IMMUNOPATHOGENESIS OF MALARIA DURING MURINE PREGNANCY

by

JAYAKUMAR POOVASSERY

(Under the Direction of Julie M Moore)

Malaria during pregnancy is detrimental to the health of both mother and her unborn child. Although malaria-induced fetal loss is one of the most severe consequences of malarial infection during pregnancy, our understanding of the molecular and immunologic mechanisms involved in fetal loss is limited. This has been due in part to the absence of an adequate system to carry out mechanistic studies during pregnancy. To overcome this limitation a model system was developed in this study by infecting pregnant C57BL/6 mice with *Plasmodium chabaudi* AS. In this model, *P. chabaudi* AS -infected pregnant B6 mice experienced parasitemia, anemia and weight change comparable to infected nonpregnant mice. Although the infection was not lethal to the mother, the fetal outcome was poor. Aborting mice experienced high placental parasitemia compared to peripheral blood. Additionally, fetal loss was associated with elevated levels of proinflammatory cytokines IFN-γ, TNF-α and IL-1β in the plasma. Since both IFN-γ and TNF-α are known embryotoxic agents, experiments using gene knockout mice and antibody ablation were performed to identify the role of these cytokines in malaria-induced fetal loss. Despite experiencing a more severe course of infection, IFN-$\gamma^{-/-}$ mice had improved pregnancy success. Since IFN-γ knockout mice also experienced fetal loss and had TNF-α, a known embryo toxic agent, in their plasma at the time of abortion, the effect of in vivo neutralization of TNF-α on pregnancy success in *P. chabaudi AS*-infected wild-type pregnant mice was tested. Whereas IgG

treated infected mice aborted their embryos by gestation day 11, infected, pregnant mice treated with anti-TNF-α antibody retained their pregnancies and had viable embryos in their uteri on day 12 post infection. It is possible that the proinflammatory cytokines are mediating their detrimental effects on the fetus through initiating a clotting cascade in the uterus. Placental sections from aborting mice had wide spread hemorrhage and fibrin thrombi formation in the maternal blood sinusoids. Furthermore, the levels of procoagulants tissue factor and plasminogen activator inhibitor-1 were upregulated in the uteri from aborting mice. In addition to maternal cells, fetal cells may also be contributing to the local placental pathology. Fetal trophoblast cells exhibited phagocytosis of iRBCs in vivo and secreted TNF-α in the culture medium following phagocytosis of iRBCs in vitro.

Taken together the results from this study suggest that TNF-α is a critical factor in malaria-induced fetal loss. TNF-α produced by both maternal and fetal cells at the placental level may be contributing to tissue injury through initiating a clotting cascade in the placenta, leading to loss of blood supply to fetus and ultimately fetal death.

INDEX WORDS: Malaria, Pregnancy, Abortion, Trophoblast, Phagocytosis.

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DEDICATION

To

My family, my wife Sunitha and my son Arnav

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CHAPTER 1

INTRODUCTION

Malaria continues to be a major public health problem in the developing world causing an estimated 300-500 million clinical cases each year and 1-2 million deaths. The majority of severe malaria cases and deaths are caused by *Plasmodium falciparum*, which is endemic in most Sub-Saharan Africa and in many other regions of the tropical world. In malaria endemic regions, the related morbidity and mortality are primarily in children and pregnant women.

Malaria during pregnancy is detrimental to the health of both mother and fetus. The clinical features of malaria in pregnancy vary depending on the endemicity or intensity of transmission. Sequestration of infected red blood cells (iRBCs) in the placental intervillous space is a characteristic feature of malarial infection during pregnancy in high endemic areas. This has been shown to be mediated through binding of iRBCs to specific receptors such as chondroitin sulphate A (CSA) and hyaluronic acid (HA) expressed on the syncytiotrophoblast cells of the placenta. *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) is the most characterized parasite ligand involved in the parasite binding. The sequestration of iRBCs along with the accompanying maternal inflammatory response in the placenta is thought to be the major reasons for the observed pathologies in malaria infected pregnant women. The clinical features of malarial infection during pregnancy in high transmission areas are severe maternal anemia and low birth weight babies who are at increased risk to die early in life.

In contrast, pregnant women living in low endemic areas experience more severe complications such severe maternal anemia, maternal death from cerebral malaria and poor fetal outcomes such as abortions, stillbirth and low birth weight babies. Although extensive studies have been done to identify the biologic basis for the increased

susceptibility of pregnant women living in high endemic areas to malarial infection, only limited studies have been done to explore the host / parasite interactions in settings of low endemicity and the effect of maternal anti-malarial immune responses on pregnancy outcome especially during the early stages of pregnancy. Experimental study of malarial infection during pregnancy is particularly problematic, as ethical and logistical constraints limit the longitudinal sampling of pregnant women and the placenta is inaccessible until delivery. Thus, without an appropriate model system, it is not possible to dissect out the protective immune responses from those that are potentially harmful to the mother and her unborn child.

Rodent models have been used extensively to study the immunity and pathogenesis of malarial infection. Four species of malarial parasites, *Plasmodium yoelii*, *P. berghei*, *P. chabaudi* and *P. vinckei* isolated from wild thicket rats in Africa have been used extensively to study the protective and pathogenic immune responses to blood stage malaria infection. Depending on the mouse strain and the species or strain of Plasmodium the infection can be either lethal or non-lethal. While *P. berghei* and *P. yoelii 17XL* infections are uniformly lethal, *P. chabaudi* and *P. vinckei petteri* infections are non-lethal in most mouse strains. However, studies employing mouse models to investigate the interactions between malarial parasites and pregnancy are limited. Earlier studies employing *P. berghei* infection in mice reported maternal death, fetal loss and reduced litter size in infected mice. This model, however, is not suitable to study the development of early maternal anti-malarial immune responses or the impact of malarial infection on early pregnancy, because the infections were initiated on day 7 of pregnancy and were lethal to the mother. Further research to characterize the immunological and molecular

basis of fetal loss in murine models for malarial infection during pregnancy has not been done.

P. chabaudi AS infection in C57BL/6 (B6) mice represents a very useful model for the study of immune responses to malaria as it shares many characteristics with the most virulent human parasite, *P. falciparum*. *P. chabaudi* AS-infected erythrocytes express variant antigens, sequestrate in the heart, lung, liver and spleen and can bind to CD36. *P. chabaudi* AS infection in B6 mice has been used extensively to dissect the immune response to blood stage malaria. Both innate and adaptive immune responses are known to be critical in clearing primary *P. chabaudi* AS infection in non pregnant mice. The suppression of parasitemia during the acute stage of infection is thought to require NK cells, CD4+ T cells and the production of proinflammatory cytokines IL-12, IFN-γ and TNF-α during the early stages of infection. Parasite clearance during the chronic stage of infection is dependent on B cells and antibodies. However, the same proinflammatory cytokines associated with protection are also implicated in malarial pathology in both mice and humans. It has been demonstrated in rodent models that normal pregnancies require a bias against a T helper 1 (Th1) / proinflammatory environment. Excessive production or administration of recombinant IFN-γ or TNF-α has been shown to induce abortion in normal pregnant mice. Furthermore, pregnancy outcome has been shown to be impaired in the case of rodent models for parasitic diseases such as leishmaniasis and trypanosomiasis that require a Th1 cytokine-biased response for protection.

Elevated levels of cytokines and chemokines in the placental and peripheral blood are also reported in the case of malarial infection during pregnancy in humans. While these cytokines may have a role in protection against malarial infection, they may also be

involved in pathology depending on the location, amount and duration of production. Thus, elevated levels of IFN-γ and TNF-α in the placental intervillous blood have been shown to be associated with poor birth outcomes and elevated mRNA levels of chemokines MCP-1, MIP-α and IL-8 were associated with enhanced monocyte infiltration to placenta and with LBW and intrauterine growth retardation in areas of high transmission. To what extent these maternal anti-malarial immune responses contribute to fetal loss during malarial infection is not clear.

In addition to maternal immune cells, fetal cells may also be involved in mediating protective or pathogenic responses at the placental level. Indeed, fetal trophoblast cells are thought to be a component of innate immune system and play important roles in providing protection against invading pathogens at the maternofetal interface. Murine trophoblast cells are shown to be capable of phagocytosing microbes and can produce various cytokines and chemokines. Human syncytiotrophoblast cells are also shown to be capable of secreting both pro- and anti- inflammatory cytokines in response to bacterial infections. Furthermore, syncytiotrophobalst cells isolated from term placenta have been shown to respond to malarial parasites or parasite products through secretion of cytokines and chemokines in vitro. However, it is not clear how murine trophoblast cells will respond to malarial infection vitro or in vivo during early stages of pregnancy.

Significance: Aside from maternal death, fetal loss is one of the most severe consequences of malarial infection during pregnancy. Only limited studies have been done to identify the role of maternal antimalarial immune responses in fetal loss. A complete understanding of the development of the immune response and its maintenance

or regulation both systemically and at the placental level is critical in developing effective interventions or immunotherapies for preventing the detrimental effects of malaria on both mother and fetus. A detailed understanding of the development of antimalarial immune response especially during the early stages of pregnancy and its effect on pregnancy outcome is only possible through employing an appropriate model system. The model system developed in this study provides an excellent platform to investigate the host parasite interactions in the context of malarial infection during pregnancy. This study assessed the role of maternal and fetal cells in malaria-induced fetal loss by performing a series of immunological and molecular investigations employing both intact and gene null mutant mice. The results from this study demonstrate that the maternal antimalarial immune responses, especially production of proinflammatory cytokines IFN-γ and TNF-α, play major roles in parthenogenesis and fetal loss during malarial infection. Furthermore, fetal trophoblast cells were also shown to be capable of responding to malarial infection through phagocytosis of iRBCs and production of cytokines.

Hypothesis: The central hypothesis of this study was that the host response to malarial infection in pregnant C57BL/6 mice will lead to compromise of pregnancy. To test the proposed hypothesis the following four specific aims were attempted:

1. To characterize the course of *P. chabaudi* AS infection in pregnant C57BL/6 mice.
2. To assess the kinetics of development of immune response in malaria-infected pregnant mice by assessing the cytokine production in the plasma, spleen, and in the local placental environment.

3. To identify the soluble effectors leading to immunopthogenesis and fetal loss in malaria infected pregnant mice by employing gene null mutant mice and molecular detection methods.
4. To determine the ability of fetal trophoblast cells to respond to malarial parasites in vitro through cytokine and chemokine production.

CHAPTER 2

LITERATURE REVIEW

Despite a worldwide effort to curb the disease, malaria continues to be a major public health problem in developing countries with at least one-third of the world's population at risk of infection. The causative agents of malaria are single celled protozoan parasites of the genus *Plasmodium* that belong to the phylum Apicomplexa. There are more than 120 species of *Plasmodium*. However, only five *Plasmodium* species (*P. malariae, P. ovale, P. vivax, P. falciparum and P. knowlesi*) are naturally infective to humans. Although *P. ovale*, *P. malariae* and *P. vivax* can cause debilitating diseases, fatal complications are rare. *P. knowlesi*, primarily a pathogen of monkeys, has recently been implicated in a large human outbreak in Malaysia (1). Most cases of severe disease and most deaths are caused by the blood-stage life cycle of *P. falciparum*, which is endemic in sub-Saharan Africa and most of the tropics, causing an estimated 300-500 million clinical cases and 1-2 million deaths each year (2). In all endemic areas, women are at higher risk of malaria and are more susceptible to infection when pregnant than either before or after pregnancy, or when compared with adult males. Malaria during pregnancy is estimated to be responsible for a third of preventable low birth weight babies in sub-Saharan Africa and to cost the lives of 100,000–200,000 infants annually (3).

LIFE CYCLE: Malarial parasites undergo a series of morphological and biochemical changes throughout their complex life cycle that includes both mammalian hosts and an insect vector. The malaria parasite is transmitted to the human host from an infected female mosquito of the genus *Anopheles* upon taking a blood meal. The sporozoites inoculated under the skin rapidly migrate to the liver via the blood stream. Sporozoites migrate through a number of hepatocytes before invading a final hepatocyte in which

they form a vacuole. Sporozoite activation induced by migration through hepatocytes is thought to be an essential step for hepatocyte infection (4). The invading sporozoites undergo exoerythrocytic schizogony within hepatocytes to produce thousands of daughter merozoites, that when released invade red blood cells (RBCs) and begin the asexual blood stage life cycle of the parasite. Within the RBCs the merozoite develops through ring, trophozoite and schizont stages and undergoes binary division to produce daughter merozoites. Each erythrocytic schizont produces 10-30 merozoites, which are then released to the host blood stream upon rupture of the RBC membrane. In non-immune individuals this stage of the life cycle can be associated with considerable morbidity ranging from recurrent fever to severe and fatal complications such as anemia, renal failure, pulmonary edema and cerebral malaria (5). The released merozoites can either reinvade new RBCs to continue the asexual erythrocytic stage of life cycle or may develop into male and female gametocytes which when taken up by another feeding *Anopheles* mosquito continue the sexual stage of life cycle within the mosquito.

HOST PARASITE INTERACTIONS: The survival of malarial parasites within the vertebrate host is absolutely dependent on their ability to recognize and invade liver cells and erythrocytes. Multiple receptor / ligand interactions are involved in malarial parasite invasion of the host cell. The sporozite invasion of hepatocytes has been shown to involve sporozoite surface proteins such as circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP) (6). Additionally, the parasite employs various tactics, ranging from exporting parasite proteins to the RBC membrane, sequestration within various internal organs, switching between ligands used for host receptor binding for cytoadherence, inhibition of dendritic cell maturation and

macrophage function, to survive within the RBCs, a terminally differentiated cell lacking machineries for protein and lipid biosynthesis, and to evade immune clearance by the spleen.

RBC invasion: Malarial parasite invasion of the RBCs is a stepwise process involving interaction between specific parasite molecules and host erythrocyte receptors. There are three types of membrane-bound organelles, namely, rhoptries, micronemes and dense granules located at the anterior end of the merozoite (7). The contents of these organelles play important roles in the binding and invasion of merozoites into host red blood cells. A number of merozoite proteins including merozoite surface protein (MSP) - 1 (8), apical membrane anigen (AMA) - 1 (9), and erythrocyte-binding antigen (EBA) - 175 (10) are all thought to be involved in the merozoite invasion process, especially in the early phase. However, the relative importance of these proteins remains to be elucidated. MSP-1 interaction with spectrin on the cytoplasmic face of RBC membrane in a sialic acid-dependent manner is thought to be involved in the initial recognition of the RBCs, as antibodies directed against MSP-1 subunits have been shown to block the RBC invasion in vitro and in vivo (11-13). The invasion process involves an initial weak interaction between the surface components of the merozoite with the RBCs, followed by re-orientation of the merozoites to allow the apical end of the merozoite to interact with the membrane of the host cell (14). This is followed by the formation of a tight junction at the interface between the merozoite surface and the RBC membrane, release of micronemes-rhoptries substances, and formation of an invagination of the RBC membrane around the merozoite accompanied by the removal of the merozoite surface coat (14, 15). This invagination eventually envelops the invading merozoite in a

membrane-lined cavity, the parasitophorous vacuole (PV) (15, 16). After the merozoite is enclosed in the PV, and during the final stage of invasion, another set of apical organelles, the dense granules, move to the surface of the parasite and release their contents into the PV, causing an expansion of the area of its membrane (15-17), and presumably causing the change in shape of the parasite to that of the ring stage.

Although the course of events involved in erythrocyte invasion are thought to be similar in all *Plasmodium* species the molecular basis of invasion appears to be distinct amongst these parasites. While *P. vivax* exclusively invades reticulocytes, *P. falciparum* is able to invade reticulocytes as well as normocytes (18, 19). Similarly, in laboratory mice, *P. berghei* and *P. yoelii* 17X preferentially invade reticulocytes, whereas *P. yoelii* 17XL and NM are capable of invading both normocytes and reticulocytes (20, 21). *P. chabaudi* invades predominanatly normocytes, but will also invade reticulocytes when reticulocyte level is elevated in mice under stress conditions (22).

RBC remodeling and Cytoadherence: Once inside the RBCs, the parasites begin to alter and modify the RBC membrane. Four types of ultrastructural modifications described in infected RBCs (iRBCs) are knobs, tubovesicular complex, cytoplasmic clefts and caveola-vesicle complexes (15). These modifications are essential for the parasite survival and proliferation within the RBCs. Knobs are cup-shaped electron dense protrusions found on the RBCs infected with *P. falciparum*, *P. malariae* and *P. ovale*. Knobs form focal junctions with the endothelial cell membrane and play important roles in mediating cytoadherence of *P. falciparum* iRBCs to vascular endothelium (23). In *P. falciparum* iRBCs, several parasite proteins such as histidine rich protein I and II (HRP-I and HRP II), erythrocyte membrane protein 1, 2 and 3 (PfEMP-1, 2, and 3), ring-infected

erythrocyte membrane surface antigen (Pf155/RESA), sequestrin and rosettins have been identified on the surface or in association with the cytoskeleton of RBCs (24). Ligands on the surface of the iRBCs can bind to a variety of receptors including CD36, thrombospondin, ICAM-1, PECAM, VCAM-1, chondroitin-4-sulfate, ELAM-1 and P-selectin (25-31).

PfEMP1 encoded by the multicopy *var* gene family is one of the major parasite ligands involved in mediating cytoadherence (32-34). Each haploid parasite possesses ~ 60 *var* genes within its genome (35). However, only a single gene is expressed at a time and all other *var* gene copies are maintained in a transcriptionally silent state (36, 37). By switching which gene is expressed, parasites alter both the cytoadherent properties of the infected cells and their antigenic phenotype, thus avoiding the antibody response of the infected individual and maintaining a persistent infection (38).

MALARIA: IMMUNE ACTIVATION AND IMMUNOPATHOLOGY

The initial interaction of the iRBCs with the host's immune system is thought to be one of the most important factors determining the nature of the subsequent innate and acquired response, and whether or not severe pathology, such as cerebral malaria, severe anaemia or hypoglycaemia, results. How the malarial parasite activates the innate immune system, and the cytokines and chemokines induced, will also influence the magnitude of the inflammatory response and the types of T cell and B cell responses elicited. An understanding of these activation processes is required to determine the level at which host immune responses contribute to malarial disease, and to differentiate between protective and pathological responses.

Since the RBCs do not express MHC class I or class II molecules necessary to initiate a T cell response, the immune responses must be generated through antigen presenting cells. However, the identity of antigen presenting cells that activate T cells during malarial infection is not clear. Bone marrow derived dendritic cells (DC), macrophages and B cells isolated from immune mice have all been shown to have capacity to present malarial antigens to T cells (39).

Dendritic Cells: Dentritic cells (DC) are a unique type of antigen presenting cell because of their ability to induce primary immune responses by efficient activation of naïve T cells. They play a central role in both innate and adaptive immune response. They frequently sample the sites of pathogen entry and uptake, and process antigens and activate both naïve and memory T cells (40). Research on DCs over the past decade has established that the DC system comprises a large collection of subpopulations with different functions. Functional differences in DC populations are related to their differentiation state as well as their specific location (41). Based on the expression of surface markers five DC subtypes are consistently found in the lymphoid tissues of uninfected mice. Spleen has three distinct DC subtypes based on the expression of T cell markers CD4 and CD8: CD4-CD8α+, CD4+CD8α-, and CD4-CD8α- DC (42). Lymph nodes (LN) contain two additional DC subtypes (CD4-CD8-CD11b+ DCs expressing moderate levels of CD205) that are not normally found in spleen (43, 44). Another distinct DC subtype found only in the skin draining LNs expresses high levels of langerin, a characteristic marker of epidermal Langerhans cells and is believed to be the mature form of these cells. Although most DCs were thought to be of myeloid origin

(45), there is evidence that some DCs share early steps of development with B cells (46, 47) and that CD8α+ DCs of thymus share early steps of development with T cells (48).

The evidence that malarial parasites interact with DCs to promote an inflammatory response is limited and conflicting. Urban et al. first showed that *P. falciparum*-iRBCs could prevent up-regulation of MHC Class II and the co-stimulatory molecules CD83 and CD86 on human DCs in response to lipopolysacharide (LPS). These DCs also failed to induce antigen-specific primary and secondary T cell responses (49, 50). CD36 and CD51 were identified as the receptors on DCs responsible for this inhibitory effect (51). These same receptors were found to mediate the inhibitory effect on macrophage function by decreasing IL-12, TNF-α and IL-1 secretion during malarial infection (52). An increase in IL-10 is also observed during infection, which could potentially directly suppress the stimulatory function of DCs as well as promote the generation of anti-inflammatory suppressor T cells (53-55).

In contrast to these observations, Seixas and colleagues reported that GM-CSF-grown bone marrow-derived DCs (BM-DCs) up-regulate surface expression of MHC II, CD40 and CD86 after exposure to *P. chabaudi*-iRBCs. Their ability to stimulate T cell responses was maintained, and increased production of TNF-α, IL-12 and IFN-γ by BM-DCs was evident (56). Furthermore, Luyendyk and colleagues found that murine splenic CD11b+ and CD11c+ DC subtypes were able to up regulate MHC II and CD80 molecules following acute infection with *P. yoelii* (57). However, these studies are in direct contradiction to a recent study in mice by Ocana-Morgner and colleagues, who showed that GM-CSF grown BM-DCs and parasitized erythrocytes from *P. yoelii* inhibit DC maturation in vitro (58). Thus, DC activation by malaria parasites seems to be normal

in some *in vitro* and *in vivo* systems but is abnormal in other experimental systems. The *in vivo* relevance of possible down regulation of DC maturation by *Plasmodium* during malaria infection is thus not clear.

Macrophages: Macrophages play important roles in both innate and adaptive immune responses to malarial infection. In addition to their function as antigen-presenting cells, they are shown to phagocytose infected erythrocytes in the absence of cytophilic or opsonizing malaria-specific antibodies. This is thought to be mediated through binding of PfEMP1 on infected erythrocytes to the scavenger receptor CD36 (59). However, during the adaptive immune response macrophages function as effector cells that can mediate antibody-dependent cellular inhibition or the production of anti-parasite molecules, such as nitric oxide, after their activation by $CD4^+$ T cell -derived IFN-γ (60).

In addition to the modulation of DC function by iRBCs, macrophage function has also been shown to be impaired especially following phagocyosis of hemozoin (heme polymer produced as a by-product of intraerythrocytic hemoglobin catabolism by malarial parasites) or mature parasites. The hemozoin induced macrophage dysfunction has been shown to include inability to repeat phagocytosis, generate an oxidative burst upon stimulation, and activate protein kinase C (61). Moreover, a defect in macrophage ability to clear parasites that selectively bind to chondroitin sulfate A (CSA) expressed on the placenta has also been reported (62).

NK Cells: NK cells originally discovered for their ability to spontaneously lyse tumor cell lines are innate effector cells that perform important functions in defense against microbes and cancer through their cytolytic activity and secretion of proinflammatory cytokines, particularly IFN-γ. NK cells were shown to be the first cells to produce IFN-γ

after in vitro exposure of human peripheral blood mononuclear cells (PBMCs) to *P. falciparum* infected erythrocytes (63). Cytokines IL-12 and IL-18 have been shown to be critical in NK cell activation and IFN-γ production (63). In rodent models, NK cell derived IFN-γ production has been shown to be essential for development of protective immunity. NK-cell-mediated cytotoxicity and IFN-γ production are shown to be induced by infection with various rodent malarial parasites including *P. chabaudi* AS (64), *P. berghei* (65), and *P. yoelii* (66). Furthermore, depletion of NK cells was associated with increased parasitemia and less efficient resolution of *P. chabaudi* AS infection in B6 mice (64).

NKT Cells: Natural killer T (NKT) cells are a heterogeneous population of lymphocytes with phenotypic and functional characteristics of both NK cells and classic T cells. NKT cells express NK1.1 and an invariant αβ - T cell Receptor (TCR) (67). Murine NKT cells express a specific TCR-α chain (Vα14-Jα18) in association with Vβ2, Vβ7, or Vβ8 (68). NKT cells recognize glycolipid antigens via a repertoire of invariant VαVβ-TCR in the context of a non-classic, MHC class-I-like molecule, the Cd1 molecule (68). Upon activation through the TCR, NKT cells produce large amounts of IFN-γ and IL-4, which may be important in regulating the downstream differentiation of CD4 + T cells into Th1 or Th2 subtypes (69). Tsuji et al showed that α-galactosylceramide (α-GalCer), when administered to mice infected with sporozoites of *P.yoelii* and *P. berghei* can inhibit the development of intrahepatocytic parasites and prevent the onset of blood stage infection, suggesting a potential role for NKT cells in developing malarial immunity, particularly against the pre-erythrocytic parasites (70). Furthermore, CD1d-restricted NKT cells from mice of distinct genetic backgrounds were shown to differentially influence Th1/Th2

polarization, proinflammatory cytokine levels, pathogenesis, and fatality in the *P. berghei*–ANKA / rodent model of cerebral malaria (67).

γδ T Cells: T cells bearing γδ-TCR represent a small fraction of circulating T cells that are distinct from αβ T cells in that they can recognize antigens in the absence of classic MHC antigen presentation. γδ T cells constitute the majority of intraepithelial lymphocytes in the gut and other mucosal tissues of adult humans and mice (71). Although an expansion of γδ T cells has been reported during blood stage malarial infection, these cells were not found to be crucial in developing blood stage immunity against *P. chabaudi* AS or *P. chabaudi adami* infection (72). Together with NK cells, γδ T cells have been shown to be an early source of IFN-γ and TNF-α before the activation of αβ T cells. The observation that γδ T cells secrete potentially harmful proinflammatory cytokines has also lead to the hypothesis that these cells play a role in malarial pathogenesis rather than protection (73). Furthermore, γδ T cells have also been shown to contribute to the pathogenesis of cerebral disease in *P. berghei* ANKA model of malarial infection (74). Thus the exact role of γδ T cells in immunity to blood stage malaria is not clear and is an open area for further investigation.

B Cells and Antibodies: The obvious role of B cells in protection against malarial infection is through antibody production. The importance of antibody in resolution of malarial infection was first demonstrated in passive transfer experiments, where parasitemia could be resolved by transfer of immune sera (75, 76). The possible effector mechanisms involved in antibody-mediated protection are thought to be interference with merozoite invasion into erythrocytes (77, 78), antibody dependent cell-mediated cytotoxicity, and phagocytosis (75, 79, 80). Antibodies specific for a number of parasite

antigens, including MSP-1 and -2 (81-83) and PfEMP-1 (84, 85) have been shown to correlate with resistance to *P. falciparum* infection. In humans, cytophilic antibodies IgG1 and IgG3 isotypes predominate in the sera of immune adults (86, 87). Furthermore, IgG1 and IgG3 from immune individuals together with monocytes were shown to be able to kill the parasites in vitro (86). Consistent with these reports in human malaria infection, passive transfer of IgG2a isotype from immune serum was demonstrated to provide stronger protection to murine malaria infection with *P. berghei* than IgG1 isotype (88, 89). This suggests that the protection is isotype specific. Additionally, Couper et al showed recently that $IgM^{-/-}$ mice are more susceptible to P. *chabaudi AS* infection than WT mice and passive transfer of wild type antiserum containing malaria specific IgM into $IgM^{-/-}$ mice can reduce the mortalities in $IgM^{-/-}$ mice, suggesting a protective role for IgM during malarial infection (90).

T Cells and Cytokines: Experiments over many years have established a central role for CD4+ T cells in the protection against blood stage malaria infection. Based on the pattern of cytokine production, CD4+ T cells are classified into two major subsets. Th1 cells produce proinflammatory cytokines such as IL-2, IFN-γ, and TNF-α and initiate cell-mediated immune responses via activation of macrophages. Th1 cells also regulate the production of opsonizing and cytophilic antibodies such as IgG2a and IgG3 in mice, and IgG1 and IgG3 in humans. Th2 cells produce cytokines such as IL-4, IL-5, IL-6 and IL-13 and provide help for B-cell maturation to plasma cells and promote the production of IgG subtypes that are associated with allergies and helminthic infections, such as IgG1 in mice and IgG4 in humans (91). It has been shown in *P. chabaudi* AS infection that both Th1 and Th2 cells are important for resolution of the infection (92-94). The induction of a

Th1 response versus Th2 response appears to be tightly regulated and can be affected by the initial interaction between T cells and the antigen presenting cells.

In order to initiate a Th1 cytokine response, Th0 precursor cells must first be given the appropriate signals in the form of IL-12 and engagement of membrane-associated costimulatory molecules. IL-12 has been identified as a critical cytokine of the early immune response that links innate and acquired immunity and initiates development of the Th1 immune response (95). IL-12 is a heterodimeric cytokine composed of p35 and p40 subunits. The two subunits together form the 70- to 75-kDa (p70) protein which accounts for the biologic actions of IL-12. This cytokine is predominantly produced by activated monocytes, macrophages and dendritic cells and heterodimers must be produced in the same cells to generate active cytokine (96-98). IL-12 signals through IL-12R which is composed of two subunits, IL-12Rβ1 and IL-12Rβ2 and is expressed by macrophages, dendritic cells, NK cells, and activated T cells (99, 100). IL-23 and IL-27, two other heterodimeric cytokines that are related to IL-12, have been identified recently. Active IL-23 is composed of IL-23p19 and IL-12p40 subunits (97), while IL-27 is made up of IL-27p28 and Epstein Barr virus-induced gene 3 (EBI3), an IL-12p40 related protein (101, 102).

