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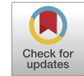
Infection with HIV-1 subtype D among acutely infected Ugandans is associated with higher median concentration of cytokines compared to subtype A



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ABSTRACT

Objective: The observation that HIV-1 subtype D progresses faster to disease than subtype A prompted us to examine cytokine levels early after infection within the predominant viral subtypes that circulate in Uganda and address the following research questions: (1) Do cytokine levels vary between subtypes A1 and D? (2) Do cytokine profiles correlate with disease outcomes?

Methods: To address these questions, HIV-1 subtypes were determined by population sequencing of the HIV-1 *pol* gene and 37 plasma cytokine concentrations were evaluated using V-Plex kits on Meso Scale Discovery platform in 65 recent sero-converters.

Results: HIV-1 subtype D (*pol*) infections exhibited significantly higher median plasma concentrations of IL-5, IL-16, IL-1α, IL-7, IL-17A, CCL11 (Eotaxin-1), CXCL10 (IP-10), CCL13 (MCP-4) and VEGF-D compared to subtype A1 (*pol*) infections. We also found that IL-12/23p40 and IL-1α were associated with faster CD4$^+$T cell count decline, while bFGF was associated with maintenance of CD4+ counts above 350 cells/microliter.

Conclusion: Our results suggest that increased production of cytokines in early HIV infection may trigger a disruption of the immune environment and contribute to pathogenic mechanisms underlying the accelerated disease progression seen in individuals infected with HIV-1 subtype D in Uganda.

Introduction

HIV infection leads to immune activation, immune cell proliferation, and production of several cytokines (Pastor et al., 2017; Salazar-Gonzalez et al., 1998, 1997; Stacey et al., 2009) and disrupts cytokine networks early in infection (Keating et al., 2012). Changes in both concentrations and profiles of cytokines in HIV-1 infected individuals affect immune functions, and can directly influence the course of HIV illness (Breen, 2002). For instance, proinflammatory cytokines such as interleukins (IL-) 1, IL-6 and tumor necrosis factor-alpha (TNF-α) alone or in combination can up-regulate HIV-1 replication (Fauci, 1996; Poli, 1999). On the other hand, interferons (IFN-) α and β are HIV-1 suppressors and inhibit HIV-1 replication in infected cells (Kedzierska and Crowe, 2001; Poli, 1999). Other cytokines such as IFN-γ, IL-10, IL-13, and IL-4 have both inhibitory and stimulatory effects on HIV-1 replication (Kedzierska and Crowe, 2001). Therefore HIV influences the balance of HIV-inductive and HIV-inhibitory cytokines, proinflammatory and anti-inflammatory cytokines, and T helper cell type 1 (Th1) and T helper cell type 2 (Th2) cytokines (Chatt et al., 2002). Imbalances between these cytokines have a significant impact on the host's capacity to regulate viral replication. In the HIV-1 acute infection phase, increased levels of inflammatory cytokines are associated with faster disease progression (Keating et al., 2016). Cytokines have been shown to impact lymphocyte migration, activation and expansion, increasing the pool of HIV-1 susceptible and activated CD4$^+$ T cells (Katsikis et al., 2011). This increased pool of susceptible CD4$^+$ T cells potentiates HIV-1 replication, viral load and in effect the systemic viral spread, leading to faster CD4+ T cell depletion (Breen, 2002; Fauci, 1996; Ford et al., 2009; Fraietta et al., 2013; Mahajan et al., 2010). Indeed, plasma concentrations of IL-1 α, IL-7, IL-12p40, and GM-CSF are predictive of

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CD4$^+$T cell depletion while high levels of IFN- γ are linked to low viral loads during acute infection (Roberts et al., 2010). While early immune events are associated with rate of disease progression in HIV-1 subtype B (Deeks et al., 2004; M. D Hazenberg et al., 2003.) and subtype C infection (de Almeida et al., 2016; Roberts et al., 2010), little is known about early immune events or their implications on disease progression in HIV subtypes A and D infections.

