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Association of Malaria-Induced Murine Pregnancy Failure with Robust Peripheral and Placental Cytokine Responses▽

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Malarial infection in nonimmune pregnant women is a major risk factor for pregnancy failure. The biological mechanisms that underlie malaria-associated fetal loss, however, are poorly understood. *Plasmodium chabaudi* AS infection during early pregnancy results in midgestational embryonic loss in naive C57BL/6 mice. To define the immunopathogenesis of this malaria-induced pregnancy compromise, cytokine production in plasma, spleen, and placenta cell culture supernatants during the first 11 days of infection and gestation was studied. In infected pregnant mice, systemic interleukin-1β and both systemic and splenic gamma interferon levels were elevated relative to those in uninfected pregnant mice, and gamma interferon was also robustly produced within the placenta 1 to 2 days before malaria-induced fetal loss. Although circulating tumor necrosis factor production was not affected by pregnancy or infection, circulating soluble tumor necrosis factor receptor II was highest in infected pregnant mice, particularly those undergoing abortion, but decreased at the placental level preceding abortion. Systemic levels of interleukin-10 were also high in infected mice at this time point, but this cytokine was not detected at the placental level. Histological examination revealed that trophoblast giant cells of aborting mice phagocytosed infected red blood cells and hemozoin. Furthermore, in vitro-cultured trophoblast cells isolated from embryos on day 7 of gestation phagocytosed *P. chabaudi* AS-infected red blood cells and secreted tumor necrosis factor. These results suggest that systemic and placenta-level proinflammatory antimalarial immune responses, in the absence of adequate and sustained counterregulatory mechanisms, contribute to pregnancy loss in this model.

Malarial infection during pregnancy is a major risk factor for maternal and infant morbidity and mortality. Epidemiological studies have shown that malarial infection during pregnancy is more severe in women with no previous exposure to malarial infection or during an epidemic. Under these circumstances, pregnancy outcome has been shown to be severely compromised, with high rates of abortion, stillbirth, and preterm deliveries (reviewed in reference 10). The precise mechanisms by which the fetoplacental unit is compromised as a result of malarial infection have not been elucidated.

In-depth studies to understand the immunologic and pathological mechanisms involved in malaria-induced fetal loss are not possible in humans due to ethical and practical constraints. We have taken advantage of our recently developed mouse model to further explore the complex interactions between antimalarial immune responses and pregnancy. In this model, C57BL/6 (B6) mice infected with 1,000 *Plasmodium chabaudi* AS-infected red blood cells (iRBCs) on day 0 of gestation develop peak parasitemia and anemia comparable to those in infected nonpregnant (INP) mice and survive the infection (36). However, infected pregnant (IP) mice fail to maintain viable pregnancies after gestation day 11 (36). Although the immune response to *P. chabaudi* AS infection is well characterized in INP mice, nothing is known about the development of immune responses in *P. chabaudi* AS-infected mice during early pregnancy or how these responses affect pregnancy outcome.

Control of acute, primary *P. chabaudi* AS infection in INP mice is dependent on the production of the proinflammatory cytokines interleukin-12 (IL-12), gamma interferon (IFN-γ), and tumor necrosis factor (TNF) (40); parasite clearance during the chronic stage is dependent on B cells and antibodies, especially those of T helper 1 (Th1)-associated subclasses immunoglobulin G2a and immunoglobulin G3 (41). In contrast, normal pregnancy requires a bias against Th1- or toward Th2-type cytokine responses (43). Production of Th2 cytokines such as IL-10 and IL-4 locally at the maternofetal interface is thought to favor the maintenance of pregnancy (34). In contrast, proinflammatory cytokines IL-2, IFN-γ, and TNF are implicated in recurrent spontaneous abortion in humans (27). Elevated levels of these cytokines have been observed in the placental blood of malaria-infected women (12), and TNF at the placental level is associated with low birth weight (12, 38). Furthermore, exogenous administration of TNF was shown to induce abortion in *Plasmodium vinckei*-infected mice (7).

Trophoblasts are fetally derived placental cells that are interposed between the fetus and the mother, in direct contact with maternal blood in the hemochorial placenta. These cells can phagocytose various microbes and produce cytokines and chemokines (13). Thus, trophoblasts can respond immunologically to invading pathogens, including malarial parasites (13, 26). This suggests that both systemic and local inflammatory responses to malaria may alter the delicate cytokine balance in the placenta and contribute to pregnancy loss.

The aim of the present study was to characterize systemic

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and placental cytokine production in *P. chabaudi* AS-IP mice and improve understanding of the development of antimalarial immune responses during early stages of pregnancy and the effects of these responses on pregnancy outcome.

MATERIALS AND METHODS

Mice and parasites. Age- and sex-matched C57BL/6 mice originally purchased from The Jackson Laboratory, Bar Harbor, ME, and IFN-γ null mutant mice (B6.129S7-*Ifng*^{tm1Ts}, obtained from Rick Tarleton, University of Georgia, Athens, GA) were used for the experiments. Mice were maintained and bred by brother-sister pairing for a maximum of 10 generations at the University of Georgia Animal Resources facility in accordance with the guidelines of the University of Georgia Institutional Animal Care and Use Committee.

P. chabaudi AS, originally obtained from Mary M. Stevenson (McGill University and the Montreal General Hospital Research Institute, Quebec, Canada), was maintained as described previously (36).

Experimental design. A serial sacrifice study (36) was performed to study the kinetics of immune response development in *P. chabaudi* AS-IP B6 mice. Briefly, 8- to 10-week-old female B6 mice were infected intravenously on gestation day 0 (hereafter referred to as experiment day [ED] 0) with 1×10^3 iRBCs per 20 g of body weight. INP mice and sham-injected, uninfected pregnant (UP) mice were used as infection and pregnancy controls, respectively. Mice were sacrificed on EDs 6, 8, 9, 10, and 11 to assess pregnancy outcome and immune responses. Development of parasitemia was monitored as described previously (36).

