Am. J. Trop. Med. Hyg., 93(2), 2015, pp. 287–292
doi:10.4269/ajtmh.14-0653

Insights Into Circulating Cytokine Dynamics during Pregnancy in HIV-Infected Beninese Exposed to *Plasmodium falciparum* Malaria

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Abstract. We investigated the circulating plasma levels of Th1- (Interleukin-2 [IL-2], tumor necrosis factor-α [TNF-α], interferon-gamma [IFN-γ]) and Th2-type (IL-4, IL-5, IL-10) cytokines in human immunodeficiency virus (HIV)-infected pregnant women living in a malaria-endemic area. We analyzed samples from 200 pregnant women included in the prevention of pregnancy-associated malaria in HIV-infected women: cotrimoxazole prophylaxis versus mefloquine (PACOME) clinical trial who were followed until delivery. Cytokine concentrations were measured by flow cytometry-based multiplex bead array. Significantly elevated levels of IL-10 and lower levels of TNF-α were observed at delivery compared with inclusion ($P = 0.005$). At inclusion, the presence of circulating IFN-γ, a higher $CD4^+$ T cell count and having initiated intermittent preventive treatment of malaria with sulfadoxine pyrimethamine (SP-IPTp) were all associated with a lower likelihood of *Plasmodium falciparum* infection. At delivery, the inverse relationship between the presence of infection and circulating IFN-γ persisted, although there was a positive association between the likelihood of infection and the presence of circulating TNF-α. Initiation of antiretroviral therapy was associated with elevated IL-5 production. Consistent with our own and others' observations in HIV seronegative subjects, this study shows circulating IL-10 to be a marker of infection with *P. falciparum* during pregnancy even in HIV-infected women, although plasma IFN-γ may be a marker of anti-malarial protection in such women.

INTRODUCTION

Pregnancy is a particular period during which the body undergoes physiological adjustments, one of them being the modulation of adaptive, pro-inflammatory immune responses to ensure fetal survival. These adjustments may decrease maternal immune defenses and hence promote infections.[1]

Both malaria and human immunodeficiency virus (HIV) are among the infectious diseases that can occur during pregnancy, with deleterious outcomes that may affect both the mother and the fetus. The two infections have bidirectional interactions, each potentially favoring the development of the other, with a consequent increased mortality risk in particular in sub-Saharan Africa.[2]

Studies have shown that by weakening host immune defenses, HIV promotes the occurrence of co-infections including malaria in regions where this is endemic.[3] Moreover, HIV-infected pregnant women have been reported to be at greater risk of placental malaria infection.[4–6] Thus, HIV infection could impair immunity to malaria by altering the cytokine profile.[7–10] The dual issues of immune modulation related to pregnancy and immunosuppression induced by HIV infection make pregnancy in HIV-infected individuals a particularly high-risk period.

Additionally, both pro- and anti-inflammatory cytokines are found at significantly increased levels in the peripheral blood and in the intervillous spaces of placentas of malaria-infected women. Production of these cytokines is responsible for the resulting Th1:Th2 imbalance observed in *Plasmodium falciparum*-infected placentas.[1,11–13] Although aspects of the cytokine imbalance have been widely explored in pregnant women without HIV, very little data exist concerning the cytokine balance during pregnancy in HIV-infected women.

It has also been shown that placental malaria is a risk factor associated with in utero mother-to-child transmission of HIV-1 through alteration of the cytokine environment.[9,14] In highly immunocompromised HIV-infected pregnant women, prophylactic coverage by administering cotrimoxazole (CTX) is advised to reduce the occurrence of clinical malaria.[15] The CTX reportedly exerts non-specific anti-inflammatory and immunomodulatory properties during rheumatoid arthritis infection.[16] However, the effect of CTX prophylaxis or antiretroviral therapy (ART) drugs on the cytokine balance in pregnant women co-infected with HIV and malaria has not been described.

