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Placental Cytokine and Chemokine Profiles Reflect Pregnancy Outcomes in Women Exposed to *Plasmodium falciparum* Infection

Arnaud Chêne,[a,b,c,d] Valérie Briand,[d,e,f] Samad Ibitokou,[g] Sébastien Dechavanne,[a,b,c,d] Achille Massougbodji,[g] Philippe Deloron,[d,e,f] Adrian J. F. Luty,[d,e,f] Benoît Gamain,[a,b,c,d] Nadine Fievet[d,e,f]

INSERM UMR_1134, Paris, France[a]; Université Paris Diderot, Sorbonne Paris Cité, UMR_S1134, Paris, France[b]; Institut National de la Transfusion Sanguine, Paris, France[c]; Laboratory of Excellence GR-Ex, Paris, France[d]; Institut de Recherche pour le Développement, UMR_216, Mère et Enfant Face aux Infections Tropicales, Paris, France[e]; PRES Sorbonne Paris Cité, Faculté de Pharmacie, Paris, France[f]; Centre d'Étude et de Recherche sur le Paludisme Associé à la Grossesse et à l'Enfance (CERPAGE), Faculté des Sciences de la Santé, Université d'Abomey-Calavi, Abomey-Calavi, Benin[g]

Pregnancy-associated malaria (PAM) can lead to severe complications for both mother and baby. Certain placental cytokine/chemokine profiles have been shown to reflect poor pregnancy outcomes, including maternal anemia and low birth weight. In intervillous plasma samples from 400 Beninese women living in an area where *Plasmodium falciparum* is endemic, we quantified 16 cytokines/chemokines. We assessed their profiles in groups with PAM, with maternal anemia, with preterm births, or with a low birth weight for gestational age. Repeated ultrasound measurements ensured that prematurity and low birth weight were highly accurate. Preliminary analyses revealed trends for lower cytokine/chemokine concentrations in placental plasma associated both with babies with low birth weight for gestational age and with *P. falciparum* infection during pregnancy, while, as a function of the latter, the concentration of gamma interferon (IFN-γ)-inducible protein 10 (IP-10) was higher. Multivariate analyses showed that (i) higher placental plasma interleukin-10 (IL-10) levels were associated with *P. falciparum* infections and (ii) independently of *P. falciparum* infections, lower concentrations of both IFN-γ and IL-5 were associated with low birth weight for gestational age. Our data further strengthen the idea that IL-10 and IP-10 could be useful diagnostic markers of *P. falciparum* infection during pregnancy. The concentrations of cytokines/chemokines in placental plasma may represent previously unrecognized markers of poor fetal growth.

The World Health Organization reported an estimated 207 million cases of malaria worldwide in 2012 and 627,000 subsequent deaths from the disease, mostly among children below 5 years of age and pregnant women (1). Among the 5 plasmodial species commonly infecting humans, *Plasmodium falciparum* is responsible for the most severe malarial cases and death. People living in areas where malaria is endemic gradually develop, over years or even decades, immunity to the most severe clinical manifestations of *P. falciparum* infection (2), with the exception of women who are pregnant for the first time, who, once they become pregnant, once again become susceptible to malaria and contract pregnancy-associated malaria (PAM), also known as placental malaria, despite previous exposure to non-PAM parasites. Thus, over 50 million women living in areas of endemicity are exposed every year to the risk of developing malaria during pregnancy (3). PAM can lead to severe complications for both the mother and the child. During pregnancy, a new organ appears in the maternal body, namely, the placenta, which displays new receptors, such as chondroitin sulfate A (CSA), mediating adherence of *P. falciparum*-infected erythrocytes (*Pf*-Es) (4). The sequestration of *Pf*-Es, accompanied by the infiltration of monocytes into the placenta, is associated with various outcomes, including low birth weight due to fetal growth restriction and preterm delivery, as well as maternal anemia, with substantial rates of morbidity and mortality (5). Each year, up to 363,000 infants die as a result of malaria-related low birth weight, and at least 10,000 maternal deaths may be attributed to malaria during pregnancy (5).

P. falciparum infection elicits a range of immune responses across the spectrum of innate and adaptive immunity (6), with cytokines playing both a protective role and a pathological role. The balance between pro- and anti-inflammatory cytokines is a key factor in the regulation of an effective immune response to malaria. For instance, gamma interferon (IFN-γ) may be beneficial for the host by promoting the killing of *Pf*-Es by activated monocytes/macrophages. On the other hand, overactivation of macrophages and the consequent release of interleukin-12 (IL-12) and/or tumor necrosis factor alpha (TNF-α) could promote a detrimental inflammatory cascade that could compromise the normal course of pregnancy (7, 8). *P. falciparum* infection increases the placental levels of IFN-γ and TNF-α (9–11), and such a Th1 immune response bias is generally considered to be incompatible with a successful pregnancy (12–14). Remarkably, *P. falciparum* infection differentially modulates the cytokine production of *in vitro*-stimulated mononuclear cells isolated from the intervillous space of women at delivery. The proportion of IFN-γ-producing NK cells appears to be decreased in parasite-infected women compared to uninfected ones (15), whereas the frequency of IFN-γ- and TNF-α-producing T lymphocytes has been reported to be increased during infection, suggesting an important role of T cell activity in protection (16). Furthermore, *P. falcipa-*

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Address correspondence to Nadine Fievet, fievet@ird.fr.

