

IFN- γ and IL-10 Mediate Parasite-Specific Immune Responses of Cord Blood Cells Induced by Pregnancy-Associated *Plasmodium falciparum* Malaria¹

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Available evidence suggests that immune cells from neonates born to mothers with placental *Plasmodium falciparum* (Pf) infection are sensitized to parasite Ag in utero but have reduced ability to generate protective Th1 responses. In this study, we detected Pf Ag-specific IFN- γ ⁺ T cells in cord blood from human neonates whose mothers had received treatment for malaria or who had active placental Pf infection at delivery, with responses being significantly reduced in the latter group. Active placental malaria at delivery was also associated with reduced expression of monocyte MHC class I and II in vivo and following short term in vitro coculture with Pf Ag compared with levels seen in neonates whose mothers had received treatment during pregnancy. Given that APC activation and Th1 responses are driven in part by IFN- γ and down-regulated by IL-10, we examined the role of these cytokines in modulating the Pf Ag-specific immune responses in cord blood samples. Exogenous recombinant human IFN- γ and neutralizing anti-human IL-10 enhanced T cell IFN- γ production, whereas recombinant human IFN- γ also restored MHC class I and II expression on monocytes from cord blood mononuclear cells cocultured with Pf Ag. Accordingly, active placental malaria at delivery was associated with increased frequencies of Pf Ag-specific IL-10⁺CD4⁺ T cells in cord blood mononuclear cell cultures from these neonates. Generation and maintenance of IL-10⁺ T cells in utero may thus contribute to suppression of APC function and Pf Ag-induced Th1 responses in newborns born to mothers with placental malaria at delivery, which may increase susceptibility to infection later in life. *The Journal of Immunology*, 2005, 174: 1738–1745.

Plasmodium falciparum (Pf)³ infection during pregnancy constitutes a widespread and significant public health problem in regions of high malaria endemicity (1, 2). Infection is coupled with the sequestration of Pf-infected erythrocytes in the placenta, which leads to placental pathologies that include syncytiotrophoblastic necrosis, increased mononuclear inflammatory cell infiltrates, and lesions (3–5). Placental pathology associated with Pf sequestration causes an increased risk of preterm delivery, low birth weight, anemia, and mortality (6–8), particularly for women experiencing their first pregnancy. Administration of antimalarial prophylaxis protects against maternal malaria and by extension alleviates infection-associated risks to the neonate, but

the resulting immunocompetence of the newborn has not been thoroughly investigated (9).

The effects of placental malaria on the development of the fetal immune response and particularly plasmodial Ag-specific T and B cell responses remain poorly understood. It has been hypothesized that Pf placental infection might result in priming of Ag-specific T cells as well as in the induction Ag-specific suppression or tolerization. Much work is needed to reach a full understanding of the nature of these responses, because such information is indispensable for the planning of a successful malaria vaccine for children and infants. On the basis of neonatal responses in rodents, it has been generally accepted that although neonatal T cells have greater requirements for costimulation (10), when provided with mature APC they are sufficiently equipped to yield Ag-specific immune responses (11, 12). Neonatal APCs, however, are limited in both numbers and costimulatory molecules, B7.1/B7.2 and MHC class II, which limits their ability to support Ag-mediated stimulation of naive T cells (13, 14). The inability of neonatal APC to produce critical cytokines, such as IFN- γ and IL-12, also contributes to the reduced APC function and MHC class I and II expression (15, 16). Because APC drive the development of Th1 responses through the production of IL-12 during priming, the lack of Th1 responses in neonates could be due to suboptimal APC function (17).

Epidemiological studies demonstrate that placental malaria and high titers of anti-pregnancy-associated malaria Abs in cord blood predict enhanced susceptibility of children to malaria (18, 19). This presumably stems from in utero exposure to Pf Ags that induce immune tolerance in the newborn, leading to down-regulated or nonprotective immune responses upon re-exposure. There is direct evidence for in utero priming of B and T cells. IgM Abs reactive with a large number of blood stage Ags including the

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³ Abbreviations used in this paper: Pf, *Plasmodium falciparum*; CBMC, cord blood mononuclear cell; rh, recombinant human; Tr, regulatory T cell; pRBC, parasitized RBC; uRBC, uninfected RBC; CM, complete medium; MFI, mean fluorescence intensity.

vaccine candidate MSP-1 (20–22), are present in up to 25% of cord samples in sub-Saharan Africa. Plasmodial Ag-specific Th2-type responses have been recalled in cord blood mononuclear cell (CBMC) cultures (23).

Apart from the evidence that Pf Ags directly interact with the immune cells of the developing fetus, whereby they modulate B cells, T cells, and macrophages, immunological changes that occur in the placental tissue and particularly the cytokine milieu also shape the immune system of the newborn. A bias toward a Th2 placental environment, e.g., IL-10, characterizes normal human pregnancy and is thought to prevent inflammatory responses that might damage the integrity of the materno-fetal placental barrier (24, 25). During placental malaria, despite the placental shift toward Th1-type cytokines (8, 26), IL-10 concentrations are elevated compared with healthy placentas (8). Active placental malaria at delivery further reduces parasite Ag-induced Th1 responsiveness in cord blood cells, as well as proliferative responses (27, 28). IL-10 is also present in the cord blood serum, and levels are inversely correlated with gestational age (29). During T cell priming, the presence of IL-10 further suppresses the generation of a Th1 response by down-regulating MHC class I and II expression, thus reducing APC function (30–32). Additionally, IL-10 may induce T cells to differentiate into regulatory, IL-10-secreting T cells (Tr cells) that suppress Ag-specific effector responses (33–37).

