Submicroscopic Infections with *Plasmodium falciparum* during Pregnancy and Their Association with Circulating Cytokine, Chemokine, and Cellular Profiles

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The immunological consequences of pregnancy-associated malaria (PAM) due to *Plasmodium falciparum* have been extensively investigated in cross-sectional studies conducted at delivery, but there have been very few longitudinal studies of changes due to PAM during pregnancy. We conducted a prospective study in Benin to investigate the changes associated with PAM in groups of 131 and 111 women at inclusion in the second trimester and at delivery, respectively. Infected women were identified by standard microscopic examinations of blood smears and by quantitative PCR (qPCR) assays and were matched to uninfected control women by age, gestational age, and gravidity. We quantified plasma levels of a panel of soluble immunological mediators and other mediators, as well as the frequencies of peripheral blood mononuclear cell types. Comparisons of these variables in infected and uninfected women used multivariate analyses, and we also assessed the predictive value of variables measured at inclusion for pregnancy outcomes at delivery. In multivariate analyses, peripheral plasma interleukin 10 (IL-10) and gamma interferon-inducible protein 10 (IP-10) levels were associated with PAM at inclusion and at delivery, while higher IL-10 levels distinguished qPCR-detectable submicroscopic infections at inclusion but not at delivery. Maternal anemia at delivery was associated with markers of proinflammatory (increased frequency of monocytes) and anti-inflammatory (increased IL-10 levels and increased activation of regulatory T cells) activity measured at inclusion. Elevated concentrations of IL-10 are associated with the majority of *P. falciparum* infections during pregnancy, but this marker alone does not identify all submicroscopic infections. Reliably identifying such occult infections will require more sensitive and specific methods.

The concept that pregnancy is associated with immune suppression for fetal allograft tolerance has been well described (1–3). In healthy pregnancies, components of the cellular arm of the immune response have been shown to populate the human decidua during the first trimester and are necessary for placental development (4, 5). In areas where *Plasmodium falciparum* is endemic, pregnancy (especially the first pregnancy) is associated with increased susceptibility to infection with this parasitic protozoan. Sequestration of *P. falciparum*-infected erythrocytes (*Pf*iEs) in the placenta, via their adherence to chondroitin sulfate A (CSA) expressed by syncytiotrophoblasts, is the major pathological characteristic of pregnancy-associated malaria (PAM), with potentially multiple detrimental outcomes (6). *P. falciparum*-infected erythrocytes induce inflammation, characterized particularly by monocytic infiltration of the infected placenta (7–10). One other well-described consequence of PAM is an altered cytokine/chemokine balance in peripheral blood and placental blood (11–17).

We recently showed that *P. falciparum* infection during pregnancy is associated with modified peripheral blood mononuclear cell (PBMC) profiles, consistently characterized by enhanced B cell activity (18), a finding consistent with the prominent B cell component of the inflammatory response to *Pf*iEs in the placenta reported by others (19). B cells are recognized as having multiple functions, not only as antigen-presenting cells and antibody producers but also as regulators of dendritic cell (DC) function and maturation through their production of the cytokine interleukin

10 (IL-10) (20, 21). The polyclonal activation of B cells is a well-recognized feature of infection with *P. falciparum* that, in the case of PAM, may be linked to the demonstration of nonspecific binding of IgM antibodies to the parasite-derived ligand for CSA expressed on *Pf*iEs (22, 23).

The concentrations of several molecules, including the cytokines IL-10, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) and the chemokines IFN-γ-inducible protein 10 (IP-10) and monokine induced by IFN-γ (MIG), have been shown in several studies to be increased in placental plasma at delivery in women with PAM (13, 14, 24). Here we used samples collected both during pregnancy and at delivery, including sam-

ples from women with submicroscopic infections, to examine in more detail the association between plasma cytokine/chemokine concentrations and PAM. We also wished to determine whether the PBMC profiles determined in the same samples retained their predictive value for infection when analyzed in parallel, following the hypothesis that both cytokines/chemokines and cells are potentially implicated in susceptibility to and protection from PAM.

(This work was presented at the 3rd Symposium of Infectious Diseases in Africa and 4th African Flow Cytometry Workshop: Measurement of Immune Responses, Cape Town, South Africa, 12 to 18 November, 2011.)

MATERIALS AND METHODS

Ethics statement. The study described here received ethical clearance from the ethics committee of the Health Science Faculty of the University of Abomey-Calavi (Cotonou, Benin).