In Uganda's HIV-1 epidemic, subtypes A and D and their inter-subtype recombinants dominate (Capoferri et al., 2020; Kapaata et al., 2013; Lee et al., 2017; Lihana et al., 2012). Compared to HIV-1 subtype A, subtype D is associated with faster decline in CD4$^+$T cell counts, higher viral load and faster progression to AIDS (Kaleebu et al., 2002; Kiwanuka et al., 2010, 2008; Ssemwanga et al., 2013). Although cytokine levels and HIV-1 subtypes have been shown to affect HIV-1 disease progression, little is known about the variation of cytokines in HIV-1 subtypes. Moreover, the effect of acute cytokine profiles and levels on HIV-1 disease progression in individuals with HIV-1 subtypes A and D or their recombinants has not been satisfactorily described. In this study we measured plasma cytokine levels in HIV-1 subtypes A, D, C and recombinants infections. We also investigated the effect of cytokine levels on HIV-1 disease progression in acutely infected individuals in Uganda.

Methodology

Ethical Statement

The parent study received ethical approvals from the UVRI Research ethics committee (REF: GC 127) and the Uganda National Council of Science and Technology (REF: HS 108). Study participants consented to their samples being used for the study as well as to their samples being stored for future use.

Study participants

This study utilized archived plasma specimens collected by a large multicenter study titled "A prospective, observational, multi-center study to evaluate Laboratory, Clinical, immunological and viral markers of disease progression in recently HIV-infected participants", also known as the International AIDS Vaccine Initiative (IAVI)Protocol C Study, conducted at clinical research centers in Uganda, Kenya, Rwanda, Zambia and South Africa between 2006 to 2011. The IAVI Protocol C study investigated the immunologic, virologic and clinical parameters in HIV-infected volunteers with a date of infection that could be defined. Details of protocol C have been published previously (Amornkul et al., 2013; Price et al., 2021). In Uganda, protocol C participants were HIV-1 heterosexual discordant couples, the negative partner was followed up until sero-conversion, thus enabling the study of immune responses that occur immediately following HIV-1 infection. Blood samples were collected at every visit (monthly) and aliquots of plasma and serum were frozen.

In this study, we used 65 plasma samples from the Ugandan IAVI Protocol C participants with known dates of seroconversion also known as estimated date of HIV infection (EDI). We used the earliest sample (<90 days) after seroconversion to measure the concentration of cytokines in the plasma. The estimated date of HIV infection (EDI) was defined as either i) the midpoint between the last negative and first positive HIV-antibody test, ii) 14 days before the first positive p24 antigen test, iii)10 days before the first positive viral load test in the absence of p24 antigen or rapid HIV antibodies, or iv) the date of a self-reported high-risk exposure event.

Subtype determination

The entire HIV-1 *pol* gene including the entire protease (PR) and partial reverse transcriptase (RT) regions were sequenced after extraction using QIAamp Viral RNA Mini Kit (Qiagen, Inc, Valencia, CA, USA) following manufacturers' instructions. Five primers and the ABI BigDye Terminator kit were used to sequence the 1.7 kb amplicon (v3.1; Applied Biosystems, Foster City, CA). Sequences were analyzed using the ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA), assembled, and manually edited with Sequencher v4.7 (Genecodes, Ann Arbor, MI). The REGA HIV-1 subtyping program in the Stanford database (http://hivdb.stanford.edu/) was used to determine the HIV-1 subtype of each volunteer's sample. Extensive phylogenetic analysis was performed on samples that could not be assigned a subtype using REGA. A multiple alignment was created using the 2008 HIV-1 reference subtype sequences from the Los Alamos HIV database (http://www.hiv.lanl.gov) using CLUSTAL X version 2.0 (Larkin et al., 2007). Neighbor-joining trees were constructed with MEGA version 4 (Tamura et al., 2007) using the Kimura two-parameter method. Recombinants were further evaluated with the Recombinant Identification Program (RIP 3.0) and jumping profile Hidden Markov Model (jpHMM-HIV; http://www.hiv.lanl.gov) (Schultz et al., 2009). Selective sequencing of the *env* region was done occasionally to resolve residual ambiguity with *pol* sequences.

