MAJOR ARTICLE

Genome-Wide Association Study of Human Immunodeficiency Virus (HIV)-1 Coreceptor Usage in Treatment-Naive Patients from An AIDS Clinical Trials Group Study

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Objectives. We conducted a genome-wide association study to explore whether common host genetic variants (>5% frequency) were associated with presence of virus able to use CXCR4 for entry.

Methods. Phenotypic determination of human immunodeficiency virus (HIV)-1 coreceptor usage was performed on pretreatment plasma HIV-1 samples from treatment-naive participants in AIDS Clinical Trials Group A5095, a study of initial antiretroviral regimens. Associations between genome-wide single-nucleotide polymorphisms (SNPs), CCR5 Δ32 genotype, and human leukocyte antigen (HLA) class I alleles and viral coreceptor usage were explored.

Results. Viral phenotypes were obtained from 593 patients with available genome-wide SNP data. Forty-four percent of subjects had virus capable of using CXCR4 for entry as determined by phenotyping. Overall, no associations, including those between polymorphisms in genes encoding viral coreceptors and their promoter regions or in *HLA* genes previously associated with HIV-1 disease progression, passed the statistical threshold for genome-wide significance ($P < 5.0 \times 10^{-8}$) in any comparison. However, the presence of viruses able to use CXCR4 for entry was marginally associated with the CCR5 Δ32 genotype in the nongenome-wide analysis.

Conclusions. No human genetic variants were significantly associated with virus able to use CXCR4 for entry at the genome-wide level. Although the sample size had limited power to definitively exclude genetic associations, these results suggest that host genetic factors, including those that influence coreceptor expression or the immune pressures leading to viral envelope diversity, are either rare or have only modest effects in determining HIV-1 coreceptor usage.

Keywords. CCR5 Δ32 mutation; genome-wide association study; HIV-1; viral coreceptor usage; viral tropism.

Human immunodeficiency virus (HIV)-1 that uses CCR5 exclusively for entry into host cells (R5 virus) is primarily responsible for viral transmission and predominates in early infection. Human immunodeficiency virus-1 that uses CXCR4, either exclusively (X4 virus) or both CXCR4 and CCR5 (dual- or mixed-tropic virus populations [D/M]), emerges in patients over time. The shift in coreceptor usage has clinical implications because X4 emergence correlates with accelerated CD4 count decline and progression to acquired immune deficiency syndrome (AIDS) [1–8]. A better understanding of the relationship between the evolution of coreceptor usage and variations in host genetics is important because modulating CCR5 expression or function is being studied as a strategy to achieve antiretroviral-free HIV-1 remission [9–12].

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Coreceptor switching and X4-D/M emergence may occur due to changes in target cell availability, replication differences among R5 and X4 variants in various immune cells, or differences in host immune responses to R5 and X4 viruses [8, 13–17]. However, there is little understanding about specific host factors that facilitate emergence of CXCR4-using viral variants. Common host genetic polymorphisms (frequency >5%) have been identified in HIV-1 coreceptor or coreceptor ligand genes that modify the rate of disease progression or susceptibility to infection [7, 18–24], but data to support the role of host factors involved in coreceptor usage switching are limited. As a result, we conducted a genome-wide association study (GWAS) to explore whether common host genetic variants are associated with the presence of X4-D/M virus in a cross-sectional study of treatment-naive patients enrolled in AIDS Clinical Trials Group (ACTG) protocol A5095 and explored the relationship between CCR5 Δ32 mutations, demographic and clinical factors, and viral coreceptor usage.

METHODS

Pretreatment, baseline plasma samples were obtained from patients enrolled in ACTG A5095, a 1147-patient randomized, double-blind trial of a triple-nucleoside regimen versus efavirenz-containing regimens for the initial treatment of HIV-1 infection, and coenrolled in A5128, a study to use stored human biological materials for genetic analyses [21, 25, 26]. When insufficient sample was available, plasma obtained 2 or 4 weeks after antiretroviral therapy (ART) initiation was used. The Partners Healthcare institutional review board approved this study.

