Disparate impact on CD4 T cell count by two distinct HIV-1 phylogenetic clusters from the same clade

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Edited by Robert C. Gallo, Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD, and approved November 21, 2018 (received for review August 28, 2018)

HIV-1 evolved into various genetic subtypes and circulating recombinant forms (CRFs) in the global epidemic. The same subtype or CRF is usually considered to have similar phenotype. Being one of the world’s major CRFs, CRF01_AE infection was reported to associate with higher prevalence of CXCR4 (X4) viruses and faster CD4 decline. However, the underlying mechanisms remain unclear. We identified eight phylogenetic clusters of CRF01_AE in China and hypothesized that they may have different phenotypes. In the National HIV Molecular Epidemiology Survey, we discovered that people infected by CRF01_AE cluster 4 had significantly lower CD4 counts (391 vs. 470, P < 0.0001) and a higher prevalence of X4-using viruses (17.1% vs. 4.4%, P < 0.0001) compared with those infected by cluster 5. In an MSM cohort, X4-using viruses were only isolated from seroconverters in cluster 4, which was associated with low a CD4 count within the first year of infection (141 vs. 440, P = 0.003). Using a coreceptor binning model, we identified unique V3 signatures in cluster 4 that favor CXCR4 use. We demonstrate that the HIV-1 phenotype and pathogenicity can be determined at the phylogenetic cluster level in the same subtype. Since its initial spread to humans from chimpanzees, estimated to be the first half of the 20th century, HIV-1 continues to undergo rapid evolution in larger and more diverse populations. The divergent phenotype evolution of two major CRF01_AE clusters highlights the importance of monitoring the genetic evolution and phenotypic shift of HIV-1 to provide early warning of the appearance of more pathogenic strains.

CRF01_AE | Coreceptor tropism | CXCR4 | CD4 count | HIV-1 pathogenesis

During global transmission, HIV-1 evolved into various subtypes and their hybrids, the so-called circulating recombinant forms (CRFs) (1). CRF01_AE, one of the major CRFs, spreads mainly in Southeast Asia and China (1, 2). Early studies in Thailand reported faster CD4 loss and shorter survival of CRF01_AE-infected people compared with infections in western countries, where subtype B predominates (3–6). In recent years, faster disease progression of CRF01_AE was also reported in China (7–9). Although past studies reported high prevalence of CXCR4 (X4) viruses and fast disease progression in CRF01_AE infections (7, 8, 10, 11), the data were mainly based on samples without known infection time and on genotype prediction without phenotypic confirmation. It is still unclear how early X4-using viruses can emerge during natural CRF01_AE infection and whether the genotype prediction is reliable. The epidemic of CRF01_AE in China was initiated by multiple phylogenetic clusters introduced from Thailand in the 1990s (2). We earlier identified eight CRF01_AE clusters, which have different geographic distributions and epidemic patterns (2, 12). Therefore, we hypothesized that they may have different phenotypes. We used the National HIV Molecular Epidemiology Survey (NHMES) dataset for screening and a men-who-have-sex-with-men (MSM) seroincidence cohort for in-depth study to determine the time of X4 virus emergence, and phenotypically confirmed viral tropism using matched viral isolates. We observed significantly lower CD4 counts in CRF01_AE cluster 4 compared with cluster 5 in the NHMES dataset. Focusing on the MSM cohort, we demonstrated higher prevalence of the X4 phenotype in cluster 4 among people within the first year of infection. We further determined the genetic and structural basis favoring X4-using coreceptor use in cluster 4. This study demonstrates that different phylogenetic clusters from the same HIV-1 subtype can cause disparate rates of disease progression, and provides the missing link between CRF01_AE genetic makeup, phenotype, and clinical outcomes.

