Cytokine profiles in peripheral, placental and cord blood in pregnant women from an area endemic for *Plasmodium falciparum*

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ABSTRACT. During gestation, inflammatory cytokines are sometimes more abundant than growth-promoting cytokines, and via direct or indirect effects, proinflammatory cytokines lead to intrauterine growth retardation. We used an enzyme-linked immunosorbent assay to measure the concentrations of three proinflammatory cytokines, tumor necrosis factor alpha (TNF-α), interleukin-12 (IL-12p40), as well as interleukin-15 (IL-15) and monocyte chemotactic protein-1 (MCP-1), in plasma from peripheral, placental and cord blood of thirty pregnant Gabonese women. All of these women lived in Libreville and Lambaréné, two malaria hyperendemic areas. IL-12p40 concentrations were higher in cord blood than in placental or peripheral blood. The MCP-1 concentration was higher in placental blood, than in peripheral or cord blood. IL-15 concentrations were similar at the three sites. MCP-1 concentrations were higher in the placentas of primiparous women than in those of multiparous women. The highest concentrations were found in infected placentas. IL-15 concentrations were significantly higher in peripheral and placental plasma from uninfected women than in plasma from infected women. Strong positive correlations were found between placental and cord IL-12p40 and IL-15 plasma concentrations. Likewise, a strong positive correlation was found between IL-12p40 and MCP-1 concentrations in cord and peripheral plasma. These results suggest that placental, maternal peripheral and cord blood present different cytokine profiles in response to *P. falciparum*.

Keywords: placental, cord blood, pregnant women, *Plasmodium falciparum*, MCP-1, cytokine

INTRODUCTION

Malaria is endemic in more than 100 countries. Over two billion people living in these countries are exposed to infection. Children and pregnant women are at increased risk of *Plasmodium falciparum* malaria and its associated complications. Approximately 24 million women become pregnant each year in malaria-endemic regions [1,2]. The complications of malaria for mother and fetus are considerable, and constitute one of Africa's main public health problems.

Acute maternal illness, maternal death and spontaneous abortions are frequent in areas of low malaria endemicity, but rare in areas of high endemicity. The immune system of pregnant women may be biased toward type 2 humoral defense mechanisms rather than towards type 1 cellular responses because the latter may compromise the viability of the fetus [3]. However, the "Th-2 phenomenon" seems now to be an oversimplification. Several studies pointed out the production of a Th-2 type cytokine (IL-4) and a Th1 type cytokine (IFN-γ) by stimulated lymphocytes *in vitro* looking for a shift towards Th-1 [4-6]. Lymphocytes, however, are only one component of the immune system. Indeed, it has been demonstrated that circulating monocytes during normal pregnancy, are primed to produce more Th-1 cytokine [7]. In areas of low endemicity, acquired immunity to malaria does not occur, and infection is controlled solely by type 1 responses. As this type of response is suppressed during pregnancy, parasite loads are higher, causing certain symptoms, including spontaneous abortion. In highly endemic areas this scenario is less likely because acquired immunity is established by the time women reach child-bearing age.

Although the relationship between maternal malaria and poor fetal outcome is recognized, the basis for the association is not clear. Placental malaria is associated with cell-mediated inflammatory responses [8]. Changes in the levels of cytokines were recently reported in women with malaria at delivery [9].The placental production of chemokines, especially monocyte chemotactic protein 1

(MCP-1), may be an important trigger for monocyte accumulation in the placenta [10]. *P. falciparum* is found in the cord blood of up to 16% of infants born to infected mothers [11], but rarely establishes a circulating congenital infection, suggesting that the parasite elicits fetal disease through other mediators.

In this study, we attempted to identify the cytokine pattern displayed by pregnant women living in Gabon, a hyperendemic region, at the time of delivery.

PATIENTS AND METHODS

Study site, participants and blood sample collection

This study took place in Libreville and Lambaréné, two cities in Gabon where *P. falciparum* malaria is hyperendemic with a perennial mode of transmission and low seasonal fluctuations. The entomological inoculation rate (EIR) measured in and around Lambaréné is about 50 infective bites per person and per year [12], and the main vectors are *Anopheles gambiae* and *Anopheles moucheti*.

