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***Plasmodium chabaudi* AS induces pregnancy loss in association with systemic pro-inflammatory immune responses in A/J and C57BL/6 mice**

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SUMMARY

The molecular mechanisms that underlie poor birth outcomes in malaria during pregnancy remain poorly defined. To assess the role of host immune responses, mice known to respond differentially to *Plasmodium chabaudi* AS infection were studied. Following infection at day 0 of pregnancy, A/J mice developed significantly higher parasitemia than C57BL/6 (B6) mice and succumbed to infection. Both strains had evidence of parasite accumulation in the placenta at mid-gestation and aborted, with significantly higher embryo loss in infected A/J mice on day 9. While infection-induced systemic tumor necrosis factor and interleukin-1 β in the latter were significantly higher at day 11, day 10 IL-10 levels were higher in B6 mice. No differences in levels of splenic lymphocyte subsets, neutrophils or monocytes between infected pregnant A/J and B6 mice were observed, with most cell types expanding in response to infection regardless of pregnancy. Antibody ablation of TNF exacerbated infection in A/J mice and did not ameliorate pregnancy outcome. Thus, malaria induces poor pregnancy outcome in both mouse strains in the context of quantitatively different systemic inflammatory responses. Further evaluation of the roles of soluble and cellular immune components, particularly at the uteroplacental level, will be required to define the most critical pregnancy-compromising mechanisms.

Keywords

placental malaria; *Plasmodium chabaudi*; pregnancy; abortion; tumor necrosis factor; A/J; C57BL/6

INTRODUCTION

Malaria during pregnancy is one of the most important health problems in the developing world (1). In malaria endemic settings, adults develop protective immunity after repeated

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exposures (2), but women are more susceptible to malarial infection when they become pregnant (1). Severe clinical manifestations associated with this infection include premature delivery and intrauterine growth restriction, contributing to low birth weight (LBW), stillbirth, abortion, and maternal mortality (3–5). Increased synthesis of inflammatory cytokines like tumor necrosis factor (TNF), interleukin (IL)-2, and interferon (IFN)- γ (6–8) has been shown in malaria during pregnancy and levels of TNF in particular have been associated with maternal anemia and LBW (6, 9). Interestingly, such pathogenic immune responses to malaria appear to be influenced by host genetic factors. For example, infants homozygous for *TNF2*, a polymorphism in the *TNF* promoter region that is associated with increased TNF production (10), are at increased risk for preterm birth and mortality, suggesting that poor birth outcomes in malaria endemic areas are precipitated by a genetically-determined maternal tendency to produce high levels of this inflammatory cytokine (11).

In mice, the immune response to malaria is complex and varies as a function of mouse genetic background (12) and anatomical sites analyzed. Moreover, it is dependent on parasite species and strain as well as route of infection. In B6 mice, early production of TNF, IFN- γ , IL-12 (13) and granulocyte-macrophage colony-stimulating factor (14) is required for resistance to blood stage *P. chabaudi* AS infection. In contrast, susceptible A/J mice mount early, predominantly Th2-biased cytokine responses (15) and succumb to infection (16). Treatment of these mice with recombinant IL-12 early in infection results in increased production of IFN- γ and TNF and facilitates elimination of parasites and survival (17). Interestingly, A/J mice overcome their Th2-cytokine bias later in infection, exhibiting increased TNF expression in the liver and high serum TNF levels coincident with the time that they begin to succumb to infection (18).

Recently, we initiated studies of malaria during pregnancy using *P. chabaudi* AS infection in B6 mice as a model platform (19–21). This model recapitulates the severe pregnancy outcomes, namely fetal loss, seen in low endemic areas and in some heavily exposed primigravidae (1). Importantly, similar to human malaria during pregnancy (6, 9), TNF plays a critical role in embryo loss in this model; antibody-mediated neutralization of this cytokine rescues mid-gestational pregnancy (21).

Because TNF is well known to have a negative impact on pregnancy outcomes even in the absence of infection (22, 23), the tendency to produce this factor in response to malarial infection may represent a common pathogenic factor that relative to other host elements is central to disease pathogenesis. In this study, we addressed the hypothesis that malarial infection in pregnant A/J mice will induce proinflammatory responses that, as in B6 mice, will result in poor pregnancy outcome. Comparative evaluation of malarial infection and pregnancy outcome in these strains showed that *P. chabaudi* AS infection leads to mid-gestational embryo loss albeit with quantitatively different systemic cytokine responses.