Results

Lower CD4 T Cell Count and Higher Prevalence of the X4-Using Virus in CRF01_AE Cluster 4. We compared the CD4 T cell counts of 1,118 CRF01_AE, 633 CRF07_BC, and 123 subtype B newly diagnosed HIV-1–positive participants in China’s NHMES study and found no differences (Fig. 1A). When analyzing the two major CRF01_AE clusters which contribute greatly to China’s MSM epidemic, however, the CD4 count in cluster 4 was significantly lower than in cluster 5 (P < 0.0001) (Fig. 1B). Since CD4 counts decline with time during natural infection, we distinguished recent infections from long-term infections by HIV-1 limiting antigen-avidity assay. CRF01_AE clusters 4 and 5 showed equally high rates of recent infection (31.3% and 31.4%), while only

Significance

Past studies on HIV-1 evolution were mainly at the genetic level. This study provides well-matched genotype and phenotype data and demonstrates the disparate pathogenicity of two major CRF01_AE clusters. While CRF01_AE cluster 4 and cluster 5 are associated with the men-who-have-sex-with-men (MSM) route of transmission, cluster 4 but not cluster 5 causes a low CD4 count, which is associated with the higher prevalence of CXCR4 viruses in cluster 4. The higher CXCR4 use tendency in cluster 4 is derived from its unique V3 loop favoring CXCR4 binding. This study demonstrates disparate HIV-1 phenotypes between different phylogenetic clusters. It is important to monitor HIV-1 evolution at both the genotype and phenotype level to identify and control more pathogenic HIV-1 strains.

Author contributions: Y.S. conceived study; H.S. and Y.S. designed research; H.S., W.O., Y.F., H.X., L.M., Q.T., B.W., and Y.S. analyzed data; and H.S., B.W., and Y.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession nos. MH672692–MH673032).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1814714116/-/DCSupplemental.

Published online December 17, 2018.

www.pnas.org/cgi/doi/10.1073/pnas.1814714116
Because CXCR4 tropism is associated with lower CD4 cell counts (13–17), we investigated the prevalence level of X4 viruses in clusters 4 and 5 using genotypic prediction. Geno2pheno prediction showed higher prevalence of the X4 genotype in cluster 4 than in cluster 5 (17.1% vs. 4.4%, \( P < 0.0001 \)) (SI Appendix, Fig. S2). In cluster 4, the X4 genotype was “concentrated” among patients with low CD4 counts (SI Appendix, Fig. S2). This result indicates a strong association between higher X4 prevalence and lower CD4 counts in CRF01_AE cluster 4.

**High Prevalence of the X4-Using Phenotype in CRF01_AE Cluster 4 Among Recently Infected Individuals.** To further characterize the mechanism of fast CD4 cell loss in CRF01_AE cluster 4, we focused on a seroconversion cohort with approximately 2,000 MSMs from Beijing’s Chaoyang District [the Chaoyang MSM (CYM) cohort]. For the 135 MSM seroconverters, we first performed deep sequencing on all 78 participants with archived first-year blood samples and successfully sequenced 71 of them (SI Appendix, Figs. S3 and S4). Among them, 60 participants purely infected by CRF01_AE, CRF07_BC, or subtype B were further analyzed for coreceptor tropism (with an average of 65,000 sequencing reads per participant) (SI Appendix, Figs. S3 and S4 and Tables S1 and S2).

To confirm the genetic prediction phenotypically, we isolated viruses using cryopreserved PBMCs from the deep-sequenced CRF01_AE participants and conducted coreceptor tropism assays using GH0ST cell lines. A total of 24 viruses were successfully isolated. The isolation success rate was similar for both clusters: A total of 16 and 8 viruses were isolated from 21 cluster 4 participants (76.2%) and 10 cluster 5 participants (80.0%) who had PBMCs available (Fig. 3A). Five isolates showed the X4-using phenotype, and the remaining 19 were the R5-only phenotype (Fig. 3A). All of the five X4-using isolates, including four dual-tropic and one exclusively X4-tropic, belonged to cluster 4 (Fig. 3A and SI Appendix, Fig. S6). This indicates a much higher prevalence of the X4-using phenotype in cluster 4 than in cluster 5 (31.3% vs. 0%), consistent with genotypic prediction.

