A ROLE FOR COAGULATION IN THE PATHOGENESIS OF PLACENTAL MALARIA

by

JOHN W. AVERY III

(Under the Direction of Julie M. Moore)

ABSTRACT

Malaria during pregnancy presents with increased rates of miscarriage, intrauterine growth restriction, premature delivery, and low birth-weight neonates. The cellular and molecular mechanisms that contribute to these poor birth outcomes are incompletely understood. A clinical feature of placental malaria (PM) that deserves greater attention is the presence of dysregulated hemostasis. The sequestration of malaria parasites within the placenta induces a proinflammatory state that we propose is accompanied by hyper-activation of the coagulation cascade. This dual homeostatic alteration likely underlies the excessive perivillous fibrin deposition known to occur in PM. Previous studies in our *P. chabaudi* AS/C57BL/6 model for malaria during pregnancy showed that mice infected in early pregnancy have complete fetal loss occurring by gestation day (GD) 12. This is the first report of aggressive anticoagulant therapy improving the pregnancy outcome in this model. Mice treated with low-molecular-weight heparin (LMWH) at a dose of 1000 IU/Kg every 12 hours displayed rescue of pregnancy at GD 12. Histopathology revealed that LMWH-treated mice have substantially decreased necrosis within the placental layers as well as within the embryo itself when compared to untreated animals. In treated mice, gross pathology indicated reduced conceptus hemorrhage with a concurrent diminution of embryonic in situ death and expulsion.

INDEX WORDS: Malaria, Pregnancy, Coagulation, Low Molecular Weight Heparin, Trophoblast

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DEDICATION

This work is dedicated to my family: Heather Hogan, John, Carolyn, and Leslie Avery, John Keating, and my boys Dakota and Loki. No one makes it without help, and this could not be more true in my case. Simple words cannot express how much I appreciate and continue to need your collective support and guidance.

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CHAPTER 1

INTRODUCTION

Throughout human history malaria has been one of the greatest health burdens and leading causes of human morbidity and mortality. At least 40% of the world's population is at risk for malaria infection annually. Even today, some 243 million people suffer clinical episodes of malaria; tragically, almost 900,000 of those afflicted die each year. Ninety percent of these deaths occur in Sub-Saharan Africa with the bulk of the burden of morbidity and mortality born by pregnant women and children under five years of age.

The clinical symptoms of malaria are observed during the erythrocytic stage of infection. The clinical outcome of malaria infection, however, occupies a spectrum from asymptomatic infection to symptomatic infection, mainly manifested by pyrexia, to severe malaria, presenting metabolic acidosis, severe anemia, cerebral malaria, and/or coma. Typically, disease severity is limited or clinically silent in otherwise healthy sub-adults and adults from high transmission areas due to acquired immunity; even semi-immune individuals may clear *Plasmodium falciparum* infection, having never progressed past symptomatic infection, without drug intervention. *P. falciparum* infected erythrocytes maintain the ability to sequester in specific tissues by adhering to macrophages, dendritic cells, endothelial cells, and placental syncytiotrophoblasts. A direct benefit for the parasite of this cytoadherent phenotype is the ability to escape splenic clearance and manipulate host immune responses. Unfortunately for the infected person, it is this cytoadherence that is believed to be responsible for the pathogenesis associated with observed severe malaria conditions. Sequestration of parasites may occur in

several organs such as the heart, lung, liver, kidney, but occur most notably in the brain or placenta.

Sequestration of parasites within the placental vascular space is fundamental to the observed placental malaria (PM) pathology. Although the morbid outcomes of malaria infection during pregnancy have been well described, the precise mechanisms of pathology are poorly understood. The clinical consequences of PM in endemic areas are maternal anemia and infant low birth weight (LBW) which is a result of intrauterine growth restriction (IUGR) and prematurity. LBW is known to be the most important risk factor for infant mortality. In areas of low endemicity, pregnant women experience more significant clinical outcomes, namely maternal death due to cerebral malaria (CM), abortion, stillbirth, and LBW neonates.

A number of unique characteristics can be ascribed to PM such as trophoblast basement membrane thickening, excess perivillous fibrin deposition, and fibrinoid necrosis of fetal villi, which comprise the basic unit for fetal circulation. It is of note that excessive fibrin deposition in the maternal blood space is indicative of dysregulation of secondary hemostasis (coagulation) or tertiary hemostasis (fibrinolysis) or both, with each capable of supporting a hypercoagulable state once dysregulated. Excessive perivillous fibrin deposition has been concurrently described with PM since 1938, but the concept that coagulopathy is a driving force of pathogenesis has been relatively ignored until recently. It is important to appreciate, however, that excessive fibrin deposition is not always found in histological examination of the malaria infected placenta, which may be one of the reasons that coagulation as a mechanism of pathology has been ignored. Recent evidence now suggests that although excessive fibrin deposition may not always be found, several of the steps within the coagulation cascade and fibrinolytic pathway are capable of initiating a myriad of events that may lead to the poor birth outcomes associated with placental

malaria. PM is also marked by substantial maternal inflammatory cell infiltrate, principally monocytes, within the maternal blood space that has been blamed for much of the pathology associated with malaria during pregnancy.