IL-12 family cytokines have been found to play important roles in NK cell activation (98, 103, 104), T cell proliferation (105), T cell cytokine production (99) and B cell antibody production (106, 107). Although IL-12, IL-23 and IL-27 can all induce IFN-γ production, IL-12 has been shown to be uniquely required for Th1 differentiation (108).

As a potent inducer of IFN-γ production, IL-12 has been shown to be critical for development of IFN-γ-mediated protection against blood-stage *P. chabaudi* AS infection.

IL-12 is one of the first cytokines initiated in this infection. Resistant B6 mice mount a strong IL-12 response within 2-3 days of infection, whereas this response is impaired in susceptible A/J mice (109, 110). Treatment of susceptible A/J mice with murine rIL-12 significantly reduces the level of parasitemia and improves host survival (111). This IL-12-induced protection is mediated by IFN-γ and TNF- α (30) and requires the presence of NK and $CD4^+$ T cells (64, 111). A similar protective effect of IL-12 treatment was also shown in mice infected with a radiation-attenuated variant of *P. berghei* (112) and liver-stage *P. yoelli* (113). In humans, low levels of plasma IL-12 have been shown to be associated with severe malarial pathology (114, 115), and IL-12 production is inversely associated with risk of infection and positively associated with hemoglobin concentration and IFN-γ and TNF-α production (116).

IFN-γ is a pluripotent cytokine that plays a central role in immune response to several infectious diseases. IFNs originally identified as agents involved in protecting the cells from viral infection (117) are classified into type I and type II according to receptor specificity and sequence homology. The type I IFNs are comprised of multiple IFN-α subtypes, IFN-β, IFN-ω, and IFN-τ, where as IFN-γ is the sole type II IFN. IFN-γ is a homodimeric protein produced by NK cells, $CD4^+$ Th1 cells, $CD8^+$ cytotoxic lymphocytes, B cells, NKT cells and antigen presenting cells (118-124) and regulates over 200 genes involved in immune responses. IFN-γ along with IL-12 plays an important role in skewing the immune response towards a Th1 phenotype. In terms of its effect on CD4+ T cells, IFN-γ stimulates secretion of IL-12 by macrophages, which binds receptors on naïve CD4+ T cells and promotes the differentiation and expansion into Th1 cells (125-127). IFN-γ also promotes Th1 cell differentiation by inducing the

transcription factor T-bet (128). Additionally, IFN-γ upregulates the class II MHC antigen presention pathway and thus promotes peptide-specific activation of CD4+ T cells (129, 130). IFN-γ affects CD8+ T cells by upregulating multiple functions within the class I MHC antigen presention pathway and thereby promoting the levels and diversity of epitopes presented for CD8+ T cell recognition in the context of class I MHC (131). The IFN-γ receptor, ubiquitously expressed on all nucleated cells, consists of two ligand binding IFN-γRI chains associated with two signal transducing IFN-γRII chains (132). Both IFN-γ receptors lack intrinsic kinase/phosphatase activity and so must associate with signaling machinery for signal transduction. Although IFN-γ secretion by NK cells and antigen presenting cells is likely to be important in early host defense against infection, T lymphocytes become the major source of IFN-γ in the adaptive immune response (121, 133).

In resistant B6 mice there is a rapid and large production of IFN-γ early in the course of *P. chabaudi* AS infection that can be detected in the plasma two to three days before peak parasitemia (134). CD4+ T cells were shown to be the major source of IFN-γ at this time point (93, 134). Depletion of IFN-γ exacerbates an acute *P. chabaudi AS* infection, and IFN- γ receptor deficient mice infected with *P. chabaudi AS* have prolonged acute phase parasitemia and greater mortality, suggesting an important role for IFN- γ in the control of malaria infection in mice (134, 135). An early Th1 and IFN- γ response have also been described for both lethal and non-lethal *P. yoelii* infections and a lethal *P. vinckei vinckei* infection (37).

IFN-γ may mediate its effect by activating macrophages to produce TNF-α and soluble mediators such as nitric oxide and reactive oxygen species. TNF-α was initially

identified on the basis of its ability to kill tumor cells *in vitro* and to cause hemorrhagic necrosis of transplantable tumors in mice (136). Since then, TNF-α is known to play an important role in immune response, inflammation, autoimmunity and pathophysiology of many diseases (137). TNF-α occurs naturally in two biologically active molecular forms, a 26-kDa type II transmembrane pro-TNF-α acting locally through cell-to-cell contact, and a 17-kDa secreted mature TNF-α that is cleaved from pro-TNF-α by proteolytic enzymes and is capable of acting on distant targets (138). TNF-α has been shown to modulate proliferation, differentiation, and apoptotic or necrotic cell death in a number of different cell types (139). These responses are mediated by TNF-α binding to two distinct TNF-α receptors, type I (TNFRI, CD120a) and type II (TNFRII, CD120b (140)). Both are type I transmembrane glycoproteins and members of the TNF receptor superfamily characterized by the presence of multiple cystine-rich repeats of about 40 amino acids in the extracellular amino-terminal domain. The TNFRI complex contains a death domain and mediates both apoptotic and antiapoptotic pathways. TNFRII lacks a cytoplasmic death domain and has been shown to induce pathways of cell activation and survival. However, TNFRII may also be involved in cytotoxicity of target cells (141). It has been proposed that the ligation of TNFRII leads to the activation of NF-κB and AP-1, which in turn will induce the transcriptional upregulation of membrane-bound TNF and FasL, leading to apoptosis in an autotropic or paratropic manner (141).

TNF-α is thought to be involved in development of immunity as well as pathology in malaria infections in experimental models and in humans. In addition to being induced by cytokines such as IFN-γ, TNF-α release by macrophages can also be directly induced by malarial parasites and their soluble antigens such as glycosylphosphatidylinositols (142)

and insoluble hemozoin (143). High levels of TNF-α in the spleen have been shown to correlate with resistance to *P. chabaudi* AS infection in mice. Administration of TNF-α to susceptible strains of mice has been shown to provide protection against *P. chabaudi AS* infection in mice (144) and administration of anti-TNF-α antibody to resistant mice abrogates protective immunity (145). However, TNF-α might not be a critical cytokine for early protective responses as mice deficient in TNFRI and II are shown to be able to control and reduce parasitemia (146).

The anti-parasitic effect of TNF-α may be through induction of soluble mediators such as ROS and NO. However the role of NO and ROS in parasite killing is still controversial. Mice deficient in inducible nitric oxide synthase (iNOS) are able to clear *P. berghei* infection at the same rate as normal mice (147). Also blocking of NO production through administration of an inhibitor of iNOS, N^G-monomethyl –L-arginine (L-NMMA) does not alter the course of *P. yoelii* infection (148). In contrast others have demonstrated an anti-parasitic effect of NO. Administration of L-NMMA has been shown to abolish the ability of mice transfused with a protective Th1 clone to control parasite growth (149). Additionally, treatment of resistant B6 mice with an iNOS inhibitor aminoguanidine has been reported to result in increased mortality indicating a protective role of NO in the early phase of blood stage *P. chabaudi AS* malaria (150).

In addition to their contribution towards eliminating the parasite and to the development of protective immunity, it has been clearly shown in rodent models that Th1 cytokines produced during acute infection, particularly TNF-α and IFN-γ, play a major role in pathogenesis (151-154). Neutralization of IFN-γ with a monoclonal antibody had been shown to reduce the level of TNF-α in the serum of *P. berghei* infected mice and

protected these mice from experimental cerebral malaria, suggesting that these cytokines contribute to pathology in *P. berghei* infection (153). TNF-α production during blood stage malaria infection can lead to splenomegaly (155), weight loss and anemia (156). TNF-α has been shown to suppress erythropoiesis both in mouse and human bone marrow cultures (152, 157, 158) and enhance erythrophagocytosis (159). High levels of TNF-α in the serum and lower plasma IL-10 to TNF-α ratio have been shown to be associated with anemia in children (159, 160). Variation in the amounts of TNF-α production has a genetic basis and is decisive for the outcome of an infection. Thus, a single nucleotide polymorphism in the TNF-promoter region (-308) has been shown to be associated with elevated TNF-α production and an increased risk of cerebral *P. falciparum* malaria (161-163). Furthermore, an association has been observed between TNF promoter allele-238A and malarial anemia in children from The Gambia (164).

In contrast, regulatory cytokines such as IL-10 and TGF-β play important roles in maintaining the balance of the inflammatory response. A deficiency of IL-10 resulted in increased mortalities in *P. chabaudi* AS infected mice and the mortalities were associated with elevated plasma levels of IL-12, IFN-γ and TNF-α (165). However, it has been shown that IL-$10^{-/-}$ mice can down regulate IFN-γ and TNF-α level, suggesting a role for other mechanisms in modulating immune responses during malarial infection. TGF-β is another anti-inflammatory and regulatory cytokine that has been shown to be capable of regulating inflammatory responses during *P. chabaudi AS* infection (166). TGF-β is produced by a wide range of cells including macrophages and T cells (167). Depending on the environment and concentration, TGF-β has shown to have both pro- and antiinflammatory properties (168). Importantly, TGF-β suppresses production of TNF-α

and nitric oxide from macrophages (169, 170) and suppresses production of IFN-γ and TNF-α from NK cells (171). It has been proposed that these effects may be mediated via enhanced IL-10 production by macrophages (172), eventually leading to a shift in the immune response away from a Th1-like response and towards a Th2-like response (173)

In rodent models, the ability to survive malarial infection is positively correlated with the ability to secrete TGF-β (174). It was observed that the administration of a neutralizing antibody to TGF-β can render otherwise resistant B6 mice susceptible to *P. chabaudi* AS infection and administration of recombinant TGF-β can prolong the survival in *P. berghei*-infected mice (174). The TGF-β response has also been shown to differ between mice infected with a virulent *Plasmodium* strain and those infected with a benign strain. Thus, Omer et al demonstrated that following infection with the avirulent *P. yoelii 17X*, TGF-β levels peaked late and correlated with reduced parasite density, whereas infection with the virulent *P. yoelii 17XL* resulted in an early TGF-β response, a downregulated Th1 response, failure to clear parasites, and death (175).

ORIGIN AND DEVELOPMENT OF MOUSE PLACENTA: The placenta is the first organ to form during mammalian embryogenesis and is composed of both maternal and fetal cells. The major function of human and mouse placenta is to promote the growth and viability of the fetus whilst preserving the maternal well-being. This is achieved in several ways which involves the development of specialized regions, the maternofetal interface, where maternal and fetal cells are brought into close association and interact with each other. The placenta ensures an adequate supply of nutrients from the mother to the fetus and the removal of toxic wastes from the fetus to the mother. In addition, the placenta acts as an important source of pregnancy-associated hormones and growth

factors, and is involved in the immune protection of the fetus. A detailed knowledge of the sequential anatomical and physiological events occurring during placentation is required to understand the maternal immune responses to placenta and how the fetus that expresses paternal antigens escapes immune-mediated rejection.

In mouse, two distinct cell lineages are formed by embryonic day 3: the outer specialized trophectodermal epithelium and the inner cell mass (ICM) (176). The trophoblast layer of the placenta arises from the outer trophectoderm layer of blastocyst. Implantation begins when blastocyst slips into a crypt formed by the thickened uterine walls and this commonly occurs at about embryonic day 4.5. The trophoblast cells overlying the ICM continue to proliferate and give rise to the diploid extra embryonic ectoderm and ectoplacental cone of the early postimplantation conceptus. The trophectoderm cells away from the ICM stop dividing but continue to endoreduplicate (repeated rounds of DNA replication in the absence of intervening mitoses) their DNA to form trophoblast giant cells. The allantois arises from mesoderm at the posterior end of the embryo and makes contact with chorion at embryonic day 8.5. The allantoic mesoderm gradually invades trophoblastic cells and establishes a network of fetal blood vessels in the labyrinthine zone. The labyrinth develops from the trophoblast and its associated fetal blood vessels. In the labyrinth trophoblast differentiates into three layers: two syncytiotrophoblast layers in contact with the fetal endothelium and one cytotrophoblast layer in contact with maternal blood. A definitive chorioallantoic placenta is developed by gestation day 12.5 (mouse gestation is 19-21 days).

Comparative Anatomy of Human and Mouse Placenta: Three physiologically and anatomically distinct regions can be distinguished in both human and murine placenta.

First is the region that is known as labyrinth in mice and fetal villous placenta in humans. This is where the maternal and fetal blood circulates in close association for physiological exchange of nutrients and gases. Based on the overall shape of the fetal placenta / labyrinth,placenta in both species is referred to as discoid and has a flat surface facing the fetus and an irregular opposite surface adjacent to the uterine wall. The fetal side of this region in both species is bounded by a layer of trophoblast cells supported by extracellular matrix and is known as the chorionic plate. In humans, from the opposite surface of the chorionic plate arises many tree like projections known as chorionic villi. The umbilical cord is typically attached to the centre of the fetal surface (177-179).

Second is the region termed basal plate in humans and junctional zone or spongiotrophoblast zone in mice. This region borders the maternal surface of the fetal placenta / labyrinth and is traversed by maternal blood channels, and is lined by fetally-derived trophoblast cells, through which maternal blood flows into and out of the fetal placenta / labyrinth (177, 178, 180). The maternal side of the murine spongiotrophoblast layer is lined by a discontinuous layer of fetally-derived trophoblast giant cells (177). Third, is the decidua basalis in both humans and mice (transformed maternal endometrium immediately bordering the basal plate in humans and trophoblast giant cell zone in mice). In humans, the decidua basalis and its underlying myometrium are collectively known as the placental bed (177, 178). A simplified diagrammatic representation of murine definitive placenta showing all three layers are presented in figure 2.1.

However, there are some differences between human and mouse placentae. In humans the definitive placental structure is visible as early as gestation day 21. In mice,

however, the definitive structure is completed only halfway through gestation. In humans, the trophoblast invasion normally extends up to the inner third of myometrium, whereas, it is limited to decidua basalis in mice (178, 181). Furthermore, the trophoblast giant cells in humans are thought to be formed by the fusion of extravillous trophoblastic cells whereas, murine trophoblast giant cells are generated by endoreduplication (182).

IMMUNOLOGY OF PREGNANCY: Pregnancy constitutes a major challenge to the maternal immune system, which must tolerate fetal alloantigens encoded by paternal genes. Several mechanisms involving both maternal and trophoblast factors are thought to be involved in the protection of the fetus from maternal immune-mediated rejection.

One of the most important immune evasion strategies is thought to be the absence of classical major histocompatibility complex (MHC) antigens on the trophoblast. MHC class II alloantigens are completely absent on extravillous trophoblast cells in both humans and mice (183). Furthermore, human trophoblast cells do not express classical MHC classs I HLA-A and HLA-B molecules but do express classical HLA-C and non-classical HLA-E and HLA-G. Trophoblast expression of monomorphic HLA-G could be another mechanism involved in fetal immune evasion. HLA-G molecules prevent cytolytic activity of NK cells by binding to killer-immunoglobulin-like receptors expressed by NK cells (184).

Another immune evasion mechanism is thought to involve production of Indolamine 2-3 dioxygenase (IDO), a tryptophan-catabolizing enzyme, by fetal trophoblast cells. Through depletion of tryptophan, an essential amino acid for rapidly dividing cells, T cell proliferation at the maternofetal interface is thought to be inhibited. A role for IDO in protecting the fetus against maternal immune-mediated rejection has been demonstrated

in rodent models where adminstration of 1-methyl tryptophan, an inhibitor of IDO, can induce abortion (185).

Trophoblast expression of high levels of complement inhibitory proteins, especially Crry, is thought to have important functions in protection against complement-mediated damage. A deficiency of complement regulatory proteins has been shown to result in excessive C3 deposition, neutrophil influx and inflammation within the developing placenta and ultimately embryonic death in mice (186). The Fas / Fas-ligand (Fas/FasL) system is also thought to be involved in protection of the fetal allograft. Trophoblast cells synthesize and express FasL, which in turn may cause apoptosis of activated maternal lymphocytes that are known to express Fas (187, 188).

The cytokine balance at the maternofetal interface is thought to be a major factor in determining fetal outcome. Thus, normal pregnancy is thought to require a bias against Th1 or towards Th2 type of cytokine responses (189, 190). Whereas production of Th2-type of cytokines such as IL-10 and IL-4 locally at the materno-fetal interface is thought to favor the maintenance of pregnany (191, 192), an excessive production of Th1/proinflammatory cytokines such as IL-2, IL-6, IFN-γ and TNF-α mediate fetal rejection (193-196).

Although the trophoblast cells do not express MHC class II molecules, macrophages and dendritic cells present in the placenta can process and present the fetal antigens to maternal T cells. Furthermore, the hypothesis that the placenta acts as a physical barrier in protecting the fetus from maternal immune attack has also been proved wrong, as there is evidence for trafficking of cells in both directions across the maternofetal interface (197, 198). Thus, despite all the immune evasion strategies, it is

generally accepted now that the maternal adaptive immune system is aware of paternal alloantigens. However, the alloantigen specific T cells are thought to be somehow anergized during pregnancy (199). Expression of regulatory T cells at the maternofetal interface (200) and antigen presentation in the absence of costimulation are thought to be the possible mechanisms involved. Thus, several factors including fetal trophoblast cells, maternal immune cells at the maternofetal interface, and a carefully regulated balance of pro and anti-inflammatory cytokine production in the placenta and periphery are all thought to be involved in preserving the fetal allograft. Factors that can affect this balance can cause poor pregnancy outcomes including fetal loss. Uterine infections are thought to be one such factor that can alter the delicate balance at the maternofetal interface. Several pregnancy complications such as preterm labor, pre-eclampsia and spontaneous abortion are all shown to have strong association with infections (201-203).

MALARIA DURING PREGNANCY: Malaria during pregnancy is a major risk factor for maternal and fetal mortality. In all endemic areas pregnant women are shown to be more susceptible to malarial infection than their nonpregnant counterparts or the general adult population. However, the severity of malarial infection can vary depending on the endemicity or intensity of transmission. Most of our understanding of the biological basis for the increased susceptibility of pregnant women to malarial infection is from studies conducted with pregnant women living in high transmission areas (204-208). Adults living in high endemic areas often develop protective immunity against severe malarial infection. However, this protection is markedly compromised in women during their first and second pregnancies (209, 210). Under these settings, malarial infection during pregnancy is characterized by severe maternal anemia and poor fetal outcomes such as

low birth weight babies and preterm labor (211-213). Pregnant women regain their protective immunity to severe malarial infection in their subsequent pregnancies. The increased susceptibility of pregnant women to malarial infection during pregnancy is thought to be largely due to accumulation of iRBCs in the placental intervillous blood space. This preferential sequestration of iRBCs in the placenta is mediated at least in part through binding of iRBCs to specific receptors expressed on the syncytiotrophoblast cells (207, 214). Placental parasites typically adhere to chondroitin sulfate A (CSA) expressed on syncytiotrophoblasts, but rarely to CD36 and intercellular cell adhesion molecule (ICAM)-1 (205, 206). In addition, binding to hyaluronic acid (HA) and non-immune immunoglobulins might also mediate sequestration of iRBCs in the placenta (215, 216). PfEMP-1 encoded by the *var* family of genes (217, 218), is the best-characterized ligand implicated in the cytoadherence. The adhesive characteristics of placental parasites from areas of very low transmission are not described. However, among women in an area of low-transmission intensity in Thailand (219), multigravidae had a greater prevalence of antibodies to CSA-binding iRBCs than men or primigravidae, suggesting that CSA-binding phenotypes are a feature of *P. falciparum* in pregnancy globally.

Immunity to malaria during pregnancy: Adults living in high endemic areas exhibit substantial immunity to malarial infection and the protection is shown to be associated with the presence of a large repertoire of variant-specific agglutinating antibodies against different *P. falciparum* isolates (220, 221). However, during their first pregnancy women lose their protection against developing severe disease suggesting the emergence of novel antigenic variants of *P. falciparum* that are poorly recognized by antibodies acquired prior to the first pregnancy (205, 206). Over successive malaria-infected pregnancies,

women living in high endemic areas acquire antibodies that can inhibit parasite adhesion to CSA and / or react with the surface of placental or CSA binding iRBCs and protect women from developing severe disease (205, 206, 222, 223).

Recent studies suggest that VAR2CSA, expressed on the surface of CSA-binding iRBCs, is the major target of antibodies associated with protection against malaria in pregnancy (224). VAR2CSA has been shown to be upregulated in parasites selected for CSA binding in vitro (225) and in placental isolates (226). Additionally, targeted disruption of *var2csa* was shown to be associated with marked reduction in the ability of parasites to adhere to CSA (227). Furthermore, recombinant proteins corresponding to VAR2CSA domains are shown to be recognized by antibodies in plasma from malaria-exposed donors according to gravidity and gender, and antibodies to these domains are associated with reduced risk of infant low birth weight (224).

Cellular Immune Responses In Malaria During Pregnancy: Apart from sequestration of iRBCs in the placental intervillous space, malarial infection is often associated with massive infiltration of immune cells in the placenta. Monocytes and macrophages, engorged with malarial parasites or hemozoin, are the most distinct cellular component of the inflammatory infiltrate [reviewed in (228, 229)](207, 230). The accumulation of macrophages in the placenta of malaria-infected pregnant women has been shown to be strongly associated with poor pregnancy outcomes (231, 232). Additionally, the placental infiltrate also includes lymphocytes and less frequently polymorphonuclear cells (233). Apart from accumulation of inflammatory infiltrates, other histological changes include excessive syncytiotrophoblast knotting, loss of microvilli, fibrin deposits, cytotrophoblast

proliferation and irregular thickening of the cytotrophoblast basement membrane (207, 232, 234).

Cytokines and Chemokines: Cytokines are thought to be involved in both protection as well as pathology during malarial infection. The proinflammatory cytokine IFN-γ has been shown to be critical in protection against malarial infection in both humans and mice. Elevated production of IFN-γ by intervillous blood mononuclear cells has been thought to be associated with protection against PM infection in multigravid women (209). However, elevated production of proinflammatory cytokines are also thought to be associated with poor pregnancy outcomes. Fried et al observed that the levels of IFN-γ, TNF-α and IL-2 were elevated in the placentas from malaria-infected women compared to their uninfected counterparts (235). Furthermore, elevated levels of TNF-α, IFN-γ and IL-8 have been shown to be associated with severe anemia and low birth weight babies (235). Macrophages are thought to be the major source of TNF-α and IL-8 in the placenta. However, syncytiotrophoblast cells are also capable of producing IL-8 and TNF-α in vitro (236). Factors that contribute to the accumulation of immune cells in the placenta are also shown to be elevated in malaria-infected pregnant women. Placental levels of macrophage inflammatory protein (MIP) -1α and β, monocyte chemoattractant protein (MCP)-1 and IL-8 are increased during PM (237-239).

However all these studies were conducted in women living in high endemic areas. Detailed studies to understand the development of immune responses in pregnant women living in settings of low endemicity or during early pregnancy have not been done. In low endemic areas exposure to malarial infection is not constant enough to promote an effective immunity in the population. Under these settings, pregnant women of all parities

are at greater risk to develop severe malarial disease compared to their nonpregnant counterparts. Thus, malarial infection in pregnant women with little or no immunity can lead to severe malarial syndromes including cerebral malaria, pulmonary edema and maternal and fetal death (240, 241). In his detailed report on the 1934-35 malaria epidemic in Sri Lanka, Wickramasuriya had reported a more severe clinical outcome in pregnant women compared to other infected adults. Pregnant women frequently experienced cerebral malaria, severe anemia and cachexia, and mortality rates were as high as 13% (242) . Apart from maternal mortalities, fetal mortalities were also reported to be higher and the combined cases of neonatal and fetal mortalities were 66.9% in cases of pregnancy malaria. Severe maternal malaria associated with poor pregnancy outcomes were also reported from other parts of the world. In India, fatal complications of malarial infection during pregnancy included, cerebral malaria, pulmonary edema and hypoglycemia. Furthermore, the rate of fetal loss was almost twice (31.1%) the rate observed in general population (240). In a low transmission area of Tanzania, the malarial infection during pregnancy was associated with high rates of still-births (33.3%) and neonatal deaths (31.3%) (243).

ANIMAL MODELS OF MALARIA DURING PREGNANCY: Detailed immunologic studies on the role of malaria in abortion and stillbirth and the effect of placental parasitization and maternal anti-malarial immune response on pregnancy success have not been done. Previous studies on the interrelationship of malaria and pregnancy in rodents have used lethal parasites such as *P. berghei.* In pregnant mice, *P. berghei* infection was shown to be more severe when compared to non-pregnant mice (244). Pregnant mice experienced a more rapid increase in parasitemia than non-pregnant

mice and had higher mortality rates (245, 246). Gestation day at which the infection is initiated may also influence the outcome. Mice infected on gestation day 6 with *P. berghei* showed a rapid rate of increase in parasitemia and succumbed to infection when compared to mice infected later in pregnancy. Litters from mice injected with 10^7 *P. berghei* iRBCs on day 16 of pregnancy showed a reduction in birth weight and malarial parasites were present in the placenta and fetal blood on delivery (247). *P berghei* infection in pregnant rats was also used as a model to study malarial infection during pregnancy. In this model pregnant Wistar rats infected with 10^6 *P. berghei* iRBCs were shown to develop more severe parasitemia and anemia compared to nonpregnant rats and succumbed to infection when infections were initiated on day 7 of gestation. Furthermore, the placental parasitemias were shown to be higher than the peripheral parasiemias at the time of delivery (248). Additionally, the placental pathologies associated with *P. berghei* infection in white rats were shown to be thickening and necrosis of cells in the labyrinthine zone, fibrosis of trophoblast cells, loss of microvilli and infiltration of maternal blood space by inflammatory cells, especially monocytes (249). Further research to characterize the immunological and molecular basis of fetal loss in rodent models for malarial infection during pregnancy has not been done.

More recently, Davison et al used *P. coatneyi* infected pregnant rhesus macaques to study the pathogenis of malarial infection during pregnancy. In this model, infected monkeys experienced increased rates of abortion and intrauterine growth retardation associated with placental pathology (250, 251). Although the leukocyte profiles were altered in infected pregnant monkeys with lower $CD4^+$ and $CD8^+$ T-cell and B cell counts suggestive of pregnancy-associated immunomodulation (252), the cytokine responses

were not altered, although fetal loss was found to be associated with elevated plasma TNF-α levels (253).

Cytokines and Fetal Loss: The exact mechanism by which the implanted embryo is killed during malarial infection is not clear. Normal pregnancy is characterized by a shift towards Th2 type immune response (189, 190). Thus a Th1 immune response mediated through the production of cytokines such as IFN-γ, TNF-α and IL-2 can have adverse effects on the conceptus either by direct embryotoxic activity or by damaging the placental trophoblast (254). It has been shown that IFN-γ can inhibit trophoblast outgrowth *in vitro* and IFN-γ and TNF-α can inhibit embryonic and fetal development as well as human trophoblast cell line growth *in vitro* (255). Administration of TNF-α, IFN-γ or IL-2 to normal pregnant mice has been shown to increase the frequency of resorptions (256, 257). In contrast to the deleterious effects of inflammatory cytokines on pregnancy, anti-inflammatory cytokines such as IL-10 and TGF-β may aid in fetal survival. IL-10 has been shown to protect against fetal death in a murine model of spontaneous resorptions (258), and reduced TGF-β levels correlate with increased spontaneous resorptions and abortions (259).

It is possible that the fetal trophoblast cells which form the interface between the embryo and the maternal tissue are damaged or killed (255). However, the trophoblast cells are not susceptible to lysis by TNF-α, NK cells, or macrophages, but may be killed by cytokine-activated NK cells (260). In a murine model for recurrent pregnancy loss the role of infiltrating NK cells and macrophages was shown to be through production of proinflammatory cytokines IL-1, TNF-α and IFN-γ (261). The mechanism by which pro-inflammatory cytokines mediate the embryotoxic effect in this model may be through the

up-regulation of procoagulant fgl2 prothrombinase, as blockade of fgl2 abrogated the observed pregnancy loss (262). Fgl2 prothrombinase is a fibrinogen-like protein that has been shown to be expressed by macrophages, vascular endothelial cells, fetal trophoblast, and a subset of uterine stromal cells (263). Proinflammatory cytokines IL-1 and TNF-α can induce the expression of fgl2 prothrombinase in the vascular endothelium (264) and IFN-γ and TNF-α can induce its expression in murine trophoblast (265). It has recently been shown that malarial parasites can induce expression of tissue factor (TF), an initiator of the clotting cascade, on activated endothelial cells in vitro (266). It is not clear whether the parasite sequestration in the placenta can induce increased TF expression on trophoblast cells. However, monocyte TF expression has been demonstrated in the placental intervillous space of malaria-infected pregnant women (267). The cytokine-induced upregulation of prothrombinase activity in the uterus may lead to termination of blood flow to the developing placenta and ultimately fetal loss via activation of thrombin, and then formation and deposition of fibrin.