The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital, Lambaréné, Gabon.

Peripheral, placental and cord blood samples were obtained from 30 women who had had uncomplicated pregnancies and gave birth via the vaginal route. All blood samples were collected in heparinized tubes. Peripheral blood was collected by venipuncture, cord blood directly after umbilical section and placental blood by the biopsy-pool method (within one hour of delivery). Briefly, a block of tissue (5 cm × 5 cm × 5 cm) was excised from the cleaned maternal surface of the placenta, resulting in a large pool of intervillous blood at the excision site. This blood was quickly aspirated with a sterile pipette and withdrawn in heparinized tube.

To assess the fetal cell contamination of the placental intervillous blood, placental blood smears were stained for fetal hemoglobin using a fetal cell stain kit (Simmler, St Louis, MO, USA), and contamination measured by counting the number of positively stained erythrocytes/1000 erythrocytes was < 4%.

Within a few hours of collection, blood samples (2 ml) from each compartment were collected, centrifuged for 10 minutes at 400 *g* using a Biofuge Pico centrifuge (Heraeus Instruments, Hanau, Germany) to separate the pellet containing the packed erythrocytes from the plasma, and were frozen at – 80°C until assays.

Malaria diagnosis

Peripheral, placental and cord blood parasitemias were determined by examination of Giemsa-stained blood smears and placental impression smears, by two microscopists following standard, quality-controlled procedures [13]. Based on the presence or absence of parasites in placental impression smears and smears of placental blood, the women were classified as positive or negative for placental malaria. Hemozoin pigments in leukocytes with parasites were found in addition in placental smears of four women. All women with peripheral parasitemia received quinine.

Measurement of cytokines and chemokines in peripheral, placental and cord plasma

Plasma samples obtained at enrollment were analyzed by standard sandwich enzyme-linked immunosorbent assay (ELISA) for interleukin-12 (IL-12 p40) and interleukin-15 (IL-15) using pairs of cytokine-specific, monoclonal antibodies according to the manufacturer's instructions (Biosource International, Nurtingen, Germany). Monocyte Chemoattractant Protein-1 (MCP-1) was measured using pairs of capture and biotinylated specific monoclonal antibodies (Beckman Coulter, Krefeld, Germany). Each plate included a standard curve of recombinant human cytokine. All specimens were measured in duplicate and the mean value was used in all analyses. Detection limits were 7 pg/ml for IL-12p40, 5 pg/ml for TNF-α 16 pg/ml for IL-15 and 10 pg/ml for MCP-1.

Statistical analysis

Non-parametric tests were used to analyze the data. Correlations between continuous variables were assessed by the Spearman rank test, corrected for ties, where a value of Rho > 0.25 (combined with $P < 0.05$) was considered significant. The non-parametric Wilcoxon signed rank test was used to test the significance of differences between paired continuous variables. The Mann-Whitney U (2-group comparisons) or Kruskal-Wallis (> 2-group comparisons) tests were used to determine the significance of differences between unpaired continuous variables. *Post-hoc* test for multiple means comparisons was used for multivariate analysis. Two-tailed *P* values of less than 0.05 were considered to be significant.

RESULTS

Patients characteristics

Samples from 30 women (10 primiparous and 20 multiparous) were analyzed in this study. The mean gravidity was three in the multiparous group. Ten women (seven primiparous women and three multiparous women) had peripheral parasitemia, eight (six primiparous and two multiparous) had placental malaria and two cord samples from two primiparous with peripheral and placental malaria were infected.

Cytokine concentrations differ between peripheral, placental and cord plasma

The concentrations of IL-12p40, IL-15, TNF-α and MCP-1 were measured in peripheral, placental and cord plasma from all the recruited women *(Table 1)*. Cord plasma contained significantly more IL-12p40 than placental or peripheral plasma ($P < 0.001$). The difference between placental and peripheral concentrations was also significant ($P < 0.001$). The MCP-1 concentration was significantly higher in the placenta plasma than in the peripheral ($P = 0.003$) or cord plasma ($P < 0.001$). The same trend was observed for TNF-α concentrations ($P = 0.07$) *(Table 1)*. IL-15 concentrations were similar in the samples from the three sites *(Table 1)*.