Recently studies have begun to consider coagulopathy in cerebral malaria but no studies have specifically investigated the role coagulation plays in the development of placental pathology during malaria in pregnancy. Performing these studies in a human population is extremely difficult both from ethical and logistical standpoints. Hemostasis during pregnancy is a dynamic process outside of the context of infection status. Observing clinical data on a daily basis is not feasible especially in the regions where malaria in pregnancy occurs with regularity. Additionally, the human placenta is only accessible after delivery and cannot provide critical information about what occurs before delivery. Rodent models of placental malaria have therefore provided an avenue to investigate the pathological mechanisms during pregnancy.

Four species of *Plasmodium*: *P. chabaudi*, *P. berghei*, *P. yoelli, and P.vicknei*, have all been adapted to grow in laboratory rodents, principally mice and rats, but were originally isolated from thicket rats in Africa. These rodent *Plasmodium spp*. are used to investigate the pathogenic and protective responses to blood stage *Plasmodium* infection. Obvious differences exist in the basic biology of each parasite; examples include their morphology and red blood cell preference. *P. chabaudi* and *P. vinckei* preferentially invade mature red blood cells, as do the human parasites *P. falciparum* and *P. malariae*, whereas *P. berghei* and *P. yoelii*, like the human parasites *P. vivax* and *P. ovale*, prefer reticulocytes. Just as biology of the parasites differs, so too do the consequences of infection. Moreover, pathological outcomes vary between rodent and parasite strains. Both *P. berghei* and *P. yoelli* 17XL or YM parasites lead to lethal infections in all mice; however, *P. chabaudi* is only lethal in certain strains of mice, and *P.*

vicknei petteri and *P. yoelli* XNL are non lethal in all strains of mice. *P. chabaudi chabaudi* AS has proven to be an excellent model for recapitulating many of the pathological features of malaria in pregnancy. *P. chabaudi chabaudi* AS shares many characteristics with the most virulent human malarial parasite, *P. falciparum.* For example, both express variant antigens, sequester in the heart, lung, spleen, brain, liver and the placenta. *P. chabaudi chabaudi* AS is well characterized in terms of its blood stage infection in mice. Most importantly, the model mimics some of the poor fetal outcomes observed in humans.

Since the identification of genes encoding mouse clotting proteins and the creation of a growing number of transgenic mice, the use of mouse models for the study of thrombotic disorders has gained increasing interest. The reasons for using mice for coagulation studies are the same as for malarial studies: ethical considerations, cost saving, direct availability, technical feasibility, ease of achieving high numbers, and the potential for creation of mice with targeted deletions (knock-out) or targeted mutations (knock-in) in their genome. These genomic modifications allow for the study of highly specific alterations in the coagulation system. The importance of mice to hemostasis research cannot be underestimated. Mice have been used to make the majority of critical discoveries in coagulation biology within the last twenty years. For example, mice were used in the discovery of the mechanisms for platelet tethering, adhesion, activation, and aggregation, as well as the mechanisms for thrombus induction and coagulation amplification. Mice are the organism of choice in studying the coagulopathy of sepsis, chronic inflammatory conditions, and tumorogenesis. For all of their benefits, however, there are also drawbacks. Unlike human assays, there is a large gap in the number of analytical methods to test coagulation activation specifically in mice. Still mice make for excellent subjects in which

to study the effects of coagulopathy in disease and provide a boost to *in vitro* data using human samples and clinical studies.

It has been shown that a model of placental malaria utilizing *P. chabaudi chabaudi* AS infection in C57BL/6 mice recapitulates the poor birth outcomes observed in severe cases of placental malaria. These conditions include elevated levels of proinflammatory cytokines such as IFN-γ, TNF-α, IL-1, and IL-8 in addition to the chemokines MCP-1 and MIP1-α. Rodent model studies have shown that high systemic and uterine production of T_H1/proinflammatory cytokines during pregnancy is deleterious to the fetus. The T_H1 cytokines IFN-γ, and TNF-α have embryotoxic effects and have been implicated in human recurrent spontaneous abortion. Additionally, the proinflammatory cytokines IFN-γ and TNF-α suppress the development of murine fetuses and induce apoptosis of human trophoblasts *in vitro*. A similar elaboration of proinflammatory cytokines has been implicated in the initiation and dysregulation of coagulation in sepsis by increased expression and exposure of the clotting initator, tissue factor (TF). TF-dependent thrombin generation has been shown to be not only coagulative but reciprocally proinflammatory and proapoptotic leading to a runaway dysregulation of both immunostatic and hemostatic systems. Abrogation of TNF-α by antibody neutralization in this model of placental malaria dramatically reduces TF expression and rescues mid-gestational pregnancy. As a result, the mechanism(s) by which coagulation contributes to the pathology associated with this model must be elucidated.

Significance: Maternal and fetal responses that contribute to IUGR, preterm delivery, placental insufficiency, and fetal loss during malaria in pregnancy are incompletely understood. Inflammatory responses have born the preponderance of culpability for the poor birth outcomes in *P. falciparum* infection in pregnancy. However, this viewpoint may be insufficient to fully

describe the mechanisms by which pathogenesis occurs and impair the ability to properly treat placental malaria sufferers. To combat this lack of information, this study used the established C57BL/6-*P. chabaudi chabaudi* AS model of placental malaria, and outbred murine trophoblasts to examine the contribution of coagulation to fetal loss. The results from this study indicate that 1) infection during pregnancy is supportive of a coagulative environment as measured by expression levels of pro/anticoagulant proteins before fetal loss begins; 2) aggressive therapy with low molecular weight heparin significantly improves mid-gestational embryonic survival; and 3) trophoblasts are induced to support coagulation in response to stimulation with *P. chabaudi* infected RBCs *in vitro*.

