Alterations in the Profile of Blood Cell Types during Malaria in Previously Unexposed Primigravid Monkeys

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Malaria in nonimmune, primigravid women threatens both mother and fetus. We used the Plasmodium coatneyi/rhesus monkey model to examine factors associated with this. Clinical and immunologic responses during the blood stage of chronic malaria (4 months) were evaluated in 8 malaria-naive primigravid (PMI) and 8 age-matched nulligravid (NMI) infected monkeys, compared with those in 8 primigravid, noninfected control monkeys. Although parasitemia levels were similar, recrudescence was more frequent and prolonged, and anemia was more severe in PMI than in NMI monkeys. During infection, CD2+, CD4+, and CD8+ lymphocyte levels were higher in NMI than in PMI monkeys. Monocyte and neutrophil levels were lower in PMI than in NMI monkeys. During chronic, untreated malaria, NMI monkeys had a B lymphocyte count 23 times greater than that of PMI monkeys. Pregnancy-induced immunomodulation, defined as a lack of appropriate cellular responses to malaria, was indiscernible until the immune system was challenged by a pathogen.

Malaria is an overwhelming public health problem—300–500 million cases and 2–3 million deaths occur annually [1]. For pregnant women and their fetuses, malaria presents a particularly severe challenge [2]. The dynamics of malaria during pregnancy vary with endemicity [3, 4]. In highly endemic areas where women have acquired immunity to malaria, primigravid and secundigravid women are more susceptible to malaria-induced pregnancy complications than are multigravid women [5–8]. In areas of low endemicity, women often lack sufficient immunity to control severe clinical malaria, and all children of women at all gravidities are susceptible to complications, including intrauterine growth retardation (IUGR), prematurity, low birth weight (LBW), failure to thrive, neonatal death, and congenital malaria [2, 3, 9–12]. Spontaneous abortion during the first trimester [10, 13–15] has been demonstrated in monkeys with malaria infection [16]. The Plasmodium coatneyi–infected monkeys that we report represent women in areas of low transmission who lack malaria immunity [17, 18]. However, unlike chronically infected, untreated monkeys, pregnant women generally receive antimalarial treatment, which limits the duration and severity of their illness. Despite treatment, many of these women’s pregnancies have serious malaria-associated complications [18].

Malaria is not unique in adversely affecting pregnancy. Pregnant women have increased susceptibility to many intracellular pathogens [19, 20]. Pregnancy-associated immunomodulation, including the downregulation of T lymphocyte responses to protect the fetus from rejection, has been hypothesized as one explanation [19, 20]. Pregnancy-associated immunomodulation has been demonstrated in rodents [21–23] but
Malaria in Naive Primigravid Rhesus

Figure 1. Daily mean parasitemia in primigravid (PMI) and age-matched nulligravid (NMI) monkeys with malaria. Parasitemia was quantified daily in Giemsa-stained thick and thin blood smears (collected by ear stick from individual monkeys) by counting the parasitized infected red blood cells (PRBCs) in 10 fields of 200 cells to determine the percentage of infected cells. The daily absolute no. of PRBCs was calculated by multiplying the daily RBC count (determined by extrapolation of the weekly RBC count determined by a complete blood cell count) by the percentage. Each point represents the daily mean of the total PRBCs occurring in each group of 8 monkeys. PMI monkeys had higher peak parasitemia and more-chronic recrudescence of parasitemia throughout the first 9 weeks of infection (gestational week [GW] 6–15) than did age-matched NMI monkeys. The second period of recrudescence (GW 12–14) was significantly different in the 2 groups (group, \( P = .04 \); time, \( P = .0001 \); group \( \times \) time, \( P = .02 \) [2-way analysis of variance for all]). The difference in the degree of severity (more-chronic recrudescence patterns) of parasitemia reflects the more-severe clinical malaria observed in the primigravid monkeys. All 10 figures follow 2 corresponding timelines on the X-axis, although, for the sake of clarity, only the gestational timeline is represented in weeks. The timeline that is not shown follows weeks after inoculation. GW 6, 8, 10, 12, 14, 16, 18, 20, and 22 (shown) are equivalent to weeks postinfection (WPI) 0, 2, 4, 6, 8, 10, 12, 14, and 16 (not shown). The first trimester ends on gestational day (GD) 55, during GW 8, WPI 2; the second trimester ends on GD 110, during GW16, WPI 10; and the third trimester ends with cesarean-section delivery on GD 155, during GW 22, WPI 16. The parasitemia graph represents daily parasite values but is also displayed along the weekly gestational timeline (X-axis). T-1, T-2, and T-3, separated by lightly dotted lines, denote trimesters of rhesus monkey gestation as they would equate to human gestation in terms of fetal development and organogenesis.

