Immunity to Placental Malaria. I. Elevated Production of Interferon-γ by Placental Blood Mononuclear Cells Is Associated with Protection in an Area with High Transmission of Malaria

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In areas in which malaria is holoendemic, primigravidae and secundigravidae, compared with multigravidae, are highly susceptible to placental malaria (PM). The nature of gravidity-dependent immune protection against PM was investigated by measuring in vitro production of cytokines by placental intervillous blood mononuclear cells (IVBMC). The results demonstrated that interferon (IFN)-γ may be a critical factor in protection against PM: production of this cytokine by PM-negative multigravid IVBMC was elevated compared with PM-negative primigravid and secundigravid and PM-positive multigravid cells. Low IFN-γ responsiveness to malarial antigen stimulation, most evident in the latter group, was balanced by increased interleukin (IL)-4 production, suggesting that counter-regulation of these two cytokines may be a crucial determinant in susceptibility to PM. A counter-regulatory relationship between IL-10 and tumor necrosis factor-α was also observed in response to malarial antigen stimulation. These data suggest that elevated production of IFN-γ, as part of a carefully regulated cytokine network, is important in the control of PM.

More than 300 million people suffer from malaria each year, and most of the mortality and morbidity is borne by children <5 years old and by pregnant women. Men and women living in high malaria-endemic areas normally develop protective immunity against severe disease and high-density parasitemia; this protection, however, appears to be partially lost during pregnancy [1]. The hallmark of malaria in pregnancy in women residing in areas of high malaria endemcity is the accumulation of malarial parasites in the intervillous spaces of the placenta (a condition defined as placental malaria [PM]) with parasitemias frequently being more dense than in the periphery [1,2]. Women in their first and second pregnancies are most susceptible to PM, demonstrating the highest prevalences and densities of parasitemia [1,3]. With the exception of severe malaria anemia [4,5], PM does not result in severe outcomes for the mother; however, it is associated with intrauterine growth retardation and low birth weight in the infant [6,7], which are significant risk factors for neonatal mortality [8].

While malaria during pregnancy in low endemic areas affects women of all gravidities equally [9-11], the gravidity-dependent resistance to PM in high endemic areas has been repeatedly confirmed in several epidemiologic studies. The biological basis of the protection acquired by multigravidae in this setting is not well understood, and several theories have been suggested. Reduced levels of immunosuppressive hormones [12,13] and higher levels of anti-malarial antibodies [14] in multigravidae have been proposed to be associated with protection in this group; however, strong supporting evidence is lacking. The most provocative hypothesis, proposed by McGregor [1], suggests that placenta-specific immunity is necessary to control placental parasitemia, and repeated exposure to malaria through successive pregnancies is required for this immunity to develop and be maintained [1]. To date, very little experimental evidence in support of this hypothesis has been generated.

Cell-mediated mechanisms are believed to play an important role in immunity against blood-stage malarial parasites. Although the relevant mechanisms for protection are not fully understood, a clear role for various cytokines in mediating this immunity has been demonstrated in several animal models and in natural human infection (reviewed in [15-17]). For example, tumor necrosis factor (TNF)-α is believed to mediate its pro-
tective effects by several mechanisms, including activation of macrophages [18] and neutrophils to kill parasites [19]. Interferon (IFN)-
macrophage-mediated killing of intraerythrocytic parasites via crisis form factor [20] and in general is an important regulator of macrophage activation and host defense [21].

It is likely that local production of these same cytokines can play an important protective role at the placental level; however, examination of cytokine expression by maternal cells in the placental blood has not been done, primarily due to a lack of optimal methods for isolating intervillous blood from the placenta. We recently developed a simple perfusion technique to isolate intervillous blood mononuclear cells (IVBMC) from human placentas [22] and have applied it to the problem of malaria in pregnancy. We report here the significance of IFN-γ, TNF-α, interleukin (IL)-4, and IL-10 in immunity to PM in an area of western Kenya that is highly malaria endemic as assessed by measurement of in vitro production of these cytokines by perfused IVBMC.

