HIV-1 Env Glycoprotein Phenotype along with Immune Activation Determines CD4 T Cell Loss in HIV Patients

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HIV-1 Env Glycoprotein Phenotype along with Immune Activation Determines CD4 T Cell Loss in HIV Patients

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The mechanism behind the selective depletion of CD4+ cells in HIV infections remains undetermined. Although HIV selectively infects CD4+ cells, the relatively few infected cells in vivo cannot account for the extent of CD4+ T cell depletion, suggesting indirect or bystander mechanisms. The role of virus replication, Env glycoprotein phenotype, and immune activation (IA) in this bystander phenomenon remains controversial. Using samples derived from HIV-infected patients, we demonstrate that, although IA in both CD4+ and CD8+ subsets correlates with CD4 decline, apoptosis in CD4+ and not CD8+ cells is associated with disease progression. Because HIV-1 Env glycoprotein has been implicated in bystander apoptosis, we cloned full-length Env from plasma of viremic patients and tested their apoptosis-inducing potential (AIP). Interestingly, AIP of HIV-1 Env glycoproteins were found to correlate inversely with CD4/CD8 ratios, suggesting a role of Env phenotype in disease progression. In vitro mitogenic stimulation of PBMCs resulted in upregulation of IA markers but failed to alter the CD4:CD8 ratio. However, coculture of normal PBMCs with Env-expressing cells resulted in selective CD4 loss that was significantly enhanced by IA. Our study demonstrates that AIP of HIV-1 Env and IA collectively determine CD4 loss in HIV infection. The Journal of Immunology, 2016, 196: 000–000.

Progressive depletion of CD4+ T cells by HIV-1 results in AIDS. Because HIV-1 selectively infects CD4+ T cells, it is not surprising that the disease is characterized by several immune manifestations. Virus replication, CD4+ T cell apoptosis, and immune activation (IA) are some of the hallmarks associated with disease progression and AIDS development. Because there is a strikingly strong correlation between IA (defined by upregulation of activation markers like CD38, HLA-DR, CCR5, and PD-1) on T cells and CD4+ loss in AIDS, it is believed that IA is the driving force behind this HIV pathology (1). Surprisingly, the mechanism of IA remains controversial, and roles for virus replication (2, 3), gut leakage, and LPS translocation (4, 5) have been proposed as mechanisms influencing CD4+ decline.

Although IA is an immunopathological hallmark of HIV infection and CD4+ T cell decline in patients correlates with this phenomenon, it is also true that suppressing virus replication with antiretroviral therapy in many cases reduces IA (2, 6–9). This suggests that some viral component or active virus replication enhances IA. Interestingly, a majority of activated cells defined as CD38HLA-DR+ are in the CD8+ compartment (10), whereas the majority of T cell loss leading to AIDS is in the CD4+ compartment. Hence the mechanism of the IA, its role in CD4+ T cell loss, and the role played by the virus in this process remains uncertain.

The HIV Envelope (Env) glycoprotein is a major determinant of virus transmission and has been implicated in HIV pathogenesis via a variety of mechanisms (11). Among these, induction of bystander apoptosis via interactions between infected Env-expressing cells and receptor/coreceptor-expressing uninfected bystander cells has been suggested as one of the mechanisms contributing to CD4+ T cell decline (12–17). We have previously demonstrated the phenomenon of bystander apoptosis mediated by HIV Env both in vitro (18, 19) and in vivo (20), and found that Env fusogenic activity correlates with bystander apoptosis and CD4 decline, but not virus replication. This phenomenon is not limited to laboratory-adapted viruses but is also seen with a variety of Envs derived from HIV-infected patients (21). The high variability in the bystander apoptosis-inducing potential (AIP) of primary Envs suggests that phenotypic variability may play a role in the differential rates of disease progression. However, whether HIV Env-mediated bystander apoptosis correlates with other immunopathological markers such as IA, and whether these factors independently or collectively determine CD4 loss remains unknown. Moreover, although it is clear that selective apoptosis of uninfected bystander CD4+ T cells is a driving force behind T cell loss, the mechanism of bystander apoptosis remains highly debated (22, 23). Halt in CD4 decline/apoptosis and partial recovery of CD4+ cells in highly active antiretroviral therapy (HAART)–suppressed patients (24, 25) further supports a role of virus and/or viral proteins in mediating CD4+ loss.

In this study, we analyzed samples from 50 HIV-infected patients for multiple immunopathological markers including those for IA and apoptosis in CD4+ and CD8+ cells. Furthermore, we cloned full-length functional env genes from 11 viremic HIV+ patients and characterized the derived Env glycoproteins for their AIP using a unique assay developed in our laboratory (21). Our results...
demonstrate that the AIP of patient Envs correlates inversely with the CD4:CD8 ratios. Interestingly, our data also demonstrate that HIV-1 Env-mediated bystander apoptosis in PBMCs is enhanced by IA. Multivariate analysis shows that the AIP of Envs in combination with IA is highly predictive of CD4 decline. We demonstrate in this article for the first time, to our knowledge, that Env glycoprotein phenotype, as assessed by AIP, in combination with IA plays a significant role in CD4 decline and HIV pathogenesis.

Materials and Methods

Ethical statement

The study was reviewed and approved by the Texas Tech University Health Sciences Center’s regional Institutional Review Board. The study design was cross-sectional and the study number recorded as Institutional Review Board no. E12092, with an approval date of July 31, 2012. All participants were provided with written and oral information about the study. Written, informed consent of all study participants in accordance with the institutional policy was documented. All participants were identified by coded numbers to assure anonymity, and all patient records were kept confidential.