MATERIALS AND METHODS

Parasites and Hosts

Plasmodium chabaudi AS (originally obtained from Dr Mary Stevenson, McGill University, Canada) was routinely passaged from frozen stocks in female A/J mice as previously described (20).

C57BL/6J (B6) and A/J mice were originally purchased from The Jackson Laboratory and were used to generate breeding stock and experimental animals in the University of Georgia Coverdell Vivarium. Infection in experimental female mice, aged 8 to 12 weeks, was initiated on day 0 of pregnancy (with evidence of a vaginal plug), referred to as experiment

day 0, and monitored as previously described (20). All infected pregnant mice were intravenously infected with 1,000 *P. chabaudi* AS-infected murine red blood cells at experiment day 0 (the day on which a vaginal plug, evidence of mating, was observed) per 20g of body weight (20). Non-pregnant (infected non-pregnant) mice were similarly infected, while uninfected pregnant control mice received a sham injection of uninfected red blood cells on experiment day 0 (20). All procedures described herein were performed in accordance with the approval of the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA.

Mice were serially sacrificed at experiment days 9, 10, and 11, corresponding to one day before *P. chabaudi* AS-induced mid-gestational abortion and ascending and peak density parasitemia in B6 mice (20). At sacrifice, anti-coagulated peripheral blood was collected by cardiac puncture, processed to yield platelet-free plasma, and preserved for cytokine and chemokine measurements by enzyme-linked immunosorbent assay (ELISA). Mice were then dissected for evaluation of conceptus status and isolation of tissues. Resorptions or non-viable embryos were identified by their necrotic and smaller size compared to viable normal embryos. Hemorrhagic embryos were identified by the presence of a dark spot of clotted blood within and/or surrounding the conceptus. The number of necrotic and hemorrhagic embryos was quantified and mice undergoing active abortion, defined as evidence of bloody, mucoid vaginal discharge and/or evidence of embryos in the open cervix or vaginal canal (20), were recorded.

Following gross pathological examination, the uterus was separated by cutting directly below the oviduct and above the cervix and the mesometrium was removed. Part of the uterus was preserved in 4% paraformaldehyde, embedded in paraffin, and 5 μ m sections Giemsa-stained for assessment of the density placental parasitemia as previously described (20).

Cytokine and chemokine detection

Plasma levels of TNF, IFN- γ , IL-6, IL-1 β , soluble TNF receptor II (sTNFR_{II}) and IL-10 were determined with two-site sandwich assays using commercially available ELISA kits (BD Biosciences, Franklin Lake, NJ or R&D Systems, Minneapolis, MN). Limits of detection for the assays were 8 pg/mL for TNF and IL-10; 15 pg/mL for IFN- γ , IL-6 and IL-1 β ; and 31 pg/mL for sTNFR_{II}.

Flow cytometric analysis

Splenocytes were isolated from infected pregnant, infected non-pregnant, and uninfected pregnant mice by passing the spleen through a 70 μ m cell strainer (BD Falcon; Fisher Scientific, Pittsburgh, PA). Staining of each sample with Trypan blue demonstrated that cell viability was routinely > 90%. Red blood cells were lysed using Tris-buffered ammonium chloride (0.14 M NH₄Cl and 0.017 M Tris (pH 7.2)). The cells were washed and Fc receptors blocked with CD16/CD32 purchased from eBiosciences (San Diego, CA) as per manufacturer specifications. Cells were stained with monoclonal antibodies purchased from eBiosciences: fluorescein isothiocyanate (FITC)-conjugated anti-CD4, FITC-conjugated anti-F4/80, phycoerythrin (PE)-conjugated anti-CD3 ϵ , PE-conjugated anti-CD115, Percp-Cy5.5-conjugated anti-B220, allophycocyanin (APC)-conjugated anti-CD8, APC-conjugated anti-NK1.1, APC-conjugated anti-CD11b, and PE-Cy5.5 anti-GR1/Ly6G using standard methodologies.

All staining reagents were first titrated to determine optimal concentrations. Following immunostaining at 4°C, cells were washed three times with staining buffer (1% BSA/1X PBS) and data were acquired using a BD FACSCalibur flow cytometer (Becton Dickinson,

San Jose, CA), with a minimum of 10,000 cells being acquired per sample. The resultant data were analyzed with FlowJo 9.0 software (TreeStar, Inc., Ashland, OR). The data shown in all figures are either gated on lymphocytes or ungated to include all cell populations as indicated. Cell numbers were calculated using total splenocyte count multiplied by percent of cells defined by staining strategy (as indicated in figure legends); for lymphocytes, total splenic count was first multiplied by number of cells falling within the lymphocyte gate defined by forward and side-scatter cell characteristics.