Cell culture. Spleens collected aseptically at sacrifice were cultured as described previously (36). Briefly, a single-cell suspension made by pressing the spleens through a sterile fine-wire mesh was cultured (1×10^6 cells/ml) in the presence of concanavalin A (ConA) (2 μg/ml) or lipopolysaccharide (LPS) (1 μg/ml) (both from Sigma), 10^6 washed iRBCs/ml as the malarial parasite antigen, or an equal number of uninfected RBCs (uRBCs) as a control for 72 h at 37°C in a humidified incubator with an atmosphere of 5% CO_2. Different cell numbers (1×10^6, 2×10^6, and 5×10^6 cells) and mitogen concentrations (1 μg/ml, 2 μg/ml, and 5 μg/ml) were tested initially for 24, 48, or 72 h to determine optimum culture conditions. Supernatants collected were stored at −85°C until use in cytokine enzyme-linked immunosorbent assays (ELISAs).

For placenta cell cultures, at EDs 10 and 11 fetoplacental units were removed aseptically from the uterus under a dissection microscope. Single-cell suspensions were prepared by pressing the whole fetoplacental units through a sterile fine-wire mesh with 10 ml of complete medium (RPMI 1640; Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine (Cellgro), and penicillin-streptomycin (Cellgro). The resulting cells (representing contributions from the embryo, the fetal placenta [trophoblasts], and maternal placental blood) were washed and cultured at a concentration of 5×10^6 cells/ml for 24 h at 37°C under 5% CO_2. The culture supernatants were preserved at −85°C until use in ELISA. On EDs 6, 8, and 9, embryos were too small to obtain sufficient cell numbers to generate single-cell suspensions. At these time points, embryos were removed from the uterus, minced with surgical scissors, washed, and cultured as explants at a concentration of 8 fetoplacental units/ml of medium. In these cases, some decidual cells, in addition to the other cell types indicated above, were likely to be present in the preparations.

Isolation and culture of EPC cells. Uteri from uninfected B6 mice on ED 7 were dissected to shell out the decidual capsules and then the embryos. Ectoplacental cone (EPC) cells were separated from surrounding embryonic tissues, pooled (three to five embryos), and disrupted to form single-cell suspensions. EPC cells were plated on tissue culture chamber slides or 18-mm glass coverslips in 12-well tissue culture plates containing 1 ml of complete medium prepared with Dulbecco's modified Eagle medium and incubated for 3 to 5 days at 37°C under 5% CO_2. To confirm EPC trophoblast purity, cells grown on coverslips were fixed using 2% paraformaldehyde in phosphate-buffered saline for 10 min and then blocked for 1 h at room temperature using 3% bovine serum albumin in phosphate-buffered saline. The monolayer was washed and incubated with a rat anti-mouse cytokeratin A antibody (1:10; TROMA, DSHB, University of Iowa) or an isotype-control antibody (Sigma). Antibody bound to cytokeratin was revealed by use of fluorescein isothiocyanate-conjugated anti-rat polyclonal secondary antibody (Sigma no. F 5262, diluted 1:400) and observation using an SP2s confocal microscope (Leica Microsystems, Inc., Bannockburn, IL). EPC cells cultured on glass coverslips exhibited the general morphology described for these cells by 48 h (2). All cells stained with a murine trophoblast-specific anticytokeratin antibody, suggesting that only trophoblast cells were isolated and cultured (data not shown).

Phagocytosis assay. iRBCs and uRBCs for the phagocytic assay were purified as described previously (16). Briefly, *P. chabaudi* AS-infected mice were housed under reverse lighting conditions to obtain mature parasites during the day. Heparinized blood obtained via cardiac puncture (30 to 40% parasitemia) from these mice was washed and loaded onto a 74% Percoll (Sigma-Aldrich) density gradient after being diluted with phosphate-buffered saline. Following centrifugation at $5{,}000 \times g$ for 20 min at room temperature, the top band containing iRBCs was collected. For uRBC controls, heparinized blood from naive mice was loaded onto a 90% Percoll gradient and centrifuged at $5000 \times g$ for 20 min at room temperature, and the top band was collected. After several washes, RBCs were resuspended in Dulbecco's modified Eagle medium at a concentration of 1×10^6 cells/ml.

EPC cells were cultured for 72 h, and then the medium was replaced with 1 ml of iRBCs or uRBCs and left for 16 h at 37°C. In some experiments, EPC cells were pretreated with cytochalasin D (Sigma-Aldrich) dissolved in dimethyl sulfoxide (Fischer Scientific) at 10 μg/ml or with 0.1% dimethyl sulfoxide as control for 1 h at 37°C. Noningested RBCs were removed by exposure to Tris-buffered 0.175 M NH_4Cl. After washing, the cells were dried and fixed in methanol. RBC phagocytosis was quantified by examining the Giemsa-stained cells by light microscopy. Trophoblast phagocytosis was estimated as the percentage of trophoblast giant cells that contained one or more iRBCs or hemozoin. The phagocytic index was calculated using the formula (number of trophoblast giant cells containing iRBCs or hemozoin/total number of trophoblast giant cells) × 100.

To determine the effect of iRBC phagocytosis on trophoblast cytokine production, trophoblast cells were incubated with iRBCs or uRBCs as mentioned above for 24 h, and culture supernatants were collected and tested for cytokine production by ELISA (see below).

Cytokine and soluble cytokine receptor ELISA. Levels of IFN-γ, TNF, IL-1β, IL-10, and soluble TNF receptor II (sTNFRII) in cryopreserved splenocyte culture supernatants and plasma samples were determined in batches using OptEIA ELISA sets according to the manufacturer's instructions (Pharmingen). To accurately assess the effect of malarial antigen on cytokine production, cytokine levels from cultures exposed to control uRBCs were subtracted from iRBC-stimulated cultures. Limits of detection for ELISA were 8 pg/ml for IL-10 and TNF, 15 pg/ml for IFN-γ and IL-1β, and 31 pg/ml for sTNFRII.