remains controversial in humans [24–28]. Aggressive Th1 responses to malaria during pregnancy may cause fetal death, IUGR, or neonatal death [29–33].

Although B lymphocytes produce antimalarial antibody [34–36], there are no reports of altered numbers of B cells in malaria during pregnancy. Suppression of B lymphopoiesis during normal pregnancy [37] and selective deletion of B lymphocytes specific for paternal histocompatibility antigens have been reported [38]. B lymphocyte levels decline in pregnant women infected with cytomegalovirus, rubella, and toxoplasmosis [39].

It is impossible to perform rigorous (controlled) studies in malaria-infected pregnant women, because they must be treated, and confounding variables—such as poor socioeconomic conditions, malnutrition, and exposure to other pathogens—are inherent to human studies. To address this problem, we used the \textit{P. coatneyi}/rhesus monkey model of malaria during pregnancy [16, 40, 41]. This model produces the same clinical consequences (severe maternal anemia, early abortion, IUGR, prematurity, LBW, high neonatal mortality, and congenital infection) [16] and placental pathology as those observed in humans [41]. The monkey model provides an opportunity to monitor malaria-infected pregnant females prospectively without confounding variables and to thus define the effects unique to malaria.

The present study was based on timed matings with 16 previously nonpregnant (nulligravid) female rhesus monkeys. Eight primigravid females were infected with \textit{P. coatneyi} on gestational day 42, during gestational week (GW) 6 (the pregnant
Figure 2.  
A, Red blood cell (RBC) counts in primigravid control (PC) monkeys and age-matched, malaria-infected primigravid (PMI) and nulligravid (NMI) monkeys. RBC counts were monitored weekly throughout infection and gestation (automated counts; Coulter T 540 or Bayer AVIDA 120). Each point represents the mean of the total weekly RBC count occurring in each group of 8 monkeys. Counts in PMI monkeys dropped to their nadir at gestational week (GW) ~12 (weeks postinfection [WPI] 6). Chronic parasite recrudescence during the entire second trimester in PMI monkeys (figure 1) resulted in the lower RBC count vs. that in NMI monkeys and failure of RBC counts to recover during the third trimester, similar to RBC counts of NMI monkeys. The gradual decline of RBCs in PC monkeys (RBCs/mL) occurred as a result of normal pregnancy and has been observed in other studies at Tulane National Primate Research Center (unpublished data).

B, Hemoglobin levels in PC, PMI, and NMI monkeys. The RBC count nadir was associated with an average hemoglobin level of 6.75 gm/dL in PMI monkeys vs. 7.87 gm/dL in NMI monkeys. Hemoglobin levels were lower in PMI than in NMI monkeys from GW 9 to 14 (WPI 3–8), which corresponded with the 2 periods of parasite recrudescence shown in figure 1. See figure 1 for description of the timeline used.

and malaria-infected [PMI] group); the other 8 monkeys received intravenous saline—the primigravid, uninfected control (PC) group. Outcomes observed in the 2 primigravid groups were compared with those in a third group of age-matched, malaria-infected nonpregnant females (the nulligravid, malaria-infected [NMI] group) throughout a timed course of chronic malaria (16 weeks), to separate the effects of *P. coatneyi* infection (malaria) from those of pregnancy (the primigravid state).