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rum infection seems to favor IL-12 production rather than TNF-α production by placental monocytes/macrophages (16).

In this context, recent studies have investigated a broader spectrum of circulating and/or placental proteins, including cytokines and chemokines, aiming to find potential biomarkers for inflammatory placental malaria (17, 18) and/or possible links with poor PAM-associated pregnancy outcomes (19, 20). For instance, increased concentrations of circulating endoglin and CXCL9 have been associated with fetal growth restriction and low-birth-weight deliveries, respectively (21–23).

Here, we assessed the levels of 16 cytokines/chemokines (IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-8, IFN-γ-inducible protein 10 [IP-10], eotaxin, monocyte chemoattractant protein 1 [MCP-1], MCP-4, macrophage inflammatory protein 1β [MIP-1β], thymus and activation-regulated chemokine [TARC], TNF-α) in a total of 400 placental plasma samples from pregnant Beninese women (a subgroup of the large Strategies to Prevent Pregnancy Associated Malaria [STOPPAM] cohort [24, 25] selected on the basis of pathological pregnancy outcomes) living in an area where malaria is mesoendemic. With the benefit of precise gestational age determination by ultrasound and of newly published African *in utero* weight charts (26), we compared the cytokine/chemokine profiles of different clinical groups segregated according to defined pregnancy outcomes, such as maternal anemia, preterm delivery, and low birth weight for gestational age.

MATERIALS AND METHODS

Study design and participants. We made use of a large subgroup drawn from the STOPPAM cohort (2008 to 2012) of >1,000 pregnant women (24, 25). The STOPPAM project was designed both to enable the accurate quantification of the effects of pregnancy-associated malaria (PAM) on maternal and fetal health in order to optimize strategies for preventive intermittent treatment and to facilitate development of a PAM vaccine. The study took place in Mono Province, located 70 km west of Cotonou, Benin. It is an area where *P. falciparum* transmission is high, with two peaks occurring during the rainy seasons: from April to June and from September to November. Malaria is mesoendemic in this area, and the entomological inoculation rate is 1 to 35 bites per person per year (27). Three dispensaries, Come, Akodeha, and Ouedeme Pedah, were involved in the study, and these dispensaries are 10 km away from each other.

For the current work, 400 Beninese women were selected at delivery according to the pathological outcomes of pregnancy in order to quantify the cytokines/chemokines in their intervillous placental plasma samples.

We selected women at delivery to construct the following groups: mothers infected with *P. falciparum* at delivery, babies with a low birth weight for gestational age (small for gestational age [SGA] babies), preterm babies (PTB), and mothers who were anemic at delivery. These groups were compared with a control group (mothers who were noninfected and nonanemic at delivery and whose babies were delivered at term and had an adequate birth weight for gestational age). In each of the four pathological groups, women were included only if they had one of the specific conditions (for instance, *P. falciparum* infection at delivery for the 1st group) but not the others (i.e., no anemia, no preterm birth, and no SGA for the 1st group). In that way, the effect of one condition on the cytokine level could be assessed independently of the effect of the other conditions. Samples from HIV-positive patients were not used in this study.

Ethics statement. The study received ethical approval from two independent institutional review boards: the Comité Consultatif de Déontologie et d'Éthique of IRD in France and the Comité d'Éthique de la Faculté des Science de la Santé, Université d'Abomey Calavi, in Benin. All the participants involved in our study, that is, the pregnant women for their follow-up and the caretakers of the minors for their follow-up, provided their written informed consent to participate in this study.

Clinical outcomes definition. Babies were classified as preterm if the gestational age, assessed by ultrasound before 24 weeks of gestation, was <37 weeks. Fetal growth restriction was approximated by low birth weight for gestational age. A baby was considered SGA if the birth weight was below the 10th percentile of fetal weight for gestational age according to sex-specific charts (26). Maternal anemia at delivery was defined as a hemoglobin level of less than 10 g/dl. Placental malaria at delivery included both peripheral infection (peripheral malaria positive [$PERI^+$]) and placental infection (placental malaria positive [PLA^+]), as they are known to be highly correlated (28). The diagnosis of peripheral and placental infection relied on routine microscopic examination of thick blood smears. *P. falciparum* infection during pregnancy was assessed by thick blood smear microscopy. All women diagnosed with *P. falciparum* infection were treated with quinine or sulfadoxine-pyrimethamine.

