Plasma Cytokine Levels in Chronic Asymptomatic HIV-1 Subtype C Infection as an Indicator of Disease Progression in Botswana: A Retrospective Case Control Study

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Abstract

HIV infects cells of the immune system causing immune activation and proliferation of immune cells, leading to alteration of production and activity of a number of cytokines. These changes in cytokine levels can affect the immune function, and have the potential to directly impact the course of HIV disease. We characterized plasma cytokine concentration profiles in HIV-1 subtype C chronically infected, antiretroviral therapy (ART)-naïve participants to establish their influence on disease progression and viremia. Plasma levels of interleukin (IL)-1 α , IL-7, IL-12p40, granulocyte macrophage-colony-stimulating factor (GM-CSF), and interferon (IFN)- γ were quantified in samples from 60 treatment-naïve participants in the placebo arm of the completed Micronutrient-HIV disease progressions study, “Dikotlana” (2004–2009) in Gaborone, Botswana. Participants were stratified into progressors (P) and nonprogressors (NP) based on their rates of CD4⁺ T cell depletion during the study period. Nonprogressors were those who had <1% CD4⁺ T cell depletion at 24 months postenrollment. Progressors were defined as those with CD4⁺ T cell depletion of >15% at 24 months postenrollment. Cytokine levels were compared between P and NP using the Mann–Whitney *U*-test. Logistic regression analysis was used to determine if cytokines predicted disease progression. Correlations of cytokines with CD4⁺ T cell counts and viral loads were determined by the Spearman rank test. Median baseline CD4⁺ T cell counts were 453 (Q1, Q3; 401, 592) and 479 (Q1, Q3; 401–592) for nonprogressors and progressors, respectively. Nonprogressors had a higher viral set point than progressors. IL-12p40 levels were significantly higher in the P than in NP at enrollment and 24 months (*p* < 0.05). Levels of IL-1 α , IL-7, IFN- γ , and GM-CSF did not differ significantly between the two groups. Except for IL-12p40, which displayed an inverse correlation with CD4⁺ T cell counts and a direct correlation with viral load, all other cytokines showed no correlations. IL-12p40 was found to be the most significant predictor of progression and its production was most likely driven by HIV replication products as evidenced by its direct correlation with viral load. In chronic HIV-1 subtype C infection, CD4⁺ T cell counts and plasma cytokine levels may not necessarily evolve in parallel, suggesting the involvement of other factors in determining the rates of CD4⁺ T cell depletion.

Introduction

THE IMMUNE SYSTEM is regulated by a complex network of cytokines, which are the soluble secreted proteins that serve as messenger molecules between cells during immune responses to antigens.¹ Cytokines play a vital role in con-

trolling the homeostasis of the immune system. Cytokines can up-regulate or accelerate antiviral immune responses and thus contribute to viral control.² HIV infects cells of the immune system causing immune activation and proliferation of immune cells, leading to the alteration of the production and activity of a number of different cytokines.³

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These changes in cytokine levels can affect the immune function, and have the potential to directly impact the course of HIV disease.⁴ Investigations into the interaction between HIV and cytokines have revealed that cytokines produced by a variety of cells can regulate HIV-1 replication.⁵ Proinflammatory cytokines such as interleukins (IL) 1 and 6 and tumor necrosis factor- α (TNF- α) alone or acting synergistically have the capacity to act on HIV-1-infected cells to up-regulate HIV-1 replication and production.^{1,5} Cytokines such as interferons (IFN) α and β are HIV-1 suppressive; they inhibit HIV-1 replication in infected cells.^{5,6} Bifunctional cytokines such as IFN- γ , transforming growth factor- β (TGF- β), IL-10, IL-13, and IL-4 have both inhibitory and stimulatory effects on HIV-1 replication depending on experimental conditions.⁶ However, these interactions are complicated further by the fact that most cytokines exert pleiotropic and sometimes contrasting effects on the immune response and viral replication.

Proinflammatory cytokines such as TNF- α that increase antiviral immunity can also induce nuclear factor-kappa beta (NF- κ B), which enhances proviral transcription and thus drives HIV replication.⁷ HIV therefore affects the balance between HIV inductive and inhibitory cytokines, between proinflammatory and antiinflammatory cytokines, and between T helper cell type 1 (Th1) and T helper cell type 2 (Th2) cytokines.⁸ The imbalances between these different cytokines have profound effects in the host's ability to control viral replication. The interaction between HIV-1 and cytokines is further complicated by the fact that cytokines often influence the synthesis and actions of other cytokines.