Study population

A cohort of 50 HIV-infected individuals was recruited from the outpatient HIV clinic at the Texas Tech University Health Sciences Center at El Paso (Supplemental Table 1). The mean age of the patient population was 37.9 ± 11.9 y. The population comprised 9 female (18.0%) and 41 male (82.0%) patients. Samples were collected randomly as per availability, and the patients were at different stages of HIV disease progression. An age- and sex-matched healthy population control group (n = 17) was also recruited from the same geographical location and comprised 3 female (17.7%) and 14 male (82.4%) patients. The mean age of the healthy control group was 35.4 ± 11.8 y. Clinical data including plasma viremia, CD4 counts, and HLA-DR status were recorded from the patient charts (Supplemental Table 1).

Sample collection and storage

Blood (20 ml) from each participant was collected in EDTA vacutainer tubes, stored cold, and transported immediately to the laboratory for processing. Blood was then separated into cell components and plasma by centrifugation at 2000 rpm for 10 min. Plasma samples were aliquoted, labeled by unique patient identification codes, and stored at −70°C until needed for further assays. Thereafter, lymphocytes were separated from whole blood using the sucrose density gradient (Ficoll) centrifugation protocol (GE Healthcare) as per the manufacturer’s recommendations. The PBMCs separated via Ficoll were washed extensively, resuspended in freezing media (90% FBS/10% DMSO), and cryopreserved for future analyses.

Immunostaining and in vitro T cell activation

Cryopreserved lymphocytes isolated from the blood samples obtained from HIV-infected or normal patients were quickly thawed in a 37°C water bath and washed with PBS. Cells were then stained for cell-surface markers using specific Abs. The IA panel consisted of Abs CD3 Cy7, CD4 Texas Red, CD8-allophycocyanin along with IA markers CD38 PE and HLA-DR FITC (BD Pharmingen) (Supplemental Fig. 1A). The apoptosis panel comprised the following Abs: CD3 Cy7, CD4 Texas Red, CD8-allophycocyanin (Beckman Coulter) along with CaspACE VTDC-VADEFMK (Promega) (Supplemental Fig. 1B). Stained cells were washed and fixed using Cytofix reagent (Beckman Coulter) and acquired on a 10-color Beckman Coulter Gallios flow cytometer. At least 20,000 events for each sample were acquired. Data were analyzed using FlowJo software (Tree Star). Cells were first gated on CD3+ population, and IA/apoptosis on CD4+ and CD8+ T cell subsets was determined (Supplemental Fig. 1A, 1B). For in vitro T cell activation, lymphocytes were cultured in RPMI 1640 medium supplemented with 20% FBS and phytomannosylglutamin (Sigma) at 2.5 µg/ml and IL-2 (Roche) at 10 U/ml for 48 h, stained, and analyzed as described earlier for IA markers and CD4:CD8 ratios (Supplemental Fig. 2).

Env cloning

Viral RNA was isolated from the plasma samples using the QIAamp viral RNA mini kit (Qiagen) following the manufacturer’s protocol followed by cDNA synthesis using the ProtoScript II first-strand cDNA synthesis kit (New England Biolabs). The full-length Env region (containing the open reading frames for the Env and Rev genes) was then amplified with subtype B–specific primers and a nested PCR using the Phusion High Fidelity PCR kit (New England Biolabs). The amplified Env region was cloned into the pCDNA3.1 vector using the pCDNA3.1 directional TOPO expression kit (Invitrogen) followed by full-length sequencing analysis to verify the authenticity of the inserts. Contigs were established using DNA star software (DNASTAR, Madison, WI) and Env Open reading frames for all constructs established. Functionality of each Env was determined by generating pseudotyped HIV particles using Env constructs and pNL4-3 R-E as described later.

Cell lines and transfections

SupT1 cells expressing CCR5 were maintained in RPMI media supplemented with 10% FBS and penicillin streptomycin (5000 U/ml) and blasticidin at a concentration of 3 µg/ml. 293T, HeLa, and T2M-bl cells (National Institutes of Health AIDS Research and Reference Reagent Program) were maintained in DMEM supplemented with 10% FBS and penicillin streptomycin (5000 U/ml). U87-CXCR4 and U87-CCR5 cells were maintained in DMEM-10% FBS supplemented with 1 µg/ml puromycin and 300 µg/ml G418. All transfections were conducted using the Turbofet Transfection reagent (Fermentas) following the manufacturer’s instructions.

Virus infectivity assays

293T cells were transfected with the pNL4-3 R-E (26) HIV backbone along with different Env constructs. Virus stocks were harvested 48 h posttransfection and used to infect the indicator T2M-bl cells in the presence of 20 µg/ml DEAE dextran (Sigma). Luciferase activity was determined 48 h postinfection using the BriteLite Plus Luciferase assay substrate (Perkin Elmer). Infectivity for each Env was calculated as percent of YU-2 Env control after subtraction of the background derived from pcDNA3.1 empty vector transfected cells. CXCR4 and CCR5 antagonists AMD-3100 and maravica (MVC), respectively, were used as described later.

Bystander apoptosis assay

Bystander AIP of cloned Envs was determined using a coculture assay described previously (21). In brief, HeLa cells transfected with the laboratory-adapted or cloned primary Env constructs were co-cultured either with SupT1-R5-H6 cells (27) or with resting or activated PBMCs. Twenty-four hours post coculture, apoptosis was determined using Caspase Glo 3/7 assay (Promega) that detects caspase 3/7 activity in culture lysates. Lai (CXCR4 tropic) or YU-2 (CCR5 tropic) Envs were used as positive controls and pCDNA 3.1 empty vector as the negative control. Percent apoptosis was determined after normalizing data to YU-2 control as 100% and pCDNA 3.1 as 0%. For some experiments, AMD-3100 (4 µM), MVC (2 µM), T-20 (8 µM), DEVD (50 µM) was added at the time of coculture. For treatment with VX765 (10 µM), the cells were preincubated for 4 h before coculture.