In this study, we investigated whether putatively heavy and chronic Ag exposure during placental malaria predisposes newborn T cells to Ag-specific immunosuppressive pathways. We hypothesize that along with generating parasite Ag-specific effector T cells, the frequency of Pf Ag-specific IL-10-producing cells increases as a consequence of placental infection. We compared Ag-specific T cell responses of 58 neonates whose mothers had either placental malaria at delivery or were diagnosed and treated for infection before delivery, thus comparing neonates who experienced either potentially long term or short term Ag exposure. Our results demonstrate that CBMC from the offspring of mothers with placental malaria at delivery had reduced Pf Ag-specific CD4⁺ and CD8⁺ T cells producing IFN- γ and reduced MHC class I and II expression on APC, which correlated with a trend toward increased Pf Ag-specific IL-10 production from CD4⁺ T cells. Moreover, we show that the immune suppression could be reversed in culture with the addition of recombinant human (rh) IFN- γ and neutralizing anti-human IL-10 Ab.

Materials and Methods

Study population

For this study we collected cord blood from a total of 135 neonates and venous blood from 27 mothers. The study was conducted at the Albert Schweitzer Hospital in Lambaréné, Gabon, and informed consent for participation was obtained before inclusion in the study. Maternal peripheral venous blood was obtained within 24 h of parturition and was collected into heparinized tubes. The umbilical venous cord blood was obtained immediately after parturition and was anticoagulated with heparin. Collection of cord blood involved direct aspiration via puncture of the ethanol-sterilized umbilical vein at a site distal to the placenta to reduce to a minimum the possibility of cross-contamination by maternal lymphocytes. The presence of Pf parasites in these compartments at the time of delivery was determined by routine microscopic examination of Giemsa-stained thick and thin smears. Giemsa-stained impression smears of placental blood were similarly examined. The medical records of uninfected mothers were examined to identify those who had had appropriately diagnosed and treated malaria episodes during their pregnancy. The specific antimalaria drugs used among 24 treated women were quinine (18), chloroquine (4), and amodiaquine (2). The median number of days of treatment before delivery was 92.5 (interquartile range, 101.5; minimum/maximum 14/203 days). To our knowledge, quinine retains 100% efficacy for the treatment of uncomplicated Pf malaria arising during pregnancy in women residing in the study area (38). For comparative analyses, the following distinct

groups were thus defined on the basis of the presence or absence of placental Pf infection by placental impression smear and on assessment of documented medical history: 1) negative: no evidence of Pf parasites/active infection in any compartment at delivery and no record of maternal malaria during pregnancy; 2) placenta positive: Pf asexual stage parasites present in placental blood; 3) treated: no evidence of Pf parasites/active infection in any compartment at delivery but recorded history of diagnosed and treated maternal malaria episode during pregnancy at least 2 wk before delivery.

For confirmation of the accuracy of these criteria, two further assessments were performed on a subgroup of 30 mother/cord pairs: 1) detection of the Pf histidine-rich protein II Ag with a commercially available assay (Malaria AG Celisa; Cellabs) used according to the manufacturer's recommendations; and 2) detection of the multicopy Pf small subunit rRNA gene using nested PCR amplification-based assay as described elsewhere (39). The results obtained with these assays validated the criteria for segregation outlined above: both assays gave negative results with samples from all mothers included in the negative and treated groups; both assays gave positive results with samples from all mothers included in the placenta-positive group.

Ethical clearance for the study was given by the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné, and the study was approved by the Institutional Review Board at Walter Reed Army Institute of Research.

Blood stage parasite Ag preparation

Blood stage Ag was prepared from schizont-infected erythrocytes using a local Pf isolate cultured and synchronized by temperature cycling through 37, 40, and 17°C. Schizont-infected erythrocytes (pRBC) or uninfected erythrocytes (uRBC) were Percoll purified, resuspended in RPMI 1640 (Sigma-Aldrich), and freeze thawed once before addition to culture.

Isolation of mononuclear cells and cell culture

CBMC and maternal PBMC were isolated by standard density gradient centrifugation on Ficoll-Paque (Amersham). For analyses of IFN- γ from *in vitro* culture supernatants, CBMC samples from 111 neonates were used. Aliquots of 0.15×10^6 cells in 100 μ l were distributed in the wells of flat-bottom Costar 96-well tissue culture plates (Integra Biosciences), pRBC or uRBC were added to the cells of triplicate wells at a dilution of 1 CBMC/PBMC to 10 U/pRBC in complete medium (CM). Control cultures were stimulated with PHA (5 ng/ml; Sigma-Aldrich). CM was composed of RPMI 1640 supplemented with 10% (w/v) human serum (AB) (Sigma-Aldrich), 1 mM L-alanylglutamine (Life Technologies), 100 U/ml penicillin, 100 μ g/ml streptomycin (Life Technologies), and 1 mM sodium

