Genetic Signatures of HIV-1 Envelope-mediated Bystander Apoptosis

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Background: Determinants of HIV-1 Env-mediated apoptosis remain poorly understood.

Results: We studied the bystander apoptosis-inducing activity of a panel of primary HIV Envs.

Conclusion: Residues Arg-476 and Asn-425 are associated with differences in HIV-1 Env-mediated bystander apoptosis induction.

Significance: We identified specific genetic signatures within the HIV-1 Env that are associated with the bystander apoptosis-inducing phenotype.

The envelope (Env) glycoprotein of HIV is an important determinant of viral pathogenesis. Several lines of evidence support the role of HIV-1 Env in inducing bystander apoptosis that may be a contributing factor in CD4+ T cell loss. However, most of the studies testing this phenomenon have been conducted with laboratory-adapted HIV-1 isolates. This raises the question of whether primary Envs derived from HIV-infected patients are capable of inducing bystander apoptosis and whether specific Env signatures are associated with this phenomenon. We developed a high throughput assay to determine the bystander apoptosis inducing activity of a panel of primary Envs. We tested 38 different Envs for bystander apoptosis, virion infectivity, neutralizing antibody sensitivity, and putative N-linked glycosylation sites along with a comprehensive sequence analysis to determine if specific sequence signatures within the viral Env are associated with bystander apoptosis. Our studies show that primary Envs vary considerably in their bystander apoptosis-inducing potential, a phenomenon that correlates inversely with putative N-linked glycosylation sites and positively with virion infectivity. By use of a novel phylogenetic analysis that avoids subtype bias coupled with structural considerations, we found specific residues like Arg-476 and Asn-425 that were associated with differences in bystander apoptosis induction. A specific role of these residues was also confirmed experimentally. These data demonstrate for the first time the potential of primary R5 Envs to mediate bystander apoptosis in CD4+ T cells. Furthermore, we identify specific genetic signatures within the Env that may be associated with the bystander apoptosis-inducing phenotype.

The mechanism behind the slow but progressive depletion of CD4+ T cells in HIV infection remains a pertinent yet unanswered question. Various hypotheses have been proposed for this HIV-induced CD4+ T cell depletion like direct cell killing due to infection (1), bystander apoptosis (2), immune activation (3), immune exhaustion (4, 5), etc. Several lines of evidence suggest that CD4+ T cells undergo apoptosis in HIV infections (6–8). A majority of these cells are shown to be uninfected, lying in close proximity to HIV-infected cells (9), suggesting a role for bystander apoptosis in this pathology. The phenomenon of bystander apoptosis has been demonstrated in both SIV (4, 10) and the humanized mouse model (11) of HIV infection, and a role of viral proteins from infected cells mediating apoptosis in bystander cells has been proposed. Among the viral proteins, the Env glycoprotein remains as the frontrunner for mediating bystander apoptosis because it is expressed on the surface of infected cells and can interact selectively with bystander CD4+ T cells (2, 12, 13).

HIV Env glycoprotein is the most dynamic viral protein that constantly evolves throughout the course of the disease (5, 14–16). The high rate of Env evolution and variability within the HIV-infected population (17) has stimulated a number of studies aimed at understanding the polymorphic nature of the Env glycoprotein (5, 18–20). One of the best studied phenomena associated with HIV Env is the evolution of virus from a CCR5-utilizing phenotype (non-syncytia-inducing) early during the infection to a CXCR4-utilizing phenotype later during the disease (15, 21, 22). This change in co-receptor use has been associated with evolution of the virus to a syncytia-inducing phenotype, leading to rapid decline in CD4 counts and accelerated disease progression (23). However, co-receptor switch is not an absolute requirement for disease progression, and in ~50% of the patients evolution of virus to X4 usage does not occur prior to AIDS development or terminal disease (24, 25). Interestingly, these AIDS-associated R5 viruses have been shown to be more fusogenic than early asymptomatic phase viruses (26, 27). Furthermore, the evolution of virus toward X4

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usage has also been variable between different subtypes of HIV with lower rates of X4 tropism seen in subtype C viruses (28). These findings emphasize the importance of the viral Env in regulating the course of disease with more fusogenic Envs being potentially more deleterious to CD4+ T cells versus the less fusogenic ones.

HIV disease progression is a complex interplay between several viral and host factors. The host immune system in most cases can suppress the virus to undetectable set point/levels after an acute phase. The CD8 cytotoxic T cell response (29, 30) and the neutralizing antibody production are largely responsible for the viral control leading into the chronic phase. During the chronic phase, the highest selection pressure due to neutralizing antibodies is on the Env glycoprotein of the virus (31), which undergoes rapid evolution via changes in the pattern of N-glycosylation and charge on the Env glycoprotein (32). Recently, several genetic signatures have been identified that can distinguish viruses from different stages of the disease that include but are not limited to changes in putative N-glycosylation sites (PNGS) (18, 32–35). Moreover, during the course of intrapatient HIV-1 evolution, it is also documented that the viral Env acquires mutations that alter CD4 binding and/or Env fusogenicity (36–39). However, how this variability in HIV Env affects bystander apoptosis and virus pathogenesis remains undetermined.

We and others have extensively studied the phenomenon of bystander apoptosis mediated by HIV Env both in vitro (40–43) and in vivo (11) in a humanized mouse model of HIV infection. Our studies demonstrate that Env fusogenic activity determines bystander apoptosis and CD4 decline but not virus replication (11). Moreover, with respect to R5 viruses, using cell lines that express different levels of CCR5, we have shown that cell surface CCR5 expression levels determine Env-mediated bystander apoptosis (44). Although experimental data from us and others demonstrate that bystander apoptosis can be induced by HIV Env in vitro, the clinical implication of these findings is limited due to a lack of data from primary HIV isolates and patient-derived Env clones.

In this study, we developed a high throughput assay to study a panel of primary patient-derived HIV Envs for their bystander apoptosis-inducing activity and determined its correlation with both phenotypic and genotypic characteristics of the Envs. These included virus infectivity, sensitivity to neutralizing antibodies, putative N-glycosylation sites, Env charge, and association of specific residues in the Env with bystander apoptosis. Our study demonstrates that although primary R5 Envs vary considerably in their bystander apoptosis-inducing potential, the phenomenon correlates inversely with PNGS and to some extent neutralizing antibody sensitivity. Moreover, using computational sequence and structure analysis, we identified specific Env signatures associated with differential bystander apoptosis-inducing phenotypes. Specific residues associated with these signatures were confirmed by site-directed mutagenesis experiments. These findings not only corroborate the role of viral Env in bystander apoptosis but take our previous studies a step further in attempting to identify specific Env signatures associated with bystander apoptosis. To the best of our knowledge, this is the first study aimed at studying the bystander apoptosis-inducing activity of a large panel of primary Envs utilizing a high throughput assay and correlating this phenomenon with specific Env signatures.

EXPERIMENTAL PROCEDURES

**Cell Lines and Transfections**—SupT1 cells were maintained in RPMI medium supplemented with 10% FBS and penicillin/streptomycin (5000 units/ml). SupT1 cells expressing CCR5 were generated by transduction of wild type SupT1 cells with a lentiviral vector expressing the CCR5 gene and have been described previously (44). These cells were maintained in RPMI medium supplemented with 10% FBS and penicillin/streptomycin (5000 units/ml) and blasticidin at a concentration of 3 μg/ml. 293T, HeLa, and TZM-bl cells (National Institutes of Health AIDS Research and Reference Reagent Program) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and penicillin/streptomycin (5000 units/ml). TZM-bl are HeLa-derived cells that express the HIV receptor CD4 and the co-receptors CXCR4 and CCR5 as well as the luciferase and β-galactosidase genes under the control of HIV LTR (45). These cells thus readily support HIV infection/repllication. All transfections were conducted using the Exgen 500 transfection reagent (Fermentas) following the manufacturer’s instructions.