To understand the immunological mechanisms occurring in pregnant women living with HIV in the context of endemic malaria, we investigated the levels of Th1 and Th2 cytokines in peripheral blood plasma of pregnant women enrolled in a prospective cohort study conducted in Benin. The objectives of this study were to describe the cytokine profiles in HIV-infected pregnant women in relation to gestational age, ART use, and CD4 T cell count, and to study the association of cytokine profiles with the risk of *P. falciparum* infection both at inclusion and delivery.

MATERIALS AND METHODS

Study population. The study population was a sub-sample of HIV-infected women enrolled in two clinical trials as part of the "PACOME" study recently conducted in Benin. The study sites consisted of five urban hospitals in Cotonou and Porto-Novo implementing the program for prevention of mother-to-child transmission of HIV. In the PACOME study, 432 HIV-infected pregnant women 18 years of age and over and permanently living in the study area were enrolled between 16 and 28 weeks of gestation, after giving written informed consent. Exclusion criteria included age < 18 years, history of a neuropsychiatric disorder, severe kidney or liver disease, serious adverse reaction with mefloquine (MQ), sulfa-drugs or quinine. The PACOME study design has been

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extensively described elsewhere.[15] A convenience sample of 200 women, for which biological and clinical data were collected from their inclusion in the study until delivery, was selected out of the larger cohort of the PACOME study for cytokine measurements.

Laboratory investigations. At inclusion, peripheral blood was collected, and plasma samples were separated and frozen at –80°C. Hemoglobin levels were determined by complete blood count (CBC) or Hemocue® colorimeter. The absolute $CD4^+$ T cell count was assessed by using a Cyflow® or Facscount® cytometer, and the HIV viral load was measured on a real-time polymerase chain reaction (PCR) thermocycler (M2000 RP Abbott®, Abbott Laboratories, Abbott Park, IL).

For malaria diagnosis, thick and thin blood smears were stained with Giemsa and read by two independent microscopists.

A highly sensitive real-time duplex PCR assay was performed on filter paper and DNA extracts collected at inclusion and delivery (in peripheral and placental blood) to capture sub-microscopic *P. falciparum* infections.[17,18] Samples were analyzed on a ViiA™ 7 Real-Time PCR system (Life Technology, Saint Aubin, France) and quantified using a DNA standard range made from a suspension of in vitro cultured 3D7 *P. falciparum* line as described previously.[19] *Plasmodium falciparum* infection at inclusion was defined by a positive PCR or microscopy, although *P. falciparum* infection at delivery was defined by a positive PCR or blood smear in either peripheral or placental blood.

Plasma cytokine measurement. In the sub-sample of 200 pregnant women, a commercially available Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit from Becton Dickinson (BD Biosciences, San Jose, CA) was used to measure plasma levels of interleukin-2 (IL-2), IL-4, IL-5, IL-10, tumor necrosis factor-α (TNF-α), and interferon-gamma (IFN-γ), according to the manufacturer's instructions. Calibration and sample acquisition were performed using a flow cytometer (FACSCalibur, Becton Dickinson, Le Pont de Claix Cedex, France) and analyzed with FCAP array software (Soft Flow, Inc., New Brighton, MN). The detection limits of cytokines were as follows: IL-2, 2.61 pg/mL; IL-4, 2.61 pg/mL; IL-5, 2.41 pg/mL; IL-10, 2.81 pg/mL; TNF-α, 2.81 pg/mL; and 7.1 pg/mL for IFN-γ.

Statistical analysis. Data analysis was performed using STATA/MP 12.0 (StataCorp, College Station, TX) and Prism 5.0 (Graph pad Inc., San Diego, CA). Data were expressed as medians with interquartile ranges (IQR). Groups were compared with non-parametric Wilcoxon-matched paired and Kruskal-Wallis tests. Categorical variables were compared with chi2 (χ^2) tests. Multiple logistic regression was performed to assess whether cytokine levels were independently associated with the likelihood of *P. falciparum* infection, transversally at inclusion and delivery. A stepwise procedure was performed to select a model that included cytokines associated with infection, after adjusting for significant covariates. Age, rank of gestation, CD4 cell count, previous IPTp intake, and ART use were introduced in the first models. Cytokine levels were dichotomized into undetectable versus detectable levels. Covariates with $P < 0.10$ were kept in final models. The undetectable cytokine levels (below the detection limit values) were arbitrarily attributed a concentration of 1 pg/mL.

