3.Luc.R+<sub>E-</sub>) and EEV DNA and Superfect Transfection Reagent. Supernatants from the transfected cells were harvested on day 2 post-transfection. Viruses were quantified using p24 and gp120 antigen ELISA.

**Viral entry assay and phenotype classification** 3T3.T4 co-receptor indicator cells, PBMC, MT-2, and MDM were infected with 60 ng of p24 equivalent virus. Infection by each virus was performed in three or more replicate wells for each experiment. Cells were assayed for entry and infection by measuring luciferase in cell lysates at 72 to 96 h post-infection. Specific inhibitors of entry were included in some experiments: AMD-3100 (a competitive antagonist of CXCR4), or monoclonal antibodies (mAb) specific for CCR5 (2D7) or CXCR4 (12G5).

**Phenotypic classification** HIV-1 classification is based on a combination of chemokine receptor usage, particularly CXCR4 and CCR5, and tropism (for macrophages and/or T-cell lines). Observed phenotypes categorized according to coreceptor usage and tropism have been designated: M-R5, macrophage (M) tropic, CCR5 using; D-X4, dual tropic using only CXCR4; and T-X4, T-cell line (T) tropic, using only CXCR4. [Briggs et al, AIDS, vol 14;2937-2939, 2000].

## SUMMARY OF RESULTS

- Viruses with D-X4 phenotypes are associated with severe immune deficiency and advance disease. D-X4 viruses evolve in vivo in tissues, including thymus.

- Envelope V3 charge is related to R5 and X4 co-receptor use on lymphocytes. In contrast, V3 sequences are necessary but insufficient to mediate X4-dependent entry into primary MDM. D-X4 envelopes are more resistant than T-X4 envelope to inhibition by AMD-3100.

- Evolution from D-X4 to T-X4 phenotype may result, at least in part, from diminished affinity for CXCR4.

- Determinants in addition to V3 play a role in X4-mediated MDM entry. V1 and V3 comprised a core region of Env gp120, which mediated efficient use of CXCR4 on CD4 T lymphocytes. Efficient entry into macrophages required D-X4 V1 and V3 core in combination with V2 and V5.

- D-X4 V2 domains have increased net positive charges, while V1 domains included unusual proline or cysteine residues localized in the region of length polymorphism. These features from D-X4 envelopes differed from either M-R5 or T-X4 envelopes, but were shared with a prototypic D-R5X4 envelope from HIV-1<sub>89.6</sub>.

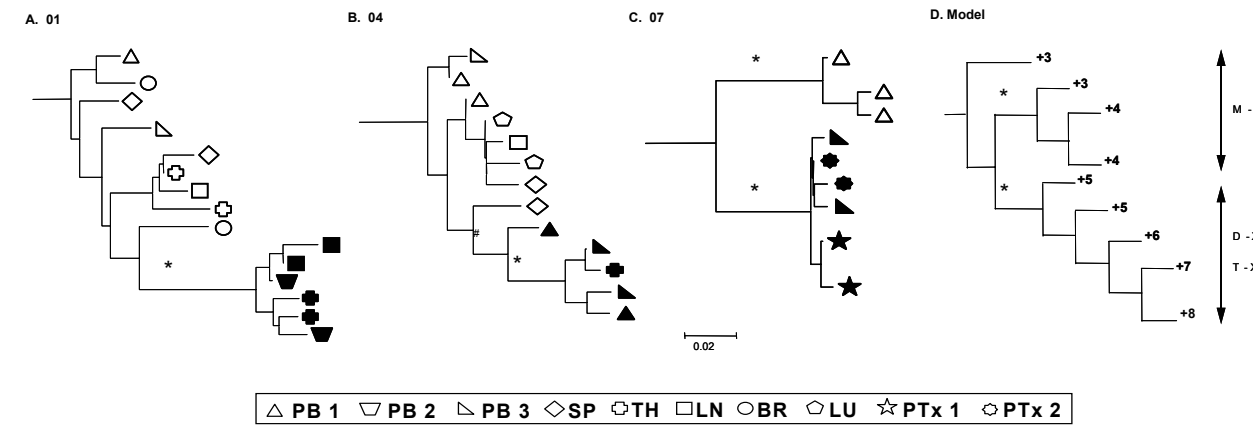
## RESULTS

**Table 1: D-[R5]X4 phenotype is related to immune suppression.**

CD4%	Number of Subjects		
	Total	M-R5	D-[R5]X4
≥ 20%	17	14	3
≤ 15%	22	3	19
Total	39	17	22

CD4% and HIV-1 dual-tropism was evaluated among 39 ART-naïve individuals. The relationship between advanced immune suppression [CD4 ≤ 15%] and dual tropic virus phenotype was significant (P < 0.001, Chi-square).