The balance between HIV-inducing and HIV-suppressive cytokines and the interaction with the host during infection is more important than any single cytokine in determining the course of the disease.^{4,9} During the chronic asymptomatic phase of HIV-1 infection, the net level of viral replication is regulated by the balance between factors that induce viral replication and factors that suppress it.¹ Because of the differences in immune responses between individuals, there may be differences in the balance of cytokines or host cytokine environment that could in part explain the differences in the ability of the host to control viral replication, hence affecting HIV disease progression.^{4,9}

Cytokines as immune modulators may contribute to the degree of CD4⁺ T cell depletion with HIV infection.^{1,4,10,11} They can increase the target cell pool for HIV by recruiting and activating CD4⁺ T cells, the primary targets for HIV infection.⁷ Plasma concentrations of IL-1 α , IL-7, IL-12p40, and granulocyte macrophage-colony-stimulating factor (GM-CSF) are predictive of CD4⁺ T cells loss whereas high IFN- γ is associated with low viral loads during acute infection.¹² We compared plasma levels of the five cytokines between two groups with distinctly diverse rates of CD4⁺ T cell depletion and evaluated associations of plasma cytokine concentrations with plasma HIV viral load and CD4⁺ T cell counts in antiretroviral (ARV)-naive patients during chronic HIV-1 subtype C infection.

Patients and Methods

Study population

A retrospective case-control study was conducted. Study participants were selected from the placebo arm of the Mi-

cronutrient study ("Dikotlana") at Botswana Harvard AIDS Institute Partnership. The Micronutrient study was a prospective randomized multifactorial double-blind placebo-controlled clinical trial to investigate the efficacy of micronutrient therapy in slowing HIV disease progression.¹³ The "Dikotlana" study had four micronutrient randomization arms, of which one was placebo. The first 30 participants who had $\geq 15\%$ CD4⁺ T cell depletion were selected for inclusion as P while the first 30 participants who had less than 1% CD4⁺ T cell depletion were selected as NP, from the placebo arm systematically ordered by CD4 depletion. In total, 60 participants from the placebo arm of the Micronutrient study were selected.

A subset of sequences generated from this cohort confirms the predominance of subtype C.¹⁴ Previous studies have also shown that the circulating subtype in Botswana is almost exclusively subtype C (>98%).^{15,16} The Human Subjects Committee at the Harvard School of Public Health and the Health Research Unit in the Ministry of Health, Botswana, approved the Micronutrient study, in which the participants were enrolled. Approval was obtained from the Kilimanjaro Christian Medical University College ethics board for this particular study utilizing stored serum specimens.

CD4⁺ T cell counts and HIV RNA quantification

CD4⁺ T cells counts were measured using the BD FACScalibur platform (BD Biosciences, San Jose, CA) at the Botswana Harvard HIV Reference Laboratory (BHHRL). Plasma HIV RNA levels were measured using COBAS AmpliPrep/COBAS AMPLICOR HIV-1 MONITOR Test, version 1.5 (Roche Molecular Systems, Branchburg, NJ). The standard assay format (PHM) with a detection range of 400–750,000 copies of HIV-1 RNA per ml was used.

BHHRL is an ISO 17025 accredited laboratory enrolled in a bimonthly external quality assurance program for CD4 and HIV RNA viral load through the United Kingdom National External Quality Assessment Service (UKNEQAS) and Rush University Virologic Quality Assurance (VQA) Program, respectively.

Quantification of cytokines

Human GM-CSF, IFN- γ , IL-1 α , and IL-12/IL-23 (p40) were quantified from stored plasma collected at enrollment, 12 months, and 24 months using the LEGEND MAX ELISA kit with precoated plates (BioLegend, San Diego, CA) according to the manufacturer's instructions. The minimum detectable concentrations of IL-1 α , IL-12p40, GM-CSF, and IFN- γ as per the manufacturer were 0.8 pg/ml, 9.5 pg/ml, 3.5 pg/ml, and 5.6 pg/ml, respectively. Human IL-7 was quantified at enrollment from plasma using the Human IL-7 Instant ELISA (eBioscience, San Diego, CA) according to the manufacturer's instructions. The kit has a lowest detection limit of 9.5 pg/ml.

