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Differential associations of IL-6 and intestinal fatty acid-binding protein with progressive untreated HIV-1 infection in Rakai, Uganda

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Abstract

The significance of HIV-associated immune activation and microbial translocation in Sub-Saharan African populations remains poorly defined. We assessed biomarkers of inflammation, microbial translocation, and cellular activation and found most factors elevated in Ugandan HIV-1 seroconverters compared to community-matched controls. In contrast to previous findings in Western cohorts, CRP, neopterin, and IFABP were not elevated. Higher T-cell activation and IL-6 were associated with faster disease progression. Surprisingly, IFABP, indicative of enterocyte turnover, was higher in slow compared to fast progressors. These data suggest differential relationships among biomarkers of intestinal barrier integrity and innate immune activation between developed countries and Sub-Saharan Africa.

Keywords

HIV-1; AIDS; immune activation; inflammation; IL-6; IFABP

Introduction

The HIV-1 epidemic continues to grow in sub-Saharan Africa where access to treatment remains limited [1]. Untreated HIV-1 disease progression is influenced by viral diversity, host genetics, and endemic diseases. In Uganda, subtype D and D containing recombinants demonstrate faster progression rates to AIDS and death compared to subtype A [2–5]. In the developed world, persistent inflammatory conditions and hyperimmune activation predict clinical outcome independent of HIV-1 viral load [6–8]. Recent findings indicate, however, that levels of T-cell activation may not be an independent predictor of HIV-1 disease progression in rural Uganda, as reported in western cohorts [9]. Still, HIV-1 viral load, CD4 T-cell count, and chronic activation are associated with HIV-1 pathogenesis in Uganda [10, 11]. However, the mechanisms driving immune activation in HIV-1 infection in different stages and disease settings remain poorly defined.

HIV-1 associated immune activation may, in part, be driven by increased enteropathy [12–15] as a consequence of HIV-induced damage to the gastrointestinal tract with increased enterocyte turnover and release of soluble intestinal fatty acid binding protein (IFABP) [16, 17]. A compromised gut-epithelial barrier may allow for microbial translocation resulting in systemic immune activation [8, 17]. Microbial products like lipopolysaccharide (LPS) bind pathogen recognition receptors such as soluble CD14 (sCD14) and in combination with LPS-binding protein (LBP) can stimulate an inflammatory response [18, 19]. Microbial products can also cause monocytes and macrophages to shed the hemoglobin scavenger receptor CD163 (sCD163) [20–23]. In addition, myeloid cells secrete cytokines (such as IL-6, IL-10, catalase and CRP) that may sustain or modulate inflammation [8, 24–26]. Similarly interferon- γ from activated T-cells may further stimulate monocytes to produce neopterin, a catabolite indicative of a pro-inflammatory environment [27]. HIV-1 infection is also associated with depletion of regulatory T-cells (Tregs) in peripheral blood, which may limit the ability to suppress systemic immune activation and inflammation [28]. Together, these biomarkers of systemic immune activation and inflammation could help identify important pathways involved in HIV-1 disease progression.

To better understand the complexities of HIV-1 disease in rural Sub-Saharan Africa, we assessed the associations between enterocyte turnover (IFABP), monocyte activation (sCD14, sCD163 and neopterin), inflammation (IL-6, IL-10, CRP and catalase), Tregs, absolute CD4 T-cell count, HIV-1 viral load and T-cell activation in ART-naïve individuals from Rakai, Uganda.

Materials and methods

Study Population

The study population was identified from a cohort in Rakai, Uganda as previously described [2, 3]. Between 1997 and 2004 prior to ART availability, HIV-seroincident participants from the Rakai Community Cohort were enrolled into a prospective study to characterize HIV-1 disease progression. Whole blood was collected, processed, and cryopreserved as previously described [29]. Plasma HIV-1 viral load was quantified using the Roche Amplicor test version 1.5 (Roche Diagnostics, Mannheim, Germany). Absolute CD4 T-cell counts were

determined using either FACS MultiSET or FACSCount (Becton Dickinson (BD), San Jose, CA). HIV-1 subtyping was determined using a multi-region hybridization assay (MHA) [30]. Study participants did not have access to ART at the time of sample collection, as previously described [2, 9]. Median follow-up time was 10 years per participant and HIV-1 positive individuals were categorized based on long-term clinical follow-up and progression to AIDS: non-progressors (NP), slow-progressors (SP), and fast-progressors (FP). Participants whose rate of progression was less than the median time of progression of 5.6 years were classified as FP. Those with greater than or equal to the median progression time of 5.6 years were regarded as SP. Those who did not progress to AIDS during the follow-up period were termed as NP. AIDS was defined as CD4 T-cell count <250 cells/ μ L or World Health Organization clinical stage 4 illness. Community matched HIV-uninfected individuals served as a control group. Ethical approval was obtained from institutional review boards of the Uganda's National Council for Science and Technology (UNCST), the Uganda Virus Research Institute's Science and Ethics Committee, The Johns Hopkins, School of Public Health Committee of Human Research, as well as the Division of Human Subjects Protection at the Walter Reed Army Institute of Research. All participants provided written informed consent.