Placental plasma collection procedure. Just after delivery and expulsion of the placenta, the cord was clamped and the placenta was kept in a sterile plate in a clean hood. The midwives rinsed the whole placenta with 1 liter of sterile physiological buffer in order to obtain a cleaned maternal side of the placenta. A 0.5-cm tissue incision and section at the maternal side of the placenta were done in order to make blood smears and to aspirate placental intervillous blood. Plasma samples were separated and were frozen at −80°C until further use.

Cytokine/chemokine quantification. Plasma cytokine/chemokine levels were determined by multiplex absolute quantification using the Meso Scale Diagnostics (MSD) multiarray technology to ensure the maximum detection sensitivity and reproducibility. MSD Multi-Spot plates were used to quantify IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, and TNF-α (human TH1/TH2 10-plex ultrasensitive kit; catalog no. K15010C) and eotaxin, MIP-1β, TARC, IP-10, IL-8, MCP-1, and MCP-4 (human chemokine 7-plex ultrasensitive kit, catalog no. K15031C). Cytokines and chemokines were detected according to the manufacturer's instructions. A 25-μl nondiluted placental plasma sample was used in each plate well. Plate readings were performed on a Sector imager, and raw data were analyzed using MSD Discovery Workbench software (version 4.0), which utilizes a 4-parameter logistic model (or a sigmoidal dose [X]-response [Y] model) and includes a $1/Y^2$ weighting function that provides a better fit of the data over a wide dynamic range.

Statistical analysis. The distribution of each cytokine/chemokine was compared between each of the four study groups (mothers with *P. falciparum* infection at delivery, SGA babies and/or PTB, anemic mothers at delivery) and the control group using the Kruskal-Wallis test (two-by-two comparisons). Cytokine and chemokine distributions were also compared between women not infected during pregnancy, women infected only once during pregnancy (but not at delivery), and those infected at least twice during pregnancy using the Kruskal-Wallis test. To correct for the high number of statistical tests, Bonferroni's correction was applied. Due to the large number of tests performed, only P values of ≤0.0006 were considered statistically significant after correction. In a second approach, we modeled the level of each cytokine/chemokine according to the four conditions (i.e., SGA, preterm birth, maternal anemia, and placental malaria at delivery). For this analysis, the crude variables rather than subgroups segregated according to pathological outcomes were used in order to assess the independent effects of each outcome, adjusted on the basis of each of the others, on the immunological parameters. For each cytokine/chemokine of interest, the analysis was performed using a two-step procedure. In the first step, all factors were included in the model, along with gravidity. A backward stepwise procedure was used to select those factors that improved the model's goodness of fit using the Akaike information criterion (AIC). At this point, no statistical tests were performed. In a second step, only the association between the cytokine/chemokine and the factor that remained from the first step of the model was tested after adjustment for gravidity and the other factors of interest (SGA, preterm birth, *P. falciparum* infection, maternal anemia). Due to the low number

TABLE 1 Women's and children's characteristics at inclusion and at delivery

Group and characteristic	Value
Women	
Mean age (yr)	26 (25–26[a])
No. (%) of women with the following:	
Primigravidity	77 (19)
No education	229 (57)
Malaria at inclusion[b]	74 (19)
Mean gestational age at inclusion[c] (days)	116 (113–119[a])
No. of malarial infections during pregnancy[b]	
0	204 (51)
1	117 (29)
≥2	79 (20)
Mean gestational age at delivery[c] (days)	277 (276–278[a])
No. of women with the following at delivery/total no. of women tested (%):	
Malaria infection (placental or peripheral)[b]	71/387 (18)
Anemia (hemoglobin concn < 10 g/dl)	82/375 (22)
Children	
No. of children with low birth wt for gestational age[d]/total no. of children tested (%)	73/390 (19)
No. of children with preterm birth[e]/total no. of children tested (%)	28/391 (7)

[a] Data in parentheses represent 95% confidence intervals.
[b] On the basis of thick blood smear microscopy.
[c] On the basis of an ultrasound examination performed before the 24th week of gestation.
[d] According to the charts of Schmiegelow et al. (26).
[e] Preterm birth was birth before 37 weeks of gestation.