Data analysis

Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA) and SAS 9.3 (SAS Institute, Cary, NC). Differences between groups were assessed using the Fisher’s exact test or the two-sample t test as appropriate. All p values were two-sided, and data were considered significant at p < 0.05. Spearman’s correlation with linear regression was used for all correlation determination using the GraphPad Prism software. For multivariate analysis of AIP, CD4 IA, and plasma viremia in predicting CD4 loss in AIDS patients, the calculation of Spearman rank correlation coefficients was followed by multiple linear regression analyses in which overall and partial F tests were performed using an α of 0.05.

Results

IA in CD8+ T cells is significantly higher than CD4+ T cells in HIV-infected individuals

IA, defined by the overexpression of CD38 and HLA-DR on both CD4+ and CD8+ T cells, is recognized as a highly correlating marker of HIV disease progression and CD4 loss (28, 29). Recently, CD4:CD8 ratios have been shown as a better laboratory predictor of combined T cell pathogenesis in HIV-infected patients (30). Hence, in our study population, we asked whether these markers were upregulated on CD4+ and CD8+ T cells and whether they correlated with CD4 decline. As expected, in our
study population, we found the CD4:CD8 ratios to be very strongly associated with CD4+ T cell counts (Fig. 1A). We also found the CD4:CD8 ratios to be inversely associated with the IA markers CD38 and HLA-DR in both CD8+ (Fig. 1B) and CD4+ T cells (Fig. 1C). We next asked whether the percentage of CD38+HLA-DR+ cells within the CD8+ and CD4+ T cell populations was different between HIV+ individuals and healthy control subjects. As evident in Fig. 1D and 1E, we found the CD38+HLA-DR+ population to be increased in both CD4+ and CD8+ T cells in the HIV-infected population. However, the level of IA in CD8+ cells was strikingly higher than that seen in CD4+ cells (Fig. 1F) ($p < 0.001$). It is important to make this distinction, because it is evident that, although IA is higher in CD8+ cells in HIV infections, the loss of T cells is largely limited to the CD4+ population. When analyzing for CD38 and HLA-DR individually, we found that, although HLA-DR upregulation on CD4+ cells was significantly different between HIV+ and healthy controls, CD38 expression was not (Fig. 1G, 1H). A similar trend was seen in CD8+ cells with differences in HLA-DR expression being more pronounced than CD38 upregulation (Fig. 1I, 1J). This suggests that the IA phenotype in HIV infection is largely driven by an upregulation of HLA-DR on CD4+, and to a greater extent on CD8+ T cells. Taken together, these findings suggest that our study population represents an IA phenotype consistent with HIV pathology and suitable for further analysis. The disconnect between the higher CD8+ IA and a greater CD4+ T cell loss in AIDS merits highlighting.

**IA correlates with plasma viremia**

One of the most extensively investigated questions in HIV research has been the mechanism behind virus-mediated IA. Many different hypotheses have been put forward including LPS translocation from the gut, role of IFNs, activation of TLRs, and so on (1). Despite these numerous hypotheses, the fact that remains undisputed is that virus replication/viremia significantly enhances IA (31). This causation hypothesis is further supported by the fact that suppression of virus replication to undetectable levels significantly reduces IA (2, 9), and that low levels of virus replication may be associated with residual CD8+ T cell activation (2, 32–34). Therefore, we asked whether IA correlates with viral load in our patient samples. A regression analysis revealed that viral load does correlate with CD38+HLA-DR+ cells in both the CD4+ and the CD8+ populations (Fig. 2A, 2B). We also compared viremia between patients on HAART versus no HAART and found that all untreated individuals had detectable viremia and some on HAART were not responding positively to therapy (Fig. 2C). We next divided our samples into three groups, normal, HIV+ nonviremic, and HIV+ viremic (viremia defined as $\geq 100$ virus copies/ml), to determine whether there were differences in IA status within these groups. Interestingly, the HIV+ viremic group showed the highest IA in both the CD4+ (Fig. 2D) and the CD8+ populations (Fig. 2E). Once again, IA in CD8+ cells was significantly higher than the CD4+ population. Taken together, this set of data supports...
the hypothesis that virus replication is fundamental to IA. Moreover, it is also clear that IA in the HIV+ nonviremic group is higher than the normal healthy control subjects (Fig. 2D, 2E) and a similar trend is seen with the CD4:CD8 ratios (Fig. 2F). Significant difference in the CD4:CD8 ratio was also seen between patients on HAART versus no HAART (Fig. 2G). Our findings are in agreement with recent reports (33–35) suggesting that virus replication correlates with T cell activation; however, the causality relationship between virus replication and IA cannot be concluded.