Study population. The study populations included subgroups drawn from the cohort of pregnant Beninese women who participated in a longitudinal study known as the Strategies to Prevent Pregnancy-Associated Malaria (STOPPAM) study. The STOPPAM study was conducted in parallel at two study sites in Benin and Tanzania. After giving written informed consent, ~1,000 pregnant women at gestational ages of ≤24 weeks were included in the district of Comé, located in the Mono province 70 km west of Cotonou, the economic capital of Benin. The STOPPAM study design in Benin has been described in detail elsewhere (25), as has the procedure used to select women for the immunological substudy described here (18). First, at inclusion in the study, we selected a subgroup of 131 women who were identified as being infected with *P. falciparum* (for methods, see below) and an uninfected control group appropriately matched for age, gravidity, and gestational age. Second, at delivery, we identified a subgroup of 111 women with varying infection histories during pregnancy, as determined from the results of our own active and passive surveillance. The latter subgroup included women with no evidence of infection from inclusion through delivery ("uninfected") and women with infections at delivery who might or might not have been infected earlier ("infected"). The women selected for inclusion in the subgroups were not the same at the two time points.

Sample collection. Venous blood samples, collected in Vacutainer tubes with citrate phosphate dextrose adenine (CPDA) anticoagulant, were transported to the research laboratories within 4 h. Plasma samples were separated and stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were isolated using Leucosep tubes (Greiner-Bio), according to the manufacturer's instructions, and were subsequently used for immunophenotyping as described previously (18).

Detection of *P. falciparum* infections. Infection status was determined using a rapid diagnostic test (RDT) (Parascreen; Zephyr Biomedical Systems). Thin and thick smears of peripheral and placental intervillous blood, to identify plasmodial parasites, were prepared using standard methods and were double-read retrospectively in routine microscopic examinations performed by two experienced technicians. Quantitative PCR (qPCR) was performed after DNA extraction from blood spots on filter paper using the Chelex method, as described elsewhere (26).

Women identified as infected had positive PCR results and had parasites detected in thin and thick smears of peripheral and/or placental blood, while uninfected women had negative results for both PCR assays and microscopic examinations of thin and thick smears. Women with a positive PCR result but a negative blood smear result constituted a separate group identified as having submicroscopic infections. In only one case was a sample defined as negative by PCR but positive by microscopy.

Cytometric bead arrays for quantitation of cytokines and chemokines. Plasma levels of IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α cytokines were analyzed using a human inflammatory kit (cytometric bead assay [CBA]; BD Biosciences, San Diego, CA, USA), and levels of regulated on activation normal T cell expressed and secreted (RANTES),

monokine induced by IFN-γ (MIG), monocyte chemotactic protein 1 (MCP-1), and IFN-γ-inducible protein 10 (IP-10) were analyzed with a human chemokines kit (CBA; BD Biosciences). The procedure used adhered to the manufacturer's recommendations and has been described in detail elsewhere (17).

ELISAs. Plasma concentrations of alpha interferon (IFN-α) and gamma interferon (IFN-γ) were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Mabtech, Stockholm, Sweden), as were those of soluble vascular endothelial growth factor receptor 1 (sVEGF-R1)/Flt1, soluble urokinase plasminogen activator receptor (suPAR), angiopoietin 1 (Ang-1), and Ang-2 (R&D, Minneapolis, MN). The methods used conformed to the manufacturer's recommendations and have been described in detail elsewhere (17).

Peripheral blood mononuclear cell immunophenotyping. PBMCs were washed and resuspended at a concentration of 10 million cells/ml. Cells were then labeled using a panel of surface and intracellular marker-specific monoclonal antibodies, as described elsewhere (18). Human anti-blood dendritic cell antigen 1 (BDCA-1)-phycoerythrin (PE) was used for myeloid dendritic cell (mDC) detection and anti-BDCA-2-PE for plasmacytoid DC (pDC) detection (all from Miltenyi Biotec). Anti-CD14-fluorescein isothiocyanate (FITC) was used for detection of monocytes, anti-CD19-FITC for detection of B cells, and the combination of anti-HLA-DR-peridinin chlorophyll protein (PerCP) and anti-CD86-allophycocyanin (APC) (all from BD Pharmingen, San Diego, CA) for assessment of the activation status of cells. Human anti-CD3-APC, anti-CD8-FITC, anti-CD4-PerCP, and anti-CD56-PE (BD Pharmingen) were used for T lymphocyte and natural killer (NK) cell labeling, while anti-CD25-FITC and anti-CD127-PE (BD Pharmingen) were used for regulatory T (Treg) cell labeling. Human anti-FoxP3-APC (BD Pharmingen) was added for Treg cell labeling, according to the manufacturer's recommendations, after permeabilization and fixation with PermFix (BD Pharmingen). Events were recorded using a BD FACSCalibur system, and data were analyzed using CellQuest Pro or FlowJo 7.6 software (TreeStar, Ashland, OR, USA).