**Env Constructs**—Reference panels for different subtype Env clones were obtained from the NIH AIDS research and reference reagent program (supplemental Table 1). These include the HIV-1 subtype A/G Env clones (catalog no. 11673), (46) the reference panel for subtype B HIV-1 Env clones (catalog no. 11227) (35, 47, 48), and clade C HIV-1 reference panel of Env clones (catalog no. 11326) (49, 50). These constructs include different patient-derived primary Envs as insert in the pDNA3.1 expression vector (Invitrogen). For the purpose of comparison and to be used as normalizing control, we used a laboratory-adapted R5-utilizing HIV-1 clone, YU-2. The YU-2 Env was thus similarly cloned into the pDNA3.1 directional Topo expression vector (Invitrogen) and contains the open reading frames for the env and rev genes. Point mutations were introduced into different Env constructs using specific mutagenic primers and the QuikChange site-directed mutagenesis kit (Stratagene). The introduced mutations were confirmed by sequencing. For detection of Env expression, HeLa cells transfected with the various Envs were cultured in growth medium devoid of methionine and cysteine and supplemented with [35S]Met/Cys. Thereafter, cell lysates were immunoprecipitated with HIV-Ig (kindly provided by the NIH AIDS Research and Reference Reagent Program) resolved by SDS-PAGE followed by PhosphorImager analysis.

**High Throughput Assay for Bystander Apoptosis**—HeLa cells were seeded in 96-well plates at 7500 cells/well and transfected with various Env constructs using the Exgen 500 transfection reagent. The next day, SupT1-R5-H6 cells were added at 40,000 cells/well. The cells were co-cultured for 24 h, following which caspase-3 substrate was added directly to the wells (Caspase-Glo 3/7 assay, Promega). The plates were incubated for 30 min at 37 °C and read on a luminescence plate reader (Fluostar Omega, BMG LabScience). The Caspase-Glo 3/7 assay is a luminescence-based assay that directly measures caspase-3/7activity.
activity in cultures. Apoptosis for each Env was calculated as the percentage of YU-2 Env-mediated apoptosis after subtraction of the background derived from pcDNA3.1 empty vector-transfected cells.

To determine if bystander apoptosis induction by primary Envs requires CCR5 binding or gp41-mediated membrane fusion, the gp41 inhibitor enfuvirtide (used at a concentration of 2 μM) or CCR5 inhibitor maraviroc (used at a concentration of 1 μM) was added at the time when SupT-R5-H6 were added to each well. After an overnight incubation, the cultures were assayed for caspase-3 activity as indicated above.

Apoptosis Assay—For the standard flow cytometry-based apoptosis assay, Env-transfected HeLa cells were co-cultured with CCR5-expressing SupT-R5-H6 cells followed by the classical method of apoptosis detection via annexin V staining. HeLa cells transfected with different Env constructs were seeded in 24-well plates at a concentration of 10⁵ cells/well. The wells were allowed to adhere for 4–6 h. Subsequently, the medium was removed, and SupT-R5-H6 cells were added at a concentration of 0.5–1 × 10⁶ cells/well. The cells were co-cultured for 24 h, following which the suspension cells were carefully collected, stained with annexin V (BD Biosciences), and analyzed by flow cytometry using a Gallios flow cytometer (Beckman Coulter). At least 10,000 events were collected and analyzed using FlowJo software (Tree Star).

Virus Infectivity—293T cells were transfected with the pNLLuc-R/E—HIV backbone along with different Env constructs. Virus stocks were harvested 48 h post-transfection and used to infect the indicator TZM-bi cell line in the presence of 20 μg/ml DEAE-dextran (Sigma). Luciferase activity was determined 72 h postinfection using the BriteLite plus luciferase assay substrate (PerkinElmer Life Sciences). Infectivity for each Env was calculated as a percentage of YU-2 Env control after subtraction of the background derived from pcDNA3.1 empty vector-transfected cells.

Neutralizing Antibody Sensitivity—pNLLuc-R/E—HIV stocks pseudotyped with different envelopes were prepared as described above. Virus stocks were then added to different concentrations of the TriMab antibody mix (IgGb12, 2F5, and 2G12) starting with a concentration of 25 μg/ml. The virus antibody mix was incubated at 37 °C 1 h and subsequently added to TZM-bi cells. Infection was determined 48 h postinfection by measuring luciferase activity in the cultures. Infectivity curves of each Env were fitted using SigmaPlot software, and IC₅₀ values were calculated from the curves.

Identification of Bystander Apoptosis Determinants in gp120—Nine pairs of genetically similar high-low apoptosis strains were chosen based on a phylogenetic tree generated using the maximum likelihood method with γ-distributed evolutionary rates (shape parameter, 0.4327) using MEGA5 (51). Mutations between these nine pairs were compared for distinctive changes in their physiochemical properties (positive charge, negative charge, aromatic groups, aliphatic groups, prolination, glycosylation, size) between the two groups. Sites that were found in at least three pairs of high-low apoptosis strains to possess distinctive differences between the two groups were considered as candidates for determinants of bystander apoptosis. We examined each of these sites in crystal structures (Protein Data Bank entries 3U2S, 2QAD, and 2B4C) (52–54) for possible structural effects that they may have in altering the function of gp120.

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HIV-1 Envs from laboratory-adapted strains. We cloned the Env and Rev region of a number of laboratory strains of HIV like YU-2, AD-8, JRCSF, Bal, and 89.6. We found that the assay showed differences in bystander apoptosis-inducing activity of these Envs with high level of reproducibility (Fig. 1C). Furthermore, the high throughput assay correlated well with the classical method of apoptosis detection via annexin V staining or by 7-aminoactinomycin D staining. 7500 HeLa cells were transfected with HIV-1 Envs from different laboratory-adapted strains of HIV-1 like YU-2, AD-8, JRCSF, Bal, and 89.6. The next day, 40,000 SupT-R5-H6 cells were added to each well. After an overnight incubation, the cells were analyzed for caspase-3 activity using the Caspase-Glo 3/7 assay. In parallel, HeLa cells were transfected with the above Envs in a 6-well plate. After an overnight incubation, the target cells were harvested and stained for surface phosphatidylserine expression using the annexin V staining kit (BD Biosciences) or by use of cell viability stain 7-aminoactinomycin D (7AAD) (BD Biosciences) followed by flow cytometry. Data are mean ± S.D. (error bars) from triplicate observations.

Primary Envs Vary Considerably in Their Bystander Apoptosis-inducing Activity—Using the assay developed above, we next asked whether there were differences in the bystander apoptosis-inducing activity of a panel of primary patient-derived HIV-1 Envs. We obtained the reference panel of HIV Envs from the National Institutes of Health AIDS Reference Reagent Program, representing subtypes B, C, and AG. A total of 37 primary Envs were tested with YU-2 Env, a laboratory-adapted R5 strain, as a normalizing control and the parent vector pcDNA3.1 as the negative control. Apoptosis was referenced as the percentage of WT or YU-2 after subtracting vector control from each sample. As seen in Fig. 3A, there was a wide variation in the bystander apoptosis-inducing activity of the primary HIV-1 Envs. Based on this variability, we classified the Envs into three groups: high (H) apoptosis-inducing, with the percentage of apoptosis more than 40; low (L) apoptosis-inducing, with the percentage of apoptosis less than 20; and medium (M) apoptosis-inducing.
sis-inducing, between 20 and 40% apoptosis (Fig. 3B). When classified based on subtypes, there was no statistically significant difference between the bystander apoptosis-inducing activity of different Envs (Fig. 3C). All of the Envs used in the study were expressed as detected after immunoprecipitation using pooled sera from HIV-infected individuals (Fig. 3D). This suggests that although primary Envs vary in their bystander apoptosis-inducing activity, this phenomenon may be universal for the subtypes tested. The lack of differences between the subtypes may be due to the selection of the reference panel to represent the most diverse Envs from each subtype. Hence, overrepresentation of certain phenotypes in the population cannot be ruled out.

Bystander Apoptosis-inducing Activity of Primary Envs Correlates with Virion Infectivity—Because both virion infectivity and apoptosis induction are dependent on Env function, we next asked whether there were differences in the virion infectivity of the Envs studied. We used a pseudotyped virus particle approach to determine the single round infectivity of the Envs tested. Here again we found that there was a huge variation in the infectivity of the Envs tested (Fig. 4A). We found that high apoptosis-inducing Envs were more infectious compared with low apoptosis ($p < 0.05$) (Fig. 4B), although subtype-based classification did not show significant differences between the Envs (Fig. 4C). This is not surprising because both virion infectivity and bystander apoptosis are dependent on the Env fusion activity. Although as a group, virus infectivity correlated with bystander apoptosis, this was not necessarily true for each Env. For instance, we found a number of Envs that showed relatively low apoptosis but very high infectivity and vice versa (Fig. 4D). Hence, the spectrum of Envs studied here also includes a number of Envs that may be highly infectious without causing significant apoptosis. This is consistent with several mutagenesis studies whereby point mutations in HIV Env significantly alter bystander apoptosis while having a limited effect on virion infectivity (40, 61).