**CD4+ but not CD8+ T cell apoptosis correlates with CD4 decline**

Apoptosis of CD4+ cells has been recognized as one of the factors leading to the progressive loss of CD4+ cells, resulting in immunodeficiency (36). Multiple studies in HIV-infected patients, SIV, and humanized mouse model have demonstrated the presence of uninfected bystander cells dying of apoptosis in HIV infections (20, 37–39). PBMCs derived from HIV-infected patients have also been shown to have a higher percentage of apoptotic phenotype (24, 25, 40, 41). Finally, caspase activation has been recognized as a critical mediator of apoptosis seen in HIV-infected patients, as well as animal model systems (20, 38). We hence asked whether apoptosis in CD4+ or CD8+ T cell populations was increased in HIV-infected patients. Apoptosis was detected in cells using FITC-VAD-FMK, a peptide that binds to active caspases, along with staining for CD3, CD4, and CD8 to distinguish the CD4+ and CD8+ T cell populations. We found that, although CD4+ apoptosis correlated inversely with CD4:CD8 ratios (\( p = 0.026 \); Fig. 3A), CD8+ apoptosis did not (\( p = 0.10 \); Fig. 3B). Furthermore, we found that CD4+ T cell apoptosis was significantly higher in HIV+ patients (\( p < 0.05 \); Fig. 3C) compared with healthy control subjects, whereas CD8+ apoptosis was not statistically different (Fig. 3D). These data are consistent with our hypothesis that selective apoptosis of CD4+ cells in HIV infections is a major contributing factor in CD4+ T cell loss and AIDS development.

Based on the observation that CD4+ apoptosis in HIV+ patients is higher, and our previous findings that CD4+ apoptosis is mediated via the HIV Env glycoprotein (18, 20, 22), we hypothesized that apoptosis should most likely be higher in viremic patients. Moreover, in the absence of significant amounts of virus, as in nonviremic patients, Env-mediated apoptosis would be irrelevant. We again divided our patient population based on viral load into normal, HIV viremic (\( > 100 \) virus copies/ml), and HIV nonviremic (\( < 100 \) virus copies/ml). Interestingly, we found that, in the viremic group, CD4+ apoptosis was significantly higher than both HIV nonviremic (\( p < 0.001 \); Fig. 3E) and the healthy controls (\( p < 0.001 \); Fig. 3E). In contrast, CD8+ apoptosis between the viremic and the nonviremic groups, although different, did not reach statistical significance (\( p = 0.058 \); Fig. 3F). Further analyses of the viremic patients showed that, although IA correlates with apoptosis for the CD8 group (\( p = 0.017 \); Fig. 3H), it does not for the CD4 group (\( p = 0.0895 \); Fig. 3G). This suggests that cell death in CD4 and CD8 cells is likely via a different mechanism in HIV patients. These findings are further supportive of the idea that selective apoptosis of CD4+ cells in viremic patients may be a
versus normal healthy control subjects. Comparison of HIV-infected nonviremic patients, and HIV-infected viremic patients. Correlation analysis of CD8+ apoptosis in PBMCs derived from HIV-infected individuals. Analysis of stained for various cell-surface markers along with CaspACE FITC-V AD-FMK. Correlation analysis of CD4:CD8 ratios with (21). This also supports the notion that Envα or viruses that lack observation that AIP and infectivity do not correlate in every case also seen for virus infectivity in a single round infectivity assay the lower end of apoptosis inducers (Fig. 4A). This variability was hence speculated that some of this variability may be a result of lower viral load, which would translate to lower Env glycoprotein expression and reduced bystander apoptosis. However, another possibility could be differences in AIP of the viruses these individuals harbor.

AIP of primary Envα is variable

Previously we have demonstrated in a panel of HIV Env glycoprotein variants derived from patients that the bystander AIP of Envα varies considerably (21). Whether this variability correlates with CD4+ decline in vivo and HIV pathogenesis remains unclear. In our set of patient samples, we observed that not all viremic patients showed high CD4+ apoptosis or low CD4+CD8+ ratios. We hence speculated that these variations were likely due to differences in the AIP of different Envα variants. To test this, we cloned full-length functional Envαs from patient plasma samples to determine the AIP of the infecting virus. We were able to clone 11 functional Envαs from a total of 18 viremic patients. The clones were sequenced and found to be unique HIV Env sequences, nonidentical to any known HIV Env in the National Center for Biotechnology Information database. The sequence accession numbers are listed in Table I. A phylogenetic tree showing Env relatedness is depicted in Fig. 4F. The Envs were characterized for various phenotypic features including AIP, infectivity, and coreceptor usage (Table I). All the Envαs found to be CCR5 tropic based on U87 tropism assay (Table I) and inhibition via MVC (Fig. 4B). Interestingly, we found that the Envs varied considerably in their AIP with clones HP029, HP025, and HP013 showing highest apoptosis, whereas clones HP003, HP038 and HP043 were toward the lower end of apoptosis inducers (Fig. 4A). This variability was also seen for virus infectivity in a single round infectivity assay (Fig. 4B). The correlation between infectivity and apoptosis was not statistically significant (Fig. 4C), consistent with our previous observation that AIP and infectivity do not correlate in every case (21). This also supports the notion that Envαs or viruses that lack apoptosis-inducing activity can still infect and replicate in vivo, as shown previously (20). Interestingly, apoptosis was inhibited in the presence of the CCR5 antagonist MVC and the Fusion inhibitor T-20, suggesting an Env-specific effect and a role of HIV fusion activity in this phenomenon (Fig. 4D). We also tested whether apoptosis in our coculture model was dependent on caspase-1. In our model, caspase 1–specific inhibitor VX765 failed to inhibit caspase 3 activity in Env-exposed cells, whereas caspase 3 inhibitor Ac-DEVD-fmk inhibited caspase 3 activity in our assay (Fig. 4E). Phylogenetic analysis showed a close relatedness among HP013, HP025, and HP029 (Fig. 4F), all high apoptosis inducers, further supporting a role of Env glycoprotein in CD4+ cell apoptosis. A complete profile analysis of the patients revealed two highly interesting phenotypes, with HP029 and HP043 representing two extreme ends of the HIV pathogenesis spectrum (Table I). Although both patients were viremic and showed similar IA, they were markedly different in AIP (109 versus 31) and in vivo CD4 apoptosis (40.17 versus 11.51). This difference was further reflected in their CD4:CD8 ratios (0.13 versus 0.57) and absolute CD4 counts (69 versus 426). This pair represents a perfect example demonstrating the role of Env phenotype in determining CD4 loss and AIDS development.

