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Distinct Th1- and Th2-Type Prenatal Cytokine Responses to *Plasmodium falciparum* Erythrocyte Invasion Ligands

Indu Malhotra,[1] Peter Mungai,[1] Eric Muchiri,[2] John Ouma,[3] Shobhona Sharma,[4] James W. Kazura,[1] and Christopher L. King[1,5]*

Center for Global Health and Diseases, Case Western Reserve University, Cleveland, Ohio[1]; Division of Vector Borne Diseases, Nairobi, Kenya[2]; Maseno University, Maseno, Kenya[3]; Tata Institute of Fundamental Research, Mumbai, India[4]; and Department of Veterans' Affairs Medical Center, Cleveland, Ohio[5]

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Prenatal immunity to *Plasmodium falciparum* merozoite proteins involved in erythrocyte invasion may contribute to the partial protection against malaria that is acquired during infancy in areas of stable malaria transmission. We examined newborn and maternal cytokine and antibody responses to merozoite surface protein-1 (MSP-1), ribosomal phosphoprotein P0 (PfP0), and region II of erythrocyte binding antigen-175 (EBA-175) in infant-mother pairs in Kenya. Overall, 82 of 167 (50%), 106 of 176 (60%), and 38 of 84 (45%) cord blood lymphocytes (CBL) from newborns produced one or more cytokines in response to MSP-1, PfP0, and EBA-175, respectively. Newborns of primigravid and/or malaria-infected women were more likely to have antigen-responsive CBL than were newborns of multigravid and/or uninfected women at delivery. Newborn cytokine responses did not match those of their mothers and fell into three distinct categories, Th1 (21 of 55 CBL donors produced only gamma interferon and/or interleukin 2 [IL-2]), Th2 (21 of 55 produced only IL-5 and/or IL-13), and mixed Th1/Th2 (13 of 55). Newborns produced more IL-10 than adults. High and low levels of cord blood IL-12 p70 production induced by anti-CD40 activation were associated with malaria-specific Th1 and Th2 responses, respectively. Antigen-responsive CBL in some newborns were detected only after depletion of IL-10-secreting CD8 cells with enrichment for CD4 cells. These data indicate that prenatal sensitization to blood-stage *Plasmodium falciparum* occurs frequently in areas where malaria is holoendemic. Modulation of this immunity, possibly by maternal parity and malaria, may affect the acquisition of protective immunity against malaria during infancy.

Falciparum malaria places a high burden of health on pregnant women and infants in sub-Saharan Africa and other regions of the world where malaria is endemic. Women experiencing their first pregnancies are especially vulnerable, and their infants may be born prematurely and have low birth weights (42). Maternal malaria and immunity may, however, indirectly benefit young infants by enhancing resistance against blood-stage infection and morbidity for the first several months after birth. The best-understood mechanism underlying this phenomenon involves immunoglobulin G (IgG) antibodies to blood-stage *Plasmodium falciparum*. These antibodies are acquired from the maternal circulation during gestation, target merozoite invasion ligands and parasite antigens transported to the surface of the infected erythrocyte, and presumably protect the young infant from severe malaria and high-density parasitemia by slowing the growth rate of intraerythrocytic parasites (5, 8, 12). IgG antibodies of maternal origin decrease progressively in the infant's circulation and are completely lost by 6 to 9 months after birth (12, 51, 59). A feature of maternal-neonatal interaction that is not as well understood involves the possible exposure of the fetus to malaria antigens in utero and the consequent immunologic sensitization or tolerance this may induce. The existence and nature of this prenatal immunity may potentially have great significance, since T- and B-cell memory and protective effector responses generally develop after repeated bouts of malaria during infancy.

It is not possible to examine human fetal immunity to malaria antigens in situ or determine directly whether in utero exposure to malaria occurs. Nevertheless, several lines of evidence suggest that such events occur, particularly in settings where maternal malaria is common. First, infected maternal erythrocytes have a propensity to be sequestered in the low-flow intervillous space of the placenta (especially in primigravidae) (2, 18, 24), thereby increasing the likelihood that infected erythrocytes and soluble blood-stage antigens will gain access to the fetal circulation. Second, newborns may be exposed to malaria parasites at or shortly before parturition. Cord blood infection rates of 5 percent or greater have been estimated on the basis of microscopic inspection of cord blood from African newborns (9, 20, 32, 57). Rates of 14 to 33 percent have been reported when more-sensitive diagnostic methods, such as PCR or circulating parasite antigen assays, are used (27, 28, 57, 61). Current evidence suggests that admixture of the maternal and fetal circulations at the time the newborn is passing through the birth canal is uncommon, since malaria genotypes examined in matched cord and maternal blood samples are often discordant with maternal peripheral blood (27, 28, 40, 57) or placental intervillous blood (27), and maternal IgE that does not cross the placenta is rarely detected in cord blood (30). Third, studies of rodent malarias demonstrate that prenatal T-cell sensitization occurs when pregnant rats are infected with *Plasmodium berghei* (10, 11, 13–15). Lymphocytes from newborn pups of malaria-infected dams proliferate in

* Corresponding author. Mailing address: Center for Global Health and Diseases, Case Western Reserve University, 2103 Cornell Rd., WRC Room 4132, Cleveland, OH 44106-7286. Phone: (216) 368-4817. Fax: (216) 368-4825. E-mail: christopher.king@case.edu.

response to extracts of blood-stage parasites, and malaria-specific immunity can be adoptively transferred to naïve rats by lymphocytes from sensitized pups.

Sensitization of human newborns to malaria and other blood-borne pathogens can be evaluated by studying antigen-driven responses by cord blood lymphocytes (CBL), since these cells reflect the pool of circulating fetal lymphocytes present at birth. Cord blood IgM or IgE antibodies also constitute prima facie evidence of in utero sensitization because, unlike IgG, the previously mentioned Ig isotypes are not transferred across the placenta from the maternal circulation. There have been relatively few studies of human CBL responses to malaria antigens (11, 19, 31, 50). Fievet and coworkers (19) observed that 9.8 and 13.2 percent of 164 Cameroonian newborns had CBL gamma interferon (IFN-γ) and interleukin 4 (IL-4) responses to native ring-infected erythrocyte surface antigen (RESA/Pf155). Response rates to RESA antigenic peptides were significantly lower, ranging from 1.3 to 6.3 percent.