Anti-TNF treatment

To assess the role of TNF in *P. chabaudi* AS-infected A/J mice, TNF was ablated by anti-TNF treatment in infected pregnant and uninfected pregnant A/J mice as previously described in B6 mice (21). Mice were i.p. injected with 100 µg of anti-TNF monoclonal antibody (clone MP6-XT22; Biolegend) or with rat IgG (Biolegend) as a control for TNF ablation on experiment days 6, 8, 9, 10, and 11. Mice were killed on experiment day 12 or immediately after evidence of abortion.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software package (version 5.01). Clinical data are expressed as mean $\pm$ SEM and were analyzed using student's unpaired t test (course of parasitemia) or ANOVA with Bonferroni's post-hoc test (for hematocrit and weight change). Immunological parameters and comparison of venous and placental parasitemia were analyzed by Kruskal-Wallis test, with Dunn's post-hoc multiple comparison test for multiple group pairwise comparisons. Unless otherwise noted, such pairwise comparisons were made between infected pregnant and uninfected pregnant, and between infected pregnant and infected non-pregnant mice within strains; and between infected pregnant mice and between infected non-pregnant mice across strains. Pregnancy outcome data were analyzed by Fisher's exact test or χ^2 as appropriate. Differences with $P < 0.05$ were considered significant.

RESULTS

Course of *P. chabaudi* AS infection in pregnant A/J and B6 mice

In agreement with previous studies of virgin mice (15, 24), pregnant A/J mice were susceptible to a lethal infection with *P. chabaudi* AS, whereas B6 mice were resistant (20). Among A/J mice, 100% of infected pregnant mice died by experiment day 12 ($n = 7$; Fig. 1A) whereas B6 mice were resistant, with only 1 out of 6 mice succumbing by experiment day 12 (Fig. 1A). Because the interest of the study was to evaluate mid-gestational pregnancy outcome in both strains, serial sacrifices were subsequently performed up to experiment day 11.

In A/J mice, a maximum peripheral parasite density of $39 \pm 2\%$ (mean $\pm$ SEM; $n = 21$) was observed in the infected pregnant group at experiment day 11, while the peak parasitemia for infected pregnant B6 mice occurred on experiment day 10 at $25 \pm 3\%$ ($n = 16$; Fig. 1B), a level significantly lower than in A/J mice. Consistent with previous reports (25, 26), parasitemia was also significantly higher in infected non-pregnant A/J mice on experiment day 9 through 11 relative to infected non-pregnant B6 mice (data not shown). Moreover, peripheral blood parasite density was significantly higher in pregnant A/J mice relative to non-pregnant mice at experiment day 6 ($0.5 \pm 0.2\%$ ($n = 64$) versus $0.1 \pm 0.0\%$ ($n = 104$), respectively; $P = 0.03$) and at peak parasitemia ($39.1 \pm 1.9\%$ ($n = 21$) versus $33.4 \pm 1.8\%$ ($n = 27$), respectively; $P = 0.04$; Fig. S1), suggesting that, as in B6 mice (20), pregnancy increases the susceptibility of A/J mice to malaria.

Clinical parameters and pregnancy outcomes in A/J and B6 mice

While anemia was not observed in uninfected pregnant A/J and B6 mice, hematocrit was substantially reduced over time in infected pregnant (Fig. 1C) and infected non-pregnant (Fig. S1 and data not shown; (20) mice of both strains. On experiment day 11, hematocrit in infected pregnant A/J mice was significantly lower than in infected pregnant B6 mice (Fig. 1C). As expected in normal pregnancy, uninfected pregnant A/J and B6 mice gained weight over the course of the experiment (Fig. 1D). In contrast, infected pregnant mice of both strains did not experience significant weight gain, and starting at experiment day 9, body weights fell steadily with reductions to below starting body weight at experiment day 11 (Fig. 1D) (20). From experiment days 9 through 11, mean body weight was significantly lower in infected pregnant relative to uninfected pregnant mice for both strains ($P < 0.05$).