**Individuals Harboring X4 Viruses Had Significantly Lower CD4 T Cell Counts.** Previous studies demonstrated the association between X4 tropism and lower CD4 counts, mainly for subtype B HIV-1 (13–17). However, a recent study based on genotypic prediction did not find such an association in CRF01_AE-infected people in China (though there is a trend that people with CD4 < 50 tend to have Gena2pheno FPR < 5) (8). Because genotypic prediction could overestimate actual X4 prevalence, we compared CD4 T cell counts based on virus phenotype (Fig. 3B). Among the 24 phenotype-confirmed participants, those with the X4-using phenotype had significantly lower CD4 counts compared with those with the R5 phenotype in cluster 4 (141 vs. 440, \( P = 0.003 \)) and in cluster 5 (141 vs. 441, \( P = 0.01 \)) (Fig. 3B). With well-matched phenotype data, we confirmed that the X4-using phenotype is associated with a significantly lower CD4 count in CRF01_AE. The higher prevalence of the X4 phenotype in cluster 4 during the first year of infection and its association with significantly lower CD4 counts explained why cluster 4 had lower CD4 counts compared with cluster 5 from the early infection stage (Fig. 1C and D).

**Genetic and Structural Determinants for Greater CXCR4 Use Propensity.** To explore the mechanism for greater X4-using propensity exhibited by cluster 4 viruses, we first compared the V3 sequences between clusters 4 and 5. We found that cluster 4 viruses have two

10.1% of recent infections were found in other minor CRF01_AE clusters (SI Appendix, Fig. S1A). Among recent infections, cluster 4 had the lowest CD4 count in comparison with cluster 5 (\( P = 0.0004 \)) and other minor clusters (\( P = 0.0037 \)), while cluster 5 and other clusters showed no statistical difference (Fig. 1C and SI Appendix, Fig. S1B). In long-term infections, the CD4 count of cluster 4 remained significantly lower than that of cluster 5 (\( P < 0.0001 \)), while the CD4 count of other clusters became the lowest (Fig. 1D and SI Appendix, Fig. S1B). Given the current HIV-1 epidemic pattern in China, the lower CD4 count of other clusters in long-term infections is likely due to a longer infection time (see Discussion). Together, the results clearly showed that CRF01_AE cluster 4 but not cluster 5 causes lower CD4 counts during early infection.
Significantly lower CD4 count in individuals harboring X4-using variant(s) from the entire viral population (that is, the exact sequence or sequences accounting for the X4-using phenotype), we further sequenced the viruses released from the GHOST X4 cell culture by single-genome amplification (SGA) for the five X4-using samples. Comparing the genetic composition between the PMBC isolates and the concurrent plasma viral population showed two different patterns: In the majority of R5 subjects (15 of 19), the V3 lineages in the PMBC isolates and in plasma were in proportion; that is, the predominant lineage in the PMBC isolates was also the predominant one in plasma. In contrast, in four of the five X4 isolates, the predominant, phenotypically confirmed X4 lineage in the PMBC isolates existed as a minor variant in plasma (SI Appendix, Fig. S9 and Table S3). All V3 lineages detected in the PMBC isolates were present in plasma, and more V3 lineages were detected in plasma than in the isolates (SI Appendix, Fig. S9 and Table S3).

Several V3 alterations were found in the phenotypically confirmed X4 sequences. First, all X4-using sequences lost the N-linked glycan site at the beginning of the V3 loop (V3 positions 6–8, HXB2 nos.301–303), mostly by T to I substitution at position 8 (Fig. 5A). Second, while residue N was invariably found in all R5 sequences at position 7, all but one X4-using sequence had residue D or E. Third, either E or D was found at position 25 in the R5 sequences; however, non-E/D substitutions (S/A/G) were present in the majority of X4-using sequences (Fig. 5A). Interestingly, none of the X4 sequences had positively charged amino acid R or K at V3 position 11 or 25. These two residues are important for X4 use in other HIV-1 subtypes (20–22). This implies a different evolutionary pathway of coreceptor switching in CRF01_AE HIV-1. In genotypic prediction, all X4-using sequences had Geno2pheno FPR values below 2% and a V3 net charge no less than 5. In contrast, all R5 sequences had FPR values no less than 5.