Virus was concentrated by centrifuging 500 μL plasma at 17 000× *g* for 1.5 h at 4°C before RNA extraction using the QIAamp Viral RNA Mini Kit (QIAGEN). After RNA extraction, full-length envelope genes were amplified using nested polymerase chain reaction with primers as described [27–29]. Polymerase chain reactions were performed in triplicate wells and combined before further processing. Bidirectional sequencing of the third variable loop (V3) of HIV-1 envelope was performed to check for potential sample cross-contamination and to perform Geno2Pheno coreceptor usage prediction using a 5% false-positive rate threshold [30, 31]. An in-house phenotypic assay able to detect minority X4 or D/M virus present at 1% or greater of the virus population using pseudoviruses incorporating a luciferase reporter gene and full-length *env* amplicons from population viral RNA was performed to determine coreceptor usage as described [32]. Phenotyping was repeated if the luciferase signal on indicator cell lines was <20-fold higher than the signal generated from envelope-deleted pseudovirus vectors alone. The assay has been optimized for the categorical determination of coreceptor-usage results (eg, X4, X4-D/M, or R5).

Covariate-adjusted analyses using binary logistic regression models were performed to identify associations between age, gender, race or ethnicity, baseline $CD4^+$ T-cell counts, baseline $\log_{10}$ HIV RNA levels with viral coreceptor usage, and the presence of a CCR5 Δ32 mutation assessed by a custom Sequenom iPLEX genotyping assay.

Genome-wide studies to identify associations between single-nucleotide polymorphisms (SNPs) and viral coreceptor usage were performed. We utilized >400 000 common SNPs genotyped using Illumina genome-wide genotyping arrays for association with HIV viral coreceptor usage (R5 virus and D/M or X4 virus) for the study population as previously described [21]. In brief, samples were genotyped using the Illumina HumanHap650Y or 1M Duo platform. Quality control and data filtering were done using the PLINK toolset [33] based on population outliers (judged by principal component analysis), signals of contamination (large deviation from expected heterozygosity), SNP missingness (missing in >5% of samples), low frequency (minor alleles frequency below 1%), and the Hardy-Weinberg equilibrium test ($P < .000005$).

Sample swaps were ruled out by using a fingerprint panel of <30 SNPs used for sample tracking. Known polymorphisms in the *CCR5* and *CXCR4* genes not represented on the GWAS chips were genotyped using a custom Sequenom iPLEX genotyping assay. Samples were split based on ancestry, and association testing was performed using logistic regression, including markers for HIV disease stage that were identified to be independently associated with coreceptor usage in addition to principal components calculated from genome-wide SNP data to correct for residual population structure. Association evidence was combined across groups using inverse-variance weighted meta-analysis. Models were performed unadjusted or included baseline $CD4^+$ T cell counts as a cofactor. High-resolution major histocompatibility complex (MHC) class I human leukocyte antigen (HLA) typing was available for a majority of patients, and separate association analyses were performed with viral coreceptor genotypes and phenotypes.

RESULTS

Stored plasma samples from 751 participants in A5095 were obtained from the ACTG specimen repository. Coreceptor usage was determined by phenotypic assay for 593 patients with available SNP data. Table 1 shows the association between patient demographic and clinical factors, including CCR5 genotype with viral coreceptor usage. The X4-D/M virus was significantly associated with a lower baseline CD4+ T cell count ($P < .001$) in the multivariate model and marginally, but not significantly, associated with the presence of the CCR5 Δ32 allele ($P = .058$). Of all X4-D/M viruses determined by phenotypic assay, only 0.9% used CXCR4 exclusively.

A large majority of patients (94.2%) with available phenotypic coreceptor usage results had HIV-1 subtype B virus predicted

Table 1. Association of Patient and Clinical Characteristics With Viral Coreceptor Usage As Determined by Phenotypic Assay

	Coreceptor Usage		Adjusted *P* Value[c]
	X4-D/M[a]	R5 Only[b]	
Number of patients	265 (44.5)[d,e]	330 (55.5)[e]	
Age (median years)	37	37	.834
Gender			
Male	210 (79.5)	263 (80.7)	.695
Female	54 (20.5)	63 (19.3)	
Race/Ethnicity			
White	113 (42.8)	149 (46.0)	.174
Black	103 (36.0)	101 (31.2)	
Hispanic	44 (16.7)	73 (22.5)	
Other/not reported	4 (1.5)	1 (0.3)	
Baseline $CD4^+$ T Cell Count (median cells/mm^3)	154	261	<.001
Baseline VL (median log_{10} copies/mL)	4.91	4.76	.993
CCR5 genotype			
Δ32 heterozygous	31 (11.7)	27 (8.3)	.058
Wild-type	233 (88.3)	299 (91.7)	

Abbreviations: D/M, dual- or mixed-tropic virus populations; GWAS, genome-wide association studies; VL, viral load.