Both A/J and B6 mice failed to maintain viable pregnancies, with evidence of conceptus loss beginning at experiment day 9 (Tables 1 and 2). Among those mice allowed to proceed to experiment day 12, all conceptuses were either hemorrhagic, resorbed, or undergoing active expulsion (data not shown). Whereas infected A/J mice had high rates of resorption as early as experiment day 9 (relative to uninfected mice), resorption in B6 mice was elevated by infection beginning one day later, on experiment day 10 (Table 1). The resorption rate in infected mice at experiment day 9 was significantly higher in A/J relative to B6 mice, but was similar between strains at experiment days 10 and 11 (Table 1). In contrast, hemorrhagic conceptuses were observed in infected B6 mice starting at experiment day 9, and hemorrhage rates were significantly higher in these mice at both experiment days 9 and 10 relative to their uninfected counterparts (Table 1). Active abortion was observed beginning at experiment day 9 in A/J mice and experiment day 10 in B6 mice, remaining elevated at experiment day 11 in both strains (Table 2). Overall, abortion rates did not differ as a function of strain (Table 2).

Accumulation of iRBCs in the maternal placental blood space

Placental malaria in humans is characterized by sequestration of infected red blood cells in the intervillous space (27), a phenomenon that may also occur in *P. chabaudi* AS-infected B6 mice (20). To verify that placental *P. chabaudi* AS iRBC accumulation occurs independently of mouse strain, parasite density was assessed in maternal blood sinusoids using Giemsa-stained placental histology sections (20). Placental parasitemia was significantly higher than peripheral parasitemia in both A/J and B6 mice at experiment day 10 (Fig. 2). Peripheral parasitemia was significantly elevated in A/J relative to B6 mice on experiment day 10, a pattern evident in both peripheral and placental blood on experiment day 11 (Fig. 2).

Systemic cytokine responses in A/J and B6 mice

Ablation of TNF with neutralizing antibodies significantly improves mid-gestational pregnancy success in *P. chabaudi* AS-infected B6 mice (21), illustrating a central role for this inflammatory factor in malaria-associated compromise of pregnancy. As a first step to assess a possible role for inflammatory cytokines in pregnancy loss in A/J mice, systemic levels of cytokines were measured by ELISA at experiment days 9 (data not shown), 10 and 11 in both strains. On experiment day 11, TNF and IL-1 β levels were statistically significantly higher in infected pregnant A/J compared to infected pregnant B6 mice (Fig. 3D, F). TNF, IFN- γ , IL-1 β , and IL-6 levels were higher in infected pregnant A/J mice relative to their uninfected pregnant counterparts on experiments days 9 (data not shown), 10 and 11 (Fig. 3). In contrast, only IFN- γ and IL-6 were consistently elevated in infected pregnant B6 mice compared to uninfected mice (Fig. 3A, B, G, H and data not shown). With the exception of TNF at experiment day 10 (Fig. 3C), at none of these time points were cytokine levels statistically significantly different between infected non-pregnant B6 and A/J

mice. Moreover, inflammatory cytokine levels were comparable between infected pregnant and infected non-pregnant mice of both strains.

IL-10 levels in infected pregnant B6 mice were statistically significantly reduced relative to infected pregnant A/J mice on experiment day 9 (median (IQR): 36 (0 – 46) pg/mL for B6 versus 550 (431– 735) pg/mL for A/J; $P = 0.001$), but this pattern was reversed on experiment day 10 (Fig. 4A). In both strains, IL-10 levels were enhanced at experiment day 11 in infected pregnant relative to uninfected pregnant mice. Levels of sTNFRII did not differ between infected pregnant A/J and B6 mice at any of the tested time points, although levels were consistently statistically significantly higher in the infected mice relative to their within strain uninfected counterparts (Fig. 4C, D and data not shown). At none of the time points were differences in IL-10 or sTNFRII observed between infected pregnant and infected non-pregnant mice of either strain nor were across-strain differences between infected non-pregnant mice found (Fig. 4).

Splenic leukocyte distribution in A/J and B6 mice

To further evaluate immune changes associated with *P. chabaudi* AS infection and pregnancy loss in A/J and B6 mice, phenotypes and levels of splenic leukocyte subsets were analyzed flow cytometrically at experiment days 9 and 10, time points at which mice of both strains retain a proportion of viable conceptuses. No statistically significant differences in B, NK or T cell counts, including T cell subsets, were observed between infected pregnant A/J and B6 mice (Fig. 5). However, malarial infection clearly stimulated expansion of all of these cell types in pregnant A/J mice, in whom splenocyte numbers for all subsets (except T cells at experiment day 9) were statistically significantly higher relative to their uninfected pregnant counterparts (Fig. 5). Similar differences in B6 mice were noted only for T, CD8+ T, B and NK cells on experiment day 9, but not 10 (Fig. 5). The total number of lymphocytes and lymphocyte subsets in general did not differ between infected pregnant and infected non-pregnant mice within each strain; only CD4+ T cells on experiment day 9 were significantly expanded in infected pregnant relative to infected non-pregnant A/J mice (Fig. 5C).