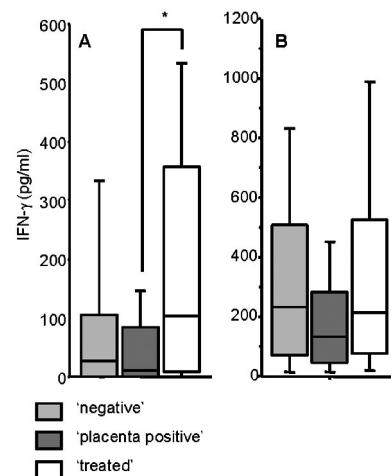


FIGURE 1. CBMC from placenta-positive neonates produce reduced levels of Pf Ag-specific IFN- γ in culture supernatants compared with treated neonates. Net IFN- γ production measured in culture supernatants from CBMC; A, pRBC stimulated ($-uRBC$ stimulated); B, PHA stimulated ($-medium$ stimulated). Groups are segregated according to maternal Pf infection status. Box plots illustrate medians with 25th, 75th, and whiskers for 10th and 90th percentiles; $n = 75$ negative, 23 placenta-positive, 13 treated. *, $p < 0.05$, Mann-Whitney U test.

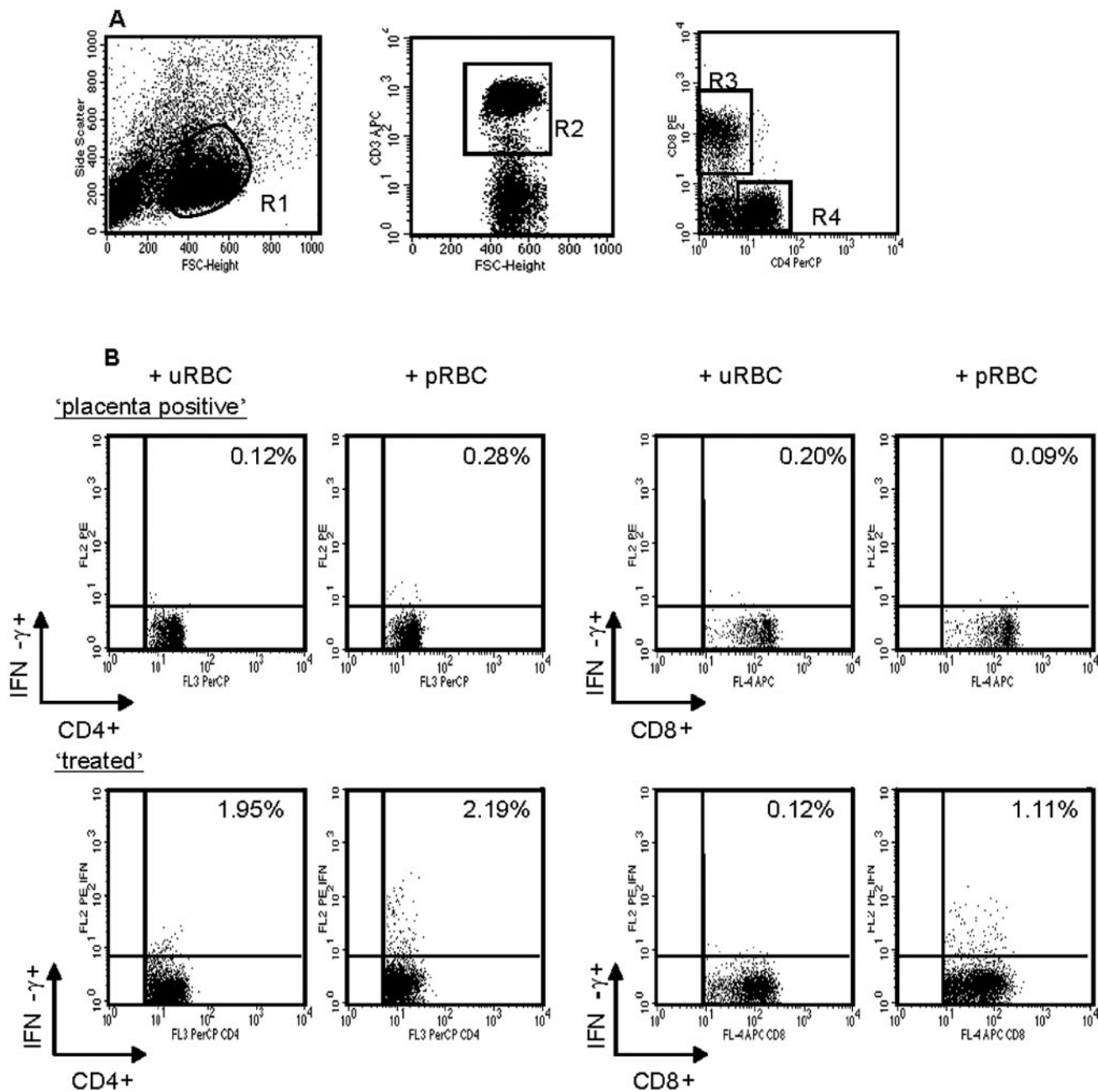


FIGURE 2. Example of IFN- γ production measured by FACS analysis from CD4⁺ and CD8⁺ cord blood CD3⁺ T cells cultured for 22 h in the presence of uRBC or pRBC lysate in two neonates, placenta-positive and treated. *A*, Side and forward scatter (FSC) of CBMC; R2, CD3⁺ gating; R3, CD4⁺; and R4, CD8⁺ gating. *B*, measurement of IFN- γ ⁺ cells from CD4⁺ and CD8⁺ T cells. FL, Fluorescence.

pyruvate (Life Technologies). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ for 6 days; and cell-free supernatants from triplicate cultures were collected, pooled, and stored frozen at -80°C until required. The concentration of IFN- γ was determined using commercial ELISA kits (Flexia; Medgenix-Biosource), with detection limits of 5.7 pg/ml. Experiments using control CBMC from German newborns born to mothers with no known exposure to Pf infection were performed. We detected only negligible, very low T cell IFN- γ activity in response to either uRBC or pRBC lysates, as was observed in our negative group of Gabonese neonates.

