Activation Status of Cord Blood γδ T Cells Reflects In Utero Exposure to Plasmodium falciparum Antigen

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Background. Placental Plasmodium falciparum infection modulates neonatal cell-mediated immune responses and is associated with increased susceptibility of infants to malaria.

Methods. By flow-cytometric analysis of maternal peripheral and cord blood samples collected at delivery, we measured and compared the activation status and proinflammatory cytokine activity of T cells from women segregated into groups according to malaria status.

Results. Stimulation with phorbol myristate acetate/ionomycin resulted in the highest percentages of tumor necrosis factor–α– and interferon-γ–positive γδ T cells in peripheral blood samples and in corresponding cord blood samples from those treated for malaria during pregnancy. Cord blood samples from this group also contained significantly higher percentages of CD69+ and CD25+ γδ T cells and CD3+ T cells, compared with samples from either the group with active placental P. falciparum infection at delivery or the group with no infection. Proinflammatory cytokine activity of cells from the group with placental P. falciparum infection at delivery was either similar to or lower than that of cells from the group with no infection.

Conclusions. The findings imply that treatment of P. falciparum malaria during pregnancy leads to enhanced innate immune T cell activation and proinflammatory cytokine responses in both maternal and fetal compartments. Ongoing placental P. falciparum infection, conversely, is associated with an absence of such activity.

Pregnancy-associated malaria (PAM) leads to low birth weights, preterm deliveries, and maternal anemia and, thus, represents a major public-health problem in sub-Saharan Africa [1–4]. PAM is associated with sequestration of Plasmodium falciparum–infected erythrocytes into the placental intervillous spaces [5]. In several studies, maternal plasmodial infections, as well as other parasitic infections, have been shown to influence neonatal immune responses [6–11]. Malaria episodes early during life are very rare, although children born to mothers with placental P. falciparum infection at delivery are more susceptible to malaria [12].

Clinical outcomes related to PAM are thought to involve proinflammatory cytokines, since (1) Th1 cytokines predominate in plasma from P. falciparum–infected placentas, (2) high levels of expression of tumor necrosis factor (TNF–α mRNA in the placenta are associated with the intrauterine retardation of growth that results from placental infection, and (3) high concentrations of both TNF–α and interferon (IFN)–γ in placental blood are associated with low birth weight [13, 14]. Nevertheless, proinflammatory cytokines are known to play a role in antimalarial immunity. TNF–α responses are related to rapid clearance of parasites, although high levels of production of TNF–α can lead to severe complications [15–19], whereas high levels of production of IFN–γ are associated with protection from malaria and/or malarial anemia [20–22]. Proinflammatory cytokines can be produced by macrophages, NK cells, γδ T cells, and other T cells [23–26]. As pivotal players in the innate immune response, NK...
SUBJECTS, MATERIALS, AND METHODS

Study population. Thirty-eight pregnant women were recruited, at delivery, from the maternity unit of Albert Schweitzer Hospital, Lambaréné, Gabon, which is situated in an area where malaria is hyperendemic [46]. Their mean age was 23 years (range, 15–42 years), and mean parity was 2 (range, 1–7). These parameters did not differ between groups of women that had been segregated according to infection history (see below). Maternal peripheral venous and cord blood samples were collected in heparinized tubes after written, informed consent to participate in the study had been obtained. The study was approved by the ethics committee of the International Foundation of Albert Schweitzer Hospital.

Determination of P. falciparum infection status. Giemsa-stained thick smears of maternal peripheral, placental, and cord blood were assessed for the presence of P. falciparum parasites. A clinical history of the women, including information documented in their personal health booklets, was also recorded. Additional criteria included the presence or absence of P. falciparum histidine-rich protein II (HRPII) and P. falciparum DNA in maternal peripheral and cord blood. As outlined in table 1, all maternal samples that were found to be parasitemic by microscopy also were found to contain both HRPII and P. falciparum DNA. None of the cord blood samples were found to be parasitemic by microscopy, and none were found to contain either HRPII or P. falciparum DNA. P. falciparum DNA was detected in the peripheral blood samples from 4 women whose blood samples were negative both by microscopy and by HRPII ELISA. Data on samples from these 4 women and their offspring were excluded from the analyses.