Ethics. The protocol received ethical clearance, both by a French (CCDE/IRD) and a Beninese (Ministry of Health) institutional review board.

RESULTS

Characteristics of the population. At inclusion, the mean gestational age of women was 21.3 weeks and 11% of these were in their first pregnancy. The mean $CD4^+$ T cell count was 374/mm^3, 42% of women were already receiving ART and viral DNA was undetectable in 34%. Regarding malaria, all women were asymptomatic, but 87 women (44%) had *P. falciparum* infection detected by PCR at inclusion, whereas 113 were uninfected (Table 1). Fifteen, i.e., 17% of the infections were also detected by microscopy (median parasite density 564 parasites per microliter, IQR [171–3612] as determined by quantitative real-time PCR), whereas 72 had submicroscopic parasitemia (median 25 parasites per microliter, IQR [17–42]). Half of the women were already receiving CTX prophylaxis for the prevention of opportunistic infections associated with HIV, and 15% had received a dose of the anti-malarial combination sulfadoxine-pyrimethamine (SP) more than 1 month before inclusion. Women with $CD4^+$ T cell count below 350/mm^3 were more likely to be infected with *P. falciparum* at inclusion, whereas having received CTX prophylaxis or SP intermittent treatment before inclusion was associated with a reduced likelihood of having *P. falciparum* infection. No significant difference was observed between *P. falciparum*-infected and uninfected pregnant women with regards to ART and HIV viral load. At delivery, 17 women (8.5%) were infected with *P. falciparum* (5 microscopically and 12 sub-microscopically), whereas 183 were uninfected.

Th1/Th2 cytokines changes during pregnancy regardless of *P. falciparum* infection. To determine Th1 and Th2 cytokine profiles in HIV-infected pregnant women, we determined the plasma levels of IL-2, TNF-α, IFN-γ, and IL-4, IL-5, IL-10 at inclusion and at delivery. We observed a significantly increased concentration of IL-10 at delivery compared with inclusion (Wilcoxon paired test, $P = 0.004$; Figure 1). A similar but non-significant trend was observed for IL-5 ($P = 0.07$). Conversely, a significant decrease of TNF-α was observed

TABLE 1

Characteristics of study patients comparing *P. falciparum*-uninfected and infected pregnant women at inclusion

Variables	Uninfected ($N = 113$)	Infected ($N = 87$)	P*
Age in year (SD)	30.0 (7.0)	28.0 (7.0)	0.31
Gestational age in weeks (SD)	22.0 (7.0)	21.0 (6.0)	0.29
Primigravidae (%)	10.6	10.3	0.95
Anemia at INC (%)	22.4	34.1	0.07
CD4 cell count < 350 at INC (%)	45.1	58.6	0.06
ART started before pregnancy (%)	40.7	42.5	0.79
CTX started before inclusion	59.3	41.2	0.01
IPT/SP taken before inclusion (%)	18.7	9.20	0.06
Viral load, copies/mL	61091 (752)	25559 (337)	0.49
Infection detected by PCR	–	72 (83%)	–
Infection detected by microscopy	–	15 (17%)	–
Parasite density, parasites/μL	–	989 (28)	–

ART = antiretroviral therapy; CTX = cotrimoxazole; IPT = intermittent preventive treatment; INC = inclusion; SP = sulfadoxine pyrimethamine; PCR = polymerase chain reaction.

Values are means (standard deviation) except for viral loads and parasite density that are mean (median).

*Mann Whitney U or χ^2 test for proportions.