Histology. Uteri were harvested on ED 11 and fixed in buffered formalin for 48 h. Tissues were subsequently paraffin embedded and processed for histology. Hematoxylin- and eosin-stained placental sections (5 μm thick) from IP mice were examined by light microscopy.

Statistical analysis. Unless otherwise noted, the SAS statistical software package (version 8.02; SAS Institute, Inc., Cary, NC) was used for data analysis. Where necessary, the data were normalized by logarithmic transformation before analysis. Proc GLM (analysis of variance [ANOVA]) or Student's *t* test was used to analyze the significance of differences among group means in the case of normally distributed data, and Tukey's Studentized range test was used to perform multiple pairwise comparisons. For these analyses, the pairwise comparisons yielding a *P* value of 0.05 are indicated in the figures, and the ANOVA *P* value for the group comparison is given in the text and is denoted as such. In cases of non-normally distributed data, the nonparametric Wilcoxon rank sum test was used. To compare the averages of the ranked data in more than two groups, the nonparametric Kruskal-Wallis test was performed; the permutation method from the MULTTEST procedure (SAS proc multtest) was used to obtain the adjusted *P* value for each pairwise comparison in multiple-group analyses. In these cases, the adjusted *P* value is reported in the figures and, where appropriate, in the text. Cytokine and soluble receptor data are presented as median and interquartile range (IQR). The relationship between cytokines and sTNFRII was assessed using linear regression. *P* values of ≤0.05 were considered to be significant.

RESULTS

Plasma cytokine levels in *P. chabaudi* AS-IP mice. IP B6 mice undergo abortion beginning on ED 10, with all mice having most or all embryos nonviable on ED 11, corresponding to ascending and peak parasitemias (36). To identify a potential association between a proinflammatory cytokine response to malaria and fetal loss, IFN-γ, TNF, and IL-1β were measured in plasma samples from IP, INP, and UP mice. Regardless of pregnancy, infected mice exhibited a robust IFN-γ response following *P. chabaudi* AS infection (Fig. 1A). IFN-γ

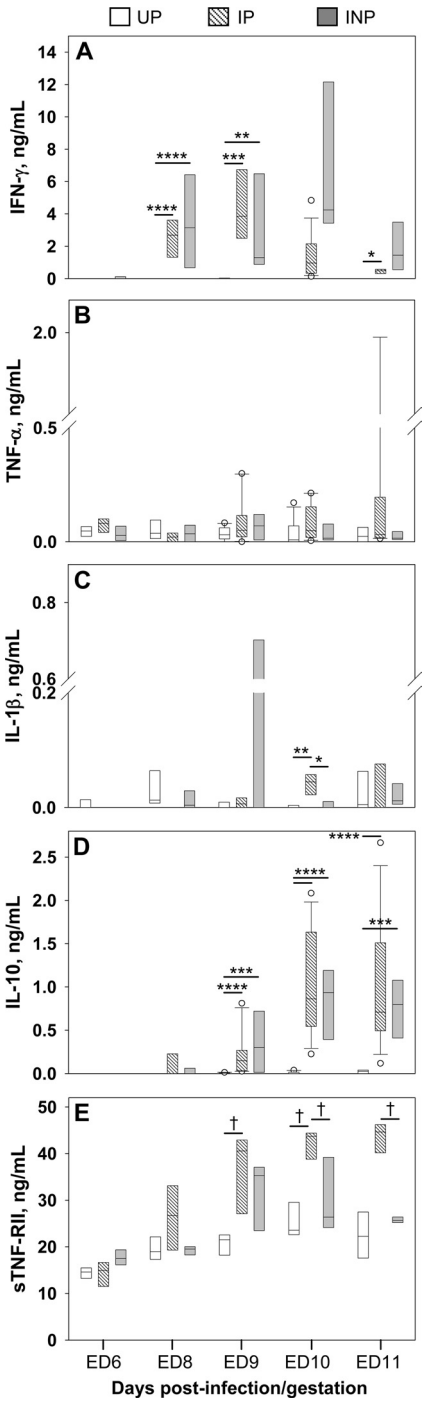

FIG. 1. IFN-γ, TNF, IL-1β, IL-10, and sTNFRII plasma levels are elevated in *Plasmodium chabaudi* AS-IP mice. IP and INP mice were infected with *P. chabaudi* AS, and UP mice were sham injected. Mice were sacrificed at the indicated time points, and plasma samples were assayed for IFN-γ (A), TNF (B), IL-1β (C), IL-10 (D), and sTNFRII (E) levels by ELISA. Results shown are data grouped from two separate experiments. Box plots show IQR with median; whiskers represent 10% and 90% percentiles. The numbers of mice sacrificed (IP, UP, and INP, respectively) were as follows. For IFN-γ, at ED 6, n = 5 for all; at ED 8, n = 7, 8, and 5; at ED 9, n = 5, 5, and 6; at ED 10, n = 15, 5, and 6; at ED 11, n = 5, 3, and 4. ED 10 includes both aborting and nonaborting mice. For TNF and IL-10, at ED 6 and 8, n = same as described for IFN-γ, at ED 9, n = 10, 10, and 6; at ED 10, n = 12, 11, and 5; at ED 11, n = 12, 8, and 5. For IL-1β, at ED 6, n = 5 for all; at ED 8, n = 6, 5, and 3; at ED 9, n = 4, 5, and 5; at ED 10, n = 6, 5, and 5; at ED 11, n = 7, 5, and 4. For sTNFRII, at ED 6, 9, and 11, n = 5 for all except INP at ED 11, where n = 4; at ED 8, n = 6, 4, and 5; at ED 10, n = 8, 5, and 5. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P \leq 0.0001$ (by Kruskal-Wallis test and proc multtest). Proc GLM with Tukey HSD, †, $P = 0.05$; ANOVA P values are given in the text.