The highly conserved basic amino acids at positions 13 and 32 in the V3 loop (R13 and K32, HXB2 nos. R308 and K327, respectively), which were present in only about 8% of the cluster 5 viruses (Fig. 4A). These two highly conserved amino acids confer on cluster 4 viruses a higher positively charged V3 loop. Because the V3 net charge is an important determinant for X4 use, the presence of R13 and K32 may decrease the threshold of R5 to X4 tropism switching in cluster 4, while in cluster 5 the genetic and functional barrier for such switching is higher. Structure analysis using V3-docking models (18) suggested that residue R13 in cluster 4 potentially forms salt bridges with D262 and E277 and a hydrogen bond with the side chain of H281 in the CXCR4 coreceptor, while the corresponding residue in cluster 5 may form hydrogen bonds with K22 and D276 in the CCR5 coreceptor (Fig. 4B). Because the ligand-binding pocket in CXCR4 is more negatively charged than that in CCR5, the positively charged residue R13 in cluster 4 viruses is more favored. The residue K32 in cluster 4 may form salt bridges with CXCR4’s N terminus, which contains more acidic residues than CCR5’s N terminus (Fig. 4B). Therefore, the highly conserved residues R13 and K32 in cluster 4 may be key determinants of the higher tendency toward X4 use. Indeed, the basic amino acid at V3 position 32 has been observed to associate with T cell tropism in subtype B HIV-1 (19). Interestingly, the V3 region of cluster 4 has fewer variations compared with the CRF01_AE ancestor sequences from Thailand—notably the preservation of K32—while cluster 5 is more divergent (Fig. 4A and SI Appendix, Fig. S7).
Genetic determinants and structural basis of the higher X4-using phenotype in CRF01_AE cluster 4. (4) The frequency of each V3 amino acid was determined using a total of 385 available sequences from cluster 4 and 328 available sequences from cluster 5. The sequences from Thailand (n = 34) were downloaded from the Los Alamos HIV sequence database before year 2000. The plots were generated using the Weblogo tool (55). The exact frequency of V3 residues R13 and K32 in clusters 4 and 5 is shown in red boxes. (B) Structural analysis for V3 positions 13 and 32 in binding of the CCR5 and CXCR4 coreceptors using the V3-docking model.

values higher than 2% and a V3 net charge no more than 5. As expected, cluster 4 had an overall higher V3 net charge than cluster 5 (Fig. 5A). Notably, five sequences in cluster 4 with FPR below 5% were in fact the R5 phenotype (Fig. 5A). Therefore, using FPR 5% as the cutoff may significantly overestimate the prevalence of the X4 phenotype in CRF01_AE. Although determining a coreceptor prediction method was not the focus of the study due to a relatively small sample size, the results showed that three common features were shared by all phenotypically confirmed X4 sequences: The loss of N301 glycan, a V3 net charge no less than 5, and an FPR lower than 5%. Thus, these three features together may strongly indicate an X4 phenotype in CRF01_AE HIV-1.

Using the CCR5-V3 and CXCR4-V3 complex models (23, 24), we also investigated the role of V3 positions 7, 8, and 25 in viral tropism from a structural perspective. In the model of the CCR5-V3 complex, residue T8 in the R5 V3 loop is surrounded by hydrophilic amino acids, suggesting that residue T8 is more favored by a hydrophilic environment. However, in the model of the CXCR4-V3 complex, residue I8 in the X4 V3 loop is surrounded by hydrophobic amino acids (Fig. 5B). This could explain why all X4 viruses have T to I/M substitutions at position 8 because residue T8 may not fit the hydrophobic environment within all X4 viruses have T to I/M substitutions at position 8 because residues, which are favored for interacting with negatively charged residues D/E25. This explained why all R5 viruses have D/E at V3 position 25. However, D/E may be less favored in the less positively charged environment in the ligand-binding pocket of CXCR4 (Fig. 5B). Therefore, non-D/E substitutions, as observed in X4-using sequences, would be required for efficient X4 binding (Fig. 5B). Taken together, genetic analysis and structural modeling showed that specific V3 substitutions at positions 7, 8, and 25 may be required to achieve an X4-using phenotype in the context of the CRF01_AE cluster 4 envelope.