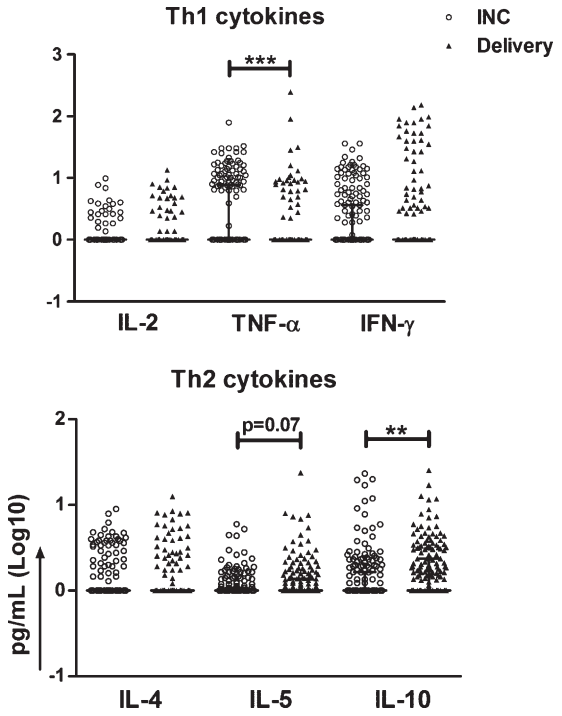

FIGURE 1. Human immunodeficiency virus (HIV) infection-related changes in plasma cytokines levels between inclusion and delivery in 200 pregnant women included in our study. Scatter plots include bars depicting medians of Th1 (IL-2, TNF-α, IFN-γ) and Th2 (IL-4, IL-5, IL-10) cytokines measured in plasma samples. Cytokine levels are represented in log10 on the left (*y*) axis. Numbers of women with detectable levels of cytokines are as follows, at Inclusion: IL-2 (n = 23), IL-4 (n = 47), IL-5 (n = 54), IL-10 (n = 62), TNF-α (n = 63), IFN-γ (n = 65, and at delivery: IL-2 (n = 25), IL-4 (n = 41), IL-5 (n = 68), IL-10 (n = 95), TNF-α (n = 30), IFN-γ (n = 44). The statistical significance of differences between inclusion and delivery were determined using the Wilcoxon paired test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

between inclusion and delivery ($P < 0.001$). No significant differences were found in IL-2, IL-4 and IFN-γ levels (Figure 1).

No significant differences were observed in the cytokine levels of primigravidae versus multigravidae either at inclusion or at delivery.

Cytokine levels at inclusion, in relation to CD4 T cell count and ART. Activated T cells can produce inflammatory cytokines during infection. We aimed to determine whether cytokine levels in our study were associated with CD4 T cell counts that are a standard marker of progression of HIV infection. For this purpose, we compared cytokine levels measured in pregnant women who had low (< 350/mm^3) CD4 T cell counts (N = 102) with the levels found in the group who had high CD4 T cell counts (N = 98) at inclusion, a period that marked the initiation of ART for more than half the women. At this stage IL-5 and IL-10 plasma concentrations showed a trend toward higher levels in the group of women with lower CD4 T cell counts compared with the group with higher CD4 counts (Kruskal-Wallis test; $P = 0.06$ for both comparisons) (Figure 2). When considering *P. falciparum* infection at inclusion as a confounder, the trend toward a negative association between IL-5 and IL-10 levels and CD4 T cell counts was confirmed only in women who were parasitemic at inclusion (data not shown).

Additionally, among women who initiated ART at inclusion into the study, the IL-5 level increased significantly between inclusion and delivery ($P = 0.015$, Paired Wilcoxon test), though this was not observed in women already on ART. Finally, at delivery, all women were on ART and the IL-5 level was similar in both groups ($P = 0.5$, Figure 3). Taken together, these results suggest that ART may be associated with increased IL-5 production in HIV-infected women.