The role of fetal cells in the immunopathogenesis of malarial infection during pregnancy is not clear. In mice and other rodents, trophoblast giant cells are the placental cells in direct contact with endometrial tissues and maternal blood throughout gestation (177, 268). These cells play crucial roles in implantation and placentation (269). Trophoblast shares many characteristics with macrophages. Phagocytic activity is an established characteristic of trophoblast during the early gestation stages of many species. Ectoplacental cone cells derived from 7.5-day-old mouse concepti can phagocytose large amounts of *P. berghei* iRBCs (270). This trophoblast mediated phagocytosis was sensitive to the inhibitory effects of cytochalasin B, suggesting actin cytoskeletal

involvement in the process (270). Trophoblast giant cells are also capable of producing or responding to various cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF) (271), colony stimulating factor (CSF)-1 (271, 272), IL-1 (273), IL-6 (274), TNF-α and β (275), and TGF-α and β (276, 277). The effect of phagocytosis of malarial parasites or hemozoin on trophoblast function is not known.

SUMMARY AND GAPS IN KNOWLEDGE: In non-immune women malarial infection during the first or second trimester is associated with high rate of abortion and during the third trimester frequently results in premature delivery (278). Although an association between maternal anti-malarial immune responses, especially accumulation of immune cells and associated production of proinflammatory cytokine TNF-α at the maternofetal interface, has been shown to be associated with low birth weight babies in high endemic areas, to what extent maternal anti-malarial immune responses contribute to fetal loss in low endemic settings is not clear. Furthermore, only limited progress has been made in our understanding of the development of immune responses against malarial infection in women living in low endemic areas or during early pregnancy. The objective of this study was to characterize the protective and pathogenic maternal immune responses to malarial infection during pregnancy and how these responses are related to the fetal outcome by employing a mouse model.

Apart from the maternal immune cells, fetal cells could also be involved in fighting against the invading pathogens at the maternofetal interface. Both human and murine trophoblast cells are shown to play important roles in innate immune protection at the maternofetal interface. This has been shown to be mediated through phagocytosis of invading microbes as well as through production of various cytokines and chemokines.

Although trophoblast production of cytokines and chemokines are shown to be essential for implanation and fetal growth and development during normal pregnancy, when produced unchecked, especially in the face of an ongoing infection, the same responses can induce placental pathology. No study has been done to explore the interaction between malarial parasites or hemozoin with murine trophoblast cells and how this interaction might affect the trophoblast function and ultimately fetal outcome. The results from this study are expected to advance our knowledge of the role of cytokine/chemokine production by maternal cells and fetal trophoblast cells in the placental pathologies and fetal loss during malarial infection. An understanding of the complex immunological mechanisms in malarial infection during pregnancy is critical in developing effective intervention or immunotherapies for preventing the detrimental effects of malaria on both mother and fetus.

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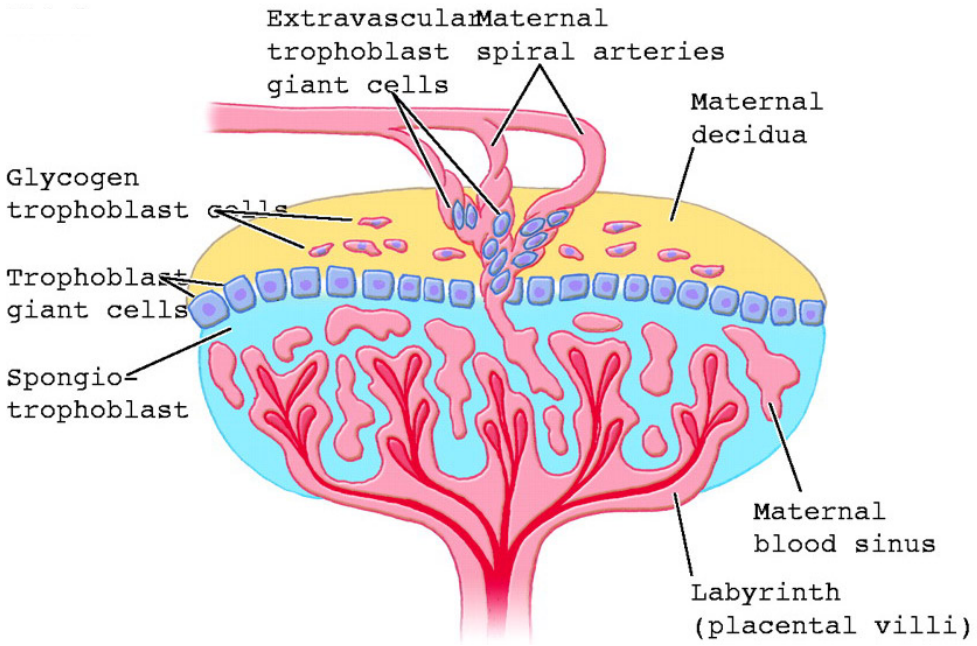
Figure 2.1: Schematic representation of murine definitive placenta

Adapted from Watson, ED and Cross, JC (2007) Physiology 20: 180-193



CHAPTER 3

THE COURSE OF *PLASMODIUM CHABAUDI* AS INFECTION IS NOT ALTERED DURING PREGNANCY BUT INDUCES FETAL LOSS ASSOCIATED WITH ACCUMULATION OF INFECTED[1] ERYTHROCYTES IN THE PLACENTA

[1] Jayakumar Poovassery and Julie M. Moore. 2006. Infect. Immun. May; 74 (5):2839-48.

ABSTRACT

Malarial infection in non-immune women is a risk factor for pregnancy loss, but the role that maternal anti-malarial immune responses play in fetal compromise is not clear. We conducted longitudinal and serial sacrifice studies to examine the pathogenesis of malaria during pregnancy using the *Plasmodium chabaudi* AS/C57BL/6 mouse model. Peak parasitemia following inoculation with 1000 parasite-infected murine erythrocytes and survival were similar in infected pregnant and non-pregnant mice, although development of parasitemia and anemia was slightly accelerated in pregnant mice. Importantly, pregnant mice failed to maintain viable pregnancies, most aborting before day 12 of gestation. At abortion, maternal placental blood parasitemia was statistically significantly higher than peripheral parasitemia. Infected mice had similar increases in spleen size and cellularity which were statistically significantly higher than uninfected mice. In contrast, splenocyte proliferation in response to mitogenic stimulation around peak parasitemia were statistically significantly reduced in both groups of infected mice compared to uninfected, non-pregnant mice, suggesting that lymphoproliferation is not a good indicator of antimalarial immune responses in pregnant or non-pregnant animals. This study suggests that while pregnant and non-pregnant C57BL/6 mice are equally capable of mounting an effective immune response to and surviving *P. chabaudi* AS infection, pregnant mice cannot produce viable pups. Fetal loss appears to be associated with placental accumulation of infected erythrocytes. Further study is required to determine to what extent maternal anti-malarial immune responses, anemia and placental accumulation of parasites contribute to compromised pregnancy in this model.

INTRODUCTION

Malaria continues to be a major public health problem in the developing world, causing an estimated 300-500 million cases each year and 1-2 million deaths (3). In malaria endemic regions, the related morbidity and mortality are primarily borne by children and pregnant women. Although women living in highly endemic areas acquire protective immunity against severe malaria, this protection is markedly compromised in the first and second pregnancies (5, 44) and is characterized by maternal anemia (33, 41, 56) and low birth weight babies (20, 60, 66) who are at increased risk to die early in life (60). In contrast, in areas with low or unstable transmission of malaria, exposure is not constant enough to result in effective immunity in the population. In these settings, pregnancy outcome is severely compromised, and pregnant women of all parities are at greater risk of developing severe disease than non-pregnant women. Pregnant women living in low endemic areas experience high rates of abortion, stillbirth and low birth weight babies (47, 55, 57, 58, 79).

Most of our understanding of the biological basis for the increased susceptibility of pregnant women to malarial infection is from studies conducted with pregnant women living in high transmission areas (4, 21, 50, 73, 74). No detailed study has been done to understand the development of the maternal anti-malarial immune responses in settings of low endemnicity or during early pregnancy and the resultant effects on the mother and fetus. Experimental study of malarial infection during pregnancy is particularly problematic as ethical and logistical constraints limit the longitudinal sampling of pregnant women, and the placenta is inaccessible until delivery. Finally, pregnant women in malaria endemic regions often do not visit antenatal clinics early during pregnancy,

making it difficult to access women who abort early in gestation. An easily manipulable rodent model for malaria in pregnancy would be of great use in overcoming these limitations and improving our understanding of the immunological basis for the poor fetal outcome in non-immune pregnant women in areas of low endemnicity.

Early studies on the interrelationship of malaria and pregnancy in mice used *Plasmodium berghei.* These studies reported a more severe clinical course in pregnant animals with maternal mortality, fetal loss and reduced litter size (67, 68). This model, however, is not suitable to study the development of early maternal anti-malarial immune responses or the impact of malarial infection on early pregnancy because the infections were initiated on day 7 of pregnancy and were lethal to the mother (29, 48, 72). Further research to characterize the immunological and molecular basis of fetal loss in murine models for malarial infection during pregnancy has not been done. Thus, the recent advances in immunology and mouse genetics have not been applied to investigate the development of immune responses in malaria during pregnancy and their effect on fetal outcome. The rodent malarial parasite *P. chabaudi* AS represents a very useful organism for the study of immune responses to malaria as it shares many characteristics with the most virulent human malarial parasite, *P. falciparum*. Both have been shown to express variant antigens (35, 59), sequestrate in the heart, lung, and liver (23, 54) and bind to CD36 (32, 46). *P. chabaudi* AS infection in non-pregnant C57BL/6 (B6) mice has been well characterized and used extensively to dissect the immune response to blood-stage malaria.

In the present study the clinical consequences of experimental infection with *P. chabaudi* AS in pregnant B6 mice was investigated. In this model system, infected

pregnant mice developed splenic immune responses comparable to infected non-pregnant mice and survived the infection, but failed to maintain their pregnancies. Also, we report here for the first time the accumulation of *P. chabaudi* AS-infected erythrocytes in the murine placenta, a phenomenon that is associated with poor fetal outcomes.

MATERIALS AND METHODS

Mice, Parasites and Experimental Design: Two types of studies, a longitudinal and serial sacrifice, were performed to develop and characterize a mouse model for the immunopathogenesis of malaria during pregnancy. In both studies, 8-9 week-old, female C57BL/6J (B6) mice were used. Original breeding pairs purchased from Jackson Laboratories, Bar Harbor, ME were maintained and bred by brother-sister pairing for a maximum of ten 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. Mice were maintained on a 10 hours dark and 14 hours light cycle with feed and water *ad libitum.* To improve breeding efficiency pregnant mice were fed with a breeder diet (5K67) purchased from LabDiet, Richmond, IN. To avoid bias in weight gain, non-pregnant control mice were also fed with the same diet. The day on which a vaginal plug was observed in timed mated mice was considered as day zero of pregnancy (gestation day/GD 0).

P. chabaudi AS originally obtained from Dr. Mary M. Stevenson (McGill University and the Montreal General Hospital Research Institute, Quebec, Canada) maintained as frozen stock and by passaging through IFN-γ knock out (B6.129S7-*Ifng*^{tm1Ts}; obtained from R. Tarleton, University of Georgia) or A/J mice were used for all the experiments.

The first experiment was performed to characterize the course of *P. chabaudi* AS infection in pregnant mice. B6 (infected pregnant, IP) mice were injected intravenously on GD 0 with 1000 *P. chabaudi* AS-infected murine red blood cells (iRBC) per 20 grams of body weight. Control, infected, non-pregnant (INP) mice were similarly infected. Uninfected pregnant (UP) and uninfected non-pregnant (UNP) mice were intravenously sham injected with 200 μl of phosphate buffered saline per 20 grams of body weight as controls for pregnancy and handling. After recording the clinical parameters such as body weight, hematocrit and parasitemia on GD 0, pregnant mice were not handled until day 6 of pregnancy to avoid stress-induced blastocyst implantation failure. For consistency, other mice were also not handled on those days. Thereafter, body weights were recorded daily and hematocrit and parasitemia on alternate days and at sacrifice at GD/experiment day (ED) 18. No IP mouse maintained pregnancy to GD18 (see Results).

Because IP mice did not maintain pregnancy to term, two prospective serial sacrifice studies were conducted to assess the dynamics of malaria-induced fetal loss. Control INP, UP and UNP mice were included. In the first study, mice were sacrificed on GD/ED 6, 9, 12 and 15 and in the second, on GD/ED 6, 8, 9, 10, and 11. Clinical measures such as body weight, hematocrit and parasitemia were recorded as described above in the initial longitudinal study. In the second study, apart from recording the routine clinical measures, IP mice were observed thrice daily beginning on GD 8 to identify those in the early stages of abortion. Mice having bloody, mucoid vaginal discharge were considered to be in the early stages of abortion and were sacrificed immediately. IP mice were continuously generated until at least five mice per time point were obtained. Thus, different numbers of mice, ranging from 3 – 14, were sacrificed at

different time points. At sacrifice, non-viable fetuses or resorptions were identified by their necrotic appearance and notably smaller size compared to normal, viable fetuses. Resorption scars were identified by examining the uterus under a dissection microscope. To assess development of splenomegaly, spleens were collected aseptically from mice sacrificed on GD/ED 6, 8, 9, 10, 11 and 12 and spleen index was calculated by dividing spleen weight by body weight.

Assessment of infection: The development of parasitemia was monitored by counting at least 1000 erythrocytes in 4-5 high power fields on Giemsa-stained tail blood thin smears. Hematocrit was used as a measure of anemia. Blood collected from the tail vein into heparinized capillary tubes was centrifuged in a microhematocrit centrifuge and percent hematocrit was calculated according to the formula: volume packed erythrocytes/total blood volume) X 100. Body weight was recorded in grams.

Placental Parasitemia: Uteri collected from mice at the time of sacrifice were fixed in 10% buffered formalin for 24 hours and then paraffin embedded. Giemsa stained 2-3 μm thick placental sections were used to determine the placental parasitemia by counting at least 1000 erythrocytes in the maternal blood spaces in the placentas of at least five embryos.

Splenocyte Proliferation Assay: To make a single cell suspension, spleens collected aseptically from mice sacrificed on GD/ED 6, 9 and 12 were pressed through a sterile fine-wire mesh with 10 ml of RPMI 1640 (Cellgro, Herndon, VA 20171 USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), 200 mM L-glutamine (Cellgro, USA), penicillin (10,000 I.U / ml) and streptomycin (10,000μg / ml) (Cellgro, USA). Cell suspensions were centrifuged at

300×g for 10 min. Erythrocytes were lysed with Tris-buffered 0.175 M NH_4Cl, and the cells were washed twice in fresh medium. The viability of the cells was determined by trypan blue exclusion. One million splenocytes were then cultured in the presence of concanavalin A (ConA; 2 μg/ml), lipopolysaccharide (LPS; 1 μg/ml) and pokeweed mitogen (PWM; 2 μg/ml) (all from Sigma) in black 96-well microtitre plates for 72 h at 37°C in a humidified CO_2 incubator with an atmosphere of 5% CO_2. During the last 18 h of the culture the cells were incubated with BrdU labeling solution (Roche). After removing the labeling medium the cells were dried and the incorporation of BrdU was detected using Cell Proliferation ELISA, BrdU (Roche Molecular Biochemicals) using chemiluminescent detection (Lmax II Luminometer, Molecular Devices, Sunnyvale CA) following manufacturer's instructions.

Statistical Analysis: Unless otherwise noted, the SAS statistical software package (version 8.02; SAS Institute, Inc., Cary, N.C.) was used for data analysis. Proc GLM was used to analyze the significance of differences among group means for parasitemia, hematocrit, body weight, spleen index, spleen cell number and proliferative stimulation index. Duncan's Multiple Range Test was used to perform multiple pairwise group comparisons in cases of equal sample size; where sample sizes were unequal, Tukey's Studentized (HSD) Range Test was used. Comparisons of two groups with unequal variances and sample sizes were performed with Welch's ANOVA. Resorptions evaluated over time and survival were analyzed by a 2×2 contingency table and the significance determined by Fisher's exact test using GraphPad Instat software (version 2.05a; San Diego, CA). Student's t test was performed to analyze the significance of

differences in the number of viable fetuses between IP and UP mice and placental and peripheral parasitemias. P values of ≤ 0.05 were considered to be significant.

RESULTS

P. chabaudi AS infection increased the incidence of resorptions and abortions in pregnant C57BL/6 mice: Our initial longitudinal experiment revealed that B6 mice infected on GD 0 of pregnancy with 1000 *P. chabaudi AS* infected erythrocytes were able to recover from infection (Fig 1) with no significant differences in survival between IP and INP mice. Mortalities were observed only after GD/ED 11. Seven of 12 IP mice and three of three INP mice survived until GD/ED18 (vs survival of IP mice, P > 0.05). GD 18 was chosen an appropriate time point for assessment of pregnancy since the duration of gestation in mice is 19-21 days. Although most IP mice recovered from infection, none of them went on to deliver live pups. Of the 7 that progressed to GD 18, only three had any evidence of pregnancy, in the form of uterine resorption scars. This suggested that fetal loss had occurred at least several days earlier. (The pregnancy status of the other 4 animals could not be confirmed; thus, they were not included in any analysis.) The weight loss (Fig 1E) and vaginal bleeding during ascending and peak parasitemia exhibited by IP mice suggested that they might have actively expelled their embryos during this time. Since fetal loss is one of the most severe and under studied consequence of malarial infection in pregnant women living in low endemic areas, the effect of malarial infection at different stages of fetal development was examined in greater depth.

To identify the stage of gestation at which mice start losing their pregnancies, an initial serial sacrifice study was performed by sacrificing mice on GD/ED 6, 9, 12 and 15. This study revealed that IP mice failed to maintain viable pregnancies up to GD 12

(Table 1) and the few IP mice that did proceed to GD 15 had only necrotic/resorbing embryos in their uteri (data not shown). To further characterize the events leading to fetal loss, a second, more detailed serial sacrifice study was performed with mice being sacrificed on GD 6, 8, 9, 10 and 11 with heightened surveillance beginning on GD 8 to identify the mice in early stages of abortion. Abortions occurred at GD 10.3 ± 1.0 (mean ± SD). The fetal outcome results pooled from the two serial sacrifice studies are presented in Table 1. At sacrifice, mice with vaginal bleeding were observed to have an open cervix with embryos in the cervix or vagina, demonstrating that they were actively expelling their embryos. IP mice had a significantly higher number of fetal resorptions and abortions compared to uninfected pregnant mice on GD 10 through 12. IP mice had 37.4% and 38.8% resorptions on GD 10 and 11, and 100% resorptions on GD 12 of pregnancy compared to 0, 5.1, and 3.3% in the UP group, respectively ($P = 0.0001$). Compared to UP mice the number of viable fetuses was significantly reduced in IP mice on GD 12 ($P = 0.0001$). Thus, *P. chabaudi* AS infection abrogated pregnancy in B6 mice, with only nonviable fetuses and resorption scars being present in IP mice from GD 12 onwards.

Development of parasitemia and anemia is accelerated in P. chabaudi AS-infected pregnant mice: To study to what extent pregnancy impacts the course of *P. chabaudi* AS infection, development of parasitemia, anemia and changes in body weight were studied in the four groups of mice. The results from the initial longitudinal study are presented in Fig 1 (A, C, E). Despite the fact that IP mice lost their pregnancies around GD 10, they were retained in this group for the purposes of analysis. Parasitemia was patent in IP mice by GD6, was more than double that of INP mice on GD/ED8 ($P > 0.05$), and peaked

at 22.35% on GD10. In contrast, peak parasitemia (19.62%) was observed on ED 12 in INP mice. However, parasitemia resolved after GD/ED 12 in all mice, decreasing to less than 2% on GD/ED 16. Since IP mice did not maintain pregnancy after GD 12, it is not possible to conclude from this study whether or not peak parasitemia and resolution of *P. chabaudi* AS infection differ in the context of pregnancy in B6 mice.

Hematocrit values measured on alternate days were used as an indicator of anemia. Both IP and INP mice experienced profound reductions in hematocrit during the course of infection (Fig. 1C). Development of malarial anemia was accelerated in the IP group compared to the INP group, with the percent hematocrit values being significantly lower in IP mice compared to the UP and UNP groups on GD/ED10. However, the hematocrits of both infected groups reached their nadir on GD/ED 12 (13.67 % for INP versus 14.0 % for IP mice, $P > 0.05$) and were significantly lower than those in the UP and UNP groups ($P < 0.05$). These significantly lower hematocrits persisted throughout the remainder of the experiment. Interestingly, the hematocrits of the UP mice were also significantly lower than those of UNP mice on GD/ED 6 ($P < 0.05$). This was likely due to hemodilution associated with normal pregnancy (18).

Body weights of the mice were recorded daily and are represented in Figure 1E. UP mice exhibited a steady increase in body weight starting on GD 10 from which time their body weights were significantly higher than all other groups ($P < 0.05$, GD/ED 10-18). IP mice also exhibited an increase in body weight during the initial stages of pregnancy presumably due to the initial fetal developments. However, both infection groups lost weight as parasitemia rose and hematocrits fell. Also, some reduction in body weight in IP mice was likely due to fetal resorption. IP and INP groups regained weight

as they started to resolve the infection. As expected, UNP mice maintained their body weights throughout the experimental period.

In the initial longitudinal study, the clinical parameters such as development of parasitemia and anemia were monitored only on alternate days. To further characterize the course of infection, the development of parasitemia and anemia were monitored daily in the serial sacrifice study starting from GD/ED 8 and the experiments were terminated on GD/ED 12, the time point at which no viable fetuses were evident in IP mice (Table 1). Similar to the longitudinal study, the development of parasitemia was accelerated in IP mice and was significantly different from INP mice on GD 8 ($P = 0.0004$) (Fig 1 B). However, peak parasitemia was observed on GD/ED 11 in both IP (27.17%) and INP (25.04%) mice ($P > 0.05$). The discrepancy in the results for peak parasitemia between this and the longitudinal study is likely due to our failure to prepare blood smears on GD/ED 11. Anemia also developed earlier in IP mice and was significantly different from all the groups on GD 8, 9 and 10 ($P < 0.05$). However, hematocrit values for all infected mice were statistically significantly lower than that of UP and UNP mice on GD/ED10 post infection and reached their nadir on day 12 post-infection (INP: 11.88 %, IP: 11.33%; versus uninfected mice, $P < 0.05$).

As in the longitudinal study, IP mice exhibited an initial increase in body weight which was significantly different from INP mice on GD/ED 6 through 9 ($P < 0.05$). By GD/ED 8, IP mice gained, on average (± SD), 1.2 ± 1 g (6.8 ± 6.3 % of average GD 0 body weight). However, as the infection progressed, both infected groups lost weight compared to UP mice on GD 9 through 12 ($P < 0.05$). The lowest body weights for IP and INP mice were recorded on GD 12 (8.8 ± 15.3 % and 13.4 ± 5.4 % loss in IP and INP

mice, respectively, compared to average GD 0 weight). Compared to peak body weight on GD 8, IP mice lost 13.1 ± 9.8 % by GD 12. This loss of body weight in IP mice could be due to a combined effect of malaria-induced cachexia and fetal loss. In contrast to IP mice, UP mice exhibited a steady increase in body weight starting at GD 6, increasing 9.1 ± 5.7 % and 31.1 ± 13.1 % at GD 8 and 12, respectively, relative to starting weight.

Accumulation of P. chabaudi A- infected erythrocytes in the placentae of pregnant mice: Sequestration of *P. chabaudi* AS-infected erythrocytes has been reported in the heart, liver, lungs and spleen of infected mice (13, 23), and sequestration of *P. falciparum* is associated with poor fetal outcome in human pregnancy (49). Thus, it was of interest to determine whether the observed pregnancy loss in *P. chabaudi* AS-infected pregnant mice is associated with an accumulation of iRBCs in placentae. To investigate this possibility, placental parasitemias scored on Giemsa-stained, 2 μm thick placental sections were compared with the corresponding peripheral parasitemias. Indeed, there was massive accumulation of *P. chabaudi* AS-infected erythrocytes in the maternal sinusoids of the placentae (Fig. 2) with placental parasitemias in GD 10 and 11 mice being statistically significantly higher than in the peripheral blood (41.9 ± 12.7 % versus 22.4 ± 8.0 %; P = 0.003). To characterize the dynamics of placental accumulation of iRBCs, parasitemias were determined in mice on GD 9 (a day before abortions are observed), GD 10 (both aborting and non-aborting) and GD 11 (all aborting) of pregnancy (Fig. 3). Whereas GD 9 parasitemias were low in both placental and peripheral blood, parasitemias in placental sections from mice undergoing abortions on GD 10 and 11 were > 40% higher than in the peripheral blood (47.1% and 42.4% versus 27.1% and 24.4%, respectively). A trend toward higher placental parasitemia in GD 10 non-aborting

mice was not statistically significant ($P > 0.05$). These data demonstrate that there is an accumulation of *P. chabaudi* AS-infected RBCs in the placentae of infected mice, with statistically significantly higher levels than in the peripheral blood being found only in mice undergoing abortion. Contrary to what has been reported in the placentae of malaria infected pregnant women (22, 45, 74), preliminary histopathological analysis revealed little accumulation of monocyte/macrophages in the placentae of IP mice (Poovassery *et al*., unpublished data).

Splenic function in P. chabaudi AS infected pregnant mice: Pregnancy-associated immunomodulation is thought to play an important role in the increased susceptibility of pregnant women to malarial infection. To assess the immunocompetence of IP mice, changes in spleen size and cellularity as well as proliferative response of splenocytes in response to mitogens at different stages of infection were evaluated. To compensate for significant differences in the body weights of the pregnant and non-pregnant mice, splenomegaly was evaluated using spleen index (see Materials and Methods). For UNP mice, spleen parameters were recorded only on ED 6, 9 and 12. A comparison of the spleen indexes among the four groups is presented in Figure 4A. Although both pregnant groups showed some level of persistent splenomegaly beginning on GD/ED 6 (both versus UNP, $P < 0.05$, days 6, 9 and 12), *P. chabaudi* AS infection, regardless of pregnancy, resulted in large, progressive increases in splenic index beginning on GD/ED 9 (compared to uninfected mice, days 9 and 12, $P < 0.05$).

Spleen cell number was also high in infected mice (Fig. 4B), increasing dramatically from GD/ED 8 compared to uninfected mice. Whereas splenocyte count in INP mice persisted at an elevated but constant level from day 8 to 11, spleen cellularity

peaked on GD 9 in IP mice ($9.46 \pm 1.3 \times 10^7$); this value was significantly different from UP ($2.93 \pm 0.4 \times 10^7$), UNP ($1.53 \pm 0.3 \times 10^7$), and INP mice ($4.93 \pm 0.8 \times 10^7$) mice ($P < 0.05$). This enhancement of spleen cell number in IP mice was at least in part pregnancy-related, since UP mice also had a higher splenocyte count compared to UNP mice, beginning at GD/ED 6 (GD/ED 6 and 12, $P < 0.05$). Overall, with the exception of a transient, accelerated expansion of spleen cellularity at GD/ED 9 in IP mice, increases in both spleen weight and cellularity were comparable in IP and INP mice.

Splenocyte function was assessed by proliferative response to mitogenic stimulation (Fig. 5). At day 6 of infection, responses were comparable among the four experimental groups. However, at time points corresponding to ascending and peak parasitemia, responses in infected mice decreased relative to uninfected mice. Proliferation in response to LPS stimulation was significantly higher in UNP mice compared to INP mice on ED 9 and 12 ($P < 0.05$), and to IP mice on GD/ED 9 ($P < 0.05$). Proliferation of splenocytes from the IP and INP groups in response to ConA and PWM was also statistically significantly lower than that of the UNP group on GD/ED ($P < 0.05$). A tendency for IP mice to have a higher LPS response compared to INP mice on GD 12 was not statistically significant ($P > 0.05$).

DISCUSSION

In non-immune pregnant women malarial infection during the first or second trimester has been reported to be associated with high rates of abortion (42). No detailed study has been done, however, to investigate anti-malarial immune responses during early pregnancy in the absence of pre-existing immunity or the effect of these responses on fetal outcome. Some progress toward this end has been made using non-immune,

pregnant rhesus monkeys infected with *Plasmodium coatneyi* (16). In this model, infected monkeys experienced increased rates of abortion and intrauterine growth retardation associated with placental pathology (15, 16). Also the leukocyte profiles were altered in infected pregnant monkeys, with lower CD4+ and CD8+ T cell and B cell counts that were suggestive of pregnancy-associated immunomodulation (17). As an alternative approach, we have developed a mouse model for the study of adverse fetal outcomes associated with malaria. The short gestation time, availability of inbred, congenic and gene knock-out mice, and the well-characterized immune system of the laboratory mouse allow us to address questions and undertake experiments that due to ethical, financial and biological constraints cannot easily be performed in humans or non-human primates.

In this model system, both IP and INP B6 mice survived *P. chabaudi* AS infection. Although the development of parasitemia and anemia was accelerated in IP mice, there was no significant increase in peak parasitemia or reduction in hematocrit as a function of pregnancy. Regardless of whether or not mice were pregnant at the beginning of the experiment, all were able to ultimately control parasitemia, reducing it to less than 2% by ED 16. The 2-3 day delay observed in development of patent and peak parasitemia in this study compared to most published literature on *P. chabaudi* AS infection in B6 mice is likely due to the difference in the size of the inoculum (10^3 versus 10^6) and the difference in the route of infection (intravenous versus intraperitoneal) (10).