**Figure 1. In vivo evolution of D-[R5]X4 viruses in tissues and PBMC.**



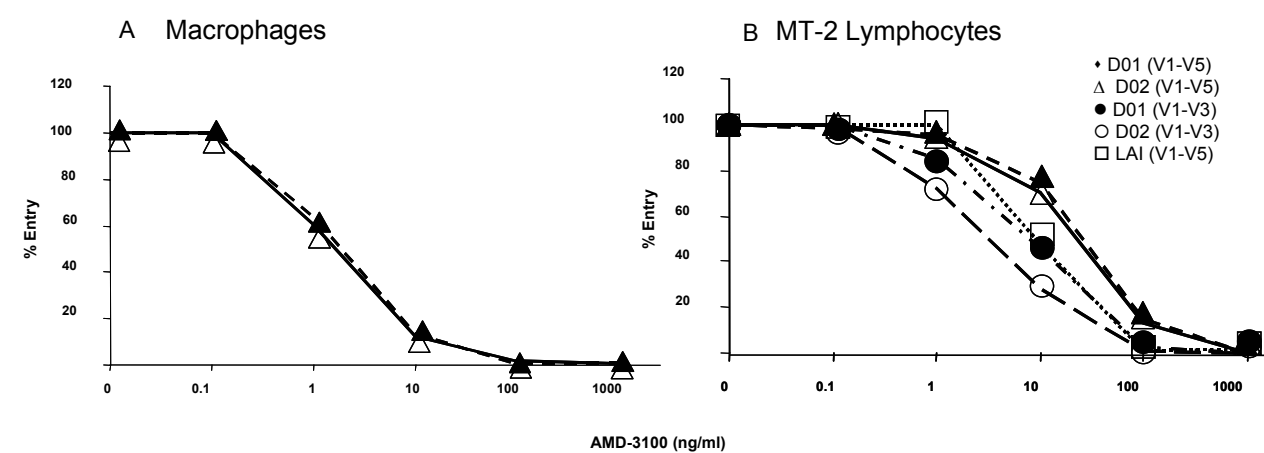
Neighbor joining trees of representative V3 nucleotide sequences from PBMC and tissues from subjects 01 [A] and 04 [B], who were ART-naïve, and 07 [C], prior to and following ART. Model of evolution based on V3 net charge and viral phenotype is shown in [D]. Symbols: open, V3 loop net charge ≤ 4; closed, V3 net charge ≥ 5; asterisk, boot strap > 90; #, boot strap > 75; PB 1, 2, or 3, PBMC at different time points; TH, thymus; SP, spleen; LN, lymph nodes, LU, lung; BR, brain; PTx1 or PTx2, PBMC at 24-weeks or 48-weeks post-initiation of ART.

**Table 2: D-X4 phenotype maps to V1 to V5.**

	V1 to V5			V3			V1 to V3		
	JRFL M03	LAI T04	D02 D02	JRFL M03	LAI T04	D02 D02	JRFL M03	LAI T04	D02 D02
<b>Tropism</b>									
PBMC	+	+	+	+	+	+	+	+	+
MT-2	-	+	+	-	+	+	-	+	+
MDM	+	-	-	+	-	-	+	-	+/-
<b>Coreceptor</b>									
CCR5	+	-	-	+	-	-	+	-	-
CXCR4	-	+	+	-	+	+	-	+	+
<b>Phenotype</b>	M-R5	T-X4	D-X4	M-R5	T-X4	T-X4	M-R5	T-X4	D-X4

Phenotype of the original virus isolates M-R5, D-X4, or T-X4, map to Env V1 to V5. Exchange of HIV-1<sub>LAI</sub> V3 with HIV-1<sub>JRFL</sub> or primary virus M03 conferred an M-R5 phenotype. V3 regions from HIV-1<sub>LAI</sub> or primary virus T04 were sufficient to convert HIV-1<sub>JRFL</sub> from an M-R5 to a T-X4 phenotype. In contrast, chimeric HIV-1<sub>LAI</sub> envelopes with V3 domains from D-X4 primary virus isolates invariably displayed a T-X4 phenotype.