Discussion

Since its initial introduction to humans in the early 20th century, HIV-1 evolved genetically and biologically at a faster pace than other viruses, due to the highly error-prone nature of its reverse transcriptase and its diverse transmission routes, such as intravenous drug use (IDU), heterosexual transmission (HT), and MSM activities. The Chinese HIV-1 epidemic, with its multiple subtypes and their genetic clusters circulating at the same time, provides a unique opportunity to monitor virus evolution at both genotype and phenotype levels.

Past studies on HIV-1 evolution were mainly focused on virus genotype, not phenotype, because the latter takes longer observation time and requires large well-matched samples. In this study, we focused on two major CRF01_AE clusters in China. In the large cross-sectional dataset from the NHMES, we discovered a significant difference in CD4 count between people infected by CRF01_AE clusters 4 and 5 at both an early and a later stage of infection. The lower CD4 count in cluster 4 is directly associated with the higher prevalence of the X4 virus based on genotypic prediction. This genotype-based observation in a large population was further confirmed by well-matched genotype and phenotype data from an MSM seroincidence cohort. We observed that, among seroconvertors, those harboring X4 viruses had significantly lower CD4 counts. Although in the NHMES dataset other minor CRF01_AE clusters had the lowest CD4 counts, this could have been mainly due to the higher proportion of chronic infections with longer infection times. Although the exact time of infection was not measurable, it could be estimated that the overall infection time in other clusters is substantially longer than in clusters 4 and 5. This is supported by the following observations: First, China’s HIV-1 epidemic has experienced a transition from IDU and HT to MSM. Second, the current HIV incidence is highest in the MSM population, followed by HT and lastly IDU. Third, the MSM epidemic is mainly caused by clusters 4 and 5, while the HT and IDU epidemics are mainly caused by other clusters. Due to both the different epidemic pattern and the low recent infection rate of other clusters, it was difficult to include them in the current study. However, the pathogenicity of other CRF01_AE clusters will be important to investigate in the future.

It is usually considered that X4 variants emerge during late stages of infection, with the development of immunodeficiency of the host (25). In subtype B infections, around 50% of patients underwent a coreceptor switch, usually after five years of infection, which correlated with rapid CD4 decline and faster progression to AIDS (16, 26–31). The exact times of coreceptor switching in different HIV-1 subtypes are not well understood. A recent study did not detect X4 variants in subtype B-infected people within two years of infection (32). We demonstrated that, in certain HIV-1 subtypes or clusters, coreceptor switching may occur much earlier than previously thought. Investigating the dynamics of CD4 loss in association with coreceptor switching using longitudinal samples will promote better understanding of the pathogenicity of the X4 virus. In the current study, we could not rule out the possibility that, in some of individuals harboring X4 viruses, the X4 variants were initially transmitted as the transmitted/founder (T/F) virus. No samples from earlier time points were available to address this possibility. However, nearly all T/F viruses characterized to date were...
exclusively CCR5-tropic except in some rare cases (33–38). In particular, a study of T/F virus envelopes from another MSM cohort in China showed that all CRF01_AE T/F envelopes tested were CCR5-tropic (38). These all support early coreceptor switching as a more plausible scenario. Nevertheless, it will be important to determine whether X4 transmission more frequently occurs in CRF01_AE than in subtypes B and C, as the existence of X4 variants in up to 30% of recent infections, as observed in cluster 4, may increase the chance of X4 transmission at the population level.