Cytokine concentrations from peripheral, placental and cord plasma based on gravidity

When the three compartments were compared according to the gravidity, similar patterns to those described above

Table 1
Plasma cytokine concentrations in 30 pregnant Gabonese women (10 primiparous and 20 multiparous) at the time of delivery

Cytokines, pg/ml, median [interquartile range]	Peripheral	Placental	Cord	*P**
IL-12p40 (total)	94 [77-120]	238 [177-329]	279 [237-415]	< 0.001
Primiparous	96 [87-111]	190 [174-312]	253 [228-473]	< 0.001
Multiparous	92 [74-121]	268 [181-329]	315 [241-413]	< 0.001
IL-15 (total)	200 [162-243]	203 [169-232]	189 [163-228]	0.69
Primiparous	175 [164-221]	189 [166-204]	192 [166-226]	0.85
Multiparous	219 [147-254]	214 [173-238]	181 [157-230]	0.52
TNF-α (total)	16 [11-26]	20 [17-31]	18 [12-24]	0.07
Primiparous	15 [10-19]	20 [17-23]	24 [16-28]	0.13
Multiparous	16 [11-28]	22 [17-36]	17 [12-20]	0.07
MCP-1 (total)	422 [268-1270]	2228 [1137-3216]	322 [255-687]	< 0.001
Primiparous	285 [260-404]	2491 [1803-3034]	326 [275-665]	0.001
Multiparous	510 [288-1566]	1985 [1130-3331]	314 [250-735]	< 0.001

* *P* value.

were observed. Nevertheless, placental plasma from multiparous women contained significantly more IL-15 than did cord plasma from the same women ($P = 0.008$). Overall, placental plasma concentrations of MCP-1 were significantly higher in primiparous women ($P = 0.001$) and multiparous ($P < 0.001$) *(Table 1)* than in peripheral and cord plasma concentrations. TNF-α concentrations in cord samples were slightly, but not significantly higher ($P = 0.07$) in primiparous women than in multiparous women. *(Table 1)*.

IL-15 and MCP-1 peripheral and placental plasma concentrations differ according to infection status

Peripheral and placental plasma IL-15 concentrations were significantly higher in uninfected women than in women infected with *P. falciparum* ($P = 0.036$ and $P = 0.045$ respectively) *(Table 2)*. In contrast, the concentration of MCP-1 was higher in placental plasma from infected women than in that from uninfected women ($P = 0.002$).

Infected primiparous women had significantly higher peripheral plasma concentrations of IL-12p40 (96 pg/ml *versus* 68 pg/ml) ($P = 0.017$) compared to infected, multiparous women. The IL-12p40 concentration was significantly higher in peripheral plasma from uninfected multiparous women (98 pg/ml) than other women ($P = 0.017$). Likewise, the peripheral IL-15 concentration was slightly but not significantly, higher in uninfected, multiparous women (224 pg/ml). MCP-1 concentrations were also higher in parasitized placentas from primiparous women (2568 pg/ml) than in those from their uninfected counterparts (2010 pg/ml). Placental IL-12p40 plasma levels were significantly higher in uninfected multipars (201pg/ml *versus* 165 pg/ml) ($P = 0.03$).

After multivariate analysis, gravidity was not associated with IL-15, MCP-1 and TNF-α concentrations when comparing primigravidae and multigravidae cytokine concentrations inside each compartment. This analysis confirmed the association between placental MCP-1 concentrations and placental malaria infection ($P = 0.028$).