The clinical outcome observed in IP mice was different from previous observations made in other rodent model systems for malaria during pregnancy, which showed a more severe course for pregnant animals (19, 29, 48, 72). Differences in rodent strain and parasite species, inoculum size, and the gestation day on which infection was

initiated may be responsible for the observed differences between those studies and the current results. *P. chabaudi* AS infection has been shown to result in non-lethal infections in mouse strains such as BALB/c, C57BL/6 and C57BL/10 mice and lethal infections in A/J and DBA/2 mice (14, 38, 61). B6 mice develop moderate levels of acute primary parasitemia and resolve the infection by 4 to 5 weeks post-infection by mounting a Th1 cytokine-biased immune response early during the course of infection (14, 63, 82). In contrast, susceptible A/J mice mount an early T helper 2 (Th2) cytokine-biased immune response and succumb to infection by 10 to 12 days post infection (53, 63).

It has been well demonstrated that successful pregnancy requires a bias against Th1-type and toward Th2-type cytokines (24, 52, 75). Since the survival rate of IP mice was comparable to INP mice, it is likely that the former also developed a proinflammatory/Th1 cytokine-biased immune response early during the infection to control the parasitemia, but at the expense of their pregnancies. It is noteworthy that malaria-induced abortion in *P. vinckei*-infected mice was shown to be dependent on pro-inflammatory tumor necrosis factor-α (11). The results of the present study are consistent with that finding in that viable pregnancy in IP mice was completely abolished. Most failed to carry their fetuses beyond mid-gestation, with higher rates of fetal resorption and abortion compared to UP mice, and none delivered live, term pups. Spontaneous abortions during the first trimester have been reported in *P. coatneyi*-infected, non-immune, pregnant rhesus monkeys (16) and in *P. falciparum*-infected, non-immune pregnant women (42, 47, 58, 79). However, the immunologic mechanisms that lead to fetal loss during such non-immune malarial infections remain to be fully elucidated.

To begin to address this issue, we assessed the development of splenomegaly as well as the splenocyte proliferative response in the presence of mitogens in infected and uninfected mice. In murine models it has been shown that during acute malarial infections the spleen plays important roles in parasite clearance (77), development of pathogen-specific T and B cell responses (36, 81), and provision of strong hematopoietic support (71, 78). Spleen cellularity and architecture also change dramatically during malarial infection (27, 62). Depending on the mouse strain and parasite species, these changes have been shown to be associated with either resistance or susceptibility to infection. Thus, development of massive splenomegaly has been found to correlate with resistance to *P. chabaudi* AS infection in resistant B6 mice but not in susceptible A/J mice (62). In agreement with this, both IP and INP B6 mice developed massive splenomegaly and, in a longitudinal study, survived the infection albeit, for IP mice, in the absence of viable pregnancy. Also, contrary to expectation, IP mice had the highest splenocyte counts at one time point corresponding to ascending parasitemia. However, after the initial peak on day 9, both IP and INP mice exhibited a decrease in splenocyte count after peak parasitemia, as reported in the case of *P chabaudi* AS-infected BALB/c mice (27).

In vitro proliferation of splenocytes isolated from both IP and INP mice during the acute phase of the infection was significantly reduced compared to uninfected mice. Malaria patients frequently show reduced immune responses not only to the malaria parasite, but also to unrelated antigens (31, 70, 80), suggesting that an active immunosuppressive mechanism may be operating during the course of malarial infection. One possibility is nitric oxide-mediated suppression of splenocyte proliferation, as was

reported for responses to ConA in *P. chabaudi* AS-infected B6 mice (34). This has also been demonstrated in other protozoan infections (28, 76). Furthermore, it is noteworthy that NO has been linked to pregnancy loss in mice (26). Recently, it was suggested that CD4+CD25+ regulatory T cells may be involved in the immunosuppression observed with *P. yoelii* strain 17XL infection in BALB/c mice (30). Apart from the mechanisms proposed in non-pregnant mice, pregnancy-specific immunosuppression may be also operating in IP mice prior to pregnancy loss (69).

Despite low splenocyte proliferative responses, both IP and INP mice survived *P. chabaudi* AS infection, suggesting that in vitro proliferative response of splenocytes is not a good indicator of the ability of infected mice to mount an effective immune response to malarial infection. As demonstrated in the case of non-pregnant B6 mice, an early proinflammatory/Th1 cytokine biased immune response may be relatively more important for protection in IP and INP mice (64). This, however, may not translate to immune responses that can clear parasites from the placenta. Ultimately, it will be necessary to investigate in detail the immunological events occurring in the placental environment and in the spleen of *P. chabaudi* AS-infected pregnant mice to fully elucidate the protective and pathogenic immune mechanisms at play in pregnant mice. Furthermore, development of an experimental system that allows IP mice to progress to term pregnancy will be necessary for investigation of pregnancy-associated alterations in disease course and immune patterns throughout gestation. In this context, it will also be of value to assess the course of infection and outcome of pregnancy in previously malaria-exposed mice, all of which are currently being addressed in our laboratory.

Although the peak peripheral parasitemia was not significantly different between IP and INP mice, placental parasitemia was > 40% higher than peripheral parasitemia in IP mice at the time of abortion, which effectively translates to a higher total parasite load in these animals. Sequestration of infected erythrocytes in several organs has been reported in *P. chabaudi* AS-infected non-pregnant mice (13, 23). Furthermore, the *in vitro* binding of the *P. chabaudi* AS-infected RBCs to endothelial cells has been shown to be mediated through CD36 (a well-characterized receptor utilized by *P. falciparum*-infected erythrocytes to bind to endothelial cells) (32), but other receptors are likely to be involved as well (46). Sequestration of *P. falciparum*-infected erythrocytes in the placental intervillous blood space is a key feature of malarial pathogenesis in pregnant women (49, 74) and is thought to be mediated largely through interaction with chondroitin sulphate A (CSA) (21). The accumulation of *P. chabaudi* AS-infected erythrocytes in the placenta of infected mice, which has not been described before, may be a manifestation of specific placental sequestration. It is noteworthy that, like human trophoblast, murine trophoblast expresses a low sulfated chondroitin sulfate (C. Gowda, personal communication). Clearly, further detailed studies of the interactions between *P. chabaudi* AS iRBCs and fetal trophoblast cells are required to define the biological mechanisms of placental parasite accumulation in mice and the pathogenic implications thereof. Our laboratory is currently investigating the impact of iRBC binding to trophoblast on immunopathological events at the human (39) and murine maternofetal interfaces.

As reported in the case of *P. chabaudi* AS-infected non-pregnant B6 mice, both IP and INP mice experienced profound anemia (14). Although the lowest hematocrit

levels and the day on which these levels were reached were not different between IP and INP mice, the development of anemia was faster in IP mice. Because UP mice also developed some anemia, it is likely that the faster rate in IP mice was not entirely malaria specific. Pregnancy-associated hemodilution has been reported in rats (18, 37) and pregnant women (7). In general, several factors may contribute to the complex process of anemia during malarial infection. Sequestration of infected RBCs (2), rupture of iRBCs during schizogony, development of autoantibodies (1, 25, 43) and ineffective erythropoiesis (12, 51, 83) may all contribute. Furthermore, it was recently suggested that the proinflammatory cytokine macrophage migration inhibitory factor (MIF), may play important role in malarial anemia (40). It is of interest that MIF expression is massively upregulated in the placentae of malaria-infected pregnant women (9) and is specifically secreted by trophoblast bound by *P. falciparum*-infected RBCs (8). Further study will be required to determine the relative roles of all of these factors, particularly that of MIF, in anemia and other protective and pathogenic immune mechanisms during pregnancy in B6 mice.

In addition to immune responses and placental accumulation of parasites, anemia may play a role in the observed pregnancy loss in this model. However, most of the abortions occurred between days 10 and 11, which is one to two days before peak anemia. Additionally, while anemia has been shown to be associated with low birth weight (6) and pre-term labor (65), it is not associated with fetal loss in rodents or in humans. Furthermore, for cases of low birth weight among women with severe malarial anemia (<7g/dL; 36% below normal level), Brabin and Piper (6) calculated that anemia alone can account for only about 10% of infant low birth weight cases, whereas malaria

(with all associated immunopathogenic effects), can account for 40%. Thus, factors other than anemia likely play dominant roles in inducing pregnancy loss in *P. chabaudi* AS-infected B6 mice. We are actively pursuing this line of investigation.

In conclusion, this study shows that *P. chabaudi* AS infection leads to poor pregnancy outcomes in B6 mice. Although the splenocytes from both IP and INP mice exhibited reduced proliferation in response to mitogens compared to UNP mice, both IP and INP mice exhibited a comparable increase in spleen size and cell number during the course of infection, and all survived the infection, albeit in the absence of viable post-midgestational pregnancy for IP. This suggests that IP mice develop peripheral anti-malarial immune responses that are sufficient to control parasitemia during primary infection. Despite effective control of peak parasitemia, IP mice experienced massive accumulation of iRBCs in their placentae and failed to maintain their pregnancies beyond GD 12. This suggests that while peripheral parasitemia is controlled immunologically, this response is not sufficient to control localized placental parasitemia, but may, paradoxically, contribute to fetal loss. Continued characterization of this model will contribute significantly to our understanding of the molecular and cellular immunological mechanisms involved in fetal loss during malarial infection.

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Figure 3.1.

Parasitemia, hematocrit and weight change in longitudinal and serial sacrifice studies of *P. chabaudi* AS-infected and control B6 mice. Eight-nine week old C57BL/6 mice were injected intravenously with 1000 *P. chabaudi* AS-infected erythrocytes or 200 µl of PBS (control mice) per 20 grams of body weight on GD/ED 0. Percent parasitemia (A, B) from Giemsa-stained thin smears, hematocrit (C, D) of tail vein blood and weight (E, F) were assessed at one-two day intervals as shown. Mice were divided into four groups: uninfected, nonpregnant (UNP), infected, nonpregnant (INP), uninfected, pregnant (UP), and infected, pregnant (IP). Groups were either followed longitudinally for 18 days (n = 3 for all groups; A, C, E), or sacrificed at days 6, 8, 9, 10, 11 and 12 in two serial sacrifice studies (B, D, F). Although IP mice aborted, resorbed or had only dead embryos in their uteri by GD 12, for the sake of clarity and illustration of point, mice in this group are retained as such for the whole of the longitudinal experiment. For serial sacrifice studies, clinical parameters were measured on mice as described in Materials and Methods until day of sacrifice. Starting sample sizes: UNP = 9, INP = 39, UP = 56, and IP = 67. Number of mice sacrificed (INP, UP, IP) at each GD/ED; GD/ED 6: 8, 12, 14; GD/ED 8: 5, 10, 8; GD/ED 9: 8, 13, 13; GD/ED 10: 5, 5, 14; GD/ED 11: 5, 9, 11; GD/ED 12: 8, 7, 7. Three UNP mice were sacrificed at ED 6, 9 and 12. All data presented are mean ± standard error of the mean (SEM). The Y axis on E, F begins at 15 g to avoid compression and poor visualization of the data. Statistical differences, all $P < 0.05$, Proc GLM, Tukey. Longitudinal study: hematocrit, GD/ED 6, UP versus UNP; GD/ED 10: IP versus uninfected; GD/ED 12, 14: infected versus uninfected; GD/ED 16: IP versus UNP; GD/ED 18: INP versus IP and UNP. Weight, GD/ED 6: UNP versus UP;

GD/ED 7, 8: UNP and INP versus UP; GD/ED 10-18, all groups versus UP. Serial sacrifice studies: parasitemia, GD/ED 8, INP versus IP, P = 0.0004, Welch's ANOVA. Hematocrit: GD/ED 8-10: IP versus all groups; GD/ED 10: INP versus all groups; GD/ED 11: infected versus UP; GD/ED 12: infected versus uninfected. Weight: GD/ED 6, 8: INP versus IP and UP; GD/ED 7: INP versus all groups; GD/ED 9: IP versus INP; GD/ED 9-12: infected versus UP; GD/ED 11, 12: INP versus UNP.

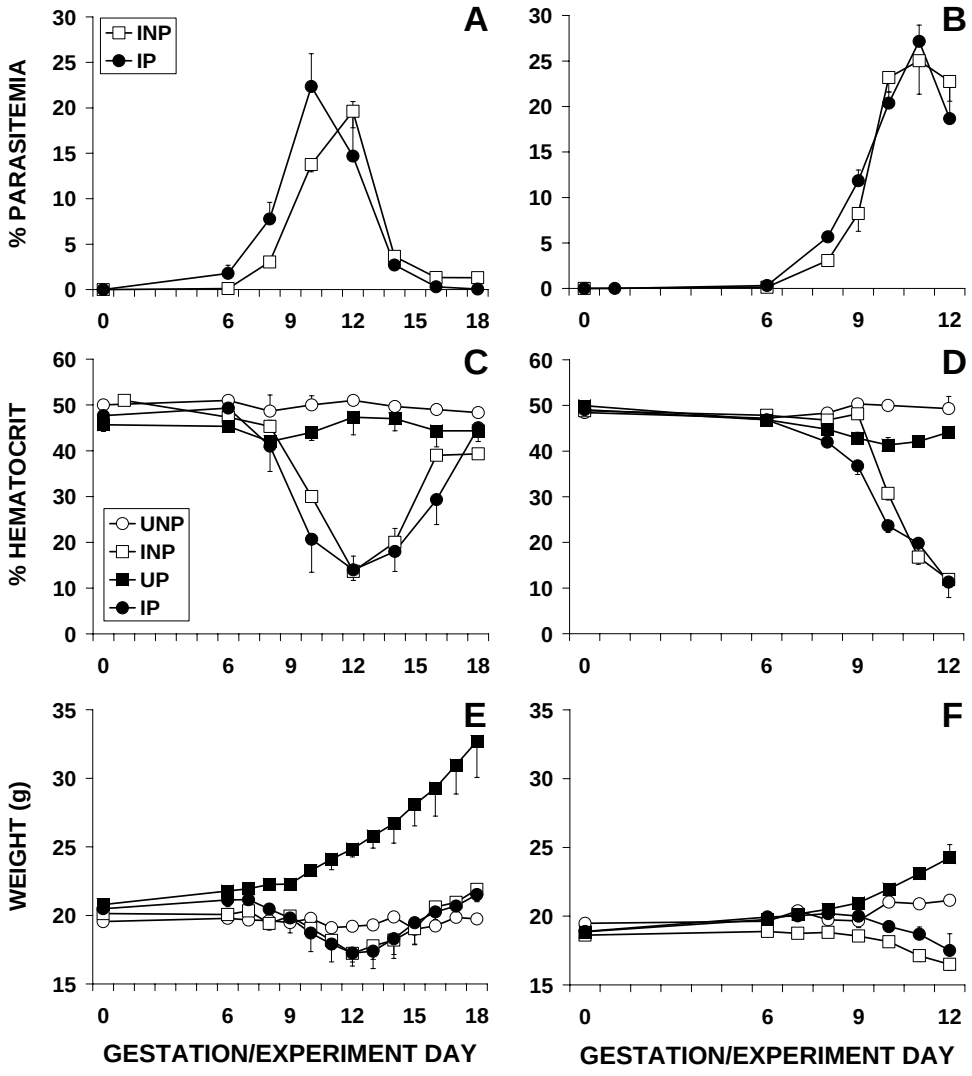
A
B
C
D
E
F
INP
IP
UNP
UP
% PARASITEMIA
% HEMATOCRIT
WEIGHT (g)
GESTATION/EXPERIMENT DAY
GESTATION/EXPERIMENT DAY

Figure 3.2.

***P. chabaudi* AS-infected erythrocytes in the placenta of an aborting mouse.**

(A) Giemsa-stained placental section (2 μm thick) from IP mouse undergoing abortion on GD/ED 11 showing infected erythrocytes in the maternal blood spaces (arrows). (B) Giemsa-stained placental section from UP mouse at the same time point showing normal RBCs in the maternal sinusoids. MS: maternal sinusoid, N: giant cell nucleus. Photographs were prepared using Adobe Photoshop, v 8.0.

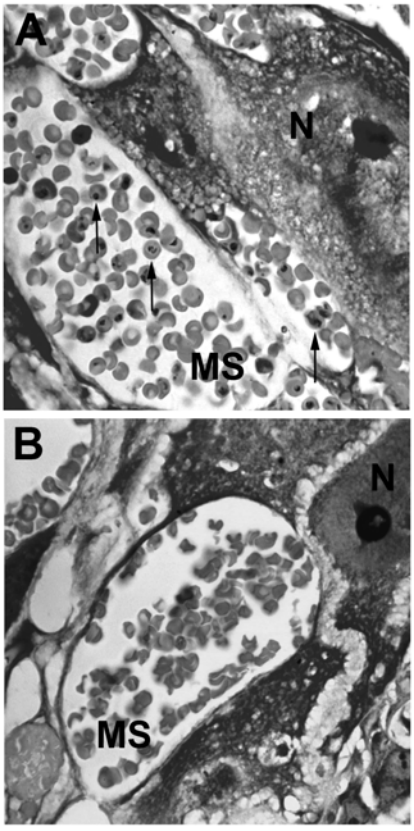
A
N
MS
B
N
MS

Figure 3.3.

Comparison of placental and peripheral parasitemia in aborting and non-aborting IP mice.

Placental parasitemias were scored in Giemsa-stained, 2 μm thick placental sections by counting at least 1000 RBCs in the maternal blood spaces. Peripheral parasitemias were determined in Giemsa-stained tail blood smears. N = 5 at each time point per group except for day 9 (n = 3). ∗ P = 0.003, Student's t test.

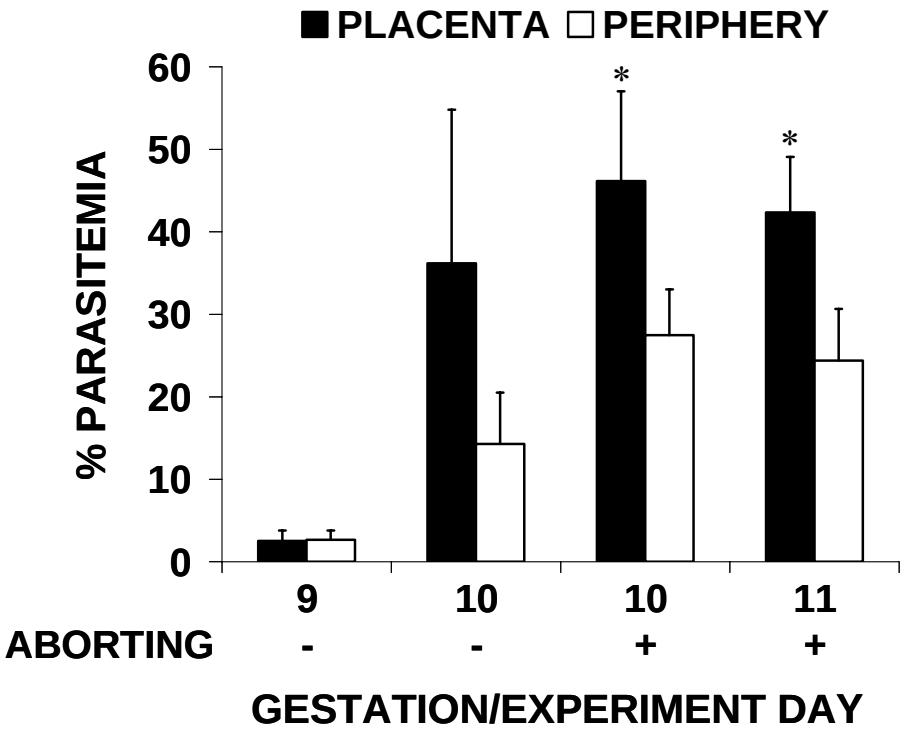
PLACENTA
PERIPHERY
% PARASITEMIA
60
50
40
30
20
10
0
*
*
9
10
10
11
ABORTING
-
-
+
+
GESTATION/EXPERIMENT DAY

Figure 3.4.

Changes in spleen index and cell number in experimental mice.

Mice were experimentally manipulated as indicated in legends to Figures 1 and 2. (A) Spleen index is calculated as a proportion of spleen weight to total body weight. (B) Cell number was calculated from isolated splenocytes enumerated by trypan blue exclusion. Data are pooled from two replicate experiments and are presented as mean ± SEM of five to ten mice for the IP, INP and UP groups (n = 4, IP day 12) and three UNP mice analyzed individually per time point. Spleen index: Day 6: UNP versus IP, UP; day 8: UP versus IP; days 9, 12: both UNP and UP versus all groups; days 10, 11: UP versus IP and INP; $P < 0.05$, Proc GLM, Tukey. Spleen number: day 6: INP and UNP versus UP, and UNP versus IP; *day 8: UP versus INP; day 9: UNP versus INP and IP versus all groups; *day 11: IP versus UP; day 12: UNP versus all groups; all $P < 0.05$, Proc GLM, Tukey and *Dunnett's T3.

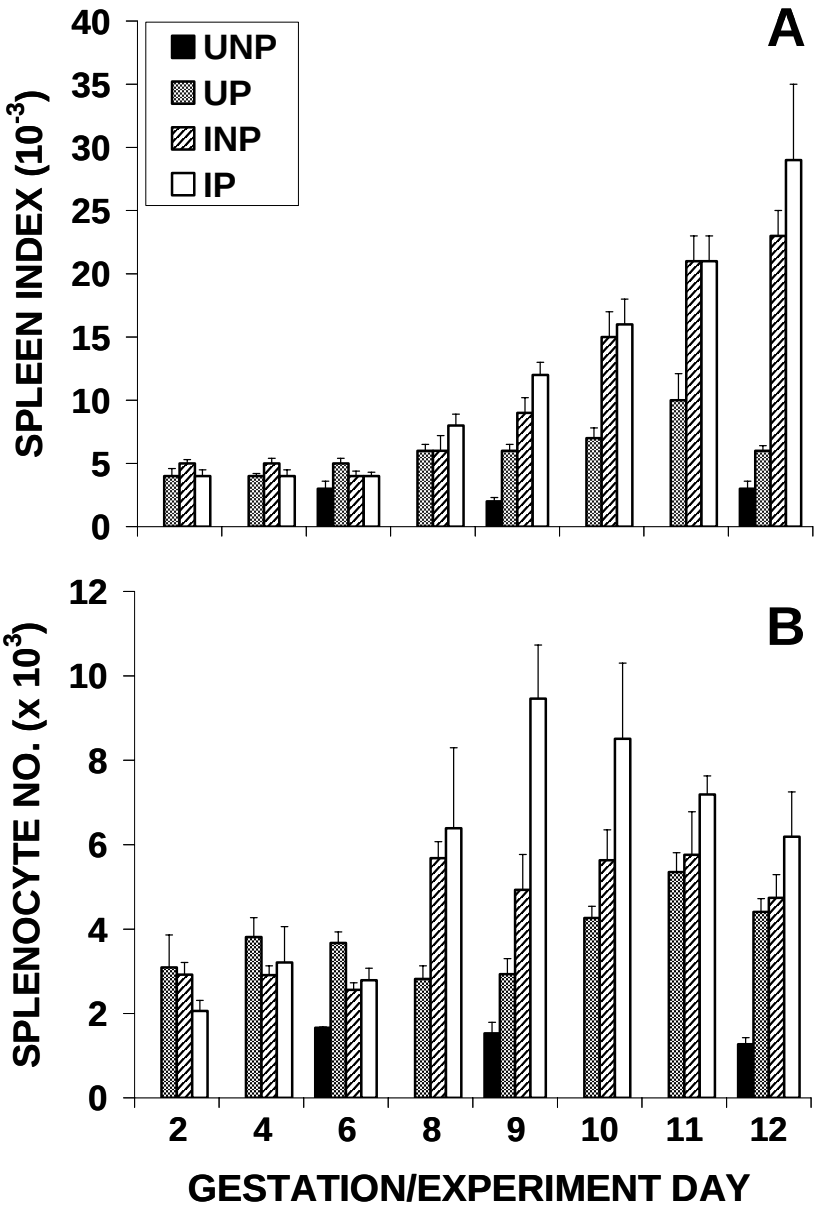
A
UNP
UP
INP
IP
SPLEEN INDEX (10-3)
40
35
30
25
20
15
10
5
0
B
SPLENOCYTE NO. (x 10^3)
12
10
8
6
4
2
0
2
4
6
8
9
10
11
12
GESTATION/EXPERIMENT DAY

Figure 3.5.

Proliferative response of spleen cells in experimental mice.

Spleen cells from *P. chabaudi A*-infected pregnant, infected non pregnant and uninfected controls collected aseptically on the days indicated were cultured with ConA (2 μg/ml), PWM (2 μg/ml) and LPS (1 μg/ml) or medium as the control. BrdU uptake was measured and data shown are means ± SEM for three mice per group. *INP and IP versus UNP, **INP versus UNP, $P < 0.05$, Proc GLM, Duncan.

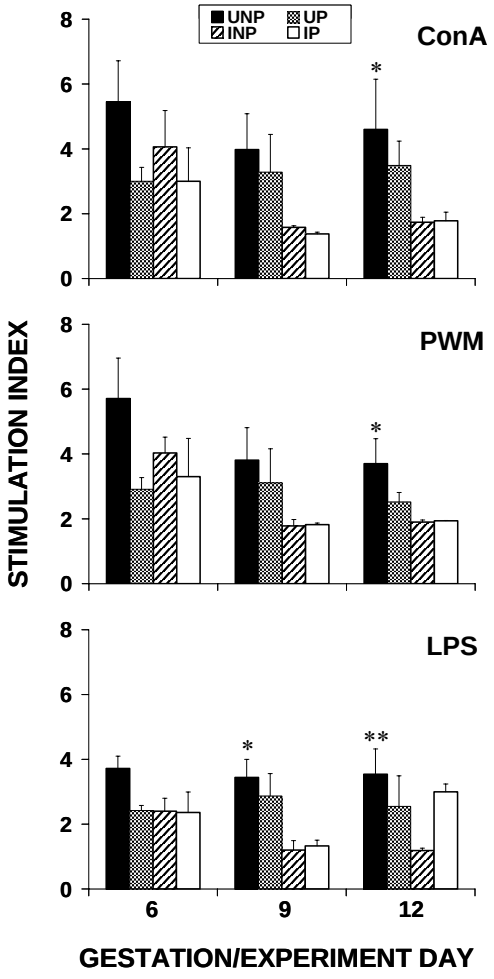
UNP
UP
INP
IP
ConA
PWM
LPS
STIMULATION INDEX
GESTATION/EXPERIMENT DAY
0
2
4
6
8
6
9
12
*
*
**

Table 3.1

Fetal outcome in *P. chabaudi* AS-infected pregnant and uninfected pregnant mice.

Mice were sacrificed on days indicated to assess pregnancy success. Resorptions were scored by examination of uteri under a dissection microscope. [a]Gestation Day; [b]total number of resorptions per total number of fetuses; [c]Fisher's exact test; [d]mean # of viable fetuses; [e]Student's t test.

Table 3.1

GD[a]	IP resorp-tions[b]	*N*	UP resorp-tions[b]	*N*	P value[c]	IP viability[d]	UP viability[d]	P value[e]
6	1/99	14	1/99	12	0.75	7.36	8.25	0.99
8	0/64	8	0/78	10	N/A	8.00	7.80	0.77
9	3/108	13	1/86	13	0.40	8.07	6.61	0.08
10	40/107	14	0/38	5	0.0001	4.40	7.60	0.11
11	38/98	11	3/59	9	0.0001	5.45	7.37	0.07
12	30/30	4	2/60	7	0.0001	0.00	8.57	<0.0001

CHAPTER 4

MALARIA-ASSOCIATED MURINE PREGNANCY FAILURE. I: ASSOCIATION WITH ROBUST PERIPHERAL AND PLACENTAL CYTOKINE RESPONSES[1]

[1] Jayakumar Poovassery and Julie M. Moore. To be submitted to Journal of Immunology.

ABSTRACT

Malarial infection in non-immune pregnant women is a major risk factor for pregnancy failure. In a recent study we have reported that experimental *Plasmodium chabaudi* AS infection completely abrogates pregnancy in C57BL/6 (B6) mice by gestation day 12. To further characterize the protective and pathogenic immune responses to malaria, the pattern of cytokine production in plasma, spleen and placenta cell culture supernatants during the first 11 days of infection and gestation were studied. Regardless of pregnancy, the systemic levels of proinflammatory cytokines IFN-γ, TNF-α and IL-1β were elevated in infected mice compared to uninfected, pregnant mice. IFN-γ and TNF-α levels peaked on gestation / experiment day 9 in infected, pregnant and infected, nonpregnant mice and the highest IL-1β response was observed in mice on gestation day / experiment day 10 preceding by 1 to 2 days the onset of malaria associated fetal loss. Furthermore, TNF-α and its soluble receptor II were higher in aborting mice and the soluble TNF receptor II level exhibited a positive correlation with parasitemia. Although infected mice also produced the anti-inflammatory cytokine IL-10 in the plasma and splenocyte cell culture supernatants, infected, pregnant mice failed to produce IL-10 at the placental level. On histological examination, trophoblast giant cells from mice undergoing abortion exhibited massive phagocytosis of infected erythrocytes and hemozoin. Furthermore, cultured trophoblast cells isolated from gestation day 7 embryos were able to phagocytose *P. chabaudi* AS-infected erythrocytes and produced TNF-α in the culture supernatants. Taken together these results suggest that a proinflammatory anti-

malarial immune response, particularly one that is not counter-regulated by IL-10 at the placental level, may contribute to fetal loss in this model.

INTRODUCTION

Malarial infection during pregnancy is a major risk factor for maternal and infant morbidity and mortality. Epidemiological studies have shown that the 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 (1, 2). The precise mechanisms involved in malaria-induced fetal loss have not been identified.