Determination of plasma cytokine concentrations

We quantified 37 soluble analytes in plasma using a combination of five Meso Scale Discovery (MSD) (Meso Scale Diagnostics, LLC) human V-PLEX panels. The panels were: 1) angiogenesis panel that included vascular endothelial growth factors (VEGF-A, VEGF-C, VEGF-D, Tyrosine kinase receptor (Tie-2), fms-related tyrosine kinase (Flt-1/ VEGFR-1) and basic fibroblast growth factor (bFGF). 2) Chemokine panel which consisted of CCL11/CCL26 (Eotaxin-1 and 3), macrophage inflammatory protein (MIP) -1 α and β also known as CCL3/CCL4 respectively, thymus-and activation -regulated chemokine, CCL17 (TARC), interferon-γ-induced protein, CXCL10 (IP-10), monocyte chemoattractant protein (MCP) -1 and 4 also known as CCL2/CCL13 respectively and macrophage-derived chemokine, CCL22 (MDC), Interleukin (IL)-8. 3) The cytokine panel included granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor-beta (TNF-β) also known as lymphotoxin-alpha, and interleukins (IL) -1α, 5, 7, 12/Il-23p40, 15, 16, 17A. 4) The pro-inflammatory panel included interferons (IFN)-γ, interleukins (IL) -1β, 2, 4, 6, 8, 10,12p70 and 13 plus TNF- α were included for their role in inflammation. 5) The vascular injury panel consisted of serum amyloid A (SAA), C-reactive protein (CRP), vascular adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1. Frozen plasma from acute/early samples was thawed and cytokine concentrations determined following the manufacturer's instructions. MSD plate reader type MESO QuickPlex SQ 120 was used to read the plates. The MSD program was used to collect data for two replicates per sample (Discovery Workbench Version 4.0). To calculate sample concentrations using standard curves, a five-parameter logistic regression method was used. Analytes below the lower limit of detection were assigned a concentration of half the lower limit of quantification (LLOQ).

CD4$^+$T cell counts and viral load measurements

These were done by the parent study (Price et al., 2021) and data was retrieved from the database. Viral load setpoint was the median viral load following acute phase HIV-1 infection (which in this case was 3-24 months).

Statistical analysis

All analyses were done using Stata version 15 (Stata Corp, USA), GraphPad Prism version 7 (GraphPad Software, Inc, USA) and JMP version 16 (SAS Institute, USA). The Mann-Whitney U-test was used to compare the median cytokine concentrations of the various subtypes. Cox proportional hazard models were used to determine relationship

Table 1
Characteristics of study participants

		Subtype A	Subtype D	Subtype C	Recombinants
Variable					
SPVL		37924 (19111-75764)	55399 (10905-145825)	24769 (2923-108325)	286379 (14528-558231)
Age		33.2(8.9)	29.5 (7.4)	30 (7.4)	27 (6)
EDI		55 (16)	44 (15)	37 (10)	62 (21)
CD4 + T cells		680 (241)	619 (242)	689 (375)	724 (347)
STI	No	9 (50%)	17(43.6%)	1 (33.3%)	4 (80%)
	Yes	9 (50%)	22 (56.4%)	2 (66.7%)	1 (20%)
Sex	F	2 (11.1%)	18 (46.2%)	0 (100%)	3 (60%)
	M	16 (88.9%)	21 (53.9%)	3 (100%)	2 (40%)

between cytokine levels and drop to CD4$^+$ T cell count less than 350 cells/μl. P values < 0.05 were considered statistically significant.

Results

Characteristics of the study participants

A total of 65 participants whose plasma was collected during the acute/early phase of HIV-1 infection were analyzed in this study. Of the 65 participants 39 were male and 26 were female. The average age of the study participants was 30.5 ± 7.7 years, the mean number of days from estimated date of infection (EDI) was 49.5 ± 6 days, the mean CD4$^+$ T cell count was 644 ± 252 cells/ μl and the median SpVL was 42,718 (IQR 14,031-96,368) copies/ml. Details of the participant demographics are in Table 1.

Subtype D was most prevalent

HIV-1 *pol* genotyping revealed that subtype D was the most prevalent (39/65, 60.0%), followed by subtype A1 (18/65, 27.7%) and subtype C (3/65, 4.6%), and 7.7% (5/65) were inter-subtype recombinants.

There are subtype-specific variations in plasma cytokine levels

Median plasma concentrations of IL-5, IL-16, IL-1α, IL-7, IL-17A, CCL11 (Eotaxin-1), CXCL10 (IP-10), CCL13 (MCP-4) and VEGF-D were significantly higher in subtype D than in subtype A1 infections (Figure 1). Additionally, subtype D had lower but non-significant concentrations of CCL3 (MIP-1α), VEGF-C, SAA, CRP, VCAM-1 and ICAM-1 compared to subtype A infections. Only CCL3 (MIP-1α) (p=0.0125) and SAA (p=0.0017) (Figure 2) were significantly higher in subtypes A than D infection. On the other hand, VCAM-1 (p=0.0163) and ICAM-1 (p=0.0002) were significantly higher in recombinants than subtype D infections. We also found that 26 of the 37 cytokines and soluble markers showed no significant differences between subtypes (supplementary data 1 and 2).