Hypothesis: The central hypothesis of this study is that *P. chabaudi* AS infection promotes a hypercoagulable state in the pregnant mouse which drives poor fetal outcomes. To test the proposed hypothesis the following specific aims were attempted:

1. Characterize the level of pre-abortive coagulation transcripts in infected pregnant mice
2. Assess the ability of therapeutic administration of anticoagulants to improve mid-gestational embryonic survival.
3. Assess the ability of trophoblasts to support coagulation by the level of pro/anti coagulant transcripts in response to stimulation by infected red blood cells.

CHAPTER 2

LITERATURE REVIEW

Plasmodium

Malaria is a disease caused by a parasitic protozoan of the genus *Plasmodium*, which is transmitted by the bite of an infected, hematophagous, female *Anopheles* mosquito. Typically, a specific mosquito vector transfers parasites between specific terrestrial vertebrate hosts. Over 200 species of *Plasmodia* have been described to date with host animals ranging from mammals through both birds and reptiles (1-3). As an infectious agent, this apicomplexan requires two hosts to complete its life cycle: the mosquito as a definitive host and a vertebrate as an intermediate host; however, the parasite uses both to amplify its number. The malaria parasite is an obligate intracellular parasite that invades several tissue types throughout its life cycle and host occupancy. Specifically within mammalian hosts, the malaria parasite exerts its most pathological effects during the erythrocytic stage of its life cycle (4-7). *Plasmodia* also lead to a loss of fitness of the mosquito vector, which has critical importance to human infection, however, the topic is beyond the scope of this thesis (8).

The life cycle of *Plasmodium* is extremely complex and occurs through distinct stages as the parasite is transferred from mosquito to vertebrate host and finally back to mosquito. Natural malaria infections are initiated by sporozoites, the progeny of sexual reproduction within the mosquito, which are injected into a vertebrate host through the salivary canal of the proboscis of a female mosquito as she probes the dermis of her prey in search of a blood meal (6, 9). Sporozoites may be injected directly into capillary lumina; however, the majority are injected

into the surrounding avascular tissue and must migrate into nearby blood vessels (9-11). The progression of development in vertebrate hosts differs, and in mammals the fate of invading sporozoites is varied: some are successful in reaching a blood vessel, some invade the lymphatic system, and still others linger in the dermis, where they are degraded, never having entered circulation (10, 12-13) . Those sporozoites that invade the lymphatic vessels are transported to the proximal draining lymph nodes where they either remain or partially develop into exo-erythrocytic forms (EEFs). Both cases encounter dendritic cells and prime the first T cell response and end in sporozoite degradation. (10, 12, 14-15). It has been demonstrated that within an hour of mosquito-mammalian delivery, of those sporozoites that escape the dermis, 70% enter blood vessels and 30% the lymphatics (12, 16). The mammalian targeted sporozoites that navigate the endothelium and invade blood vessels are carried to the liver where they mature into the first of two vertebrate multiplicative life stages, merozoites.

Migration of the sporozoite from the point of entry to the liver happens rather rapidly for those parasites that make it to blood vessels, with the time lapse on the order of minutes to hours (17-18). The limiting factor for migration time is the proximity of the capillaries to the bite site (18). The explanation for this swift travel through the dermis lies in the ability for *Plasmodium* parasites to traverse many tissue types during its life stages. Sporozoites utilize gliding motility to pass through the endothelium. When sporozoites arrive at the liver, they enter through the liver sinusoids, an area of slow circulation where oxygen rich blood from the hepatic artery and nutrient rich blood from the hepatic portal vein merge (19). The flow disturbance is caused by Kupffer cell (liver macrophage) migration and leukocyte-vessel wall interactions (19). In order to gain access to their final destination, hepatocytes, the sporozoite must traverse the endothelium and survive interaction with Kupffer cells. The sporozoite may utilize this low flow

environment to its advantage; the sporozoite contains an unique Apicomplexan protein on its surface, the circumsporozoite protein (CSP), which interacts with liver and Kupffer cells' heparan sulphate proteoglycans (HSPGs) to aid in its arrest (16, 20-21). Additional recognition and adhesion to liver and Kupffer cells is mediated by sporozoite thrombospondin-related adhesive protein (TRAP) (16-17, 22-23). Evidence has been presented, which demonstrates that sporozoites use their encounter with Kupffer cells as the primary means of traversing the sinusoidal cell layer for access to hepatocytes and are neither recognized nor attacked by them (16, 23-28). Entry into Kupffer cells is mediated by formation of a parasitophorous vacuole which is forced through the membrane of a host cell through a moving junction formed between the membranes of the sporozoite and the host cell using the same actin-myosin motor used in gliding motility (16, 20, 23-28). Once sporozoites have migrated across the sinusoidal cell layer, they encounter their final target, but do not take up residence in the first hepatocyte they invade. Invasion and exit of several hepatocytes takes place prior to establishment of final residence. To do this sporozoites use yet another method of cell invasion, transmigration. Transmigration is unlike vacuole invasion in that the plasma membrane of the host cell is disrupted as the sporozoite passes through the plasma membrane, a vacuole is not formed, and the sporozoite then exits to invade a subsequent neighboring hepatocyte (29). A number of the damaged hepatocytes are able to repair themselves, however, many undergo necrosis and lead to disruption of the liver's architectural microenvironment (16, 29-30). The exact mechanism for final hepatocyte selection is unclear. The prevailing evidence suggests that transmigration provides sporozoite signals to prepare for development into merozoites and also makes adjacent hepatocytes more susceptible to infection and may prepare the final cell for the final invasion, which occurs via formation of another parasitophorous vacuole (16, 31-34). The invasion of the