CD4 loss in vivo is a combination of AIP of Envα and IA

Because of the variability in the AIP of the cloned Envαs, we next asked whether AIP is associated with in vivo CD4+ T cell loss and disease progression. We hence correlated AIP of Envαs with CD4:CD8 ratios and found a strong inverse correlation between the two phenomena, Spearman’s $r = -0.70$, $p = 0.020$ (Fig. 5A). The fact that Envαs with higher AIP were associated with lower CD4:CD8 counts and vice versa suggests that AIP of the Envαs may have a causal effect on CD4:CD8 ratios during HIV disease progression. This is consistent with our hypothesis that Env glycoprotein phenotype is one of the determinants of CD4 loss and AIDS development. Having characterized the AIP of Envαs and establishing a correlation between AIP and CD4:CD8 ratios, we next asked whether AIP alone is responsible for the CD4 loss seen in patients or whether other factors like IA and viremia also contribute to
### Table I. Clinical and laboratory data for the 11 Env clones obtained from viremic HIV+ patients

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age, y</th>
<th>Sex</th>
<th>CD4 Counts</th>
<th>CD8 Counts</th>
<th>In Vivo CD4 Apoptosis</th>
<th>Tropism</th>
<th>In Vitro CD4 Apoptosis</th>
<th>CD4:CD8 Ratio</th>
<th>Viremia</th>
<th>AIP</th>
<th>Infectivity</th>
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<th>Subtype</th>
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<tr>
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<td>R5</td>
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</table>

Several lines of evidence suggest that IA, specifically in CD4+ cells, can enhance virus replication by creating additional targets for HIV infection (31). Our data suggest that AIP of primary Envs in combination with IA correlates strongly with in vivo CD4+ T cell loss. We hence asked whether IA can enhance bystander apoptosis mediated by HIV Env and whether the presence of viral Env in the culture conditions in Fig. 6C would induce apoptosis in activated PBMCs. To address this, we used an assay where PBMCs from healthy donors were cocultured with cells expressing Lai Env (X4), YU-2 Env (R5), or a vector control (pcDNA3.1). Apoptosis was determined 24 h later via caspase 3 activity measurement. Interestingly, we found that both X4 (Lai) (Fig. 7A) and an R5 Env (YU-2) (Fig. 7B) were able to induce caspase 3 activation in resting PBMCs. Apoptosis induction by YU-2 Env, although...
higher than vector control in five of the eight samples, was not comparable with Lai Env. This is most likely due to the paucity of CD4+ CCR5+ cells in peripheral blood (Supplemental Fig. 3B), consistent with our previous report where X4 Env induced higher apoptosis than R5 Env in peripheral blood purified CD4+ T cells (21). More interestingly, when cells were activated with PHA and IL-2 and then cocultured with Lai Env-expressing cells, apoptosis induction in the cocultures was significantly higher than in resting cells for all the donors, \( p < 0.005 \) (Fig. 7C). These studies suggest that, although IA is a pathological hallmark associated with AIDS, this phenomenon per se in the absence of HIV Env fails to induce specific loss of CD4+ cells as seen in HIV infections. However, from the earlier experiment, it remains uncertain whether CD4+ cells were the actual source of caspase 3 activation and whether

**FIGURE 4.** AIP of primary Envs correlates with CD4 decline. (A) HeLa cells transfected with the cloned primary Env constructs were cocultured with SupT-R5-H6 cells. Twenty-four hours after coculture, apoptosis was determined using the caspase glo 3/7 assay. YU-2 Env was used as a positive control and pcDNA 3.1+ empty vector as the negative control. Percent apoptosis was determined after normalizing data to YU-2 control as 100% and pcDNA 3.1 as 0%. (B) Infectivity of the cloned primary Env constructs. Virus stocks were prepared by cotransfecting 293T cells with the pNL-Luc backbone along with the indicated Env constructs. Equal reverse transcriptase cpm of culture supernatants were used to infect TZM-bl cells in the presence of AMD-3100 (4 \( \mu \)M) or MVC (2 \( \mu \)M), and luciferase activity was determined. Infectivity is normalized to YU-2 Env as 100%. (C) Correlation of AIP of the primary Env constructs with percent infectivity. (D) The coculture assay described in (A) was conducted with selected primary Envs in the presence of media, AMD-3100 (4 \( \mu \)M), MVC (2 \( \mu \)M), or T-20 (8 \( \mu \)M) added at the time of coculture. (E) YU-2 or Lai Env-expressing HeLa cells were cocultured with SupT-R5-H6 cells in the presence of indicated inhibitors, and apoptosis was determined using the caspase glo 3/7 assay. (F) Phylogenetic analysis of the primary HIV Env clones used in the study. A phylogenetic tree showing Env relatedness was derived using the DNA Star software.