Materials and Methods

Study site, participants, and samples. This study was conducted in Kisumu, western Kenya. Malaria transmission is perennial in this area with two peak transmission periods: November to December and May to July. Transmission of malaria is intense, with persons residing in this area receiving 100–300 infective bites each year [23].

Study participants were recruited from women attending the antenatal clinic and delivery ward in the New Nyanza Provincial General Hospital (NNPGH) in Kisumu, the major public hospital in this area, which serves residents of Kisumu and the surrounding areas.

Primigravidae and secundigravidae in malaria holoendemic areas like Kisumu are highly susceptible to PM. In their third and later pregnancies, women tend to be protected. Thus, whereas 35% of primigravidae and 28% of secundigravidae delivering in NNPGH in Kisumu have PM at delivery, only 11% of multigravidae are similarly affected (M. Parise, B. Nahlen, and R. Steketee, unpublished data). It is assumed that the underlying biologic (immunologic) basis for the susceptibility of primigravidae and secundigravidae is the same; thus, these 2 groups were joined into 1 group in this study and will be designated “primi/secundigravidae.”

A portion of the placentas collected for this study were from women recruited from the antenatal clinic at NNPGH as part of a large ongoing Centers for Disease Control and Prevention (CDC)/Kenya Medical Research Institute (KEMRI) study. Those women who, after providing written consent for counseling and serum testing, tested negative for human immunodeficiency virus (HIV) type 1 were selected specifically for this study. HIV-1–positive women were excluded. Placentas were also collected at delivery from women who were not part of the aforementioned large study. These women were not tested for HIV serostatus and thus are of unknown HIV-1–infection status. In all, 36% of primi/secundigravida placentas and 35% of multigravida placentas were from this latter untested group. Cytokine expression by IVBMC from HIV-1–seronegative subjects did not differ significantly from that of untested persons (data not shown).

Only healthy mothers with uncomplicated labor and singleton, vaginal deliveries were included. Placentas from 112 such women (gravida 1–gravida 7) were collected and processed. The placenta collection procedures and the perfusion method used to obtain maternal intervillous blood from the intervillous spaces of the placenta have been described in detail elsewhere [22]. A complete gross pathologic examination was performed on each placenta; any tissue with extensive tearing, damage, or gross abnormalities was excluded from the study. Frequently more placentas than could be processed were collected; in these cases, efforts were made to perfuse equal numbers from each group, with preference given to more recently delivered placentas.

Placental parasitemia status and ages of primi/secundigravidae and multigravidae are summarized in table 1. Although not statistically significant, parasite density tended to be higher in primi/secundigravidae than in multigravidae placentas (P > .05, Wilcoxon rank sum test [WRS]), which is in agreement with numerous other studies in high malaria-endemic areas [1, 3]. Overall, primi/secundigravidae women were significantly younger than multigravidae women (P = .000, WRS). These age differences were maintained when the data for each gravidity group were stratified by PM status (P < .004, WRS; table 1),
IFN-γ production by primi/secundigravid (P/S) and multigravid (M) intervillous blood mononuclear cells (IVBMC), stratified by placental malaria (PM; PLAC MAL) status. Supernatants from IVBMC cultured for 48 h at 10^6 cells/mL alone (medium, MED) or in presence of phytohemagglutinin (PHA), anti-CD3 monoclonal antibody (CD3), purified protein derivative (PPD), and soluble malarial antigen (Mal Ag) were assayed for IFN-γ by ELISA. Values measured for exogenously stimulated cells were adjusted for background cytokine production (see Materials and Methods). Resulting values were grouped by gravidity and PM status and arithmetic means ± SE calculated. Values at bar tops (SE) are arithmetic mean and (n). Scales for individual panels are not equivalent. By Wilcoxon rank sum test: P/S vs. M, PM-negative: MED, ; PHA, ; PPD, P < .013 P < .045 P < .045. M, PM-negative vs. PM-positive: MED, ; PHA,.007 P < .045 P < .008 demonstrating that the incidence of PM was not an age-dependent phenomenon in this study population.