Chemokines positively correlated with CD4$^+$T cell counts

We investigated the relationship between plasma concentrations of cytokines and CD4$^+$T cell counts (Figure 3). TARC (r=0.3577, p=0.0034), MDC (r=0.303, p=0.0141) and VEGF-A (r=0.322, p=0.0089) exhibited a significant positive correlation with CD4$^+$T cell counts. On the other hand, IL-8 (r=0.273, p=0.0280) showed only a low positive correlation with CD4+ T cell counts while a low level negative correlation was observed with IL-12/23p40 (r=-0.214, p=0.087) but this was not statistically significant (Figure 3). Other cytokines had no significant correlation with CD4$^+$ T cell counts.

IL-12/23p40 and IL-1α predict HIV-1 disease progression

We also explored the prognostic value of cytokines in early infection on CD4$^+$ T cell decline below 350 cells/μl using a Cox proportional hazard model. Bivariate analysis showed that only IL-12/23p40 (hazard ratio (HR) =5.42, 95%confidence interval (CI)1.74-16.83, p=0.003) and IL-1α (HR=1.69, 95%CI 0.92-3.09, p=0.090) were associated with an increased risk of CD4$^+$ T cell counts decline below 350cells/μl. On the other hand, bFGF levels (HR=0.42, 95%CI 0.17-1.06, p=0.067) were associated with a 58% reduced risk of CD4+ T cell decline below the 350 cells/μL threshold. However, after multivariate analysis none of the cytokines, namely bFGF (HR= 0.42, 95% CI 0.15-1.16, p= 0.093), IL-12/23p40 (HR= 2.63, 95%CI 0.62-11.19, p= 0.191,) and IL-1α (HR=1.73, 95%CI 0.79-3.76 p= 0.169) remained significantly associated with a drop in CD4$^+$T cell counts to less than 350cells/μl (Table 2). IL-12/23p40 and IL-1α increased the risk of CD4$^+$T cell count decline by 2.63 and 1.73 times respectively , while bFGF was associated with maintenance of CD4$^+$T cell count above 350 cells/μl. Possible confounders in the model were age, sex, presence of sexually transmitted infections (STIs) and HIV-1 pol subtype, and the contribution of other cytokines.

Table 2
Adjusted cox proportional hazard model for cytokines that are a risk factor for CD4$^+$T cell count drop to below 350 cells/μl

Variable		Hazard Ratio (95%CI)	P value
bFGF		0.42 (0.15 -1.16)	0.093
IL-12/23p40		2.63 (0.62-11.19)	0.191
IL-1 α		1.73 (0.79-3.76)	0.169
age		0.96 (0.92-1.02)	0.165
Sex(Male)		1.08 (0.51-2.31)	0.833
No STI		1.86 (0.94-3.68)	0.076
Pol subtype	C	1.13 (0.24-5.41)	0.876
	D	1.02 (0.47-2.21)	0.957
	Recombinant	0.67 (0.13-3.39)	0.627

Adhesion molecules correlated with increase with time after initial HIV-1 infection (EDI)

Because a cytokine storm is often intense and transient occurring within the first ~20 days after infection, we examined if there was a temporal association of cytokine changes within EDI of 90 days. Increase in expression of cellular adhesion molecules is an important and integral component of immune cell trafficking to sites of the body where there is sequestered replication of pathogens. We observed a significant positive correlation in levels of soluble adhesion markers VCAM-1 (r=0.371, p=0.0024) and ICAM-1(r=0.342, p=0.0052) with EDI (Figure 4). A pairwise spearman correlation analysis of all 37 cytokines found VCAM-1 and ICAM-1 to have the greatest positive pairwise correlation (supplementary data 3).

Discussion

Our study found that subtype D (*pol*) infections had significantly higher median plasma concentration of IL-5, IL-16, IL-1α, IL-7, IL-17A, CCL11 (Eotaxin-1), CXCL10 (IP-10), CCL13 (MCP-4) and VEGF-D compared to subtype A1 (*pol*) infections. There is a paucity of data comparing cytokine expression by the different HIV-1 subtype. Hassan et al