Data analysis. Data analysis was performed using Stata/MP 12.0 (StataCorp, College Station, TX, USA) and GraphPad Prism version 5.00 for Windows (GraphPad, San Diego, CA, USA). Data are expressed as medians with interquartile ranges (IQRs). At both time points, data from study participants with missing values were excluded, with consequent reductions in the numbers of matched pairs remaining, and the use of qPCR assays for parasite detection resulted in data for purportedly uninfected individuals being moved to the infected group, again disrupting the originally defined pair matching. Initial univariate comparisons between groups were therefore conducted using nonparametric Kruskal-Wallis and Mann-Whitney tests, rather than pairwise analyses. Categorical variables were compared with χ^2 tests. Multiple logistic regressions were performed using logit commands in Stata, in order to identify factors associated with the risk of *P. falciparum* infections in pregnant women at inclusion and at delivery. Segregation of values into low and high categories used the median values of the corresponding variables for the uninfected groups as references. In order to assess whether any of the cytokines, chemokines, or other factors measured were independently associated with the risk of anemia (defined as a concentration of hemoglobin below 11 g/dl) or with particular PBMC profiles during pregnancy, multiple logistic regressions were used. A stepwise procedure was performed to select a model including factors associated with infection. Prospective analysis of the associations between factors measured at inclusion and maternal anemia identified at delivery was also conducted using multiple logistic regressions with data collected at inclusion. Two-tailed P values of <0.05 were considered significant.

RESULTS

Detection of *P. falciparum* infections. The study described here included subgroups of Beninese women participating in the STOPPAM study. These subgroups were shown previously not to

TABLE 1 Characteristics of study subjects

Variable	At inclusion				At delivery			
	Uninfected (n = 52)	Submicroscopic (n = 18)	Infected (n = 61)	P^a	Uninfected (n = 54)	Submicroscopic (n = 20)	Infected (n = 37)	P^a
Age in yrs (median [IQR])	$25.5 (8.0)^b$	23.5 (7.0)	$22.0 (8.0)^b$	0.047	27 (9)	25 (5.5)	25 (10)	0.41
Primigravid (%)	30.7	27.8	26.2	0.86	12.9	10.0	24.32	0.24
Gestational age in wks (median [IQR])	18.5 (4.6)	16.9 (4.8)	17.4 (5.1)	0.12	39.6 (1.3)	40.1 (1.4)	39.4 (2.3)	0.36
Possessing bednet (%)	36.5^c	33.3	19.6^c	0.12	38.9	30.0	29.7	0.60
Hemoglobin level of <11 g/dl (%)	50.0^d	66.6	81.6^d	0.002	31.5	35.0	45.9	0.36
No. of parasites/µl by qPCR (median [IQR])	0	52.0 (210.7)	714.6 (2,981.4)		0	5.3 (105.8)	4,119.8 (24,088.9)	

^{a} Kruskal-Wallis and χ^2 tests.
^{b} Mann-Whitney test, $P = 0.015$.
^{c} χ^2 test, $P = 0.045$.
^{d} χ^2 test, $P < 0.001$.

differ in demographic characteristics from the whole cohort (18). Here we wished to determine specifically whether and how circulating plasma levels of cytokines and chemokines during pregnancy might be associated with peripheral blood cell profiles in the same women and/or with the presence or absence of infection with *P. falciparum*. Since such infections may remain below the level of detection of the routinely used microscopic method of diagnosis, here we performed additional analyses using a sensitive, species-specific, PCR-based method to identify "occult" infections. The latter were detected in a total of 38/242 women. In a subgroup of 131 women assessed at inclusion, the majority of whom were in the second trimester of pregnancy, 52 were uninfected, 61 had infections identified by microscopy, and 18 had submicroscopic infections identified by PCR; among 111 women assessed at delivery, 54 were uninfected, 37 had infections detected by microscopy, and 20 had submicroscopic infections (Table 1

and Fig. 1). In the subgroup of women assessed at inclusion, those with infections detectable by microscopy were significantly younger and significantly less likely to possess a bednet, and a significantly greater proportion were anemic, compared with women who were uninfected (Table 1). Of note, the same variables in those harboring submicroscopic infections at inclusion did not differ significantly with respect to either of the other two groups, while there were no differences in any variable among all of the groups in the subgroup assessed at delivery.