Bystander Apoptosis-inducing Activity of Primary Envs Correlates Negatively with PNGS—One of the characteristics of HIV evolution in patients over the course of the infection is acquisition of variations in PNGS (35). It has been demonstrated that PNGS tend to increase during the course of infection, with the highest PNGS seen during chronic infection (33) associated with a minimal decline in CD4 T cell counts. The PNGS have been shown to decrease at the late stages of the disease, where there is an accelerated CD4 cell decline (32). Because we hypothesize that bystander apoptosis mediated by Env may be one of the contributing factors in CD4 T cell decline, we asked whether PNGS in Env correlate with bystander apoptosis. PNGS were estimated using the sequence information for each Env and the N-Glycosite software available at the LANL database (Fig. 5A). When the Envs were classified based on high, medium, or low bystander apoptosis activity and PNGS were determined for the Env region (gp160) in each group, there was a significant difference in PNGS between
the high and low apoptosis groups (Fig. 5B). The high apoptosis group shows significantly lower PNGS compared with the low apoptosis group ($p < 0.05$). We also determined whether there were differences between PNGS in the gp120 and gp41 region. Interestingly, most of the differences in PNGS were seen in the gp120 region (Fig. 5C) and not gp41 (Fig. 5D). This suggests that adaptation of the virus via an increase in PNGS may be counterproductive to bystander apoptosis induction. However, when the Envs were grouped on the basis of subtype, no significant differences were seen (Fig. 5, E–G). Once again, this may be due to the diverse set of Envs selected for the reference panel.

Neutralizing Antibody Sensitivity of Primary Envs Correlates with Bystander Apoptosis Induction—Primary Envs derived from HIV-infected patients are known to vary considerably in their sensitivity to neutralizing antibodies (62). Interestingly, the sensitivity to broadly neutralizing antibodies also varies with the stage of the disease as well as PNGS, with end stage viruses being more sensitive and having lower PNGS (32). Because we saw a strong correlation between PNGS and apoptosis induction, we next asked whether there was a correlation between bystander apoptosis activity of primary Envs and sensitivity to broadly neutralizing antibodies. For this purpose, we
used a mixture of the three well characterized broadly neutralizing antibodies 2F5, 2G12, and IgGb12 commonly referred to as TriMab mix. We utilized HIV particles pseudotyped with the panel of HIV Envs to infect the TZM-bl target cell line in the presence of serial dilutions of the TriMab antibody mix (Table 1). Interestingly, the low apoptosis-inducing Envs showed a trend toward being more resistant to inhibition via neutralizing antibodies (Table 1 and Fig. 6A). In this analysis, the Envs that showed no inhibition to the TriMab mix were excluded because an IC50 could not be calculated. Interestingly, when classified based on virus subtype, the non-BC (comprising largely of AG subtype) type isolates showed higher resistance to neutralization when compared with the B subtype (Fig. 6B). It is worth noting that subtype B has been shown to be more susceptible to broadly neutralizing antibodies like b12 (62) used in our study, and hence it is not surprising that the IC50 values for this subtype were low. Several previous studies have reported a correlation between PNGS and neutralizing sensitivity, whereby higher PNGS in Env correlated with increased resistance to neutralizing antibodies (32–34, 63). Although the most significant association of bystander apoptosis was found to be with PNGS, the indirect correlation of PNGS and neutralizing antibody sensitivity reported by others (32–34, 63) is indicative of possible selective pressure on the virus altering its bystander apoptosis-inducing phenotype. However, this is largely speculative because data are limited in this regard in our study.

Sequence Analysis of Primary Envs Reveals Several Residues Associated with Bystander Apoptosis-inducing Activity—Using genotypic information to predict the co-receptor usage of primary HIV Envs is a routine practice (64). The co-receptor prediction software programs utilize sequence information gathered from numerous laboratory experiments using pseudotyped virus-based infection assays as a standard. Based on early findings, a set of rules has been established for prediction of CXCR4/CCR5 co-receptor usage by HIV isolates. These rules include the V3 loop sequence and charge rules (65). However, there is very limited information on the Env sequence changes that can predict or may be associated with pathogenicity of primary Envs. Recently, Sterjovski et al. (66) demonstrated that the presence of Asn-362 in the HIV gp120 region was associated with increased cell-to-cell fusion activity. We hence asked whether there were specific amino acids in the HIV Env that could be associated with bystander apoptosis induction. A search for distinctive physicochemical properties...
between pairs of phylogenetically close high-low apoptosis strains (Fig. 7 and Supplemental Fig. 1) revealed several important residues that may be relevant to the bystander apoptosis-inducing phenotype. Twelve sites (134, 139, and 189 (in the V1 loop); 328, 330, 332, and 334 (V3 loop and vicinity); and 362, 363, 425, 462, and 476 (adjacent to the CD4 binding site) (HXB2 numbering)) were found (Table 2) in at least three pairs of high-low apoptosis strains with distinctive differences in amino acid residues in the above positions. Notably, we found that Asn-362, which has previously been shown to be associated with...
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mutational analysis shows that R476K and N425R may be relevant to bystander apoptosis-inducing activity of env glycoprotein—Our sequence analysis data and molecular modeling identified specific amino acid residues that may be linked to apoptosis induction by the primary HIV Env used in this study. Of those, the most noticeable ones were Asn-425 and Arg-476 because these sites are probably involved in CD4 binding based on our structure analysis. To confirm the involvement of these residues in apoptosis induction, we used a site-directed mutagenesis approach to induce R476K and N425R mutations in the high apoptosis-inducing strains, converting them to low apoptosis inducers, were also expected to affect CD4 binding via steric hindrance and also lead to charge alterations in the Env (Fig. 9).

increased fusogenicity of the Env glycoproteins (66), was present in a number of high apoptosis inducers in our analysis (Fig. 7 and supplemental Fig. 1).

To further our analysis and to avoid complications from founder effects through amino acid differences that may arise as a result of separate evolution of the different subtypes, we considered the relative phylogenetic positions of all analyzed strains. Thereafter, we selected genetically closely related pairs with differing apoptosis-inducing results to increase the specificity of identifying mutations directly related to this phenotype (Fig. 8A). In essence, this approach identifies Envs that are most closely related in sequence but varied in phenotypic characteristics, thus classifying them as nearest pair neighbors. For instance, using this approach, Envs 11034 and 11035 were found to be closely related in sequence but widely varied in apoptosis induction. This novel analysis revealed a number of sites that may be associated with bystander apoptosis induction with two sites that stood out in particular. These include amino acid positions 425 and 476. Interestingly, four of the nine high apoptosis inducers selectively showed the presence of an asparagine at position 425 by our pairwise analysis approach (Fig. 8B). Additionally, position 476 was of particular interest (Fig. 8C) because five of nine high-low apoptosis pairs possessed a lysine in the low apoptosis inducer that was not found in any of the high apoptosis strains examined. In all nine pairs, the high apoptosis group had an arginine at this position. In addition to our semimanual phylogeny and structure-aware candidate selection procedure, we also compared the results with the related sequence harmony approach (67), which confirms our two studied candidates as being highly significant (Fig. 8D and supplemental Fig. 2). Using this comprehensive analysis, we were able to identify several potential residues important for bystander apoptosis, of which residues 425 and 476 were most promising due to the reasons described below and hence chosen for further in depth analysis.