For intracellular cytokine and MHC expression studies, mononuclear cells were isolated as above and immediately resuspended at 5×10^6 cells/ml in CM and 0.5 μ g/ml each of mAbs with specificity for the human T cell-costimulatory molecules CD28 and CD49d (BD Biosciences). A total of 0.2 ml of cell suspension was placed in 96-well round-bottom tissue culture plates (Falcon; BD Biosciences). uRBC and pRBC were added to the cells at a dilution of 1 CBMC/PBMC to 10 U/pRBC. The cells were

then incubated at 37°C for 18 h in a humidified 5% CO₂ atmosphere. Brefeldin A (Sigma-Aldrich) at 10 μ g/ml was added, and the cells were incubated for a further 4 h before fixation. For cytokine neutralization and exogenous cytokine supplementation experiments, 1 μ g/ml anti-human IL-10 (JES3-9D7; BD Biosciences) or 100 U/ml rhIFN- γ (Roche Diagnostics) was added at the beginning of cell cultures. Cultured cells were harvested and washed in PBS, fixed with 2% formaldehyde for 20 min, washed, resuspended in HBSS, 0.1% azide, 0.1% BSA, and stored at 4°C before staining for flow cytometric analysis.

Flow cytometric analyses of APC surface MHC class I and II and T cell intracellular cytokines

Cells were washed twice in cold PBS and incubated on ice for 15 min with PBS-10% FCS containing 2 μ l of FcR Blocking Reagent (Miltenyi Biotec). Cells (5×10^5 /ml) were surface-stained for four-color flow cytometry with the following panel of fluorescently labeled antibodies, all supplied by BD

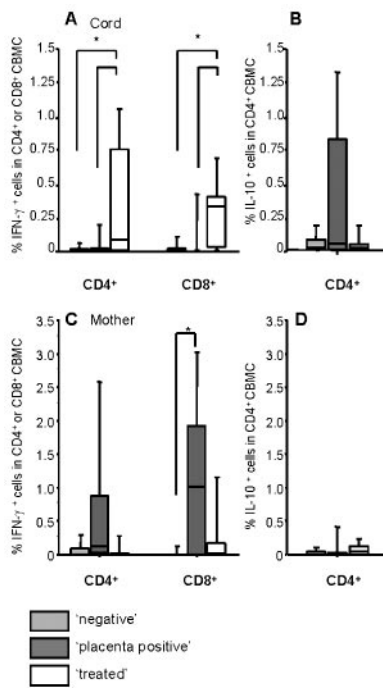


FIGURE 3. CBMC from placenta-positive neonates contain a lower percentage of Pf Ag-specific IFN- γ CD4 $^{+}$ and CD8 $^{+}$ T cells and a higher percentage of IL-10 $^{+}$ CD4 $^{+}$ T cells compared with treated neonates. Net (pRBC – uRBC lysate stimulated) IFN- γ (A and C) and IL-10 (B and D) production measured in CD3 $^{+}$ T cell subsets from CBMC (A and B) and maternal PBMC (C and D). Neonates are segregated by placental Pf infection status at the time of delivery. Box plots illustrate medians with 25th, 75th, and whiskers for 10th and 90th percentiles; cord, $n = 12$ negative, 11 placenta-positive, 11 treated; mother, $n = 9$ negative, 9 placenta-positive, 8 treated. *, $p < 0.05$, Mann-Whitney U test.

Biosciences (clone): CD4-PerCP (SK3), CD8-APC (RPA-T8), CD3-FITC (UHCT1), CD14-FITC (M5E2), CD19-PerCP (HIB19), HLA-A,B,C-APC (G46-2.6), HLA-DR-PerCP (L243), or isotype control Abs mouse IgG1, -FITC, -PE, -PerCP, -APC (MOPC-21), and mouse IgG2a, -FITC, -PE, -PerCP (G155–178). The frequencies of IFN- γ - and IL-10-producing cells were determined by intracellular cytokine staining using Cytotfix/Cytoperm Plus kits according to the manufacturer’s protocol (BD Biosciences) using Abs IFN- γ -FITC (25723.11), IL-10-PE (JES3-9D7), or isotype control Abs mouse IgG1-PE (MOPC-21) and mouse IgG2b-FITC (27–35). Flow cytometry was performed on a FACScan flow cytometer with CellQuest data analysis software (BD Biosciences). A minimum of 50,000 lymphocytes were gated.

Statistical analyses

The significance of differences between continuous variables was assessed using the Mann-Whitney U test. The level of statistical significance in all cases was set at $p < 0.05$.