Assessment of parasitemia status. Thick blood films were made with blood obtained from a shallow incision in the maternal side (basal plate) of the placenta from two areas distal to the perfused quadrant. Parasites per 300 leukocytes were counted on Giemsa-stained slides, and parasite densities were calculated with an assumed 8000 leukocytes/μL of blood.

Isolation of IVBMC. The IVBMC were isolated from perfused placental blood samples within 24 h after collection by centrifugation over ficoll-hypaque (0.16 mM Nycograde polysucrose 400; Nycomed, Oslo), 0.16 M sodium diatrizoate (Sigma, St. Louis), and 12 mM NaCl (Sigma; density = 0.977 g/L). Cells were washed once in a large excess volume of PBS and then resuspended in complete medium (RPMI 1640; Gibco BRL, Grand Island, NY) with 5% fetal bovine serum (Gibco), 5% AB-positive pooled human serum (CDC Blood Bank, Atlanta), 1 mM L-glutamine (Gibco), and 1 mM penicillin-streptomycin (Gibco). IVBMC yields, calculated as described [22], ranged from 8.1 × 10^6 to 222.4 × 10^6 cells/mL of packed red blood cells (RBC).

Culture of IVBMC. IVBMC were cultured in 48-well plates (Costar, Cambridge, MA) at a concentration of 10^6 cells/mL and were maintained at 37°C with an atmosphere of 6% CO2. Supernatants from cells cultured in complete medium alone to serve as controls and in medium supplemented with phytohemagglutinin (PHA; Sigma; final concentration, 10 μg/mL), anti-CD3 monoclonal antibodies (MAbs) [22] (final concentration, 1 μg/mL), purified protein derivative (PPD; Evans Medical, Leatherhead, UK; final concentration, 100 U/mL), and soluble malarial antigen were collected after 48 h and stored at −80°C until use.

Soluble malarial antigen was in the form of supernatants from high-density cultures of Plasmodium falciparum isolate 3D7 diluted from 1:8 to 1:16, depending on the original parasitemia. Parasite cultures were maintained essentially as described [24]. Cultures were allowed to grow to >8% parasitemia with most parasites in the ring stage, indicating that a significant portion of parasites had undergone rupture (and antigen release) within the previous 12 h; supernatants were collected, centrifuged at 400 g to remove RBC, and recentrifuged at 15,000 g to remove smaller debris. Control supernatants from uninfected RBC were processed similarly. These antigen preparations were aliquoted and stored in liquid nitrogen until use, and aliquots were not refrozen.

Measurement of cytokine levels in supernatants by ELISA. Preliminary studies showed that maximal cytokine expression occurred as early as 48 h after stimulation [22], so all supernatants were collected at this time point. Double sandwich ELISAs were done as previously described [22]. A minor proportion of supernatants were assayed using matched pairs of MAbs, one of which was biotinylated (R&D Systems, Minneapolis).
IL-4 production by primi/secundigravid (P/S) and multigravid (M) intervillous blood mononuclear cells (IVBMC) stratified by placental malaria (PM; PLAC MAL) status. Supernatants from IVBMC cultured as described in the legend to figure 1 were assayed for IL-4. Values are as in figure 1. Scales for individual panels are not equivalent. By Wilcoxon rank sum test: P/S, PM-negative vs. PM-positive: soluble malarial antigen (Mal Ag), \( P < .041 \), medium; PHA, phytohemagglutinin; CD3, anti-CD3 monoclonal antibody; PPD, purified protein derivative.

Limits of detection were 5 pg/mL for IL-4 and IFN-\( \gamma \) and 30 pg/mL for IL-10 and TNF-\( \alpha \).

In order to accurately assess the effect of the individual stimulants on cytokine production, endogenously produced cytokine levels from unstimulated culture supernatants were subtracted from stimulated culture values for each individual for the purpose of comparison. In the case of soluble malarial antigen, cytokine levels from cultures exposed to control RBC antigen were subtracted from malarial antigen–stimulated cultures.

Statistical analyses. A statistical software package (version 6.0; SAS Institute, Cary, NC) was used for data analysis. The nonparametric WRS was used to assess differences between groups. Proportions were compared by Fisher’s exact test. \( P < .05 \) was considered significant.