Similar to lymphocyte subsets, numbers of neutrophils, monocytes and monocytes with an inflammatory phenotype (CD11b+/CD115+/Gr1^{high}) were similar in infected pregnant B6 and A/J mouse spleens on experiment days 9 and 10 (Fig. 6). Neutrophil levels were enhanced in infected B6 mice at experiment day 9 relative to uninfected pregnant B6 mice (Fig. 6A), a difference that did not reach statistical significance on experiment day 10 (Fig. 6B; Kruskal-Wallis, $P = 0.0024$, Dunn's pairwise comparisons, all $P > 0.05$). Monocytes levels were increased in infected pregnant B6 mice compared to their uninfected counterparts on experiment days 9 and 10, and on the former day were also higher than in infected non-pregnant mice (Fig. 6C, D). Infected pregnant mice of both strains also showed a significant enhancement of splenic monocytes relative to their uninfected pregnant counterparts, but only on experiment day 10 (Fig. 6D). Inflammatory monocytes trended upward in some infected groups on experiment day 9 (Fig. 6E; Kruskal-Wallis, $P = 0.0062$, Dunn's pairwise comparisons, all $P > 0.05$), and at experiment day 10 infected pregnant A/J mouse spleens had higher numbers of these cells than uninfected pregnant A/J mice (Fig. 6F).

TNF ablation does not improve outcome in A/J mice

Although TNF antibody ablation provides dramatic preservation of B6 conceptuses up to experiment day 12 (21), the same treatment protocol was not successful in improving pregnancy outcome in A/J mice. In this case, all embryos were expelled by experiment day 11 (Fig. 7A). Course of parasitemia was not altered by TNF ablation (Fig. 7B), and neither

hematocrit levels nor weight change differed significantly at any time point between control and antibody-ablated infected mice (Fig. 7C, D).

DISCUSSION

It has become clear that immune responses elicited by malaria during pregnancy can have significant adverse effects on the placenta and fetus (28). However, detailed examination of underlying mechanisms in humans is difficult due to a myriad of practical and ethical barriers, making mouse models an important tool for advancing understanding of gestational malaria pathogenesis. An extension of previous work that revealed a critical role for maternal immune responses in *P. chabaudi* AS pathogenesis in the B6 mouse (19–21), the present work addressed the hypothesis that malaria during pregnancy in A/J mice will induce proinflammatory responses that, as in B6 mice, will result in poor pregnancy outcome. The results show that while immune responses to this infection during pregnancy vary as a function of genetic background, pregnancy is compromised in both mouse strains.

B6 and A/J mice have been used extensively to explore immunoprotective and immunopathogenic responses to *P. chabaudi* AS infection (12, 29, 30), and thus were an attractive choice to assess strain-dependent immune responses to this infection during pregnancy. Like virgin females and males (15, 31–33), pregnant A/J mice are more susceptible to *P. chabaudi* AS infection than their B6 counterparts. Whereas B6 mice ultimately control *P. chabaudi* AS infection (20), infected pregnant A/J mice are highly susceptible and succumb to infection by experiment day 12. Nonetheless, consistent with the well-reported epidemiology of malaria during human pregnancy (1), both infected pregnant B6 (20) and A/J mice display higher density peak peripheral parasitemia compared to their non-pregnant counterparts. In addition, *P. chabaudi* AS accumulates in the maternal blood sinusoids of both B6 (20) and A/J mice. Although definitive demonstration of specific placental sequestration and identification of the host receptor and parasite ligand remain to be achieved, it is noteworthy that a family of adherence receptors, *cir*, has been identified in *P. chabaudi* AS (34). Similarly, *P. berghei*, which has a homologous gene family, *bir* (35), has been shown to sequester via specific interaction with placental chondroitin sulfate A (36), the best described receptor for *P. falciparum* in the human placenta (27).

Severe anemia in pregnancy is an important contributor to maternal morbidity and mortality (37, 38), and in malaria endemic settings accounts for 7% to 18% of malaria-associated LBW (39). Significant anemia is observed in both B6 (20, 21) and A/J mice, but ultimately is more severe in the latter, likely contributing to the lethality of the infection (40). Although anemia may contribute to compromise of pregnancy in A/J mice, it is noteworthy that infected pregnant IFN- $\gamma^{-/-}$ B6 mice develop severe anemia, but abort later than their IFN- $\gamma^{+/+}$ counterparts, suggesting that anemia may play a minor role in malaria-induced murine pregnancy loss (21).