Statistical analysis

The nonparametric Mann-Whitney *U*-test was used to compare the median cytokine levels of the two groups by time point and the χ^2 -test was used for categorical variables. The Spearman rank test was used for correlations between plasma cytokine levels and CD4⁺ T cell counts and between

TABLE 1. PARTICIPANT'S BASELINE CHARACTERISTICS

Variable	Nonprogressors (N = 30)	Progressors (N = 30)	p-value
Age years, median (IQR)	37.5 (33–45)	37 (32–43)	0.563 ^a
Sex, male/female	6/24	4/26	0.317 ^b
Prevalence of STIs and opportunistic infections	10	10	1.00 ^b
Viral load, median baseline log ₁₀ copies/ml (IQR)	3.96 (2.97–4.26)	3.95 (3.59–4.99)	0.148 ^a
CD4 ⁺ T cells, median baseline cells/ μ l (IQR)	453 (399–592)	479 (396–597)	0.734 ^a

^aFor quantitative measures, median (interquartile range). Statistical analyses performed by Wilcoxon rank-sum (Mann–Whitney) test.

^bFor categorical parameters, statistical analysis performed by χ^2 -test.

plasma cytokine levels and HIV-1 RNA levels. Logistic regression was used to further determine whether cytokine levels were predictors of progression adjusted for possible confounding effects of other factors such as age, time point, gender, and the presence of opportunistic infections. *p* values <0.05 were considered significant. All analysis was performed using STATA version 12.0 statistical software (STATA, College Station, TX).

Results

Baseline characteristics of the participants

Sixty participants were included in the study and were divided into two arms, each composed of 30 participants for P and NP, respectively. P and NP presented similar baseline CD4⁺ T cell counts (*p*=0.734; Table 1). There were no differences between the two groups in median age and baseline viral load (*p*=0.563 and *p*=0.148, respectively; Table 1). The two groups were also comparable in prevalence of sexually transmitted infections (STIs) and opportunistic infections and male-to-female ratio (*p*>0.05; Table 1).

Evolution of viral loads and CD4⁺ T cell counts

Over time, viral loads and CD4⁺ T cell counts evolved differently between the two groups. Both groups seem to have had a bleep in CD4⁺ T cell counts at the 3 month time point. Overall, progressors experienced CD4⁺ T cell count

depletion over time whereas nonprogressors maintained a CD4⁺ T cell count that was more or less the same throughout the observation period. The median CD4⁺ T cell counts were significantly different at most time points except for baseline and 6 months (Table 2). The two groups had similar viral loads at baseline, but over time, progressors experienced a significant increase in viral loads compared to non-progressors, who maintained almost stable viral loads over time as shown in Table 2.

Comparison of plasma cytokine levels between P and NP

IL-12p40 levels were significantly higher in the P than in NP at enrollment and 24 months (*p*<0.05), but comparable at 12 months (*p*=0.1276) (Fig. 1). Levels of IL-1 α , IFN- γ , and GM-CSF did not differ significantly between the two groups over time. There was a trend toward increased levels of IL-1 α levels in P compared to NP through baseline, 12 months, and 24 months; however, the difference between the two groups was not statistically significant. IL-7 levels were also comparable between the two groups at the one time point that was assayed.

Association between cytokine levels and CD4⁺ T cell counts

IFN- γ exhibited a significant inverse correlation with CD4⁺ T cell counts at 24 months (*r*=−0.2770; *p*=0.0491) (Fig. 2A). IL-1 α displayed a significant direct correlation

TABLE 2. COMPARISON OF CD4⁺ T CELL COUNTS AND VIRAL LOADS BETWEEN THE PROGRESSORS AND NONPROGRESSORS OVER TIME

Variable	Time point	Nonprogressors	Progressors	p-value
CD4 ⁺ T cell counts, cells/ μ l median (IQR)	Baseline	453 (399–592)	479 (396–597)	0.734
	3 months	996 (794–1172)	777 (603–952)	0.0196*
	6 months	502 (427–618)	444 (351–522)	0.062
	9 months	540 (446–728)	405 (306–505)	0.0015*
	12 months	547 (439–676)	426 (300–490)	0.0053*
	15 months	552 (408–646)	365 (311–514)	0.0077*
	18 months	509 (407–634)	390 (299–519)	0.0236*
	21 months	597 (432–704)	341 (293–410)	0.0001*
	24 months	545 (482–678)	332 (269–409)	<0.0001*
HIV-1 viral load, log ₁₀ copies/ml median (IQR)	Baseline	3.96 (2.97–4.26)	3.95 (3.59–4.99)	0.148
	6 months	3.63 (3.00–4.15)	4.16 (3.77–4.70)	0.012*
	12 months	3.93 (3.44–4.28)	4.39 (3.79–4.88)	0.041*
	18 months	3.72 (3.25–4.25)	4.49 (3.84–5.05)	0.003*
	24 months	3.69 (3.09–4.43)	4.70 (3.72–5.35)	0.003*

Statistical analyses performed by Wilcoxon rank-sum (Mann–Whitney) test. IQR, interquartile ranges.

*Statistically significant.