Results

Pf Ag-specific IFN- γ and IL-10 production from CBMC

Our first objective in this study was to assess CBMC cytokine responses from neonates sensitized to Pf Ag, as a result of either active placental infection at delivery or maternal Pf infection that was cleared during pregnancy. Examination of immune responses in neonates born to mothers who were treated for infection during pregnancy has, to our knowledge, not previously been reported. Others have reported reduced Th1 responsiveness in cord blood from neonates whose mothers had active placental malaria at delivery compared with neonates born to uninfected mothers from the same region (28). In support of these findings, the median levels of IFN- γ (10 vs 27 pg/ml) in CBMC from placenta-positive

and -negative neonates were similarly low (Fig. 1). The highest median level of IFN- γ (102 pg/ml) in 6-day culture supernatants was in CBMC from neonates whose mothers had been drug treated for infection during pregnancy and did not have placental malaria at delivery. The levels of IFN- γ in supernatants of CBMC stimulated with PHA did not differ among the groups (Fig. 1).

To better define effector T cell activity, we then used flow cytometric methods to analyze induction of IFN- γ and IL-10 in CD4 $^{+}$ and CD8 $^{+}$ CD3 $^{+}$ T cell subsets after short term (22 h) co-culture of pRBC with CBMC, as demonstrated in Fig. 2, and for comparative purposes with maternal venous PBMC. The levels of intracellular IFN- γ detected confirmed our earlier results of IFN- γ production in long term culture supernatants in that parasite Ag stimulated the highest frequencies of IFN- γ -producing CD4 $^{+}$ and CD8 $^{+}$ CD3 $^{+}$ T cells in CBMC from neonates of treated mothers (Fig. 3A). In marked contrast to the pattern observed in CBMC, the highest frequencies of pRBC-induced IFN- γ -producing CD4 $^{+}$ and CD8 $^{+}$ CD3 $^{+}$ T cells were detected in maternal PBMC from those in the placenta-positive group (Fig. 3C). Parallel analyses of pRBC-induced IL-10 production revealed that the frequency of IL-10 $^{+}$ CD4 $^{+}$ T cells was elevated in CBMC of placenta-positive neonates, whereas the frequency of IL-10 $^{+}$ CD4 $^{+}$ cells was similarly low in PBMC of mothers in all three groups (Fig. 3, B and D). Parasite Ag-specific PBMC IFN- γ activity was comparatively strong in the placenta-positive and weak in the treated groups of mothers, in complete contrast to the profiles in the equivalent CBMC samples, suggesting that no contamination of CBMC with maternal PBMC occurred during cord blood collection.

It is well established that IFN- γ activates APC by increasing the expression of the costimulatory molecules CD80/86 as well as of MHC class I and II, all of which lead to enhancement of T cell responses, whereas IL-10 reduces APC MHC expression and thus down-modulates T cell responsiveness. To determine whether the

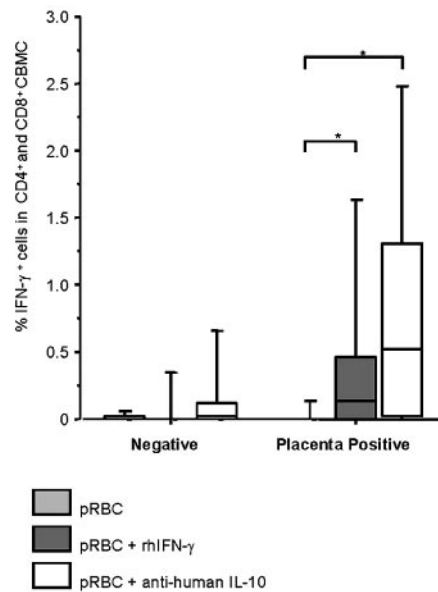


FIGURE 4. Exogenous rhIFN- γ or neutralizing anti-human IL-10 Ab added at the beginning of cell culture induces Pf Ag-specific IFN- γ production from T cells obtained from placenta-positive neonates. Bars represent net percentages of IFN- γ -producing CD4 $^{+}$ and CD8 $^{+}$ CD3 $^{+}$ T cells from negative and placenta-positive CBMC cultured for 22 h in medium with pRBC (vs uRBC) in the presence of exogenous IFN- γ or anti-human IL-10. Box plots illustrate medians with 25th, 75th, and whiskers for 10th and 90th percentiles; $n = 9$ negative, 9 placenta-positive. *, $p < 0.05$, Mann-Whitney U test.

Table I. *In vivo* activation status of APC in CBMC and PBMC segregated by maternal infection status

	Cord Blood			Maternal Venous Blood		
	Negative (<i>n</i> = 12)	Placenta positive (<i>n</i> = 11)	Treated (<i>n</i> = 11)	Negative (<i>n</i> = 9)	Placenta positive (<i>n</i> = 9)	Treated (<i>n</i> = 8)
MHC class I (MFI)						
CD14 ⁺ monocytes	6.7 (24.4) ^a	15.8 (8.8)	20.2 (11.6)	6.8 (0.4)	29.5 (36.0)	64.7 (39.5) ^b
CD19 ⁺ B cells	10.4 (17.2)	19.4 (19.1)	31.4 (26.5) ^b	11.1 (6.1)	18.7 (70.0)	64.1 (68.0) ^b
MHC class II (MFI)						
CD14 ⁺ monocytes	6.6 (6.9)	10.8 (4.9)	13.6 (4.8) ^b	7.7 (0.8)	10.2 (5.4)	15.3 (7.5) ^b
CD19 ⁺ B cells	5.3 (6.5)	16.3 (4.7) ^b	14.2 (8.8) ^b	9.4 (7.2)	19.8 (19.6)	18.4 (21.9) ^b

^a Values are median (interquartile ranges).