Circulating levels of cytokines and chemokines associated with *P. falciparum* infections at inclusion and at delivery. There are few published studies concerning the effects of submicroscopic infections during pregnancy and their potential association with poor pregnancy outcomes (27, 28). Therefore, we first addressed the changes in the levels of circulating cytokines and chemokines as a function of the women's infection status.
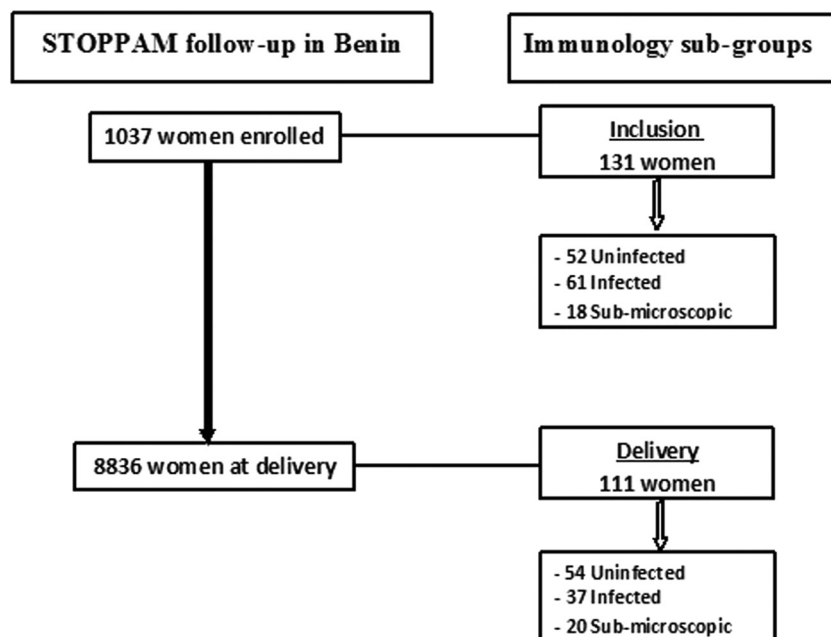

FIG 1 Study profile.

TABLE 2 Plasma cytokine and chemokine concentrations at inclusion and at delivery

Variable	Concn in pg/ml (median [IQR]) at inclusion			Concn in pg/ml (median [IQR]) at delivery		
	Uninfected ($n = 52$)	Infected ($n = 79$)	P^a	Uninfected ($n = 54$)	Infected ($n = 57$)	P^a
Il-1β	1.0 (0.0)	1.0 (0.0)	0.91	1.0 (0.0)	1.0 (0.5)	0.32
IL-6	1.0 (0.0)	1.0 (1.5)	0.002	12.1 (15.0)	13.8 (31.5)	0.15
IL-8	9.6 (12.2)	11.8 (17.0)	0.24	114.0 (423.2)	99.73 (40.7)	0.81
IL-10	1.0 (0.0)	12.3 (19.9)	<0.0001	1.8 (2.6)	6.0 (26.3)	<0.0001
IL-12p70	1.0 (0.0)	1.0 (0.0)	0.59	1.0 (0.0)	1.0 (0.0)	0.11
TNF-α	1.0 (0.0)	1.0 (0.0)	0.71	1.0 (0.0)	1.0 (0.0)	0.27
RANTES	58,383 (61,130)	63,393 (67,573)	0.84	64,620 (63,760)	51,957 (55,947)	0.19
MIG	2,272 (1,701)	3,494 (2,618)	0.0001	124 (746)	2,649 (4,813)	0.0001
MCP-1	1 (2.3)	1 (3.8)	0.38	10.4 (10.0)	13.1 (24.3)	0.029
IP-10	1,806 (969)	2,634 (1,803)	<0.0001	1,790 (1,165)	2,871 (3,423)	0.0010
Ang-1	18,844 (4,459)	19,190 (6,183)	0.77	20,005 (21,454)	14,853 (25,661)	0.050
suPAR	2,047 (754)	2,150 (937)	0.36	2,014 (1,215)	2,562 (1,829)	0.17
IFN-α	3.5 (0.0)	3.5 (0.0)	0.50	3.5 (5.5)	3.5 (5.8)	0.69
IFN-γ	1.0 (0.0)	1.0 (0.0)	0.51	1.0 (6.5)	1.0 (0.0)	0.18
sVEGF-R1/Flt1	748 (1,244)	747 (1,347)	0.80	4,547 (4,759)	4,222 (5,851)	0.91

[a] Comparisons between groups used the nonparametric Mann-Whitney test.

At inclusion, there were significantly higher levels of IL-10 in women with submicroscopic infections than in uninfected women, but otherwise there were no differences (see Table S1 in the supplemental material). This evidence, albeit limited, of modified immune status in women with submicroscopic infections led us to combine them with the group of women harboring microscopically detectable infections, as one group, in all subsequent analyses. Univariate analyses revealed significantly increased levels of IL-6, IL-10, MIG, and IP-10 at inclusion among the infected women, in comparison with the uninfected women, while significantly higher levels of IL-10, MIG, MCP-1, and IP-10 and lower levels of Ang-1 were observed in infected versus uninfected women at delivery (Table 2 and Fig. 2).

P. falciparum infection-altered cellular profiles during pregnancy. As we showed previously, *P. falciparum* infection is associated with altered peripheral blood mononuclear cell (PBMC) profiles at inclusion and at delivery in our study population (18). Here, PCR-based methods that identified women who were previously classified as uninfected but who actually harbored submicroscopic *P. falciparum* infections were used to determine the effects of such infections, if any, on PBMC profiles at inclusion and at delivery, as for the cytokines/chemokines described above. For the full range of variables analyzed, we found no significant differences at either time point between women with submicroscopic infections and either of the other two groups (see Table S2 in the supplemental material).