Results

IFN-\( \gamma \) production by primi/secundigravid and multigravid IVBMC. IFN-\( \gamma \) levels in IVBMC cultures stimulated with mitogens, PPD, and malarial antigen and in control cultures were determined. Mitogenic stimulation was used as a positive control to assess the overall responsiveness and cytokine production potential of the IVBMC. PPD stimulation served as a marker for recall antigen-specific responsiveness. In general, IVBMC from PM-negative multigravidae produced more IFN-\( \gamma \) than those from primi/secundigravidae, regardless of parasitemia status (figure 1). The endogenous production of IFN-\( \gamma \) in unstimulated cultures was more than 7-fold higher in uninfected multigravid samples as compared with those from uninfected primi/secundigravidae (\( P = .013 \), WRS). Likewise, stimulation with PHA, anti-CD3 MAb, PPD, and, to a lesser extent, soluble malarial antigen (figure 1) resulted in variably elevated levels of IFN-\( \gamma \) production by IVBMC from PM-negative multigravidae as compared with their primi/secundigravid counterparts.

Regardless of antigenic stimulus, PM-positive multigravid IVBMC were consistently low IFN-\( \gamma \) responders, producing up to 60-fold less IFN-\( \gamma \) than uninfected multigravid cultures. This reduction in IFN-\( \gamma \) production was not observed in PM-positive primi/secundigravid IVBMC supernatants; instead, parasitemia in this group was associated with slightly elevated levels of IFN-\( \gamma \) (figure 1).

IL-4 production by primi/secundigravid and multigravid IVBMC. In contrast to IFN-\( \gamma \), there was no substantial difference in endogenous IL-4 expression levels between PM-negative primi/secundigravid and multigravid IVBMC; this was uniformly observed following PHA, anti-CD3 MAb, and PPD stimulation as well (figure 2). In PM-positive primi/secundigravid cultures, IL-4 production tended to be lower than in their uninfected counterparts, although this difference was less dramatic in the context of PHA and anti-CD3 MAb stimulation (figure 2). IL-4 and IFN-\( \gamma \) expression in response to soluble...
malarial antigen stimulation tended to be exclusive: Elevated IFN-γ by PM-positive primi/secundigravid IVBMC was accompanied by barely detectable IL-4, and higher IL-4 in PM-positive multigravid cultures was associated with very low levels of IFN-γ (compare Mal Ag panels in figure 1 and figure 2).

PM-positive multigravid IVBMC produced low levels of IL-4 and IFN-γ both endogenously and in response to PPD stimulation; however, they generated an IL-4 response comparable to the other groups following stimulation with PHA and anti-CD3 MAb and also responded relatively well to soluble malarial antigen.

**TNF-α production by primi/secundigravid and multigravid IVBMC.** TNF-α production by primi/secundigravid cultures appeared to follow the same pattern as observed with IFN-γ but with some key differences: TNF-α production by IVBMC from PM-positive placenta was significantly higher than that by cells from uninfected placenta (P < 0.045, WRS) under all stimulation conditions except with malarial antigen. The latter resulted in a significant decrease in TNF-α production by PM-positive IVBMC (P = 0.04, WRS; figure 3).

For the most part, PM-positive multigravid cultures remained consistently low responders, although the decreased production of TNF-α by this group compared with their PM-negative counterparts was not as dramatic as with IFN-γ. The single exception was in the response to malarial antigen stimulation: PM-positive multigravid IVBMC produced slightly more TNF-α than cells from nonparasitized multigravid placentas (figure 3).

Similarities between the IFN-γ and TNF-α responses were also observed in the relationship between PM-negative primi/secundigravid and multigravid IVBMC. The latter spontaneously produced more TNF-α than their primi/secundigravid counterparts, a relationship that also held following PHA stimulation (figure 3). These same groups generated comparable levels of TNF-α in response to anti-CD3 MAb and PPD stimulation, and the primi/secundigravid IVBMC were higher responders after malarial antigen stimulation (figure 3), a pattern opposite to that seen for IFN-γ (figure 1).