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 response and pregnancy. In this model C57BL/6 (B6) mice that are infected on day 0 of gestation (GD 0) with 1000 *Plasmodium chabaudi* AS-infected erythrocytes developed peak parasitemia and anemia comparable to infected nonpregnant (INP) mice and survived the infection. However, infected pregnant (IP) mice failed to maintain viable pregnancies after GD 11 (3). Although the immune responses to *P. chabaudi* AS infection are well characterized in INP mice, nothing is known about the development of immune responses in *P. chabaudi* AS-infected pregnant mice or how this may affect the pregnancy outcome.

Both $CD4^+$ T cell and B cell responses are shown to be critical in protection against primary *P. chabaudi* AS infection in INP mice. While production of

proinflammatory cytokines IL-12, IFN-γ and TNF-α (4) early during the infection is thought to be essential to control the parasitemia, parasite clearance during the chronic stage is dependent on B cells and antibodies, especially of Th1-associated subclassess IgG2a and IgG3 (5). However, it has been demonstrated in rodent models that normal pregnancy requires a bias against Th1-or towards Th2-type cytokine responses (6, 7). While production of Th2-type cytokines such as IL-10 and IL-4 locally at the materno-fetal interface is thought to favor the maintenance of pregnancy (8, 9), an excessive production of Th1/proinflammatory cytokines such as IL-2, IL-6, IFN-γ and TNF-α can mediate fetal rejection (10-13). Proinflammatory cytokines IL-2, IFN-γ and TNF-α are implicated in recurrent spontaneous abortion in humans (14).

Several pregnancy complications such as preterm labor and pre-eclampsia are shown to be strongly associated with infections (15, 16), and infection during pregnancy, including malaria, is a major factor that can alter the cytokine balance at the placental level. Elevated levels of IFN-γ, TNF-α and IL-2 have been observed in the placental blood from malaria-infected women compared to uninfected women (17) and a higher expression of TNF-α at the placental level is associated with low birth weight babies high malaria endemic areas (18). Furthermore, TNF-α was shown to induce abortion in *Plasmodium vinckei*-infected mice (19). High rates of abortion or fetal resorptions associated with systemic and placental production of IFN-γ and TNF-α have also been reported in rodent models for *Leishmania major* (10) and *Trypanosoma cruzi* (20) infection.

In addition to maternal immune cells, trophoblast cells are also thought to play important roles in innate immune defense against invading pathogens at the maternofetal

interface (21). Trophoblast cells are fetally-derived placental cells that are interposed between the fetus and the mother, in direct contact with maternal blood in the hemochorial placenta. Trophoblasts share many characteristics with macrophages. They can phagocytose various microbes and produce or respond to various cytokines and chemokines (21, 22). Thus development of a local and systemic inflammatory response to control the infection may alter the delicate cytokine balance in the placenta and result in abortion.

The aim of the present study was to characterize the systemic and placental cytokine production in *P. chabaudi AS*-infected pregnant mice in an attempt to improve understanding of the development of anti-malarial immune responses during early stages of pregnancy and their effect on pregnancy outcome.

MATERIALS AND METHODS

Mice and parasites: Age-and-sex matched C57BL/6 mice originally purchased from Jackson Laboratories, Bar Harbor, ME and IFN-γ KO mice (B6.129S7-*Ifng*^{tm1Ts}, obtained from Dr. Rick Tarleton) 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) maintained as described previously (3) was used for all the experiments.

Experimental design: A serial sacrifice study was performed as described earlier (3) to study the kinetics of immune response development in *P.chabaudi AS*-infected, pregnant

(IP) B6 mice. Briefly, 8- to 9-week-old female B6 mice were infected intravenously on GD 0 with 1000 *P. chabaudi* AS-infected erythrocytes (iRBC) per 20 grams of body weight. Infected, non-pregnant (INP) mice, and sham-injected uninfected, pregnant (UP) mice were used as infection and pregnancy controls respectively. Mice were sacrificed on GD 6, 8, 9, 10 and 11 to assess the pregnancy outcome and development of immune response. Development of parasitemia was monitored as described previously (3).

Cell culture: Spleens collected aseptically at sacrifice were cultured as described (3). Briefly, a single cell suspension made by pressing the spleens through a sterile fine-wire mesh was cultured ($1x10^6$ cells/ml) in the presence of concanavalin A (ConA; 2 μg/ml), lipopolysaccharide (LPS; 1 μg/ml) (both from Sigma), 10^6 washed iRBC/ml as the malaria parasite antigen or equal number uninfected RBCs as control for 72 h at 37°C in a humidified CO_2 incubator with an atmosphere of 5% CO_2. (Different cell (1, 2 and 5 million cells) and mitogen concentrations (1μg/ml, 2μg/ml and 5μg/ml) were tested initially for 24, 48 or 72 hrs before determining the optimum culture conditions). Supernatants collected were stored at -85^0C until used for cytokine assays.

For placenta cell culture, at GD 10 and 11 uteri removed aseptically at sacrifice by cutting at the cervix and at the uterotubal junction were dissected under a dissection microscope. The isolated fetoplacental units were then used to make a single cell suspension by pressing through a sterile fine-wire mesh with 10 ml of RPMI 1640 (Cellgro, Herndon, VA 20171 USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), 2 mM L-glutamine (Cellgro, USA) and Penicillin-Streptomycin (Cellgro, USA). The cells were washed and cultured at a concentration of $5x10^6$ cells/ml for 24 hours at 37^0C under 5% CO_2. The culture

supernatants were used to detect the spontaneous production of cytokines by a standard sandwich ELISA. On GD 6, 8 and 9 fetoplacental units were too small to obtain sufficient cell numbers in single cell suspension. Therefore, the whole fetoplacental units were minced into small pieces and used for explant culturing at a concentration of 8 embryos / ml of medium.

Isolation and Culture of Ectoplacental Cone: Uteri removed intact from uninfected B6 mice on GD 7 by cutting across the cervix and at the utero-tubal junctions were dissected to shell out the desidual capsule. Embryos gently extracted from the decidual capsules were then dissected to separate the ectoplacental cones (EPC) from the remaining embryonic tissues. EPCs from 3-5 embryos were then transferred to tissue culture chamber slides or 18 mm glass coverslips in 12-well tissue culture plates containing 1 ml Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), 200 mM L-glutamine (Cellgro, USA), penicillin (10,000 I.U / ml) and streptomycin (10,000μg / ml) (Cellgro, USA) and cultured for 3-5 days at 37^0 C under 5% CO_2.

Phagocytosis Assay: Infected and uninfected RBCs for the phagocytic assay were purified by a method described by Ing et al (23). Briefly, heparinized blood obtained via cardiac puncture from *P. chabaudi* AS-infected B6 mice with 30–40% parasitemia was washed and loaded onto a 74% Percoll (Sigma-Aldrich) density gradient after diluting with PBS. Following centrifugation at 5000 x *g* for 20 min at room temperature, the top band containing iRBCs was collected. For normal RBC (nRBC) controls, heparinized blood from naive mice was loaded onto a 90% Percoll gradient, centrifuged at 5000 x *g*

for 20 min at room temperature, and the top band was collected. After several washes, RBCs were resuspended in DMEM at a concentration of $1x10^6$ iRBCs / ml.

EPCs were cultured for 72 hours prior to addition of infected or uninfected erythrocytes. The medium was then replaced with $1x10^6$ iRBCs or an equal number of nRBCs for 16 hours at 37^0C. In some experiments, EPCs were pretreated with cytochalasin D (Sigma-Aldrich) dissolved in DMSO (Fischer Scientific**)** at 10μg/ml or with 0.1% DMSO control at 37°C for 1 hour before the addition of pRBCs or nRBCs. At the end of the assay, the non-ingested RBCs were removed by extensive washing with Hanks Balanced Salt Solution followed by exposure to Tris buffered 0.175 M NH_4Cl to lyse remaining RBCs. After drying, the slides were fixed in methanol and the phagocytic activity 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 malarial pigment. The phagocytic index (PI) was calculated using the formula (number of trophoblast giant cells containing iRBCs or hemozoin / total number of trophoblast giant cells) x 100.

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

Immunofluorescence: Ectoplacental cone cells grown on cover slips were fixed using 2% paraformaldehyde in PBS for 10 minutes 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 (1:10,

TROMA, DSHB, University of IOWA, Iowa city) or an isotype-control antibody (Sigma). Antibody bound to cytokeratin was revealed by use of fluorescein isothiocyanate (FITC)-conjugated anti-rat polyclonal secondary antibody (Sigma # F 5262, diluted 1:400) and observation using a SP2s confocal microscope (Leica Microsystems, Inc., Bannockburn, IL, USA).

Ectoplacental cone cells cultured on glass cover slips exhibited the general morphology described for these cells by 48 hours (24). Also all the cells cultured were positive for staining with a murine trophoblast specific anti-cytokeratin antibody (as described above) suggesting that only trophoblast cells were isolated and cultured (data not shown).

Cytokine and soluble cytokine receptor ELISA: Levels of IFN-γ, TNF-α, IL-1β, IL-10 and soluble TNFR-II (sTNFRII) in the culture supernatants and plasma samples were determined using OptEIA enzyme-linked immunosorbent assay (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 RBC antigens were subtracted from malarial antigen-stimulated cultures. Limits of detection were 8 pg/ml for IL-10 and TNF-α, 15 pg/ml for IFN-γ and IL-1β and 31 pg/ml for sTNFR-II.

Statistical Analysis: Unless otherwise noted, the SAS statistical software package (version 8.02; SAS Institute, Inc., Cary, N.C.) was used for data analysis. Proc GLM was used to analyze the significance of differences among group means in the case of normally distributed data and Tukey's Studentized (HSD) range test was used to perform multiple pairwise group comparisons in cases of unequal sample size. In cases of non-

normally distributed data the nonparametric Wilcoxon rank sum test was used for comparisons. To compare the averages of the ranked data in more than two groups, the nonparametric Kruskal-Wallis (KW) test was performed. If the result from a comparison of multiple groups by KW test yielded statistical significance, the permutation method from the MULTTEST procedure (SAS proc multtest) was used to obtain the adjusted *P* value for each pair of groups in multiple comparisons. The relationship between cytokines or sTNFRII was assessed using Spearman's rank correlation (GraphPad Prism, version 4.01). Where necessary the data were normalized by logarithmic transformation before analysis. *P* values of ≤ 0.05 were considered to be significant.

RESULTS

Plasma cytokine levels in P. chabaudi AS infected pregnant mice: *P. chabaudi* AS infection in B6 mice was shown to be associated with high rates of abortion or resorptions during the ascending or peak parasitemia (GD / experiment day (ED) 10 and 11) and a complete loss of viable pregnancies by GD / ED 12 (3). To investigate whether the observed fetal loss in IP mice is associated with elevated levels of proinflammatory cytokines, levels of IFN-γ, TNF-α IL-1β and also an anti-inflammatory cytokine, IL-10, were measured in the plasma samples from IP, INP and UP mice. Since in this model system IP mice abort between GD / ED 10 and 11, comparisons were also made between aborting and nonaborting IP mice on GD / ED 10 to determine whether the plasma cytokine levels correlate with pregnancy outcome.

Figure 4.1A represents the kinetics of IFN-γ production in the plasma from malaria-infected pregnant and control mice at different stages of infection. IP mice exhibited a robust IFN-γ response following *P. chabaudi AS* infection. IFN-γ was

detectable in the plasma samples from IP mice starting from GD / ED 8 and the peak response was observed on GD / ED 9, which corresponds to the stage of ascending parasitemia in this model. Compared to UP mice, the IFN-γ level was significantly higher in IP mice on GD / ED 8 through 11 ($P < 0.04$), thereafter exhibiting a downward trend. It is noteworthy that there was no IFN-γ production by UP mice at any of the stages tested. In comparison to INP mice, IFN-γ production by IP mice, except on GD 9, was lower and more transient (INP versus IP; GD / ED 10; $P = 0.0002$). The IFN-γ level was not significantly different between aborting and nonaborting IP mice on GD 10. The intermediate pattern of response observed in IP mice suggests that pregnancy may modulate IFN-γ production in *P. chabaudi* AS-infected pregnant B6 mice.

Similar to IFN-γ response, the plasma TNF-α level also exhibited a tendency to be higher in IP mice relative to UP mice (Figure 4.1B). Compared to UP mice, IP mice exhibited a sustained high level of TNF-α in their plasma samples on GD / ED 9, 10 and 11 which corresponds to ascending and peak parasitemia. Although the average TNF-α level was higher in mice undergoing abortion compared to nonaborting mice on GD / ED 10 (mean ± SEM: 57.61 ± 28.6 pg/ml versus 33.5 ± 8.6 pg/ml) the differences did not reach statistical significance. INP mice also exhibited higher plasma TNF-α level in comparison to UP mice on GD / ED 9 and 10.

TNF-α has been shown to function in synergy with IL-1β in inducing malarial pathogenesis (25). Similar to TNF-α, IL-1β was detectable in the plasma samples from IP mice beginning GD / ED 10 (Figure 4.1C) and the peak response observed on GD / ED 10 was significantly higher than that observed in INP and UP mice ($P < 0.05$).

Comparisons were not done between aborting and nonaborting mice due to insufficient sample size.

The counterregulatory cytokine IL-10 exhibited different kinetics compared to IFN-γ, TNF-α, and IL-1β (Figure 4.1D). Production peaked on GD / ED 10 in IP and INP mice, a day after peak IFN-γ response, and remained high on GD / ED 11, a time point at which IFN-γ, TNF-α and IL-1β responses exhibited a downward trend. Compared to UP mice, IP mice had significantly higher levels of IL-10 in their plasma samples on GD / ED 9 through 11 (IP versus UP; $P < 0.0002$). Furthermore, the average IL-10 level tended to be lower in IP mice undergoing abortion on GD / ED 10 (mean ± SEM: 787.2 ± 224.3 pg/ml) compared to non aborting mice (mean ± SEM: 1064 ± 311 pg/ml; $P > 0.05$). The IL-10 level in IP mice overall was not significantly different from INP mice.

Although both IP and INP mice had higher amounts of TNF-α in the plasma in comparison to UP mice, the levels were not significantly different. To examine whether this is due to increased soluble TNF-α receptor levels, which could bind and sequester soluble TNF-α, the level of sTNFRII in the plasma samples from mice at different stages of infection / gestation was measured. As represented in Figure 4.1E, sTNFRII levels were significantly higher in the plasma samples from IP mice compared to UP (GD / ED 9, 10 and 11; $P < 0.007$) and INP mice (GD / ED 10 and 11; $P < 0.012$). The highest plasma sTNFRII level in IP mice was observed on GD / ED 11 which corresponds to peak parasitemia. INP mice also exhibited higher plasma sTNFRII levels compared to UP mice (GD / ED 10, $P= 0.012$). Importantly, a significant positive association between plasma sTNFRII level and parasitemia was observed in mice undergoing abortion on GD / ED 10 and 11 (Figure 4.2; $P = 0.036$, $R^2 = 0.37$).

Both TNF-α and IL-10 have been shown to influence the shedding of sTNFRII. Thus the associations between plasma sTNFRII, TNF-α and IL-10 levels in aborting mice were determined by fitting the data to a linear regression model. A significant positive association between plasma sTNFRII and TNF-α was observed in mice undergoing abortion on GD / ED 10 ($r^2 = 0.59$, $P = 0.02$). Plasma sTNFRII and IL-10 levels did not exhibit a significant association ($r^2 = 0.21$, $P = 0.253$).

In vitro cytokine production by spleen cells from P. chabaudi AS infected pregnant and non-pregnant mice:

To examine the ability of spleen cells from IP or control mice to produce cytokines in response to malarial antigen or mitogens in vitro, the levels of IFN-γ, TNF-α and IL-10 in the spleen cell culture supernatants from mice sacrificed at different stages of infection were tested. As observed in the case of plasma samples, the level of IFN-γ production was intermediate in IP mice compared to INP and UP mice (Figure 4.3). Spleen cells from IP mice were able to produce significantly higher amounts of IFN-γ in the culture supernatants in comparison to UP mice either spontaneously or in response to malarial antigen and LPS stimulation during ascending and peak parasitemia which corresponds to GD / ED 8 through 11 in this model (IP versus UP; spontaneous, $P < 0.015$; malarial antigen, $P < 0.019$; LPS, $P < 0.002$). There was no spontaneous, LPS stimulated, or antigen specific IFN-γ production by UP mice at any of the time points tested. IFN-γ production by IP mice in comparison to INP mice was either lower or for shorter duration. Both infected and uninfected mice had robust IFN-γ production in response to ConA and the levels were not significantly different between IP and UP mice.

Figure 4.4 represents TNF-α production by splenocytes from IP and control mice cultured in the presence or absence of stimulants as described above. The spontaneous and malarial antigen specific TNF-α production was higher in the culture supernatants from IP mice when compared to UP mice. IP mice tended to produce higher amounts than UP mice in response to malarial antigen on GD / ED 8 ($P = 0.06$). In response to ConA, however, IP mice produced significantly lower amounts of TNF-α compared to UP (GD 9, 10 and 11, $P < 0.0273$) and INP mice (GD / ED 10, $P = 0.0145$). Additionally, the spontaneous and malarial antigen specific responses were also lower in IP mice in comparison to INP mice ($P > 0.05$). Thus pregnancy-associated as well malaria-induced immunomodulatory mechanisms operating in the spleen might be contributing the reduced or intermediate responses observed in IP mice.

The intermediate responses observed in IP mice could be due to high production of the anti-inflammatory cytokine, IL-10. The spleen cells from IP mice exhibited higher spontaneous as well as antigen specific IL-10 production compared to UP mice on GD / ED 8, 9 and 10 (Figure 4.5; GD / ED 9, $P = 0.009$, antigen specific and $P = 0.0284$, spontaneous). However, IL-10 production in response to ConA and LPS was not significantly different between IP and UP mice. The splenocytes from INP mice also produced significantly higher amounts of IL-10 than UP mice spontaneously (GD / ED 9 and 10, $P < 0.022$), in response to ConA (GD / ED 10, $P = 0.032$) and malarial antigen stimulation (GD / ED 8, 9 and 10, $P < 0.03$). The splenocyte IL-10 production was not different between IP and INP mice. Additionally, the levels of IFN-γ, TNF-α and IL-10 were not significantly different between aborting and nonaborting IP mice on GD 10.

Cytokine production by cultured fetoplacental units from P. chabaudi AS infected pregnant and uninfected pregnant mice.

Because plasma and spleen cytokine levels may not reflect the events happening in the uterine environment, IFN-γ, TNF-α and IL-10 secretion by cultured fetoplacental units from IP mice were tested. The kinetics of IFN-γ production in the culture supernatants paralleled the IFN-γ production in the periphery. The fetoplacental units from IP mice produced high amounts of this cytokine but there were no detectable amounts of IFN-γ in the culture supernatants from UP mice (GD / ED 8 and 9; versus UP, $P < 0.05$; Table 4.1). Contrary to expectation, TNF-α production was either comparable or lower in IP mice in comparison to UP mice (GD 11, $P < 0.05$ by GLM Tukey). This was not due to high IL-10 production in IP mice because IL-10 production was minimal in both IP and UP mice. However, as observed in the plasma, the sTNFRII level was significantly higher in the placenta cell culture supernatants from IP mice in comparison to UP mice (GD / ED 9, $P < 0.05$; Table 4.2). The significance of the elevated levels of sTNFRII in immune regulation or pathology at the placental level remains to be investigated.

Role of fetal cells in the malaria induced fetal loss:

Fetal loss in IP mice was associated with increased accumulation of infected erythrocytes in the maternal blood sinusoids of the placenta (3). Contrary to what has been reported in the placentae of malaria-infected pregnant women (26), the accumulation of monocyte/macrophages in the placentae of IP mice was minimal (Poovassery, J and Moore JM, unpublished data). However, on histological examination there was massive phagocytosis of iRBCs or hemozoin by the trophoblast giant cells

(Figure 4.6A). The impact of phagocytosis of iRBCs by trophoblast giant cell on trophoblast function or how this may affect the local placental pathology is an understudied problem. To address this, an in vitro system was used as described earlier by Pavia (27). To study whether the trophoblast cells can phagocytose *P. chabaudi* AS-infected erythrocytes in vitro, EPCs from GD 7 embryos were cocultured with infected or uninfected erythrocytes. Indeed, there was massive phagocytosis of iRBCs by trophoblast cells (Figure 4.6B), which was evident as early as 1 hour after introducing iRBCs into the culture system (data not shown). Following an incubation period of 16 hours, the EPC cells exhibited an enhanced phagocytosis of iRBCs compared to uRBCs (PI (mean ± SEM) = 30.86 ± 2.75 % versus 4.29 ± 0.46 %; P = 0.015). Furthermore, the trophoblast cells treated with cytochalasin D, a known inhibitor of phagocytosis, exhibited marked reduction in phagocytosis of both iRBCs and uRBCs (88 % and 58 % reduction, respectively).

Although IFN-γ was detected in placenta cell culture supernatants (as mentioned above), the cellular source for IFN-γ in this model is not known. Furthermore, the fetal loss in this model was associated with minimal mononuclear cell infiltration to the placenta. Since trophoblast cells can produce IFN-γ (28), we hypothesized that the fetal cells could be the source of IFN-γ and potentially other pathogenic and protective cytokines in the placenta cell culture supernatants. To investigate this possibility, trophoblast cells were incubated with iRBCs in vitro for 24 hours and the levels of IFN-γ, TNF-α, IL-10 and sTNFRII in the cell culture supernatants were assayed by ELISA. There was no detectable amount of IFN-γ in the trophoblast cell culture supernatants (data not shown). As an alternate approach, infected IFN-γ gene null mutant females were

crossed with wild type B6 males such that any detectable IFN-γ is due to production by trophoblasts. The fetoplacental units were cultured as described above and the cell culture supernatants were assayed for IFN-γ level by ELISA. The placenta cell culture supernatants from heterozygous embryos failed to produce any detectable amounts of IFN-γ (data not shown), suggesting that the source of IFN-γ in *P. chabaudi AS*-infected pregnant mice is maternal. However, incubation of trophoblast cells with iRBCs resulted in robust TNF-α production (mean ± SEM: 44.05 ± 17.42 pg / ml; P = 0.04 versus 0 ± 0 nRBC). This suggests that the fetal trophoblast cells could be immunoactive during malarial infection. There was no detectable amount of IL-10 or sTNFRII in the culture medium.

DISCUSSION

High rates of abortion, preterm labor and stillbirths have been reported in *P. falciparum*-infected non-immune pregnant women (2). Abortions associated with high density parasitemia were also reported in a monkey model for malaria during pregnancy (29). Although an immunological basis has been attributed to malarial pathogenesis, few studies have been done to explore this possibility during pregnancy. We have previously shown that the *P. chabaudi* AS infection can completely abrogate pregnancy in B6 mice (3). To begin to investigate the possible role maternal anti-malarial immune responses play in the observed fetal loss, the pattern of cytokine production in the plasma, spleen and placenta cells from malaria infected pregnant mice was studied.

The immune response to *P. chabaudi* AS infection in nonpregnant B6 mice has been well characterized. The resolution of primary infection with *P. chabaudi* AS requires IFN-γ (4, 30, 31). IFN-γ along with TNF-α induces downstream effector

molecules such as nitric oxide and reactive oxygen intermediates (32, 33) which are thought to be directly involved in parasite killing (34). However, all these responses could be harmful for the pregnancy (10). Because IP mice developed peak parasitemia and anemia comparable to INP mice and survived the infection it is possible that the IP mice would also develop an early Th1-cytokine biased immune response to survive the infection at the expense of their pregnancies.

In agreement with this IP mice exhibited a robust Th1 / proinflammatory response against primary *P. chabaudi* AS infection. The systemic levels of proinflammatory cytokines IFN-γ, TNF-α and IL-1β were elevated in IP mice compared to UP mice. IP mice exhibited a persistent high TNF-α level in the plasma and the levels were higher in aborting mice compared to nonaborting mice, suggesting a possible association with fetal loss. Furthermore, the level of IL-1β, a cytokine which has been shown to function in synergy with TNF-α in inducing malarial pathogenesis was also higher in IP mice on GD / ED 10, a day at which IP mice start aborting in this model.

IFN-γ is a cytokine that has been shown to be essential for protection against malarial infection in both mice and in humans (4, 35). Maximum IFN-γ response in the serum of *P. chabaudi* AS infected non-pregnant mice has been shown to occur 1-2 days before peak parasitemia (36). Consistent with this, IFN-γ level peaked in the plasma on GD / ED 9 in IP mice, which is 2 days before peak parasitemia (3). Although TNF-α level has been shown to peak during highest parasitemia levels in nonpregnant mice (37), in this model system peak TNF-α response was observed 1-2 days before peak parasitemia. This could be due to the differences in the infective dose (10^3 versus 10^6) as well as in the route of infection (intravenous versus intraperitoneal) (38, 39).

Following an appearance during the early stages of infection there is a down regulation of systemic production of proinflammatory cytokines IFN-γ and TNF-α in infected mice during the later stages of gestation / infection. Thus, as has been suggested previously (40), a regulatory mechanism could be operating in the periphery to avoid pathologies associated with uncontrolled production of proinflammatory cytokines. Consistent with this, IL-10, which is a regulatory cytokine that has been shown to play important roles in downregulating IFN-γ and TNF-α responses (41), appeared in the plasma two days after peak IFN-γ and TNF-α response. Additionally, *P. chabaudi* AS infection was shown to be associated with increased mortality in IL-$10^{-/-}$ mice, and an excessive production of TNF-α was thought to be responsible for the pathologies in this model as anti-TNF-α treatment was able to abolish the observed mortalities (42). Furthermore, a lower IL-10 plasma concentration and low IL-10 / TNF-α ratios have been shown to be associated with anemia in humans (43, 44).

Consistent with plasma and spleen cell culture supernatants, the placenta cells from IP mice spontaneously secreted large amounts of IFN-γ, peaking 1-2 days before abortion. It is important to note that there was no IFN-γ production by placenta cells from UP mice. Despite the presence of high levels of IFN-γ at the placental level, TNF-α levels were reduced in the placenta cell culture supernatants from IP mice. This was not due to the induction of the regulatory cytokine IL-10, as the level of IL-10 was not significantly different between IP and UP mice. However, the level of soluble TNFRII was significantly higher in the plasma and placenta cell culture supernatants from IP mice. Although both infected and uninfected mice had high sTNFRII expression, a progressive increase in sTNFRII level corresponding to disease progression was observed

only in IP mice. Furthermore, the plasma sTNFRII level also exhibited a positive association with plasma TNF-α and was significantly higher in aborting mice compared to nonaborting mice. An association between disease severity and elevated sTNFRII level has been reported during malarial infection in human (45) and monkeys (46). Increased levels of sTNFRs have also been reported in a number of clinical conditions ranging from infectious (47), malignant (48) and autoimmune disorders (49). Although soluble TNF-α receptors can act as TNF-α antagonists by competing for ligand with membrane-bound TNFR (50), at low concentration sTNFR can actually enhance TNF-α activity by protecting TNF-α from degradation and prolonging its availability for binding to membrane bound receptors (51). It is possible that the elevated sTNFRII levels in IP mice could be a regulatory mechanism to protect the mother from the harmful effects of uncontrolled TNF-α production. However, it seems, in the context of malaria, it is not enough to protect the fetus. The fact that the neutralization of TNF-α rescued pregnancy in IP mice (see chapter 5) further confirms the embryotoxic effect of this cytokine. Although the trophoblast cells are shown to be positive for the expression of sTNFRI (52), the placental source of sTNFRII in this model remains to be investigated.

Studies in rodent models have demonstrated that high systemic and local production of Th1 / proinflammatory cytokines during pregnancy can be harmful to the fetus. Th1 cytokines IL-2, IFN-γ and TNF-α have embryotoxic effects (53, 54) and are implicated in recurrent spontaneous abortion in women (14). Administration of IFN-γ, TNF-α and IL-2 to normal pregnant mice can increase the frequency of resorptions (55, 56). IFN-γ along with TNF-α can suppress the development of murine fetuses and can induce apoptosis of human trophoblasts *in vitro* (54). On the other hand, Th2 cytokines

such as IL-10 and IL-4 have been shown to promote proliferation and differentiation of trophoblast cells (57, 58). Furthermore, administration of IL-10 can reverse fetal resorption in a murine model of recurrent spontaneous abortion (59). Thus the local and systemic release of IFN-γ along with TNF-α and IL-1β in the absence of the regulatory cytokine IL-10 at the placental level may be contributing to the fetal loss in this model system (54). In the context of malaria, elevated placental levels of IFN-γ, TNF-α and IL-2 have been reported in pregnant women (17), and high expression levels of TNF-α are associated with low birth weight infants (18, 60). Furthermore, TNF-α can induce abortion in *P. vincknei*-infected pregnant mice (19) and fetal death in *P. coatneyi*-infected monkeys (46). High rates of fetal loss associated with increased systemic and placental production TNF-α has also been reported in *Trypanosoma cruzi*-infected mice (20).

Although the placental cells from IP mice produced elevated amounts of IFN-γ ex vivo, the cellular source of IFN-γ in this model is not known. 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 maternal. It is possible that the uterine NK cells which constitute the majority of decidual leukocytes could be the major source of IFN-γ at the maternofetal interface (61). Since the whole fetoplacental units were used for culturing in this study, the possibility for the presence of decidual leukocytes in the cell suspension cannot be ruled out.