We show that, in the context of nonimmune, primigravid pregnancy challenged by malaria, decreased levels of mono-
Monocytes, CD4+ and CD8+ lymphocytes, and, surprisingly, B lymphocytes are associated with more-severe systemic parasitemia and clinical malaria in the mother. Whether this failure leads to aberrant increases in Th1 immunity at the level of the placenta is currently under investigation by our laboratory and by others [30, 32, 41, 42].

MATERIALS AND METHODS

Experimental design. The 3 groups of 8 spleen-intact, age-matched female rhesus monkeys (Macaca mulatta) had no previous exposure to malaria. They were housed individually under the same conditions, in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services, National Institutes of Health).

Before each weekly examination, monkeys were anesthetized with 10 mg/kg of ketamine hydrochloride. Eight time-bred primigravid monkeys were inoculated intravenously with 10^6 P. coatneyi parasites at GW 6 (late first trimester) (PMI group), 8 non-pregnant monkeys were inoculated with the same inoculum (NMI group), and 8 primigravid monkeys were inoculated with saline for use as controls (PC group). Antimalarial treatments were withheld until 1 week after delivery. The timeline follows the gestational age of the infant in weeks (GW 6–22) in the pregnant monkeys, and infection in NMI monkeys was monitored along the same timeline as weeks postinfection (WPI; 0–16). GW 6 corresponds to infection time 0 on all graphs presented. Physical examinations were performed weekly, and blood samples were obtained for serum collection, complete blood cell (CBC) counts, and monthly flow-cytometric (FACS) analysis. Daily ear sticks were performed for thick and thin blood smears for the determination of parasite counts.

Malaria parasite inoculum. The inoculum, obtained from a simian retrovirus–free monkey infected with P. coatneyi, was stored in liquid nitrogen. It contained ~10^6 parasites/mL and was thawed in water at 37°C before administration into the saphenous vein. P. coatneyi is a falciparum-like parasite that produces serious clinical malaria in rhesus monkeys and has recrudescence patterns similar to those of Plasmodium falciparum malaria [43]. Because P. coatneyi sequesters in the blood vascular system, P. coatneyi–infected rhesus monkeys have been used elsewhere as models for cerebral malaria [44] and malaria during pregnancy [16].

Parasite counts. Parasitemia levels were estimated by examination of Giemsa-stained blood smears prepared from daily ear sticks from the time of inoculation (GW 6) until 1 week after delivery. Parasitized red blood cells (PRBCs) and unparasitized RBCs were counted in 10 microscopic fields (magnification, 1000) and recorded as the percentage of PRBCs. Whole-blood smears were obtained for serum collection, complete blood cell (CBC) counts, and monthly flow-cytometric (FACS) analysis. Daily ear sticks were performed for thick and thin blood smears for the determination of parasite counts.

Blood counts. CBC counts were obtained weekly by use of either the Coulter T 540 (before September 2001) or the Bayer AVIDA 120 (after September 2001) devices. Whole-blood sam-
Figure 4. Nos. of polymorphonuclear neutrophils (PMNs) in primigravid control (PC) monkeys and age-matched, malaria-infected primigravid (PMI) and nulligravid (NMI) monkeys. Nos. of PMNs were significantly decreased in both malaria-infected groups through gestational week (GW) 19, with PC monkeys having ~1.75 times the nos. of PMNs as PMI or NMI monkeys. Each point represents the mean of the total weekly PMN counts occurring in each group of 8 monkeys (group, ; time, ; group/time, [2-way analysis of variance for all]). See figure 1 for description of the timeline used.

ples in K, EDTA (Vacutainer; Becton Dickinson) were evaluated for RBC count, hemoglobin (Hb) concentration, hematocrit level, leukocyte count, and platelet count. Lymphocyte, monocyte, and neutrophil counts were obtained from these CBC counts. The Coulter T 540 was calibrated by use of normal and abnormal controls and fresh whole blood; the Bayer AVIDA 120 was normalized by use of the Coulter T 540.