**FIGURE 5.** CD4 loss in vivo is a combination of AIP of Envs and IA. (A) Correlation analysis of AIP of Envs with CD4:CD8 ratios shows an inverse correlation between the two phenomenon. (B) Correlation of CD4:CD8 ratios with CD4+ IA. (C) Correlation analysis between plasma viremia and CD4:CD8 ratios. (D) Correlation analysis between patient plasma viremia and CD4+ IA.
Env-mediated apoptosis leads to a specific loss of CD4+ cells. Hence we repeated the earlier experiments to perform staining for CD4+ and CD8+ cells in parallel with caspase 3 activity determinations. Once again, we found that both X4 (Lai) and an R5 (YU-2) Env were able to induce caspase 3 activation in unstimulated PBMCs, which was enhanced when cells were activated with PHA and IL-2 (Fig. 7D). Interestingly, we found that coculture with Env-expressing cells resulted in a decrease in CD4:CD8 ratio much in line with what is seen in HIV-infected patients (Fig. 7E). Furthermore, the decrease in CD4:CD8 ratio was more pronounced in activated cells than unstimulated PBMCs (Fig. 7D). We also found that increase in CD4+ apoptosis and decrease in CD4:CD8 ratios were both inhibited by the HIV Env fusion inhibitor T20 (Fig. 7F, 7G, Supplemental Fig. 3A), confirming that apoptosis was specific to CD4+ cells and mediated by the fusion activity of Env glycoprotein. Surprisingly, these in vitro assays, whereby PBMCs were exposed to Env-expressing cells, reproduced many of the pathological features seen in HIV infection in vivo including a specific loss of CD4 cells and increased CD4 depletion via IA. Taken together, these findings support the hypothesis that Env-mediated bystander apoptosis is the major cause of CD4+ loss during AIDS progression, with IA playing a significant role in enhancing this phenomenon (Fig. 8).

Discussion

After >30 y of research, the mechanism via which HIV infection leads to CD4+ T cell depletion and AIDS development remains unclear. Two critical immunopathological markers of HIV infections are bystander apoptosis (37) and IA (1). Although both apoptosis and IA correlate with disease progression and AIDS development, the interdependence of these factors remains unclear. Whether IA leads to apoptosis of CD4+ cells or loss of CD4+ cells by apoptosis and/or virus replication drives IA remains a fundamental yet unanswered question. Another critical player in HIV pathogenesis is the viral Env glycoprotein, which is known to vary both between and within individuals and with the stage of the disease (43–46). The higher rate of disease progression in animal models in many cases can be traced back to the phenotype of Env glycoprotein in both simian HIV model in Macaques (47–49) and HIV infection in humanized mice (20).

Apoptosis of uninfected bystander cells has long been believed to be the cause of CD4+ decline and AIDS development (11, 22, 37). The Env glycoprotein comes forth as a likely mediator in the process because it specifically binds to the CD4 receptor and is expressed on the surface of infected cells whereby it can interact with bystander cells. Interestingly, Env glycoprotein expressed on the surface of effector cells has the potential to mediate bystander apoptosis in CD4+ T cells (14, 16, 18, 46, 50). This phenomenon is dependent on membrane fusion potential of the Env glycoprotein (11, 12) and varies considerably between primary Envs (21). We have previously demonstrated that altering the fusion capacity of Env glycoprotein can alter bystander apoptosis and CD4+ decline in humanized mouse model without affecting virus replication (20). Based on these model systems, it is reasonable to speculate that the AIP of Env glycoprotein could potentially be related to CD4+ decline and AIDS development, and explains why some individuals progress to AIDS faster than others (45, 46, 51, 52).

Although most studies support the hypothesis that bystander cells are lost via classical apoptosis (36, 37, 53, 54) in HIV infection, recently a different mechanism of cell death termed pyroptosis has been implicated in HIV-mediated CD4+ T cell loss (55). Early studies suggested that abortive infection by HIV particles initiates caspase-1–dependent pyroptosis of bystander cells (56), as well as IL-1 production (55). In our system, we have measured apoptosis using FITC-VAD-fmk reagent that binds all active caspases and not caspase 3 specifically. Whether some cells in our assays are dying via caspase-1–dependent pyroptosis cannot be ruled out. However, a recent study suggests that close cell-to-cell contact is essential even for pyroptotic death in human lymphoid aggregate cultures (57), which is consistent with our model of close contact between Env-expressing and bystander target cells. Differences in the mechanism of cell death in vitro may be a consequence of different cell systems used as demonstrated by other groups (15). Nevertheless, in vivo evidence for a role of caspase-3–dependent apoptosis in CD4 T cell loss in HIV/SIV infections is overwhelming (12, 20, 38, 39, 58, 59).

The fundamental question in our study was how IA, CD4+ T cell apoptosis, and Env glycoprotein phenotype collectively determine AIDS progression. First, we found that CD8+ IA was higher than CD4+ T cell activation in HIV+ individuals. More interestingly, viremic patients showed higher IA than nonviremic patients, indicating a role for virus replication in driving IA, which is consistent with several recent reports linking viremia to IA in CD8+ cells (32, 33, 60). The mechanism by which viremia may induce IA could be via plasmacytoid dendritic cells, as recently suggested.