By use of clinical histories and data on parasitemia, women and their offspring were classified according to both the presence or absence of placental parasitemia at delivery and the documented history of maternal malaria:

1. The infected group (n = 9) comprised those with placental parasitemia at delivery, regardless of clinical history;
2. The treated group (n = 5) comprised women who had a documented, treated malaria infection during pregnancy and who had no placental or peripheral parasitemia at delivery; and
3. The negative group (n = 20) comprised those who had neither placental parasitemia at delivery nor a history of malaria during pregnancy.

Women who reported a malaria episode but had no documentation of proper diagnosis and treatment in their health booklets were excluded from the study.

Antigens. For preparation of parasite antigen, a P. falciparum isolate (designated cys007) that was originally obtained from a child presenting with severe malaria to the pediatric ward of Albert Schweitzer Hospital was adapted for in vitro

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<th>Table 1. Confirmation of microscopical diagnosis of Plasmodium falciparum infection, by detection of parasite antigen and DNA.</th>
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<td><strong>Group</strong></td>
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a Mothers and cords were classified according to (1) the presence or absence of placental parasitemia at delivery, detected by examination of blood smears by microscopy, and (2) documented history of a treated malaria episode during pregnancy (negative, no placental infection at delivery and no history of malaria; infected, placental P. falciparum infection detected at delivery; treated, no placental infection at delivery but a history of a treated malaria episode during pregnancy).
b P. falciparum histidine-rich protein II (HRPII) antigenemia was assessed by ELISA; presence of DNA coding for P. falciparum ribosomal RNA was determined by polymerase chain reaction (PCR) of maternal peripheral and cord blood.
culture, in accordance with the method of Trager and Jensen [47], by use of RPMI 1640 medium supplemented with HEPES (25 mmol/L), sodium bicarbonate (25 mmol/L), L-glutamine (2 mmol/L), and hypoxanthine (300 mmol/L) (all from Sigma); gentamycin (50 μg/mL) and albumax (5 mg/mL) (both from Life Technologies); and 5% human serum (blood bank of the University Hospital, Tübingen). Parasites were grown in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂, and were subcultured with O⁺ erythrocytes depleted of lymphocytes (blood bank of the University Hospital, Tübingen). Cultures were grown to a parasitemia of 3%–5% and then were synchronized by selective high-power magnetic sorting (SuperMACS; Miltenyi Biotech). Briefly, cultures were diluted in RPMI 1640 medium containing 2% fetal calf serum (Sigma) and were applied to a prewashed magnetic cell sorting column in a magnetic field, followed by intensive washing. Schizont-infected red blood cells (pRBCs) captured on the column were eluted after the column had been removed from the magnetic field. The schizont-enriched eluates were then washed twice with PBS and freeze-thawed thrice, to obtain a crude schizont lysate. Uninfected red blood cells (uRBCs) from the same donors were used to prepare control lysate accordingly. Parasite cultures were continuously tested for the presence of Mycoplasm species by polymerase chain reaction (PCR) (primers: 5'-GTG CCA GCA GCC GCG GTA ATA C-3' and 5'-TAC CTT GTT ACG ACT TCA CCC CA-3' [48]), and only Mycoplasma-negative cultures were used.

Peripheral blood mononuclear cell (PBMC)/cord blood mononuclear cell (CBMC) cultures. Samples were processed within 4 h of collection. PBMCs or CBMCs were isolated from heparinized blood by use of routine density centrifugation methods with Ficoll (Amersham Biosciences). For stimulation, they were cultured at a concentration of 1.25 × 10⁶ cells/mL in medium (Ultra Culture; BioWhittaker) supplemented with l-glutamine (2 mmol/L), 2-mercaptoethanol (3.5 μL/L; Sigma), and gentamycin (170 μg/mL).