Evaluations of lymphocyte subsets. Whole-blood samples in EDTA were evaluated for CD2+, CD20+, CD4+, and CD8+ lymphocytes by staining with fluorochrome-conjugated monoclonal antibodies, followed by flow cytometry [45]. The percentages of CD4+ lymphocytes were determined by staining with CD4 fluorescein isothiocyanate (BD Clone M-T477; Pharmingen) mouse IgG2a. The percentages of CD2+ T lymphocytes and CD20+ B lymphocytes were determined by use of double staining with RD1-T11 and FI-B1 (Coulter). The percentage of CD8+ lymphocytes was determined by use of FI-Leu 2a (Becton Dickinson). Erythrocytes were lysed and leukocytes fixed by use of the TQ-Prep machine and ImmunoPrep reagents (Coulter). Data acquisition and analysis for flow cytometry were performed on the FACSCalibur instrument by use of CellQuest software (Becton Dickinson). Samples from the 3 different groups (PMI, NMI, and PC) were evaluated at the same time points, beginning at GW 6 (day 0 of infection or of saline administration). Absolute counts for individual lymphocyte subsets were estimated by multiplying the total (CBC count) lymphocyte count by the percentages determined by FACS analysis.

Ultrasound examinations. All fetuses were monitored weekly by ultrasound and were delivered by cesarean section at GW 22, before labor and delivery. The examinations were performed by use of a digital real-time ultrasound system (Toshiba PowerVision; Toshiba) with Toshiba microtransducers (PVK-738F/738H/745V) linked to a database and software program (Sonultra Ultra32-OB; Sonultra). This software is based on established normal values for intrauterine fetal growth in rhesus monkeys [46, 47]. Gestational age and expected delivery dates were based on timed breeding dates and ultrasound measurements.

Cesarean sections. Because the gestation of rhesus monkey averages 23.5 weeks, elective cesarean sections were performed at GW 22, to ensure the collection of placental and cord-blood samples and to avoid the confounding effects of labor and delivery. Immediately before surgery, blood samples were obtained for CBC count, FACS analysis, standard chemistry panels, and blood smears. Preanesthesia consisted of glycopyrrolate followed by ketamine hydrochloride; this was maintained by use of isofluorane. The fetus and placenta were removed after exteriorizing the uterus through a vertical midline incision. A sample of amniotic fluid was obtained with a needle and syringe before the removal of the infant, and a cord-blood sample was
Figure 5. Total blood platelet counts in primigravid control (PC) monkeys and age-matched, malaria-infected primigravid (PMI) and nulligravid (NMI) monkeys. Total nos. of blood platelets were significantly lower in PMI than in NMI monkeys and in both groups than in PC monkeys. Each point represents the mean of the total weekly platelet counts occurring in each group of 8 monkeys (group, ; time ; group /H11003 time, P! .005 P! .0001 P! .0001 [2-way analysis of variance for all]). PC monkeys consistently had platelet counts >500,000 cells/mL after gestational week (GW) 10 vs. PMI monkeys, which had levels of 300,000–350,000 cells/mL. The lower levels in PMI monkeys lasted throughout the entire final 2 trimesters of pregnancy. The platelet counts in PC monkeys steadily increased during the last 4 weeks of gestation, to reach their highest level on the day of delivery. See figure 1 for description of the timeline used.

obtained before extraction of the placenta. Postsurgical recovery of the dams was uneventful.

Statistical analysis. RBC, platelet, lymphocyte, monocyte, and neutrophil counts were analyzed use of the STATISTICA program for Macintosh computers (StatSoft). Normally distributed continuous data were compared between groups by use of a 2-way analysis of variance (ANOVA) with repeated measures. Post hoc analyses were performed by use of the Newman-Keuls test. Analyses of data at individual time points (between or among groups) were performed by use of 1-way ANOVA.

Parasitemia and FACS data were analyzed with general linear model repeated-measures procedures by use of the statistical program SPSS for Windows (SPSS). The daily mean parasitemia levels of PMI and NMI were compared. There were 6 consecutive measurements of each lymphocyte subset for each monkey during the period beginning 2 weeks before inoculation and continuing until the end of pregnancy (GW 22) and/or infection (WPI 16). The main effects of time and grouping (PMI, PC, and NMI) were tested, as was the interaction between the 2 factors.

