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Infection with different HIV subtypes is associated with CD4 activation-associated dysfunction and apoptosis ^{1,2,3,4}

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Abstract

Determination of HIV-1 subtype may be important in the management of HIV infected individuals, particularly with regard to deciding the CD4 cell count at which to initiate ART. Non-B subtypes A and D are prevalent in Uganda and individuals infected with subtype D appear to have faster disease progression compared to those infected with subtype A. We examined the level of apoptosis in CD4⁺ T cells in a study cohort of volunteers infected with subtype A and D infection. Although the levels of apoptosis in the activated CD4⁺ cells significantly decreased with viral suppression, CD4⁺ apoptosis in individuals infected with subtype D were found to be significantly higher compared to those infected with subtype A prior to antiretroviral treatment. Surface expression of PD-1 on CD4 cells in subtype D was substantially higher compared to subtype A ($p=0.03$). This difference was not observed in the CD8 population ($p>0.05$). Our findings suggest that the infecting HIV subtypes exert an independent influence on the disease outcome in response to antiretroviral treatment.

BACKGROUND

Differences in the genetic characteristics of HIV likely influence the rate of disease progression and the response to antiretroviral therapy. One important unanswered question is the biological basis for the apparent higher virulence of HIV subtype D compared to subtype A [1–6]. Surprisingly, knowledge in this area is limited and no conclusive recommendations currently exist regarding subtype and treatment guidelines or therapeutic monitoring. The level of viral replication and its subsequent effect on the immune system influences CD4⁺ T cell depletion. During chronic HIV infection, more CD4⁺ T cells die than can be accounted for by direct infection [7]. This bystander loss of uninfected T cells is believed to be associated with the generalized HIV-induced immune activation [8]. We have previously reported higher T cell activation in Uganda compared to western cohorts (HIV subtype B) [9]. We hypothesize that the infecting HIV subtypes exert independent influence on the outcome of immunity in response to antiretroviral treatment in our study population.

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MATERIALS AND METHODS

Study Subjects and Samples

HIV positive ART naive volunteers were randomly recruited from a prospective observational cohort study from individuals receiving care at the Mbarara Hospital Uganda AIDS Rural Treatment Outcomes (UARTO) in Mbarara, Uganda [10]. Demographic information, CD4+ T cell count, and HIV viral load were obtained at the time of enrollment, and then again at 3 and six months after initiation of ART. All patients initiated ART within 2 weeks of being enrolled into the study. HIV-1 RNA levels were determined from plasma using the Roche Amplicor 1.5 (Roche, Branchburg, New Jersey), as per manufacturer's recommendations. Peripheral blood mononuclear cells (PBMCs) were obtained from enrolled volunteers. PBMC samples were isolated from whole blood by the use of density gradient centrifugation over ficoll-hypaque solution, cryopreserved immediately, and stored in liquid nitrogen. Institutional Review Board approvals were obtained from the Uganda National Council of Sciences and Technologies, the UCSF committee on Human Research, and the California Department of Public Health. All study patients participated voluntarily and gave written informed consent.

HIV Subtyping

Subtyping was based on plasma sequencing for Pol as previously reported [11]. Briefly, RNA was extracted from plasma using the Qiagen viral RNA kit with a BioRobot 9600/9604. HIV Pol genes were amplified using nested reverse transcriptase polymerase chain reaction and sequenced in both the 5' and 3' directions. The National Institutes of Health's HIV-1 Genotyping Tool website (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.html>) in conjunction with the Los Alamos HIV sequence database (<http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html>) was used to determine the HIV-1 subtype.

Apoptosis Assays

HIV-1 infection-induced cleavage of procaspases results in cell apoptosis. Flow cytometry analysis approach allows for the detection of apoptotic cells at the single cell level. We measured caspase activity by using the Vybrant FAM Poly Caspases Assay Kit (Molecular Probes). The kit employs a fluorescently labeled inhibitor of caspases (FLICA) reagent containing a fluoromethyl ketone (FMK) group, a caspase specific recognition sequence (VAD), and a carboxyfluorescein (FAM) group as a reporter. Cryopreserved PBMCs were thawed, washed, and resuspended at 1×10^6 cells/mL in 300 μ l RPMI media with 10% FBS. After adding 10 μ l of 30X FLICA working solution to each culture, the suspensions were incubated for 1 hour at 37°C and 5% CO₂. The cells were washed twice with 1X wash buffer supplied by the manufacturer before proceeding with subsequent surface staining. Apoptosis was analyzed based on caspase positive and negative populations, with gating based on a fluorescence minus one (FMO) control.