Correlation between peripheral, placental and cord plasma concentrations of cytokines

We found strong positive correlations between placental and cord IL-12p40 (rho = 0.70 ; $P = 0.002$) and IL-15 (rho = 0.74 ; $P < 0.001$) plasma concentrations. Similarly, significant positive correlations were found between IL-12p40 and MCP-1 concentrations in cord and peripheral plasma (rho = 0.38 and $P = 0.045$ for IL-12p40 and

Table 2
Plasma cytokine levels evaluated in uninfected ($n = 20$) and *P. falciparum*-infected ($n = 10$) women at the delivery

Cytokines, pg/ml, median [interquartile range]	Peripheral	Placental	Cord	*P**
IL-12p40				
Infected	89 [70-111]	204 [161-321]	326 [180-473]	< 0.001
Uninfected	97 [81-121]	259 [177-329]	315 [239-413]	< 0.001
IL-15				
Infected	163 [155-182]	174 [165-202]	159 [156-163]	0.2
Uninfected	224 [172-254]	213 [177-236]	192 [166-228]	0.6
TNF-α				
Infected	14 [11-22]	20 [17-28]	21 [16-27]	0.33
Uninfected	16 [12-27]	21 [17-32]	18 [12-24]	0.17
MCP-1				
Infected	285 [260-404]	2491 [1429-3366]	322 [246-1193]	0.002
Uninfected	510 [336-1566]	1971 [1130-2935]	315 [253-593]	< 0.001

* *P* value.

rho = 0.50 and $P = 0.011$ for MCP-1). There was no correlation between TNF concentrations at the three sites.

DISCUSSION

Pregnancy is associated with local and systemic bias of the maternal immune response towards a Th-2 response that may leave the mother more vulnerable to Th-1-dependent infections such as malaria. It was recently suggested that the ability of *P. falciparum* to adhere to the chondroitin sulfate A expressed in the trophoblast plays a role in the susceptibility of pregnant women to malaria [14, 15]. However, it has been postulated that an immunosuppressed status, either general or local, is the major underlying determinant, although the basis of suppression has not been clearly defined [16, 17]. We therefore determined the endogenous cytokine concentrations in peripheral, placental and cord blood from *P. falciparum*-infected and uninfected pregnant women living in G abon, a malaria hyperendemic country, during single, uncomplicated, vaginal delivery.

High levels of IL-12 play an important role in the defense against *P. falciparum* infection and in the protection against systemic damage induced by the presence of parasites [18, 19] The fact that IL-12p40 concentrations were higher in uninfected multiparous women might suggest that this cytokine is involved in the control of parasitemia in peripheral blood and in placenta. The low amounts of IL-12p40 found in *P. falciparum*-infected multiparous women further supports this idea. Nevertheless, Stevenson *et al.*, in a review focusing on the role of IL-12 in malaria, reported that, although this pleiotropic cytokine has been demonstrated to induce protection in response to *P. falciparum* malaria, it can also mediate pathology during blood-stage malaria [20]. The highest levels of peripheral IL-12 found in infected primiparous women could be explained by its controversial association with the infection. High levels of IL-12p40 in cord plasma may explain the low prevalence of *P. falciparum* antigens in infants born to infected mothers. Indeed, this cytokine not only increases the cell-mediated immune response, but also affects humoral immunity by inducing isotype-switching through both IFNγ-dependent and independent mechanisms [21]. It has been demonstrated that cord blood-derived cell-mediated cytotoxic activity could be increased by IL-12 [22]. Moreover, the low prevalence of malaria antigens in infants born of infected mothers is also explained by the physical efficacy of the placental barrier. However, the presence of *P. falciparum* IgM in cord blood [23] suggests that the fetus was infected *in utero*. It is unclear how malarial parasites or antigens cross the placental barrier and enter the circulation of the fetus. Alternatively, malarial antigens, perhaps as immune complexes, could cross the placental barrier and stimulate the immune response [24]. The transplacentally transmitted antibodies may afford transient protection for the infants and thus delay the onset of clinical manifestations.