In univariate analyses at inclusion, the frequency of mDCs expressing HLA-DR in infected versus uninfected women was significantly lower, while there were nonsignificant trends for (i) more cells with a high expression level of CD86 (CD86^{hi} B cells), (ii) more effector T (Teff) cells (CD4$^+$ CD25$^+$ CD127$^+$) (and consequently a lower Treg cell [CD4$^+$ CD25$^+$ CD127$^-$]/Teff cell ratio), and (iii) fewer NK cells (CD56$^+$) in infected women (Table 3). Similar analyses at delivery revealed significantly higher frequencies of both Teff cells and CD86^{hi} B cells in infected women but significantly lower frequencies of pDCs (BDCA-1$^+$) and NK T cells (CD3$^+$ CD56$^+$) than in uninfected women, as described previously (Table 3).

Multiple regression analyses of associations of cytokine levels, chemokine levels, and peripheral blood cell profiles with *P. falciparum* infections during pregnancy. In order to determine the independent associations between PAM and the different cytokines, chemokines, and peripheral blood cell populations identified by univariate analyses, we next included all of the variables in multiple logistic regression analyses. The models tested also included the variables anemia, bednet possession, gravidity, and maternal ages at inclusion and at delivery. The model including cytokine and chemokine levels at inclusion showed an increased risk of infection with *P. falciparum* at delivery to be associated with elevated levels of IL-6, IL-10, and IP-10. The same model with variables at delivery showed that the risk of PAM increased with higher IL-10 and IP-10 levels, primigravidity, and younger age (data not shown). In the same model with cytokines replaced with PBMCs, anemia, primigravidity, maternal age below the median, and a higher frequency of CD86^{hi} B cells were associated with PAM at inclusion. For PAM at delivery, the model showed that the risk was increased with a higher frequency of CD86^{hi} B cells but also with fewer pDCs and NK cells and with a lower Treg cell/Teff cell ratio (data not shown).

The final model, which combined cytokines, chemokines, and PBMCs, showed that the risk of infection increased with higher levels of IL-6, IL-10, and IP-10 at inclusion (Table 4) and with higher levels of IL-10 and IP-10 and a lower Treg cell/Teff cell ratio at delivery (Table 5). The levels of the cytokines and chemokines included in the final model are correlated (see Tables S4 and S5 in the supplemental material). A discussion of the relevance of these correlations to the interpretation of the results of the analyses is presented below.

Factors predicting maternal anemia at delivery. Various cytokines and chemokines have been proposed to have predictive values for the identification of infection with *P. falciparum* during pregnancy. Therefore, we were interested to know to what extent the different variables we measured at inclusion, including the PBMC profiles, might be predictive of maternal anemia, as one of the primary clinical outcomes identifiable at delivery. For this purpose, we included a range of factors measured at inclusion in multiple logistic regression analyses. Table 6 shows that higher
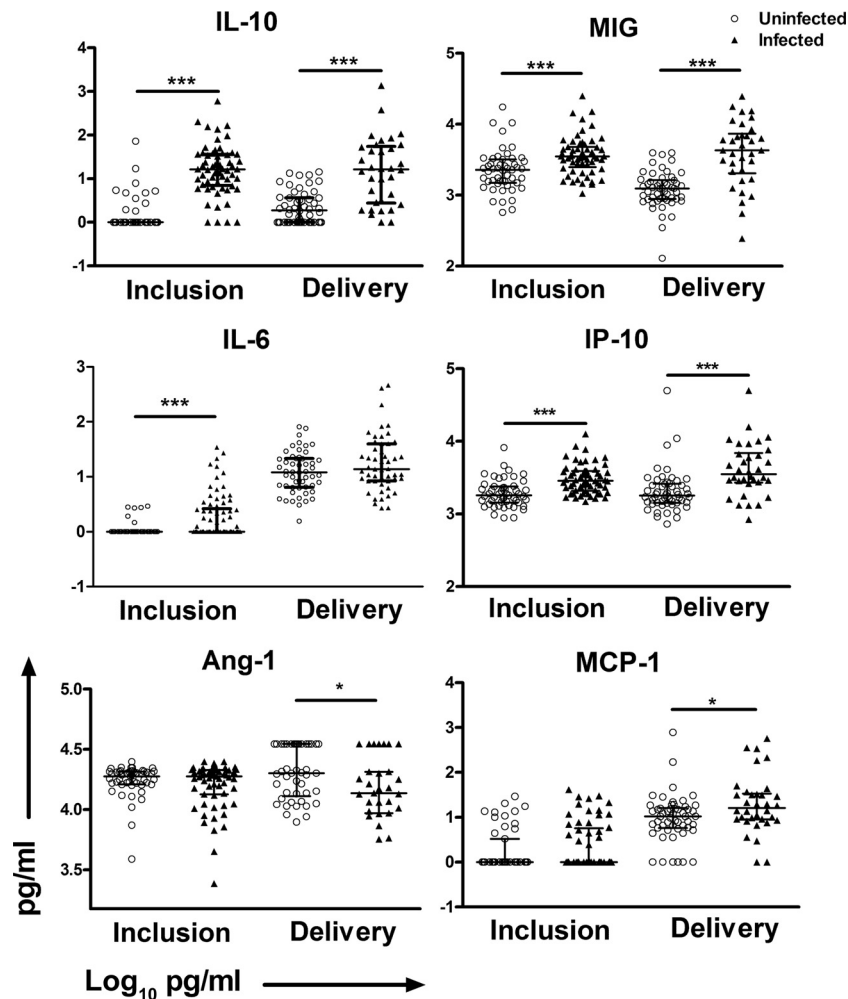
FIG 2 Plasma levels of IL-10, IL-6, Ang-1, MIG, IP-10, and MCP-1 with *P. falciparum* infections at inclusion and at delivery. The scatter plots include bars depicting the medians with interquartile ranges of plasma levels of IL-10, IL-6, Ang-1, MIG, IP-10, and MCP-1 from 52 uninfected women and 79 infected women at inclusion and from 54 uninfected and 57 infected women at delivery. The statistical significance of differences between groups was determined using the nonparametric Mann-Whitney *U* test. *, $P < 0.05$; ***, $P < 0.001$.