We recently showed that antigenic peptides corresponding to the C-terminal region of merozoite surface protein-1 (MSP-1), a leading candidate for a human malaria vaccine, stimulated CBL IFN-γ responses in 26 percent of 92 newborns from an area of Kenya in which malaria is holoendemic (30). In contrast, responses to the preerythrocytic *P. falciparum* antigen liver stage antigen-1 were not detected, and CBL from control newborns of mothers who had never been exposed to malaria failed to respond to MSP-1, confirming the specificity of this immunity for malaria. Importantly, lymphocytes from newborns that failed to respond to MSP-1 in bulk cultures produced IFN-γ when $CD4^+$ T cells were purified, suggesting that prenatal sensitization to malaria may be more common than that estimated by experiments using bulk CBL alone and that a subset of neonatal lymphocytes may produce immunoregulatory cytokines that suppress IFN-γ and IL-2 production (19, 30, 50). In the current study, we examined maternal peripheral blood mononuclear cell (PBMC), bulk CBL, and neonatal $CD4^+$ and $CD8^+$ T-cell cytokine responses to MSP-1 and two additional blood-stage antigens, *P. falciparum* ribosomal phosphoprotein P0 (PfP0) and erythrocyte binding antigen-175 (EBA-175). T-cell cytokine profiles were correlated with evidence of prenatal exposure to malaria antigens. In addition, we determined whether there is a bias toward malaria-driven Th2 cytokine and increased IL-10 production by a subset of neonatal $CD4^+$ T cells, as reported for other microbial antigens (1, 49).

MATERIALS AND METHODS

Study population. Healthy pregnant women living in a region of Coast Province, Kenya, in which malaria is holoendemic were recruited from the antenatal clinic at Msambweni District Hospital. Maternal venous blood and umbilical cord blood from full-term newborns of uncomplicated pregnancies were collected at the time of vaginal delivery. Blood samples from healthy adults and newborns who had never been exposed to malaria were collected from 11 non-pregnant residents of Cleveland, Ohio, and 20 healthy infants delivered vaginally at University Hospitals of Cleveland. Ethical approval was obtained from the Human Investigations Institutional Review Boards of University Hospitals of Cleveland and the Kenya Medical Research Institute in Nairobi.

Of note, we were occasionally unable to perform all the described assays for all individuals because of unavailability of some samples. This results in different sample sizes for the different groups, although all assays were performed for the majority of newborns and their mothers ($n = 90$).

Determination of malaria infection status and in utero exposure to *P. falciparum* antigens. In addition to maternal and cord blood, intervillous blood for the diagnosis of placental malaria was obtained by aspiration through a cannula inserted into the maternal side of the placenta through the basal plate. For blood smear diagnosis, thick and thin films were stained with 4 percent Giemsa and examined microscopically for asexual *P. falciparum* organisms under 100 oil immersion fields. For diagnosis by detection of circulating malaria antigen in maternal peripheral, placental intervillous, and/or cord blood, histidine-rich protein-II (HRP-II) and malaria-specific lactate dehydrogenase (LDH) were measured with commercial kits (Cellabs Pty. Ltd., Brookvale, New South Wales, Australia, and FlowLabs, Portland, OR, respectively). An individual was considered positive if circulating antigen was present, by one and/or both assays, in at least one of the blood samples collected from the mother or her newborn. Plasma separated from fresh blood was stored at −70°C until assays were performed according to the manufacturer's instructions.

PCR-based diagnosis of malaria. DNA was extracted from 200 μl of whole blood by use of individual spin blood kits (QIAGEN Inc., Valencia, CA). A volume of 2.5 μl of DNA was used for amplification of the multicopy 18S (small subunit) rRNA genes of *P. falciparum* by real-time quantitative PCR as described previously (23). PCR was performed using an ABI 3600 quantitative thermocycler (GeneAmp 5700 sequence detector; ABI Research, Oyster Bay, NY). Each run included no-template DNA (negative control) and 0.5-log-fold differences in the estimated number of the small subunit rRNA amplicons (GenBank accession no. AF145334) (positive control). The sensitivity of the real-time quantitative PCR was between 10 and 20 *P. falciparum*-infected red blood cells/μl.

Antigens and mitogens. Cytokine responses to three blood-stage antigens were examined. Peptides corresponding to previously identified T-cell epitopes in the C-terminal 83-kDa fragment of MSP-1, designated P2 (GYRKPLDNIKDNVG KMEDYIKK; codons 250 to 271) and P3 (KLNSLNNPHNVLQNFSVFFNK; codons 1101 to 1121), were synthesized by ResGen (Carlsbad, CA) and purified to >95% (48). Three peptides corresponding to N- and C-terminal regions of PfP0 were synthesized and purified to 70 to 80% (Chiron Corp., Clayton, Victoria, Australia). The peptides were designated N1 (DNVGSNQMASVRKSLR; codons 33 to 48), N2 (SVRKSLRGKATILMGKNT; codons 42 to 59), and C1 (AKADEPKKEEAKKVE; codons 285 to 299), and correspond to T-cell epitopes identified by lymphocyte proliferation responses of immunized mice (6). A purified GMP grade recombinant protein corresponding to region II of EBA-175 (codons 144 to 753) was expressed in baculovirus (36) and kindly provided by David Narum, Malaria Vaccine Development Unit, National Institutes of Health, Bethesda, Md. Recombinant EBA-175 does not contain a tag used for purification. Phorbol 12-myristate 13-acetate (PMA) plus ionomycin (Calbiochem, La Jolla, CA) were used as positive mitogen controls in parallel cultures. Endotoxin in these preparations was <0.5 ng/ml; this concentration is 5- to 50-fold less than that required for lipopolysaccharide stimulation of cytokine production by human lymphocytes.

Culture conditions and measurements of cytokine production by enzyme-linked immunospot (ELISPOT) assay and enzyme-linked immunosorbent assay (ELISA). All studies were performed with freshly isolated mononuclear cells/lymphocytes separated from maternal venous (PBMC) or cord blood (CBL) by density gradient centrifugation on Ficoll-Hypaque. After washing, cells were finally suspended to a density of 2×10^6/ml to a total volume of 1 ml in RPMI 1640 supplemented with heat-inactivated 10% autologous plasma, 4 mM L-glutamine, 25 mM HEPES, and 80 μg/ml gentamicin (Biowhittaker, Gaithersburg, MD). In preliminary studies, heat-inactivated autologous plasma was not suppressive compared to serum-free media in activated lymphocyte cultures (data not shown). By contrast, we observed high background and nonspecific lymphocyte reactivity by use of fetal calf serum or pooled human sera (data not shown). Media alone (negative control), 10 μg/ml MSP-1 P2 or P3 peptide, 10 μg/ml PfP0 N1, N2, or C1 peptide, 5 μg/ml EBA-175, and PMA (50 pg/ml) plus ionomycin (1 μg/ml) were added to duplicate wells in 0.5-ml cultures in 48-well flat-bottomed plates (Costar) after optimal concentrations were determined in pilot experiments.