[a] X4-D/M, viral population that uses either CXCR4 or both CXCR4 or CCR5 for entry.

[b] R5, viral population that uses only CCR5 for entry.

[c] Adjusted *P* value from logistic regression models including all variables listed.

[d] Number and percent within coreceptor usage group.

[e] *N* = 593 included in GWAS analyses; 1 X4-D/M and 6 R5 patients with missing clinical information excluded in regression modeling; 2 patients with missing data included in regression analysis were excluded from the GWAS.

by V3 loop genotype, and 46% of these patients had X4-D/M virus by phenotypic assay. Subtype C and A (or AG) represented 2.1% and 2.7% of patient viruses, respectively, but only 23.1% and 17.6% of patients with these subtypes had X4-D/M virus. Two patients each had D, G, and F subtypes with 1 subtype D patient having X4-D/M virus. Of samples with phenotypic coreceptor usage results, only 10 (1.7%) were obtained 2 or 4 weeks after ART initiation.

No associations between any SNPs and viral coreceptor usage passed the genome-wide threshold for significance ($P < 5 \times 10^{-8}$) in any comparison. Table 2 shows the genome-side association results for each ancestral population and a meta-analysis across ancestral groups for disease-modifying polymorphisms identified in CCR5, CCR2, and stromal cell-derived factor 1 (SDF1)-3′A (A5095 consisted of participants with European, African-American, and Mexican ethnicities). Table 3 shows association results for disease-modifying polymorphisms adjusted for baseline $CD4^+$ T-cell counts to minimize potential disease stage bias, because $CD4^+$ count was strongly associated with the presence of X4-D/M virus in non-GWAS regression modeling. The presence of CXCR4-using virus was not significantly associated with any of these polymorphisms in either analysis, although presence of the CCR5 Δ32 allele approached marginal nongenome-wide significance ($P = .080$) in the $CD4^+$ T-cell count adjusted model. Figure 1 shows Manhattan plots of all SNPs in the $CD4^+$ T cell unadjusted analyses for each ancestral group and the meta-analysis across ancestry; the 100 polymorphisms with the lowest *P* values in the meta-analysis across ancestral association data in the $CD4^+$ T cell unadjusted model are shown in the Supplementary Table. None of these polymorphisms was related to CXCR4 or CCR5, or the MHC. Assuming an additive genetic model and a variant frequency of 10%, the present sample size provides >80% power to detect an odds ratio (OR) of 3 or greater. Stand-alone meta-analyses across ancestral association

Table 2. List of SNPs Included in the GWAS Previously Associated With HIV-1 Disease Progression or Presence of X4-D/M Virus Determined by Phenotypic Assay

				OR (*P* value) for Association Analysis by Ancestry			
SNPa	Gene	Marker	Prior Reported Effect	European (n = 266)	African American (n = 209)	Hispanic (n = 118)	Meta-Analysis[a] (n = 593)
rs333	*CCR5*	Δ32	Decreased susceptibility [22]	1.45 (0.252)	4.03 (0.213)	1.9 (0.548)	1.59 (0.120)
rs1799987	*CCR5*	P1	Fast progression [20]	0.92 (0.677)	0.79 (0.309)	0.76 (0.375)	0.84 (0.205)
rs1800023	*CCR5*	A676G	Slow progression [19]	1.04 (0.845)	1.00 (1.000)	0.66 (0.224)	0.94 (0.674)
rs1800024	*CCR5*	C927T	Slow progression [19]	0.99 (0.976)	1.15 (0.575)	0.82 (0.581)	1.02 (0.908)
rs2734648	*CCR5*	G280T	Slow progression [19]	1.13 (0.530)	0.84 (0.440)	0.81 (0.510)	0.96 (0.758)
rs1799988	*CCR5*	T627C	Slow progression [19]	1.05 (0.795)	0.84 (0.384)	0.67 (0.183)	0.89 (0.353)
rs1799864	*CCR2*	V64I	Slow progression [7]	0.97 (0.945)	1.03 (0.897)	0.84 (0.624)	0.97 (0.852)
rs1801157	*SDF1*	G801A (3′A)	Fast progression (previously associated with X4 virus) [18]	1.17 (0.506)	0.58 (0.241)	0.81 (0.642)	0.97 (0.888)

Abbreviations: D/M, dual- or mixed-tropic virus populations; GWAS, genome-wide association studies; HIV, human immunodeficiency virus; OR, odds ratio; SDF1, stromal cell-derived factor 1, a CXCR4 ligand; SNP, single-nucleotide polymorphism.