In addition to NK cells and macrophages, trophoblast cells are also shown to have the potential to function as a component of the innate immune system at the uteroplacental level (21). The results from this study suggests that the fetal cells are

involved in the local defense mechanism, while at the same time might contribute to the local pathological events indirectly through cytokine production. Trophoblast cells exhibited massive phagocystosis of iRBCs or hemozoin both in vivo and in vitro and produced TNF-α in the culture supernatants. Although the trophoblast cells also phagocytosed nRBCs in vitro it was significantly lower compared to iRBCs. Additionally, TNF-α production was observed only after phagocytosis of iRBCs. Trophoblast giant cells in the uninfected placenta are shown to express TNF-α (62) and can produce nitric oxide and reactive oxygen species following phagocytosis of microbial organisms (63). Furthermore, the phagocytic activity has been shown to be increased in the presence of IFN-γ (24).

The exact mechanism involved in fetal loss during malarial infection is not known. It is possible that the TNF-α produced by the fetal cells may cause the maternal uterine NK cells in the decidua to produce IFN-γ in vivo, which in turn may induce increased phagocytosis of iRBCs / hemozoin and more TNF-α production by trophoblast giant cells. TNF-α along with IFN-γ may then induce the apoptosis of trophoblast cells and ultimately fetal death. Additionally, thrombotic events initiated in the placenta by proinflammatory cytokines IFN-γ and TNF-α (64) or TNF-α induced proinflammatory lipid mediators may be also involved in malaria-induced fetal death. It is also possible that there could be an insufficient expansion of pregnancy-induced regulatory T cells in IP mice as reported in the case of a murine model for spontaneous abortion (65). All of these mechanisms are currently under investigation in our laboratory.

The molecular mechanisms leading to trophoblast cell activation and cytokine production following phagocytosis of iRBCs or hemozoin is not known. Although

hemozoin has been shown to be immunoactive and capable of inducing cytokine and chemokine production by mouse innate immune cells by interacting through TLR 9 (66), it is not known whether TLRs are involved in the interaction between murine trophoblast cells and hemozoin.

Overall, the data presented here further confirm the complex nature of immune responses to malarial infection during pregnancy. In the *P. chabaudi* AS/B6 model system, the systemic production of proinflammatory cytokines during the ascending stages of infection is followed by the production of IL-10 in the plasma and splenocyte culture supernatants. These data suggest that the cytokine responses are tightly regulated at the periphery to avoid pathologies associated with uncontrolled production of proinflammatory cytokines. However, as reported in the case of missed abortions in humans (67), a failure to produce IL-10 at the local placental level in the context of high IFN-γ production may be contributing to cytokine-mediated trophoblast damage and finally fetal death in *P. chabaudi* AS-infected pregnant mice.

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Figure 4.1.

IFN-γ (A), TNF-α (B), IL-1β (C), IL-10 (D) and soluble TNF receptor II (sTNFRII) (E) levels in the plasma samples from *Plasmodium chabaudi* AS infected, pregnant (IP), infected, nonpregnant (INP), and uninfected, nonpregnant (UP) mice.

IP and INP mice infected with *P. chabaudi* AS and UP mice sham injected. Mice were sacrificed at indicated time points and plasma samples were assayed for cytokines or cytokine receptor level by ELISA. Results shown are data grouped from two separate experiments. Data from individual mice and median for each group are presented. Number of mice sacrificed (IP, UP, and INP, respectively) was as follows: for IFN-γ, at GD/ED 6, *n* = 5, 5, and 5; at GD/ED 8, *n* = 7, 8, and 5; at GD/ED 9, *n* = 5, 5, and 6; at GD/ED 10, *n* = 15, 5, and 6; at GD/ED 11, *n* = 6, 3, and 4. GD / ED 10 include both aborting and non aborting mice. For TNF-α and IL-10, GD / ED 6 and 8, n = same as described for IFN-γ, GD / ED 9, *n* = 10, 10 and 6; at GD/ED 10, *n* = 12, 11, and 5; at GD/ED 11, *n* = 12, 8, and 5; for IL-1β, at GD/ED 6, *n* = 5, 5, and 5; at GD/ED 8, *n* = 6, 5, and 3; at GD/ED 9, *n* = 4, 5, and 5; at GD/ED 10, *n* = 6, 5, and 5; at GD/ED 11, *n* = 7, 5, and 4; for sTNFRII, at GD/ED 6, 9 and 11, *n* = 5, 5, and 5; at GD/ED 8, *n* = 6, 5, and 5; at GD/ED 10, *n* = 15, 5, and 5. The following statistically significant differences were observed for comparisons by Wilcoxon rank sum test For IFN-γ, IP and INP versus UP (GD / ED 8, 9 and 10) $P < 0.022$; for IL-1β, IP versus UP and INP (GD / ED 10) $P < 0.05$; for IL-10, IP versus UP (GD / ED 9 through 11) $P < 0.0002$; INP versus UP, GD / ED 9 and 11, $P < 0.0021$, GD / ED 10, $P = 0.0002$; for sTNFRIIIP versus UP (GD / ED 9, 10, 11) $P < 0.0079$; IP versus INP (GD / ED 10, 11) $P < 0.012$; INP versus UP (GD / ED 9) $P = 0.029$.

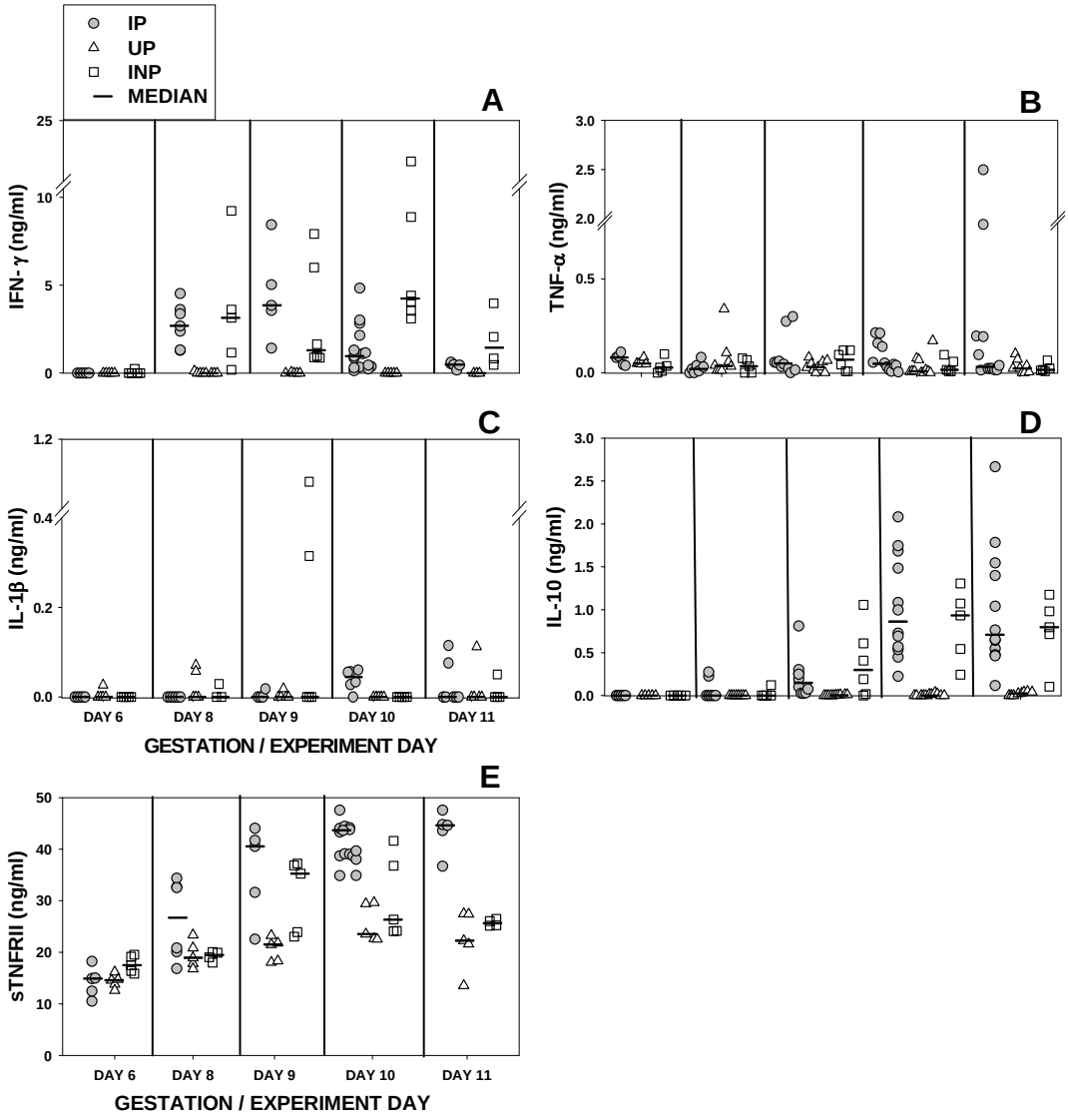
IP
UP
INP
MEDIAN
A
B
C
D
E
IFN-γ (ng/ml)
TNF-α (ng/ml)
IL-1β (ng/ml)
IL-10 (ng/ml)
sTNFRII (ng/ml)
DAY 6
DAY 8
DAY 9
DAY 10
DAY 11
GESTATION / EXPERIMENT DAY

Figure 4.2.

Association of plasma sTNFRII level with parasitemia in IP mice. Plasma cytokine or cytokine receptor levels were determined by ELISA. Parasitemia was determined by counting at least 1,000 erythrocytes in four to five high-power fields on Giemsa-stained tail blood thin smears. $R^2 = 0.37$; $P = 0.036$.

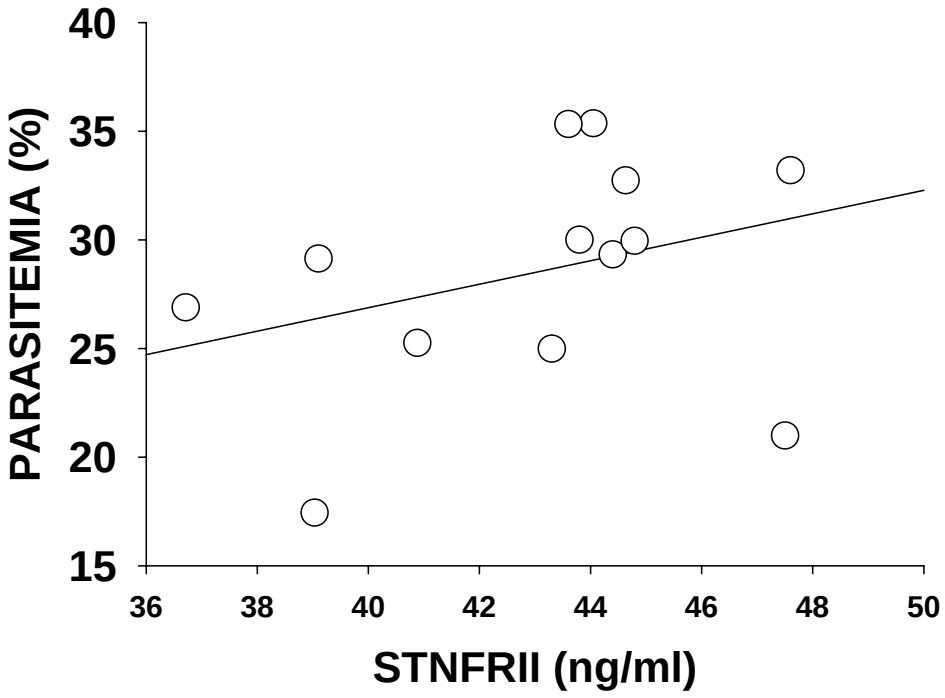
PARASITEMIA (%)
40
35
30
25
20
15
36
38
40
42
44
46
48
50
STNFRII (ng/ml)

Figure 4.3.

IFN-γ production by spleen cells in vitro.

Spleens collected aseptically from *P. chabaudi* AS infected, pregnant (•), infected, nonpregnant (□) and uninfected, pregnant (Δ) mice at sacrifice were cultured for 72 h at 5 x 10^6 cells/ml alone (medium) or in the presence of Concanavalin A (ConA), Lipopolysaccharide (LPS) and *Plasmodium chabaudi* AS infected erythrocytes (Antigen). Culture supernatants were then assayed for IFN-γ by ELISA. Data from individual mice and median for each group are presented. Number of mice sacrificed (IP, UP, and INP, respectively) at each GD/ED was as follows: at GD/ED 6, n = 5, 5, and 5; at GD/ED 8, n = 5, 5, and 5; at GD/ED 9, n = 5, 5, and 5; at GD/ED 10, n = 10, 5, and 5; at GD/ED 11, n = 5, 5, and 5. Results shown are representative of two independent experiments. The following P values were observed by Wilcoxon rank sum test: Medium: IP and INP versus UP (GD / ED 9 and 10), $P < 0.013$; LPS: IP versus UP (GD / ED 10 and 11), $P < 0.002$, INP versus UP (GD / ED 8, 9, 10 and 11), $P < 0.0275$, INP versus IP (GD / ED 11), $P = 0.0085$; Antigen: IP and INP versus UP (GD / ED 8 and 9), $P < 0.018$, INP versus UP (GD / ED 10), $P = 0.0375$.

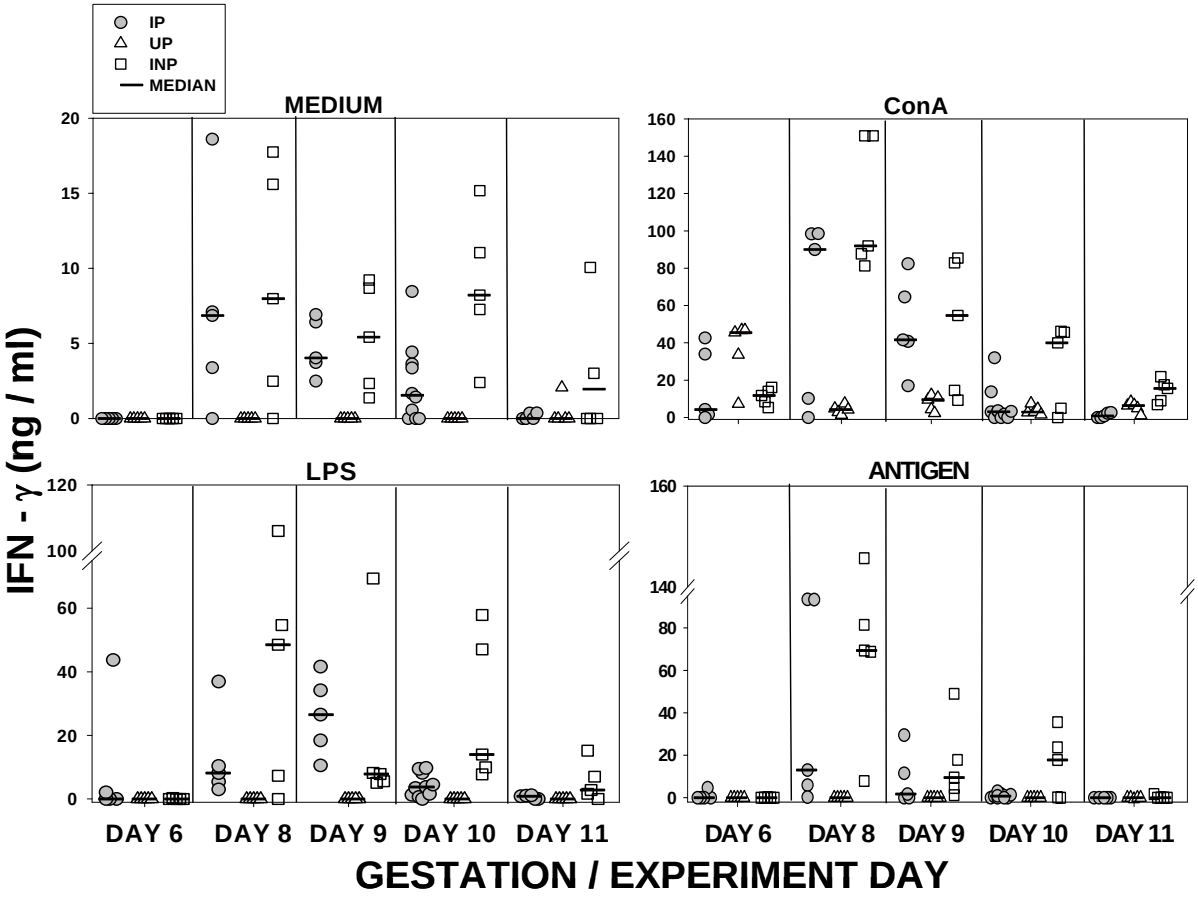
IP
UP
INP
MEDIAN
MEDIUM
ConA
LPS
ANTIGEN
IFN - γ (ng / ml)
DAY 6
DAY 8
DAY 9
DAY 10
DAY 11
GESTATION / EXPERIMENT DAY

Figure 4.4.

TNF-α production by spleen cells in vitro.

Supernatants from splenocytes cultured as described in the legends to figure 2 were assayed for TNF-α by ELISA. Data from individual mice and median for each group are presented. Sample size same as described in the legends to figure 2. Results shown are representative of two independent experiments. By Wilcoxon rank sum test: Medium: INP versus UP (GD / ED 10), $P = 0.034$; ConA: UP versus IP (GD / ED 9, 10 and 11), $P < 0.02$, INP versus IP (GD / ED 10), $P = 0.0145$; Antigen: INP versus UP (GD / ED 8 and 10), $P < 0.03$.

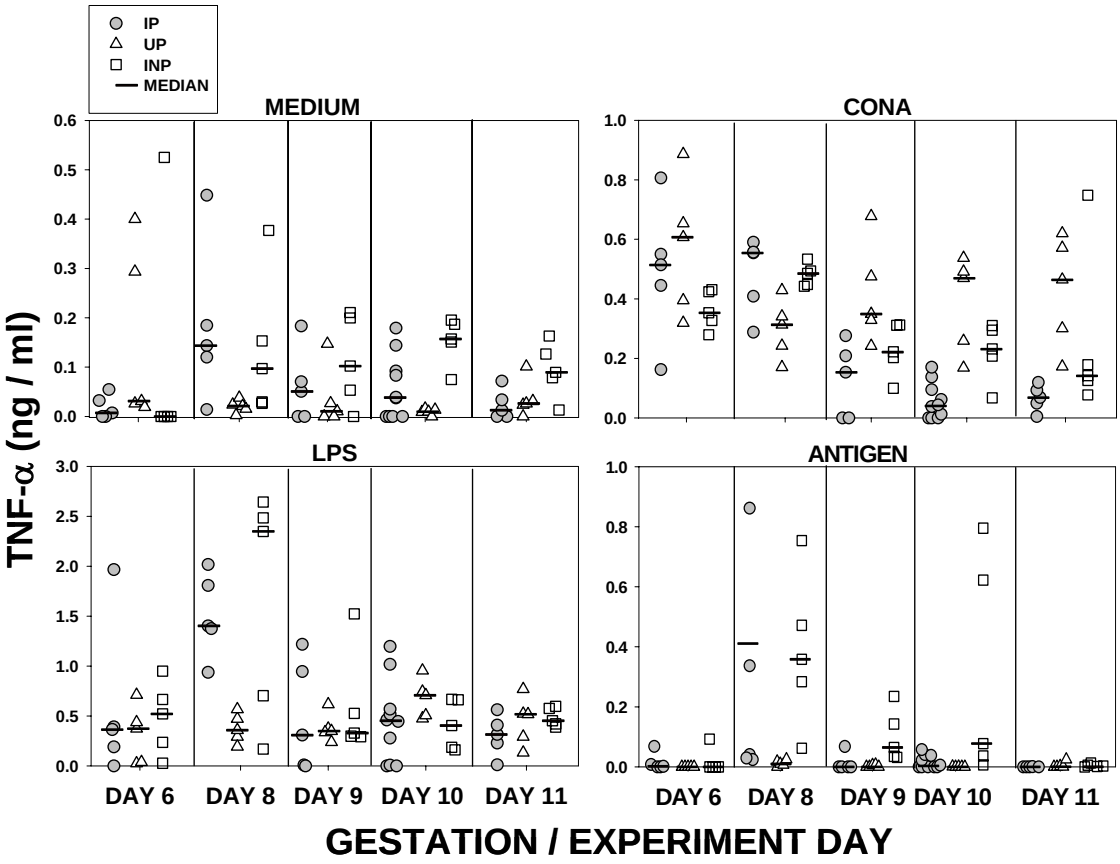
IP
UP
INP
MEDIAN
MEDIUM
CONA
LPS
ANTIGEN
TNF-α (ng / ml)
DAY 6
DAY 8
DAY 9
DAY 10
DAY 11
GESTATION / EXPERIMENT DAY

Figure 4.5.

IL-10 production by spleen cells in vitro.

Supernatants from splenocytes cultured as described in the legends to figure 2 were assayed for IL-10 by ELISA. Data from individual mice and median for each group are presented. Sample size same as described in the legends to figure 2. Results shown are representative of two independent experiments. By Wilcoxon rank sum test: Medium: IP and INP versus UP (GD / ED 9), $P = 0.022$, INP versus UP (GD / ED 10), $P = 0.0032$; ConA: INP versus UP (GD / ED 10), $P = 0.0326$; Antigen: INP versus UP (GD / ED 8, 9 and 10), $P < 0.03$, IP versus UP (GD / ED 9), $P = 0.0284$.

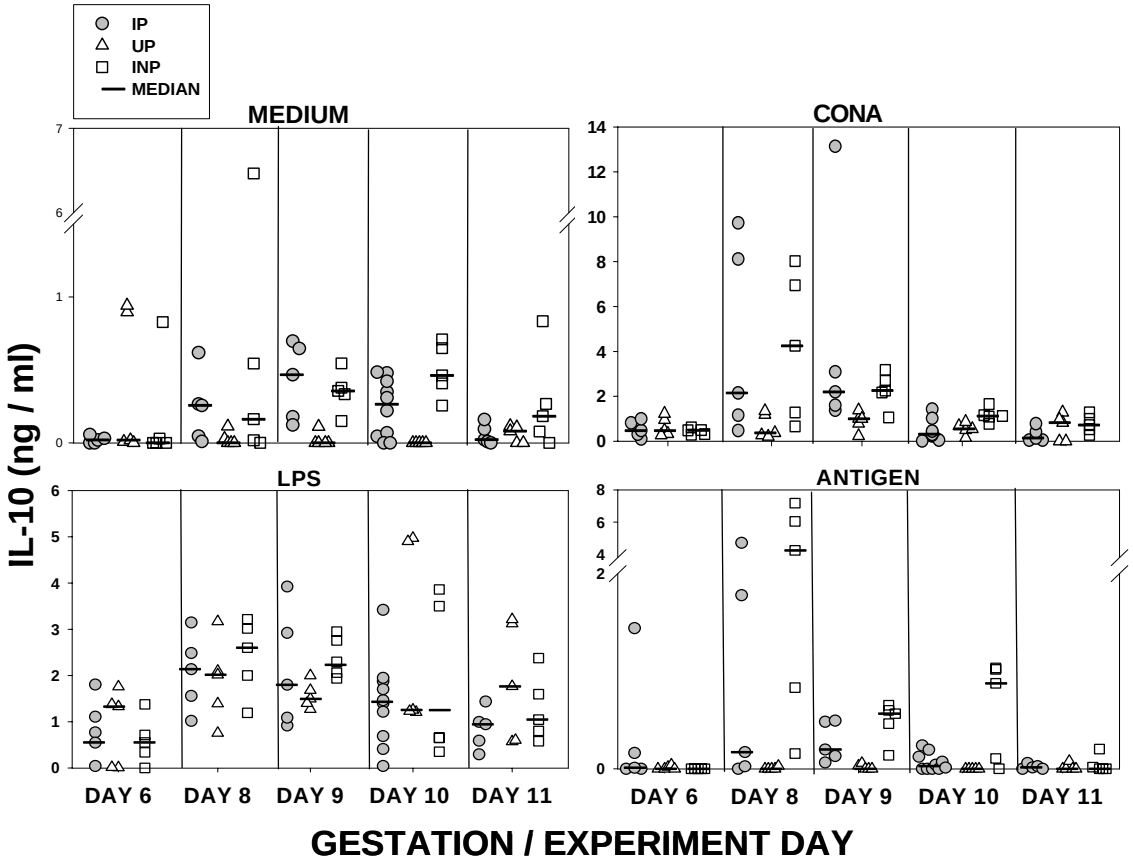
IP
UP
INP
MEDIAN
MEDIUM
CONA
LPS
ANTIGEN
IL-10 (ng / ml)
DAY 6
DAY 8
DAY 9
DAY 10
DAY 11
GESTATION / EXPERIMENT DAY

Figure 4.6.

Trophoblast giant cells phagocytose *P. chabaudi* AS-infected erythrocytes (iRBCs) in vivo (A) and in vitro (B).

Giemsa stained placental sections from an IP mice undergoing abortion on GD/ED 11 (A). Trophoblast giant cell with phagocytosed iRBCs (arrow heads) and hemozoin (arrows) are presented. B. Phagocytosis of iRBCs by murine trophoblast cells in vitro. Ectoplacental cone cells cocultured as desribed under the materials and method section were fixed and stained with Geimsa stain. *P. chabaudi* AS infected erythrocytes (arrow heads) internalized by a multinucleated (N) trophoblast cell is presented.

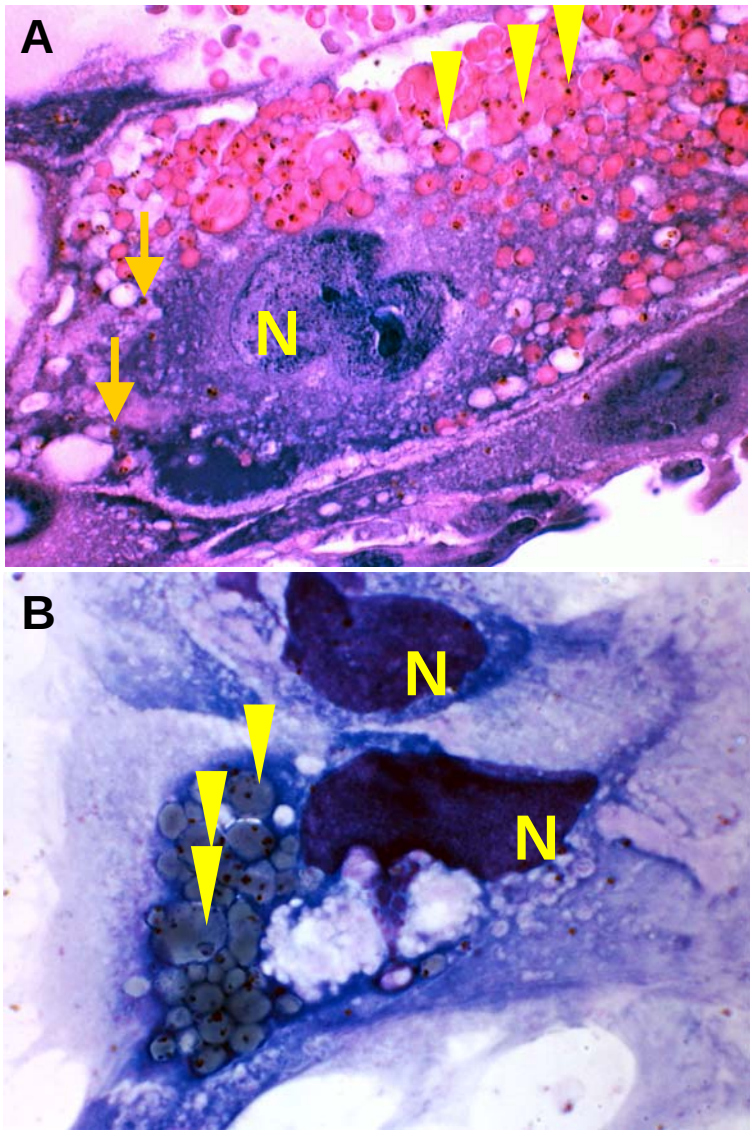
A
N
B
N
N

Table 4.1.

Kinetics of IFN-γ, TNF-α and IL-10 production by placenta cells from IP and UP mice.

Initiation of infection and sacrifice were performed as described in the legends for figure 1. Fetoplacental units isolated from mice sacrificed on GD 6, 8 and 9 were minced and cultured directly in RPMI with 10% FBS. On GD 10 and 11, single cell suspension of placental and decidual tissues was made as described in the materials and method section. Supernatants from cells cultured without any external stimuli at a concentration of $5x10^6$ cells/ml for 24 hours at 37^0C were then assayed for IFN-γ level by ELISA. Data from individual mice and median and interquartile range for each group is presented. Number of mice sacrificed (IP and UP respectively) at each GD/ED was as follows: at GD/ED 6, = 5 and 4; at GD/ED 8, n = 6 and 4; at GD/ED 9, n = 5 and 5; at GD/ED 10, n = 5 and 9; at GD/ED 11, n = 5 and 8. GD / ED 10 include both aborting and nonaborting mice. For IFN-γ; IP versus UP (GD 8 and 9), $P < 0.05$ (GLM Tukey), TNF-α; UP versus IP (GD 11), $P < 0.05$. ∗N = nonaborting, A = Aborting.

Table 4.1.