The IFN-γ ELISPOT assay was performed at 48 h as described previously (30). A positive response was scored when one of the following conditions was met: (i) >4 IFN-γ-secreting cells/10^6 PBMC or CBL were present in response to recombinant EBA-175 plus similar responses to least one PfP0 or MSP-1 antigenic peptide when no IFN-γ-secreting cells were present in negative-control wells (media alone); (ii) >4 IFN-γ-secreting cells were present in response to two or more PfP0 or MSP-1 peptides when no IFN-γ-secreting cells were present in negative-control wells containing media alone; or (iii) in a case in which IFN-γ-secreting cells in negative-control wells were observed, the number of spots generated by antigen-driven CBL was at least twofold greater. On the basis of these criteria, no malaria antigen-driven IFN-γ-secreting cells were detected in CBL from 16 healthy North American newborns or PBMC from 10 malaria-naïve adults.

Quantification of IFN-γ and other cytokines by ELISA was performed on

culture supernatants collected at 72 h. Results were expressed in pg/ml by interpolation from standard curves based on recombinant lymphokines (29). Antibody pairs for capture and detection (all biotinylated) for the cytokines studied were as follows: for IL-5, 18051D and 18522D (BD-Pharmingen, San Diego, CA); for IFN-γ, M-700A and M-701B (Endogen, Cambridge, MA); for IL-13, P-130-E and M-130-B (Endogen); for IL-2, MAB-602 and BAF-202 (R&D Systems Inc., Minneapolis, MN); for IL-10, 18551D and 18652D (BD-Pharmingen); and for tumor necrosis factor alpha (TNF-α), 18631D and 18642D (BD-Pharmingen). A positive response was scored when the following two criteria were fulfilled: (i) there was a net value for antigen-stimulated cells that was at least twofold greater than that of parallel cultures containing media alone (if cytokine production was not detectable in the negative-control cultures, then >40 pg/ml cytokine was considered to be a positive response) and (ii) there were responses to two or more PfP0 or MSP-1 peptides or recombinant EBA-175 plus one or more PfP0 or MSP-1 peptides.

Depletion and enrichment of $CD4^+$ and $CD8^+$ T cells. CBL were washed once, and $CD4^+$ and $CD8^+$ T-cell enrichment was performed using magnetic beads directly conjugated to anti-CD4 or anti-CD8 antibodies (Miltenyi Biotec, Auburn, CA). Immunomagnetic bead enrichment was performed according to the manufacturer's instructions. The purity of positively selected $CD4^+$ or $CD8^+$ T cells was >95% (data not shown) ascertained by fluorescence-activated cell sorter analysis. Autologous bulk CBL (10^5 cells/ml) were added as antigen-presenting cells (APC) to purified $CD4^+$ or $CD8^+$ T cells (1.5×10^6/ml). Preliminary studies demonstrated that autologous bulk APC produced malaria antigen-driven cytokine responses similar to those of adherent cells as APC.

Anti-CD40-induced IL-12 production. Whole blood anticoagulated with 2 U/ml of sodium heparin was mixed with an equal volume of culture medium to a total volume of 1 ml. Anti-CD40 monoclonal antibody (obtained from ATCC HB-9110, clone G28-5) (33) was added at a concentration previously determined to be optimal (16 μg/ml), and incubation continued for 48 h. An identical amount of mouse Ig isotype control (IgG1; Jackson ImmunoResearch, West Grove, PA) was added to parallel cultures. ELISA was used to quantify IL-12 p70 production (R&D Systems, Inc., Minneapolis, MN) by use of frozen supernatants.

IgM antibodies to MSP-1_{19}, PfP0, and EBA-175. IgM antibody to recombinant MSP-1_{19} expressed in *Saccharomyces cerevisiae* (Q-KNG variant; MR4, Bethesda, MD) was measured by ELISA as described previously (30). A similar protocol was followed for measuring antibodies to recombinant PfP0 and EBA-175 (22, 36). The recombinant PfP0 is a glutathione *S*-transferase (GST) fusion protein. Therefore, all samples were run with GST alone. Optical densities were recorded as that of the fusion protein minus that of GST alone. Briefly, Immunolon 1 plates (Dynatech, Sterling, VA) were coated with 200 ng/ml of recombinant EBA-175 or 100 ng/ml PfP0 (22) in phosphate-buffered saline overnight at 4°C. After being blocked and washed, freshly thawed plasma diluted to 1/100 was added, and incubation continued overnight at 4°C. The plates were then washed, and alkaline phosphatase-conjugated anti-human IgM (Jackson ImmunoResearch, Malvern, PA) diluted 1/2,000 was added for 1 h at room temperature. Substrate *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) was added after the final wash. The reaction was stopped by the addition of 5% EDTA, and absorbance was read at 405 nm with a V_{max} ELISA reader (Molecular Devices, Sunnyvale, CA). Plasma samples from the North American adults and newborns were used as controls.

Statistics. Cytokine data were log-normally distributed. The values were therefore expressed as geometric means ± standard errors of the means, and statistical tests were performed on log-transformed values. The significances of differences between groups were evaluated by using Student's *t* test, and relationships between variables were examined by simple linear regression. Comparisons of the proportions of responders of various groups of donors were evaluated by chi-square analysis.