RESULTS

Parasitemia levels, RBC counts, and Hb levels. There were no significant differences in mean peak parasitemia levels between PMI and NMI monkeys (figure 1). The duration of each recrudescence was longer, with higher parasite density, in PMI monkeys; this, however, resulted in more-chronic parasitemia that led to more-severe anemia. In humans, the prevalence and density of P. falciparum are highest during the first half of pregnancy, and levels decrease progressively until delivery [17, 48]. This pattern has also been observed in monkeys.

Although a similar decline in RBC counts occurred in PMI and NMI monkeys (figure 2A), the chronic parasitemia in PMI monkeys resulted in more-severe and -persistent anemia and lower Hb levels (figure 2B) in this group, compared with those in NMI monkeys (group, P< .0001; time, P< .0001; group × time, P< .0001 [2-way ANOVA for all]). The nadir at GW 14 in PMI monkeys was ~1 million RBCs less than those of NMI monkeys, and Hb levels were 6.75 and 7.87 g/dL, respectively. Although Hb levels were similar during the recovery period, NMI monkeys had more-rapid and -complete recoveries of their RBC counts than did PMI monkeys at GW 18–22. The gradual decline of RBCs in PC monkeys (from 5.32 to 4.8 × 106) (figure 2A) occurred as a result of normal pregnancy; this has been observed in other studies at Tulane National Primate Research Center (unpublished data).

Monocytes and polymorphonuclear neutrophils. PMI monkeys had significantly fewer circulating monocytes than did NMI monkeys, despite chronic parasitemia in PMI monkeys throughout the second trimester (group, P< .0001; time, P<
Figure 6. Absolute no. of total lymphocytes in primigravid control (PC) monkeys and age-matched, malaria-infected primigravid (PMI) and nulligravid (NMI) monkeys. The absolute no. of lymphocytes was significantly lower in PMI than in NMI monkeys, starting at gestational week (GW) 14 (weeks postinfection [WPI] 8). Post hoc Newman-Keuls evaluation for each time point tested at GW 14 was \( P = .002 \), that for GW 18 (WPI 12) was \( P = .0002 \), and that for GW 22 (WPI 16) was \( P = .0005 \). Differences in counts in PMI vs. PC monkeys were not significantly different \( (P = NS) \) for any time point tested. Although PMI monkeys had severe clinical malaria, their lymphocyte counts were similar to those of PC monkeys. Each point represents the mean of the monthly lymphocyte counts occurring in each group of 8 monkeys. See figure 1 for description of the timeline used.

\[ .0001; \text{group} \times \text{time}, P < .0001 \] (2-way ANOVA for all) (figure 3). During parasite recrudescence at GW 16, monocytes peaked in NMI but not PMI monkeys, although PMI monkeys had 65,223 PRBCs/\( \mu \)L, compared with 32,472 PRBCs/\( \mu \)L in NMI monkeys (figure 1). *P. coatneyi* infection was also associated with decreased levels of neutrophils (group, \( P < .05 \); time, \( P < .002 \) [2-way ANOVA for both]) (figure 4) and platelets (group, \( P < .0007 \); time, \( P < .0001 \); group \( \times \) time, \( P < .001 \) [2-way ANOVA for all]) (figure 5) in both PMI and NMI monkeys, compared with PC monkeys.

**Total lymphocyte counts.** Primigravid females inoculated with *P. coatneyi* had strikingly different lymphocyte patterns than did age-matched NMI monkeys. The most significant differences began late in trimester 2 (GW 14) \( (P = .002) \) (figure 6), concurrently with the RBC nadir (figure 2A). Moreover, by GW 18 and at delivery (GW 22), total lymphocyte counts were lower in PMI than in NMI monkeys \( (P = .0002 \text{ and } P = .0005, \text{ respectively}) \). Interestingly, despite the severe clinical malaria observed in PMI monkeys, their lymphocyte counts did not differ from those of PC monkeys.