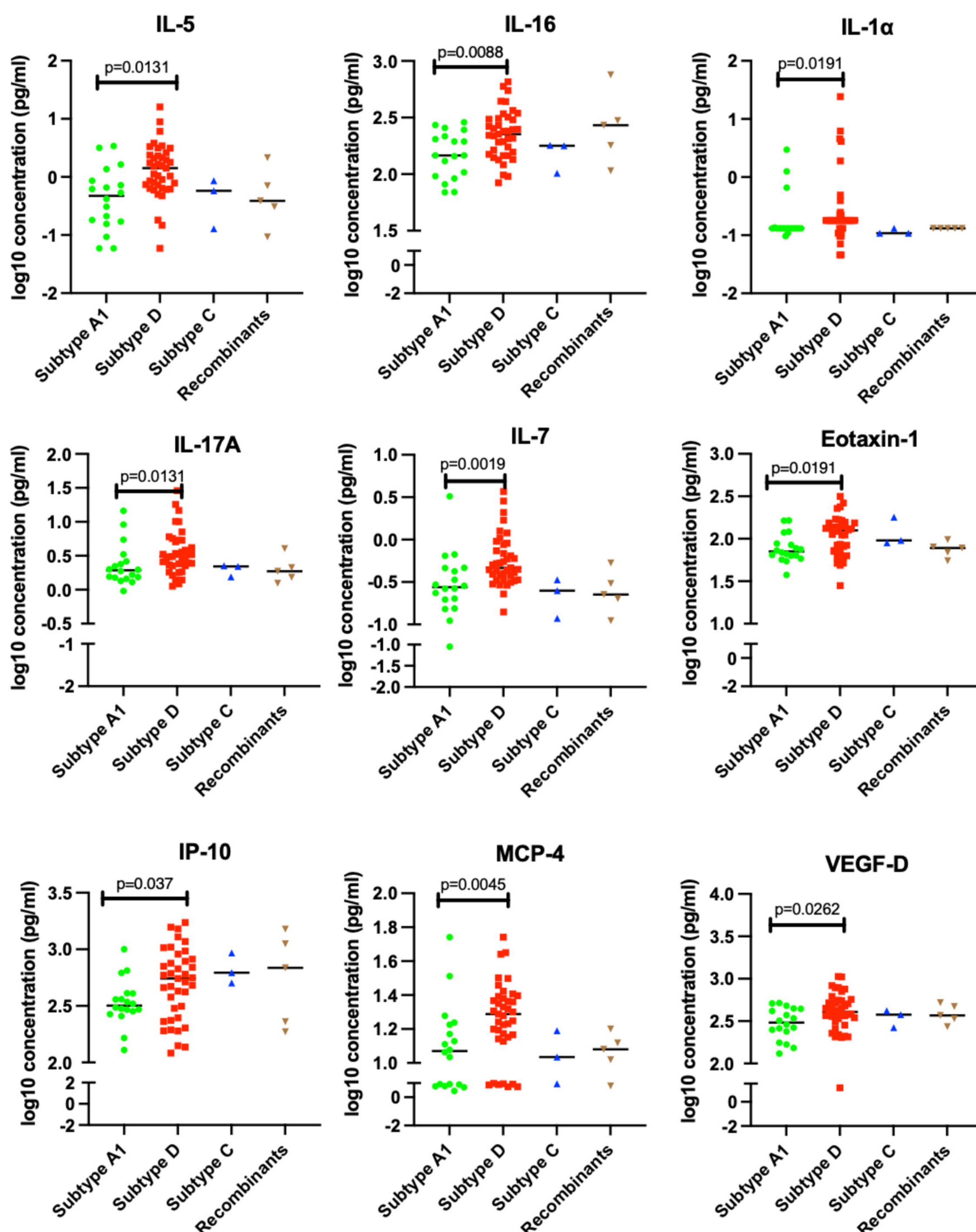

Figure 1. Cytokine concentrations that were significantly higher among those infected by HIV-1 subtype D. Cytokine expressions were measured using a combination of five Meso Scale Discovery (MSD) (Meso Scale Diagnostics, LLC) human V-PLEX panels. Mann-Whitney test was used to compare the median cytokine concentrations by subtype. The bars represent median values. IL-5,IL-16,IL-1α, IL17A,IL-7,Eotaxin,IP-10, MCP-4 and VEGF-D were significantly increased among subtype D infections. Abbreviations: IL, interleukin; MCP, monocyte chemoattractant protein; VEGF, vascular endothelial growth factor; IP, interferon gamma-induced protein.