TABLE 2 Overall detection of cytokines/chemokines[a]

Cytokine or chemokine	No. of placental plasma samples	Concn (pg/ml)			Mean (SD) lower detection limit (pg/ml)
		Mean	Median	Range	
Eotaxin	400	193.9	194.6	6.0–513.6	1.36 (0.58)
IFN-γ	400	46.8	23.0	2.1–415.8	1.33 (0.20)
IL-10	400	70.2	36.8	1.0–853.1	0.81 (0.35)
IL-12p70	400	21.8	15.1	0.4–102.5	1.01 (0.35)
IL-13	400	77.8	63.2	2–250.2	6.37 (1.77)
IL-1β	374	1,314.7	73.7	1.3–9,968.3	0.30 (0.05)
IL-2	400	20.2	11.9	0.9–153.8	0.34 (0.05)
IL-4	400	11.6	7.7	0.2–54.4	0.78 (0.53)
IL-5	400	11.1	6.3	0.1–45.8	0.28 (0.06)
IL-8	243	2,567.4	1,531.2	11.2–9,982.1	0.26 (0.04)
IP-10	399	923.1	491.0	23.5–9,428.5	2.74 (0.69)
MCP-1	390	1,337.7	478.7	23.1–9,455.7	0.37 (0.05)
MCP-4	400	361.7	311.3	16.1-1,572.2	3.54 (1.41)
MIP-1β	396	4,857.2	740.2	17.7–39,287.4	2.43 (1.04)
TARC	400	993.3	669.8	23.9–17,573.5	5.67 (3.97)
TNF-α	400	211.7	46.6	2.6–5,660.0	0.67 (0.28)

[a] The mean and median concentrations and the range of concentrations of each cytokine/chemokine, along with the lower detection limits of each assay, are displayed.

of statistical tests performed at this step, Bonferroni's correction was not applied.

RESULTS

Women's and children's characteristics. The STOPPAM cohort subgroup used in this work comprised 400 Beninese pregnant women (mean age, 26 years) followed up during the course of pregnancy (Table 1). The selected placental plasma samples collected, combined with the detailed clinical characteristics of the women (Table 1), represent a unique resource to investigate if a particular cytokine/chemokine profile present in the placenta at the time of delivery was linked to the clinical outcomes of the mother and the child.

Among the 400 women, 77 (19%) were primigravid. The mean gestational age at inclusion was 116 days, and the mean gestational age at delivery was 277 days. During the course of pregnancy, 204 (51%) women had no detectable evidence of plasmodial infection, whereas 117 (29%) women had one infection and 79 (20%) women had two or more infections. Eighteen percent of women were infected with *P. falciparum* at delivery, and 22% were anemic. Preterm births represented 7% of cases, whereas 19% of newborns had a low birth weight for gestational age.

Cytokine/chemokine distribution by clinical group. In order to determine an absolute quantification of the cytokines/chemokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, TNF-α, eotaxin, MIP-1β, TARC, IP-10, MCP-1, MCP-4) present in placental plasma samples at the time of delivery, we performed multiplex quantification of nondiluted samples using ultrasensitive Multi-Spot plates with detection limits ranging from 0.26 to 6.37 pg/ml, depending on the cytokine/chemokine (Table 2). The overall distribution of each detected cytokine/chemokine is given in Fig. 1 and Table 2.

We then compared the distribution of each cytokine/chemokine between the controls, defined as mothers who were *P. falciparum* uninfected and nonanemic at the time of delivery of term babies with an adequate birth weight for gestational age, and each of the other groups presenting different clinical outcomes for either the mother or the offspring ($PLA^+/PERI^+$, SGA, preterm birth, and maternal anemia) (Table 3). We observed a trend for the distribution of some cytokines/chemokines among the different groups. That is, the levels of the anti-inflammatory/regulatory cytokine IL-10 tended to be higher in the placental plasma of women with *P. falciparum* infection at the time of delivery than in the placental plasma of women in the control group. Of particular note, the concentrations of a number of cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-12, IL-13) were lower in the SGA group than in the control group. Even though the associated *P* values did not reach statistical significance after Bonferroni's correction, these trends merit discussion. In the case of preterm birth (data not shown) and of anemia, there were no differences in the concentrations of any cytokine or chemokine compared to those in the control group.

Cytokine/chemokine distribution by number of *P. falciparum* infections during the course of pregnancy. After comparing the distribution of each cytokine/chemokine between the control and the $PLA^+/PERI^+$, SGA, preterm birth, and maternal anemia groups, we then investigated the association between the number of *P. falciparum* infections during the course of pregnancy (none, one infection, two or more infections) and the cytokine/chemokine profile in the placental plasma collected at the time of delivery. As described above, this analysis did not lead to any statistically significant findings after Bonferroni's correction. Nevertheless, we observed a trend for some cytokines/chemokines. The concentrations of IFN-γ, IL-1β, IL-2, IL-4, IL-12, and IL-13 all tended to be lower in the groups with *P. falciparum* in-