Biomarker assays and flow cytometry

Plasma and PBMC from HIV infected participants at a chronic timepoint, median 923 days after seroconversion (range 307 – 2284 days), was used for analysis. Soluble factors were measured using commercial kits as per manufacturer's instructions. Enzyme-linked immunosorbent assays (ELISA) were used to quantify neopterin (Immuno-Biological Laboratories America, Minneapolis, MN) and catalase (ABCAM®, Cambridge, MA). CRP, sCD14 and sCD163 levels were measured by Quantikine ELISA (R&D Systems, Minneapolis, MN). The R&D systems human IFABP2 Duo Set ELISA kit was used to measure IFABP. Optical density was determined using a BioTek ELX800 plate reader and final concentrations were calculated from standard curves using KC4 software (BioTek, Winooski, VT). IL-6 and IL-10 levels were measured from plasma using BD human inflammatory cytokine bead array (BD Biosciences, San Jose, CA). T-cell activation (the percentage of CD4 or CD8 T-cells co-expressing HLA-DR and CD38) and Treg frequency (FoxP3+ regulatory CD4 T-cells) were determined as previously described [31]. Briefly PBMCs were thawed, washed and counted. T-cell activation was assessed using CD3, CD4, CD8, HLA-DR and CD38 antibodies (BD Biosciences). Viability assessments were performed using the aqua live dead amine reactive dye (Invitrogen, Carlsbad, CA). Tregs were identified based on surface staining with CD25, CD3, CD127 and CD4 (BD Bioscience) and intranuclear expression of FoxP3 (eBioscience, San Diego, CA). Samples were acquired on a BD FACS Canto II and data analyzed using FlowJo version 9.8 (Treestar, Ashland, OR).

Statistical analysis

GraphPad Prism version 6 (GraphPad, La Jolla, CA) was used for statistical analysis. Differences comparing ranks within groups were determined using a two-tailed Mann-Whitney test whilst the Kruskal Wallis one-way analysis of variance measured differences across multiple groups. Dunn's test was used to correct for multiple comparisons. Spearman

rank correlation analysis was used to determine the association between two continuous variables. All $p < 0.05$ were considered statistically significant.

Results

Virologic and immunologic indices in HIV-1 infected and uninfected participants from Rakai District, Uganda

The HIV-1 infected ($n=62$) and uninfected ($n=33$) participant groups displayed similar age and sex distribution (Table 1). The HIV-1 subtype distribution of infected participants was 77% HIV-1 subtype D and 23% subtype A, and none of the clinical or immunological parameters measured differed between HIV-1 subtype A and D infected individuals. Median (range) time to AIDS was 5.6 (1.1–12.2) years. As expected, HIV-1 infected individuals had higher levels of CD4 and CD8 T-cell activation, (both $p < 0.001$) and lower Treg frequency and CD4/CD8 ratio (both $p < 0.001$) compared to uninfected study participants. In contrast, the levels of IL-10, IFABP, CRP and neopterin in plasma were not significantly different between the groups, although there were trends towards higher CRP levels ($p=0.06$) and lower IFABP levels ($p=0.07$) in HIV-1 infected donors. However, HIV-1 infection was associated with a nearly two-fold increase in sCD14 ($p < 0.001$). Similarly, the levels of sCD163 ($p=0.012$), IL-6 ($p < 0.001$), and catalase ($p < 0.001$) were all elevated in HIV-1 positive individuals compared to negative controls.