Cells were stimulated with PMA (10 ng/mL) and ionomycin (1.25 μmol/L) (both from Sigma) for 4 h at 37°C in 5% CO₂, in the presence of brefeldin A (10 μg/mL; Sigma). For antigen-specific stimulation, crude schizont lysate was used at a concentration equivalent to a ratio of 10 pRBCs:1 PBMC/CBMC, which has been shown to be optimal for antigen-specific stimulation (authors’ unpublished data and [25, 49]). Cultures were incubated for 18 h in the presence of anti-CD28 antibodies (2.5 μg/mL), and brefeldin A (10 μg/mL) was added for the last 12 h.

Cells were then harvested on ice, washed twice in PBS, and fixed with a 2% solution of formaldehyde (Merck) in PBS for 20 min. Next, they were washed twice in PBS and stored in Hanks’ balanced salt solution containing 0.1% sodium azide (both from Sigma) and supplemented with 0.3% bovine serum albumin (Serva), at 4°C in the dark, until being used for staining. These procedures are in accordance with those originally described by Winkler et al. [49, 50]. The percentage of cytokine-producing cells in cultures incubated with uRBC lysate was subtracted from the percentage of cytokine-producing cells incubated with pRBCs, to obtain the net stimulation induced by the parasite lysate.

Flow-cytometric analysis. Cells were permeabilized in PBS/0.1% saponin (Sigma). Four-color staining was performed in PBS/0.1% saponin for 25 min at room temperature by use of appropriate combinations of the antibodies described below. Up to 2 × 10⁹ cells (R1) were acquired on a FACSCalibur flow cytometer (Becton Dickinson), and gates were set according to the profiles obtained with antibody isotype controls. Figure 1 shows an example of the flow-cytometric analysis. For measurement of the expression levels of cell surface proteins, the mean fluorescence intensity (MFI) was used. The MFI of isotype controls was subtracted from the MFI of the sample of interest. Data were analyzed by use of CellQuest software (version 3.3; Becton Dickinson).

Statistical analysis. Statistical analysis was performed by use of the Statview software program (version 5.0.1). Unpaired comparisons of continuous variables were made by use of the nonparametric Kruskal-Wallis and the Mann-Whitney U tests; P < .05 was considered to be significant. The significance of associations between continuous variables was assessed by use of Spearman’s rank correlation test; P > 0.25 with P < .05 was considered to be significant.

Antibodies. The following monoclonal antibodies (MAbs) were used: fluorescein isothiocyanate (FITC)–anti–IFN-γ MAb, FITC–anti–CD69 MAb, phycoerythrin (PE)–anti–CD25 MAb, peridinin chlorophyll protein–anti–CD3 MAb, and allophycocyanin (APC)–anti–T cell receptor γδ MAb (pan specific) (all from Becton Dickinson); and PE–anti–TNF-α MAb, APC–anti–CD94 MAb, and purified antihuman CD28 (all from Pharmingen). Isotype controls were included as appropriate. For technical reasons, the conventional NK cell surface marker CD56 was replaced by staining for CD94 on the population of CD3⁺ cells.

HRPII-antigen ELISA. A commercially available kit (malaria antigen CELISA; Cellabs) was used, in accordance with the manufacturer’s instructions, for detection of P. falciparum HRPII in maternal peripheral and cord blood samples.

PCR detection of P. falciparum DNA. Genomic DNA was purified from 200 μL of packed erythrocytes from maternal peripheral and cord blood samples by use of a commercially available kit (Qiagen), in accordance with the manufacturer’s instructions. A nested PCR procedure was used to detect the P. falciparum small subunit ribosomal RNA gene [51]. The PCR products of the amplifications were analyzed by gel electrophoresis and staining with ethidium bromide.
RESULTS

Proinflammatory cytokine activity of PBMCs and CBMCs.