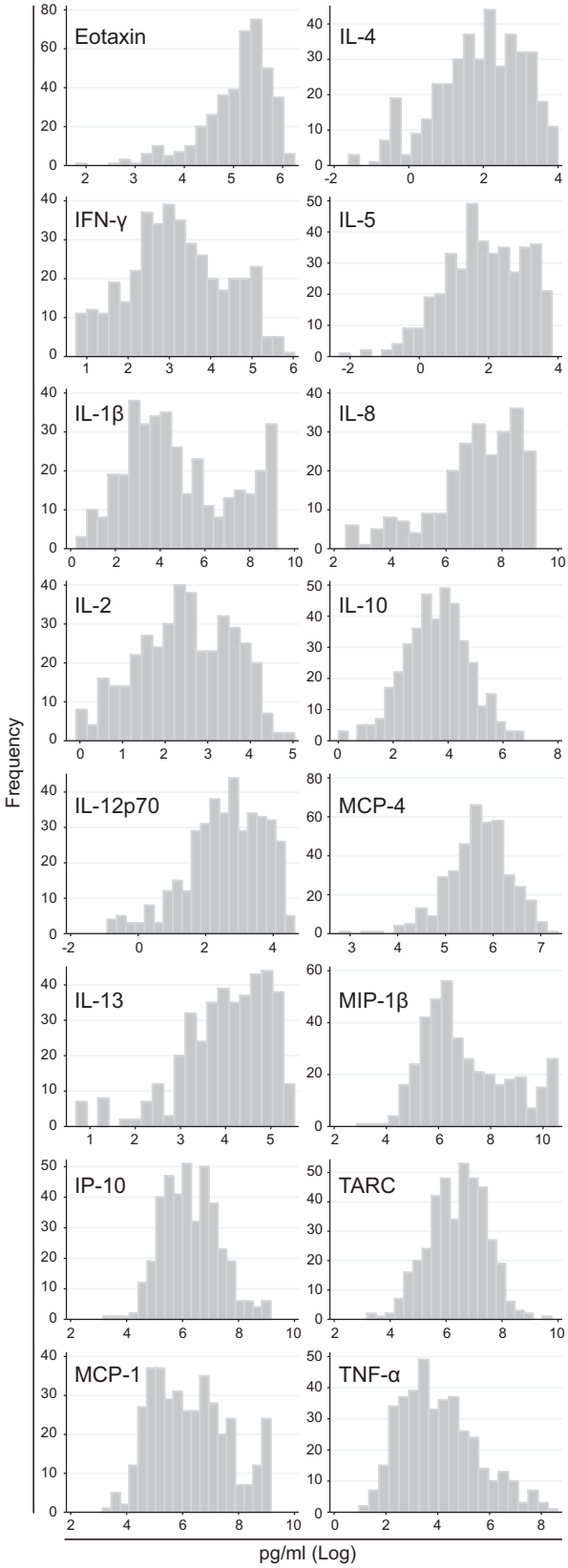

FIG 1 Overall distribution of cytokines/chemokines. For each cytokine/chemokine, the frequency distribution of individuals is illustrated according to the plasma concentration.

fection during pregnancy, contrasting notably with the IP-10 concentration, which appeared to increase as a function of infection (Table 4).

Clinical factors associated with cytokine/chemokine concentrations using multivariate analysis. Our initial statistical analyses exploring the potential association between specific cytokine/chemokine profiles and clinical outcomes did not reveal any significant results after correction for multiple tests, although some distinct trends were evident (Tables 3 and 4). In order to assess the independent effect of each clinical parameter and to bypass the need to use the strict Bonferroni's correction, which could hide real associations, we used novel and original multivariate analyses to reveal factors linked to particular cytokine/chemokine signatures. After adjusting for SGA, preterm birth, and maternal anemia and gravidity, we observed that IL-10 concentrations were significantly higher in the placental plasma of *P. falciparum*-infected women ($PLA^+/PERI^+$) than in the placental plasma of uninfected women at the time of delivery ($P = 0.006$). In addition, the concentrations of both IFN-γ and IL-5 were significantly lower in the placental plasma of mothers who gave birth to babies with a low birth weight for gestational age ($P = 0.007$ and $P = 0.003$, respectively) after adjustment for preterm birth, *P. falciparum* infection at delivery, maternal anemia, and gravidity (Table 5).