levels of circulating IL-10, lower levels of IL-1β, and increased frequencies of monocytes and of activated Treg cells (FoxP3^{hi}) detectable at inclusion were all associated with an increased risk of maternal anemia at delivery.

DISCUSSION

Defining alterations in the profiles of soluble factors and cells in peripheral blood associated with *P. falciparum* infections in pregnant women serves multiple purposes. From a fundamental biological point of view, such information contributes to our understanding of the pathophysiological effects of infection, particularly with respect to the host's response to it. Studies with a longitudinal design, as was the case in our STOPPAM project, are beginning to broaden our knowledge of the immunological components of that response. From a translational viewpoint, such studies can reveal potentially novel ways of detecting PAM events that might otherwise go unidentified, thus helping to clarify issues such as the timing of occurrence of infections and the need to adjust or to improve existing preventive measures.

In a previously published study of women in the Tanzanian cohort of the STOPPAM project, we revealed that the levels of most of the plasma-derived factors we studied here were stable during pregnancy, regardless of the women's *P. falciparum*-specific infection status (17). In the Beninese cohort assessed here, we focused on assessments of samples from only two time points, i.e., inclusion and delivery, for a larger number of women, rather than samples from multiple consecutive time points for a comparatively smaller number of women, as in the study of the Tanzanian cohort. Importantly, in this regard, we know from our own parasite genotypic data (N. Tuikue Ndam, unpublished observations) that the overwhelming majority of *P. falciparum* infections detected at delivery in the Beninese cohort were acute, i.e., they had been acquired within 4 weeks of delivery. Our own work, as well as others' work, has shown that infections with *P. falciparum* during pregnancy are associated with increased levels of cytokines and chemokines such as TNF-α, IFN-γ, IL-10, MCP-1, and IP-10 in peripheral or placental plasma (11, 13, 15, 17, 29, 30). Studies of the placenta have revealed inflammatory immune infiltrates that are likely a direct reflection of the altered cytokine/chemokine profiles, although causality in this respect is difficult to prove