was detectable in IP mice beginning on ED 8 and peaked on ED 9, corresponding to ascending parasitemia in this model (36). IFN-γ was significantly higher in IP mice on EDs 8, 9, and 11 than in UP mice (ED 8, $P < 0.0001$; ED 9, $P = 0.0009$; ED 11, $P = 0.0338$), which had no detectable IFN-γ production at any of the time points tested. Although IFN-γ production by IP mice declined 1 day earlier than that by INP mice (ED 9 versus ED 10), plasma levels of this cytokine did not differ between the two groups. On ED 10, when IP mice begin to abort (36), IFN-γ levels did not differ in aborting and nonaborting mice (median [IQR] of 1.0 [0.4 to 1.9] ng/ml [n = 10] versus 1.0 [0.3 to 1.1] ng/ml [n = 5]).

Contrary to expectation, plasma TNF levels were not significantly different among the infection groups, although a nonstatistically significant tendency for higher levels in IP mice relative to INP and UP mice on ED 10 was observed (Fig. 1B) ($P > 0.05$). Median TNF levels were not different in mice undergoing abortion compared to nonaborting mice on ED 10 (median [IQR] of 16 [10 to 46] pg/ml [n = 11] versus 56 [45 to 139] pg/ml [n = 5]).

TNF has been shown to function synergistically with IL-1β to induce malarial pathogenesis (37). IL-1β was detected in IP mouse plasma beginning on ED 9 (Fig. 1C) and peaked on ED 10, at which time the level was significantly higher than in INP ($P = 0.014$) and UP mice ($P = 0.0037$). Comparisons between aborting and nonaborting mice were not done due to insufficient sample sizes.

To assess a potential role for anti-inflammatory, counterregulatory factors, plasma IL-10 was also measured. As shown in Fig. 1D, infected mice exhibited robust IL-10 production. The response increased dramatically on ED 10 in IP mice, a day after the peak IFN-γ response, and, similar to the case for INP mice, remained elevated on ED 11. Compared to UP mice, IP mice had significantly higher levels of plasma IL-10 on EDs 9 through 11 (all $P < 0.0001$). Nonetheless, IL-10 did not appear to have a protective effect against abortion; plasma levels were not different in mice undergoing abortion compared to nonaborting mice on ED 10 (median [IQR] of 0.6 [0.4 to 1.4] ng/ml [n = 11] versus 0.7 [0.5 to 0.7] ng/ml [n = 5]).

Previous studies of *P. chabaudi* AS infection have reported elevated levels of circulating TNF (18). Because neither IP nor INP mice exhibited systemic TNF expression patterns substantially different from those of UP mice, it was of interest to determine whether sTNFRII, which binds and sequesters soluble TNF, was differentially elaborated in these mice. As shown in Fig. 1E, sTNFRII levels were significantly higher in the plasma of IP mice than in that of UP mice (on EDs 9 and 10, $P = 0.0099$ and $P = 0.0002$, respectively, by ANOVA) or INP mice (on EDs 10 and 11, $P = 0.0002$ and $P < 0.0001$, respectively, by ANOVA). The median sTNFRII level was