IL-12 plus IL-15 enhance the production in peripheral blood of cytokines such as TNF-α, IL-10, IFN-γ, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β at the protein and transcript levels [25-27]. The IFN-γ production by natural killer (NK) cells in response to *P. falciparum* infection is highly IL-12-dependent [28]. The high levels of IL-15 found in uninfected women may reflect the early activation of the innate immune response. Indeed, infectious agents up-regulate IL-15 expression, thus enhancing natural killer cell activity, which is implicated in early host responses against plasmodial infection through the lysis of infected erythrocytes [29, 30]; and IL-15 can function with IL-2 to enhance the capacity of γδ-T cells to inhibit parasite replication [31].

The concentrations of TNF-α were low and did not significantly differ according to *P. falciparum* infection status in the three compartments. Similar TNF-α concentrations in peripheral and cord blood were reported by Roncarolo *et al.* [32], in keeping with our results. The levels of TNF-α detected in the three compartments were very low compared to the levels of the other cytokines and to those (spontaneously produced) reported in Kenya [33]. Lower placental TNF-α concentrations in serum than in tissue cultures have been reported by others [9, 34], suggesting a poor secretion *in vivo* or important uptake during labor [35]. Nevertheless, placental blood tends to contain more TNF-α than peripheral or cord blood, and the same pattern has been found in malawian women [36]; this cytokine is involved in the mediated activation of macrophages in the placenta [37]. The accumulation of monocytes and macrophages within the intervillous space is a hallmark of placental malaria, and activated lymphocytes also release potent antimicrobial molecules such as TNF-α to aid in the elimination of parasites. Malarial placental infection induces an alteration in the cytokine balance and elicites Th-1 type cytokines (including TNF-α) at the placental level [34, 36, 38]. The lack of association between placental TNF-α levels and malaria infection in our study could be explained by the small sample size, although even with a larger sample size, Suguitan *et al.* did not find a strong association between placental plasma TNF-α concentrations and placental malaria [9]. It is also important to notice that high TNF-α concentrations reported by Fried *et al.* were associated with presence of severe maternal anemia [38].

The monocyte recruitment during placental malaria has also been associated with elevated β chemokine expression [10]. In our study, the MCP-1 concentration was five- or six-fold higher in placental plasma than in peripheral or cord plasma. This has not been compared before, but similar results were obtained with macrophage migration inhibitory factor (MIF) [39], a lymphokine that mediates macrophage activation, and which is involved in the killing of intracellular parasites. Abrams *et al.* observed a strong relationship between the quantity of MCP-1 mRNA and density of monocytes in the placentas of malaria-infected women [10]. The high MCP-1 plasma levels found in infected placenta seem to confirm the involvement of MCP-1 in placental monocyte recruitment during placental malaria. *P. falciparum*-infected erythrocytes activate macrophages to produce adhesion molecules and cytokines. Macrophages secrete β chemokines including MCP-1 in response to these cytokines [40]. Activated macrophages and monocytes, through TNF, are partly involved in the immune process that results in low birth weight [41]. Further investigations are needed to clarify these relationships.

IL-12p40 and IL-15 might be transported from placenta to cord or vice versa or be produced locally in each of the sites. However in general, it is unclear whether cytokines and chemokines are transported from the maternal circu-

lation to the fetal circulation or whether the fetal blood cells produce these soluble factors themselves or following the activation of cord cells by the passively acquired malarial antigens from the maternal side, or both. We found no correlation between the peripheral and placenta plasma concentrations of cytokines, suggesting that anti-malaria immune responses occurring in the placenta are not influenced by the cytokines from mother's blood, and that the immune response during *P. falciparum* infection is different in the peripheral and placental compartments.

In conclusion, understanding the cytokine interactions that underlie both control and disease should be helpful when designing anti-malarial treatments in pregnancy, as the levels of some cytokine are associated with poor pregnancy outcomes [36, 41, 42]. We have tried to clarify the patterns of the immune responses that occur in placental, peripheral and cord blood, suggesting that MCP-1, IL-12 and TNFα are key mediators in the host response to *P. falciparum* infection during pregnancy in women living in malaria hyperendemic areas. The small size of sample makes our conclusion provisional; further studies are required.

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