RESULTS

Malaria infection of maternal, placental, and cord blood. *P. falciparum* infection was diagnosed by blood smear, real-time PCR, and malaria antigen immunoassay for the three blood compartments examined (Fig. 1). As expected, PCR yielded a higher proportion of positive tests than blood smear for each of the blood compartments (~2- to 5-fold greater). A second, more striking finding emerged when the results were stratified according to maternal parity. Samples from primigravidae had higher infection rates than those from multigravidae regardless

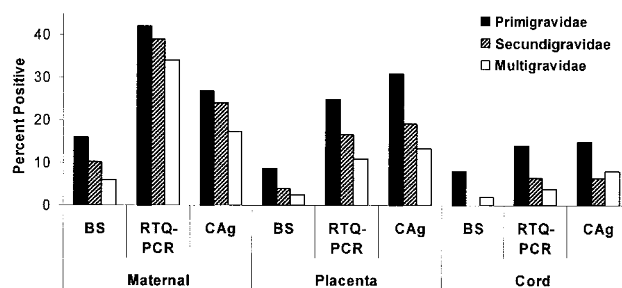

FIG. 1. Maternal venous, placental, and cord blood infection statuses determined by blood smear (BS), real-time quantitative PCR (RTQ-PCR), and detection of circulating malaria antigens (CAg). *P. falciparum* infection was determined by microscopic inspection of blood smear or real-time quantitative PCR and stratified according to parity. Malaria-specific HRP-II and LDH in plasma were measured by immunoassay as described in Materials and Methods. The numbers of maternal venous blood samples of primigravid, secundigravid, and multigravid donors analyzed were 79, 29, and 98, respectively; for placental intervillous blood, the numbers of samples in each group were 58, 26, and 82, respectively; and for cord blood samples, the numbers of samples in each group were 74, 31, and 102, respectively.

of the blood compartment analyzed. For example, malaria diagnosed by microscopic inspection of maternal blood smears was observed for 18 percent of primigravidae, as opposed to 6 percent of multigravidae ($P = 0.02$); cord blood smears from the former group were also more frequently positive than those from the latter group ($P < 0.01$). Infection rates of samples from cord blood of secundigravidae were intermediate. Blood smears positive by microscopy were concordant with PCR results for maternal blood (81% of blood smear-positive samples were also PCR positive) and placental blood (100% agreement) but less so for cord blood (50% agreement). Infection status of cord blood determined by microscopy and PCR was similar to that of maternal or placental blood, i.e., 80% agreement when results of blood smear and PCR were combined. PCR is more sensitive than blood smear analysis, and it shows that malaria infections are diagnosed more commonly by PCR.

We also examined whether soluble malaria antigens released into the circulation by infected erythrocytes were present by measuring *Plasmodium* HRP-II and LDH (35, 47). Both antigens were present more frequently in maternal and placental blood from primigravidae than in blood from multigravidae (Fig. 1). The presence of circulating malaria antigen in these two blood compartments was 77% concordant with infection diagnosed by blood smear or PCR. HRP-II and/or LDH was also detected in 14 percent of cord blood samples from primigravidae, as opposed to 6.7 percent of multigravidae. Concordance of malaria antigen immunoassay and blood smear/PCR results for cord blood was lower than for maternal venous and placental blood (32% versus 77%). In sum, the results of blood smear, PCR, and circulating antigen immunoassay demonstrate that newborns of primigravidae are more likely those of multigravidae to have prenatal exposure to malaria antigens.

Malaria antigen-specific cytokine responses by newborn and maternal lymphocytes. Prenatal exposure to malaria-infected erythrocytes or soluble malaria antigens may be associated with immune responses in newborns. To determine this, we first examined malaria antigen-stimulated IFN-γ CBL re-

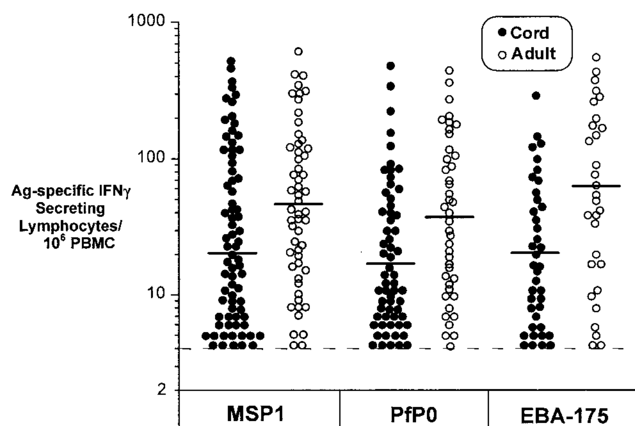

FIG. 2. CBL and maternal PBMC ELISPOT assay IFN-γ responses to malaria blood-stage antigens (Ag). Only the geometric means and ranges of frequencies of IFN-γ-secreting cells per 10^6 CBL or maternal PBMC for individuals responding to MSP-1, PfP0 peptides, and recombinant EBA-175 are shown. The maximum responses to MSP-1 and PfP0 peptides are shown and were used to calculate the geometric means. The proportion of newborns with IFN-γ-secreting cells was lower than that for their mothers ($P < 0.05$ to 0.01). A total of 169 CBL and 116 maternal PBMC donors were examined in response to MSP-1 and PfP0 and 132 CBL and 114 PBMC in response to EBA-175. The horizontal bars indicate the geometric means.

sponses by ELISPOT assay. The percentage of MSP-1-driven responses by ELISPOT assay was the highest (74 of 169 donors responded; 44%), followed by PfP0 (61 of 169; 36%) and EBA-175 (39 of 132; 29%). The proportions of maternal PBMC (obtained within 24 h of parturition) responding were similar to those for newborns (42 of 116 [36%] for MSP-1, 55 of 116 [47%] for PfP0, and 30 of 114 [26%] for EBA-175). Overall, 53 percent of newborns (89 of 169) and 57 percent of mothers (66 of 116) had malaria-specific IFN-γ-secreting lymphocytes to one or more of the three antigens tested. All CBL and maternal PBMC preparations responded vigorously to PMA plus ionomycin (data not shown).

Figure 2 shows the actual frequencies of IFN-γ-secreting cells by individual donors that responded to malaria blood-stage antigen for both maternal and cord blood lymphocytes. The geometric mean frequency of IFN-γ-secreting lymphocytes was twofold greater for maternal PBMC than for CBL ($P < 0.01$). There was no correlation between the frequencies of IFN-γ-secreting cells for paired maternal PBMC and CBL ($r^2 = 0.07$; $P = 0.3$), indicating that maternal lymphocytes were unlikely to have contaminated cord blood.

In order to evaluate additional cytokines produced by CBL, we measured IL-2, IL-13, IL-5, IL-10, TNF-α, and IFN-γ by ELISA. Fifty-five of 170 CBL donors (32%) had malaria antigen-driven responses. This lower frequency of cytokine production in CBL as detected by ELISA is due to ELISA having a lower sensitivity than the ELISPOT assay for detecting antigen-specific responses (e.g., comparison of IFN-γ assays; data not shown). When the results for IFN-γ ELISPOT assay and all cytokine ELISAs were combined, 66 percent of CBL donors were calculated to respond to one or more of the malaria antigens.