GD	Pregnancy Status	Culture Method	IFN-γ (pg/ml)		TNF-α (pg/ml)		IL-10 (pg/ml)	
			IP (n)	UP	IP (n)	UP	IP (n)	UP
6	**N***	**Explant**	**0, 0-0**	**0,** 0-0	**14,** 8-25	**17,** 6-41	**0,** 0-0	**0,** 0-0
8	**N**	**Explant**	**261,** 51-550	**0,** 0-0	**25,** 13-45	**37,** 32-44	**0,** 0-0	**0,** 0-0
9	**N**	**Explant**	**535,** 419-643	**0,** 0-0	**25,** 18-31	**28,** 28-30	**0,** 0-0	**0,** 0-0
10	**N**	**Single cell**	**0 (3)**	**0**	**23 (3)** 19-28	**30,** 27-30	**0 (3)**	**0,** 0-0
10	**A***	**Single cell**	**0 (2)**		**23 (2)** 14, 32		**0 (2)**	
11	**A**	**Single cell**	**0,** 0-0	**0,** 0-0	**13,** 9-18	**35,** 29-44	**0,** 0-0	**0,** 0-0

Table 4.2.

Levels of sTNFRII in the placenta cell culture supernatants from *Plasmodium chabaudi* AS-infected, pregnant and uninfected, pregnant mice.

Initiation of infection and sacrifice were performed as described in the legend to figure 1A. Placenta cells were cultured as described in the legends to Table 1. The number of mice sacrificed (IP and UP respectively) were: at GD/ED 6, 8 and 11, n = 5 and 5; at GD/ED 9, n = 5 and 4; at GD/ED 10, n = 4 and 4. GD / ED 10 include both aborting and nonaborting mice. ∗N = nonaborting, A = Aborting

Table 4.2.

GD	Pregnancy Status	Culture Method	sTNFRII (ng/ml)	
			IP (n)	UP (n)
6	**N***	**Explant**	**11,** 7-17	**8,** 6-8
8	**N**	**Explant**	**56**, 7-59	**86,** 37-96
9	**N**	**Explant**	**110,** 95-131	**34,** 18-66
10	**N**	**Single cell**	**9 (2)** **8, 10**	**2,** 1-2
10	**A***	**Single cell**	**16 (2)** **28, 3**	
11	**A**	**Single cell**	**2**, 1-4	**3,** 0.6-5

CHAPTER 5

MALARIA-ASSOCIATED MURINE PREGNANCY FAILURE. II: DISTINCT ROLES FOR IFN-γ AND TNF-α IN MALARIA-INDUCED PREGNANCY LOSS IN MICE[1]

[1] Jayakumar Poovassery and Julie M. Moore. To be submitted to Journal of Immunology.

ABSTRACT

The immunological basis for malaria-induced fetal loss is not clear. In this study, the role of proinflammtory cytokines IFN-γ and TNF-α in *Plasmodium chabaudi* AS-induced fetal loss in mice was investigated by employing gene knockout mice and antibody ablation experiments. In the first set of experiments IFN-γ gene knockout mice on the malaria resistant C57BL/6 background were used to study the impact of this cytokine on pregnancy success. As expected, *P. chabaudi* AS-infected, pregnant knock out mice experienced a more severe course of infection characterized by high parasitemia, severe anemia and marked loss of weight. However, the pregnancy progression was improved these mice compared to wild type mice. In comparison to 79% resorptions observed in wild type mice, knock out mice had only 11% resorptions on gestation day 11. However, like wild type mice, the knockout mice also failed to carry viable pregnancies to term. Fetal resorptions in knockout mice were associated with persistently high levels of TNF-α in the plasma. In the second set of experiments, C57BL/6 mice treated with TNF-α neutralizing antibodies were used to dissect out the role of this cytokine in fetal loss. The course of infection was not different in infected, pregnant mice treated with anti-TNF-α antibody compared to infected, pregnant mice treated with control IgG. While infected, pregnant IgG controls aborted or resorbed all of their embryos by gestation day 11, anti-TNF-α antibody-treated infected, pregnant mice retained their pregnancies and had only 15% resorptions on gestation day 12. The mechanism by which TNF-α may mediate fetal loss is not known, but it is noteworthy that fetal loss in infected, pregnant mice was associated with an upregulation of procoagulants, tissue factor and plasminogen activator inhibitor–1, in the uterus.

Moreover, placentae from aborting mice had wide spread hemorrhage and fibrin thrombi formation in the maternal blood sinusoids on histological examination. Results from this study suggest that TNF-α is a critical factor in malaria-induced fetal loss in mice and TNF-α-induced fetal loss may be through upregulation of procoagulants in the uterus.

INTRODUCTION

The immunological basis for the poor pregnancy outcome during malarial infection is not clear. Accumulation of maternal immune cells as well as production of proinflammatory cytokines and chemokines at the maternofetal interface is thought to contribute to poor pregnancy outcomes, especially low birth weight babies in the case of malarial infection during pregnancy in women living in high endemic areas (1-3). Both maternal and fetal cells are shown to be involved in production of cytokines and chemokines at the maternofetal interface (4, 5). In contrast, *Plasmodium falciparum* infection in nonimmune pregnant women or during an epidemic has been shown to be more severe and can cause high rates of abortion, still-birth and preterm labor (6, 7). However, the immunologic basis for the fetal loss in malaria-infected nonimmune women is not clear and no definitive studies have been performed to provide causative evidence for proinflammatory cells or soluble factors in malaria-induced fetal loss. We have recently developed a mouse model to investigate the immunologic and molecular mechanisms involved in malaria-induced fetal loss. In this model, C57BL/6 (B6) mice infected on gestation day (GD) 0 abort their fetuses at mid-gestation (8). Furthermore, the fetal loss in this model was associated with high levels of proinflammatory cytokines, IFN-γ and TNF-α, in the plasma and spleen cell culture supernatants (please see chapter 4).

Production of IFN-γ during early stages of infection is essential for protection against primary *P. chabaudi AS* infection in B6 mice (9). IFN-γ, primarily produced by NK cells and T cells, is a pluripotent cytokine that has been shown to regulate over 200 genes in a wide variety of cells and tissues (10-16). In association with IL-12, IFN-γ plays an important role in skewing the immune response towards a Th1 phenotype. Additionally, IFN-γ modulates $CD8^+$ T cell and $CD4^+$ T cell function by regulating major histocompatability complex gene expression (17-19). Although IFN-γ produced in small quantities at appropriate locations is thought to be beneficial for normal pregnancy (20), when produced in excess this cytokine can have an abortifacient effect (21).

During malarial infection, IFN-γ is thought to mediate its functions through activating macrophages to produce TNF-α and other soluble parasitocidal mediators such as nitric oxide and reactive oxygen species. TNF-α is a multifunctional cytokine produced by macrophages, T and B cells and mast cells and is involved in protective immunity, inflammation, autoimmunity and pathophysiology of many diseases (22, 23). TNF-α exists either as membrane-bound or soluble homotrimer (24) and can modulate proliferation, differentiation, and apoptotic or necrotic cell death in a number of different cell types (25). These functions are mediated by signaling through two distinct receptors, namely, TNF receptor I (TNFRI, CD120a) and TNF receptor II (TNFRII, CD120b) (26). While the TNFRI complex contains a death domain and mediates both pro-apoptotic and anti-apoptotic pathways, TNFRII lacks a cytoplasmic death domain and is involved in inducing pathways of cell activation and survival. During malarial infection TNF-α has been implicated in both protection and pathology. In humans, high levels of TNF-α have been shown to be associated with severe anemia and cerebral malaria in children (27, 28).

High levels of TNF-α in the serum and lower plasma IL-10 to TNF-α ratio have been shown to be associated with anemia in children (29, 30). Furthermore, TNF-α production during blood stage malaria-infection in mice is associated with splenomegaly (31), weight loss, and anemia (32).

During pregnancy TNF-α is expressed by a variety of cell types, including maternal decidual cells and fetal trophoblast cells in humans and mice (33-35), but its role during normal pregnancy is not clear. TNF-α is thought to be involved in normal embryonic growth and development (36) and also in smooth muscle contraction during labor (37). However, TNF-α or TNF-α receptor knockout mice can reproduce normally, suggesting that this cytokine may not be essential for normal pregnancy. Nonetheless, aberrant production of TNF-α during pregnancy is detrimental to the fetus (38). In humans, a high level of TNF-α is associated with several pregnancy complications such as preterm labor (39), preeclampsia (40), and recurrent spontaneous abortion (41). An elevated systemic and placental level of TNF-α is also thought to be associated with fetal loss in rodent models of parasitic infections (42, 43), including malaria (see chapter 4). To identify the role of proinflammatory cytokines IFN-γ and TNF-α in malaria-induced fetal loss, both IFN-γ gene knock out mice and selective depletion of TNF-α in *P. chabaudi* AS-infected pregnant (IP) and control mice using a TNF-α neutralizing antibody were employed. The results from this study revealed that IFN-γ deficiency alone was not enough to rescue the pregnancy in *P. chabaudi* AS-infected mice. However, TNF-α neutralization resulted in reduced number of resorptions in IP mice suggesting that TNF-α is a major factor involved in malaria-induced fetal loss.

MATERIALS AND METHODS

Mice and parasites: Wild type (WT) C57BL/6 (B6) mice originally purchased from Jackson Laboratories, Bar Harbor, ME and IFN-γ gene knockout (KO) mice (B6.129S7-*Ifng*^{tm1Ts}; obtained from Dr. Rick Tarleton) 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. Eight to nine week old female mice were used for all the experiments. *P. chabaudi* AS originally obtained from Mary M. Stevenson (McGill University and the Montreal General Hospital Research Institute, Quebec, Canada) maintained as described previously (8) was used for all the experiments.

Experimental design: WT or KO female mice were infected intravenously on GD 0 with 1000 *P. chabaudi* AS-infected erythrocytes (iRBC) per 20 grams of body weight. Infected, non-pregnant (INP) mice, and sham-injected uninfected, pregnant (UP) mice were used as infection and pregnancy controls, respectively. Mice were sacrificed on GD / experiment days (ED) 6, 8, 9, 10, 11and 12 to assess the pregnancy outcome and immune response development. Parasitemia, anemia and changes in body weight were monitored as described previously (8). At sacrifice, uteri for histological examination were collected in 10 % buffered formalin and for gene expression studies uteri were collected in RNA later (Ambion) and stored at -20^{0}C until used for RNA extraction.

Anti-TNF-α antibody treatment: B6 mice infected on day 0 of pregnancy with *P. chabaudi* AS were either treated with 100μg of anti-TNF-α antibody (Clone MPG-XT3, Upstate, Lake Placid, NY) or control rat IgG (Sigma) on GD / ED 6, 8, 9, 10 and 11. by intraperitoneal injection. Control, uninfected pregnant mice were also treated with anti-

TNF-α antibodies or rat IgG. Mice were sacrificed on GD / ED 12 or immediately on signs of abortion (namely, bloody, mucoid discharge from the vagina).

Cytokine ELISA: Levels of IFN-γ, TNF-α and IL-10 in the plasma samples were determined using OptEIA enzyme-linked immunosorbent assay (ELISA) sets (Pharmingen) or DuoSets (R&D Systems) according to the manufacturer's instructions. Limits of detection were 8 pg/ml for IL-10 and TNF-α and 15 pg/ml for IFN-γ.

Real-time reverse-transcription PCR: Total RNA was isolated from fetoplacental units using the RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol and stored at −85 °C. Genomic DNA contamination was removed by digesting with RNAse-free DNAse (RQ1 RNAse free DNAse, Promega Madison, WI , USA) as recommended by the manufacturer. First strand cDNA was synthesized from 1μg of obtained total RNA using the Omniscript reverse-transcription kit (Qiagen). Real-time PCR was carried out using specific primers for tissue factor (TF) (forward: 5′-tcagttcatggagacggagac-3′ and reverse: 5'-ggtttgtgtctcggtaaggtaa-3'), Plasminogen activator inhibitor (PAI)-1 (forward: 5′- atcagacaatggaagggcaac-3′ and reverse: 5'-gaacttaggcaggatgagga-3') and 18s RNA (forward: 5′-gtaacccgttgaaccccatt -3′ and reverse: 5′- ccatccaatcggtagtagcg-3′) (MWG-Biotech Inc., High Point, NC, USA). All the primers used were first validated for use in comparative real-time PCR. Real-time PCR was performed using the Mx3000P thermocycler and program (Stratagene, Cedar Creek, TX, USA). No template controls and no reverse-transcription controls were included. The 2^ ΔΔ CT method of analysis was used with the 18S RNA gene as normalizing gene and RNA from UP mice as the calibrator. Results are expressed as fold increase over UP controls.

Statistical Analysis: Data analysis was performed using SAS statistical software package (version 8.02; SAS Institute, Inc., Cary, N.C.). The significance of differences among group means in the case of normally distributed data was determined using Proc GLM. Tukey's Studentized (HSD) range test was used to perform multiple pairwise group comparisons in cases of unequal sample size. In cases of non-normally distributed data the nonparametric Wilcoxon rank sum test was used for comparisons and the nonparametric MULTTEST (SAS proc multtest) was used to obtain the adjusted *P* value for each pair of groups in multiple comparisons. *P* values of ≤0.05 were considered to be significant.

RESULTS

P. chabaudi *AS infected IFN-γ KO mice experienced more severe course of infection:*

Studies with *P. chabaudi AS* infection in WT B6 mice revealed a significant increase in the level of Th1/proinflammatory cytokine IFN-γ in plasma, spleen, and placental cell culture supernatants from IP mice relative to UP mice, at time points corresponding to ascending and peak parasitemia (which also corresponds to the timing of fetal loss in this model) (see chapters 3 and 4). To dissect out the role of IFN-γ in the observed fetal loss, experiments were performed using KO mice. First, to characterize the course of *P. chabaudi* AS infection in pregnant KO mice, the development of parasitemia, anemia and changes in body weight were monitored in IP and INP KO mice. The development of parasitemia was accelerated in KO mice (figure 5.1A) compared to WT mice (figure 5.1B) regardless of pregnancy and was significantly higher than that observed in WT mice on GD / ED 8 through 10 (IP KO versus IP WT, $P < 0.014$; IP KO versus INP WT, $P < 0.0007$). Furthermore, the parasitemia peaked on GD / ED 10 in IP KO mice, one day

earlier than that observed in WT mice. The parasitemia was also slightly higher in IP KO mice compared to INP KO mice on GD / ED 8 through 10 ($P > 0.05$). At peak, the mean parasitemias were 41.96% and 39.62% in IP and INP KO mice compared to 30.28 and 25.57% observed in IP and INP WT mice respectively. Although the parasitemia levels in IP and INP KO mice dropped to 5.80% and 6.20%, respectively, on GD / ED 14, these mice failed to survive beyond day 15 (data not shown). One probable reason for the observed mortality could be the severe anemia experienced by KO mice (Figure 5.1 C and D). It has been shown previously that WT mice can regain their hematocrit values as they recover from parasitemia (8). In contrast, KO mice failed to recover from anemia even after experiencing a decline in parasitemia on GD / ED 11. IP KO mice exhibited significantly lower hematocrit values on GD / ED 10 in comparison to UP KO and all WT groups ($P < 0.05$), but were equivalent to INP KO mice. However, in IP KO mice the nadir of hematocrit occurred on GD / ED 11 compared to GD / ED 12 in INP KO mice and at peak anemia the hematocrit values were 8.2% in IP KO and 9.07 % in INP KO mice. Thus, in addition to malarial infection, pregnancy-associated hemodilution (44), as observed previously in this model (8), may also be contributing to the accelerated anemia observed in IP KO mice.

IP KO mice also experienced changes in their body weight during the course of infection (Figure 5.1 E and F). As in the case of IP WT mice, IP KO mice initially gained weight until GD / ED 8. However, all infected mice lost weight corresponding to ascending and peak parasitemia and anemia. Although infected WT mice regained their weight as they started clearing the parasitemia and regaining their hematocrit values (data

not shown and (8)), IP and INP KO mice exhibited a downward trend in their body weight until they succumbed to infection.

Fetal Outcome: Contrary to the expectation, IP KO mice also failed to maintain viable pregnancies to term. However, the pattern of pregnancy loss observed in IP KO mice was different from WT mice (Table 5.1). In agreement with our previous report (8), pregnancy in none of the IP WT controls progressed beyond GD / ED 11. Compared to 79% resorbing embryos observed in IP WT mice, IP KO mice had only 11 % resorbing embryos on GD / ED 11, a level that was indistinguishable from UP KO mice. However, this dropped dramatically on GD 12 (40 % viable embryos in IP KO versus 100 % in UP KO mice; Table 5.2). Thus, although absence of IFN-γ has some positive impact on pregnancy progression, the data suggest that mechanisms other than those directly involving IFN-γ may induce the observed pregnancy loss in IP mice.

IP KO mice exhibited sustained high levels of TNF-α in the plasma: It has been shown previously that *P. chabaudi* AS-infected non pregnant KO mice are capable of producing TNF-α, an abortifacient cytokine, though in smaller amounts compared to WT mice (9). To investigate whether the observed fetal loss in IP KO mice is associated with TNF-α production, the level of TNF-α in the plasma samples from IP KO and control mice at various stages of infection was determined by ELISA. Indeed, IP KO mice exhibited a sustained high plasma level of TNF-α in comparison to UP KO and INP KO mice (Table 5.3) and the levels were significantly higher on GD / ED 8, 9, and 12 relative to UP KO ($P < 0.043$) and on GD / ED 9, versus INP KO ($P = 0.023$). Thus the observed mortalities in IP KO mice could be due to dysregulated TNF-α production.

To study whether the uncontrolled production of TNF-α is due to a defect in IL-10 production, a cytokine that has been shown to suppress TNF-α production and function (45), the level of IL-10 in the plasma samples from IP and UP KO mice was assayed by ELISA. Table 5.3 represents the kinetics of appearance of IL-10 in the plasma from IP, INP and UP KO mice. In comparison to INP KO mice, IP KO mice exhibited a lower plasma IL-10 level. Its level peaked on GD / ED 9 in IP KO mice and thereafter exhibited a downward trend. By GD / ED 12 there was a total suppression of IL-10 production in IP KO mice. The IL-10 level was either very low or absent in the plasma samples from UP KO mice.

The course of infection is not altered in anti-TNF-α antibody treated mice: As a next logical approach, the question whether the pregnancy can be rescued in IP mice by neutralizing TNF-α was addressed. For this IP mice were treated on GD / ED 6, 8, 9, 10 and 11 with 100μg of TNF-α neutralizing antibody. To identify the effect of neutralizing TNF-α on normal pregnancy, UP mice were also treated with anti-TNF-α antibodies. Figure 5.2A represents the clinical outcome in anti-TNF-α treated and control mice. Although the parasitemia developed slightly slower and peaked slightly higher in anti-TNF-α treated mice compared to IP IgG mice (31.59 % in IP anti-TNF and 29.20 % in IP IgG group), both treatment groups peaked on GD / ED 11 and the differences in parasitemia levels did not reach statistical significance.

Figure 5.2 B shows the development of anemia in IP anti-TNF-α group and controls. Here again, the anti-TNF-α antibody treatment did not significantly alter the course of anemia in IP mice. Other than a slight delay observed in the IP anti-TNF-α group, both infection groups exhibited progressive development of anemia until sacrifice

(GD 11, IP IgG and GD12, anti-TNF-α group). Malarial infection also induced weight loss in infected mice (figure 5.2 C). However, in comparison to the IP anti-TNF-α group, IP IgG group exhibited a marked loss in body weight which was significantly different from the anti-TNF-α group on GD / ED 11 ($P < 0.05$), suggesting a possible role for TNF-α in malaria-induced anorexia and wasting.

***Anti-TNF-α treatment rescues pregnancy*:** Although the IP anti-TNF-α and IP IgG groups experienced similar courses of infection, the pregnancy progression was greatly improved in mice treated with anti-TNF-α antibody (Table 5.4). While all the mice in IP IgG group aborted or resorbed their embryos by GD / ED 11, the IP anti-TNF-α group had only 15 % resorptions on GD / ED 12, which was comparable to that observed in UP anti-TNF-α (15 %) and UP IgG (13 %) group. These results demonstrate that removal of TNF-α can significantly improve fetal development in malaria infected mice.

To study the effect of anti-TNF-α on production of other critical cytokines, the level of IFN-γ and IL-10 in the plasma samples from IP anti-TNF-α group and controls were measured at the time of sacrifice (GD 11 for IP IgG and GD 12 for other groups). As observed previously (see chapter 4), UP mice did not have detectable levels of IFN-γ or IL-10 in the plasma (Table 5.5). However, the IP anti-TNF-α group had significantly higher levels of IFN-γ (versus uninfected, $P < 0.004$) and IL-10 (versus uninfected, $P < 0.020$) in the plasma. TNF-α was not detected in the plasma from mice treated with TNF-α neutralizing antibodies (data not shown).

Tissue Factor and Plasminogen Activator-1 genes are upregulated in the uterus of aborting mice: Malaria infection has been shown to be associated with an enhanced

systemic procoagulant state (46, 47). To investigate whether the observed fetal loss in WT IP mice is related to any disturbances in the coagulation cascade the levels of TF and PAI-1 in the fetoplacental units from IP mice were measured by real time PCR (Figure 5.3). TF mRNA levels were upregulated in IP mice on GD / ED 10 and 11, with greater than 5 fold increase in TF mRNA levels in IP mice on GD / ED 10 in comparison to UP mice. Similarly, the PAI-1 level also exhibited a two fold increase in IP mice undergoing abortion on GD / ED 11.

Placenta sections from aborting mice had massive hemorrhage and fibrin thrombi formation: To further investigate the potential role of coagulopathies on fetal loss, histological examinations were performed on placental sections from mice undergoing abortion. Mice were sacrificed at different stages of infection and 5μm thick placental sections were stained with hematoxylin and eosin (H & E). As presented in figure 5.4, IP mice undergoing abortion had wide spread hemorrhage in the placenta (5.4A) and the hemorrhage was associated with thrombosis in maternal blood sinusoids (5.4C).

DISCUSSION

We have shown previously that *P. chabaudi* AS infection completely abrogates pregnancy in B6 mice (8). Pregnancy loss in IP mice was associated with increased production of proinflammatory cytokines in the plasma, spleen and placenta cell culture supernatants (see chapter 4). Proinflammatory cytokines are shown to be involved in the protection as well as pathology during malarial infection in humans and mice (9, 48, 49). The results from this study demonstrated that both IFN-γ and TNF-α are involved in malaria-induced pathology and fetal loss.

IFN-γ is a critical cytokine involved in protection against primary *P. chabaudi* AS infection in B6 mice (9). Depletion of IFN-γ or IFN-γ receptor deficiency have been shown to be associated with prolonged acute phase parasitemia and greater mortalities in *P. chabaudi AS*-infected mice suggesting an important role for IFN-γ in the control of malaria infection in mice (50, 51). An early Th1 and IFN-γ response have also been described for both lethal and non-lethal *P. yoelii* infections and a lethal *P. vinckei vinckei* infection (37). Furthermore, IFN-γ production correlated with protection against *P. falciparum* infection in humans (52). In agreement with these observations, WT mice regardless of pregnancy status were able to clear the parasitemia and survive the infection. In contrast, deficiency of IFN-γ was associated with severe clinical outcome in IP and INP mice. IP KO mice experienced a significantly higher parasitemia, severe anemia and marked loss of weight compared to IP WT mice and succumbed to infection by GD / ED 15. Although the differences were not statistically significantly different, the parasitemia and anemia levels were higher in IP KO mice compared to INP KO mice during the early stages of infection, suggesting a role for pregnancy in modulating the course of malarial infection. This was consistent with the accelerated development of parasitemia and anemia reported in IP WT mice (8). In contrast to WT mice, which regained their hematocrit values and body weights as they started clearing the parasitemia, IP and INP KO exhibited a continuous downward trend in their body weights and hematocrit values until they succumbed to infection. None of the IP KO mice survived beyond GD / ED 15. Paradoxically, although IP KO mice experienced a more severe clinical course, their pregnancy progression was improved. Compared to

wild type mice that failed to maintain any viable fetuses beyond GD / ED 11, IP mice had only 11% resorptions at the same time.

During normal pregnancy IFN-γ is produced by different cell types in the uterus (53-55). Uterine NK cells are thought to be the major source of placental IFN-γ in mice (20, 56). When produced in appropriate quantities, uteroplacental IFN-γ production is thought to function in normal implantation and fetal development (20). However, excessive production of this cytokine could be detrimental to the fetus (57, 58). Exogenous administration of IFN-γ to normal pregnant mice has been shown to induce fetal resorptions (58). The exact mechanism by which IFN-γ induces fetal loss is not clear. In humans, IFN-γ has been shown to be capable of inducing the apoptosis of cultured term trophoblast cells (59, 60). It is also possible that IFN-γ may be mediating pathology through induction of downstream effector molecules. The fact that IP KO mice failed to maintain viable pregnancies beyond GD / ED 15 indicates a possible role for other factors in malaria-induced fetal loss. In agreement with this, elevated levels of TNF-α, a known embryotoxic factor, were observed in the plasma samples from IP KO mice. In addition to IFN-γ, a major cytokine implicated in activating macrophages to produce TNF-α, nonpregnant infected KO mice are capable of producing TNF-α (9). This is thought to be mediated through direct activation of monocytes or macrophages by malarial parasites and their soluble antigens such as glycosylphosphatidylinositols (61) and insoluble hemozoin (heme polymer produced as a by-product of intraerythrocytic hemoglobin catabolism by malarial parasites) (62).

In this study, IP KO mice exhibited a sustained high level of TNF-α in the plasma, which is in contrast to an upward and then downward pattern observed in WT

mice during the later stages of infection (see chapter 4). One probable reason for this could be the dysregulated kinetics of IL-10 production. The IL-10 level peaked on GD / ED 9 in IP KO mice, two days earlier than the peak observed in INP KO mice. By GD / ED 12 IP KO mice exhibited a complete suppression of plasma IL-10 level. Thus the high level of TNF-α, in the absence of IL-10, may contribute to the pathologies observed in IP KO mice, including fetal loss. IL-10 is a cytokine that plays an important role in preserving the fetal allograft through suppression of abortifacient cytokines TNF-α and IFN-γ (45). Administration of IL-10 reversed fetal resorptions in a mouse model for stress-induced abortion (63), and missed abortions in humans are thought to be associated with low IL-10 with high IFN-γ production by the decidual cells (64).

The abortifacient effect of TNF-α has been demonstrated in a number of studies. Administration of high levels of exogenous TNF-α or LPS to normal pregnant mice can induce embryonic death (65, 66), and blockade of TNF prevented fetal loss in murine models of stress-induced abortion (67). However, the exact mechanism by which TNF-α induces fetal loss is not clear. It is possible that TNF-α may be inducing a thrombotic event in the uterus through upregulation of procoagulants (68). In agreement with this there was more than five-fold increase in the expression of TF gene in the fetoplacental units from mice undergoing abortion. Furthermore, the placentae from aborting mice had severe hemorrhage and fibrin thrombi formation in the maternal blood sinusoids. However, it remains to be determined if these events precede (and thus induce) fetal loss, or are simply markers of other local pathological events.

TF is a 47- kD protein that initiates the clotting cascade by binding to coagulation factor VIIa and ultimately yielding thrombin which converts fibrinogen to fibrin. In

addition to proinflammatory cytokines, *P. falciparum*-infected erythrocytes can also induce TF expression on activated endothelial cells (69). Furthermore, accumulation of macrophages positive for TF staining is also reported in the placentas from *P. falciparum*-infected women (70). Constitutive expression of TF has been demonstrated in syncytiotrophobalst cells and perivascular cells and is thought to be involved in normal hemostasis (71-73). However, induced expression of TF is associated with life-threatening thrombosis in a variety of pathological conditions including sepsis, atherosclerosis and cancer (74-76), and at the maternofetal interface TF activation was associated with fetal loss in thrombomodulin-deficent mice (77).

In this model, the identity of cells expressing TF in the uterus of aborting mice is not known. Since the fetal loss in this model was not associated with mononuclear cell accumulation in the placenta, it is possible that the TF expressing cells could be fetal trophoblast cells. Furthermore, whether there are any alterations in the systemic levels of coagulation factors in IP mice as reported in the case of *P. falciparum* infection (47) is also not known. Our laboratory is currently investigating this possibility. However, there was an upregulation of PAI-1 mRNA in the fetoplacental sections from aborting IP mice. PAI-1 blocks fibrinolysis by suppressing tissue type plasminogen activator (tPA) (78). Moreover, placental PAI-1 levels are shown to positively correlate with the severity of preeclampsia and intrauterine growth retardation in humans (79, 80). Thus, an increase in PAI-1 along with elevated TF level may contribute to clot formation and accumulation in the placenta.

Accumulation of iRBCs in the maternal intervillous space is a characteristic feature of malarial infection during pregnancy in humans. An increased accumulation of

P. chabaudi AS iRBCs in maternal blood sinusoids of the placentae from mice undergoing abortion is also observed (8). Furthermore, *P. falciparum*-infected erythrocytes expose phosphatidyl serine on their surface which has been shown to be important for clot formation (81). Thus, it is possible that the accumulation of iRBCs in the placenta along with high levels of proinflammatory cytokines may provide a favorable environment for the progression of coagulation cascade in the placenta leading to the formation of fibrin thrombi and ultimately fetal loss through loss of blood supply.