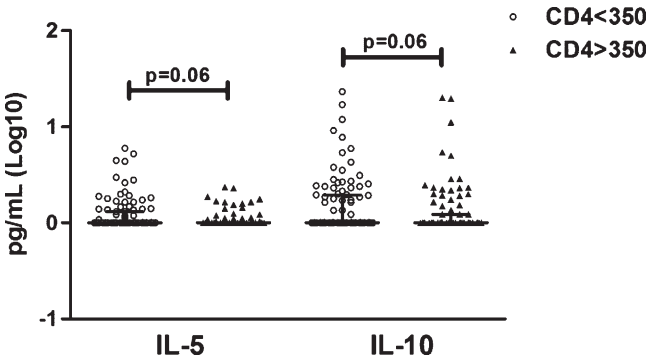

FIGURE 2. IL-5 and IL-10 levels in pregnant women with low and high CD4 T cell counts at inclusion. Scatter plots include bars depicting medians with interquartile ranges of IL-5 and IL-10 measured in 98 pregnant women with CD4$^+$ T cell count > 350/mm^3 compared to 102 women with CD4$^+$ T cell count < 350/mm^3. Cytokine levels are represented in log10 on the left (*y*) axis. The non-parametric Mann Whitney *U* test was used to determine the significance of differences in cytokine levels.

Cytokine levels in HIV-infected pregnant women in relation to *P. falciparum* infection. To assess the impact of *P. falciparum* infection on cytokine profiles, we compared

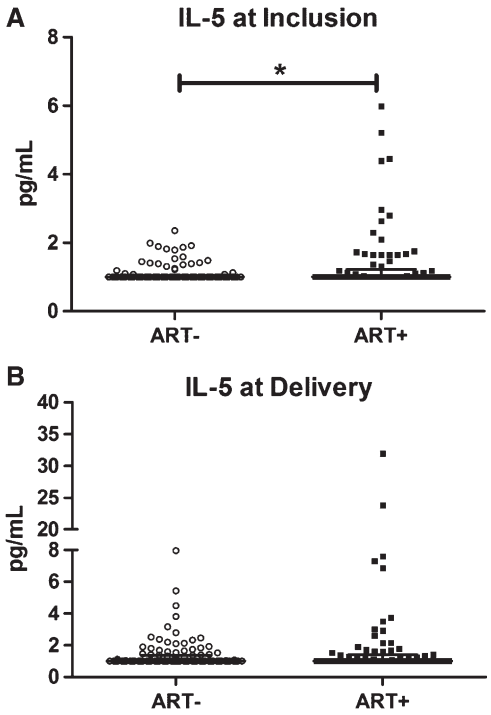

FIGURE 3. IL-5 level at inclusion and delivery in pregnant women with and without ART at Inclusion. Scatter plots include bars depicting medians with interquartile ranges of IL-5 measured at Inclusion (**A**) and at delivery (**B**) in 117 pregnant women without ART compared to 83 under ART at inclusion. IL-5 concentration was presented as the absolute level of cytokine on the left (*y*) axis. The non-parametric Mann Whitney *U* test was used to determine the significance of differences in cytokine levels (* $P < 0.05$).

TABLE 2
Multiple logistic regression analysis for independent associations of cytokines with *P. falciparum* infection at the time of inclusion

Variables inclusion	Odds ratio	95% CI	*P*
Detectable IFN-γ*	0.45	0.21–0.97	0.044
Detectable IL-10*	1.95	0.91–4.21	0.086
CD4 count (> 350)	0.60	0.34–1.08	0.091
IPT/SP taken before INC	0.43	0.17–1.03	0.060

IFN-γ = tumor necrosis factor α; tumor necrosis factor α; IL-10 = interleukin-10; IPT = intermittent preventive treatment; SP = sulfadoxine pyrimethamine.
*Cytokine levels were dichotomized into undetectable vs. detectable levels.

cytokine levels measured in the sub-groups of malaria infected and uninfected women at two time points of pregnancy, i.e., inclusion and delivery. In univariate analysis, we found no significant changes in the levels of IL-2, IL-4, IL-5, TNF-α, and IFN-γ measured between *P. falciparum*-infected compared with uninfected women, either at inclusion or at delivery. However, IL-10 plasma levels tended to be higher among parasitemic women than those who had no parasitemia at inclusion (Kruskal-Wallis test; $P = 0.07$).