FIG. 1. Circulating IL-12p40 levels of progressors (P) and nonprogressors (NP). The values are shown using box-and-whisker plots representing the minimum, 25th percentile, median, 75th percentile, maximum, and outlying values. Differences between the progressors ($n=30$) and non-progressors ($n=30$) at baseline, 12 months, and 24 months were examined using the Mann-Whitney rank-sum test. A p -value <0.05 is significant.

with $CD4^+$ T cell counts at 12 months ($r=0.3082$; $p=0.0233$) (Fig. 2B). The two cytokines did not show significant correlations at other time points and overall. GM-CSF and IL-7 showed no significant correlations with $CD4^+$ T cell counts, although IL-7 was measured only at baseline. IL-12p40 displayed a significant inverse correlation with $CD4^+$ T cell counts at baseline ($r=-0.3710$; $p<0.0001$), 12 months ($r=-0.2635$; $p=0.0441$), and 24 months ($r=-0.3980$; $p=0.0032$). Combining all the time points, IL-12p40 still displayed a significant inverse correlation with $CD4^+$ T cell counts ($r=-0.4513$; $p=0.0009$) (Fig. 2C).

Association between cytokine levels and viral load

IFN- γ had a significant direct correlation with HIV-1 viral load at 24 months ($r=0.3835$; $p=0.0055$) (Fig. 3A). IL-1 α ,

GM-CSF, and IL-7 showed no significant correlations with HIV-1 viral load. There were significant direct correlations for IL-12p40 with HIV-1 viral load at baseline ($r=0.4532$; $p=0.0005$), 12 months ($r=0.5056$; $p=0.0001$), and 24 months ($r=0.5050$; $p=0.0002$) (Fig. 3B). Overall, at all the time points together, IL-12p40 had an even stronger direct correlation with HIV-1 viral load ($r=0.4926$; $p<0.0001$) (Fig. 3B).

Determining which cytokines are predictors of progression

Adjusting for possible confounding by age, gender, on-going infections, and taking into account the contribution of each cytokine, IL-7, GM-CSF, and IFN- γ were not significant predictors of HIV-1 disease progression. IL-1 α and IL-12p40 were significant predictors of disease progression [adjusted odds ratio (aOR)=3.77; 95% confidence interval (95% CI) 0.66–21.49; aOR=3.21 (95% CI 1.39–7.45), respectively] (Table 3).

Discussion

Circulating levels of IL-1 α , IL-7, IL-12p40, GM-CSF, and IFN- γ in plasma were examined and compared over time in HIV-1 subtype C-infected treatment-naïve participants with different rates of $CD4^+$ T cell depletion. Relationships between cytokines and $CD4^+$ T cell counts, then between cytokines and HIV-1 viral load, were determined using correlations. IL-12p40 levels were significantly higher in the P than in NP at enrollment and 24 months and were significantly associated with HIV-1 viral load. IL-1 α and IL-12p40 were significant predictors of disease progression, with IL-12p40 being the strongest predictor. IL-1 α , GM-CSF, and IFN- γ levels were similar between the two groups over time. IL-7 levels were also comparable between the two groups at the one time point examined (baseline). IL-1 α , IL-7, GM-CSF, and IFN- γ did not show significant correlations overall with $CD4^+$ T cell counts and HIV-1 viral loads.

IL-12p40 levels differed between P and NP over time; P had significantly higher IL-12p40 levels than NP. IL-12p40 levels displayed an inverse correlation with $CD4^+$ T cell

FIG. 2. Association of plasma interferon (IFN)- γ (A), interleukin (IL)-1 α (B), and IL-12p40 (C) levels at baseline, 12 months, 24 months, and overall with $CD4^+$ T cell counts. The Spearman rank test was used for correlation (r : Spearman's correlation coefficient) with p -value for correlations.

FIG. 3. Association of plasma IFN- γ (A) and IL-12p40 (B) levels at baseline, 12 months, 24 months, and overall with viral load. The spear rank test was used for correlation (r : Spearman's correlation coefficient) with p -value for correlations.

counts and an even stronger direct correlation with viral load. Changes in HIV RNA levels during untreated HIV infection have been shown to have implications for CD4⁺ T cell count depletion.¹⁷ In particular, higher HIV RNA increments from baseline to the current time were associated with greater subsequent rates of CD4⁺ T cell count decline.¹⁷ The stronger direct correlation with viral load as compared to the inverse correlation with CD4⁺ T lymphocyte cell counts probably indicates that IL-12p40 levels have more to do with the immune responses to HIV-1 replication products than to declining CD4⁺ T cell counts.