Structural Analysis of Candidates for Determinants of HIV Env-mediated Bystander Apoptosis—Having identified specific amino acid residues that may be involved in bystander apoptosis induction, we next conducted structural analysis to determine the location of these residues with respect to the viral Env. Structural mapping of the above identified 12 candidate sites onto Protein Data Bank structure 2QAD (52) revealed that these sites could be found in the V1 loop (positions 134, 139, and 189), V3 loop and its vicinity (positions 328, 330, 332, and 334), and the CD4 binding site region (positions 362, 363, 425, 462, and 476). For this study, we chose candidate sites that could play a role in directly affecting CD4 binding (positions 362, 363, 425, and 476) for further experimental analysis. Because position 362 has already been shown to affect HIV cell fusion (66) and position 363 may possibly play a similar role because it lies next to residue 362, we tested the other promising positions, 425 and 476, for association with bystander apoptosis. Because both of these positions are within 5 Å of the CD4 binding site, residue changes R476K and N425R in the high apoptosis-inducing strains, converting them to low apoptosis inducers, were also expected to affect CD4 binding via steric hindrance and also lead to charge alterations in the Env (Fig. 9).
N425R and R476K are associated with bystander apoptosis in our study, R476K was also confirmed to be a bystander apoptosis-regulating site via gain of function mutations. Env expression between each pair was found to be identical (Fig. 10H), confirming that changes in Env bystander apoptosis are not likely to be due to differences in expression levels.
TABLE 2
Amino acid residues that may be associated with bystander apoptosis induction using the approach delineated in Figs. 7 and 8

| Position | Region | Amino Acid distribution | Structure |
|----------|--------|-------------------------|-----------|
| 134      | V1 loop| Low A1 tends to have aromatic rings (YW) | In PDB structure 3U2S, codon 134 is missing and the last residue before which the structure is truncated is at codon position 132, which extends towards the antibody structure. Codon 134 is likely to be even closer to the antibody. |
| 139      | V1 loop| Low A1 is glycosylated | Found in V1. loop. |
| 189      | V1 loop| The found only in low A1 | In PDB structure 3U2S, codon 189 is missing and the last residue before the missing V1 loop region is codon 190, extending away from the antibody. Codon 189 is likely to be even closer to the antibody. |
| 328      | V3 loop| High A1 tends to have positive charge | In PDB structure 2GAD, codon 328 lies on the opposite side of CD4 binding site, next to an NAG. |
| 330      | V3 loop| High A1 have Tyr instead of His | In PDB structure 2GAD, codon 330 lies on the opposite side of CD4 binding site, next to an NAG. |
| 332      | after V3| Low A1 are glycosylated | In PDB structure 2GAD, codon 332 lies on the opposite side of CD4 binding site, next to an NAG. |
| 334      | after V3| High A1 are glycosylated | In PDB structure 2GAD, codon 334 lies on the opposite side of CD4 binding site, next to an NAG. |
| 362      | adjacent to CD4| High A1 are glycosylated | In PDB structure 2B4C, codon 362 is an N-glycosylation site, lying next to CD4 molecule. |
| 363      | adjacent to CD4| Low A1 are proximated | Found beside codon 362 and may exert an effect on CD4 indirectly via codon 362. |
| 425      | adjacent to CD4| High A1 has Arg (not a glyco site) instead of Asp | Adjacent to CD4 binding site. |
| 462      | adjacent to CD4| High A1 tends to be glycosylated | Within the V5 loop region. |
| 476      | adjacent to CD4| High A1 have Arg instead of Lys | Binds to CD4 molecule. |

Because our approach involves a pairwise analysis of the nearest neighbors that are genetically similar but with differing apoptosis induction capability, we tested the various characteristics of 11034 and 11035 (R476K pair). A side by side comparison of this pair revealed maximum differences with respect to all of the characteristics studied like apoptosis (Fig. 10D), virus infectivity (Fig. 10E), antibody neutralization (Fig. 10F), and PNGS (Fig. 10G). Thus, this pair represents the perfect scenario for identification of characteristics associated with low versus high apoptosis-inducing Envs. Also this pair corroborates many of the associations revealed in our study, including virion infectivity, PNGS, neutralizing antibody sensitivity, and specific amino acids, in determining bystander apoptosis activity. This approach also reveals that the Env phenotype in terms of bystander apoptosis is probably quite complex, and multiple Env regions and characteristics may collectively determine the apoptosis outcome. Thus, studying genotypically closely related Envs that show a diverse apoptosis phenotype is probably the best approach for identifying the residues/genetic signatures important for this phenomenon.

**Bystander Apoptosis Induction by Primary Envs Requires CCR5 Binding as Well as gp41-mediated Membrane Fusion—**
The membrane fusing activity of HIV Env has long been suspected to have a role in HIV pathogenesis (68). Specifically, the role of the gp41 subunit in mediating bystander apoptosis has only recently been demonstrated (41, 59). Studies by our group and others have shown that gp41-mediated fusion/hemifusion is critical for apoptosis induction, and binding of gp120, although required, cannot alone induce apoptotic signaling by HIV Env (40, 41, 59). Although studies with laboratory viruses have provided proof of principle for this hypothesis (69), there is a lack of data from primary Envs derived directly from patients to corroborate the physiological relevance of these findings. We therefore asked whether bystander apoptosis induction by primary Envs is also gp41-dependent. To answer this question, we selected the highest apoptosis-inducing Envs from our panel and tested bystander apoptosis induction in the presence or absence of CCR5 antagonist maraviroc and gp41 inhibitor enfuvirtide. As seen in Fig. 11, both maraviroc and enfuvirtide inhibited bystander apoptosis by all of the primary Envs. Inhibition by CCR5 inhibitor confirms a role of gp120 binding to the co-receptor as a requirement for apoptosis induction, whereas inhibition by enfuvirtide is indicative of a critical role played by the gp41 unit in this phenomenon. These findings are consistent with previous studies and further support the role of HIV gp41-mediated fusion in apoptosis induction not only with laboratory isolates but also primary viruses. Furthermore, this also suggests that the mechanism of bystander apoptosis by primary and laboratory variants of HIV is probably the same.

**DISCUSSION**
HIV infection leads to a selective depletion of CD4+ T helper cells, resulting in immunodeficiency. However, the mechanism by which HIV mediates this process remains highly debated (2, 6, 12, 70–73) and forms the basis of our study. It is proposed that the loss of CD4 cells during HIV infection is due to the process of bystander apoptosis (2). This is supported by early studies conducted by Finkel et al. (9), who demonstrated that the majority of cells undergoing apoptosis during HIV infection are not infected but are in close proximity to infected cells. A role of direct infection in the loss of CD4 cells is also refuted by natural infections in sooty mangabeys with the simian SIVsm, where high levels of infection and viremia do not result in CD4 loss or AIDS development (74). Hence, bystander apoptosis is believed to be one of the major causes of CD4 loss leading to AIDS (6, 70, 75). Bystander apoptosis is a phenomenon that has been demonstrated in various animal models of HIV, including the SIV model in rhesus macaques (4, 10) and more recently in the humanized mouse model (11). Interestingly, bystander apoptosis induction in these models correlates with CD4 loss, further supporting the role of this phenomenon in disease progression.

The mechanism of bystander apoptosis of CD4 T cells in HIV infection remains unresolved. Interestingly, the role of the Env glycoprotein in this process is becoming increasingly evident (12, 13). This is largely supported by the arguments that 1) as cell death in HIV infection outnumbers the infected cell population, a role of bystander cell death in the progression to AIDS is likely; 2) as the depletion of immune cells is restricted to CD4+ helper phenotype and as the Env glycoprotein of HIV binds to CD4, it probably plays a role either directly or indirectly in CD4 T cell death; and 3) the Env glycoprotein is the only viral protein expressed on the surface of infected cells and...
has been shown to interact with and mediate apoptosis in bystander cells, albeit only in in vitro studies (41, 59, 60, 76).

With support from in vitro studies, understanding the genetic signatures associated with the bystander apoptosis-inducing phenotype of the Env glycoprotein is of increasing importance. Although the phenotypic and genotypic characterization of HIV Env has been well documented for co-receptor usage and neutralizing antibody sensitivity, there is virtually no information on the phenotypic characterization of HIV Env for the phenomenon of bystander apoptosis induction. We have previously developed cell lines that express varying levels of the HIV co-receptor CCR5 (44). Using these cell lines and a laboratory-adapted YU-2 clone of HIV, we studied the bystander apoptosis-inducing activity of HIV Env and the role of CCR5 co-receptor levels on this phenomenon. Our findings suggest that the bystander apoptosis induction is dependent on the Env fusogenic activity as well as the surface CCR5 expression levels on cells (44). Although this study provided valuable insights into the mechanism of R5 Env-mediated apoptosis, the physiological relevance of these findings remains untested. Hence, we undertook this study with the objectives to understand whether and how primary HIV Env variants induce apoptosis in bystander cells, whether there is variability in bystander apoptosis induction between different HIV subtypes, and whether specific genetic signatures in the Env glycoprotein are associated with increased bystander apoptosis phenotype.

To achieve these objectives, we developed a high throughput assay that can determine the relative bystander apoptosis-inducing activity of primary HIV-1 Envs. Our caspase-based assay was highly reproducible and correlated well with other methods of apoptosis determination. More importantly, the assay could distinguish small changes in bystander apoptosis-inducing activity between various HIV Env variants tested. With the help of this assay, an important characteristic of HIV Env can be added to the profile of patient viruses.