Biomarkers of immune activation and inflammation across HIV-1 disease progression categories

The HIV-1 infected participants were next stratified by disease progression categories (Figure 1A). FP had higher HIV-1 viral load ($p=0.003$), reduced absolute CD4 T-cell counts ($p=0.048$), and lower CD4/CD8 T cell ratio ($p=0.012$) compared to NP. FP also displayed an increased frequency of CD38+HLADR+ CD8 T-cells ($p=0.007$) and CD38+HLADR+ CD4 T-cells ($p=0.008$) compared to NP. With regard to soluble markers of inflammation, FP exhibited elevated levels of IL-6 ($p=0.045$) and a trend toward increased IL-10 ($p=0.05$) compared to NP. Surprisingly, levels of sCD14 were not different across AIDS progression groups ($p=0.180$), whereas levels of IFABP were higher in SP compared to FP groups ($p=0.008$).

Associations between markers of HIV-1 disease, inflammation, and immune activation

To understand the relationships between the different measures of immune activation, inflammation, and HIV disease progression we next examined possible correlations between these measures (Figure 1B). The proinflammatory cytokine IL-6 was associated with multiple markers of HIV disease progression, including HIV-1 viral load ($\rho=0.393$, $p=0.002$), absolute CD4 T-cell counts ($\rho=-0.432$, $p=0.010$), CD4 T-cell activation ($\rho=0.406$, $p=0.001$) and CD8 T-cell activation ($\rho=0.367$ and $p=0.004$). IL-6 also correlated positively with CRP ($\rho=0.480$ and $p < 0.001$). In contrast, we observed a weak negative correlation between IFABP and HIV-1 viral load ($\rho=-0.279$, $p=0.028$), as well as an inverse relationship between IFABP and sCD14 ($\rho=-0.296$, $p=0.019$). Additional correlations were evident between sCD14 and sCD163 ($\rho=0.337$, $p=0.007$), absolute CD4

T-cell count ($\rho=-0.552$, $p<0.001$), CD4 T-cell activation ($\rho=0.389$, $p=0.002$) and CD8 T-cell activation ($\rho=0.385$ and $p=0.002$).

Discussion

In this study, we examined indices of immune activation and inflammation in untreated HIV-1 seroconverters from Rakai district, Uganda and found that the majority of biomarkers were elevated in HIV-1 infected study participants compared to uninfected individuals, except for CRP, IFABP, IL-10, and neopterin. Non-specific markers of infection and inflammation like neopterin and CRP predict HIV disease progression and AIDS mortality in industrialized countries [27, 32–35], but our data do not recapitulate these patterns in this rural African population. Consistent with findings in Western cohorts, HIV-1 infected study participants' viral load, T-cell activation, and IL-6 levels were associated with faster progression to AIDS [36, 37].

A trend toward lower levels of IFABP was observed in HIV-positive participants compared to HIV-uninfected individuals, contrary to findings from other regions, which have indicated increased IFABP levels in HIV-1 infection [17, 38, 39]. Levels of IFABP in HIV-1 uninfected participants from Rakai are similar to those in HIV-1 infected individuals from the US [17], suggesting that endemic infections may stimulate an inflammatory state independent of HIV-1 infection. Additionally, healthy Sub-Saharan Africans and HIV infected US populations have been reported to share similar features of their gut microbiota closely resembling patterns observed in inflammatory disorders [40]. Notably in Sub Saharan Africa, prevalent enteric infections also cause gastrointestinal tract damage [41–43]. The elevated levels of IFABP in SP compared to FP and the unexpected inverse correlation with viral load suggest that in the Ugandan context HIV-1 infected individuals with high enterocyte turnover may display delayed disease progression. Accelerated enterocyte turnover facilitates expulsion of gut parasites [44]. It is possible that humans adapt to these endemic diseases by continuously remodeling their guts. Thus it is also possible that loss of this protective effect could be a potential mechanism behind the association between low IFABP levels and rapid HIV disease progression. It is important to note that no difference was observed in IFABP levels between HIV-infected NP and FP, which highlight the need to study IFABP and intestinal barrier permeability in other African cohorts.

Levels of IL-6, sCD14, sCD163 and catalase were, as expected, all increased in HIV positive individuals compared to negative participants, in line with substantial increases in monocyte activation and inflammation. It is possible that HIV-1 associated reduction of Tregs leads to reduced ability to suppress inflammation thus enabling a highly activated microenvironment [28, 45]. Despite sCD14 being associated with another marker of monocyte activation (sCD163) and increased T-cell activation, it displayed an inverse correlation with IFABP levels. IFABP and sCD14 are often interchangeably used as markers of microbial translocation [17, 39, 46]. However our findings suggest they could be reflective of independent biological processes in this cohort. Statistically significant associations were observed between IL-6 and HIV-1 viral load as well as IL-6 and CRP, but not between CRP and HIV-1 viral load, supporting the hypothesis of independent biological processes, again reflecting multiple drivers of inflammation. The presence of co-infections has been

associated with increased levels of immune activation in the African setting [11], which supports the idea that endemic co-infections may affect disease progression. However, in our study, prevalence of Hepatitis B (10%), Hepatitis C (13%), and active Syphilis (8%) was relatively low and rates did not differ between HIV-1 infected and uninfected individuals, or between the three HIV-1 disease progression categories. Additional studies are needed to better understand the contribution of co-infections to inflammation in rural African populations.