Another interesting finding is the outgrowth of highly replication-competent X4 variants in primary viral isolates from CRF01_AE cluster 4. The low frequency of these X4 variants in plasma may not be explained by their low replication fitness, as they rapidly outcompete other lineages in the in vitro setting. Instead, it is more likely due to the compartmentalization of the R5 and X4 viruses in different cell subsets or tissues in vivo. Due to the differential expressions of CCR5 and CXCR4 coreceptors in memory and naive CD4 T cell subsets (39, 40), R5 and X4 viruses are considered to preferentially replicate in the memory and naive CD4 subsets, respectively (41–45). It has been shown that naive CD4 T cells produce viruses at a lower propagation rate than memory T cells (46, 47), possibly due to the relatively low division rate (48, 49). Therefore, in vivo those minor X4 lineages might compartmentalize in cell subsets or tissues that shield the viruses less efficiently into the blood. Although the plasma viral population is most widely studied, the relative contribution of different tissues/compartments to plasma viremia and immunopathogenesis remains an unresolved question in the field. The mechanism of CD4 loss caused by such minor X4 lineages in plasma will be an important question to address in the future. Regardless of the mechanism, this observation has a clinical implication: Because conventional sequencing methods may not be sensitive enough to capture those minor X4 variants in plasma, deep sequencing or phenotypic assay will be required to determine the existence of X4 variants in vivo, especially when using treatment regimens with the CCR5 inhibitor.

In summary, we demonstrated that various phylogenetic clusters of the same HIV-1 subtype can have disparate pathogeneticities and cause different disease outcomes, providing the missing link between HIV-1 genetic evolution and viral phenotypes. At the phenotype level, CRF01_AE cluster 4 evolved with enhanced X4 tropism and viral pathogenesis, while cluster 5 became more attenuated due to a decreased potential of using CXCR4. Whether the process of “phenotype divergence” occurred as a random founder event from the initial seeding clusters or was due to adaptation to different hosts or transmission routes remains to be studied. Our study emphasizes the importance of monitoring HIV-1 genetic drift and phenotype shift at the phylogenetic cluster level to control the spread of more pathogenic viruses like CRF01_AE cluster 4.

Materials and Methods

Study Participants. The study participants were from the NHMES and the Beijing Chaoyang District MSM cohort (the CYM cohort) (SI Appendix). Written informed consent was obtained from all study participants. All experiments involving human subjects from the NHMES (project no.: X140617334) and CYM cohort (project no: X080216132) were approved by the Institutional Review Board of the National Center for AIDSSTD Control and Prevention, Chinese Center for Disease Control and Prevention.

Enzyme Immunoassay. To distinguish recent HIV-1 infections from long-term HIV-1 infections, enzyme immunoassay (EIA) was performed using the Maxim HIV-1 Limiting Antigen Avidity (Lag-Avidity) EIA Kit (Maxim Biomedical). The experiment and the data analysis were performed according to manufacturer’s instructions (Maxim Biomedical).

Viral RNA Extraction and cDNA Synthesis. Viral RNA was extracted from 200 μL of plasma sample using the QIAamp Viral RNA Mini Kit (Qiagen). RNA was eluted into 50 μL of RNase-free water. A total of 17 μL of viral RNA was used for cDNA synthesis using Invitrogen SuperScript III reverse transcriptase (Thermo Fisher Scientific) with Oligo (dt) primer. The cDNA was immediately used for PCR amplification.

Deep Sequencing. The Illumina MiSeq library was prepared using a nested PCR approach. The 8-nt Illumina index and adaptors (PS and P7) were added to both ends of the second-round PCR primers. The pooled DNA library was sequenced on an Illumina MiSeq system using the Reagent Kit V2 (500 cycles) as previously described (50). Data analysis was performed as previously described (51) (SI Appendix).
Genotypic Prediction of Coreceptor Use. Genotypic prediction of coreceptor use was performed using the Geno2pheno clonal model (52).

Primary Virus Isolation from PBMCs. Primary viruses were isolated by cocultivation of infected PBMCs with normal PBMCs from healthy donors, after CD8 T cells were depleted from both the infected and normal PBMC samples (SI Appendix).

Coreceptor Tropism Determination. Coreceptor tropism of the primary viral isolates was determined using the GHOST(3) CCR5 and GHOST(3) CXCR4 cell lines (53). Both GFP expression in the GHOST cell lines and viral p24 production were used to determine coreceptor use (SI Appendix).

Single Genome Amplification. The SGA was performed as previously described (54). The sequences were aligned using the Los Alamos National Laboratory’s GeneCutter tool followed by manual adjustment to obtain the optimal alignment (SI Appendix).

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