Immune activation and functional immunophenotype

Activated T cells were measured as previously described [9]. Further immunophenotyping was performed on cryopreserved PBMCs, which were thawed and subsequently stained with antibody panels for activation and PD-1. For activation panels, cells were stained with the following antibodies: HLADR FITC, CD38 PE, CD3 PerCp Cy5.5, CD4 APC CY7, CD27 APC, CD45RA PECy7, CD8 Pacific Blue (BD BioSciences, San Jose, CA, USA). For the PD-1 panels, cells were stained with antibodies including CD3 PerCP Cy5.5, CD4 PeCy7, CD8 Pacific Blue, HLADR FITC, CD38 PE, and PD-1 APC (BD BioSciences). Data was collected on an 8 color flow cytometer (LSRII, BD Biosciences) and analyzed using FLOWJO software (TreeStar, San Carlos, CA). A minimum of 30,000 CD4+ cells per sample was acquired on the

LSRII. Dead cells were first gated out using a violet excited viability dye (LIVE/DEAD Fixable Dead Cell Stain; Invitrogen). Immune activation was defined as the percent of CD38+ HLADR + T cells. PD-1 level was defined as the percent expression of PD-1 APC on CD3+ CD8+ T cells. Gating was standardized and set using fluorescence controls for HLADR, CD38 and PD-1.

Statistical Analysis

Groups were compared using the *Mann-Whitney U* test or the paired t test using PRISM software version 4.02 (GraphPad, San Diego, CA). Statistical significance was defined as $p < 0.05$.

RESULTS

Subtype distribution

We analyzed subtype distribution in our study population based on the Pol region of the genome and valid data was obtained for 172 samples. We found that subtype distribution in our study population (Figure 1) is comprised primarily of subtype A and D (N=84 and 76, respectively). No correlation between viral subtypes (A and D) for CD4 count (mean values 95 and 70 count/ml, $p > 0.05$, subtype A and D, respectively) or viral load (mean values 203, 000 and 151,000 copies/ml, $p > 0.05$, subtype A and D, respectively) was observed prior to ART initiation (Table 1). In addition, CD4 cell gain following ART suppression did not differ significantly between the two infecting subtypes within six months (Figure 1C). There was no significant difference in subtype distribution by gender or age ($p > 0.05$, data not shown).

CD4 immune activation decreases post ART suppression in subtypes A and D

Different HIV-1 subtypes may vary in pathogenesis resulting in distinct profiles of immune activation. We and others have described heightened immune activation in HIV-infected individuals from Africa compared to those from Europe and the U.S [9,12]. Activation staining was performed as previously described [9,13]. CD8 and CD4 activation was defined as the percentage of CD3+ CD8+ CD38+ HLADR+ or CD3+ CD4+ CD38+ HLADR+ lymphocytes, respectively. Both CD8 and CD4 T cell immune activation were examined separately at baseline (when the patients were naïve) and again at 6 months following the initiation of antiretroviral treatment. Because the majority of our population was infected with subtypes A and D, we focused our immune activation analysis on these two subtypes. We observed no significant differences in CD4 and CD8 immune activation between subtypes (Figure 2 and data not shown).

Differential levels of apoptosis in activated cells between subtype A and D

We next examined the level of apoptosis in CD4+ T cells between the two subgroups. Overall levels of apoptosis were highest in the activated CD4 cells compared to the total fraction of CD4+ caspase+ T cells and significantly decreased with viral suppression (Figure 3A, $p < 0.0001$). Interestingly, the apoptosis of activated CD4 cells in individuals infected with subtype D was significantly higher compared to those infected with subtype A (Figure 3B, $p = 0.028$) prior to antiretroviral treatment. After ART mediated viral suppression we observed normalization of activation to similar levels between subtypes.

PD-1 expression on CD4+ T cells

The inhibitory programmed cell death receptor (PD-1) has been compared to activation marker CD38 and viral load as a predictor for clinical progression. PD-1 is upregulated in chronic HIV infection, and its expression correlates positively with plasma viral load and inversely with CD4+ T-cell count. We found that levels of expression of PD-1 on CD4 cells in subtype D was

substantially higher compared to subtype A (Figure 4A, $p=0.01$). This difference was not observed in the CD8 population (Figure 4B, $p=0.97$). The level of PD-1 expression in the two populations was not significantly different after antiretroviral suppression ($p=0.25$, data not shown).

DISCUSSION

The mechanism by which HIV causes immune destruction is linked to its ability to induce a generalized immune hyperactivation. Discordant responses to antiretroviral therapy have been linked to the level of cellular activation [14–16]. How chronic activation might be detrimental to the immune system remains unclear. Bystander activation of uninfected CD4⁺ T cells has been demonstrated to increase susceptibility to infection and subsequent virus production *ex vivo*. T cell apoptosis and subsequent immune exhaustion are likely key factors in the eventual failure of viral control.