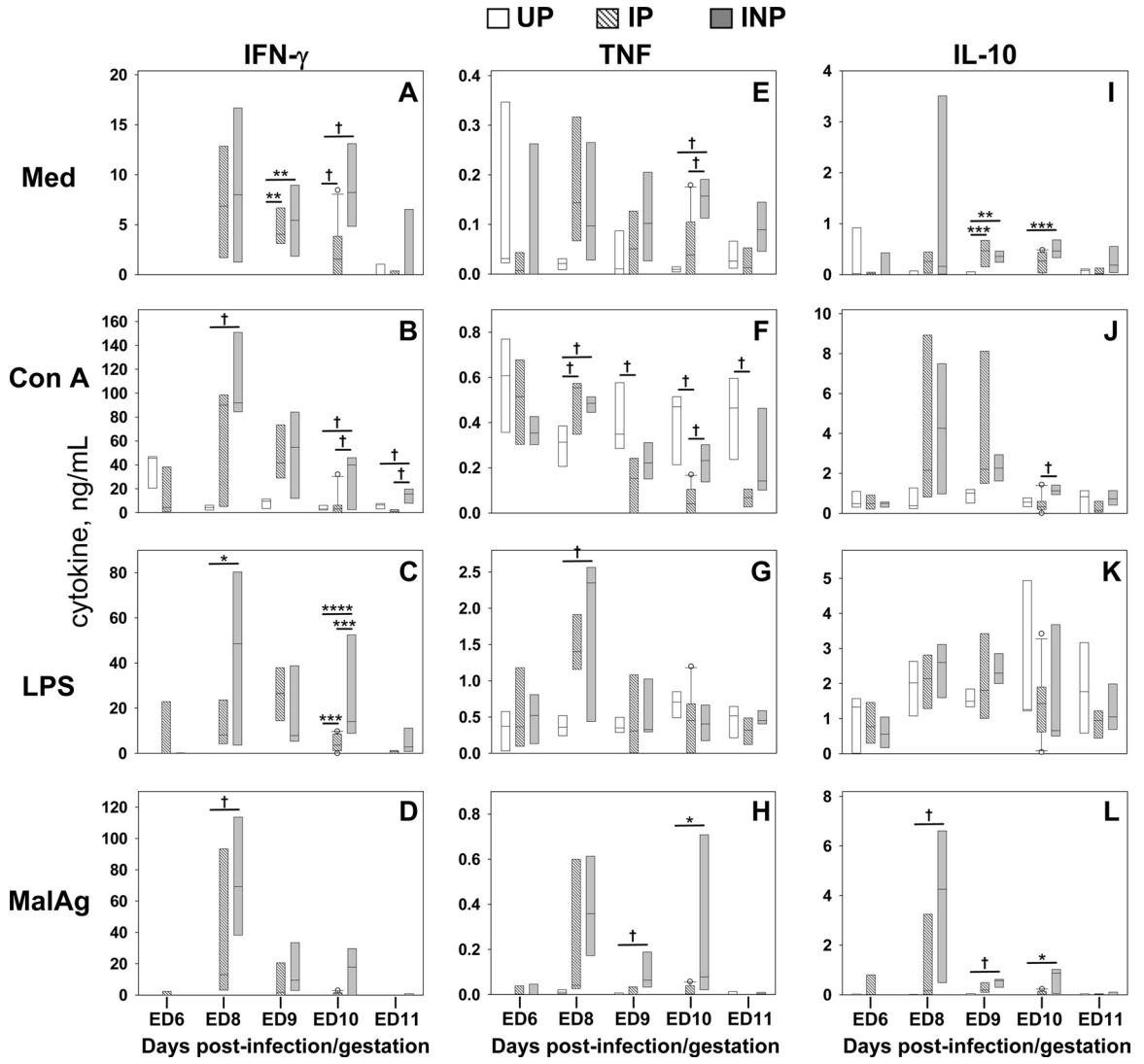

FIG. 2. Splenocytes from IP mice produce increased amounts of cytokines in response to mitogens or malarial antigen (MalAg) in vitro. Spleens collected aseptically from *P. chabaudi* AS-IP, INP, and UP mice at the time points shown were cultured without stimulation (Med) or in the presence of ConA, LPS, or iRBCs. Culture supernatants were assayed for IFN-γ, TNF, and IL-10 by ELISA. Box plots are as in Fig. 1 and are representative of two independent experiments. The numbers of mice sacrificed (IP, UP, and INP, respectively) at each ED were as follows: at ED 6, 8, 9, and 11, $n = 5$ for all; at ED 10, $n = 10$, 5, and 5. *, $P < 0.05$; **, $P < 0.05$; ***, $P < 0.025$; **** P, 0.001 (by Kruskal-Wallis test and proc multtest). Proc GLM with Tukey HSD, †, $P = 0.05$; ANOVA P values are given in the text.

significantly higher in IP mice undergoing abortion on ED 10 (median [IQR] of 43.7 [40.0 to 44.1] ng/ml [$n = 11$]) than in nonaborting mice (38.7 [38.4 to 39.2] ng/ml [$n = 3$]) ($P = 0.01$). Furthermore, in ED 10 and 11 aborting mice, plasma TNF was significantly positively predictive of circulating sTNFRII levels ($r^2 = 0.45$, $P = 0.014$).

In vitro cytokine production by spleen cells from *P. chabaudi* AS-IP and nonpregnant mice. To further characterize immune responses in IP mice, the ability of splenocytes from these mice to produce IFN-γ, TNF, and IL-10 in response to iRBCs or mitogens (LPS and ConA) in vitro was examined by ELISA and compared to results for INP and UP mice. As observed in plasma (Fig. 1), IFN-γ production for the most part was intermediate in IP mice compared to INP and UP mice under all conditions tested (Fig. 2). Splenocytes from IP mice produced larger amounts of IFN-γ than those from UP mice spontaneously (ED 9, $P = 0.0147$; ED 10, $P = 0.0006$ [by ANOVA]) and in response to LPS stimulation (ED 10, $P = 0.0020$ [by ANOVA]) during ascending parasitemia. In fact, there was little to no spontaneous, LPS-stimulated, or malaria-specific IFN-γ production by UP mice at the time points tested. However, IFN-γ secretion by ConA-stimulated splenocytes from IP and UP mice was comparable and reduced relative to results for INP mice on EDs 10 ($P = 0.0176$ by ANOVA) and 11 ($P = 0.0007$ by ANOVA). Overall, the levels of this cytokine in IP mice relative to INP mice either were lower or were produced more transiently.

TNF production by IP splenocytes was similar to that by both UP and INP splenocytes following LPS and iRBC stimulation. With the exception of ED 10, at which time IP mice

TABLE 1. Kinetics of IFN-γ, TNF, IL-10, and sTNFRII production by placental cells[a]

ED (IP *n*, UP *n*)	Pregnancy status	Culture method	Median, IQR							
			IFN-γ (pg/ml)		TNF (pg/ml)		IL-10 (pg/ml)		sTNFRII (ng/ml)	
			IP	UP	IP	UP	IP	UP	IP (*n*)	UP (*n*)
6 (5, 4)	Nonaborting	Explant	0, 0–0	0, 0–0	14, 8–25	17, 6–41	0, 0–0	0, 0–0	11, 7–17	8, 6–8 (5)
8 (6, 4)	Nonaborting	Explant	261, 51–550[b]	0, 0–0	25, 13–45	37, 32–44	0, 0–0	0, 0–0	56, 7–59 (5)	86, 37–96 (5)
9 (5, 5)	Nonaborting	Explant	535, 419–643[c]	0, 0–0	25, 18–31	28, 28–30	0, 0–0	0, 0–0	110, 95–131[d]	34, 18–66 (4)
10 (3, 9)	Nonaborting	Single cell	0	0	23, 19–28	30, 27–30	0	0, 0–0	9, 8–10 (2)	2, 1–2 (4)
10 (2, 0)	Aborting	Single cell	0		23, 14–32		0		16, 3–28	
11 (0, 8)	Nonaborting	Single cell		0, 0–0		35, 29–44		0, 0–0		3, 1–5 (5)
11 (5, 0)	Aborting	Single cell	0, 0–0		13, 9–18[e]		0, 0–0		2, 1–4	