After stimulation with PMA/ionomycin, PBMCs from women in the treated group contained the highest percentages of TNF-α- and IFN-γ-producing γδ T cells, in both cases at levels significantly higher than those in PBMCs from women in the infected group (figure 2A). A similar pattern was observed with respect to the percentages of TNF-α-producing γδ T cells in PMA/ionomycin-stimulated CBMCs, but with only nonsignificant trends toward higher percentages in CBMCs from the treated group, compared with those in CBMCs from the other 2 groups (P = .079, for treated vs. infected; P = .50, for treated vs. negative) (figure 2B). CBMCs from the negative group had a significantly lower percentage of IFN-γ-producing γδ T cells than did CBMCs from the other 2 groups (figure 2B).

For separate assessments of proinflammatory cytokines in NK cells, we analyzed populations of CD94+ CD3− cells. The percentages of cytokine-positive CD94+ CD3− cells in maternal PBMC and CBMC populations did not differ significantly when the samples were segregated according to maternal plasmodial infection history (data not shown), although there was a nonsignificant trend toward a higher percentage of IFN-γ-producing NK cells in PBMCs from women in the treated group (P = .089, for treated vs. negative; data not shown).

In addition to the cells of the innate immune response, the proinflammatory cytokine activity of CD3+ T cells was exam-
Figure 2. Percentages of proinflammatory cytokine (interferon-γ/tumor necrosis factor-α)–positive γδ T cells detected by flow-cytometric analysis after stimulation of maternal peripheral blood mononuclear cells (PBMCs) (A) and cord blood mononuclear cells (CBMCs) (B) with PMA/ionomycin for 4 h in the presence of brefeldin A, followed by fixation, surface and intracellular staining, and gating on CD3+ T cell receptor γδ cells. Samples were segregated according to maternal malaria status (INF: placental malaria at delivery; NEG: neither placental parasitemia at delivery nor a history of malaria during pregnancy; and TRT: documented, treated malaria infection during pregnancy and no parasitemia at delivery). Box-whisker plots show medians of percentages, with 25th and 75th percentiles in boxes and 10th and 90th percentiles as whiskers. P values refer to the significance of differences between percentages considered as continuous variables and compared between groups by use of the nonparametric Mann-Whitney U test.

ined after stimulation with PMA/ionomycin. The activity of pro-inflammatory cytokines in CD3+ PBMCs was similar between the 3 groups of women, whereas, in CBMCs, a significantly higher percentage of IFN-γ–positive CD3+ cells was detected in samples from the treated group than in samples from the negative group, with a nonsignificant trend (P = 0.074) toward a difference between the percentages of TNF-α–positive cells in samples from the same groups (figure 3A and 3B).

Correlations between proinflammatory cytokine activity of the innate and the acquired arms of the immune response. The percentages of lymphocytes in the innate (γδ/NK) and the acquired (CD3+) immune populations that exhibited activity
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**Figure 3.** Percentages of proinflammatory cytokine (interferon-γ/tumor necrosis factor-α)–positive CD3+ cells detected by flow-cytometric analysis after stimulation of maternal peripheral blood mononuclear cells (PBMCs) (A) and cord blood mononuclear cells (CBMCs) (B) with PMA/ionomycin for 4 h in the presence of brefeldin A, followed by fixation, surface and intracellular staining, and gating on CD3+ cells. Samples were segregated according to maternal malaria status (INF, placental malaria at delivery; NEG, neither placental parasitemia at delivery nor a history of malaria during pregnancy; and TRT, documented, treated malaria infection during pregnancy and no parasitemia at delivery). Box-whisker plots show medians of percentages, with 25th and 75th percentiles in boxes and 10th and 90th percentiles as whiskers. *P* values refer to the significance of differences between percentages considered as continuous variables and compared between groups by use of the nonparametric Mann-Whitney *U* test.

of either IFN-γ or TNF-α after stimulation with PMA/ionomycin showed strong positive correlations, associations that were of a similar magnitude between maternal PBMC and CBMC populations (table 2).