High rates of abortion have been associated with malaria infection in nonimmune pregnant women during the first or second trimester (41). Pregnant malaria-naïve rhesus monkeys infected with *P. coatneyi* have increased rates of abortion and intrauterine growth retardation associated with significant malaria-associated placental pathology (42). Mid-gestational and pregnancy-associated recrudescence *P. berghei* infection in BALB/c mice results in reduced gestation time (36), reduced litter size (43), and reduced birth weight (36, 43). Consistent with these observations, both B6 and A/J mice experience poor pregnancy outcomes as a result of *P. chabaudi* AS infection. As evidenced by a higher rate of embryo resorption at experiment day 9, A/J mice experience accelerated pregnancy loss relative to B6 mice (20). Interestingly, the presence of hemorrhaging in embryos is more frequent and occurs earlier

in B6 mice, suggesting that the precipitating mechanisms that drive embryo loss in these two mouse strains are complex and multifactorial.

Increased systemic inflammatory cytokines like TNF and IFN- γ have been observed in malaria during pregnancy (6). Levels of TNF in particular have been associated with maternal anemia and LBW (6, 9) and this cytokine is sufficient to drive mid-gestational pregnancy loss in *P. chabaudi* AS-infected B6 mice (21). In this study, systemic levels of TNF and IL-1 β were significantly elevated only in infected pregnant A/J mice, as early as experiment day 9, at which time resorption rates are increased. Thus, while pregnancy-protective anti-inflammatory responses may prevail early during infection in this strain (15), including elevated IL-10 production at experiment day 9, the tendency for this strain to subsequently produce inflammatory cytokines (18) is intact in pregnant mice. Interestingly however, whereas antibody ablation of TNF successfully restored mid-gestational pregnancy in B6 mice (21), the same treatment was unsuccessful in A/J mice. While it is plausible that TNF is only a marker of disease severity rather than a causal mediator of poor pregnancy outcome in A/J mice, the positive outcome in B6 mice following TNF neutralization suggests this is not the case. Rather, it is more likely that the treatment failed to effectively neutralize the relatively higher amount of TNF in A/J mice. Future studies will be required to assess the extent to which TNF drives pregnancy loss in A/J mice and the pathogenic pathways activated by this cytokine in both strains. Current evidence implicates the inflammation-coagulation cycle as a central mediator for malaria-induced pregnancy compromise in B6 mice (21) (51). However, it is known that inflammatory cytokines like TNF are directly embryotoxic (44), inducing trophoblast apoptosis via TNF receptors (45), especially if the cytokine is released by monocytes in direct contact with trophoblast (46). A potential role for apoptosis in the pathogenesis of placental malaria is currently being assessed in both mouse strains.

In the context of high levels of high pro-inflammatory cytokines, IL-10 plays a regulatory role (7, 47), blocking malaria-associated immunopathology and *P. chabaudi* virulence (48). In this study, as pro-inflammatory cytokine levels increased in infected pregnant A/J mice, regulatory IL-10 decreased, at experiment day 10 reaching levels significantly lower than in infected pregnant B6 mice. While elevated IL-10 may serve to partially dampen inflammatory damage in *P. chabaudi* AS-infected pregnant mice (20), it is inadequate to prevent pregnancy loss in both A/J and B6 mice. In humans, this cytokine is significantly higher in infected primigravidae compared to their uninfected counterparts, and has been proposed to be a marker for inflammatory placental malaria (49). Elevated levels of sTNFR_{II}, which can serve to bind and sequester TNF, are likewise apparently inadequate to control TNF-mediated pathogenesis; however, the specific role played by this solubilized receptor in infected mice and women with placental malaria (49, 50) remains to be established.