<table>
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<td>AIP, CD4 IA, log viremia</td>
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**Figure 6.** IA per se does not alter the CD4:CD8 ratios. Lymphocytes obtained from HIV− healthy donors were cultured in RPMI 1640 medium supplemented with 20% FBS and phytohemagglutinin at 2.5 μg/ml and IL-2 at 10 U/ml for 48 h. Cells were then stained for IA markers as described in Fig. 1. Increase in percentage of CD38+HLA-DR+ cells in the (A) CD4+ and (B) CD8+ populations after 48 h of activation. (C) No significant alteration in the CD4:CD8 ratio after 48 h of IA in vitro.
by Li et al. (3), or direct damage of intestinal integrity, or depletion of Th17 cells in the gut mucosa by HIV leading to microbial translocation (9, 58). Intensification of HAART therapy using raltegravir has recently been shown to reduce CD8+ T cell activation, further supporting a causal relationship between viremia and IA (2). Second, we found that, although CD4+ apoptosis was higher in HIV+ individuals compared with normal control subjects, CD8+ apoptosis was not statistically different. This in turn indicates that IA may not be the direct cause of HIV-mediated CD4 loss, because the cells dying in HIV infection are CD4+, although activation is higher in CD8+ cells.

To specifically address the role of Env phenotype in CD4+ loss and AIDS progression, we cloned 11 full-length functional Envs from viremic patients and asked whether the AIP of the Envs correlated with CD4+ decline. We show in this article for the first time, to our knowledge, that indeed the bystander AIP of Envs is inversely related to CD4:CD8 ratios, a well-characterized marker of HIV-mediated immunopathology and CD4+ decline (30). Although AIP alone was a strong predictor of CD4+ T cell pathology (CD4:CD8 ratio), addition of IA in a multivariate analysis significantly enhanced the correlation. This supports the idea that IA and Env glycoprotein may synergize in mediating bystander CD4+ T cell apoptosis. In the same analysis, IA and viremia strongly correlated in the selected group of high viremic patients. This further emphasizes the multifactorial nature of HIV-mediated CD4+ loss and supports the hypothesis that IA and Env phenotype collectively determine bystander apoptosis.

To further investigate the issue of Env glycoprotein and IA synergy, we first determined whether IA per se would alter CD4:CD8 ratios. In vitro stimulation of normal PBMCs failed to induce a specific CD4+ decline, although the cells exhibited CD38+HLA-DR+ upregulation similar to that seen in HIV infections. In other more relevant in vivo models, experimental induction of IA failed to induce a specific CD4+ decline in both humanized mice (42).
and Macaques (61) in the absence of HIV infection. Hence we hypothesized that presence of virus/Env glycoprotein in the cultures would be essential for mediating specific CD4\(^+\) loss. Interestingly, although HIV Env (specifically Lai Env) was capable of inducing bystander apoptosis and CD4\(^-\) decline in unstimulated cells, IA significantly enhanced apoptosis and CD4\(^-\) decline in this model. Interestingly, this model system recapitulates many of the immunopathological changes seen in HIV infection including specific loss of CD4 cells, inversion of CD4/CD8 ratios, and increased pathology via IA. In this same model, we were able to demonstrate the requirement of Env glycoprotein-mediated membrane fusion in inducing CD4\(^+\) cell loss by specifically inhibiting the process via the gp41 inhibitor T20 as seen previously (18, 19). Although similar, the effects were less dramatic with the R5 Env (YU-2), which was expected because of a paucity of CCR5\(^+\) cells in the peripheral blood, also seen previously (21). In this regard, one must keep in mind that most of the pathology of R5 viruses is in the gut-associated lymphoid tissue (62) because of an abundance of CCR5\(^+\) cells, and the same mechanisms would be far more dramatic in the gut-associated lymphoid tissue.

Based on these findings, we provide a simplistic model of HIV pathology (Fig. 8). In this model, virus replication or viremia is responsible for IA and for the Env phenotype or AIP to be manifested. In the absence of viremia, Env-mediated apoptosis would be irrelevant. Once there is viremia, in the presence of an apoptosis-inducing Env (high AIP), this would lead to bystander apoptosis in CD4\(^+\) T cells, which in turn would be significantly enhanced by IA, resulting in reduced CD4/CD8 ratios. Another important manifestation would be enhancement of virus replication via IA and consequently an increase in the number of Env-expressing cells. Hence IA in vivo is likely working on two fronts: 1) by enhancing the number of infected cells expressing Env, and 2) by rendering the uninfected bystander cells more vulnerable to apoptosis via the Env glycoprotein (Fig. 8). This trifecta of virus replication, IA, and Env-mediated apoptosis would result in progressive loss of CD4\(^+\) cells leading to AIDS.

Although this model is simplistic, it explains why patients HP029 and HP043 have different outcomes in terms of CD4\(^+\) cells despite similar virus replication and IA. A lack of AIP (as determined in our study) for HP043 Env is manifested by preservation of CD4\(^+\) cells despite the presence of high viremia, whereas HP029 represents the opposite scenario. This pair is also reminiscent of our humanized mouse study where the V38E Env mutant virus (similar to HP043 Env) that lacks AIP (19) replicated to high levels and induced IA without causing CD4 apoptosis and decline (20). Our model may also provide an explanation for a number of recent clinical findings. For example, in viremic slow progressors, virus replication and IA are not sufficient for CD4\(^+\) loss as reported by Shaw et al. (63) and Klatt et al. (64). In fact, Shaw et al. (63) suggested a possible role of virological features in preservation of CD4\(^+\) cells, which is in agreement with our findings. Interestingly, differences in the nef gene do not account for the preservation of CD4\(^+\) cells in these patients (64, 65). It is hence tempting to speculate that differences in AIP of the infecting virus or differences in the ability of CD4\(^+\) T cells from viremic slow progressors to undergo bystander apoptosis may be responsible for these effects. In this context, one must also consider the CCR5 gene and promter polymorphisms in these patients, which have been found to be associated with disease progression (66, 67). We have previously shown that lower levels of CCR5 can limit bystander apoptosis of Env while maintaining the virus replication potential (27). Our findings also suggest that, in highly pathogenic SIV models like SIVsab in pig tailed macaques, the high AIP of the infecting virus may overcome the IA dependence for CD4 loss. Hence altering IA in these animals would have little effect on CD4 loss as seen by Kristoff et al. (68). Furthermore, in natural infections in SIV (SIV African green monkeys, SIV sooty mangabeys), a low AIP of the SIV variants in combination with lack of IA may protect the uninfected CD4\(^+\) cells from bystander apoptosis resulting in a nonpathogenic infection. Reduced cell-surface expression of CCR5 coreceptor in natural SIV hosts (69, 70) might also protect against bystander apoptosis in certain subsets of CD4\(^+\) T cells. In fact, lack of bystander apoptosis in natural SIV infections is a prominent differentiating marker from pathogenic SIV infections in macaques (38, 39).