DISCUSSION

Among the 10 cytokines and 6 chemokines assessed in this study, multivariate analyses showed that IL-10 was the only one present at significantly higher concentrations in the intervillous plasma of *P. falciparum*-infected women than in that of uninfected women at delivery, while lower concentrations of both IFN-γ and IL-5 were detected in the intervillous plasma of mothers who gave birth to babies with a low birth weight for gestational age (SGA babies) independently of the occurrence of *P. falciparum* infection. Low levels of placental IFN-γ and IL-5 at delivery could thus represent a cytokine signature reflecting the pathological development/induction of SGA during pregnancy. The higher placental IL-10 and IP-10 concentrations associated with PAM that we observed here are consistent with our own earlier observations concerning cytokine/chemokine levels in the peripheral plasma of STOPPAM cohort subgroups (18, 29) and confirm the findings of other studies (17, 30). The present results thus further strengthen the idea that IL-10 and IP-10 could serve as useful diagnostic markers of *P. falciparum* infection during pregnancy.

That we did not observe radically altered (elevated) proinflammatory cytokine/chemokine profiles (e.g., those for TNF-α, IFN-γ, and MCP-1) that have previously been reported to be associated with PAM-related outcomes (9, 10, 13), including low birth weight, may at first sight appear to be contradictory, if not illusory. Our use of state-of-the-art equipment allied with longitudinal monitoring that allowed the precise measurement of gestational age might explain, at least in part, the differences from the findings of other studies that we observed. Furthermore, differences in the transmission characteristics of malaria between our site in Benin and those at other study sites should also be taken into consideration. Our own interpretation of our data is also based on the fact that, in our STOPPAM cohort, parasite genotypic analyses have proven that infections at delivery were recently acquired, i.e., within 4 weeks of delivery (N. T. Ndam, unpublished data). The comparatively low parasitemias present, coupled

TABLE 3 Cytokine/chemokine concentrations by clinical group[a]

Cytokine or chemokine	Controls ($n = 190$)		PLA$^+$/PERI$^+$ mothers at delivery ($n = 43$)			PTB ($n = 24$)			SGA babies ($n = 80$)			Anemic mothers ($n = 59$)		
	Median	IQ	Median	IQ	*P* value	Median	IQ	*P* value	Median	IQ	*P* value	Median	IQ	*P* value
Eotaxin	5.31	4.80–5.53	5.29	4.81–5.55	0.99	5.12	4.77–5.37	0.18	5.19	4.53–5.57	0.42	5.29	4.82–5.55	0.84
IFN-γ	3.32	2.52–4.08	3.25	2.61–4.65	0.67	2.87	2.03–4.10	0.41	2.73	2.27–3.43	0.002	3.14	2.45–4.30	0.89
IL-10	3.63	2.79–4.27	4.16	3.16–4.60	0.02	3.32	2.81–4.51	0.89	3.31	2.52–4.07	0.07	3.6	2.93–4.68	0.74
IL-12p70	2.87	1.97–3.54	2.65	2.03–3.62	0.85	2.52	1.88–3.43	0.41	2.23	1.72–3.02	0.005	2.84	2.06–3.64	0.98
IL-13	4.24	3.51–4.81	4.26	3.48–4.87	0.55	3.94	3.56–4.75	0.48	3.85	3.31–4.44	0.009	4.26	3.32–4.90	0.68
IL-1β	4.36	3.12–6.95	4.52	3.20–7.92	0.66	5.01	2.99–7.41	0.8	3.91	2.90–5.85	0.08	4.41	3.09–6.63	0.98
IL-2	2.57	1.74–3.39	2.65	1.61–3.58	0.71	2.23	1.51–3.30	0.46	2.06	1.49–2.77	0.009	2.6	1.81–3.66	0.88
IL-4	2.12	1.30–2.89	1.93	1.41–2.88	0.78	1.85	0.90–2.88	0.34	1.58	1.29–2.88	0.005	2.17	1.30–3.07	0.69
IL-5	1.99	1.30–2.90	2.19	1.12–3.03	0.54	1.81	0.90–2.74	0.83	1.43	0.84–2.20	0.002	2.07	1.30–3.02	0.6
IL-8	7.37	6.34–8.30	6.88	5.19–8.42	0.54	7.21	6.37–8.07	0.65	7.33	6.41–8.13	0.7	7.57	6.46–8.45	0.61
IP-10	6.08	5.38–6.86	6.12	5.64–6.85	0.57	6.86	5.90–7.32	0.01	6.39	5.77–7.06	0.04	6.23	5.44–6.86	0.58
MCP-1	6	5.06–7.18	6.53	5.39–6.98	0.5	5.99	5.06–7.17	0.09	6.17	5.01–7.26	0.9	6.13	5.13–7.20	0.75
MCP-4	5.73	5.36–6.08	5.72	5.28–6.14	0.97	5.72	5.28–6.24	0.91	5.75	5.26–6.14	0.83	5.81	5.38–6.12	0.55
MIP-1β	6.61	5.67–8.41	7.51	6.26–8.38	0.11	6.69	5.98–8.66	0.56	6.27	5.76–7.02	0.1	6.77	6.06–8.59	0.33
TARC	6.58	5.75–7.11	6.49	5.69–7.42	0.92	6	5.34–6.69	0.04	6.24	5.39–7.26	0.17	6.7	5.99–7.37	0.28
TNF-α	3.96	2.96–4.94	4.26	2.96–5.46	0.32	4.06	2.80–4.93	0.81	3.48	2.72–4.68	0.13	3.81	3.11–5.27	0.56