The different dynamics of cytokine expression in infected A/J and B6 mice prompted an examination of the potential cell types that may contribute to these differences at the splenic level. In general, lymphocyte and myeloid cell levels were influenced only by infection status, with strain and pregnancy having no significant impact, although only infected pregnant B6 mice show early elevation of neutrophils and monocytes (at experiment day 9). Interestingly, however, one day later, infected pregnant A/J mice showed elevated monocyte and inflammatory monocyte levels relative to uninfected pregnant mice. While these observations clearly demonstrate that pregnancy does not alter infection-induced splenic cellular expansion in either strains, they do not shed any light on the differential dynamics of embryo loss in A/J and B6 mice. However, expansion of inflammatory monocytes in the former may be an important source of the observed high levels of circulating TNF; the liver may also be critical in this regard (18). Nonetheless, since the splenic expansion of

inflammatory monocytes in A/J mice is modest and monocytes in general expand in both strains, it is tempting to speculate that expansion of inflammatory cells in other tissues is a more important determinant for pregnancy outcome. In particular, it will be important in future studies to examine whether differential cell accumulation occurs at the level of the conceptus in A/J and B6 mice. Such studies are in fact underway. Ultimately, examination of the role of different cell types in determining host response and pregnancy outcome in these mouse strains will require use of adoptive transfer experiments, cell ablation techniques, and appropriate null mutant mice.

In summary, *P. chabaudi* AS infection in B6 and A/J mice results in pregnancy loss in association with systemic pro-inflammatory cytokine responses and infection-induced splenic cellular responses. Although the dynamics of anti-inflammatory responses differ between the two strains, they appear in both cases to be inadequate to provide protection for the conceptus. The extent to which these responses overall shape events occurring at the uterine level and lead to pregnancy loss remains to be explored. Because these two genetically disparate mouse strains ultimately exhibit enhanced inflammatory responses in association with pregnancy loss (21), patterns that have been identified in genetically complex human populations, continued study promises to reveal common and critical mechanisms that contribute universally to malaria-induced compromise of pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Comparative course of *P. chabaudi* AS infection in pregnant A/J and B6 mice
Survival (A), percent parasitemia in infected pregnant (IP) mice (B); percent hematocrit in uninfected pregnant (UP) and IP mice (C); and weight change in UP and IP mice (D) from both strains are depicted. Sample sizes start with $n = 70$ for IP A/J, $n = 23$ for IP B6, $n = 56$ for UP A/J, and $n = 33$ for UP B6. Data represent mean $\pm$ SEM. Parasitemia data were analyzed by unpaired Student's *t* test and by ANOVA with Bonferroni's post-hoc test for hematocrit and weight change. * $P < 0.05$, ** $P < 0.01$.

Figure 2. Accumulation of *P. chabaudi* AS in A/J and B6 placentae

Placental (P) and peripheral (venous; V) parasitemia in A/J and B6 mice. Giemsa-stained 5 μ m sections of placenta were used to determine maternal placental parasitemia; corresponding Giemsa-stained thin smears of peripheral blood were used to calculate venous parasitemia. Data for at least for 5 mice per group at each experiment day (ED) are shown and are shown as boxplots with whiskers representing the 10th and 90th percentiles. Data were analyzed by Kruskal-Wallis test, with Dunn's post-hoc multiple comparisons test. * $P < 0.05$, ** $P < 0.01$.

Figure 3. Proinflammatory cytokine levels in *P. chabaudi* AS-infected and uninfected pregnant (IP and UP) and infected non-pregnant (INP) A/J and B6 mice

Measurement by ELISA of peripheral plasma levels of TNF, IFN- γ , IL-1 β and IL-6 at experiment day (ED) 10 and 11 are shown for individual A/J and B6 as indicated in the legend; median concentrations are indicated by a horizontal bar. Data were analyzed as in Figure 2. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 4. Anti-inflammatory factor levels in *P. chabaudi* AS-infected and uninfected pregnant (IP and UP) and infected non-pregnant (INP) A/J and B6 mice

Measurements by ELISA of peripheral plasma levels of IL-10 and sTNFR II at experiment day (ED) 10 and 11 are shown for individual A/J and B6 mice as indicated in the legend; median concentrations are indicated by a horizontal bar. Statistical comparisons were performed as in Figure 2. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 5. Comparative flow cytometry analysis of splenic T cell subsets and B cells in *P. chabaudi* AS-infected and uninfected pregnant (IP and UP) and infected non-pregnant (INP) A/J and B6 mice

The numbers of splenocytes at experiment day (ED) 9 and 10 positive for CD3 ϵ (T cells; A, B), CD3 ϵ /CD4 (CD4+ T cells; C, D), CD3 ϵ /CD8 (CD8+ T cells; E, F), B220 (B cells; G, H), and NK1.1 (NK cells; I, J) are shown for A/J and B6 mice as indicated in the legend; horizontal bar indicates median value. Statistical comparisons were performed as in Figure 2. * P < 0.05, ** P < 0.01, and *** P < 0.001.