**Figure 2. AMD3100 inhibition of CXCR4-mediated entry into macrophages and T-lymphocytic cells.**



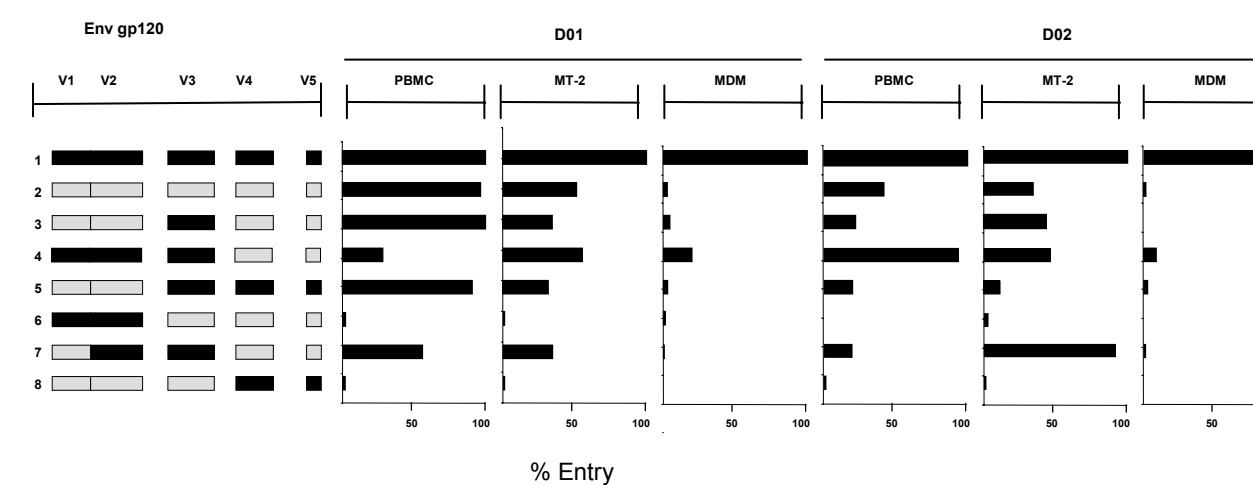
Relative sensitivity by luciferase tagged, single-cycle pseudotype viruses to CXCR4-specific antagonist AMD3100 (0.1 to 1000 ng per ml). In MT-2 cells, viruses pseudotyped by chimeric envelopes with D-X4 V1-V3 regions displayed ~3- to 8-fold increased sensitivity to inhibition [IC<sub>50</sub> 3 or 8.5 ng per ml for D01 or D02, respectively] versus D-X4 V1-V5. Viruses pseudotyped with T-X4 V1-V5 envelope were inhibited [IC<sub>50</sub> 9 ng per ml] at similar levels required for D-X4 V1-V3 chimeric envelopes.

**Figure 3. Strategy to construct chimeric envelope gp120 regions.**



Map of hypervariable domains (white boxes) and conserved regions (black boxes) is shown with the restriction endonuclease sites used for construction of chimeric envelopes. Abbreviations: K, KpnI; B, BstAPI; S, StuI; Bu, Bsu36I; M, MfeI; P\*, PstI site introduced by mutagenesis without changing the amino acid sequence. Bars below indicate the actual regions evaluated in the chimeric constructs.

**Figure 4. Determinants in gp120 for use of CXCR4 to enter MDM**



One, two, or three adjacent domains from D-X4 envelopes were introduced into the T-X4 V1-V5 background of HIV-1<sub>LAI</sub>. Entry into PBMC, MT-2 cells, or macrophages mediated by chimeric envelopes was compared to D-X4 V1-V5 parental envelopes by luciferase relative sensitivity.

**Figure 5. Mapping determinants in V1 to V5 required for X4-mediated infection of macrophages.**

Env ID	gp120					No. D-X4	D01			gp120/p24	Pheno-type	D02			gp120/p24	Pheno-type
	V1	V2	V3	V4	V5		PBMC	MT-2	MDM			PBMC	MT-2	MDM		
1	D	D	D	D	D	5	H	H	H	673	D-X4	H	H	H	990	D-X4
2	T	T	T	T	T	2	H	I	NA	1148	T-X4	I	I	NA	1148	T-X4
3	T	T	D	T	T	2	H	I	NA	1058	T-X4	I	I	NA	1086	T-X4
4	D	D	D	T	T	3	L	I	L	1967	D-X4	H	I	NA	1440	T-X4
5	T	T	D	D	D	3	H	I	NA	789	T-X4	L	L	NA	566	T-X4
6	D	D	T	T	T	2	NA	NA	NA	762	N	NA	NA	NA	475	N
7	T	D	D	T	T	2	I	I	NA	845	T-X4	L	H	NA	1153	T-X4
8	T	T	T	D	D	2	NA	NA	NA	480	N	NA	NA	NA	437	N