TABLE 3 Peripheral blood cellular profiles at inclusion and at delivery

Variable	Median (IQR) at inclusion			Median (IQR) at delivery		
	Uninfected ($n = 52$)	Infected ($n = 79$)	P^a	Uninfected ($n = 54$)	Infected ($n = 57$)	P^a
Monocytes (%)	7.6 (7.7)	8.9 (5.5)	0.47	9.4 (7.4)	9.8 (7.4)	0.70
B cells (%)	12.5 (6.5)	13.2 (6.3)	0.13	11.8 (4.9)	12.0 (7.0)	0.53
mDCs (%)	0.3 (0.2)	0.3 (0.2)	0.88	0.28 (0.1)	0.2 (0.2)	0.26
pDCs (%)	0.41 (0.3)	0.4 (0.3)	0.49	0.3 (0.3)	0.2 (0.3)	0.040
CD86$^+$ monocytes (%)	7.0 (3.8)	6.1 (4.1)	0.58	5.05 (3.2)	5.4 (4.4)	0.89
CD86$^+$ B cells (%)	0.9 (0.9)	1.1 (1.0)	0.072	0.93 (0.8)	1.3 (1.5)	0.025
CD86$^+$ mDCs (%)	0.8 (1.3)	0.8 (1.4)	0.69	1.3 (2.2)	1.2 (2.2)	0.58
CD86$^+$ pDCs (%)	0.8 (1.3)	1.1 (1.1)	0.27	1.1 (1.2)	1.3 (1.6)	0.45
HLA-DR$^+$ monocytes (%)	39.2 (20.3)	34.0 (20.7)	0.20	32. 8 (24.1)	35.2 (32.5)	0.42
HLA-DR$^+$ B cells (%)	56.2 (20.3)	56.2 (26.1)	0.82	62.6 (30.7)	54.2 (39.8)	0.42
HLA-DR$^+$ mDCs (%)	148.6 (49.1)	125.2 (67.4)	0.004	139.2 (74.3)	121.8 (94.5)	0.40
HLA-DR$^+$ pDCs (%)	54.2 (31.7)	52.3 (31.6)	0.75	40.6 (31.5)	48.6 (30.9)	0.23
Treg cells (%)	3.8 (2.05)	3.6 (2.1)	0.30	3.6 (2.4)	3.6 (2.1)	0.92
Teff cells (%)	4.0 (5.2)	5.1 (6.5)	0.07	1.6 (2.0)	2.6 (4.02)	0.002
Treg cell/Teff cell ratio	0.9 (1.8)	0.7 (1.1)	0.06	2.3 (2.3)	1.4 (1.4)	0.001
Activated Treg cells (%)	2.3 (2.7)	2.1 (3.0)	0.54	1.6 (0.6)	1.7 (0.8)	0.12
Activated Teff cells (%)	1.2 (0.2)	1.2 (0.41)	0.57	1.3 (0.23)	1.3 (0.4)	0.78
NK cells (%)	4.8 (4.3)	3.9 (3.4)	0.052	5.3 (5.3)	4.8 (5.4)	0.14
CD3$^+$ cells (%)	67.9 (14.6)	68.1 (12.1)	0.38	65.0 (11.7)	62.2 (17.4)	0.55
NK T cells (%)	1.4 (1.6)	1.6 (1.6)	0.77	2.4 (1.5)	1.88 (2.0)	0.034
CD4$^+$ cells (%)	63.6 (10.4)	63.6 (8.8)	0.42	56.7 (12.7)	58.9 (12.1)	0.42
CD8$^+$ cells (%)	27.4 (9.1)	27.1 (8.3)	0.88	31.8 (13.5)	31.0 (9.2)	0.40
CD4$^+$ cell/CD8$^+$ cell ratio	2.3 (1.2)	2.3 (1.0)	0.69	1.8 (1.2)	1.9 (1.1)	0.38

[a] Comparisons between groups used the nonparametric Mann-Whitney test.

(7–10, 19). The study we present here represents an attempt to address this issue by combining assessments of soluble peripheral blood plasma-borne factors with assessments of peripheral blood cellular profiles for infected and uninfected pregnant women. One potential limitation of the study concerns the fact that the women in the different subgroups were not the same at inclusion and at delivery, which precludes the possibility of pairwise comparisons between the two time points. It should be noted in this context that, in such a study and in such a setting, optimal study design and conduct are not always realistic practical possibilities, and compromises often must be sought.

In one important respect, our analyses here differ from those we conducted with data from the Tanzanian arm of the STOPPAM study (18). That difference concerns the identification of submicroscopic *P. falciparum* infections that we performed here. We and others have consistently shown that IL-10 is present at higher concentrations in the plasma of infected women (31). In this context, it is perhaps not surprising that we found significantly increased levels of IL-10 in women with submicroscopic

infections, in comparison with uninfected women, but this finding held only for comparisons between the groups at inclusion and not between those at delivery. We conjecture that this is a reflection of the fact that the infections present at delivery, as outlined above, were recently acquired and therefore pathological inflammatory changes in the placenta were not as well established as would be expected in what we assume to be the persistent (chronic) infections detected at inclusion. For delivery samples, this conjecture is supported by the lack of malarial pigment (hemozoin)-containing cells in the placental blood of women with placental infections in the current study (N. Fievet, unpublished observations). We speculate that the presence of submicroscopic parasitemia at delivery may thus reflect the most recently acquired infections, with the least detectable changes in terms of soluble blood-borne factors. In the same context, the significantly elevated levels of IL-10 in women with submicroscopic infections at inclusion, in the absence of changes in any other parameter we measured, could be an indication of the fact that such infections were persistent in nature, with a more chronic evolution. Sepa-

TABLE 4 Multiple logistic regression analysis of independent associations between selected parameters and *P. falciparum* infections at inclusion

Factor at inclusion[a]	Odds ratio (95% confidence interval)	P^b
IL-6 level above median	3.72 (1.09–12.64)	0.035
IL-10 level above median	17.29 (6.23–47.96)	<0.0001
IP-10 level above median	3.03 (1.01–9.12)	0.048
Anemia	2.92 (0.98–8.73)	0.054

[a] The median value for the uninfected group was used for dichotomization.
[b] Hosmer-Lemeshow test (4 degrees of freedom [df]), $\chi^2 = 0.61$; $P = 0.96$. The model included bednet possession, gravidity, and age.