Figure 6. Comparative flow cytometry analysis of splenic neutrophils and monocytes in *P. chabaudi* AS-infected and uninfected pregnant (IP and UP) and infected non-pregnant (INP) A/J and B6 mice

The numbers of splenocytes at experiment day (ED) 9 and 10 with a CD11b⁺/CD115⁻/Gr-1⁺ phenotype (neutrophils; A, B), CD11b⁺/CD115⁺/Gr-1⁺ phenotype (monocyte/macrophages; C, D), CD11b⁺/CD115⁺/Gr-1^{bright} phenotype (inflammatory monocytes; E, F) are shown for A/J and B6 as indicated in the legend; horizontal bar indicates median value. Statistical comparisons were performed as in Figure 2. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 7. Pregnancy outcome, density peripheral parasitemia, hematocrit, and body weight in anti-TNF antibody treated A/J mice

The incidence of abortion (A) and course of parasitemia (B) are depicted for infected pregnant (IP) mice treated with neutralizing anti-TNF antibody or control IgG. Percent hematocrit (C), and body weight change (D) in these animals and in likewise treated uninfected pregnant (UP) mice are also shown. Statistical comparisons were performed as in Figure 1; in (C) and (D), the IP α -TNF group was compared against all other groups. Results of ANOVA are shown in the panels: * $P < 0.05$, ** $P < 0.0014$, and *** $P < 0.0001$. By Bonferroni, in (C), at experiment day 8: IP α -TNF versus UP α -TNF, $P < 0.05$; versus UP

IgG, $P<0.01$; at experiment days 9 and 10: IP α -TNF versus both UP groups, $P<0.001$. In (D), at experiment day 9, IP α -TNF versus both UP groups, $P<0.05$.

Comparative pregnancy outcome in *P. chabaudi* AS-infected pregnant (IP) and uninfected pregnant (UP) A/J and B6 mice.

Table 1

Strain	Resorptions ^a					Hemorrhagic conceptuses ^b				
	ED	IP (%)	n	UP (%)	n	p ^c	p ^d	IP (%)	UP (%)	p ^e
A/J	9	49/130 (38)	19	6/91 (6)	12	<0.0001	0.02	14/130 (11)	7/91 (8)	NS ^e
	10	69/123 (56)	23	16/181 (9)	21	<0.0001	NS	29/123 (23)	14/181 (8)	0.0002
	11	49/80 (61)	16	17/96 (18)	14	<0.0001	NS	30/80 (37)	12/96 (12)	0.0002
B6	9	4/46 (9)	7	0/48 (0)	5	NS		19/80 (24)	0/48 (0)	0.0007
	10	33/58 (57)	10	5/80 (6)	10	<0.0001		25/58 (43)	5/80 (6)	<0.0001
	11	12/24 (50)	5	2/85 (2)	10	<0.0001		10/24 (42)	6/85 (7)	<0.0001

Mice (n = number of pregnant mice) were sacrificed and status of conceptuses in experiment day (ED) 9, 10, and 11 was assessed by scoring the number of ^aresorbing (necrotic appearance and smaller size compared to normal embryos), and

^b hemorrhagic conceptuses among the total.

^c P indicates level of significance for comparison between infected pregnant (IP) and uninfected pregnant (UP) mice within each strain;

^d P indicates level of significance for comparison between infected pregnant (IP) A/J and infected pregnant (IP) B6, by Fisher's exact test or χ^2 test as appropriate.

^e NS, not statistically significant.

Table 2

Comparative proportions of A/J and B6 mice actively aborting during *P. chabaudi* AS infection.

Strain	ED	IP	n/N ^a (%)	UP	n/N (%)	P ^b	P ^c
A/J	9	7/20 (35)	0/12 (0)	0.0605	NS ^d		
	10	18/27 (64)	0/22 (0)	<0.0001	NS		
	11	17/21 (76)	0/14 (0)	<0.0001	NS		
B6	9	2/10 (20)	0/6 (0)	NS			
	10	7/11 (64)	0/10 (0)	0.0086			
	11	4/6 (67)	0/10 (0)	0.0082			

^a n = number of mice undergoing active abortion (mice with bloody, mucoid vaginal discharge were considered actively aborting and were sacrificed immediately along with their corresponding controls); N = total number of mice.

^b P indicates level of significance for comparison between infected pregnant (IP) and uninfected pregnant (UP) mice within each strain;

^c P indicates level of significance for comparison between infected pregnant (IP) A/J and B6, by Fisher's exact test or χ^2 test as appropriate.

^d NS, Not significant.