[a] Initiation of infection and sacrifice were performed as described in the legend for Fig. 1. Fetoplacental units isolated from mice on the days shown were processed and cultured without any external stimuli for 24 h. Culture supernatants were assayed by ELISA. The number of mice is as indicated with ED except as noted for the sTNFRII results.
[b] $P = 0.023$ compared to UP on the same ED (by Kruskal-Wallis test).
[c] $P = 0.005$ compared to UP on the same ED (by Kruskal-Wallis test).
[d] $P = 0.014$ compared to UP on the same ED (by Kruskal-Wallis test).
[e] $P = 0.019$ compared to UP on the same ED (by Kruskal-Wallis test).

had significantly reduced spontaneous secretion relative to INP mice ($P = 0.0015$), unstimulated TNF production was similar among the groups. Interestingly, whereas ConA-stimulated TNF production by IP mice was significantly higher than that by UP mice on ED 8 ($P = 0.0158$ by ANOVA), at the later time points this production was significantly reduced relative to that by the pregnancy controls (ED 9, $P = 0.0147$; ED 11, $P = 0.0409$ [by ANOVA]) and was also significantly lower than that by INP mice at ED 10 ($P < 0.0001$ by ANOVA). iRBC-stimulated responses overall were poor, and IP responses did not differ from UP or INP responses, being intermediate between the two.

The intermediate TNF responses observed in IP mice could be due to high production of the regulatory cytokine IL-10. Splenocytes from IP mice exhibited higher spontaneous, ConA-specific, and iRBC-specific IL-10 production than those from UP mice during ascending parasitemia, but this reached statistical significance only for unstimulated production on ED 9 ($P = 0.0219$). Overall, IP splenocytes produced amounts of this cytokine comparable to those for INP mice, producing significantly less only on ED 10 following ConA stimulation ($P = 0.01$ by ANOVA). LPS-stimulated IL-10 production was equivalent regardless of infection or pregnancy. Finally, splenocyte production of IFN-γ, TNF, and IL-10 was not significantly different between aborting and nonaborting IP mice on ED 10 (data not shown).

Cytokine production by cultured fetoplacental units from *P. chabaudi* AS-infected IP and UP mice. Cytokine production at the placental level may be different from that observed in the plasma and spleen, especially in light of the significant accumulation of iRBCs in the maternal blood sinusoids of *P. chabaudi* AS-infected B6 mice (36). To assess this directly, IFN-γ, TNF, and IL-10 secretion by cultured cells derived from whole embryo/placenta preparations from IP mice was tested. The kinetics of IFN-γ production in the culture supernatants paralleled those of IFN-γ production in the periphery (Table 1). Cells isolated from IP mice produced large amounts of this cytokine, but none was detected in UP mice (EDs 8 and 9, $P \leq 0.023$). Contrary to expectation, TNF production was either comparable or lower in IP mice relative to UP mice (ED 11, $P = 0.019$). This was not due to elevated IL-10 production in IP mice, because IL-10 production was below the level of detection in both groups. However, as observed in the plasma, sTNFRII production was significantly higher in the placenta cell culture supernatants from IP mice than in those from UP mice on ED 9 ($P = 0.014$).

Trophoblast phagocytosis of *P. chabaudi* AS iRBCs. Although fetal loss in IP mice is associated with placental iRBC accumulation (36), the infiltration of monocytes/macrophages into the placentae of IP mice was minimal (J. Poovassery et al., unpublished data). This is in contrast to what has been reported for the placentae of malaria-IP women (32) and mice infected midgestationally with *Plasmodium berghei* (31). However, histological examination revealed massive phagocytosis of iRBCs and hemozoin by trophoblast giant cells (Fig. 3A). The impact of iRBC phagocytosis by these cells on placental function or how this may affect local pathology is an understudied problem. To address this, EPC cells from gestation day 7 embryos were dissected (33) and cultured in vitro. EPC cells cocultured with iRBCs began phagocytosing these cells (Fig. 3B) within 1 h (data not shown). Following an incubation period of 16 h, the EPC cells exhibited enhanced phagocytosis of iRBCs compared to uRBCs (phagocytosis index [mean ± standard error of the mean] = 31% ± 3% versus 4% ± 0.5%, respectively; $P = 0.015$, $n = 3$ independent experiments). Furthermore, treatment with cytochalasin D significantly suppressed phagocytosis of both iRBCs and uRBCs (88% and 58% reductions, respectively).

Production of cytokines by *P. chabaudi* AS-exposed murine trophoblasts. Although IFN-γ was detected in placental cell culture supernatants (Table 1), the cellular makeup of these preparations is a combination of embryonic, fetal placental (trophoblast), and maternal blood cells, and thus the precise cellular source of this cytokine is not known. Because abortion in this model occurs with minimal maternal mononuclear cell infiltration into the placenta and murine trophoblast cells are known to produce IFN-γ (35), we hypothesized that fetal cells are a local source of IFN-γ and potentially other pathogenic and protective cytokines. To investigate this, EPC cells were incubated with iRBCs in vitro for 24 h. Contrary to expectation, this treatment did not result in increased IFN-γ secretion (median [IQR] of 0 [0 to 24] pg/ml for iRBCs [$n = 13$] versus