**Expression of surface CD69 and CD25 on PBMCs and CBMCs after stimulation with PMA/ionomycin.** In CBMCs, the highest percentages of both γδ T cells and CD3+ T cells expressing CD69 and CD25, concurrent with the highest MFI, were detected in cells from the treated group (figure 4A–4D) (data not shown). In the case of CD69, these percentages were significantly higher in cells from the treated group than in cells from either of the other 2 groups (figure 4A and 4C). Similarly,
the percentages of CD25+ γδ and CD3+ T cells in CBMCs from the treated group were significantly higher than those in CBMCs from the negative group and showed nonsignificant trends toward higher percentages of CD25+ T cells, compared with those in CBMCs from the infected group (figure 4B and 4D).

According to plasmodial infection histories, no significant differences in the profiles of these activation markers in PBMCs were observed, either in terms of percentages of cells or of MFI, between the γδ T cell populations (data not shown). With respect to expression of activation markers on CD3+ T cells from maternal PBMCs, the only significant difference observed was the higher percentage of CD25+ cells in PBMCs from women in the treated group, compared with that in PBMCs from women in the infected group (1.56% vs 0.25%; P = .042) (data not shown).

Cytokine responses after stimulation with plasmodial schizont lysate. Stimulation with P. falciparum schizont lysate enhanced the percentage of γδ T cells in maternal PBMCs that had detectable activity of IFN-γ and TNF-α, compared with stimulation with the control lysate (data not shown), but no parasite antigen–specific proinflammatory cytokine activity was detectable in cord γδ T cells or in maternal or cord CD94+ CD3+ cells (data not shown). Segregation of maternal γδ T cell parasite antigen–specific activity of cytokines, according to maternal plasmodial infection history, revealed no significant differences between the 3 groups of women (data not shown).

DISCUSSION

The levels and ratios of pro- and anti-inflammatory cytokines represent measures that have recently received widespread attention, with respect to pathogenesis of and protection in P. falciparum malaria [15–18, 21, 52–56]. In this context, pivotal roles are played by IFN-γ and TNF-α, both of which are produced by cells of the innate immune system early after contact with pRBCs (reviewed in [26]). Since innate responses early during life represent a particularly important line of defense against infectious pathogens, in the present study, we chose to focus on the effects that plasmodial infection during pregnancy had on maternal and neonatal γδ T cell and NK cell responses, which were exemplified by production of IFN-γ and TNF-α and also by expression of the T cell activation markers CD69 and CD25, in the case of γδ T cells. CD69 and CD25 (interleukin [IL]–2 receptor–α) are considered to be early and late T cell activation markers, respectively.

Our observation of enhanced proinflammatory cytokine activity after nonspecific stimulation of both maternal peripheral and cord blood γδ T cells, specifically those from the treated group, is interesting for 2 principal reasons. First, this finding implies that chemotherapeutic clearance of maternal P. falciparum infection induces a population of γδ T cells that is primed for activation and displays a bias for production of proinflammatory Th1 cytokines; second, this effect extends to cells of the fetal innate immune system. Notable in this context are the kinetic studies that indicated that IFN-γ–producing γδ T cells predominate in posttreatment samples collected from patients with malaria [57]. Of further interest are the data we have presented, which show that the enhanced activation status of cord blood cells from women in the treated group is characterized by a higher level of expression of activation markers, not only on γδ T cells but also on CD3+ T cells, as well as by enhanced proinflammatory cytokine activity. These findings add to the burgeoning body of evidence of infection-associated in utero sensitization of the acquired arm of the fetal immune system [58, 59]. Of equal importance, they highlight the fact that γδ T cells—which, classically, have been considered to be effectors of innate immunity—display a capacity for activation that is maintained over an extended period of fetal development. It should be noted, however, that we were unable to detect enhanced parasite antigen–specific activity of cord blood γδ T or CD94+ cells. A requirement for longer periods of stimulation of γδ T cells with parasite antigen lysate preparations, to elicit detectable responses, may explain this finding [25, 60]. Recent observations do suggest that γδ T cells can exhibit a “memory/recall” phenotype and can thus act as effective intermediaries between innate and acquired immune responses [31, 61, 62]. The close associations we found between the per-
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Figure 4. Expression of activation markers on cord blood mononuclear cell (CBMC) γδ T cells (A and B) and CD3⁺ T cells (C and D) stimulated with PMA/ionomycin for 4 h in the presence of brefeldin A, followed by fixation and staining for CD3, T cell receptor (TCR)γδ, CD25, and CD69. A gate was set on CD3⁺ TCRγδ cells or CD3⁺ T cells. Samples were segregated according to maternal malaria status (INF, placental malaria at delivery; NEG, neither placental parasitemia at delivery nor a history of malaria during pregnancy; and TRT, documented, treated malaria infection during pregnancy and no parasitemia at delivery). Box-whisker plots show medians of percentages of cells expressing either CD69 (A and C) or CD25 (B and D), with 25th and 75th percentiles in boxes and 10th and 90th percentiles as whiskers. *P* values refer to the significance of differences between percentages considered as continuous variables and compared between groups by use of the nonparametric Mann-Whitney *U* test.