[a] The concentration of each cytokine/chemokine in the control group (*P. falciparum*-uninfected, nonanemic mothers at the time of delivery of term babies with an adequate birth weight for gestational age) was compared with the concentration of each cytokine/chemokine in the four defined groups (PLA$^+$/PERI$^+$ mothers, SGA babies, PTB, and anemic mothers) using the Kruskal-Wallis test. The cytokine and chemokine data are presented as the log-transformed median value with the interquartile range (IQ). *P* values refer to separate comparisons with the control group. *P* values of ≤0.0006 were regarded as statistically significant.

with the absence of malarial pigment (hemozoin)-containing cells in the intervillous placental blood of these women (N. Fievet et al., unpublished data), is also entirely consistent with these infections being of relatively recent origin. In this context, the close follow-up of mothers that was integral to the STOPPAM project's design did not allow the establishment of chronic infection at delivery, as the women were treated each time that infection was detected. We conclude, then, that the commonly reported pathological inflammatory changes, including a range of altered cytokine/chemokine profiles in the placentas of *P. falciparum*-infected women presenting with chronic infections, had not had time to occur in our cohort, and thus, the associations with poor pregnancy outcomes previously reported in the literature could not be shown (9–11, 13). Our data in this regard thus serve to highlight the benefits of frequent antimalarial treatment during pregnancy, since inflammatory responses that are potentially detrimental for the fetus are minimized.

Interestingly, we observed that, although the differences were not statistically significant at a strict level, the concentrations of both Th1- and Th2-type cytokines in the placenta at delivery

TABLE 4 Cytokine/chemokine concentrations segregated according to the number of *P. falciparum* infections during the course of pregnancy[a]

Cytokine or chemokine	No infection ($n = 204$)		One infection ($n = 117$)		Two or more infections ($n = 79$)		
	Median	IQ	Median	IQ	Median	IQ	*P* value
Eotaxin	5.20	4.70–5.50	5.29	4.76–5.55	5.41	4.87–5.63	0.14
IFN-γ	3.31	2.53–4.21	2.89	2.17–3.84	2.84	2.24–3.77	0.03
IL-10	3.68	2.82–4.43	3.62	2.80–4.22	3.40	2.63–4.29	0.56
IL-12p70	2.90	2.06–3.53	2.48	1.77–3.42	2.44	1.84–3.16	0.05
IL-13	4.29	3.51–4.83	3.97	3.31–4.65	4.04	3.51–4.53	0.09
IL-1β	4.72	3.06–7.56	3.92	2.87–5.18	4.47	3.32–5.84	0.05
IL-2	2.71	1.72–3.48	2.24	1.60–3.21	2.24	1.50–2.98	0.02
IL-4	2.22	1.39–2.92	1.70	1.14–2.74	1.83	1.09–2.48	0.01
IL-5	2.09	1.19–2.90	1.65	0.91–2.67	1.69	0.96–2.53	0.17
IL-8	7.12	6.19–8.15	7.39	6.34–8.47	7.77	6.81–8.38	0.22
IP-10	6.09	5.37–6.79	6.65	5.84–7.22	6.16	5.61–7.10	0.001
MCP-1	5.97	5.07–7.20	6.44	5.11–7.20	6.30	5.24–7.22	0.40
MCP-4	5.75	5.27–6.12	5.67	5.38–6.17	5.82	5.46–6.18	0.56
MIP-1β	6.77	5.79–8.59	6.39	5.78–7.71	6.57	6.06–7.92	0.39
TARC	6.52	5.77–7.13	6.58	5.61–7.15	6.21	5.57–7.27	0.67
TNF-α	4.01	3.06–5.04	3.58	2.71–4.61	3.62	3.07–4.66	0.12

[a] The concentration of each cytokine/chemokine was compared between women not infected during pregnancy (no infection), women infected only once during pregnancy but not at delivery (one infection), and women infected at least twice during pregnancy (two or more infections) using the Kruskal-Wallis test. The cytokine and chemokine data are presented as the log-transformed median value with the interquartile range (IQ). The *P* values refer to the global comparison of the three groups. *P* values of ≤0.0006 were regarded as statistically significant.