In summary, in the Rakai cohort, faster HIV-1 disease progression is associated with elevated levels of inflammation markers, as well as chronic immune activation and increased viral load. In addition, continuous enterocyte turnover may be associated with more favorable HIV-1 disease outcome. Additional studies are required to assess gut damage and its impact on HIV-1 disease progression in Sub-Saharan Africa as well as determine major determinants driving immune activation and inflammation. Future work should examine a more diverse array of biomarkers and systematically evaluate other factors that may associate with faster disease progression.

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Figure 1. Immunologic biomarkers associated with HIV-1 disease progression.

Soluble and cellular factors associated with immune activation were compared in chronic untreated HIV-1 infected study participants categorized based on rate of HIV-1 disease progression. (A) Non-progressors (strike through circles), slow-progressors (empty circles), and fast-progressors (gray filled circles) were compared for plasma HIV-1 viral load, CD4 T-cell count, CD4/CD8 ratio, CD4 T-cell activation, CD8 T-cell activation, IL-6, IFABP, and sCD14. Biomarkers were compared for all HIV-1 infected participants to see which factors associated with each other. (B) Comparisons of IL-6 and plasma HIV-1 viral load, CD4 absolute T-cell count, CD8 T-cell activation, and CRP levels for the total HIV-1 infected population were calculated using Spearman rank order analysis. Comparisons were also displayed for IFABP and plasma HIV-1 viral load and IFABP and sCD14. Correlation coefficients (ρ) for biomarkers are shown with corresponding p values (significance was determined to be $p < 0.05$).

Table 1:

Clinical, demographic, and immunologic characteristics of study participants by HIV status

	HIV-1 negative (n=33)	HIV-1 positive (n=62)	<i>P-value</i>
Female, no. (%)	17 (51%)	41 (66%)	NA
HIV Subtype D, no. (%)	NA	48 (77%)	NA
HIV Subtype A, no. (%)	NA	14 (23%)	NA
Age in years, median (range)	29 (21 - 40)	31 (20 - 53)	0.083
Progression to AIDS(years), median (range) *	NA	5.6 (1.1-12.2)	NA
HIV-1 viral load (copies/ml), median (range)	NA	47,571 (528-1.68 × 10 ⁶)	NA
CD4 T-cell counts (cells/mm ³), median (range)	875 (530-1450)	489 (2-1092)	<0.001
CD4/CD8 T-cell Ratio	2.1 (1.2 - 3.5)	0.76 (0.07 - 3.75)	<0.001
%CD8/CD38+HLA-DR+ (cells), median (range)	10.3 (3.2-29.6)	45.3 (16.8-74.1)	<0.001
%CD4/CD38+HLA-DR+(cells), median (range)	2.6 (0.9-6.1)	10.8 (2.6-41.0)	<0.001
Treg frequency (% of CD3)	0.65 (0.1-1.24)	0.17 (0.02-0.95)	<0.001
IL-6 (pg/ml), median (range)	2.2 (1.2-6.6)	3.4 (1.0-10.9)	<0.001
IL-10 (pg/ml), median (range)	3.3 (1.2-12.7)	4.1 (1.1-41.5)	=0.22
Catalase (ng/ml), median (range)	124 (56-525)	185 (61-525)	<0.001
Neopterin (nmole/ l), median (range)	2.37 (0.09-11.88)	3.6 (0.03-20.7)	=0.22
sCD163 (ng/ml), median (range)	1201 (371-1691)	1485 (251-1721)	=0.012
sCD14 (ng/ml), median (range)	1288 (132.9-2663)	2171 (774-3600)	<0.001
CRP (ng/ml), median (range)	1300 (13-10545)	2474 (65-10545)	=0.06
IFABP (pg/ml), median (range)	330 (68-2861)	160 (100-1592)	=0.07

n = number; NA – not applicable;

* 26 Individuals progressed to AIDS defined by possession of a CD4 count less than 200 cells/mm³. No differences in innate soluble factors were observed between HIV Subtype A and D individual