final hepatocyte initiates the maturation of the sporozoite to undertake exo-erythrocytic schizogony, a form of protozoan mitotic asexual reproduction. The resultant daughters of this reproductive event, merozoites, number in the tens of thousands and displace the hepatocyte organelles, eventually causing the hepatocyte to rupture and release the merozoites into the liver sinusoid (17, 35). Released merozoites find themselves naked in the circulation and must quickly invade host red blood cells (RBCs).

Invasion of host RBCs happens rapidly and efficiently. Merozoites are capable of distinguishing between competent RBCs to invade and other cell types from long distances (36-37). Recognition and attachment of the merozoite to the RBC surface is also species specific, suggesting the involvement of specific merozoite/host ligand-receptor interactions (38-39). The merozoite maintains a battery of proteins to initiate attachment to the erythrocyte plasma membrane: erythrocyte binding antigens (EBA), merozoite surface proteins (MSP), apical membrane antigens (AMA), and Duffy binding proteins (DBP) (38). These proteins serve to attach and orient the invading merozoite to the RBC. The process of merozoite attachment and invasion proceeds in a stepwise fashion. Initial attachment is reversible and is mediate by low affinity contacts until the merozoite juxtaposes its apical end with the plasma membrane of the erythrocyte. At the apical surface, high affinity interactions form a tight junction between the erythrocyte and merozoite; using the aforementioned actin-myosin motor, the tight junction moves from the apical end to the posterior end, forcing the merozoite into the RBC's plasma membrane. The merozoite never fully penetrates the plasma membrane but rather causes an invagination, which circumscribes the merozoite and establishes the borders of the parasitophorous vacuole; the merozoite removes its own surface coat, and finally the plasma membrane of the erythrocyte is resealed as is the parasitophorous vacuole membrane (6, 36-39).

Once safely inside the RBC, the merozoite develops through an erythrocytic maturation cycle through ring, trophozoite, and schizont stages. The ring form is a young trophozoite, so named by its characteristic staining with Giemsa. The parasite begins to increase in size and mature into a trophozoite as it ingests RBC cytoplasm. Hemoglobin that resides within the cytoplasm of the RBC is a critical source of amino acids for the developing trophozoite; hemoglobin is internalized in the parasite's food vacuole and is degraded. During degradation, free heme is liberated. Free heme is toxic to the parasite so, as a protective measure, the trophozoite polymerizes the heme into an inert crystalline structure known as hemozoin (Hz), or malaria pigment (40-42). The trophozoite matures into a schizont, a stage marked by 3-5 rounds of nuclear replication and heterogeneous distribution of nuclear material. Termination of schizogony results in condensed and segmented merozoites, which burst from the RBC and are free to infect other RBCs and repeat asexual replication or proceed into gametocytogenesis. Each schizont can produce between 10-30 merozoites (species dependent) that are capable of invading another RBC. The entire intraerythrocytic cycle may range from 24-72 hours in species specific manner (6, 43). The rapid amplification of parasites, destruction of RBCs, and resultant release of waste and massive antigen exposure are chief reasons the erythrocytic stage of malaria is the most pathogenic (7, 44). Gametocytogenesis, by contrast, is not pathogenic and is a committed differentiation step; merozoites from a schizont either all invade new RBCs or all undergo sexual differentiation into microgametocytes, which are taken up by the mosquito during her blood meal to begin sexual reproduction in the insect's midgut and culminates with the eventual release of more sporozoites into the next host, repeating the cycle of disease transmission (6, 10). The signals which result in merozoite/gametocyte shift are as yet unresolved; however, evidence suggests that host immune pressure is partly responsible (45).

Malaria Epidemiology and Social Impact

Throughout human history malaria has been one of the greatest health burdens and leading causes of human morbidity and mortality (46). At least 40% of the world's population is at risk for malaria infection annually (47-48). Even today, some 243 million people suffer clinical episodes of malaria; tragically, almost 900,000 of those afflicted die each year (49). Ninety percent of these deaths occur in Sub-Saharan Africa with the bulk of the burden borne by pregnant women and children under five years of age (46-47, 50-51). These numbers may underestimate the burden born by sufferers on the Asian continent, however. As Snow et al. reported in 2005, the WHO relies too heavily on passive reporting in regions that are entrenched in poverty and where reporting is unreliable. Their empirical approach, an attempt to more accurately delineate the cartography of malaria risk, showed that the WHO underestimates the global occurrence of *Plasmodium falciparum* infection, the most virulent human malaria, by up to 50%. Moreover, the WHO's estimates for areas outside Africa were underestimated by as much as 200% (52). In addition to *P. falciparum*, four other *Plasmodium* species infect humans: *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Along with *P. falciparum*, *P. vivax* may lead to severe disease and death. Morbidity and mortality are not the only impacts malaria infection has on an endemic populace. It has been proposed in the "malaria hypothesis" that no other pathogen has had such an intense impact on human evolution; consider the appearance of phenotypes such as sickle cell, thalassemias, glucose-6-phosphate dehydrogenase deficiency, ovalocytosis, and RBC Duffy negativity as examples of potentially debilitating hematological conditions that persist due to some survival advantage in the face of malarial pathogenesis (46, 53-54). It may also be, as manifested in West Africa among other locations, that the seasonal outbreaks of malaria, which require the parasite to persist in the host for months, have provided residents with