Polarization of Th-cell cytokine responses. Figure 3 summarizes the results for individual cytokines produced by CBL and maternal PBMC. The lowest proportions and magnitudes of CBL responses were for IL-5, IL-13, and IL-2 (response rates were <10%), and the highest were for IL-10 (20, 18, and 25% for MSP-1, PfP0, and EBA-175, respectively). The proportions and magnitudes of IL-5, IL-13, IL-2, and IFN-γ responses by maternal PBMC did not differ significantly from those by CBL. In contrast, IL-10 responses by CBL were greater than those by maternal PBMC ($P < 0.05$), and TNF-α responses by CBL were lower than those by maternal PBMC in responses to

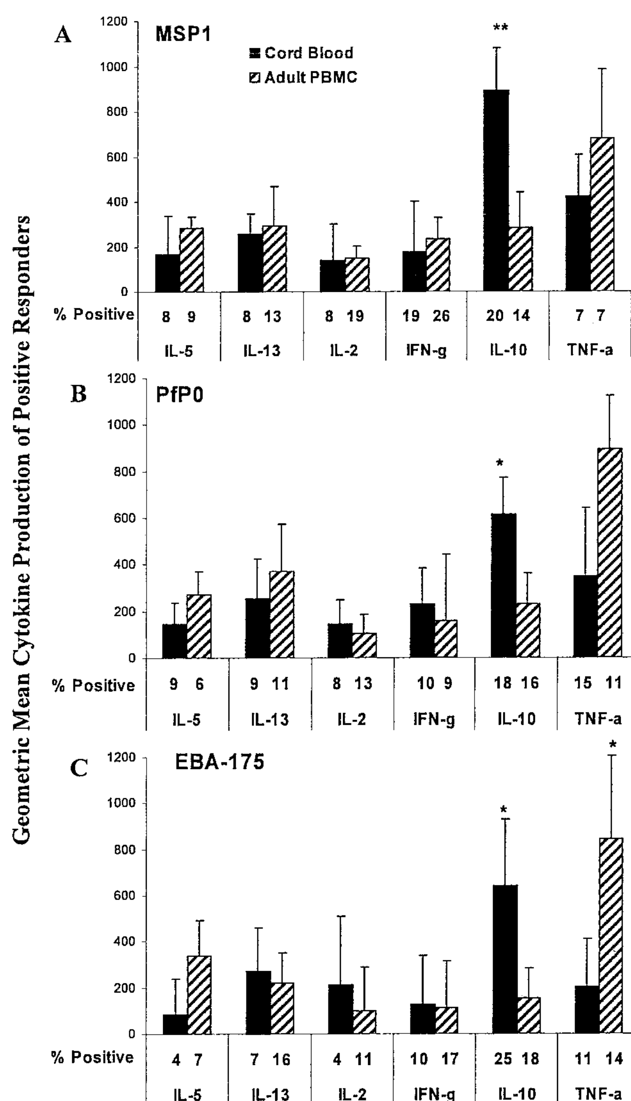

FIG. 3. The geometric mean levels of cytokine produced by positive responders to malaria antigens for CBL and maternal PBMC. The percentages of individuals with a positive response for a given cytokine are represented by the values immediately below each bar. Values are in picograms/ml. A total of 170 CBL samples were evaluated for responses to MSP-1 (A) and PfP0 (B); 90 CBL samples were evaluated for responses to EBA-175 (C). Sixty-eight maternal PBMC samples were evaluated for responses to all three antigens. Bars indicate the geometric means ± 95% confidence intervals for CBL (solid bars) and maternal PBMC (striped bars). An asterisk above a bar indicates a significant difference between CBL and maternal PBMC, with one asterisk (*) meaning that P was <0.05 and two asterisks (**) meaning that P was <0.01. TNFa, TNF-α; IFN-g, IFN-γ.

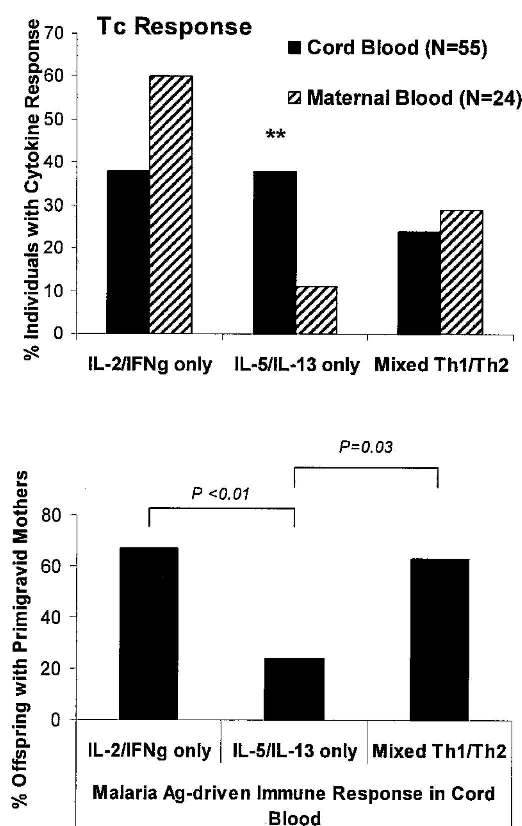

FIG. 4. Th-cell (Tc) cytokine pattern of malaria antigen (Ag)-driven CBL and maternal PBMC responses and distribution of CBL Th responses for newborns of primigravidae. Results of cytokine ELISAs were categorized as Th1 type (IFN-γ and/or IL-2 only), Th2 type (IL-5 and/or IL-13 only), or mixed Th1/Th2 type (combinations of the two groups). The percentages of CBL (solid bars) and maternal PBMC (striped bars) donors with responses in a given category are represented in the upper panel. The percentages of CBL donors of primigravidae with a given Th pattern are shown in the lower panel. The significance of differences between groups was evaluated using the chi-square test. IFNg, IFN-γ.

EBA-175 ($P < 0.05$) and with a trend similar to those of MSP1 and PfP0. No North American control CBL ($n = 20$) or adult PBMC ($n = 11$) cytokine responses to malaria antigens were detected, although these preparations responded vigorously to PMA plus ionomycin (data not shown).