Multiple logistic regression analysis further investigated which cytokines were associated with *P. falciparum* infection after adjustment for cofactors, transversally at inclusion then at delivery. At inclusion, a detectable plasma concentration of IFN-γ was significantly associated with a reduced likelihood of infection (odds ratio [OR] = 0.45; $P = 0.04$, Table 2). In contrast, though not significant, a detectable concentration of IL-10 showed a trend towards a positive association with infection (OR = 1.96; $P = 0.08$; Table 2), whereas associations with other cytokines and adjustment covariates were not found. At delivery, a reduced likelihood of infection was again associated with detectable IFN-γ (OR = 0.10, $P = 0.024$) but at this time-point detectable TNF-α was associated with a stronger likelihood of infection (OR = 8.5; $P = 0.023$) (Table 3).

DISCUSSION

This study aimed to characterize the Th1 and Th2 cytokine profiles among malaria-exposed and HIV-infected Beninese pregnant women. This study was conducted as an ancillary study to the PACOME clinical trials, and was based on longitudinal observations of the plasma levels of cytokines, measurements that are rare in the literature for this particular population.

Consistent with previous reports, our data showed that infection with *P. falciparum* at inclusion was less frequent in those who reported as already being under CTX prophylaxis, suggesting a protective effect of this antibiotic against infection with *P. falciparum*.[15,20]

Not unexpectedly, our study shows that the presence of infection with *P. falciparum* at inclusion is associated with the low $CD4^+$ T cell counts, themselves primarily related to the concurrent HIV infection, as well with the absence of any previous anti-malarial preventive treatment (IPTp), both of which lead to a stronger likelihood that pregnant women have plasmodial infections (Tables 1–3). Nevertheless, it should be stressed in this context that our sample size is small, and that some of our data only revealed trends that show borderline significance. Multivariate analyses adjusted for multiple confounders however confirmed most of the findings obtained in univariate analyses.

TABLE 3
Multiple logistic regression analysis for independent associations of cytokines with *P. falciparum* infection at the time of delivery

Variables delivery	Odds ratio	95% CI	*P*
Detectable IFN-γ*	0.96	0.01–0.73	0.024
Detectable TNF-α*	8.52	1.35–53.8	0.023

IFN-γ = tumor necrosis factor α; IL-10 = interlelukin-10; TNF-α = tumor necrosis factor α.
*Cytokine levels were dichotomized into undetectable vs. detectable levels.

Interleukin-10 plays an important immunoregulatory role during pregnancy by impeding inflammatory responses.[21] During pregnancy in women without HIV, we and others have consistently shown an association between elevated plasma IL-10 levels and *P. falciparum* infection.[11,22,23] The data we present here suggest that even in immunosuppressed women *P. falciparum* infection is associated with an elevated plasma IL-10 concentration, perhaps yet another indication of the essential anti-inflammatory role this cytokine plays in the context of maintenance of the pregnancy when exposed to inflammatory insults. Parasite-induced IL-10 may also contribute, by suppression of inflammatory activity, to reducing HIV viral load,[24,25] with possibly a further indirect effect on viral replication.[26] The absence of an association between plasma IL-10 and infection with *P. falciparum* at delivery may reflect the longevity of the infections in the sense that those present at delivery were likely—given the fact that all women were subjected to malaria chemoprevention—to have been recently acquired and therefore to have had comparatively little time to induce immunological alterations detectable at the systemic level. Moreover, in our study a clear induction of plasma IL-5 upon starting ART was observed between inclusion and delivery. It is possible that this is linked to the immune restoration reflecting a recovery of $CD4^+$ T cell function associated with ART. Such observations were reported by Oliver and others[27] in HIV patients co-infected with *Mycobacterium tuberculosis*.