Finally, our data suggest that suppressing virus replication would be the best way to inhibit Env-mediated bystander apoptosis and slow disease progression in line with recent clinical findings (24, 25). Our findings also suggest that reducing IA may have mixed results in terms of clinical benefits (71–73) because of the high variability in the AIP of Env glycoprotein, and viruses with different AIP may benefit differentially from IA reduction.

Overall, our study is the first, to our knowledge, to demonstrate a clear correlation between the AIP of primary Envs and CD4 decline. Our study is also the first, to our knowledge, to demonstrate the synergy between IA and Env glycoprotein–mediated bystander apoptosis. Hence the role of Env-mediated bystander effects and the role of IA in disease progression may not be exclusive but work in concert to mediate CD4\(^+\) loss. Our data support a hybrid hypothesis whereby the primary mediator of T cell apoptosis is most likely the Env glycoprotein, whereas IA acts as an accelerator by enhancing Env-mediated apoptosis and virus replication, and consequently disease progression.

Acknowledgments

We thank the patients who agreed to participate in the study by providing valuable samples and the National Institutes of Health AIDS Research and Reference Reagent Program for providing necessary reagents.

Disclosures

The authors have no financial conflicts of interest.

References


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ENV GLYCOPROTEIN AND IMMUNE ACTIVATION IN HIV PATHOGENESIS


Figure S1: (A) Strategy for flow cytometry analysis for determination of immune activation status in CD4+ and CD8+ T cells. Cryopreserved lymphocytes from HIV infected or normal patients were thawed in a 37°C water bath and washed with PBS. Cells were then stained with the antibodies CD3-Cy7, CD4-Tx red, CD8-APC along with immune activation markers CD38 PE and HLA-DR FITC (BD Pharmingen). Stained cells were washed with PBS and fixed using cytofix reagent (Beckman coulter) and acquired on a 10 color Beckman Coulter Gallios flow cytometer. At least 20,000 events for each sample were acquired. Data was analyzed using FlowJo software (Tree Star). Cells were first gated on CD3+ population (top) and immune activation on CD4+ and CD8+ T cell subsets determined. A representative staining for CD4+ and CD8+ T cell activation as determined by dual positive CD38+HLADR+ cells in normal (middle) and HIV infected individuals (bottom) is shown. (B) Strategy for flow cytometry analysis for determination of percent apoptosis in CD4+ and CD8+ T cells. Cryopreserved lymphocytes were thawed and washed as above. Cells were then stained with a cocktail of antibodies comprising of CD3-Cy7, CD4- Tx Red, CD8-APC (Beckman Coulter) and CCR5-PE (BD Pharmingen) along with FITC-VAD-FMK (Promega). 20,000 events for each sample were acquired and data analyzed using the FlowJo software. Representative dot plot of apoptosis staining in CD4+ T cells from a normal control (left) or HIV infected individual (right).
Figure S2: Analysis of CD4:CD8 ratio, CD4 and CD8 immune activation in resting versus activated PBMCs in vitro. Lymphocytes obtained from HIV negative healthy donors were cultured in RPMI-1640 medium supplemented with 20% FBS and phytohaemagglutinin at 2.5 µg/ml and IL-2 at 10U/ml for 48h. Cells were then stained for surface CD3, CD4 and CD8 along with the immune activation markers as described in Supplemental Figure 1. The CD4:CD8 ratio and increase in percentage of CD38+HLADR+ cells in the CD4+ and CD8+ population was determined after 48h of in vitro activation via flow cytometry analysis. For the resting panel, cells were stained directly after thawing without any in vitro culture. Representative contour plots of CD4:CD8 ratio (left) and increase in percentage of CD38+HLADR+ cells in the CD4+ (middle) and CD8+ population (right) are depicted.
Figure S3: (A) Analysis of CD4:CD8 ratios after co-culture of PBMCs with HIV Envelope expressing HeLa cells. PBMCs derived from healthy donors were co-cultured with HeLa cells expressing Lai Env, YU-2 Env or pCDNA3.1 vector control. The CD4:CD8 ratios were determined after 24h of co-culture via specific antibody staining followed by flow cytometry analysis. For some wells, the fusion inhibitor T-20 was added at the time of co-culture. Analysis of CD4:CD8 ratios in the presence of media (top) or fusion inhibitor T-20 (bottom). (B) Analysis of CD4 and CCR5 expression in PBMCs derived from healthy donors. PBMCs from normal volunteers NR013-NR016 were stained for surface CD4 and CCR5 expression followed by flow cytometry analysis.
Table S1. Characteristics of the Study Population (n=67).

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Note: SD=standard deviation, min=minimum value, max=maximum value

† From a Fisher’s exact test for the sex variable, otherwise from two-sample t-tests.