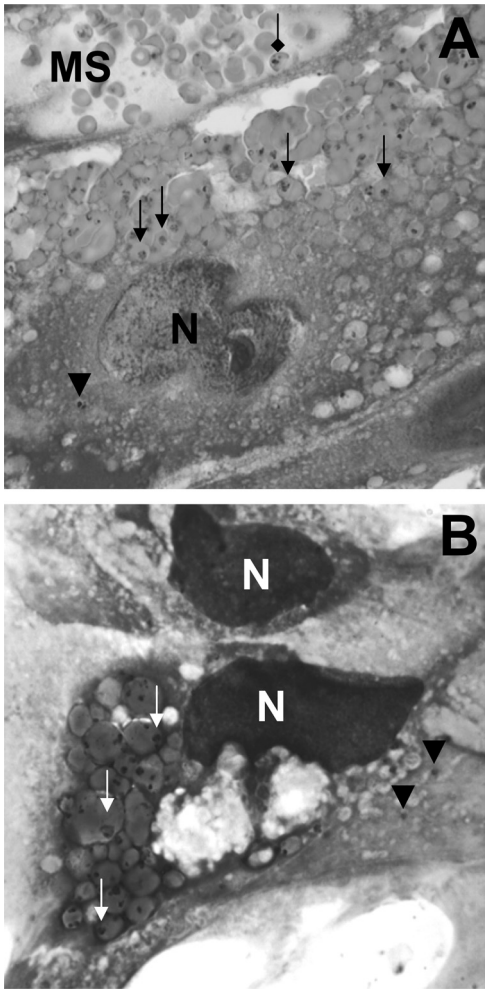

FIG. 3. Trophoblast giant cells phagocytose *P. chabaudi* AS-iRBCs. (A) Hematoxylin- and eosin-stained placental section from an IP mouse undergoing abortion on ED 11, showing a trophoblast giant cell in contact with an iRBC (diamond-headed arrow) in a maternal blood sinusoid (MS). Numerous iRBCs and uRBCs have been phagocytosed. (B) Syncytialized EPC cells cocultured for 16 h with iRBCs, fixed, and stained with Giemsa stain. In both images, phagocytosed iRBCs are depicted with arrows and free cytoplasmic hemozoin with arrowheads. N, nucleus. Both photomicrographs are at a magnification of ×100.

14 [7 to 24] pg/ml for uRBCs [$n = 3$] [$P > 0.05$]). To confirm this result, IFN-γ null mutant females were mated with intact B6 males and infected. The resultant heterozygous embryos were isolated and processed on ED 9 (corresponding to peak systemic and fetoplacental production in B6 mice) and cultured for 24 h, and supernatants were assayed for IFN-γ. Production of this cytokine was minimal (median [IQR] of 21 [15 to 41] pg/ml [$n = 4$]), more than 25-fold lower than production by embryo/placental cell preparations from intact B6 mice (Table 1), indicating that the source of IFN-γ in *P. chabaudi* AS-IP mice, be it systemic, splenic, or placental, is essentially maternal. Nonetheless, incubation of B6 EPC cells with iRBCs resulted in robust TNF production (median [IQR] of 9 [4 to 52] pg/ml for iRBCs [$n = 13$] versus 0 [0 to 1] pg/ml for uRBCs [$n = 3$]; $P = 0.023$). These results suggest that fetal trophoblast cells have the potential to be immunoactive during malarial infection and contribute to a locally pathogenic environment in the uterus.

DISCUSSION

High rates of preterm labor, abortion, and stillbirth have been reported in nonimmune, *P. falciparum*-IP women (10). Abortions associated with malaria have also been reported in *Plasmodium coatneyi*-infected rhesus monkeys (8) and in late gestational *P. berghei* murine infection (31). Although an immunological basis has been attributed to malarial pathogenesis, few studies have been done to explore this possibility in severe pregnancy outcomes such as abortion and stillbirth, particularly during early gestation. We have previously shown that *P. chabaudi* AS infection invariably results in midgestational abortion in B6 mice infected in early pregnancy (36). To investigate the role that maternal antimalarial immune responses play in this phenomenon, the patterns of cytokine production in the plasma and by in vitro-cultured spleen and embryo/placenta cells from *P. chabaudi* AS-IP mice were studied.

The immune response to *P. chabaudi* AS infection in nonpregnant B6 mice has been well characterized. Resolution of primary infection with *P. chabaudi* AS requires IFN-γ (40), which, along with TNF, induces downstream effector molecules such as nitric oxide (NO) and reactive oxygen intermediates that participate in parasite killing (17, 39). However, these responses can be harmful for pregnancy (21). Because IP B6 mice develop peak parasitemia and anemia comparable to those in INP mice and survive the infection (36), we anticipated that IP mice would also develop an early Th1 cytokine-biased immune response at the expense of their pregnancies.

Studies with rodent models have demonstrated that high systemic and uterine production of Th1/proinflammatory cytokines during pregnancy can be harmful to the fetus. The Th1 cytokines IL-2, IFN-γ, and TNF have embryotoxic effects (15, 44) and are implicated in human recurrent spontaneous abortion (27). IFN-γ along with TNF can suppress the development of murine fetuses and can induce apoptosis of human trophoblasts in vitro (15). On the other hand, exogenous administration of the Th2/immunoregulatory cytokine IL-10 can reverse fetal resorption in a murine model of recurrent spontaneous abortion (5). In the context of human malaria, elevated placental levels of IFN-γ, TNF, and IL-2 have been reported (12), and high mRNA and protein levels of TNF are associated with low birth weight (30, 38). Furthermore, exogenous administration of TNF induces abortion in *P. vinckei*-IP mice (7) and is associated with fetal death in *P. coatneyi*-infected monkeys (9). Consistent with the paradigm of a negative effect of proinflammatory immune responses on pregnancy, IP mice exhibited robust systemic IFN-γ and IL-1β responses against primary *P. chabaudi* AS infection preceding or at the time of abortion. Likewise, cultured splenocytes and cells isolated from fetoplacental units of IP mice produced elevated amounts of IFN-γ ex vivo, whereas these responses by UP mice were considerably lower or absent. In light of these data, it was of interest to assess the source of IFN-γ in more detail. Results from a heterozygous crossing experiment as well as from in vitro experiments suggest that the source of IFN-γ at the placental level is largely maternal. Uterine NK cells, which constitute the

majority of decidual leukocytes, could be a source of IFN-γ (3) in this context, although it is more likely that maternal lymphocytes (T cells and NK cells) circulating in the maternal placental blood at the time of sacrifice were the major contributors. These two cells types are known to be potent producers of IFN-γ in *P. chabaudi* AS infection (22, 28), but further study will be required to definitively establish the responsible cell types in IP mice.