These results differ from the findings among acute infections that showed that elevated levels of IL-12p40 were associated with the ability to maintain CD4⁺ T cell counts above 350 cells/ μ l.¹² In our study, CD4⁺ T cell counts decreased as IL-12p40 increased. The differences in the results are most likely due to the different HIV-1 infection stages examined in the two studies. In acute infection, elevated levels of IL-12p40 may be associated with elevated levels of IL-12p70, which is important in the induction of cell-mediated immune responses

and control of viremia; however, in later stages of the disease, as in this study, elevated levels of IL-12p40 may largely be caused by the overproduction of this subunit resulting in its existence as a homodimer.

The role of IL-12p40 in HIV-1 infection has not been widely researched; however, in other diseases such as cutaneous *Leishmania*, in which cell-mediated immunity is critical in the severity of the disease as in HIV, transgenic mice overexpressing p40 showed enhanced disease susceptibility.¹⁸ IL-12p40 is known as a component of the bioactive IL-12 and IL-23 without widely recognized functional activity.¹⁹ However, recent publications have supported an independent role for IL-12p40.¹⁹ The IL-12p40 homodimer, IL-12p80, has been established as a chemoattractant for macrophages and an inducer of DC migration.¹⁹ The cytokine also mediates inflammatory responses in the lung and high levels of IL-12p40 have been associated with damaging inflammatory responses within the lung.¹⁹ The IL-12p40 homodimer has been shown to induce the production of IL-16; a cytokine that promotes lymphocyte migration induces expression of proinflammatory molecules and modulates apoptosis.

Recent studies imply that IL-12p40 possibly exerts its effect by enhancing or contributing to the inflammatory conditions during HIV-1C infection. The IL-12p40 homodimer may also interfere with the induction of cell-mediated immunity by competitive binding to the IL-12 receptor, thereby inhibiting effective control of the viremia.^{20,21}

In the logistic regression model, IL-12p40 and IL-1 α were the two significant predictors of P, IL-12p40 being the strongest predictor. This perhaps underscores the importance of inflammation in HIV-1C disease progression, as these two are proinflammatory cytokines. They both recruit other immune cells including CD4⁺ T cells to areas of infection.

IL-12p40 was found to be most significant marker of progression and its production may likely is driven by HIV replication as evidenced by its direct correlation with viral load. IL-12p40 was significantly higher in P compared to NP

TABLE 3. THE INFLUENCE OF CYTOKINES ON HIV DISEASE PROGRESSION ADJUSTED FOR POSSIBLE CONFOUNDING BY PRESENCE OF OPPORTUNISTIC AND SEXUALLY TRANSMITTED INFECTIONS, AGE, GENDER, AND VIRAL LOAD

Cytokine (log ₁₀)	aOR (95% CI)	p-value
IL-1 α	3.77 (0.66–21.49)	0.036
IL-7	0.94 (0.15–5.96)	0.949
IL-12p40	3.21 (1.39–7.45)	0.007
IFN- γ	1.70 (0.51–5.63)	0.386
GM-CSF	0.77 (0.08–7.31)	0.819

Analysis done by logistic regression: adjusted odds ratios (aOR) and 95% confidence intervals (CI) shown.

IL, interleukin; IFN, interferon; GM-CSF, granulocyte macrophage-colony-stimulating factor.

at baseline when their viral loads and CD4⁺ T cell counts were still comparable. IL-12p40 could therefore be a promising marker for impending CD4⁺ T cell decline or increase in viral replication that could be used as an additional marker. In chronic HIV with a moderate degree of immunodeficiency as in our study, CD4⁺ T cell counts and plasma cytokine levels may not necessarily evolve in parallel as evidenced by the lack of correlation between CD4⁺ T cell counts and almost all the cytokines examined, suggesting the involvement of other factors in determining the rates of CD4⁺ T cell depletion. However, more studies are required to further understand the role of cytokines in CD4⁺ T cell depletion and how immune modulation by cytokines could be taken advantage of to aid immune recovery in HIV-1 subtype C infection.

Because plasma cytokines were measured at 12 month intervals the results may not be a true reflection of the cytokine dynamics at play in these participants. Many changes may have occurred in between that we were unable to capture simply because our intervals were too far apart. The estimated time of infection for the participants was unknown, therefore the stratification of participants into P and NP may be a reflection of the stage of infection at enrollment rather than the rate of disease progression. Viral load measurements were done at 6 month intervals; the absence of viral load measurements at the 3 month time point did not permit us to explain the presence of a bleep in the CD4⁺ T cell count at 3 months from enrollment but generally a declining trend thereafter.

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Author Disclosure Statement

No competing financial interests exist.

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