2021 explored the expression of different cytokines pre and post HIV-1 infection among adults from Sweden and Africa and found a post infection elevation of cytokines CXCL10 (IP-10) and IL-1α. Muema et al reported elevated CXCL10 (IP-10), CCL2(MCP-1) and IL-12 among those hyper-acutely HIV-1 infected woman in South Africa. Stacey also reported an increased expression of CXCL10 (IP-10) in early HIV-1 infection (Hassan et al., 2021; Muema et al., 2020; Stacey et al., 2009). However, all the above studies did not compare the cytokine expression among the different subtypes. Interferon-γ-induced protein, CXCL10 (IP-10) is a chemokine involved in trafficking immune cells to the site of inflammation and has been considered a pro-inflammatory factor in HIV infection that is correlated with HIV disease progression (Lei et al., 2019). In another study, acute HIV-1 infection was associated with elevated levels of CCL2 (MCP-1) and CCL11 (Eotaxin-1) among people co-infected with hepatitis C virus infection (Lamoury et al., 2016). This study only compared cytokine expression between individuals with and without HIV/HCV co-infection. Since we found higher expression of CCL11 (Eotaxin-1), CXCL10 (IP-10), and CCL13 (MCP-4) among subtype

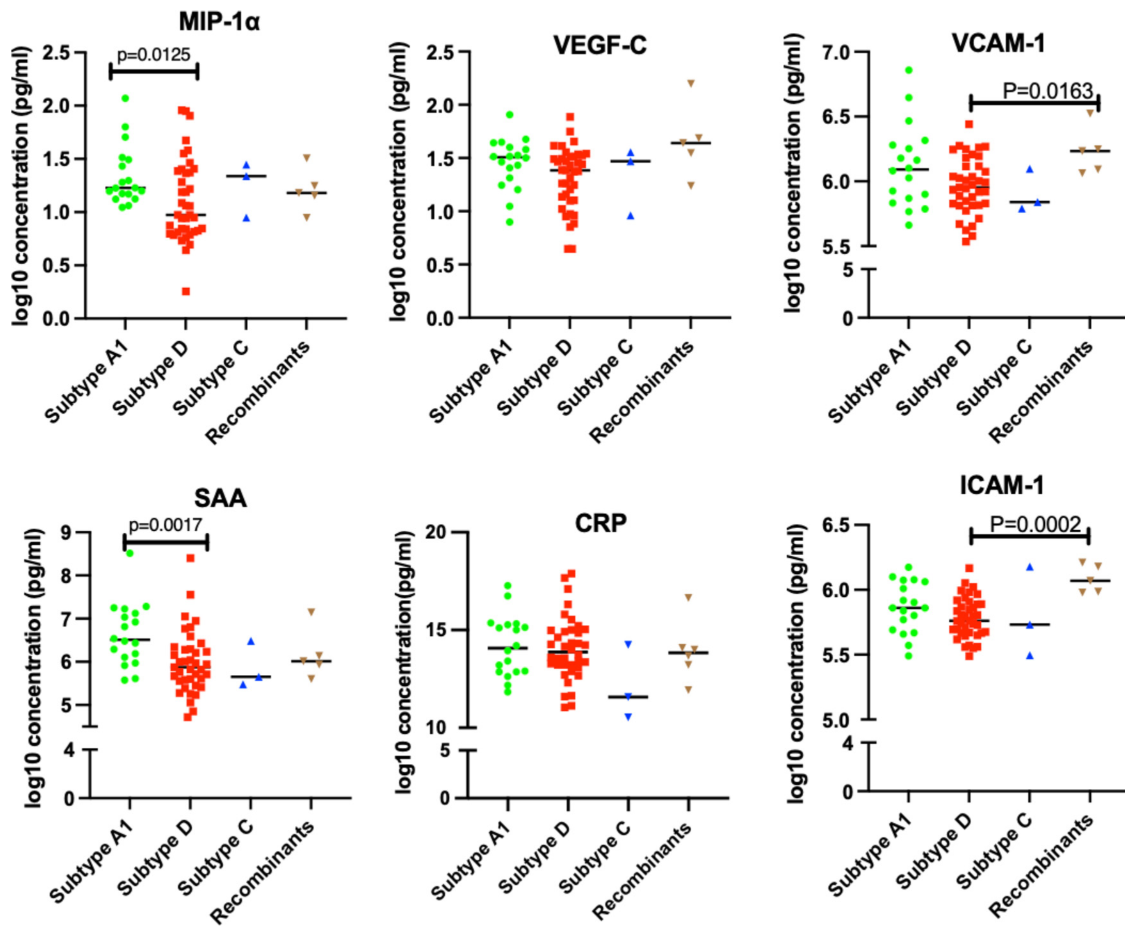

Figure 2. Cytokine concentrations that were higher among those infected by HIV-1 subtype A and recombinants. Cytokine expressions were measured using a combination of five Meso Scale Discovery (MSD) (Meso Scale Diagnostics, LLC) human V-PLEX panels. Mann-Whitney test was used to determine differences between two group medians. MIP-1α, SAA, VEGF-C, VCAM-1, ICAM-1 and CRP were increased in subtype A although statistical significance was only observed among SAA and MIP-1α. Abbreviations: MIP, macrophage inflammatory proteins; SAA, serum amyloid A; VEGF, vascular endothelial growth factor; VCAM, vascular cell adhesion molecule; ICAM, Intercellular adhesion molecule; CRP, C-reactive protein.

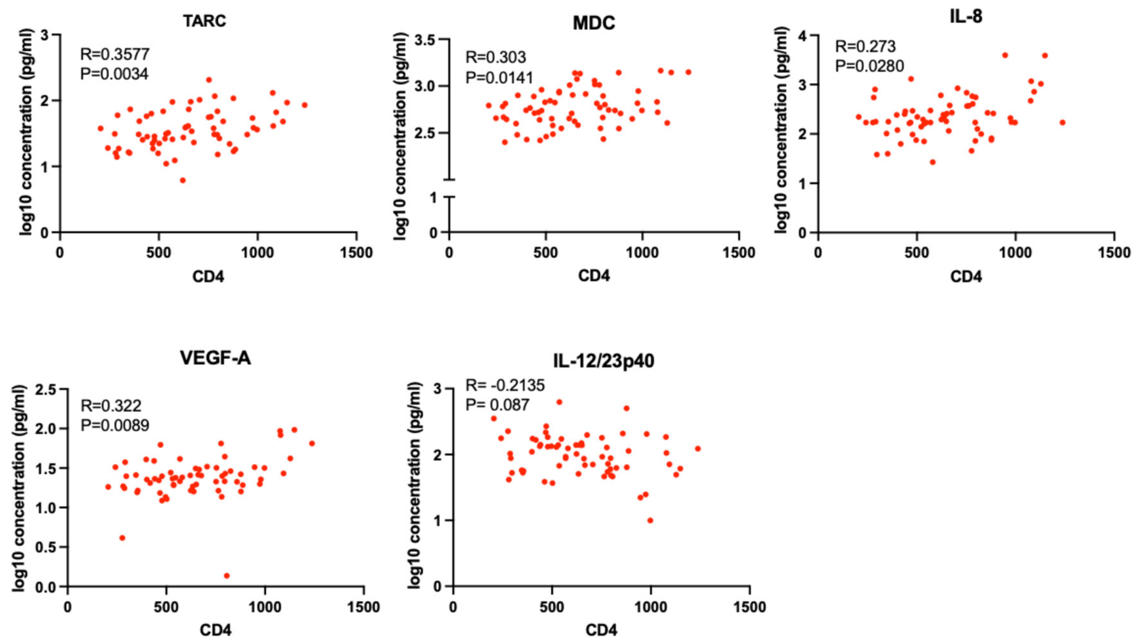

Figure 3. The correlation between cytokine concentrations and CD4^{+}T cell counts. Spearman's rank-order correlation was used to measure the correlation between the cytokines and CD4+ T cell counts. A positive R value indicated a positive correlation, and a P value <0.05 was considered significant. TARC, MDC, IL-8, and VEGF-A were positively correlated with CD4+ T cells , while IL-12/23p40 was negatively correlated with CD4+ T cells