**IL-10 production by primi/secundigravid and multigravid IVBMC.** The tendency for PM-positive primi/secundigravid IVBMC, compared with their PM-negative counterparts, to produce slightly elevated levels of cytokine (as observed with IFN-γ and TNF-α) was not observed for IL-10 production (figure 4); rather, the pattern of expression by PM-positive primi/secundigravid IVBMC was reminiscent of that observed for IL-4 (figure 2). This was not the case, however, following malarial antigen stimulation: the pattern of IL-10 production paralleled that of IFN-γ for all groups (figure 4). Also, as with IL-4 and IFN-γ, IL-10 and TNF-α appeared to have a reciprocal (counter-regulatory) relationship in the context of malarial antigen stimulation (compare Mal Ag panels in figure 3 and figure 4).

PM-positive multigravid IVBMC continued their trend to-
Figure 4. Interleukin (IL)-10 production by primi/secundigravid (P/S) and multigravid (M) intervillous blood mononuclear cells (IVBMC), stratified by placental malaria (PLAC MAL) status. Supernatants from IVBMC cultured as described in legend to figure 1 were assayed for IL-10. Values are as in figure 1. MED, medium; PHA, phytohemagglutinin; CD3, anti-CD3 monoclonal antibody; PPD, purified protein derivative; Mal Ag, malarial antigen.

ward very low cytokine production, generating levels of IL-10 comparable to primi/secundigravid IVBMC only following stimulation with malarial antigen. As with IFN-γ, PM-negative multigravid IVBMC tended to produce more IL-10 than PM-negative primi/secundigravid cells, although the differences were not significant.

Discussion

In this study, we systematically investigated cytokine production by perfused IVBMC isolated from placentas of women of different gravidity groups living in malaria holoendemic western Kenya. The data provide the first evidence that the gravidity-based differences in susceptibility to PM in a malaria holoendemic area are associated with the differential ability of mothers to mount local cytokine responses in the placenta.

The elevated expression of IFN-γ by PM-negative multigravid IVBMC compared with PM-negative primi/secundigravid IVBMC suggests that this cytokine is likely to be important in the control of parasitemia in the placenta. The lack of IFN-γ production by PM-positive multigravid IVBMC, especially after stimulation with malarial antigen, lends further support to this hypothesis. Also, primi/secundigravid cells mounted a slightly elevated IFN-γ response in the presence of PM infection, yet they were unable to clear their parasitemia. Taken together, these data suggest that women whose IVBMC are committed to constitutively produce high levels of IFN-γ (i.e., PM-negative multigravidae) can effectively control parasitemia upon exposure, and those who are low producers tend to be susceptible to PM.

An alternative interpretation of our findings is that PM results in a gravidity-dependent depression of cytokine production, as has been suggested to occur in acutely infected persons [25]. However, while high-density parasitemias are associated with the most significant depression in cell-mediated immunity [26], we observed a significant effect on IFN-γ production in PM-positive multigravid IVBMC and not in PM-positive primi/secundigravid cells (who generally had higher density parasitemias; table 1). Thus, it is unlikely that this interpretation is valid. Furthermore, the decreased production of IFN-γ by malarial antigen–stimulated multigravid IVBMC was observed together with an increased production of IL-4, showing that this group does not experience a generalized malaria-induced immunosuppression.

Our hypothesis that IFN-γ plays a key role in protection against PM is consistent with several previous experimental studies that have demonstrated the importance of this cytokine in the control of blood-stage asexual malarial parasitemia [27–29]. For example, an IFN-γ–dominated Th1 response to Plasmodium chabaudi AS in C57BL/6 mice was essential for protection against high-level parasitemia and death, whereas susceptible A/J mice, which develop a strong Th2 (IL-4) response to the same infection, experienced higher peak parasitemia.
temias and a high rate of mortality [28]. This mutual exclusion of IL-4 and IFN-γ has not been reported in malaria-exposed humans; however, the IVBMC cytokine production patterns, particularly in response to malarial antigen stimulation, suggest that, as in mice, a high IFN-γ-to-IL-4 ratio may be important in controlling P. falciparum infection in the placenta. It is an intriguing possibility that it may be the tendency to maintain local production of IL-4, or more accurately, the failure to generate IFN-γ, as seen in PM-positive multigravid IVBMC cultures, that renders women susceptible to PM.