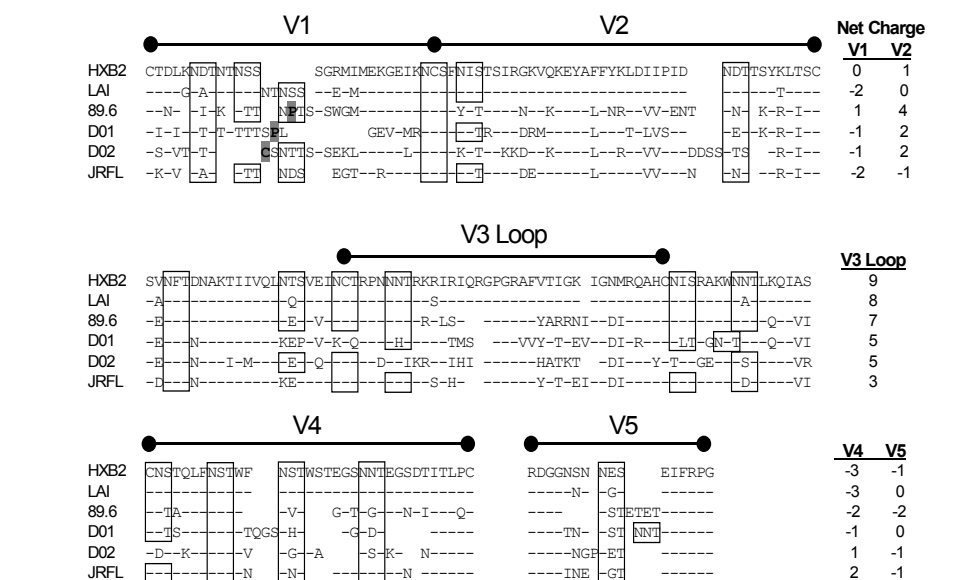
Infection of PBMC and macrophages from eight independent donors and MT-2 cells using discontinuous hypervariable domains. Mean percent luciferase activity for each chimeric envelope relative to D-X4 parental envelope in each cell type was calculated and classified as: NA [no activity] = ≤10%; L [low] = >10% to 30%; I [intermediate] = >30% to 60%; or H [high] = >60% to 100%. No relationship between the ratio of gp120 to p24 with phenotype.

**Figure 6. Mapping discontinuous determinants in V1 to V5 required for X4-mediated infection of macrophages.**

Env ID	Env constructs					No. D-X4	D02			gp120/p24	Pheno-type
	V1	V2	V3	V4	V5		PBMC	MT-2	MDM		
1	D	D	D	D	D	5	H	H	H	990	D-X4
2	T	T	T	T	T	0	I	I	NA	1148	T-X4
9	D	T	D	T	T	2	H	H	NA	920	T-X4
10	D	T	D	D	T	3	I	I	I	608	D-X4
11	D	T	D	T	D	3	I	H	I	575	D-X4
12	D	D	D	D	T	4	I	I	I	532	D-X4
13	D	T	D	D	D	4	H	I	I	349	D-X4
14	D	D	D	T	D	4	H	H	H	939	D-X4

Infection of PBMC and macrophages from three independent donors and MT-2 cells using discontinuous hypervariable domains. For classification of symbols see figure 5 legend.

**Figure 7. Characterization of gp120 V1 to V5 amino acids from D-X4 envelopes.**



Amino acid sequences from V1-V5 regions from primary D-X4 viruses from subjects 01 or 02 were aligned with reference strains of HIV-1 [LAI, 89.6, and JRFL] relative to HIV-1<sub>HXB2</sub>. N-linked glycosylation motifs are indicated by boxes. Novel cysteine and proline residues in V1 are highlighted in gray. Net positive charge of each domain is indicated.

## CONCLUSIONS

- Cell type specific regulation of viral entry is comprised of complex discontinuous genetic determinants in gp120, including charged and uncharged amino acid residues in V3, the V5 hypervariable domain, and novel V1/V2 regions distinct from prototypic M-R5 or T-X4 viruses.
- Unique gp120 determinants required for CXCR4 usage on macrophages, in contrast to cells of lymphocytic lineage, can provide targets for development of novel strategies, including therapeutic vaccines, to block emergence of pathogenic quasispecies of HIV-1.

## ACKNOWLEDGEMENTS

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