some survival advantages as the parasite and host coadapt over time (14, 55-57). Still, physiologic impacts are not the only foot prints left by malaria infection; as a result of health consequences, malaria infection has shaped human social and economic developments throughout our history and continues to do so today (58). In modern times, for example, the per capita gross national product (GNP) of sub-Saharan Africa declined by 44.3% between 1960 and 1999 (46, 51, 59). Two of the causes for this decline have been the emergence of resistance to insecticides and the antimalarial drug chloroquine, both of which have lead to increases in the rate of infection and deaths due to malaria (51, 60). Moreover, because malaria has such a severe impact on children, especially in endemic areas, the accumulation of human and physical capital is retarded. What is the significance of these factors? Infected neonates and children born to infected mothers, generally have increased incidence of cognitive and neurosensory impairment; these disadvantages result in a general decline in the education of a large number of individuals and place a significant burden on families to not only rear but care for these children once they become adults (58, 61-63). The direct loss of man hour productivity in Africa alone is 800 million days of incapacity due to malaria infection, and these numbers do not account for indirect loss due to caring for the ill (64-65). The increased burden of infection on families leads to a loss in productivity, both in terms of working man hours lost and a general decline in household savings. Household economics limit economic and physical migration, that is, the ability to advance socio-economic status or relocate to support such changes (58). Malaria is a yoke on all segments of society.

Malaria Clinical Presentation, Immunology, Protection, and Pathogenesis

The clinical symptoms of malaria are observed during the erythrocytic stage of infection. The clinical outcome of malaria infection, however, occupies a spectrum from asymptomatic

infection to symptomatic infection, mainly manifested by pyrexia, to severe malaria, presenting metabolic acidosis, severe anemia, cerebral malaria, and/or coma. Accordingly, severe malaria can, and most often will, involve the dysfunction of multiple organs (5). If treatment cannot be acquired, severe malaria can lead to death, especially in children, the non-immune, and immuno-compromised individuals (66). Even still, treatment does not guarantee positive clinical outcomes as these are intimately tied to host genetics, pregnancy, co-infection, nutritional status, immunological experience, and parasite density in addition to age and immunocompetency (7, 66). Typically, disease severity is limited or clinically silent in otherwise healthy sub-adults and adults from high transmission areas due to acquired immunity; even semi-immune individuals may clear *P. falciparum* infection, having never progressed past symptomatic infection, without drug intervention (5, 66). Non-immune and individuals from low transmission areas are most at risk from complications due to malaria. Many of the clinical complications of malaria infection are related to the destruction of infected red blood cells (iRBCs) and subsequent anemia. Anemia is not the only physiological consequence of iRBC rupture, however. As stated, the rupture of iRBCs after schizogony results in the liberation of parasite antigens, which stimulate the expression and release of a host of inflammatory cytokines (5, 7, 67-70). The classical symptoms of symptomatic malaria are fever, chills, nausea, headaches, and general malaise. Presentation of severe disease may include: sustained impaired consciousness, seizure, coma, and long term neurologic sequelae associated with cerebral malaria (CM); lethargy due to severe anemia (hemoglobin $< 5g\ dl^{-1}$); respiratory distress and hypovolemia due to metabolic acidosis (also the single most important determinant of survival); shock, hemodynamic changes, renal failure, impaired organ perfusion, and disseminated intravascular coagulation (DIC), discussed later, all as a response to severe anemia and shock-like syndrome; infected pregnant women may

have placental insufficiency (failure of the placenta to supply appropriate nutrient and gas exchange to the fetus), low birth weight neonates due to prematurity or intrauterine growth restriction (IUGR), and loss of pregnancy or stillbirth (5, 7, 71-72). These syndromes can occur mutually exclusively of one another but may also occur concomitantly; severe malarial anemia, CM, and respiratory distress lead to the most severe cases and cause the largest proportion of deaths during malaria infection (73-74).

Human immunological responses to *Plasmodium* infections are extremely complex and developmental delayed. Certainly, individuals from high transmission areas can, and do, develop protective responses, but the development and persistence of protection requires frequent and habitual infection throughout life (72, 75). Therefore, immunity is never complete and is rather slow to develop, with onset of naturally acquired immunity (NAI) usually occurring during or after adolescence in endemic areas (75). To complicate matters, NAI is both parasite stage and species specific (72). The slow gradual development of NAI comes as a result of evasion and possibly disabling mechanisms possessed by the invading parasite.