[a] Association data combined across groups using inverse-variance weighted meta-analysis.

Table 3. List of SNPs Included in the GWAS Previously Associated With HIV-1 Disease Progression or Presence of X4-D/M Virus Determined by Phenotypic Assay in CD4$^+$ T Cell-Adjusted Models[a]

				OR (*P* value) for Association Analysis by Ancestry			
SNPa	Gene	Marker	Prior Reported Effect	European (n = 266)	African American (n = 209)	Hispanic (n = 118)	Meta-Analysis[a] (n = 593)
rs333	*CCR5*	Δ32	Decreased susceptibility [22]	1.56 (0.175)	5.01 (0.159)	1.63 (0.645)	1.70 (0.080)
rs1799987	*CCR5*	P1	Fast progression [20]	0.93 (0.716)	0.82 (0.408)	0.79 (0.439)	0.87 (0.287)
rs1800023	*CCR5*	A676G	Slow progression [19]	1.03 (0.877)	0.92 (0.803)	0.70 (0.312)	0.92 (0.638)
rs1800024	*CCR5*	C927T	Slow progression [19]	0.96 (0.918)	1.06 (0.827)	0.87 (0.701)	0.98 (0.929)
rs2734648	*CCR5*	G280T	Slow progression [19]	1.14 (0.516)	0.80 (0.323)	0.90 (0.744)	0.96 (0.783)
rs1799988	*CCR5*	T627C	Slow progression [19]	1.06 (0.757)	0.90 (0.609)	0.66 (0.183)	0.92 (0.505)
rs1799864	*CCR2*	V64I	Slow progression [7]	0.93 (0.851)	0.87 (0.627)	0.88 (0.736)	0.89 (0.546)
rs1801157	*SDF1*	G801A (3′A)	Fast progression (previously associated with X4 virus) [18]	1.06 (0.705)	0.71 (0.494)	0.87 (0.770)	0.98 (0.930)

Abbreviations: D/M, dual- or mixed-tropic virus populations; GWAS, genome-wide association studies; HIV, human immunodeficiency virus; OR, odds ratio; SDF1, stromal cell-derived factor 1, a CXCR4 ligand; SNP, single-nucleotide polymorphism.

[a] Model adjusted for baseline absolute CD4$^+$ T cell counts.

[b] Association data combined across groups using inverse-variance weighted meta-analysis.

data for HLA class 1 alleles and viral coreceptor usage adjusted or not adjusted for baseline CD4$^+$ T-cell count were performed. The association between phenotypic coreceptor usage and several HLA type 1 alleles had *P* values <.05, but none met the