TABLE 5 Factors associated with cytokine/chemokine concentrations using multivariate analysis[a]

Cytokine or chemokine	Factor(s) significantly associated at 5% level	Coefficient	*P* value
IFN-γ ($n = 337$)	SGA	−0.43	0.007
IL-10 ($n = 337$)	$PLA^+/PERI^+$	0.46	0.006
IL-5 ($n = 337$)	SGA	−0.47	0.003

[a] The analyses employed a two-step multivariate analysis procedure. In the first step, all factors were included in the model, along with gravidity. A backward stepwise procedure was used to select those factors that improved the model's goodness of fit using the AIC. At this point, no statistical tests were performed. In a second step, only the association between the cytokine/chemokine and the factor that remained from the first step was tested using a multivariable linear model after adjustment for gravidity and the other factors of interest (SGA, preterm birth, *P. falciparum* infection, maternal anemia). The coefficient represents the average variation of the logarithm of the cytokine in women with (versus without) a baby who was SGA or *P. falciparum* infection at delivery, after adjustment for preterm birth, anemia at delivery, gravidity, infection at delivery (for IFN-γ and IL-5), and having a baby who was SGA (for IL-10). *P* values of ≤0.05 were regarded as statistically significant.

tended to decrease as a function of *P. falciparum* infections occurring during the course of pregnancy, whereas chemokine levels, best illustrated by the profile of IP-10, were either stable or tended to increase. That several T cell-derived cytokines appeared to be affected (diminished) in this way as a result of *P. falciparum* infection is intriguing. A speculative interpretation would be that T cells present in the placenta are rendered less active as a result of (treated) infections, perhaps reflecting the sustained levels of IL-10 present. If this interpretation is true, the consequences of these changes in T cell activity for the pregnancy merit further investigation (see the discussion of SGA below). The results of separate analyses of peripheral blood cellular profiles during the course of pregnancy in the STOPPAM cohort, results that we have published elsewhere, revealed a significant decrease in the number of circulating $CD4^+$ T cells in peripheral blood, along with a trend toward reduced monocyte activation, at delivery (31). Whether or not these findings are linked in any way to the placental T cell cytokine-specific effects that we report here remains to be determined.

It should be noted that application of Bonferroni's correction is one of the most commonly used methods to overcome the difficulties linked to the occurrence of multiple comparisons during statistical analyses. When variables are highly correlated, as was the case here, this stringent method could obscure real associations due to the very low *P* values set as the cutoff for significance. We therefore reported the results of initial analyses highlighting the associations, before Bonferroni's correction, that could be regarded, though with caution, as possible trends. To obviate the severe penalties from the use of Bonferroni's correction and to take into consideration the highly linked variables, we used a novel two-step multivariate analytical method. This revealed the presence of a statistically significant cytokine signature (elevated levels of IL-10 and diminished levels of IFN-γ and IL-5) in placental plasma at delivery that reflected the pathological development/induction of SGA during pregnancy.

As was the case for *P. falciparum* infection, our initial analyses revealed decreased placental plasma concentrations of a broad range of cytokines associated with SGA. It is known that deregulation of the immune system in early pregnancy is associated with adverse pregnancy outcomes, such as intrauterine growth retardation (IUGR), that can result in babies who are SGA (32). A generally stronger proinflammatory bias has been reported in a study of peripheral blood cells, tested at delivery, of women with babies with IUGR (33). However, the same study reported a relatively lower IFN-γ/IL-10 ratio from cells from women with IUGR and normal placental blood flow than from cells from those with normal pregnancy, as well as the production of lower levels of IL-13 from the cells of women in the IUGR group overall. Our own findings concerning the significantly altered profiles of IFN-γ and IL-5 (a Th2′-type anti-inflammatory cytokine in the same family as IL-13) in association with SGA thus appear to be consistent with those of Raghupathy et al. (33). Nevertheless, the particular functional relevance of these associations remains unclear. However, the suggestion from our data, as discussed above, of a suppressive effect of *P. falciparum* infection, reflected by the concentrations of different T cell-derived cytokines in placental plasma at delivery, requires further investigation in this context. There are striking similarities, furthermore, between our findings concerning cytokine concentrations and those of a recently published study using a murine model of placental malaria infection (34). This appears to strengthen the idea that infection-related suppression of T cell activity—regardless of whether it is the cause or the effect—may play a role in determining pregnancy outcomes.

Confirmation of the findings that we report here will, in any case, require both more detailed analyses of the current data set and further more detailed studies of IUGR in our setting to determine the proportions of individuals with and without abnormal placental blood flow. Clearly warranted, in the same context, are assessments of maternal cellular immunological activity during the whole course of pregnancy. Such assessments should be performed as a function of the spectrum of poor pregnancy outcomes, which may or may not result directly from infection with the parasite, encountered in areas where *P. falciparum* is endemic.

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