We found that the bystander apoptosis-inducing activity of primary Envs of R5 tropism varies considerably. Although we did not find subtype-specific differences in our panel, it should be noted that the assay was done using a reference panel of Envs that represents a highly diverse set of Envs. Whether certain subtypes contain high apoptosis-inducing Envs that are over-represented in the population can only be determined by studying larger populations of Envs directly from patients. We also found that virion infectivity correlated with bystander apoptosis-inducing activity in our panel. This does not come as a surprise because both virion infectivity and bystander apoptosis are dependent on the function of the Env glycoprotein. Although as a group, the Envs showed these differences, the bystander apoptosis and virion infectivity of individual Envs did not necessarily correlate in every case. We did find some Envs that showed high infectivity but low bystander apoptosis and vice versa. These findings underscore both the genotypic and phenotypic variability of Envs seen in patients. This also suggests that in some cases, the viruses may be capable of infection/replication in the absence of inducing bystander apoptosis. Such non-pathogenic viruses probably contain changes in the Env glycoprotein that alter their apoptosis phenotype while maintaining virion infectivity. This is consistent with our previous findings, whereby a single amino acid change in the Env glycoprotein altered bystander apoptosis and CD4 loss in a humanized mouse model of HIV infection (11) while maintaining virus infectivity. However, from a viral evolution point of view, the acquisition of bystander apoptosis-inducing activity by a virus must come with some selective advantage, and in this case, it might be increased fitness/infectivity. This increased fitness may help in virus dissemination via infection with cell-free virus in different anatomical sites.

Putative N-glycosylation sites have been shown to vary considerably in Env glycoproteins from HIV-infected patients based on the stage of the disease (18, 33). During the chronic phase, HIV Env has been shown to contain more PNGS compared with the acute phase of infection (33). This same characteristic is reflected in viruses isolated from late stages of the disease, whereby loss of PNGS is observed during late versus chronic infections (32). Furthermore, the presence of higher glycosylation or PNGS has also been associated with reduced viral fitness (32). The question that arises is what mediates the increase in PNGS during the chronic phase of HIV infections. The leading hypothesis is that the neutralizing antibody pressure is the primary driving force behind PNGS evolution in HIV. This is supported by the fact that the presence of PNGS on
HIV Env is responsible for masking the epitopes and increasing neutralization resistance (33–35). However, this phenomenon may also come at the price of reduced viral fitness (77), and hence, when the opportunity arises, as in the case of late stages where the immunocompromised patients are no longer able to mount an effective neutralizing antibody response, the virus reverts to low PNGS, increased fitness, and possibly increased bystander apoptosis phenotype.

Our study demonstrates that many of the characteristics of Env glycoprotein, including PNGS, neutralizing antibody sensitivity, and viral fitness, that have previously been shown to correlate with CD4 loss and disease progression are also incidentally associated with bystander apoptosis induction. The preliminary hypothesis that emerges from our study is that during the chronic phase of the disease, immune pressure selects for Envs with higher PNGS. This potentially results in a loss/reduction in bystander apoptosis, which may be reflected in the slower depletion of CD4+ T cells seen during the chronic phase. Subsequently, the gradual loss of CD4 cells results in an immunocompromised state that paves the way for the viruses with...
lower PNGS, increased fitness, and possibly higher bystander apoptosis-inducing phenotype to emerge and precipitate a rapid CD4 loss. Although our study only provides circumstantial evidence of this correlation, it opens the door to further studies focused on this aspect.

Interestingly, most of the PNGS differences as well as the 12 most relevant sites found to be associated with the apoptosis phenotype were in the gp120 region, with many mapping around the CD4 binding site. The fusion process mediated by Env is a concerted effort between both the gp120 and the gp41 subunits of HIV Env. Because the gp120 subunit is exposed to host defense mechanisms, it is not surprising that the largest variability is seen in this subunit, possibly via antibody pressure. It is also not surprising that most of the changes associated with bystander apoptosis induction also map to gp120. Supporting these findings, Holm et al. (78) demonstrated that changes in the gp120 co-receptor binding domain can affect Env function and bystander apoptosis.

As mentioned above, one of our major objectives was also to determine whether certain HIV phenotypic and genotypic signatures correlate with bystander apoptosis and, more specifically, whether sequence analysis alone may be able to predict the bystander apoptosis-inducing phenotype of HIV Envs. To this end, we adopted a novel pairwise analysis approach that focused on phylogenetically closely related Env pairs with differing apoptosis activity in an attempt to identify candidate amino acid signatures related to this unique phenotype. The Env fusogenic activity has been associated with a specific stage of disease and specific residues like Asn-362 (66). Recently, Wade et al. (27) demonstrated that Env glycoproteins derived from late stage patients are more fusogenic and induce enhanced CD4+ T cell apoptosis compared with early stage Envs. In our study, we identified a total of 12 amino acid positions that were potentially associated with high bystander apoptosis-inducing Envs, including the previously mapped Asn-362 linked to increased fusogenicity. Among these, R476K and N425R were identified as novel sites through our pairwise analysis and molecular modeling. We also confirmed the potential role of these amino acid signatures experimentally using site-directed mutagenesis.

The primary function of HIV Env is to mediate fusion of the viral and cellular membranes to facilitate entry of the virus core into cells. However, the same phenomenon can also mediate fusion between an infected and uninfected bystander cell. This fusion process and the intermediate of this phenomenon referred to as hemifusion (kiss of death) have been shown to be the primary cause of bystander apoptosis in \textit{in vitro} culture systems (40, 41, 59, 79). Most of the data supporting the role of Env-mediated fusion/hemifusion in bystander apoptosis comes from laboratory-adapted viruses. Hence, in this study, we also asked whether bystander apoptosis mediated by primary HIV-1 Envs is dependent on the Env fusogenic activity or gp41 function. We indeed demonstrate that bystander apoptosis induction by primary Envs is dependent on gp41 function (Env fusogenic activity). Interestingly, fusogenic activity of Env glycoprotein has been indirectly associated with HIV pathogenesis in clinical studies where the presence of a highly fusogenic syncytia-inducing phenotype has been associated with poor prognosis in patients (80, 81), increased pathogenesis (23), and CXCR4 (82) tropism of viruses. The syncytia-inducing phenotype has also been associated with increased pathogenesis in the SCID-hu mouse model. In SCID-hu mice, the lack of CD4 T cell loss by certain CCR5 tropic laboratory strains underscores this difference (83). However, it has also been demonstrated that CCR5 tropic viruses from late stages of disease are more fusogenic, which correlates with pathogenicity (26). This is further supported by recent studies by Wade et al. (27), demonstrating the apoptotic potential of late stage R5 viruses. Probably the strongest evidence for a role of Env membrane-fusing activity and HIV pathogenesis and CD4 decline comes from SHIV studies in rhesus macaques, where the pathogenicity of the SHIV-KB9 variant can be linked directly to the membrane fusion activity of the virus (84–88). The fact that primary Env-mediated bystander apoptosis in our study could be inhibited by inhibiting Env-mediated fusion using a gp41 inhibitor adds further support for the role of membrane fusion in this phenomenon. Recently, Hunt et al. (89) have shown the clinical benefits of maraviroc and ENF therapy that target Env, supporting the role of Env in HIV pathogenesis.

Our findings suggest that bystander apoptosis-inducing activity of HIV Env may be an important phenotypic characteristic that warrants studies in a larger panel of Envs for two reasons. 1) It will help strengthen the hypothesis that bystander apoptosis is an important phenomenon for CD4 loss in HIV infection. 2) With a larger panel of Envs, we will be able to obtain enough preliminary data to determine whether certain genetic signatures are associated with bystander apoptosis, much in line with the current methods for determining co-receptor tropism using sequence information alone.