These differences were even more apparent when lymphocyte subsets were examined separately. Unfortunately, because of the small group size with individual and daily variation, PMI monkeys started with lower numbers of T and CD4+ lymphocytes than did the PC monkeys at inoculation. However, 2 weeks before this sample was obtained, another baseline value showed the 3 groups to be more comparable. Regardless, numbers of T lymphocytes decreased significantly in PMI monkeys during the second trimester (by GW 14) and remained significantly lower, whereas levels increased in NMI monkeys \( (P = .004) \) (figure 7). By repeated-measures analysis, levels of CD4+ lymphocytes were significantly lower throughout the course of infection in PMI monkeys than in NMI monkeys \( (P < .003) \) (figure 8). Moreover, there was a negative trend in absolute numbers of CD4+ lymphocytes in PMI monkeys, compared with that in PC monkeys; levels of CD4+ lymphocytes were significantly lower in PMI monkeys \( (P = .003 \text{ and } P = .001) \) (figure 8). In contrast to the increase in numbers of CD8+ lymphocytes in NMI monkeys, PMI monkeys had almost no change \( (P = .006) \), and their CD8+ lymphocyte profiles were similar to those of PC monkeys (figure 9). Levels of CD8+ lymphocytes were significantly lower in PMI monkeys than in NMI monkeys at GW 14 \( (P < .007 \text{ and } P = .005) \) (figure 8). Pregnancy itself, represented by PC monkeys, was associated with little change in levels of CD4+ and CD8+ lymphocytes from baseline until delivery. Despite malaria, the cellular profiles of PMI monkeys were more similar to those of PC monkeys than to those of NMI monkeys.

Our most striking observation was the failure of B lymphocyte (CD20+) counts to increase in PMI monkeys to levels observed in NMI monkeys, despite chronic malaria infection of similar duration (figure 10). Even given our small groups, the difference between PMI and NMI monkeys was significant throughout gestation and malaria infection. The average CD20+ lymphocyte count in NMI monkeys was ~23 times that of PMI monkeys.
monkeys (time, group, and time × group, \( P < .001 \)). Although the absolute number of circulating B lymphocytes in PMI monkeys was 5-fold greater than that in PC monkeys, the difference was not significant.

**DISCUSSION**

Because we examined leukocyte populations in long-term (16 week) asexual or blood-stage malaria infection in monkeys with intact spleens and without antimalarial treatment, we obtained a complete leukocyte profile uncompromised by confounding variables. Thus, the effects could be identified of primigravid pregnancy and chronic malaria, the 2 defining variables in the experiment, on peripheral blood leukocyte levels. Even in severe malaria, the absolute numbers of peripheral blood monocytes, neutrophils, and lymphocytes in PMI monkeys resembled those of PC monkeys more than those of NMI monkeys.

Unlike NMI monkeys, T lymphocyte levels did not increase in pregnant monkeys in response to malaria. Malaria infection during pregnancy was associated with lower CD4+ and CD8+ lymphocyte counts, similar to PC monkeys. The higher group mean CD2+ and CD4+ lymphocyte counts in PC monkeys than in PMI and NMI monkeys on the day of inoculation was likely due to individual variation resulting from early unidentified pregnancy-associated events in specific individuals. However, levels were similar for all cell types in the 3 groups before inoculation, at GW 4. Studies have suggested that the numbers and types of circulating lymphocytes in healthy pregnant women are similar to those in nonpregnant women [25, 49], although some studies have shown changes in various cell types [24–26, 37, 49–54]. Overall, our findings are consistent with healthy pregnant women and nonpregnant women having similar cellular profiles. Immunomodulation—a decline in CD4+ and CD8+ and B lymphocyte counts during pregnancy—in pregnant monkeys was apparent only when they were challenged with *Plasmodium* infection.

Compared with PC monkeys, pregnant monkeys with malaria also showed a significant decrease in neutrophil levels starting at GW 9. Throughout normal human pregnancy, there is a consistent increase in neutrophil levels [55]. Declining neutrophil levels might interfere with a malaria-infected pregnant woman’s ability to control bacterial infections associated with chorioamnionitis (CA). CA is the most common cause of preterm delivery [56], is often associated with malaria [57], and, like concomitant HIV and malaria [58, 59], has been linked to increased vertical transmission of HIV [60].