TABLE 5 Multiple logistic regression analysis of independent associations between selected parameters and *P. falciparum* infections at delivery

Factor at delivery[a]	Odds ratio (95% confidence interval)	P^b
IL-10 level above median	7.82 (2.38–25.67)	0.001
IP-10 level above median	3.37 (1.11–10.22)	0.032
Treg cell/Teff cell ratio above median	2.60 (0.87–7.80)	0.086

[a] The median value for the uninfected group was used for dichotomization.
[b] Hosmer-Lemeshow test (6 df), $\chi^2 = 9.75$; $P = 0.13$. The model included bednet possession, gravidity, and age.

TABLE 6 Predictive value for maternal anemia at delivery of factors measured at inclusion

Factor at inclusion	Odds ratio (95% confidence interval)	P^a
IL-10 level above median	5.3 (1.49–19.3)	0.010
IL-1β level above median	0.05 (0.004–0.59)	0.017
HLA-DR$^+$ B cell proportion above median	3.0 (0.85–10.6)	0.088
Activated Treg cell proportion above median	4.5 (1.19–16.7)	0.026
Monocyte proportion above median	7.0 (1.86–26.0)	0.004

^{a} Hosmer-Lemeshow test (7 df), $\chi^2 = 4.35$; $P = 0.74$.

rately, the lack of any significant increase in the levels of TNF-α in the peripheral plasma of women with *P. falciparum* infections of any density and at either time point is consistent with our own data from Tanzania (17) and with data in a study from Ghana (31) but not with data in a study from Tanzania (24), although it should be noted that the latter study assessed only samples taken at delivery.

The pattern observed with submicroscopic infections aside, the combination of increased levels of IL-10 and IP-10 associated with infections during pregnancy that we report here after multivariate analyses is again consistent with our data from Tanzania (17). Notably, all of these data are derived from samples taken from women without any symptoms of malaria, and the findings thus serve to emphasize the fact that infections with *P. falciparum* that, for the most part, are subclinical do have measurable effects on some recognized markers of PAM, although their specificity requires further verification. The increased peripheral blood plasma concentrations of IL-6 associated with *P. falciparum* infections at inclusion we report here also echo our findings for the Tanzanian cohort of the STOPPAM project (17), although in the latter case this association did not survive multivariate analyses. With respect to our findings concerning altered cellular profiles associated with PAM, our earlier report of increased frequencies of activated B cells (CD86^{hi}), regardless of the timing of the infection, was confirmed here. It is important to note the fact that, in the multivariate analyses we performed, none of the cellular profiles was shown to have independent associations with infection when combined with cytokine/chemokine profiles, suggesting that the latter likely represent the most robust markers among the broad panel that we measured here. It is also important to note that the levels of IL-6, IL-10, and IP-10 retained in the final multivariate models were all highly correlated with each other (see Tables S4 and S5 in the supplemental material). Nevertheless, the fact that IL-10 was the variable with by far the strongest association with infection at both time points is noteworthy, perhaps reflecting the need to optimally control placental inflammation to ensure survival of the fetus.

In the context of their predictive value for maternal anemia at delivery, the variables measured at inclusion that we identified here included, as in our earlier study (18), increased frequencies of both monocytes and HLA-DR-expressing B cells. In addition, here we identified women with significantly higher plasma levels of IL-10 and with higher frequencies of activated Treg cells at inclusion as having a higher risk of anemia at delivery. Although these are independent associations, they may reflect a common cause in the sense that activated Treg cells could themselves be a source of IL-10. Such anti-inflammatory activity may in itself reflect a response to the proinflammatory activity represented by the elevated numbers of monocytes, but exactly how, mechanistically, the ensemble of these factors may lead to a heightened risk of anemia later in pregnancy must remain in the realm of speculation. Infections that arose between inclusion and delivery likely played a role in this context, but this aspect requires further study. It is also entirely unclear to us how the independent association of a lower risk of anemia at delivery with significantly lower plasma concentrations of the cytokine IL-1β at inclusion might be explained. In any case, it is noteworthy that the levels of IL-1β we detected at either time point were very low, frequently at the limit of detection of the assay employed, and thus any interpretation of these findings must necessarily be done with great caution.

In conclusion, the findings we report here add to our knowledge of the pathophysiological changes associated with *P. falciparum* infections associated with pregnancy. Overall, they strengthen the evidence base for the utility of the concentrations of circulating plasma-borne cytokines (IL-10) and chemokines (IP-10) as biomarkers of such infections, but a reliable specific means of detection of occult submicroscopic infections remains problematic.

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