Although the Env glycoprotein has previously been characterized for numerous phenotypic properties like infectivity, co-receptor use, neutralizing antibody sensitivity, and PNGS, bystander apoptosis induction is a unique phenotype of HIV that has previously not been studied, probably due to a lack of an appropriate high throughput assay. Because a number of these characteristics of the Env glycoprotein are associated with
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disease progression and CD4 loss, our findings provide further support for the phenomenon of bystander apoptosis in CD4 loss and the role of Env glycoprotein in this process. The actual apoptotic potential of Env variants may best be calculated using our assay and pairwise computational analysis. We believe that using this strategy, we will be able to generate a database that can be used for developing a methodology for predicting bystander apoptosis. Recently, a machine-learning method has been developed for identifying genetic signatures in HIV Env predictive of HIV-associated dementia (90) based on a database of HIV Env sequences from the brain (91). Using similar strategies, our study should lead to development of an HIV Env sequence database characterizing the bystander apoptosis potential of Envs. Our study establishes a first set of preliminary rules like PINGS and specific amino acid signatures that can be further developed into an algorithm. Ultimately, this should lead to determination of bystander apoptosis phenotypes of Envs by sequence analysis alone. Whether these findings would have a diagnostic value remains to be seen.

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REFERENCES

1. Gandhi, R. T., Chen, B. K., Straus, S. E., Dale, J. K., Lenardo, M. J., and Baltimore, D. (1998) HIV-1 directly kills CD4+ T cells by a Fas-independent mechanism. J. Exp. Med. 187, 1113–1122.
2. Garg, H., Mohl, J., and Joshi, A. (2012) HIV-1 induced bystander apoptosis. Viruses 4, 3020–3043.
3. Douek, D. (2007) HIV disease progression. Immune activation, microbes, and a leaky gut. Top. HIV Med. 15, 114–117.
4. Meythaler, M., Martinot, A., Wang, Z., Prpyptwiznieck, S., Kasheta, M., Ling, B., Marx, P. A., O’Neill, S., and Kaur, A. (2009) Differential CD4+ T-lymphocyte apoptosis and bystander T-cell activation in rhesus macaques and sooty mangabeys during acute simian immunodeficiency virus infection. J. Virol. 83, 572–583.
5. van Gils, M. J., Edo-Matas, D., Bowles, E. J., Burger, J. A., Stewart-Jones, G. B., and Schuitemaker, H. (2011) Evolution of human immunodeficiency virus type 1 in a patient with cross-reactive neutralizing activity in serum. J. Virol. 85, 8443–8448.
6. Gougeon, M. L., and Montagnier, L. (1993) Apoptosis in AIDS. Science 260, 1269–1270.
7. Oyaizu, N., McCloskey, T. W., Than, S., Hu, R., and Pahwa, S. (1995) Mechanism of apoptosis in peripheral blood mononuclear cells of HIV-infected patients. Adv. Exp. Med. Biol. 374, 101–114.
8. Oyaizu, N., McCloskey, T. W., Coronesi, M., Chirmule, N., Kalyanaraman, V. S., and Pahwa, S. (1993) Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. Blood 82, 3392–3400.
9. Finkol, T. H., Tudor-Williams, G., Banda, N. K., Cotton, M. F., Curiel, T., Monks, C., Baba, T. W., Ruprecht, R. M., and Kupfer, A. (1995) Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat. Med. 1, 129–134.
10. Meythaler, M., Prpyptwiznieck, S., and Kaur, A. (2008) Kinetics of T lymphocyte apoptosis and the cellular immune response in SIVmac239-infected rhesus macaques. J. Med. Primatol. 37, 33–45.
11. Garg, H., Joshi, A., Ye, C., Shankar, P., and Manjunath, N. (2011) Single amino acid change in gp41 region of HIV-1 alters bystander apoptosis and CD4 decline in humanized mice. Virol J. 8, 34.
12. Perfettini, J. L., Castedo, M., Roumier, T., Andreau, K., Nardacci, R., Piacentini, M., and Kroemer, G. (2005) Mechanisms of apoptosis induction by the HIV-1 envelope. Cell Death Differ. 12, 916–923.
13. Abt, B., Robert-Hebmann, V., Devaux, C., and Biard-Piechaczyk, M. (2004) Apoptosis of uninfected cells induced by HIV envelope glycoproteins. Retrovirology 1, 12.
14. Shankarappa, R., Margolick, J. B., Gange, S. J., Rodrigo, A. G., Upchurch, D., Farzadegan, H., Gupta, P., Rinaldo, C. R., Learn, G. H., He, X., Huang, X. L., and Mullins, J. I. (1999) Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J. Virol. 73, 10489–10502.
15. Edo-Matas, D., Rachinger, A., Setiawan, L. C., Boeser-Nunnink, B. D., van’t Wout, A. B., Lemey, P., and Schuitemaker, H. (2012) The evolution of human immunodeficiency virus type-1 (HIV-1) envelope molecular properties and coreceptor use at all stages of infection in an HIV-1 donor-recipient pair. Virology 422, 70–80.
16. Navis, M., Matas, D. E., Rachinger, A., Koning, F. A., van Swieten, P., Kootstra, N. A., and Schuitemaker, H. (2008) Molecular evolution of human immunodeficiency virus type 1 upon transmission between human leukocyte antigen disparate donor-recipient pairs. PLoS One 3, e2422.
17. Hahn, B. H., Gonda, M. A., Shaw, G. M., Popovic, M., Hoxie, J. A., Gallo, R. C., and Wong-Staal, F. (1985) Genomic diversity of the acquired immune deficiency syndrome virus HTLV-III. Different viruses exhibit greatest divergence in their envelope genes. Proc. Natl. Acad. Sci. U.S.A. 82, 4813–4817.
18. Curlin, M. E., Zioni, R., Hawes, S. E., Liu, Y., Deng, W., Gottlieb, G. S., Zhu, T., and Mullins, J. I. (2010) HIV-1 envelope subregion length variation during disease progression. PLoS Pathog. 6, e1001228.
19. Shankarappa, R., Kaur, A., Golub, G. H., Jr., Rodrigo, A. G., Rinaldo, C. R., Jr., Gorry, M. C., Mullins, J. I., Nara, P. L., and Ehrlich, G. D. (1998) Evolution of human immunodeficiency virus type 1 envelope sequences in infected individuals with differing disease progression profiles. Virolology 241, 251–259.
20. Gray, L., Roche, M., Churchill, M. J., Sterjovski, J., Ellett, A., Pombouriuos, P., Sherief, S., Wang, B., Saksena, N., Purcell, D. F., Wesselingh, S., Cunnigham, A. L., Brew, B. J., Gabuzda, D., and Gorry, P. R. (2009) Tissue-specific sequence alterations in the human immunodeficiency virus type 1 envelope favoring CCR5 usage contribute to persistence of dual-tropic virus in the brain. J. Virol. 83, 5430–5441.
21. Schuitemaker, H., van’t Wout, A. B., and Luzo, P. (2011) Clinical significance of HIV-1 coreceptor usage. J. Transl. Med. 9, S5.
22. Edo-Matas, D., van Dort, K. A., Setiawan, L. C., Schuitemaker, H., and Koestra, N. A. (2011) Comparison of in vivo and in vitro evolution of CCR5 to CXC4 coreceptor use of primary human immunodeficiency virus type 1 variants. Virolology 412, 269–277.
23. Schuitemaker, H., Koot, M., Koestra, N. A., Dercksen, M. W., de Goede, R. E., van Steenwijk, R. P., Lange, J. M., Schattenkerk, J. K., Miedema, F., and Tersmette, M. (1992) Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection. Progression of disease is associated with a shift from monocytotropic to T-cell-tropic human immunodeficiency virus population. J. Virol. 66, 1354–1360.
24. Jansson, M., Backström, E., Björndal, A., Holmgren, V., Rossi, P., Fenýö, E. M., Popovic, M., Albert, J., and Wigzell, H. (1999) Coreceptor usage and RANTES sensitivity of non-syncytium-inducing HIV-1 isolates obtained from patients with AIDS. J. Hum. Virol. 2, 325–338.