Three patterns of Kenyan CBL and maternal PBMC cytokine responses were discernible. Of the 55 antigen-reactive CBL preparations (Fig. 4, upper panel), 21 (38%) produced exclusively IFN-γ and/or IL-2, i.e., polarized Th1 response; 21 (38%) produced exclusively IL-5 and/or IL-13, i.e., polarized Th2 response; and 13 (24%) had a mixed Th1/Th2 pattern. IL-10 and TNF-α responses were equally frequent for CBL with a polarized Th1 or Th2 pattern. In contrast to CBL, maternal PBMC (Fig. 4, upper panel) had a predominant Th1 cytokine response (60% of all donors) or mixed Th1/Th2 response (29%). Malaria antigen-driven cytokine responses by paired maternal and cord blood samples did not correlate with each other ($r^2 = 0.13$; $P = 0.21$).

Predictors of cord blood Th-cell cytokine bias. To understand the basis for the spectrum of cytokine responses observed in newborns, we examined the relationship between CBL cytokine pattern and evidence for prenatal exposure to malaria. CBL from newborns with polarized Th1 responses were twice as likely to have *P. falciparum* detectable in cord blood by microscopy or PCR (12 of 21; 57%) relative to newborns with Th2 responses (6 of 21; 28.6%; chi-square = 3.5; $P = 0.07$). This difference became statistically significant when CBL that produced any Th1 cytokine response (mixed Th1/Th2 patterns) were included in the Th1 group (20 of 34 for the Th1 responders versus 6 of 21 for the Th2 responders; chi-square = 4.7; $P = 0.02$).

We next analyzed the relationship between maternal parity and CBL Th pattern. We reasoned that primigravidae would be more likely than multigravidae to expose their fetuses to malaria antigens during gestation, since the former are at a higher risk of placental malaria (16, 17, 42). Sixty-seven and sixty-two percent of newborns with exclusive Th1 or mixed Th1/Th2 responses, as opposed to 24 percent with exclusive Th2 responses, had primigravid mothers (Fig. 4, lower panel). In contrast, this association was reversed for newborns of multigravid mothers: 51% had a polarized Th2 response and 19% a Th1 response ($P < 0.05$).

Enhanced IL-12 production by cord blood from newborns of primigravidae. The observed spectrum of fetal cytokine responses may be due in part to differences in IL-12 production, since this cytokine is critical to Th1 differentiation. For example, immune cells of newborns are known to produce less IL-12 than those of adults (21) and are consequently less likely to generate Th1 cytokines. Malaria infection of the fetus or transplacental passage of proinflammatory malaria molecules may also prime CD40-expressing neonatal antigen-presenting cells to produce IL-12, thereby favoring development of Th1-type responses. Since there is no direct means of ascertaining malaria infection or exposure to malaria antigens in utero throughout pregnancy, we stratified cord blood IL-12 responses according to maternal parity and compared them to those from whole blood obtained from the newborn's mother.

Cord blood produced less IL-12 p70 than maternal blood regardless of the mother's parity. Cord blood from newborns of primigravidae released significantly greater amounts of IL-12 p70 than newborns of multigravidae (Fig. 5). Newborns in the former group were also more likely to have a polarized Th1 response (7 of 12 donors) than newborns in the latter group (2 of 13 donors; chi-square = 5.0; $P < 0.05$). IL-12 p70 production was not detected in parallel cultures of maternal or cord blood incubated with control mouse IgG1 (data not shown).

Cord blood T-cell subsets contributing to cytokine production. As a first step to determine the T-cell subsets that contribute to or are required for cytokine production by neonatal lymphocytes, $CD4^+$ and $CD8^+$ T cells were purified by positive selection and antigen-driven cytokine production measured. Sufficient cord blood lymphocytes were obtained from 15 newborns to examine T-cell-subset responses. The cord blood samples examined for T-cell-subset responses were similar in several respects to samples that were not selected for subset analysis; birth weights were similar, and the two groups had the same proportion of mothers that were infected with malaria at delivery and were primigravidae. Eight individuals produced IL-10 and/or IFN-γ to one or more of the three malaria blood-

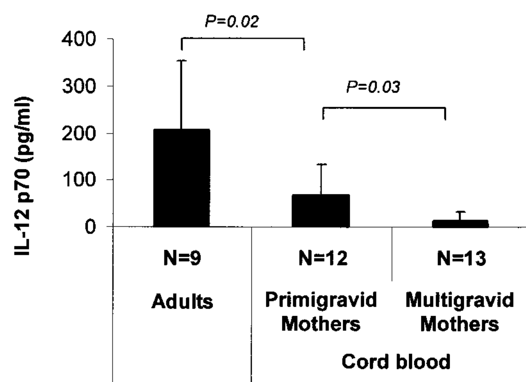

FIG. 5. Anti-CD40-induced IL-12 p70 production by maternal and cord blood. Whole blood diluted 1:1 in culture medium was incubated with 16 μg/ml of cross-linking anti-CD40 for 48 h. Supernatants were harvested, and IL-12 p70 was measured by ELISA. Bars represent means ± standard deviations. Significant differences were determined by Student's *t* test. Five of the cord blood samples from primigravid women had evidence of malaria infection based on blood smear (n = 1), PCR (n = 2), or HRP-II/LDH (n = 2). Cord blood from one multigravida had evidence of malaria by PCR.

stage antigens tested (Fig. 6). There are two basic observations. First, $CD4^+$ and not $CD8^+$ T cells produced IFN-γ in response to the malaria antigens tested, whereas both $CD4^+$ and $CD8^+$ T cells secrete IL-10 in response to malaria antigens. Second, both IFN-γ and IL-10 responses not detected in cultures of bulk CBL were revealed when enriched $CD4^+$ and/or $CD8^+$ T cells were incubated with malaria antigens. IL-2, IL-5, and IL-13 were detected only in culture supernatants of enriched $CD4^+$ T cells (data not shown).

Relationship of cord blood and maternal T-cell responses to malaria infection and parity. Prenatal sensitization to malaria antigens estimated by CBL cytokine responses was more likely when *P. falciparum* infection coexisted in maternal peripheral blood (76% of CBL samples from offspring of infected women versus 38% from those of offspring of uninfected women; $P <$ 0.001), placental intervillous blood (75% from samples from offspring of women with placental malaria versus 41% of those from offspring of woman without placental malaria; $P <$ 0.001), and/or cord blood (80% of samples from offspring of women with malaria infection versus 33% of those from offspring of women without infection; $P < 0.001$). Similarly, malaria-specific PBMC responses were detected more frequently when maternal blood-stage infection coexisted (a frequency of 81% with concurrent malaria documented by smear or PCR versus 30% for the women without concurrent malaria; $P <$ 0.001). Primigravidae or secundigravidae were more likely to have newborns with malaria-specific CBL cytokine responses than multigravidae (35 of 104 [33.6%] versus 20 of 102 [19.6%]; chi-square = 5.1; $P = 0.021$).