Percentages of activated lymphocytes in the innate and acquired immune cell populations are consistent with this role. The important question of whether the activation of such cells in utero has any bearing on the outcome of plasmodial infection early during life is the subject of ongoing studies in our laboratories.

The pattern of the cytokine- and T cell activation–related profiles in cells from the infected group and the negative group—in the majority of cases, they were substantially lower than those observed in cells from the treated group, as discussed above—lead us to conclude that differences with respect to the duration, the quantity, and, possibly, the quality of exposure to parasite antigens in utero might explain the differences in cellular responses in those exposed during and after chemotherapeutic termination of a maternal malaria episode. Importantly, in this context, we found no evidence of cord blood plasmodial infection, either by microscopy or by the use of very sensitive techniques that efficiently detect “cryptic” maternal infections. Our observations thus imply that the enhanced activity of CBMCs that we have reported here did not result from direct stimulation by either parasites or parasitized erythrocytes, but rather by transplacentally transferred parasite antigen. Expression of CD69 on T cells is up-regulated during acute malaria episodes, the expression of both CD69 and CD25 is enhanced after in vitro parasite antigen–specific stimulation of γδ T cells, and parasite-derived phosphoantigens have been reported to activate γδ T cells [57, 63, 64]. Parasite antigenemia, furthermore, is detectable in a proportion of cord blood samples [65]. Our results could thus plausibly be considered to reflect parasite antigen–induced activation of CBMCs. The results of a recently published Gambian study reveal evidence of reduced IFN-γ responses in CBMCs, possibly because of modified antigen-presenting cell function related to active placental *P. falciparum* infection.
infection [66]. These observations are consistent with the results of our own detailed investigations of cytokine-mediated antigen-presenting cell and T cell activity and function in the same context [45].

Placental plasmoidal infection per se is associated with increased susceptibility of infants to malaria as well as with suppressed immune responses in the placental compartment, which are thought to result from elevated levels of anti-inflammatory IL-10 in the placental compartment and may exert similar effects on cord blood cellular immune responses [12, 65, 67, 68]. In addition, the lack of a comparable parasite antigen–specific response in cord blood γδ T cells might be the result of differential distribution of γδ T cell subpopulations and possibly of different requirements for their activation. The Vγ9Vδ2 subset predominates in adult peripheral blood, whereas the Vδ1 subset predominates in neonatal and infant peripheral blood [69, 70]. The parasite antigen–specific responses that we did detect lie in the same range as those reported by others [25]. The lack of parasite antigen–specific NK cell responses, on the other hand, is consistent with the finding that live Plasmodium–infected erythrocytes induce stronger responses than do lysate preparations [25, 26].

The present study has highlighted the in utero modulation of fetal innate immune responses specifically associated with chemotherapeutic termination of a maternal Plasmodium infection during pregnancy. The resulting sustained, heightened activation status of γδ T cells and its clinical relevance, with respect to the outcome of plasmoidal infection early during life, remain open questions. We are actively pursuing the answers in ongoing field-based longitudinal studies of pregnant women and infants.

Acknowledgments

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References