25. Esbjörnsson, I., Månsson, F., Martínez-Arias, W., Vincic, E., Biague, A. J., da Silva, Z. I., Fenýö, E. M., Norrgren, H., and Medstrand, P. (2010) Frequent CCRX4 tropism of HIV-1 subtype A and CRF02_AG during late-stage disease. Indication of an evolving epidemic in West Africa. Retrovirology 7, 23.
26. Olivier, K., Scoggin, R. M., Bor, Y. C., Matthews, A., Mark, D., Taylor, J. R. Jr., Chernauska, D., Hammerskjold, M. L., Rekosh, D., and Camerini, D. (2007) The envelope gene is a cytopathic determinant of CCR5 tropic HIV-1. Virology 358, 23–38.
27. Wade, J., Sterjovski, J., Gray, L., Roche, M., Chiavaroli, L., Ellett, A., Jakobsen, M. R., Cowley, D., Pereira Cda, F., Saksena, N., Wang, B., Purcell, D. F., Karlsson, I., Fenýö, E. M., Churchill, M., and Gorry, P. R. (2010) Enhanced CD4+ cellular apoptosis by CCR5-restricted HIV-1 envelope glycoprotein variants from patients with progressive HIV-1 infection. Virology 396, 246–255.
28. Björndal, A., Sönnerborg, A., Tscherning, C., Albert, J., and Fenyo, E. M. (1999) Phenotypic characteristics of human immunodeficiency virus type 1 subtype C isolates of Ethiopian AIDS patients. AIDS Res. Hum. Retroviruses 15, 647–653
29. McDermott, A. B., and Koup, R. A. (2012) CD8+ T cells in preventing HIV infection and disease. AIDS 26, 1281–1292
30. Streeck, H., and Nixon, D. F. (2010) T cell immunity in acute HIV-1 infection. J. Infect. Dis. 202, S302–S308
31. Mosier, D. E. (2005) HIV-1 envelope evolution and vaccine efficacy. Curr. Drug Targets Infect. Disord. 5, 171–177
32. Borrgren, M., Repits, J., Sterjovski, J., Uchtemanen, H., Churchill, M. I., Karlsson, A., Albert, J., Achour, A., Gorry, P. R., Fenyo, E. M., and Jansson, M. (2011) Increased sensitivity to broadly neutralizing antibodies of end-stage disease R5 HIV-1 correlates with evolution in Env glycosylation and charge. PLoS One 6, e20135
33. Sagar, M., Wu, X., Lee, S., and Overbaugh, J. (2006) Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity. J. Virol. 80, 9586–9598
34. van Gils, M. J., Bunnik, E. M., Boeser-Nunnink, B. D., Burger, J. A., Terwisscha van Scheltinga, C. R., Albert, J., and Fenyo, E. M. (2005) Thymic pathogenicity of CCR5-restricted HIV-1 isolates from patients with acquired immunodeficiency syndrome. Virology 337, 384–398
35. Sterjovski, J., Churchill, M., Ellery, P., Nasr, N., Lewin, S. R., Crowe, S. M., Wesselingh, S. L., Cunningham, A. L., and Gorry, P. R. (2005) Uncoupling coreceptor usage of human immunodeficiency virus type 1 (HIV-1) from macrophage tropism reveals biological properties of CCR5-restricted HIV-1 isolates from patients with acquired immunodeficiency syndrome. Virology 343, 310–319
36. Thomas, E. R., Dunfee, R. L., Stanton, J., Bogdan, D., Taylor, J., Kunstman, K., Bell, J. E., Wolinsky, S. M., and Gabuzda, D. (2007) Macrophage entry mediated by HIV Envs from brain and lymphoid tissues is determined by the capacity to use low CD4 levels and overall efficiency of fusion. Virology 360, 105–119
37. Sterjovski, J., Churchill, M. I., Roche, M., Elliet, A., Farrugia, W., Wesselingh, S. L., Cunningham, A. L., and Gorry, P. R. (2011) CD4-binding site alterations in CCR5-utilizing HIV-1 envelopes influencing gp120-CD4 interactions and fusogenicity. Virology 410, 418–428
38. Garg, H., Joshi, A., Freed, E. O., and Blumenthal, R. (2007) Site-specific mutations in HIV-1 gp41 reveal a correlation between HIV-1-mediated bystander apoptosis and fusion/hemifusion. J. Biol. Chem. 282, 16899–16906
39. Garg, H., and Blumenthal, R. (2006) HIV gp41-induced apoptosis is mediated by caspase-3-dependent mitochondrial depolarization, which is inhibited by HIV protease inhibitor nelfinavir. J. Leukoc. Biol. 79, 351–362
40. Meissner, S., Goff, J., and Saag, M. S., Wu, X., Shaw, G. M., and Kappes, J. C. (2002) Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. 46, 1896–1905
41. Derdeyn, C. A., Decker, J. M., Bibolette-Ruche, F., Mokili, J. L., Muldooan, M., Denham, S. A., Heil, M. L., Kasolo, F., Musonda, R., Hahn, B. H., Shaw, G. M., Korber, B. T., Allen, S., and Hunter, E. (2004) Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. Science 303, 2019–2022
42. Williamson, C., Morris, L., Maughan, M. F., Ping, L. H., Dryga, S. A., Thomas, R., Reap, E. A., Cilliers, T., van Harmelen, J., Pascual, A., Ramjee, G., Gray, G., Johnston, R., Karim, S. A., and Swanstrom, R. (2003) Characterization and selection of HIV-1 subtype C isolates for use in vaccine development. AIDS Res. Hum. Retroviruses 19, 133–144
43. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5. Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2739–2749
44. Huang, C. C., Lam, S. N., Acharya, P., Tang, M., Xiang, S. H., Hussain, S. S., Stanfield, R. L., Robinson, J., Sodroski, J., Wilson, I. A., Wyatt, R., Baley, C. A., and Kwong, P. D. (2007) Structures of the CCR5 N terminus and of a tyrosine-sulfated antibody with HIV-1 gp120 and CD4. Science 317, 1930–1934
45. Huang, C. C., Tang, M., Zhang, M. Y., Majeed, S., Montabana, E., Stanfield, R. L., Dimitrov, D. S., Korber, B., Sodroski, J., Wilson, I. A., Wyatt, R., and Kwong, P. D. (2005) Structure of a V3-containing HIV-1 gp120 core. Science 310, 1025–1028
46. McElhaney, J. E., Pandera, M., Carrio, C., Gorman, J., Julien, J. P., Khayat, R., Louder, R., Pejchal, R., Sastry, M., Dai, K., O’Dell, S., Patel, N., Shahzad-ul-Hussan, S., Yang, Y., Zhang, B., Zhou, T., Zhu, J., Boyington, J. C., Chiang, G. Y., Dianji, D., Georgiev, I., Kwon, Y. D., Lee, D., Louder, M. K., Moquin, S., Schmidt, S. D., Yang, Z. Y., Bonsignori, M., Crump, J. A., Kapiga, S. H., Sam, N. E., Haynes, B. F., Burton, D. R., Koff, W. C., Walker, L. M., Phogat, S., Wyatt, R., Orwenyo, I., Wang, L. X., Arthos, J., Baley, C. A., Mascola, J. R., Nabel, G. J., Schief, W. R., Ward, A. B., Wilson, I. A., and Kwong, P. D. (2011) Structure of HIV-1 gp120 V1/V2 domain with broad neutralizing antibody PG9. Nature 480, 336–343
47. Krieger, E., Koraimann, G., and Vriend, G. (2002) Increasing the precision of comparative models with YASARA NOVA. A self-parameterizing force field. Proteins 47, 393–402
48. Eswar, N., Eramian, D., Webb, B., Shen, M. Y., and Sali, A. (2008) Protein structure modeling with MODELLER. Methods Mol. Biol. 426, 145–159
49. Li, H., Robertson, A. D., and Jensen, J. H. (2005) Very fast empirical prediction and rationalization of protein pKa values. Proteins 61, 704–721
50. Katoh, K., Kuma, K. T., Ohn, H., and Miyata, T. (2005) MAFFT version 5. Improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 33, 511–518
51. Blanco, J., Barretina, J., Ferri, K. F., Jacotet, E., Gutiérrez, A., Armand-Ugón, M., Cabrera, C., Kroemer, G., Clotet, B., and Esté, J. A. (2003) Cell-surface-expressed HIV-1 envelope induces the death of CD4+ T cells during gp41-mediated hemifusion-like events. Virology 305, 318–329
52. Biard-Piechaczek, M., Robert-Hembert, V., Richard, V., Roland, J., Hipskind, R. A., and Devaux, C. (2000) Caspase-dependent apoptosis of cells
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expressing the chemokine receptor CXCR4 is induced by cell membrane-associated human immunodeficiency virus type 1 envelope glycoprotein (gp120). *Virology* **268**, 329–344

61. Garg, H., Ishii, A., and Blumenthal, R. (2009) Altered bystander apoptosis induction and pathogenesis of enfuvirtide-resistant HIV type 1Env mutants. *AIDS Res. Hum. Retroviruses* **25**, 811–817