^b *p* < 0.05, Mann-Whitney *U* test for comparison to negative (uninfected) group.

low pRBC-specific IFN- γ responsiveness of T cells in CBMC of placenta-positive neonates was mediated through actions of these regulatory cytokines, we added rhIFN- γ or neutralizing anti-human IL-10 at the start of CBMC cultures. The addition of both rhIFN- γ and anti-IL-10 significantly enhanced Pf Ag-specific T cell IFN- γ responses in CBMC of placenta-positive neonates, whereas those in CBMC of negative neonates were unaffected (Fig. 4).

MHC class I and II expression on cord APC *in vivo* and in response to Pf Ag

Because cord blood T cell activity has been shown to depend on the maturation status of cord blood APC (15, 40), we next assessed the effects of maternal Pf infection history on surface expression of both MHC class I and class II on CBMC and maternal PBMC-derived populations of CD14⁺ and CD19⁺ APC. Treatment for malaria during pregnancy, and to a lesser extent active placental Pf infection at delivery, increased APC MHC class I and II expression compared with APC from negative neonates (Table I). Overall (data from both monocytes and B cells), MHC class I expression was significantly lower on APC from placenta-positive neonates vs treated neonates (median mean fluorescence intensity (MFI) (interquartile range), 17.2 (13.5) and 23.4 (18.9), respectively, *p* = 0.02). MHC class I and II expression on maternal APC showed a profile similar to that observed in neonates, in that APC from treated mothers expressed the highest levels of MHC class I and II (Table I). Overall, there was a trend toward higher MHC class I expression on maternal APC than cord APC (median MFI (interquartile range), 28.6 (56.9) and 13.2 (11.4), respectively, *p* = 0.07).

We cocultured CBMC with medium, uRBC, or pRBC and examined the relative percent change in the MFI of MHC class I and II expression levels on APC after 22 h of incubation (100 \times pRBC MFI - uRBC MFI/uRBC MFI). Representative results from CBMC of a single individual showing the surface expression of MHC class I and II on gated monocytes are depicted as histograms in Fig. 5. Relative to incubation with uRBC, incubation of CBMC with pRBC resulted in a marked decrease of surface MHC class I and II expression on monocytes of placenta-positive neonates while having little effect on MHC expression on monocytes of the negative and treated groups and no marked effect on B cells in CBMC of any group (Fig. 6A). Maternal APC showed very different profiles compared with neonates in that MHC class I and II expression on APC from placenta-positive and treated mothers was generally enhanced compared with negative mothers in response to pRBC (Fig. 6B). Overall, pRBC induced a significant reduction of MHC class I and II expression on APC of neonates compared with those of the corresponding mothers (median (interquartile range), -10.7% (25.2) and 12.0% (42.7), respectively, *p* = 0.0001).

Incubation of CBMC in CM alone did not cause down-regulation of MHC class I and II on APC from placenta-positive neonates (Fig. 6C). MHC class I and II expression levels were highest in placenta-positive neonates, in contrast to the high level of expression of MHC molecules observed *ex vivo* on APC in treated neonates (Table I). This suggests a recovery of MHC surface expression in the absence of plasmodial Ags. Incubation in CM induced the highest levels of MHC class I and II expression on maternal B cells in the placenta-positive group (Fig. 6D).

The pRBC-induced suppression of MHC class I and II on monocytes in CBMC of placenta-positive neonates was reversed by addition of rhIFN- γ but was unaffected by addition of neutralizing anti-IL-10 (Fig. 7).