P. falciparum maintains a vast repertoire of immune evasion tactics. The first evasion tactic involves its ability to hide from the immune system as an intracellular parasite. During its most protracted stage, the erythrocytic stage, the parasite remains mainly hidden from detection by cellular mediators of immunity. Unlike nucleated cells, RBCs do not express MHC antigens and evade $CD8^+$ T cell detection. As parasites migrate from the bite site as sporozoites or emerge from developmental stages as exoerythrocytic merozoites, they are susceptible to antibody neutralization of proteins required for invasion, opsonization, complement activation, and antibody dependent cell cytotoxicity (ADCC). However, sporozoites shed proteins, such as CSP, which limit their recognition by antibodies (Abs). The sheer number of parasites produced

during exponential multiplication of erythrocytic stages virtually guarantees survivors. Moreover, as parasites enter new cells, the Abs are rendered ineffective (76-77).

Another mechanism of avoidance that is maintained by *P. falciparum* is the capacity to enable iRBCs to adhere to endothelial cells (EC) and syncytiotrophoblasts (ST) and thereby sequester in various tissues. A direct benefit for the parasite of this cytoadherent phenotype is the ability to escape splenic clearance (7, 76-77). Sequestration of parasites may occur in several organs such as the heart, lung, liver, kidney, but most notably occurs in the brain or placenta. Adherence is mediated by receptor recognition and binding by iRBCs. Amazingly, the parasite utilizes a single protein, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), expressed on the surface of the iRBC to bind to various types of host receptors, which vary from tissue to tissue. Beyond its EC and syncytiotrophoblast (ST) binding capabilities, PfEMP1 also permits iRBCs to adhere to non-parasitized RBCs in a phenomenon known as rosetting. The primary purpose for rosetting has not been established; however, the current consensus is that the process creates a "ready to invade" environment of uninfected RBCs, presumably permitting higher parasite densities (78). Infected erythrocytes (IEs) may also bind to platelets in a process called clumping. The exact mechanism of platelet adherence is unknown as of yet, but PfEMP1 is the most likely candidate. Similarly, the purpose of clumping is not understood and does not occur in all severe cases of malaria, but it is posited that the binding of platelets by IEs serves as a bridge for EC adherence in tissues that have reduced or nonexistent expression of malarial adhesion receptors, allowing the IEs to sequester in different vascular beds (79). The promiscuous nature of PfEMP1and the ability of IEs to sequester in different vascular locations leads to the variety of observed clinical manifestations of severe malaria. In cerebral malaria, IEs bind to ECs of the cerebral microvasculature principally via PfEMP1 interaction with CD36,

intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and E-selectin (76, 79). In PM, the major ligand for PfEMP1 is chondroitin sulphate A, a glycosaminoglycan highly expressed on the surface of trophoblastic and extracellular villi as well as syncytial bridges (80).

While adhesion to ECs, uninfected RBCs, and platelets may allow for splenic avoidance and increased parasite multiplication, *P. falciparum* utilizes yet another strategy to evade the effects of the human immune system: direct immunosupression. Professional antigen presenting cells (APCs) such as dendritic cells (DCs) express CD36 on their surface. *P. falciparum* IEs can interact with these CD36 molecules via PfEMP1 and arrest maturation of the DCs, thereby weakening T cell responses (79, 81). This immune evasion mechanism does not stop with just maturational development; the IL-12/IL-10 cytokine secretion pattern is also reversed so that protective IL-12 secretion is diminished and the expression of suppressor IL-10 favored (82). Dendritic cells are not the only host cells to have their function modulated. Macrophages phagocytose mature iRBCs as well as free Hz released from ruptured RBCs following merozoite release. As Hz accumulates within the macrophage, this cell's ability to perform as a phagocyte is compromised. First, the Hz laden macrophage loses the capacity to repeat phagocytosis; subsequently, its intrinsic activities are halted: oxidative burst is inhibited as is activation of protein kinase C. Furthermore, Hz ingestion by macrophages impairs expression of major histocompatibility complex II, CD54, and, CD11c and the consequent generation of proper $CD4^+$ T cell responses (83-88). Blood stage infection initiatives by the parasite also alter subsequent reinfection. *Plasmodium* erythrocytic stage parasites induce DCs to express soluble factors that actually inhibit $CD8^+$ T cell responses (89-90). In this way, parasites introduced

upon reinfection are somewhat protected by host immune responses toward the liver stage of infection due to impaired host cellular responses.

It seems counter intuitive that a strategy for parasite survival, which employs the use of only a single protein (PfEMP1) fails to elicit early and rapid recognition by the host immune machinery. This facet of *Plasmodium* infection dynamics only serves to deepen the immune evasion repertoire within its magic hat of tricks. PfEMP1 is expressed on the surface of IEs, as such, this protein should be a target of Ab responses. However, PfEMP1 is a *var* gene product; the *Plasmodium var* gene family is a group of genes present in approximately 60 distinct loci found on different chromosomes that are singly expressed by individual parasites. Low levels of *var* gene switching occurs in clonal populations of parasites and is expected to be greater in a natural infection, especially under conditions of high transmission (14, 76, 91-93). The direct affect of this antigenic diversity is selection of parasite clones that have switched their PfEMP1 var gene to express a variant for which Ab recognition is either poor or nonexistent. The parasites expressing the variant(s) that are unrecognized by Abs receive a head start until an Ab response for the new variant(s) can be mounted (14). This process persists throughout an infection or reinfection creating so-called "waves of antigenic diversity."