62. Gnanakaran, S., Daniels, M. G., Bhattacharya, T., Lapedes, A. S., Sethi, A., Li, M., Tang, H., Greene, K., Gao, H., Haynes, B. F., Cohen, M. S., Shaw, G. M., Seaman, M. S., Kumar, A., Gao, F., Montefiori, D. C., and Korber, B. (2010) Genetic signatures in the envelope glycoproteins of HIV-1 that associate with broadly neutralizing antibodies. *PLoS Comput. Biol.* **6**, e1000955

63. Rong, R., Li, B., Lynch, R. M., Haaland, R. E., Murphy, M. K., Mulenga, J., Roberts, S., Rong, R., Li, B., Lynch, R. M., Letvin, N., and Silvestri, G. (2008) Short-lived infected cells support virus replication in uninfected CD4+ T lymphocytes. *J. Exp. Med.* **206**, 1159–1171

64. de Mendoza, C., Van Baelen, K., Poveda, E., Rondelez, E., Zahonero, N., de Roda Husman AM, and Schuitemaker, H. (2000) Genetic signatures in the envelope glycoproteins of HIV-1 that associate with broadly neutralizing antibodies. *Virology* **268**, 325–335

65. Bunnik, E. M., Lobbrecht, M. S., van Nuenen, A. C., and Schuitemaker, H. (2000) Differential coreceptor binding site exposure. *J. Virol.* **74**, 4541–4551

66. Garg, H., Ishii, A., and Blumenthal, R. (2009) Altered bystander apoptosis induction and pathogenesis of enfuvirtide-resistant HIV type 1 Env mutants. *AIDS Res. Hum. Retroviruses* **25**, 811–817

67. Gnanakaran, S., Daniels, M. G., Bhattacharya, T., Lapedes, A. S., Sethi, A., Li, M., Tang, H., Greene, K., Gao, H., Haynes, B. F., Cohen, M. S., Shaw, G. M., Seaman, M. S., Kumar, A., Gao, F., Montefiori, D. C., and Korber, B. (2010) Genetic signatures in the envelope glycoproteins of HIV-1 that associate with broadly neutralizing antibodies. *PLoS Comput. Biol.* **6**, e1000955

68. Rong, R., Li, B., Lynch, R. M., Haaland, R. E., Murphy, M. K., Mulenga, J., Roberts, S., Rong, R., Li, B., Lynch, R. M., Letvin, N., and Silvestri, G. (2008) Short-lived infected cells support virus replication in uninfected CD4+ T lymphocytes. *J. Exp. Med.* **206**, 1159–1171

69. Denizot, M., Varbanov, M., Espert, L., Robert-Hebmann, V., Sagnier, S., Gustafson, K., and Viel, J. (2009) Escape from autologous neutralizing antibodies in acute/early subtype C HIV-1 infection requires multiple pathways. *PLoS Pathog.* **5**, e1000594

70. de Mendoza, C., Van Baelen, K., Poveda, E., Rondelez, E., Zahonero, N., Stuyver, L., Garrido, C., Villacian, J., and Soriano, V. (2008) Performance of a population-based HIV-1 tropism phenotypic assay and correlation of V3 genotypic prediction tools in recent HIV-1 seroconverters. *J. Acquir. Immune Defic. Syndr.* **48**, 214–219

71. Castedo, M., Perfettini, J. L., Andreau, K., Roumier, T., Piacentini, M., and Soriano, V. (2008) A short-lived infected cell is a powerful inducer of cell death in uninfected CD4+ target cells. *Res. Virol.* **146**, 5–17

72. Bunnik, E. M., Lobbecht, M. S., van Nuenen, A. C., and Schuitemaker, H. (2010) Escape from autologous humoral immunity of HIV-1 is not associated with a decrease in replicative capacity. *Virology* **397**, 224–230

73. Espert, L., Denizot, M., Grimaldi, M., Robert-Hebmann, V., Gay, B., and Gabuzda, D. (2004) Apoptosis of bystander T cells induced by human immunodeficiency virus type 1 with increased envelope/receptor affinity and coreceptor binding site exposure. *J. Virol.* **78**, 4541–4551

74. Meissner, E. G., Zhang, L., Jiang, S., and Su, L. (2006) Fusion-induced apoptosis contributes to thymocyte depletion by a pathogenic human immunodeficiency virus type 1 envelope in the human thymus. *J. Virol.* **80**, 11019–11030

75. Koot, M., van ’t Wout, A. B., Kooistra, N. A., de Goede, R. E., Tersteme, M., and Schuitemaker, H. (1996) Relation between changes in cellular load, evolution of viral phenotype, and the clonal composition of virus populations in the course of human immunodeficiency virus type 1 infection. *J. Infect. Dis.* **173**, 349–354

76. Spijkerman, I., de Wolf, F., Langendam, M., Schuitemaker, H., and Coutinho, R. (1998) Emergence of syncytium-inducing human immunodeficiency virus type 1 variants coincides with a transient increase in viral RNA level and is an independent predictor for progression to AIDS. *J. Infect. Dis.* **178**, 397–403

77. van Rij RP, Blaak, H., Visser, J. A., Brouwer, M., Rientsma, R., Broersen, S., de Roda Husman AM, and Schuitemaker, H. (2000) Differential coreceptor expression allows for independent evolution of non-syncytium-inducing and syncytium-inducing HIV-1. *J. Clin. Invest.* **106**, 1569

78. Kaneshima, H., Su, L., Bonyhadi, M. L., Connolly, R. I., Ho, D. O., and McCune, J. M. (1994) Rapid high, syncytium-inducing isolates of human immunodeficiency virus type 1 induce cytopathicity in the human thymus of the SCID-hu mouse. *J. Virol.* **68**, 8188–8192

79. Karlsson, G. B., Halloran, M., Schenten, D., Lee, J., Racz, P., Tenner-Racz, K., Manola, J., Gelman, R., Etemad-Moghadam, B., Desjardins, E., Wyatt, R., Gerard, N. P., Marcon, L., Marqin, D., Fenton, J., Axelthelm, M. K., Letvin, N. L., and Sodroski, I. (1998) The envelope glycoprotein ectodomains determine the efficiency of CD4+ T lymphocyte depletion in simian-human immunodeficiency virus-infected macaques. *J. Exp. Med.* **188**, 1159–1171

80. Cayabyab, M., Karlsson, G. B., Etemad-Moghadam, B. A., Hofmann, W., Steenbeke, T., Halloran, M., Fanon, J. W., Axelthelm, M. K., Letvin, N. L., and Sodroski, I. (1999) Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXB2). *J. Virol.* **73**, 976–984

81. de Roda Husman AM, and Schuitemaker, H. (2000) Differential coreceptor binding site exposure. *J. Virol.* **74**, 10690–10698

82. Etemad-Moghadam, B., Sun, Y., Nicholson, E. K., Fernandes, M., Liou, K., Gomila, R., Lee, J., and Sodroski, I. (2000) Envelope glycoprotein determinants of increased fusogenicity in a pathogenic simian-human immunodeficiency virus (SHIV-KB9) passaged in vivo. *J. Virol.* **74**, 4433–4440

83. Etemad-Moghadam, B., Rhone, D., Steenbeke, T., Sun, Y., Manola, J., Gelman, R., Fenton, J. W., Racz, P., Tenner-Racz, K., Axelthelm, M. K., Letvin, N. L., and Sodroski, I. (2001) Membrane-fusing capacity of the human immunodeficiency virus envelope in the human thymus. *J. Virol.* **75**, 5646–5655

84. Hunt, P. W., Shulman, N. S., Hayes, T. L., Dahl, V., Somsouk, M., Funderburg, N. T., McLoughlin, B., Landay, A. L., Adeyemi, O., Gilman, I. E., Glagov, S., Rodriguez, B., Martin, J. N., Schacker, T. W., Shaklett, B. L., Palmer, S., Lederman, M. M., and Deeks, S. G. (2013) The immunologic effects of maraviroc intensification in treated HIV-infected individuals with incomplete CD4+ T-cell recovery. A randomized trial. *Blood* **121**, 4635–4646

85. Holman, A. G., and Gabuzda, D. (2012) A machine learning approach for identifying amino acid signatures in the HIV env gene predictive of dementia. *PloS One* **7**, e49538

86. Holman, A. G., Mefford, M. E., O’Connor, N., and Gabuzda, D. (2010) HIVBrainSeqDB. A database of annotated HIV envelope sequences from brain and other anatomical sites. *AIDS Res. Ther.* **7**, 43