Malaria-specific IgM antibody and its relationship to cord blood cytokine responses. Maternal IgM does not normally cross the human placenta and is thus presumed to originate from fetal B cells when detected in cord blood. We assayed plasma from cord blood for malaria-specific IgM antibodies. A total of 6.6 percent of samples (5 of 75) contained IgM antibodies to recombinant MSP-1_{19} (Q-KNG variant, the most common variant in the population [30]), 17.2 percent (21 of 122) contained these antibodies to PfP0, and 14.9 percent (22 of 147) contained these antibodies to EBA-175. A higher proportion of maternal plasma samples had IgM antibodies: 16 percent (15 of 93), 61 percent (37 of 61), and 26 percent (36 of 138) had these antibodies to PfP0, EBA-175, and MSP-1_{19}, respectively. The presences or levels of IgM antibodies in paired maternal and newborn plasma samples did not correlate with each other (r^2 = 0.1; P = 0.6). Newborns with IgM antibodies tended to have CBL cytokine responses to the same malaria antigens. Four of five with MSP-1_{19}-specific IgM antibody had MSP-1-driven CBL cytokine responses, 18 of 22 (82%) with PfP0-specific IgM had PfP0-driven cytokine responses, and 18 of 21 (76%) with EBA-175-specific IgM had EBA-175-driven cytokine responses.

DISCUSSION

Results presented here show that prenatal T- and B-cell immunity to *P. falciparum* blood-stage antigens commonly develops in settings where malaria during pregnancy is a major health problem. Sixty-six percent of the 176 Kenyan newborns examined had CBL cytokine responses to MSP-1, PfP0, or EBA-175, and 7 to 17 percent had IgM antibodies to the same antigens. More than one-third of CBL cytokine responses had an exclusive Th1 pattern, and one-third had a Th2 pattern that differed from that of maternal PBMC, which were skewed mainly to the Th1 pattern. Common features of newborn cytokine responses were increased IL-10 production and diminished TNF-α production, indicating an anti-inflammatory cytokine bias by neonates. Consistent with this, newborns whose CBL did not respond to malaria antigens in bulk culture had detectable responses revealed after enrichment of the $CD4^+$ cells or $CD8^+$ T cells (Fig. 6), suggesting that active suppression or T-cell anergy to malaria develops prenatally in some individuals.

Our observations support the concept that prenatal exposure to maternal erythrocytes infected with *P. falciparum* or soluble malaria antigens transported across the placenta occurs with some regularity. Nine percent of cord blood samples contained parasites detectable by blood smear or PCR, and malaria-specific HRP-II and LDH were detected in plasma samples from eight percent of cord blood donors. Other studies using a dual perfusion assay of the maternal and fetal circulation with interposed viable human placenta have demonstrated transplacental transfer of tetanus toxoid and ovalbumin (38, 39). CBL from newborns of mothers with evidence of active malaria infection at parturition (by blood smear or PCR) were also nearly twice as likely to contain malaria-responsive lymphocytes relative to CBL from newborns of mothers without active malaria infection. Maternal infection ascertained at a single time point (parturition) represents the minimal likelihood of the unborn child being exposed to malaria. We therefore attempted to estimate the malaria infection status of these women during earlier periods of their pregnancies. First, blood smears during earlier antenatal visits from 102 maternal participants were available. Fifteen (14.7%) were positive for *P. falciparum* during the second or third trimester. At parturition, 9 of these 15 women had negative blood smears and PCR assays. Second, reasoning that primigravidae experience placental malaria more frequently than multigravidae

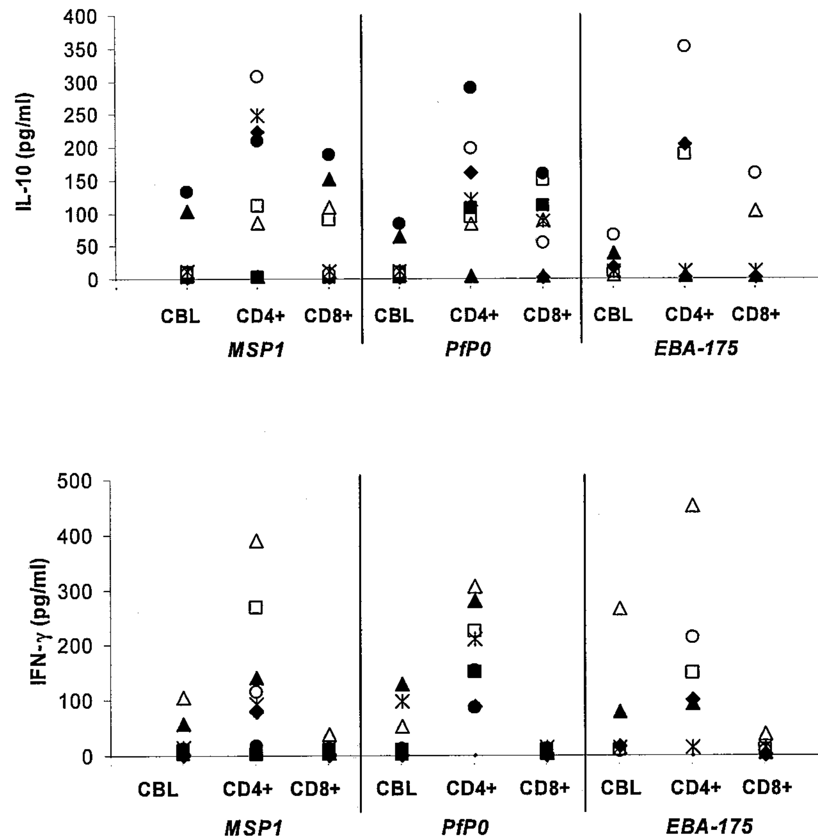

FIG. 6. Malaria antigen-driven IL-10 and IFN-γ by bulk CBL and enriched $CD4^+$ and $CD8^+$ T cells. CBL were cultured at 2×10^6/ml in a volume of 1 ml in 48-well culture plates with or without antigen for 72 h as described in Materials and Methods. Parallel cultures of $CD4^+$ and $CD8^+$ T cells (enriched by immunomagnetic positive selection) at a cell concentration of 1.5×10^6/ml with 1×10^5 bulk CBL as antigen-presenting cells were performed. Each symbol represents the levels of peak net antigen-driven cytokine production (antigen − spontaneous) for the same individual for both cytokines ($n = 8$).