Thrombocytopenia was noted in both malaria-infected groups. PMI monkeys had one-half the platelet count of PC monkeys. Low platelet levels have been associated with severe preeclampsia and other pregnancy complications that cause premature delivery and LBW [61, 62], and they may contribute to malaria-associated pregnancy complications.

Levels of monocytes, which are essential to innate immunity...
and effective immune responses to malaria, were lower in PMI than in NMI monkeys. Monocytes and macrophages are key cells in controlling malaria through the phagocytosis of PRBCs, resulting in parasite killing and antigen presentation to CD4+ lymphocytes [63, 64]. Peripheral blood monocyte responses have not been reported in prospective studies of pregnant women with malaria. The decrease that we observed may contribute to the increased susceptibility of primigravid women to severe clinical malaria. Increased macrophage levels within the placenta have been associated with poor fetal outcome [65, 66], but it remains to be shown whether decreasing monocyte levels in the peripheral blood of the monkeys is associated with a shifting of these cells into the uterine or placental compartment.

Importantly, our studies show a profound affect of pregnancy on the host’s ability to increase numbers of B lymphocytes, as does the nongravid host who is chronically infected with malaria. Antimalarial antibody–producing B cells are essential to clear blood-stage parasites [67]. Antibody studies under way in monkeys should determine whether B cells effectively produce antimalarial antibodies. We suspect, as has been reported for humans [68], that this is a malaria-induced polyclonal activation of B lymphocytes; perhaps, if it is not suppressed during pregnancy, it could lead to increased maternal hyperreactivity to fetal antigens. Alterations in the reactivity of the maternal immune system toward paternal alloantigens or foreign antigens might contribute to the reduction in the intrapartum development of the placenta and fetus [69]. Conceivably, even the 5-fold increase in B lymphocyte levels observed in PMI versus PC monkeys may cause malaria-associated pregnancy complications. Studies of human and rodent pregnancy have correlated increased maternal B lymphocyte counts with detrimental effects on the fetus, such as IUGR, preterm delivery, and LBW [26, 27, 37, 38, 69–71]. Individual differences in the reactivity of the maternal immune system to malaria antigens may account for variability in fetal outcome, with poor outcomes correlating with high B cell production. Importantly, we hypothesize that the individual reactivity of the maternal immune system to malaria antigens may change in successive pregnancies. Thus, our findings with respect to B lymphocytes may bear directly on the poor fetal outcome of a woman’s first pregnancy, compared with those of later pregnancies. Examination of these same monkeys, reinfected with malaria during subsequent pregnancies, will be the subject of future reports to verify or repudiate this possibility.

The general pancytopenia we observed, along with thrombocytopenia and anemia, suggests generalized bone marrow suppression (BMS). In adult mammals, steady-state production of B cells occurs within the bone marrow [71], so the decreased B lymphocyte levels in the pregnant monkeys challenged with malaria also suggest BMS. In mice, B lymphopoiesis in the bone marrow is markedly down-regulated during pregnancy, which affects all precursor populations beyond the pro–B cell stage,
Figure 9. Absolute no. of cytotoxic/suppressor (CD8+) lymphocytes in primigravid control (PC) monkeys and age-matched, malaria-infected primigravid (PMI) and nulligravid (NMI) monkeys, determined by flow-cytometric analysis. In contrast to the increase observed in the CD8+ lymphocytes in NMI monkeys, PMI monkeys showed no change or a slight decrease, and their CD8+ lymphocyte profile was similar to that of PC monkeys (time, $P = .074$; group, $P = .211$; time $\times$ group, $P = .006$). See figure 1 for description of the timeline used. GW, gestational week.