Spontaneous cytokine production by IVBMC, particularly among primi/secondigravid and PM-negative multigravid samples, was dominated by IL-10, IFN-γ, and TNF-α, the latter two of which are classic Th1-type cytokines. This prevalence of Th1-type cytokines in the placenta contradicts the generally held notion that this tissue is dominated by Th2 cytokines, including IL-4. Although the cytokine profiles of placentas from a malaria-nonendemic area were not evaluated in this investigation, the lack of a predominant Th1 cytokine environment in placentas of Kenyan women from a nonmalarious urban center has been reported [30]. These findings, together with the data obtained in this study, suggest that exposure to PM may induce up-regulation of IFN-γ and TNF-α production by IVBMC, potentially inducing a switch away from a Th2 cytokine-dominated environment. Such a switch occurs in Leishmania major-infected C57BL/6 pregnant mice [31].

Cytokine production by IVBMC was not entirely biased toward Th1-type cytokine production; instead, the IL-10 production pattern, especially that by multigravid IVBMC, paralleled that of IFN-γ, and to a lesser extent, TNF-α. Elevated plasma IL-10 levels in the placenta have also been reported in malaria-exposed Gambian women [32]. We speculate that the close parallel between IFN-γ and IL-10 production by placental IVBMC may serve to tightly regulate the detrimental effects that IFN-γ and TNF-α can have on the developing fetus [33] as suggested by others [34]. A role for IL-10 as a regulator of IFN-γ-mediated pathogenesis has been demonstrated in P. chabaudi chabaudi AS-infected IL-10 knockout mice [35], and a high IL-10-to–TNF-α plasma ratio has been associated with protection against malaria anemia [36].

On the basis of the results shown here, we propose a model in which IFN-γ plays a key role in the control of PM, and IL-10 plays a regulatory role to protect the fetus from any potentially detrimental effects of IFN-γ and TNF-α. According to this scheme, exposure to parasites at the placental level in the first and second pregnancies induces an elevated, albeit weak, primary IFN-γ response by IVBMC. Although this initial response may help to control the parasitemia, it is apparently not sufficient to eliminate or reduce the density of placentorial parasitemia in most women. In a subset of women, exposure to PM fails to elicit a strong IFN-γ response (low IFN-γ producers), perhaps due to the tendency of their IVBMC to maintain an IL-4-dominated Th2 response. In subsequent malaria-exposed pregnancies, the high IFN-γ producers mount an amplified IFN-γ response when exposed to PM and are able to control and eliminate the parasitemia. On the other hand, the low IFN-γ responders (who are likely to be high IL-4 producers) do not produce levels of this cytokine sufficient to completely clear placentorial parasitemia and thus remain susceptible. Furthermore, in protected women, IL-10 production is up-regulated synchronously with IFN-γ, thereby keeping IFN-γ and TNF-α in check and preventing cytokine-mediated pathogenesis. How this placenta-specific, anti-malarial immunity will be maintained from 1 pregnancy to the next remains to be determined.

It is not known whether the pattern of placental immune responses observed in this study parallel immune responses that might be found in the peripheral blood. Due to ethical and logistical difficulties related to collection of venous blood from women at the time of delivery, such a comparison could not be included here. This type of study will be necessary to demonstrate the extent to which the placenta represents a unique immunologic compartment, and, more importantly, whether cytokine responses measured in the peripheral blood will be predictive of a pregnant woman’s susceptibility to PM.

In summary, we have presented data and formulated a model that provide a cellular immunologic explanation for the gravidity-based differences in susceptibility to PM in areas where malaria is highly endemic. Although other factors may be important, IFN-γ appears to play a key role in protecting against placental parasitemia, as does IL-10, in preventing pathogenesis. Further studies using IVBMC will provide additional insights into the mechanisms of immunity to malaria at the placental level and will allow the validity of our proposed model to be determined.

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