HIV-1 subtypes may confer distinct pathogenesis and impact HIV disease progression. Subtypes A and D account for the majority of infection in our treatment-naïve study population in Uganda. CD4⁺ T cell immune activation significantly decreased after ART-mediated viral suppression as we have previously reported [17]. Although subtypes did not correlate with generalized levels of immune activation, we observed significant differences in CD4 dysfunction in individuals infected with subtype D, as demonstrated by higher level of apoptosis and PD-1 expression. This data supports the hypothesis that subtype D is more pathogenic [14,15], and is consistent with previous cohort studies demonstrating faster disease progression in subtype D that is independent of viral load levels [1].

The role of HIV subtype and viral gene products may be critical in determining the level of immunopathogenesis associated with HIV disease progression to AIDS. We propose that the differential subtype-induced activation and subsequent apoptosis is one such mechanism that leads to differential clinical disease outcome in Uganda [1]. The difference in the level of activation-associated apoptosis between subtype A and D becomes insignificant after six months of antiretroviral therapy. This finding may reflect on the specific subtype's ability to induce the expression of apoptosis ligands and disrupt the effective generation of T cell immunity. However, the full explorations of all mechanisms involved in treatment failure and immune recovery cannot be assessed in this relatively short follow-up and the small sample size of our study limits definitive long-term outcome. The true clinical impact of T cell dysfunction in association with HIV subtypes in predicting disease outcome will require a larger, longitudinal cohort study. The recognition of the different HIV-1 subtypes has led many to question whether properties of HIV-1 infection and its consequences as a whole can be generalized among different subtypes. Our findings suggest that infection with one subtype may be more aggressive than others with regard to disease progression. Such diversity could potentially affect the overall efficacy of HIV therapy or intervention strategies in the affected population.

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A **B**

Figure 1.

Viral load level (A) and CD4 count (B) distribution by subtype in the study cohort as determined by subgene analyses. CD4 recovery after ART between subtype A and D (C). Horizontal lines represent medians and interquartile range (25th and 75th percentiles). No statistical significance was found between subgroups ($p>0.05$).

A **B** **Figure 2.**

PBMCs were stained with HLADR FITC, CD38 PE, CD3 PerCP CY5.5, CD8 Pacific Blue, CD4 APC Cy7, CD45RA PECy7, and CD27 APC, and analyzed by flow cytometry. Samples were first gated on the CD3+/CD8+ and CD3+/CD4+ lymphocyte populations, then the percent of CD38 and HLADR positive cells were determined. Immune activation was defined as the percentage of CD4+ or CD8+ T cells expressing both HLADR and CD38. Immune activation before (A) and after (B) ART-mediated viral suppression are shown. A significant decrease was observed in immune activation of the CD4+ T cell subpopulation after ART in both subtype A and D ($p<0.001$), although no statistical differences were seen between the distribution of CD4 activation between subtypes ($p>0.05$).

Figure 3.

Caspase activity was measured in PBMCs using the Vybrant FAM Poly Caspases Assay Kit (Molecular Probes). Cells were also stained with HLADR PE Cy7, CD38 APC, CD8 PerCP CY5.5, CD3 Amcyan, and CD4 APC Cy7. (A) The caspase positive, activated cell subpopulation significantly decreased after antiretroviral treatment ($p < 0.0001$). (B) Interestingly, the caspase positive, activated cell populations were significantly higher in subtype D individuals ($p = 0.028$). These differences normalized with ART ($p > 0.05$).

Figure 4.

PBMCs were stained with CD3 PerCP CY5.5, PD-1 APC, CD8 Pacific Blue, CD4 PE Cy7, CD38 PE, and HLA DR FITC, then analyzed for expression of PD-1 on CD4+ (A) and CD8 + (B) T cells. Samples were first gated on the CD3+/CD4+ (and CD3+/CD8+) lymphocyte population, then the percent of PD-1 positive cells was subsequently determined. Gating on PD-1 positive cells was performed using the fluorescence minus one (FMO) control for PD-1. Results were expressed as percent of CD4+ and CD8+ T cells expressing PD-1 after subtraction of the background. Expression of PD-1 on CD4 cells in subtype D was substantially higher compared to subtype A ($p=0.01$), although this difference was not observed in the CD8 population ($p=0.97$).

Table 1

Summary of clinical characteristics of study participants pre enrollment*

Study participants	Age (years)	CD4 (count)	HIV plasma RNA (copies per milliliter)
Subtype A N=84	35 (29–40)	95 (63–216)	203,000 (46,179–315,184)
Subtype D N=76	34 (28–37)	70 (66–280)	151,000 (47,982–518,060)
P values	NS	NS	NS

*
mean values and ranges