The plasma concentration of IFN-γ in this cohort of HIV-infected pregnant women was higher at delivery compared with inclusion irrespective of the presence or absence of *P. falciparum* infection. Women who reported using ART before pregnancy were those with the highest IFN-γ levels. Our data are thus consistent with an earlier study showing expansion of IFN-γ secreting HIV-specific T cells in HIV-infected individuals[24] including those under ART.[28] Altogether, our data suggest that two mechanisms could participate in ART efficacy for reducing maternal-HIV transmission: the direct impact of ART on HIV replication and the increased IFN-γ production during pregnancy.[29]

It has been reported that HIV-malaria co-infection can modulate immune responses in pregnant women.[30] The HIV infection in pregnant women has been shown to impair pro-inflammatory cytokine production,[31,32] which could be considered as being beneficial for the persistence of malaria parasites. In our study, we showed that lower plasma levels of IFN-γ were found in women infected by *P. falciparum* during pregnancy. This result again highlights the importance of IFN-γ in controlling plasmodial infection during pregnancy.

In contrast to the consistent negative association found between plasma IFN-γ concentrations and infection with *P. falciparum* both at inclusion and at delivery, we found that the presence of TNF-α in plasma was associated with an increased likelihood of infection at delivery. We speculate that it is most probably the infection itself that is the likely

cause of the presence of this cytokine, as is the case for IL-10. The TNF-α has been shown, along with IL-8, to be involved in viral transplacental transmission,[33] and, perhaps most importantly, HIV replication during *P. falciparum* infection has been reported to be mediated by TNF-alpha;[34] The significantly lower plasma level of TNF-α found in our study at delivery compared with inclusion may reflect 1) the direct influence of IL-10 on this cytokine[24,35] or 2) the markedly lower overall prevalence of plasmodial infection, or 3) the decreased immune activation associated with institution of ART.

In conclusion, our data indicate that cytokine activity during pregnancy in HIV seropositive women, as reflected by plasma cytokine concentrations, varies as a function of both anti-retroviral therapy and co-infection with *P. falciparum*. The latter is strongly associated with an elevated level of IL-10 that itself may limit the progression of HIV disease through its anti-inflammatory properties. The control of viral replication, whether through anti-retroviral therapy or through the "bystander" effects of IL-10, is associated with augmented plasma Th1 (IFN-γ) and Th2 (IL-5) concentrations. The latter likely reflect an overall improvement in $CD4^+$ T cell activity because of the treatment received, with a beneficial outcome in terms of reduced incidence of infection with *P. falciparum* associated with the restoration of IFN-γ-mediated immunological responses. How and why the concentration of plasma TNF-α, in contrast to plasma IFN-γ, declines during pregnancy in these women is unclear. Both these cytokines are pro-inflammatory in nature, but the cellular sources, although potentially the same (e.g., T cells), are more diverse in the case of TNF-α. Consistent with our finding, it is noteworthy that, in our previous study of HIV seronegative Tanzanians, the plasma concentration of TNF-α fluctuated little in the course of pregnancy and, if anything, tended to decline from inclusion (2nd trimester) to delivery.[22]

Received October 18, 2014. Accepted for publication January 20, 2015.

Published online June 22, 2015.

Acknowledgments: We are extremely grateful to all the women who participated in the study. We especially thank Nadine Fievet for her fruitful advice on FACS. We are also grateful to Gilles Cottrell in providing fruitful advice on the design of the study.

Financial support: The PACOME trial received funding from SIDACTION (research grant no. AI19-3-01528).

Disclosure: The funding sources had no involvement in the design, collection, analysis, and interpretation of data, writing of the manuscript or decision to publish.

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