Although *Plasmodium* maintains a capable arsenal of evasion strategies and education of an effective human immune system is slow in response to malaria infection, the human host is not without its own armaments and sometimes that may be the problem. Far from being complete, the current body of knowledge concerning human immune responses indicates that both arms of the immune system are involved in combating infection. Because the erythrocytic stage of *Plasmodium* infection is the most protracted of the human host stages, it would seem likely that host responses would be most successful both in terms of generation and effectiveness

during this time and longitudinal human studies have borne this out (73, 94-97). Furthermore, experiments using rodent malarias have demonstrated that survival is connected to managing blood stage parasitism and associated responses within the first two weeks of infection; control is accomplished through T_H1 type responses that occur well before specific production of IgG Abs (72, 98-101). In humans erythrocyte stage parasitism, in areas of moderate to high transmission, drives the activation of innate responses that bridge long term adaptive responses, which when combined with continuous exposure, educates both humoral and cellular immunities (72-73, 102). It must be appreciated that sterile immunity to malaria does not exist in a natural setting; however, clinical immunity is quite common given sufficient transmission intensity (75, 102).

Similar to the results of experimental rodent malarias, clinical data of human cases demonstrate that, indeed, primary infection control is mediated by innate mechanisms (72, 103-104). Monocytes play an important part in controlling blood stage infection. During both *in vitro* and *in vivo* experiments macrophages are activated and secrete potent pro-inflammatory cytokines, tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) in a myeloid differentiation factor 88 (Myd88)-dependent fashion when exposed to malarial glycosylphosphatidylinositol (GPI)-anchored antigens. GPI-anchored proteins are thought to function as the principal malarial toxin during infection with *P. falciparum* in humans and *P. berghei* in mice (105-110). In a Machiavellian twist, both peripheral monocytes and macrophages have turned an assumed parasite advantage against the invader; non-opsonized IEs can be, and are, readily phagocytosed by monocytes and macrophages via CD36-PfEMP1 interactions (83, 111). It has not yet been demonstrated that these non-opsonic events lead to Hz dysfunction in these monocytes and macrophages (83). If their capacities are not impeded, it may be that macrophages' roles as

adaptive immune driven mediators of Ab-dependent cellular inhibition or anti-parasite nitric oxide production are more important than their clearance of IEs (72).

Natural killer (NK) cells have also been found to play a role in protection against *Plasmodium spp*. in both mice and humans. Like monocytes and macrophages, NK cells are mainly found in the peripheral blood in addition to the spleen and bone marrow, and like monocytes and macrophages they play a large part in developing the adaptive response to infection. Experiments with multiple mouse strains and multiple species of rodent malarias have demonstrated that the production of interferon gamma (IFN-γ) and NK-cell mediated cytotoxicity are induced upon infection, and that the production of IFN-γ is required for protective immunity, specifically by way of activating the appropriate $CD4^+$ T_H-cell subset (112-115). In human studies, NK cells have been shown to be the first responders to *in vitro* exposure of peripheral blood mononuclear cells to *P. falciparum* IEs, implicating a role for IE expressed ligands recognized by NK cells. In the same study, data emerged, which bolstered earlier murine experiments in regards to the requirement of IFN-γ production for protection (72, 116). Appropriate IFN-γ production is dependent on NK cell activation, which is reliant upon two factors: (i) binding of NK cells to IEs and (ii) sufficient IL-12 and IL-18 release by unimpaired macrophages and DCs (72, 117-118). Perhaps the most convincing data for the involvement of NK cell responses are those from NK cell depleted B6 mice (a resistant strain) using *P. chabaudi* AS infection to demonstrate that although the mice survived infection, peak parasitemia was increased and resolution was significantly protracted (112).

Two other cells types, natural killer T (NKT) cells and γδT cells appear to also play a function in malaria control; however, the scope of their immunologic pressure has not been completely elucidated (72). Tsuji et al. demonstrated that NKT cells were capable of inhibiting

the development of intrahepatocytic parasites, prohibiting blood stage infection in mice infected *P. yoelli* and *P. berghei* (72, 119). γδT cells have also been shown to be critical for pre-erythrocytic-stage immunity. When αβ T cell-deficient mice were immunized with *P. yoelli* irradiated sporozoites, Tsuji et al. discovered that these mice were still capable of mounting a protective response, despite their lack of $CD8^+$ and $CD4^+$ T cells. However, when these mice were depleted of the γδ T cell subset, the protective immune response was abolished. The conclusion was that the γδ T cells were responsible the protective immunity against the pre-erythrocytic stages. γδ T cell clones from these mice were isolated and adoptively transferred into naïve, wild-type (WT) mice. It was discovered that the WT receiving the transfer were capable of mediating protective immunity to sporozoite challenge, demonstrating the direct anti-malaria effect of γδ T cells. It was also found that γδ T cell-deficient mice presented with more severe liver-stage infection after sporozoite challenge as compared to WT controls (119-121). Along with NK cells, γδT T cells are a major source of IFN-γ long before the activation of Ag specific T cells (72, 122).