(41), we assessed the relationship between parity and CBL cytokine responses. Newborns of primigravidae and secundigravidae were more likely to have malaria-specific CBL responses than multigravidae (33.6% versus 19.6%).

The spectrum of cytokines generated by neonates differed from that of their mothers. Maternal PBMC produced mostly IFN-γ and IL-2, indicative of a Th1 pattern. In contrast, newborn lymphocytes were less likely to produce IFN-γ and IL-2 and instead had a propensity to produce solely IL-5 and IL-13, indicative of a Th2 response. Our studies did not include nonpregnant Kenyan women and men, so we do not yet know whether responses by these groups are different from those of women in the third trimester of pregnancy. CBL of newborns from areas where malaria is not endemic have previously been shown to have preferential Th2 responses to exogenous antigens due, in part, to depressed IL-12 production by antigen-presenting cells which drive Th1 differentiation (34, 60).

To begin to understand why a subset of neonates expressed malaria-specific Th1 as opposed to Th2 cytokines, we assessed the relationship between Th-cell cytokine bias and presumptive prenatal exposure to malaria. Newborns of uninfected and multigravid mothers, i.e., pregnant women anticipated to have relatively light malaria burdens during pregnancy, were more likely to have CBL IL-5 and IL-13 responses. Although many of these women and their offspring were uninfected at birth, it is likely that many had malaria infections earlier during pregnancy to allow sufficient amounts of malaria antigen to cross the placenta and have exposure to the fetus. By contrast, offspring of malaria-infected and primigravid/secundigravid mothers, i.e., groups anticipated to have heavier malaria burdens during pregnancy, were likely to have IFN-γ and IL-2 responses. These associations suggest that the magnitude of fetal exposure to infected erythrocytes or their soluble blood-stage antigens may regulate, in part, whether the fetus acquires a Th1- or Th2-type response to blood-stage antigens. With heavier infections during pregnancy, infected erythrocytes may cross the placenta, resulting in the release of malaria toxins in addition to malaria antigens (53). In this regard, proinflammatory *Plasmodium* glycosylphosphatidylinositol molecules (54) might favor the differentiation of fetal antigen-presenting cells to produce IL-12. In support of this, we found that the level of IL-12 produced by cord blood incubated with anti-CD40, which activates CD40 expressed on dendritic cells and monocytes, was greater for newborns with evidence of prenatal infection and/or of primigravidae than for newborns without evidence of prenatal infection and/or of multigravidae. Further, robust IL-12 responses correlated with CBL IL-2/IFN-γ production, whereas weak IL-12 responses correlated with IL-5/IL-13 production. Results of recent studies of Gambian newborns (25) are broadly similar to those reported here in that

both suggest that exposure to malaria antigens in utero affects the innate response as well as the adaptive immune response. There are, however, several differences between the two studies. The Gambian data showed that lipopolysaccharide induced less IL-12 p40 production by cord blood cells from newborns of women with placental malaria than those of women without placental malaria. These findings were interpreted to indicate that malaria-infected erythrocytes suppress IL-12 responses by fetal antigen-presenting cells (58). Several factors might account for differences between the Gambian and Kenyan data. First, the production of the IL-12 p40 subunit may not parallel that of the p70 subunit, particularly in the case of neonates in whom the p35 subunit is deficient (21).

Malaria antigen-driven IL-10 responses were observed more frequently for newborn than maternal lymphocytes. Polyclonal activation of newborn T cells has been shown previously to lead to greater production of IL-10 than that of adult T cells (4, 49), indicating a greater intrinsic ability to produce this cytokine. IL-10 might be produced by regulatory lymphocytes, such as $CD4^+$ $IL\text{-}10^{hi}$ $TGF\text{-}\beta^{hi}$ $IFN\text{-}\gamma^{low}$ cells (52), natural $CD4^+CD25^+$ regulatory cells (46, 55), or antigen-responsive $CD8^+$ T cells. This possibility is supported by experiments that revealed antigen-responsive cells in bulk CBL only after enrichment of various lymphocyte subsets (Fig. 6).

The malaria blood-stage antigens studied here are thought to be involved in merozoite invasion of erythrocytes. Protein domains of MSP-1 and EBA-175 are currently being considered for vaccine strategies. The C-terminal 19-kDa portion of MSP-1 is one of several possible targets of invasion-inhibitory antibodies in vivo (3, 26, 43). The MSP-1 T-cell epitopes used in the current study are contained in the 83-kDa portion of MSP-1 that is processed prior to the appearance of the membrane-anchored 19-kDa fragment which is ultimately carried into the newly infected erythrocyte (48). Neonatal T-cell responses to this region of MSP-1 have not, to our knowledge, been examined. Region II of EBA-175 contains motifs involved in binding to glycophorin A on the erythrocyte surface (56). Epidemiological studies have shown that antibodies to EBA-175 correlate inversely with age-related changes in parasitemia (44). T-cell responses to region II of EBA-175 in malaria-infected adults or newborns of malaria-exposed pregnant women have not been previously reported. PfP0 is the least studied of the three antigens. The protein is expressed on the merozoite surface and polyclonal antibodies to its N- and C-terminal domains inhibit *P. falciparum* growth in vitro (6, 7). Naturally acquired antibodies to PfP0 in adults residing in areas of India and Africa in which malaria is endemic are detectable (37). This report is the first to describe T-cell responses to PfP0 in humans. These responses were on average higher than those stimulated by MSP-1 and EBA-175.

In conclusion, the data presented here demonstrate that newborns from an area of Kenya where malaria is holoendemic are commonly sensitized to blood-stage antigens in utero. The presence and cytokine pattern of this immunity is not uniform, exhibiting a pattern indicative of a predominant Th1 or Th2 immunity that correlates with maternal malaria and parity. Future work to understand the mechanisms involved in regulating the expression of neonatal immunity to malaria and the risk factors that influence these outcomes needs to be directed at defining the neonatal T-cell regulatory subsets and antigen-presenting cell maturation pathways affected by maternal and placental malaria. These studies should not only advance understanding of the immune basis of the heterogeneity of infant malaria susceptibility but also be highly relevant to the evaluation and deployment of childhood malaria vaccines.

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