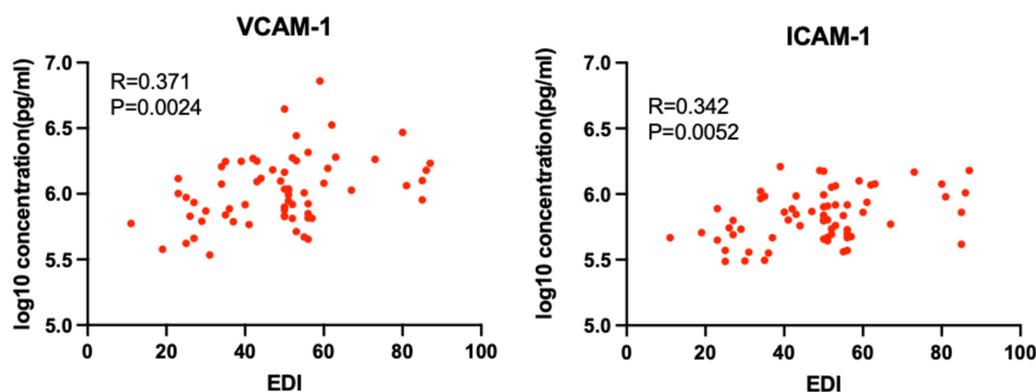

Figure 4. The correlation between adhesion molecules VCAM-1 and ICAM-1 and EDI. The correlation between VCAM-1, ICAM-1 and days post HIV-1 infection (EDI) was measured using Spearman's rank-order correlation. VCAM-1 and ICAM-1 were both positively correlated with EDI and P values < 0.05 were considered significant.

D infections, it therefore implies that subtype D could be associated with higher inflammation leading to recruitment of immune cells including CD4+ T cells that provide a target pool for HIV-1 to replicate. This may further provide an explanation to the already observed fast disease progression observed in those infected with subtype D in Uganda.

In our study cytokines IL-12/23p40 and IL-1α were associated with an increased risk of CD4+ T cell count drop while bFGF was associated with maintenance of the same. Interestingly, IL-12/23p40 and IL-1α were more elevated among those infected with subtype D and IL-12/23p40 was negatively correlated with $CD4^+$T cell counts. Similar to our study, IL-12p40 was shown to be a predictor of disease progression in chronically infected, ART-naïve HIV-1 subtype C individuals (Iketleng et al., 2016). Additionally, Roberts et al.,2010 reported that IL-7, IL-α and CCL11 (Eotaxin-1) were associated with more rapid loss of $CD4^+$T cells among acute HIV-1 subtype C infected women (Roberts et al., 2010). This potentially emphasizes the importance of inflammation in HIV-1 disease progression, as these two cytokines (IL-12/23p40 and IL-1α) are pro-inflammatory cytokines and recruit other immune cells, including $CD4^+$T cells, to the areas of infection. Moreover, IL-12p40 induces production of IL-16, a cytokine that promotes lymphocyte migration and induces expression of other pro-inflammatory molecules.

In our study we also found that adhesion molecules VCAM-1 and ICAM-1 were significantly higher among individuals infected with HIV-1 recombinants compared to those with subtype D. In a correlation analysis the adhesion molecules increased with time within the first 90 days of initial HIV-1 infection. These adhesion molecules can be shed from cell surfaces in soluble forms, which indicate the level of cellular activation (Blankenberg et al., 2003). ICAM-1 and VCAM-1 concentrations have previously been found to be elevated in HIV infection (Constans and Conri, 2006; Fisher et al., 2006; GRAHAM et al., 2013; Wu and Wu, 2006). Because VCAM-1 and ICAM-1 might disrupt adhesion events that lead to immunological functions, it's possible that the high levels of these circulating adhesion molecules found in the plasma of HIV-1 recombinants in our study will further disrupt these patients' immune systems. Furthermore, because VCAM-1 has been found to be prognostic, the significantly higher levels of VCAM-1 among the HIV-1 recombinants in our study show that these adhesion molecules may be important in driving disease progression. Elevations of these vascular cell adhesion molecules VCAM-1 and IACM-1 among the HIV-1 recombinants in the present study and their increase with time (as indicated by the positive correlation with days post infection-EDI) may indicate that these adhesion molecules may also play a role in pathogenesis of HIV-1 among A/D recombinants.

The main limitation of this study was that cytokine concentrations were not measured in HIV uninfected individuals to enable comparison of the cytokine concentrations prior to sero-conversion. Instead, the comparisons were based on cytokine concentrations in individuals with divergent viral characteristics including subtypes, $CD4^+$T cell counts and viral load. The small sub-genomic fragments analyzed here also impeded us in generalizing for biological differences in cytokine levels between different subtypes and inter-subtype recombinants.

In conclusion, our results show that acute infection with HIV-1 subtype D is associated with higher cytokine concentrations than infection with subtype A. This suggests that infection with subtype D may disrupt the immune environment during early infection and dysregulate cytokine production and that subtype-specific differences in cytokines production occur during acute HIV-1 infection. Moreover, IL12/23p40 may be an additional marker of disease progress in our population. Additionally, VCAM-1 and ICAM-1 could also play a role in disease progression. More studies are required to further understand the role of these molecules in disease progression in our population of subtypes A and D.

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Conflicts of Interest

The authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijregi.2022.03.007.

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