Figure 10. Absolute no. of B lymphocytes (CD20+) in primigravid control (PC) monkeys and age-matched, malaria-infected primigravid (PMI) and nulligravid (NMI) monkeys, determined by flow-cytometric analysis. Nos. of CD20+ B lymphocytes were different throughout gestation/infection in all 3 groups. PMI monkeys had significantly fewer B cells than did NMI monkeys throughout gestation, starting at gestational week (GW) 10. Toward the end of pregnancy, the average no. of CD20+ B lymphocytes in the PMI monkeys was $\sim$5 times that in PC monkeys. In comparison, the average CD20+ B lymphocyte count in NMI monkeys was $\sim$23 times that in PC monkeys (for time, group and time $\times$ group, $P < .001$). See figure 1 for description of the timeline used.

[37] whereas most mature B cells are exempt. BMS occurs in malaria and contributes to anemia, but the mechanisms are unclear [72]. Pregnancy, in combination with malaria, may exacerbate BMS though undefined processes. Bone-marrow biopsies in the pregnant monkey model may answer these questions.

Thus, in the face of Plasmodium infection, levels of granulocytes, monocytes, and lymphocytes decrease throughout gestation. PMI monkeys apparently tried to maintain a cellular profile similar to that of PC monkeys, allowing fetal survival. However, decreased leukocyte levels may contribute to severe recrudescent parasitemia, thus affecting fetal outcome. Future reports will present data not only correlating maternal malaria
and leukocyte populations with fetal/infant/placental growth, development, and well being but also examining the monkeys’ peripheral and placental cytokine patterns and their association with differing numbers of pregnancies and levels of immunity.

Given the complex patterns observed, conflicting reports of increased or decreased levels of lymphocyte subsets during “normal” human pregnancy are not surprising. We have demonstrated that it is the system under challenge by a pathogen that reveals the nature of the modifications induced by pregnancy, not as a loss of leukocytes through a generalized pregnancy-induced phenomenon but as a lack of appropriate leukocyte increases in response to disease. Both successful pregnancy and survival of the malaria parasite depend on a carefully balanced immune system. A selective species-survival advantage is likely when pregnancy dominates, ensuring that immune responses do not harm fetal growth, development, and survival. Because severe clinical malaria is manifested by immunopathologic symptoms, perhaps the inappropriately low leukocyte numbers in response to malaria are “protective,” allowing fetal survival. The same protection afforded to the fetus may also be advantageous to both host and parasite survival, thus making the primigravid woman a perfect host for *Plasmodium* species.

The results reported here demonstrate one aspect of the immunomodulation occurring in primates challenged with malaria during pregnancy. This was illustrated by the failure of pregnant monkeys, compared with age-matched nulligravid monkeys under the same conditions of severe *Plasmodium* infection, to appropriately increase cell numbers in multiple leukocyte populations. In our study, it was the host immune system under the influence of pregnancy that was responsible for the increased severity of clinical malaria. These findings could have important ramifications for the treatment strategies for maternal malaria and other diseases.

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**References**

26. Bartha JL, Comino-Delgado R. Lymphocyte subpopulations in intra-


28. Sacks GP, Redman CW, Sargent IL. Monocytes are primed to produce the Th1 type cytokine IL-12 in normal human pregnancy: an intra-


33. Suguitan AL Jr, Cadigan TJ, Nguyen TA, et al. Malaria-associated cy-


36. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal an-


39. Poblete A, Roberts A, Trespivi L, Guarnieri D, Nicolini U. Fetal and maternal white cells and B- and T-lymphocyte subpopula-


45. Martin L, Murphey-Corb M, Soike K, Davison-Fairburn B, Baskin GB. Effects of initiation of 3′-azido,3′-deoxythymidine (zidovudine) treat-
ment at different times after infection of rhesus monkeys with simian immunodeficiency virus. J Infect Dis 1993; 168:825–35.


49. Kuhnt M, Schmidt S. Changes in lymphocyte subsets during preg-


60. Temmerman M, Nyong’o AO, Bwayo J, Fransen K, Coppens M, Piot P. Risk factors for mother-to-child transmission of human immuno-


63. Taylor-Robinson AW. Regulation of immunity to malaria: valuable les-


68. Banic DM, Viana-Martins FS, De Souza JM, Peixoto TD, Daniel-Ri-