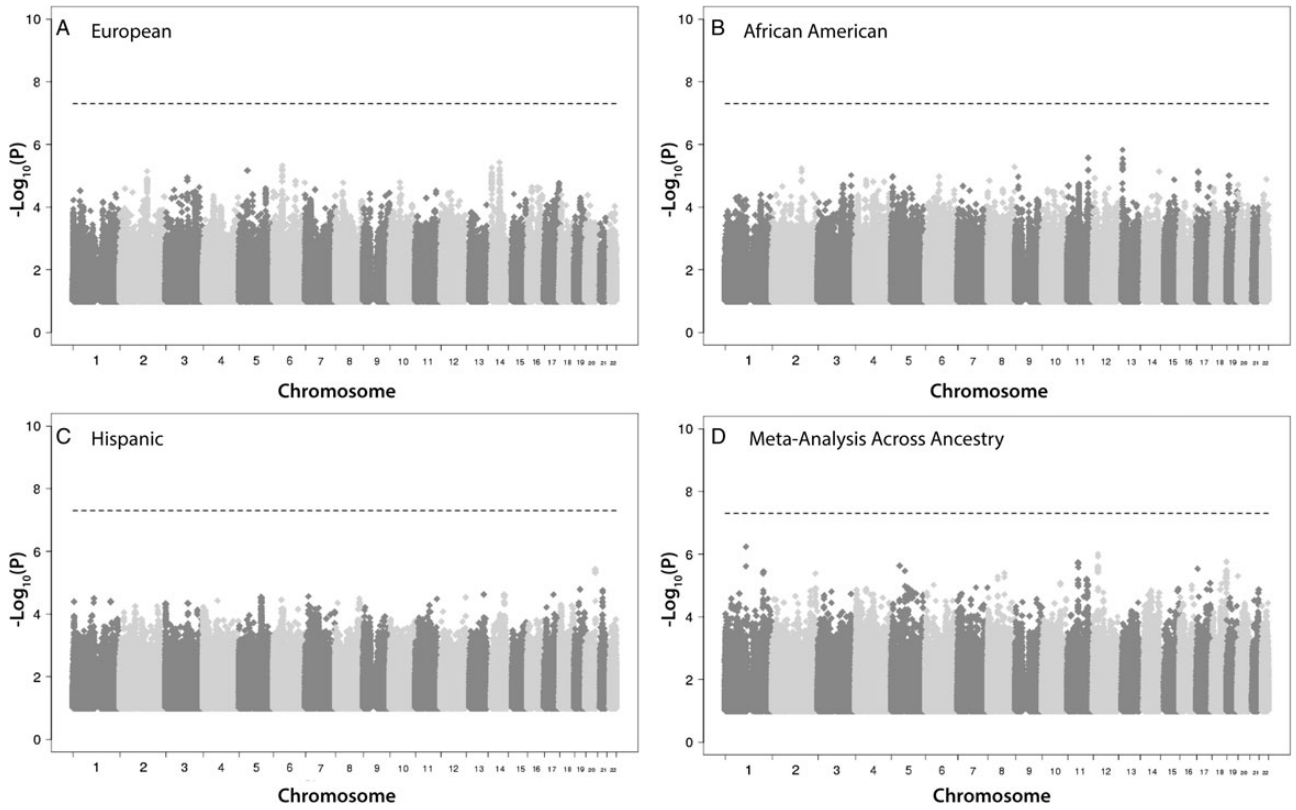

Figure 1. Manhattan plots of inverse $\log_{10}$ *P* values of association data from unadjusted models for each ancestral group (*A*–*C*), and combined across ancestry (*D*) by inverse-variance weighted meta-analyses. No single-nucleotide polymorphisms were significantly associated with coreceptor usage phenotype at the genome-wide significance level (represented by the dashed lines) in any model. The genes encoding CXCR4, CCR5, and the major histocompatibility complex are located on chromosomes 2, 3, and 6, respectively.

genome-wide threshold of significance: A*30:01 (OR, 0.40; $P = .024$), B*57:01 (OR, 4.41; $P = .014$). In addition, the association between phenotypic coreceptor usage and several HLA type 1 alleles had P values <.05 in the $CD4^+$ T cell adjusted model: A*30:01 (OR, 0.36; $P = .017$), A*33:01 (OR, 3.71; $P = 0.032$), B*57:01 (OR, 5.25; $P = .007$), and C*17:01 (OR, 0.39; $P = .05$).

Association analyses between genotypically predicted viral coreceptor usage and clinical factors and SNPs were also performed on 612 samples. As with phenotypic coreceptor usage, no significant associations between SNPs and the presence of X4-D/M virus were identified at the genome-wide level, but there was a significant association between X4-D/M and the presence of a CCR5 Δ32 allele ($P = .006$) in non-GWAS regression analysis. Compared with phenotypic methods, Geno2Pheno was 95.3% specific but only 31.6% sensitive for detecting X4-D/M virus.

DISCUSSION

A genome-wide association analysis found no significant association between any common human genetic variants and the presence of X4-D/M virus in a large cohort of patients initiating first-line ART in ACTG protocol A5095. Although the sample size had limited power to definitively exclude genetic associations, our findings suggest that host genetic factors, including those that influence HIV-1 coreceptor expression, are either rare or have only modest effects in determining HIV-1 coreceptor usage. The presence of the HLA B*57:01 was associated with the presence X4-D/M phenotype in both the $CD4^+$ T cell adjusted and unadjusted models but failed to reach statistical significance at the genome-wide level. It is interesting to note that there is a known correlation between B*57:01 and slower disease progression [34, 35], despite the fact that in this study patients with this allele were more likely to have CXCR4-using virus.