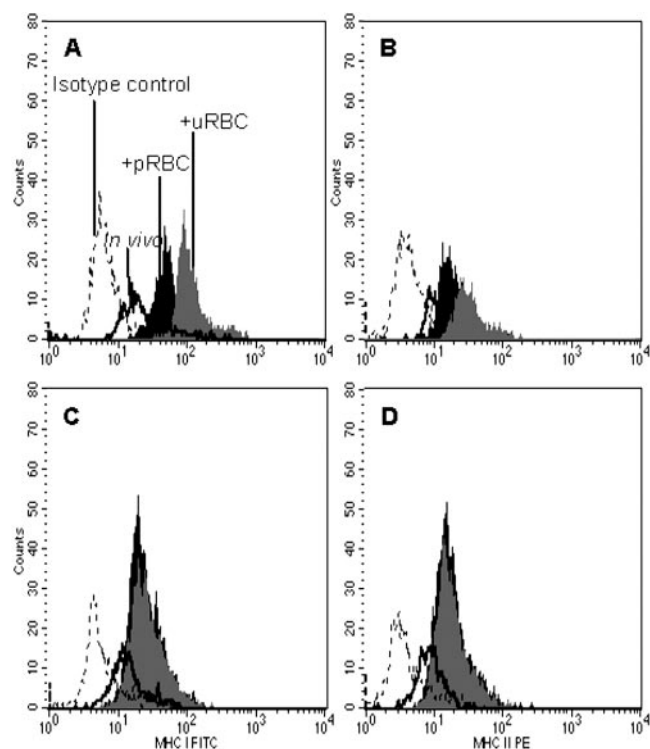
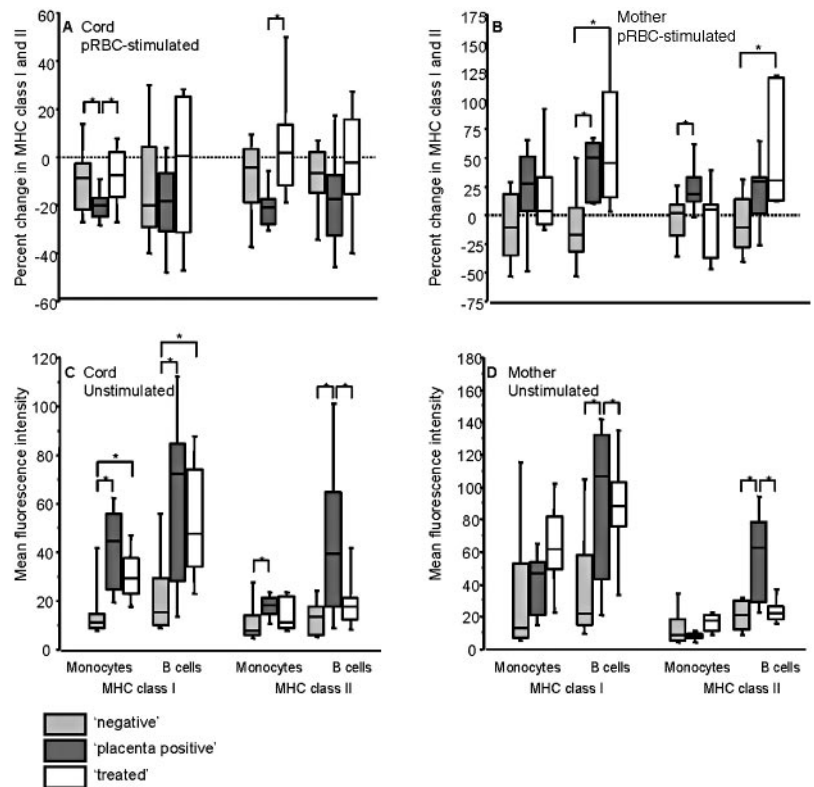


FIGURE 5. Active placental Pf infection is associated with reduced MHC class I and II expression on cord blood monocytes cultured with pRBC. Histogram analyses of MHC class I (A and C) and II (B and D) surface expression on monocytes *in vivo* and after 22 h of culture in the presence of uRBC or pRBC in placenta-positive (A and B) and negative (C and D) CBMC. ---, Isotype control; —, *in vivo*; ■, + pRBC; ▨, + uRBC.

FIGURE 6. MHC class I and II expression on monocytes from placenta-positive neonates is down-regulated in response to Pf Ag. Bars represent the percent change in the MFI of MHC class I and II on monocytes and B cells from CBMC (A) and maternal PBMC (B) cultured for 22 h in CM with pRBC vs uRBC. A reduction in MHC expression was not observed in APC from placenta-positive neonates after incubation of CBMC (C) and PBMC (D) in CM for 22 h. Box plots illustrate medians with 25th, 75th, and whiskers for 10th and 90th percentiles; cord, $n = 12$ negative, 11 placenta-positive, 11 pregnancy-positive; mother, $n = 9$ negative, 9 placenta-positive, 8 treated. *, $p < 0.05$, Mann-Whitney U test.



Discussion

In this study, we determined that IL-10 and IFN- γ play a role in regulating Th1-type immune responses in neonates born to mothers with placental malaria at delivery. Placental malaria at delivery was associated with increased numbers of IL-10⁺CD4⁺ T cells and diminished Pf Ag-specific Th1 responsiveness, whereas chemotherapeutic cure of pregnancy-associated malaria several weeks before delivery resulted in substantially increased frequencies of Pf Ag-specific IFN- γ -producing T cells, particularly CD8⁺ cells, in cord blood. These data reflect response profiles observed in children treated for Pf malaria in that CD8⁺ IFN- γ responses were also predominant (41). Neonates exposed to Pf Ag in utero are thus able to produce Th1-type immune responses in a manner, although not necessarily intensity, similar to responses observed in malaria-exposed children. Cord blood immune responsiveness is dependent on factors related to maternal infection history, and possibly cytokine environment before and at the time of sample collection. To determine whether cytokine-mediated events induced suppression of Th1-type immune responses associated with placental malaria, we manipulated the cytokine milieu of in vitro cultures of CBMC by addition of rhIFN- γ or neutralizing anti-IL-10 Ab. IFN- γ increases expression of MHC class I and II on cord blood dendritic cells (16, 42), leading to increased T cell activation, whereas IL-10 has counterregulatory properties to IFN- γ that include blocking IFN- γ synthesis as well as down-modulating MHC class I and II expression (30, 32, 43). Accordingly, we found that these manipulations revealed the existence of a pool of parasite Ag-specific IFN- γ -producing T cells similar in magnitude to that found in CBMC from treated neonates. Although addition of neutralizing anti-IL-10 Ab increased parasite Ag-specific IFN- γ responses in short term in vitro cultures, it did not reverse the suppression of surface MHC expression, suggesting that IL-10 suppresses effector T cell functions directly (44). IL-10 plays a role in immune suppression and the long term persistence of the protozoan parasite *Leishmania major* (45), although it is not yet established whether

IL-10 acts directly on T cells and/or through down-regulation of APC function (46). From our findings, we conclude that active placental Pf infection leads to suppression of fetal parasite Ag-specific Th1-type immune responses via an IL-10-mediated pathway that reduces IFN- γ availability.