Taken together, the results from this study demonstrated that both IFN-γ and TNF-α are involved in fetal loss observed during the acute stage of malarial infection in pregnant B6 mice. The fact that the IFN-γ deficiency improved the fetal progression even as the mother dies of the infection further confirms the detrimental effect this cytokine can have on the fetus. However, it seems in this model system the IFN-γ is mediating its embryotoxic effects indirectly through induction of downstream effector molecules, especially TNF-α. Furthermore, the increased expression of procoagulants in the uterus of aborting mice is consistent with the procoagulant state reported in human malarial infection (70), making this model an ideal choice for further investigation of complex interactions between proinflammatory cytokines and coagulation factors in malarial pathogenesis and poor pregnancy outcome.

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Figure 5.1.

Parasitemia*, hematocrit and weight change in P. chabaudi AS-infected IFN-γ gene knock out (KO) and control B6 (WT) mice.*

Eight-nine week old KO or WT mice were injected intravenously with 1000 *P. chabaudi* AS-infected erythrocytes or 200 µl of PBS (control mice) per 20 grams of body weight on GD/ED 0. Percent parasitemia (A, B) from Giemsa-stained thin smears, hematocrit (C, D) of tail vein blood and weight (E, F) were assessed at one-two day intervals as shown. Mice were divided into six groups: infected, nonpregnant knock out (INP KO), uninfected, pregnant knock out (UP KO), infected, pregnant knock out (IP KO) (Figure 1 A, C and E) and infected, nonpregnant wild type (INP WT), uninfected, pregnant wild type (UP WT), and infected, pregnant wild type (IP WT) (Figure 1 B, D and F). KO mice were sacrificed at days 6, 8, 9, 10, 11 and 12 in two serial sacrifice studies. WT mice were sacrificed at the time of abortion. Starting sample sizes: INP KO, n = 30; UP KO, n = 23 and IP KO, n = 28. Number of mice sacrificed (INP KO, UP KO, IP KO) at each GD/ED; GD/ED 6: 4, 5, 4; GD/ED 8: 4, 4, 4; GD/ED 9: 4, 5, 4; GD/ED 10: 3, 3, 3; GD/ED 11: 7, 3, 5; GD/ED 12: 8, 3, 8. The number of mice sacrificed for WT mice (INP, UP and IP) on GD/ ED 11 = 6, 8 and 7. All data presented are mean ± standard error of the mean (SEM). The Y axis on E and F begins at 15 g to avoid compression and poor visualization of the data. Statistical differences, all $P < 0.05$, Proc GLM, Tukey. HEMATOCRIT: GD/ED 9: IP WT < UP KO; GD/ED 10: IP KO < all, INP KO < INP WT, IP WT < uninfected; GD/ED 11: IP KO < uninfected and INP WT, INP KO < uninfected, INP WT < uninfected, IP WT < uninfected; GD / ED 12: IP KO < UP KO, INP KO < UP KO. WEIGHT: GD/ED 7, 8: INP KO < IP KO, INP WT and INP KO <

UP; GD / ED 9: IP KO, INP KO and INP WT < UP KO; GD/ED 10, 11: infected < uninfected; GD/ED 12, IP and INP KO < UP KO. PARASITEMIA: Proc MULT TEST; GD/ED 8: IP and INP WT < IP KO, $P < 0.014$, INP WT < INP KO, $P = 0.014$; GD / ED 9: IP and INP WT < IP KO, $P < 0.010$, INP WT < INP KO, $P = 0.029$; GD / ED 10: IP and INP WT < IP KO, $P < 0.003$, IP and INP WT < INP KO, $P < 0.010$.

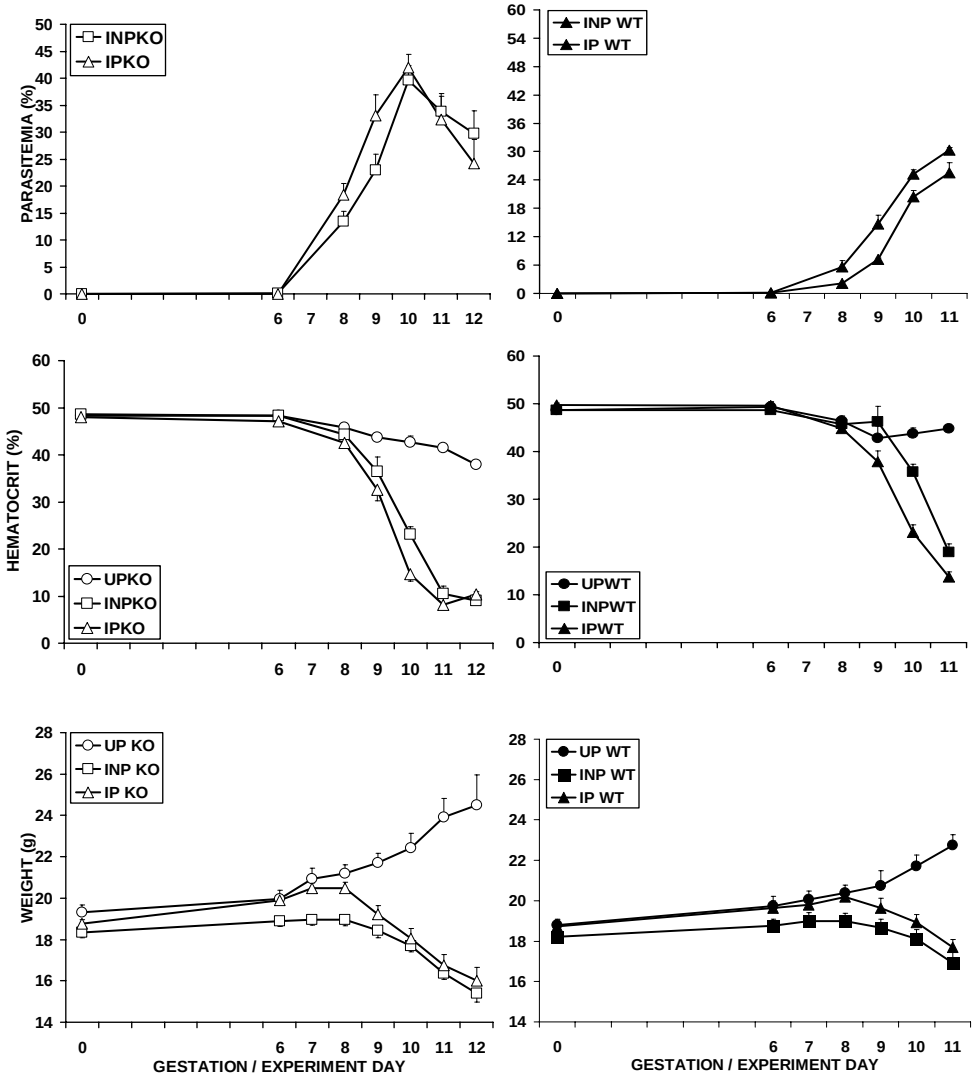

PARASITEMIA (%)
INPKO
IPKO
INP WT
IP WT
HEMATOCRIT (%)
UPKO
INPKO
IPKO
UPWT
INPWT
IPWT
WEIGHT (g)
UP KO
INP KO
IP KO
UP WT
INP WT
IP WT
GESTATION / EXPERIMENT DAY
GESTATION / EXPERIMENT DAY

Figure 5.2.

Development of parasitemia, anemia and weight chanage in *P. chabaudi* AS-infected B6 mice treated with anti-TNF-α antibody or IgG.

Infections were initiated as described under the legend to figure 1. IP and UP mice were treated with antiTNF-α antibodies or rat IgG. Percent parasitemia (A) from Giemsa-stained thin smears, hematocrit (B) of tail vein blood, and weight (C) were assessed at one-two day intervals as shown. Mice were divided into four groups: infected, pregnant anti-TNF (IP anti-TNF), infected, pregnant IgG (IP IgG), uninfected, pregnant anti-TNF (UP antiTNF), and uninfected, pregnant IgG (UP IgG). IP IgG mice were sacrificed at the time of abortion and IP anti-TNF, and UP mice were sacrificed on GD / ED 12. Sample sizes were for anti-TNF and IP IgG group, n = 5, for UP IgG group, n = 4; for UP anti-TNF, n = 3. All data presented are mean ± standard error of the mean (SEM). The Y axis on E, F begins at 15 g to avoid compression and poor visualization of the data. Statistical differences, all $P < 0.05$, Proc GLM, Tukey unless otherwise noted. HEMATOCRIT: GD / ED 10: infected < uninfected, GD / ED 11: infected < UP anti-TNF, GD / ED 12: IP anti-TNF < uninfected. WEIGHT: GD / ED 10: IP IgG < UP IgG; GD / ED 11: infected < UP IgG, IP IgG < UP anti-TNF, IP IgG < IP anti-TNF; GD / ED 12: IP anti-TNF < uninfected.

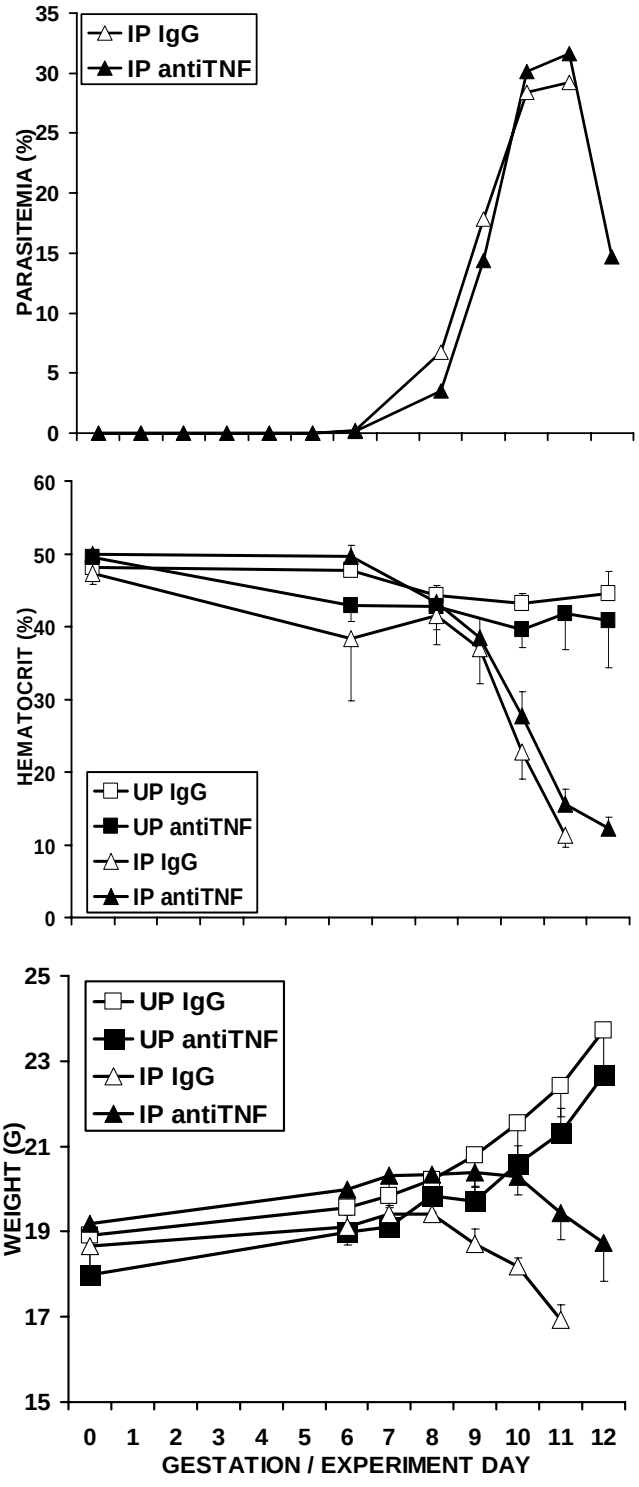

IP IgG
IP antiTNF
PARASITEMIA (%)
0
5
10
15
20
25
30
35
HEMATOCRIT (%)
0
10
20
30
40
50
60
UP IgG
UP antiTNF
IP IgG
IP antiTNF
WEIGHT (G)
15
17
19
21
23
25
UP IgG
UP antiTNF
IP IgG
IP antiTNF
0 1 2 3 4 5 6 7 8 9 10 11 12
GESTATION / EXPERIMENT DAY

Figure 5.3.

Upregulation of tissue factor (TF) and plasminogen activator inhibitor (PAI) -1 gene in the uterus of aborting mice.

Infections were initiated as described under the legends to figure 1. Mice were sacrificed on GD / ED 9, 10 and 11. RNA was extracted and gene expression changes assayed by real-time reverse-transcription PCR. n = 3 for GD / ED 9, n = 1, GD / ED 10 and n = 4 GD / ED 11.

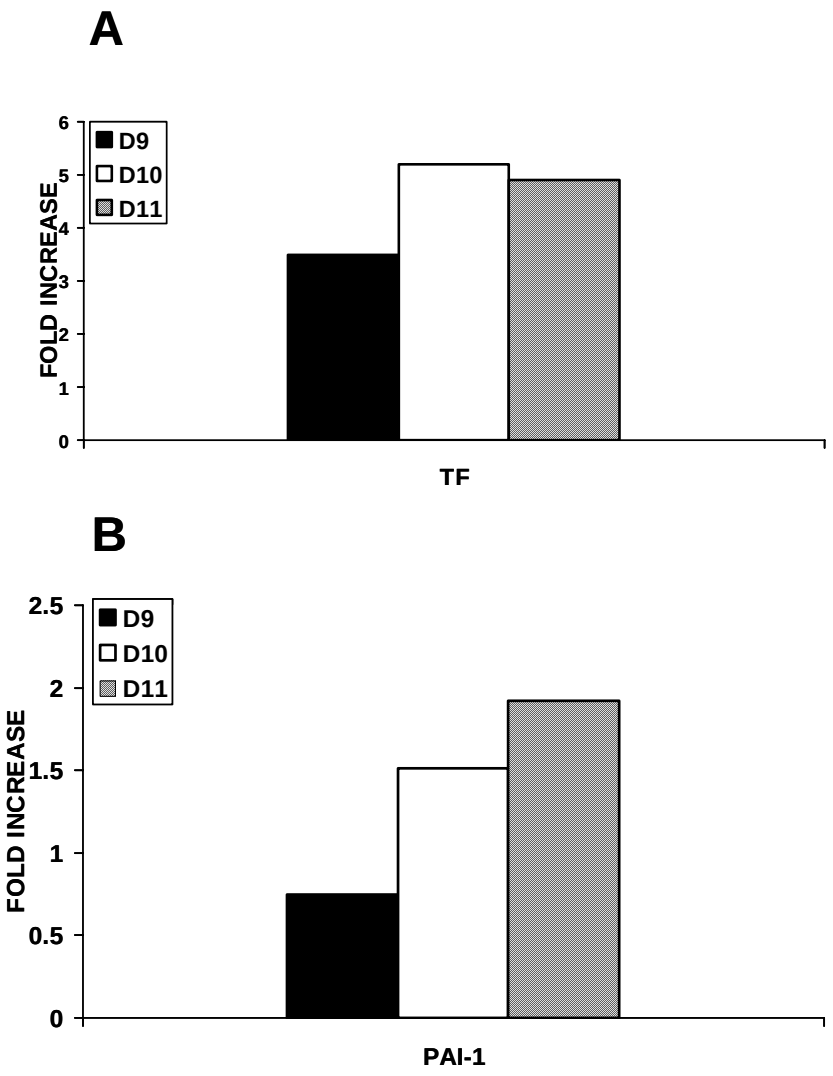
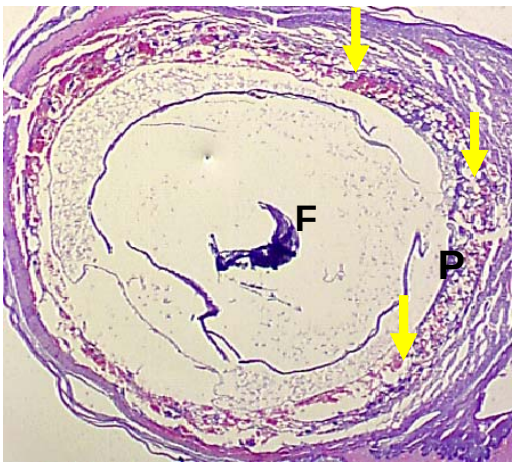
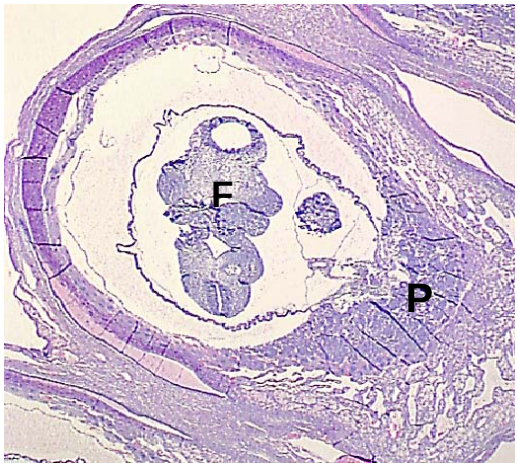

A
D9
D10
D11
FOLD INCREASE
0
1
2
3
4
5
6
TF
B
D9
D10
D11
FOLD INCREASE
0
0.5
1
1.5
2
2.5
PAI-1

Figure 5.4.

Histopathological lesions in the placenta from aborting, *P. chabaudi* AS-infected mice

Mice were infected as indicated in legends to Figure 1. A-C: photomicrograph of hematoxylin and eosin stained 5μm sections. A. Widespread hemorrhage (arrows) in placental tissue from a day 10 aborting mice (fetus, (F)). B. Cross section of day 10 UP embryo. C. Fibrin thrombus (Th) in maternal blood sinusoid in placenta section from day 9 IP embryo. P = Placenta.


F
P


F
P

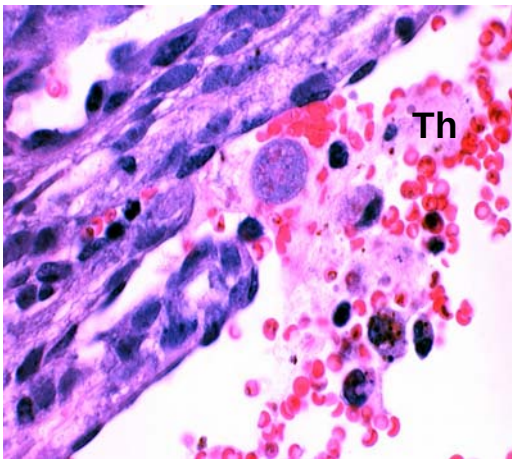

Th

Table 5.1.

Pregnancy progression in *P. chabaudi* AS-infected, pregnant and uninfected, pregnant knockout and wild type mice.

Mice were sacrificed on GD / ED 11 to assess the pregnancy success. Resorptions were scored by examination of uteri under a dissection microscope. For IP KO group, $n = 4$; for IP WT group, $n = 8$, for UP KO group, $n = 3$; for UP WT group, $n = 7$.

INFECTION GROUP	Mean # of Viable / Total Fetuses	% Resorption
IP KO	8 / 9	11.26
IP WT	1 / 7	79
UP KO	8 / 8	4.16
UP WT	6 / 6	0.02

Table 5.2.

Pregnancy progression in *P. chabaudi* AS-infected, pregnant and uninfected, pregnant knockout mice.

Mice were sacrificed on days indicated to assess pregnancy success to that point. Resorptions were scored by examination of uteri under a dissection microscope. [a]Gestation day; [b]Total number of resorptions per total number of fetuses; [c]Fisher's exact test.

GD[a]	IP KO resorp-tions[a]	*N*	UP KO resorp-tions[b]	*N*	P value[c]
6	0/44	6	0/29	5	NA
8	0/48	6	0/30	4	NA
9	0/33	4	0/40	5	NA
10	6/52	6	0/22	3	0.17
11	4/34	4	1/24	3	0.39
12	39/65	9	0/19	3	0.0001

Table 5.3.

TNF-α and IL-10 level in the plasma samples from IP KO, INP KO and UP KO mice.

Mice were infected as indicated in the legends to figure 1.Mice were sacrificed at indicated time points and plasma samples were assayed for cytokine level by ELISA. Median and interquartile range are presented. Number of mice sacrificed (IP KO, UP KO, and INP KO, respectively) at each GD/ED was as follows: at GD/ED 6, n = 5, 3, and 4; at GD/ED 8, n = 4, 4, and 3; at GD/ED 9, n = 4, 5, and 4; at GD/ED 10, n = 5, 3, and 3; at GD/ED 11, n = 4, 4, and 3; at GD/ED 12, n = 6, 3, and 6. The following statistically significant differences were observed for comparisons by Wilcoxon rank sum test: for TNF-α, IP KO versus UP KO (GD / ED 8, 9 and 12), $P < 0.043$; IP KO versus INP KO (GD / ED 9), $P = 0.023$; INP KO versus UP KO (GD/ED 8), $P = 0.021$. For IL-10: IP KO versus UP KO (GD / ED 9 and 10), $P < 0.049$; INP KO versus UP KO (GD / ED 10 and 11), $P < 0.037$.

GD	TNF (pg/ml)			IL-10 (pg/ml)		
	IP KO	UP KO	INP KO	IP KO	UP KO	INP KO
6	**0,** 0-0	**2** 0-17.5	**10** 0.4-21	**0,** 0-10	**0**, 0-0	**0,** 0-0
8	**35,** 9-102	**0,** 0-0	**19**, 11-25	**224**, 117-293	**0,** 0-0	**894,** 0-1745
9	**43,** 37-100	**19**, 5-20	**16,** 4-30	**640,** 522-1042	**0,** 0-0	**728,** 357-937
10	**31**, 19-46	**23,** 0-45	**47,** 15-1695	**217,** 182-294	**0,** 0-0	**343,** 53-770
11	**82,** 9-397	**0.19,** 0-26	**10,** 8-11	**44,** 30-61	**9,** 4-48	**941,** 826-1057
12	**42,** 6.5-81	**0,** 0-0	**16,** 8-30	**0,** 0-0	**0,** 0-0	**35,** 0-118

Table 5.4.

Pregnancy progression in *P. chabaudi* AS-infected B6 mice treated with anti-TNF-α antibody or IgG.

Infections were initiated as described in the legends to figure 1. IP and UP mice were treated with anti-TNF-α antibodies or rat IgG as described under materials and method section. IP IgG mice were sacrificed at the time of abortion (GD / ED 11). IP anti-TNF, UP anti-TNF and UP IgG groups were sacrificed on GD / ED 12 to assess the pregnancy success. For IP anti-TNF group, $n = 5$; for IP IgG group, $n = 5$, for UP antiTNF group, $n = 3$; for UP IgG group, $n = 4$.

Treatment Group (day12)	Mean # of Viable / Total Fetuses	% Resorption
IP IgG (at abortion)	0 / 4	100
IP anti-TNF-α	7 / 8	15
UP IgG	7 / 8	13
UP anti-TNF-α	7 / 9	15

Table 5.5.

IFN-γ and IL-10 levels in the plasma samples from *P. chabaudi* AS-infected B6 mice treated with anti-TNF-α antibody or IgG.

Infections were initiated as described in the legends to figure 1. IP and UP mice were treated with anti-TNF-α antibodies or rat IgG as described under materials and method section. IP IgG mice were sacrificed at the time of abortion (GD / ED 11). IP anti-TNF, UP anti-TNF and UP IgG groups were sacrificed on GD / ED 12. For IP anti-TNF group, $n = 5$; for IP IgG group, $n = 5$, for UP anti-TNF group, $n = 3$; for UP IgG group, $n = 4$.

Treatment Group (day12)	IFN-γ (pg / ml)	IL-10 (pg / ml)
IP IgG (at abortion)	110, 92-368	106, 19-161
IP anti-TNF-α	186, 138-645	103, 69-344
UP IgG	0, 0-19	0, 0-0
UP anti-TNF-α	0, 0-50	0, 0-0

CHAPTER 6

SUMMARY AND CONCLUSION

It is generally accepted that the physiological, anatomical and immunological changes happening in pregnant women to accommodate the developing embryo also make her more susceptible to various disease conditions. Malaria during pregnancy is one such condition that has been shown to be more severe in pregnant women compared to their nonpregnant counterparts or men. About 30 million pregnancies occurring each year among women living in high malaria endemic areas of Africa are at risk of developing the disease. The majority of the morbidity and almost all of the mortalities are due to the blood stage life cycle of *P. falciparum* and pregnant women and children living in sub Saharan Africa are the major risk group for developing severe disease. An understanding of the basic cellular and molecular mechanisms involved in immune activation and its regulation in malaria-infected pregnant women is critical in developing competent interventions for use against malarial infection during pregnancy.

Normal pregnancy is thought to require a bias against a Th1 or proinflammatory cytokine environment and a delicate balance between Th1 and Th2 cytokines both systemically and at the placental level is essential for successful pregnancies. However, several parasitic infections, including malaria, that require the development of a Th1 cytokine response for protection against the infection can alter this balance and can lead to poor pregnancy outcomes. Indeed, malarial infection during pregnancy has been shown to be associated with poor pregnancy outcomes such as abortion, stillbirth and preterm labor in the case of women living in low endemic areas. Although fetal loss is one of the most severe consequences of malarial infection, our understanding of the molecular and immunologic mechanisms of malaria-induced fetal loss is limited. One major reason for this is the lack of an appropriate, highly manipulable model system to

perform the necessary experiments. The current study was undertaken with an aim to develop an animal model to characterize the protective and pathogenic immune responses to malarial infection during pregnancy. For this, *Plasmodium chabaudi* AS, a rodent malarial parasite that closely resembles *P. falciparum,* was used to infect pregnant C57BL/6 mice. This model system allowed us to take advantage of the recent advances in immunology and mouse genetics to address the immunopathogenesis of malarial infection during pregnancy.

The small dose of *P. chabaudi* AS (1000 iRBCs per 20 gram of body weight intravenously) used in this study was not lethal to pregnant mice. IP mice experienced parasitemia, anemia and weight change comparable to INP mice. However, successful pregnancies were totally compromised. All IP mice failed to maintain viable pregnancies beyond gestation day 11. Additionally the fetal loss was associated with an accumulation of iRBCs in the maternal blood sinusoids in the placenta.

Since IP mice survived the infection, we hypothesized that the IP mice also develop an early Th1-cytokine biased-immune response as reported in the case of nonpregnant mice. The second study was undertaken to investigate this possibility. As predicted, IP mice exhibited elevated levels of proinflmmatory cytokines IFN-γ, TNF-α, IL-1β and also a regulatory cytokine IL-10 in the plasma. Although malarial infection impaired the ability of spleen cells from IP and INP mice to proliferate in response to mitogens, they produced IFN-γ, TNF-α and IL-10 in the culture medium either spontaneously or in response to malarial antigen or mitogens. Additionally, placenta cells from IP mice produced elevated amounts of IFN-γ in the culture medium spontaneously. However, the source of IFN-γ at the placental level during malarial infection is not

known. Since placenta cells from heterozygous embryos failed to produce IFN-γ in the culture supernatants, the source of this cytokine could be maternal.

Both IFN-γ and TNF-α are produced during normal pregnancy and are thought to have functions in normal implanation and fetal development. However, the same cytokines are also known embryotoxic agents. The role of IFN-γ and TNF-α in *P. chabaudi* AS-infected pregnant mice was investigated in the next study. IFN-γ deficiency resulted in a more severe clinical outcome characterized by high peak parasitemia, severe anemia and marked loss of weight in IP KO mice. Infected KO mice regardless of pregnancy status succumbed to infection by GD / ED 15. However, in comparison to WT mice, the time to fetal death was improved in IP KO mice. The deficiency of IFN-γ also altered the kinetics of other important cytokines such as TNF-α and IL-10 in IP KO mice. On the other hand, within the parameters of this study neutralization of TNF-α rescued the pregnancy in IP mice. While IP IgG group failed to maintain viable pregnancies beyond GD / ED 11, antibody-treated mice had viable fetuses in their uteri at sacrifice on GD / ED 12. The fetal loss in IP mice was also associated with widespread hemorrhage and fibrin thrombi formation in the maternal blood sinusoids of the placenta. These observations suggest that the proinflammatory cytokines may be inducing a coagulation cascade in the uterus of aborting mice. Indeed, there was an upregulation of TF and PAI-1 genes in the fetoplacental units from aborting mice.

The final experiment was conducted to study whether the fetus is playing any role in the observed placental pathology. For this an in vitro system was developed by culturing ectoplacental cone cells from GD 7 embryos. The trophoblast cells cultured in the presence of iRBCs exhibited phagocytosis of iRBCs and produced TNF-α in the

culture medium suggesting that the fetal cells may also be playing a role in the fight against the malarial infection at the maternofetal interface and may indirectly contribute to the fetal loss through production of harmful cytokines.

In conclusion, *P. chabaudi* AS infected naïve mice experienced accumulation of iRBCs in the placenta and aborted or resorbed their embryos by mid gestation. The fetal loss was associated with enhanced production of proinflammatory cytokines in the plasma, spleen and placenta cell culture supernatants. Placentae from mice undergoing abortion exhibited an upregulation of procoagulants and on histological examination had severe hemorrhage and fibrin thrombi formation in the maternal blood sinusoids. In addition, fetal trophoblast cells exhibited phagoytosis of iRBCs in vivo and produced TNF-α in the culture medium following phagocytosis of iRBCs in vitro suggesting that the fetus is also actively contributing to the immune defense against malarial infection at the placental level.