Despite the finding that placental IFN-γ is likely to be maternally derived in *P. chabaudi* AS-infected mice, trophoblast cells are known to have the potential to function as a component of the innate immune system at the uteroplacental level (13), including release of cytokines such as TNF (14). Our recent studies have shown that cytoadherent *P. falciparum*-iRBCs and hemozoin induce the phosphorylation of several proteins, including mitogen-activated protein kinases, in human primary syncytiotrophoblasts and also stimulate these cells to secrete cytokines and chemokines and to promote chemotaxis of peripheral blood mononuclear cells (25, 26; N. Lucchi et al., submitted for publication). The results from the present study suggest that murine fetal cells can also respond immunologically to malarial infection. Trophoblast cells exhibited massive phagocytosis of iRBCs and hemozoin both in vivo and in vitro, which stimulated significant secretion of TNF.

The latter finding was intriguing, since aside from a tendency to be higher in IP mice at ED 10, TNF did not differ significantly among the experimental groups at the time points evaluated (ED 6 through 11). Although TNF levels have been shown to be highest at peak parasitemia in INP mice (18), plasma TNF reached its maximum 2 days before peak parasitemia and was sustained from ED 9 to 11 in IP mice. This difference in the dynamics of cytokine production could be due to differences in the infective dose (10^3 versus 10^6) as well as in the route of infection (intravenous versus intraperitoneal) (4, 42). Splenic TNF in response to ConA stimulation was stronger in infected mice than in UP mice at ED 8, preceding by 2 days initiation of malaria-associated abortion (36). Strikingly, however, ConA-stimulated TNF production was significantly reduced in IP mice compared to UP mice from ED 9 to 11. This result suggests that T cells with TNF production capacity either are profoundly suppressed in IP mice or emigrate to other tissues. The placenta is an unlikely candidate given that placental cell cultures from IP mice did not reveal elevated TNF levels relative to those from UP mice. It has been shown that NO produced during acute *P. chabaudi* AS infection in B6 mice inhibits proliferation of splenocytes in response to ConA stimulation (40) and suppresses IFN-γ, TNF, and IL-12 production by $CD4^+$ T cells from *P. chabaudi* AS-infected mice during acute infection; thus, NO-mediated immunosuppression could be also operative in IP mice. Nonetheless, the fact that sTNFRII levels were significantly higher in the plasma and embryo/placenta cell culture supernatants from IP mice than in those from the other groups also deserves consideration. An association between disease severity and elevated sTNFRII level has been reported during malarial infection in humans (19) and monkeys (9). Although sTNFRs can act as TNF antagonists by competing for ligand with membrane-bound TNFR (20), at low concentrations sTNFRs can enhance TNF activity by protecting the cytokine from degradation and prolonging its availability for binding to membrane-bound receptors (1). In IP mice, elevated sTNFRII levels may reflect a failed physiological attempt to counter the harmful effects of ongoing TNF production, which is established before a significant rise in sTNFRII occurs (at ED 9). The positive association between plasma sTNFRII and TNF at EDs 10 and 11 thus serves as an indicator of an ongoing inflammatory process, which is likely to underlie placental damage; the significantly higher plasma sTNFRII levels in aborting compared to nonaborting IP mice at ED 10 also support this supposition. It is interesting that local sTNFRII release in the placenta dropped off precipitously at this same time point, concurrently with malaria-induced abortions. Trophoblast cells are known to express TNFRs (14), and EPC cells secrete TNF and sTNFRII (data not shown) in response to malarial parasites. Thus, reduced sTNFRII in the placental environment could be an indicator of TNF-induced trophoblast cell death. Additional, detailed studies will be required to delineate the relationships between TNF and sTNFRs, as well as other soluble factors, and their respective roles in malaria-induced pregnancy loss in this model. A distinct role for TNF seems certain, however, given our recent observation that neutralization of TNF rescues pregnancy in *P. chabaudi* AS-infected mice (36a).

Following the response to acute *P. chabaudi* AS infection, systemic and splenic production of proinflammatory cytokines IFN-γ and TNF in IP and INP mice was downregulated. IL-10 is a regulatory cytokine that has been shown to play important roles in controlling IFN-γ and TNF responses (29), and it has recently been argued to be important for blocking both malaria-associated immunopathology and *P. chabaudi* virulence (24). *P. chabaudi* AS infection is associated with increased mortality in IL-$10^{-/-}$ mice, with excessive production of TNF likely to be responsible, since anti-TNF treatment was able to abolish the observed mortalities (23). IP mice elaborated a robust systemic IL-10 response that was indistinguishable from that of INP mice during peak parasitemia. This likely served to protect the dams from mortality (23) but, because it was not produced at the placental level, was insufficient to preserve fetal viability (36). Thus, local and systemic release of IFN-γ, TNF, and IL-1β in the absence of the regulatory cytokine IL-10 at the placental level (15) may contribute to pregnancy loss in *P. chabaudi* AS-infected mice.

Despite the observed associations between cytokine production in this model and malaria-induced abortion, the precise mechanisms that compromise pregnancy during malarial infection remain to be elucidated. It is possible that TNF produced by splenocytes and fetal cells, even at low levels, may induce maternal NK cells to produce IFN-γ, which in turn may increase trophoblastic phagocytosis (2) of iRBCs and hemozoin. This could result in more TNF production by trophoblasts in a pathogenic feedback loop, ultimately inducing apoptosis of the trophoblasts, placental destruction, and embryonic death. We are currently investigating this possibility. Additionally, thrombotic events initiated in the placenta by proinflammatory cytokines IFN-γ and TNF (6, 36a) may also be involved in malaria-induced fetal death, similar to what has recently been proposed for cerebral malaria (11).

In summary, *P. chabaudi* AS infection during early pregnancy in B6 mice results in midgestational pregnancy loss (36)

in association with systemic as well as local placental proinflammatory cytokine responses. Continued use of this model should prove to be valuable in identifying the mechanisms by which malaria-induced maternal and trophoblastic immune responses result in compromise of the placenta and embryo and loss of pregnancy.

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