Cord blood APC expressed reduced levels of MHC class I, and to a lesser extent MHC class II, compared with maternal APC which gives an indication that these cord blood APC were less mature and were not able to express MHC at levels comparable with those of adults. During early development, fetal APC are

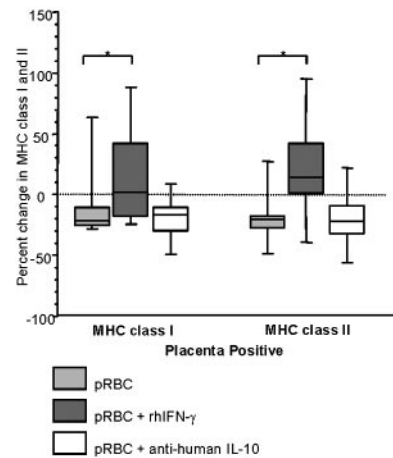


FIGURE 7. Exogenous rhIFN- γ added at the beginning of cell culture up-regulates Pf Ag-driven MHC class I and II expression on monocytes from placenta-positive neonates. Percent change in the MFI of MHC class I and II on CD14⁺ monocytes from placenta-positive CBMC cultured for 22 h in medium with pRBC (vs uRBC) in the presence of exogenous rhIFN- γ or anti-human IL-10 Ab. Box plots illustrate medians with 25th, 75th, and whiskers for 10th and 90th percentiles; $n = 9$ negative, 9 placenta-positive. *, $p < 0.05$, Mann-Whitney U test.

deficient in costimulatory capacity and hence, when loaded with Ag, may lead to induction of Ag-specific IL-10-producing CD4⁺ T cells with suppressive properties (47). Our data demonstrate that placental malaria was associated with increased frequencies of Pf Ag-specific IL-10⁺CD4⁺ cells in cord blood. It is interesting to note that cord blood from the treated group of neonates did not contain Pf Ag-specific IL-10⁺CD4⁺ T cells considering that this group showed the strongest evidence of in utero exposure to Ag in terms of the frequency of Pf Ag-specific IFN- γ ⁺CD4⁺ and CD8⁺ T cells. The Ag dose, duration of exposure, and time since last exposure may provide some explanation. Type 1 regulatory T cells (Tr1), which are characterized as CD4⁺IL-10^{high}TGF- β ^{high}IFN- γ ^{low}, require the presence of chronic low levels of Ags, have relatively limited effect in the absence of persistent Ag (48), and become fully mature after repeated Ag stimulation (47, 49). Mothers from the treated group received drug treatment at least 2 wk before delivery and were confirmed to be Pf free at delivery, in both the periphery and the placenta, thus making it likely that fetal exposure to Pf Ag was markedly diminished at delivery as compared with exposure in neonates from the placenta-positive group. Another explanation is that the cytokine environment, e.g., reduced IL-10, in the treated group at the time of sample collection reduced the frequency of activated IL-10⁺CD4⁺ T cells. In this case, we postulate that further stimulation and/or exogenous cytokines, such as IL-2, IL-10, and/or IL-15, are required for in vitro expansion and activation (50), thus possibly distinguishing active vs memory IL-10⁺CD4⁺ T cells.

Several types of CD4⁺ suppressor, or regulatory T cells, have been described, and the mechanisms by which these cells exert their suppressive effects are not limited to Ag-driven IL-10 and TGF- β (Tr1, Th3) (34) but may also include Ag-independent cell-to-cell interactions (51, 52). There is also evidence that CD4⁻regulatory cells mediate immunosuppression. Acute Pf episodes in children are associated with reduced T cell proliferation and cytokine production in vitro that can be partially abrogated by removal of CD8⁺ lymphocytes (53–55). Clearly, our data do not aim to identify a specific regulatory T cell phenotype in the cord blood samples; instead we sought to determine whether Ag-induced IL-10 plays a role in regulating cord blood APC function and T cell responsiveness. The presence of immature APC and IL-10 in the fetal circulation would support the generation of Tr cells.

This study provides evidence that the absence of IFN- γ and the presence of IL-10 mediate down-regulation of Ag-specific Th1 responses in neonates whose mothers had placental malaria. We postulate that placental Pf infection can subject the fetal immune system to prolonged, continuous and possibly high-grade Ag exposure which enhances the generation of regulatory cells required to reduce the pathological consequences of persistent activation of proinflammatory Th1 cells. More detailed assessments of this particular aspect are the subject of ongoing studies that aim to clarify whether the higher susceptibility to Pf infection of infants born to mothers with active placental Pf infection at delivery is related to the presence of a Tr cell population. These data also provide supportive evidence that treatment for pregnancy-associated malaria enhances Th1 immunoresponsiveness of newborns to Pf Ags, thus possibly leading to enhanced protection from malaria early in life.

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