A longitudinal study of HIV-1 evolution in 9 men with progressive HIV disease before ART showed that coreceptor usage followed a predictable course, with X4-D/M viral variants emerging in all patients during early to intermediate stages of HIV-1 disease [36]. In larger cohorts, the timing of X4-D/M emergence varies between individuals and R5 viruses have been isolated from patients with advanced disease or AIDS [1–7]. We identified an independent association between lower baseline $CD4^+$ T-cell counts and presence of X4-D/M virus, consistent with the findings of previous studies [37, 38]. However, several previous studies are either cross-sectional or determined coreceptor usage only at baseline. Host factors not directly linked to genetic polymorphisms, such as changes in the availability of HIV-1 target cells expressing different amounts of CCR5 and CXCR4, may play an important role in the evolution of viral coreceptor usage [14]. Cells with high levels of CCR5 expression are reduced early in infection [39], driving virus to evolve an enhanced ability to enter cells expressing low levels of CCR5 [40, 41]. CXCR4-using viruses may emerge once the virus is unable to increase any further its capacity to enter cells expressing low levels of CCR5.

We identified a marginal correlation between the presence of X4-D/M virus and the presence of at least 1 copy of the CCR5 Δ32 mutation. A significantly higher proportion of X4-D/M virus in patients with decreased expression of functional CCR5 has been observed in a prior study, and these data support the hypothesis that target cell selection guides the evolution of HIV-1 *env* and coreceptor usage [17, 37]. Gene editing of CCR5 in autologous $CD4^+$ T cells conferred a selective advantage for the genetically modified T cells when ART was interrupted; the longest time to virologic rebound was observed in a patient heterozygous for the CCR5 Δ32 mutation [12]. Whether this approach will select for emergence of X4-D/M virus over time will require careful monitoring.

In the current analysis, the presence of X4-D/M virus was not significantly associated with the SDF1-3′A SNP in the genome-wide analysis or in a stand-alone analysis. Stromal cell-derived factor 1 is a CXCR4 ligand and endogenous inhibitor of CXCR4 and viral entry [42]. Presence of the SDF1-3′A polymorphism was associated in a smaller study with faster disease progression and with the presence of X4 virus [18]. Previous studies have suggested that interleukin (IL)-7, which increases the density of CXCR4 expression on the surface of $CD4^+$ T cells surface, may be associated with the emergence of X4 virus [43]. Likewise, homozygosity of the IL-4 promoter region polymorphism 589T has been correlated with increased rates of X4 virus conversion [43, 44]. Single-nucleotide polymorphisms in these IL genes were not included in our analysis, but our failure to find significant associations between coreceptor usage and the SDF1-3′A polymorphism highlights the importance of using large cohorts to explore associations of host genetic factors on viral evolution.

Although this study included 593 patients with phenotypic data on coreceptor usage, a limitation of this study was the relatively limited power to detect associations in the genome-wide context. Moreover, this study does not address the role of rare sequence variants or copy number polymorphisms. Although a number of low frequency variants could be imputed that may be related to HIV-1 coreceptor usage [45], we were unable to detect effects of variants <1% at genome-wide significance in this study due to the limited sample size.

Approximately 45% of pseudoviruses were X4-D/M by our phenotypic assay, which is higher than the 18% X4-D/M prevalence by commercial phenotyping from a previous study of coreceptor usage in treatment-naive individuals with baseline CD4 counts and viral loads similar to those in the A5095 trial [37]. The reason for this difference is not known but may reflect differences in assay sensitivity. Dual-mixed HIV type 1 isolates have varied considerably in their utilization of CCR5 and CXCR4 coreceptors, with some isolates using low-levels of CXCR4 [46]. Our assay may detect relatively low levels of

CXCR4 usage. To minimize overcalling X4-D/M phenotype, the mean luciferase relative luciferase units (RLUs) from pseudoviral entry into cells expressing CXCR4 had to be significantly higher than the background RLU on the same cells from envelope-deleted pseudoviral controls by *t* test, and RLUs had to decrease by at least 50% in the presence of a small-molecule CXCR4 antagonist [32]. The assay was designed and optimized for the categorical determination of coreceptor-usage results, and it was not possible to directly compare the strength of coreceptor usage on the assay cell lines with the genomic data.

Despite these limitations, our findings suggest that host genetic factors, including those that influence coreceptor expression or the immune pressures leading to viral envelope diversity, are either rare or of modest effect in determining HIV-1 coreceptor usage. Pooled analyses of larger patient cohorts with available genome-wide SNP data and measured coreceptor usage are needed to increase study power and to better understand the interplay between host genetics and viral coreceptor usage.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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