Thus, cell mediated and humoral immunities participate in malarial protection. Innate responses provide a bridge of communication which drive the education of both cellular and humoral action. For example, in mice infected with *P. chabaudi*, T cells were shown to induce and maintain cell mediated immunity to infection (123-124). These mechanisms are principally driven by release of various cytokines upon stage specific antigen presentation (125). Fonseca *et al.* demonstrated that *P. chabaudi* infections initiated by mosquito bite were characterized by lower parasitemia of shorter duration than those observed after direct blood challenge; splenomegaly was equivalent signifying that parasitemia alone does not account for the increase in spleen size. Overall numbers of $CD4^+$ T cells (inclusive of those producing IFN-γ, IL-10 and

IL-2) were reduced in comparison to direct blood challenge. By contrast, the diminution in IL-4 producing cells was less distinct signifying a proportionally lower T_H1-like response in mice infected via natural route. Remarkably, pre-exposure to bites of uninfected mosquitoes reduced the magnitude and duration of the subsequent mosquito-transmitted infection still further, and enhanced the response of $CD4^+$ T cells producing IFN-γ and IL-4 (125). The polarization towards a cell mediated response is driven by IFN-γ, while IL-4 drives a humoral response; antibody dependent mechanisms of parasite clearance are reliant on T_H2 $CD4^+$ T cell action. Regulatory cytokines such as IL-10 delivered later during infection may serve to limit proinflammatory responses of excessive IFN-γ and subsequent TNF-α release and consequent pathology; during liver stage reinfection, IL-10 may improve antibody-dependent cellular inhibition (ADCI) activity against *P. falciparum*. ADCI is carried out by the cooperative activity of both antibodies and monocytes, which culminates in arrested parasite development (126-128). Furthermore, human IL-10 induces activated B cells to secrete immunoglobulin (Ig) and supports the retention of Fcγ on the surface of monocytes augmenting neutralization and opsonization (126, 129-131). Beyond humoral responses, T cell release of IL-10 may enhance the activity of $CD8^+$ CTL mediated killing of liver-stage parasites as evidenced in animal models of malaria (126, 132). IL-10 has been shown to enhance IL-2-induced differentiation, proliferation, and cytotoxicity of $CD4^-$ $CD8^+$ cells in mice (126, 133). In humans, IL-10 is a chemoattractant for $CD8^+$ lymphocytes and may recruit $CD8^+$ CTLs to the site of infected hepatocytes.(126, 134). B cell responses owe most of their protective role to the production of polyclonal and specific Igs, which aid in, amongst other things, opsonization, invasion neutralization, and encouraging splenic clearance (96-97, 127, 135-139). Their protective ability has been demonstrated in

human studies: passive transfer of Abs from immune individuals to naturally and experimentally challenged individuals is well documented (97, 140).

The clinical continuum of malaria, from asymptomatic through severe disease, is rooted in the balance of immunoprotection and immunopathology. Cytokines, particularly proinflammatory cytokines, are critical to protection, however, if dysregulated, the mechanisms that drive protection also drive pathogenesis. The majority of evidence concerning the pathogenesis of severe malaria has arisen from murine CM experiments and clinical data from CM sufferers. In an excellent review by Schofield and Grau, the progression from symptomatic malaria to the intersection of various syndromes of severe malaria were attributed to four basic processes of infection and immunity: (i) site-specific localization of IEs among target organs; (ii) local and systemic action of bioactive parasite products, such as HZ and GPI; (iii) the local and systemic production of pro-inflammatory and counter-regulatory cytokines and chemokines by the innate and adaptive immune systems in response to parasite products; and (iv) the activation, recruitment and infiltration of inflammatory cells (5). According to their review, Schofield and Grau assert that severe malaria and its associated syndromes are "end-stage processes of atypical inflammatory cascades that are initiated in target organs by pathogen products and are maintained by infiltrating cells through positive-feedback" loops run amuck and inadequately controlled (5).

The first process of severe malaria pathogenesis, site-specific localization of IEs, was discussed previously. To review, sequestration is a basic parasitic immune evasion tactic, whereby IEs expressing PfEMP1 are able to recognize and bind to a suite of diverse host receptors on various tissues to avoid clearance by the spleen. The accumulation of parasites in these locales leads to mechanical blockage of the microvasculature. This blockage is not solely a

consequence of parasites alone; platelets, rosettes, and microparticles (physiologically active membrane particles shed from activated platelets, monocytes, and endothelial cells) accompany sequestered parasites to the microvasculature. Compounding obstruction, these physiologically active entities are also capable of cytokine/chemokine release, further activation of EC and PfEMP1 target ligand expression, the generation of thrombi, and in CM specifically, alteration of the architecture of the blood brain barrier (BBB) (141-152).

Both Hz and GPI were previously discussed in the context of their immunomodulatory functions. Additionally, GPI specifically acts as a pathogen associated molecular pattern (PAMP) of malaria in addition to its role as a toxin (5, 153). Upon recognition of GPI through toll-like receptors (TLR) 2 and 4 on unimpaired macrophages and DCs, the potent proinflammatory cytokines TNF-α, IL-1, IL-6, and IL-12 are produced and released (107, 109, 154-155). GPI is also responsible for the stimulation of inducible nitric oxide synthase (iNOS, a potent free radical generator via nitric oxide production and interaction with cellular superoxides) by both macrophages and vascular endothelium as well as the upregulation of adhesion molecules recognized by PfEMP1 on both leukocytes and ECs (108, 153, 156). The significance of iNOS production lies in its association with mitochondrial dysfunction in both cardiac and skeletal myocytes; this leads to poor oxygen supply, contractile failure, and eventually respiratory acidosis (70, 157-162). It has been shown that purified GPI alone is able to recapitulate symptoms of acute, primary infections in non-immune individuals, namely pyrexia, hypoglycemia, TNF-α induced coagulopathy, and even death, as occurs in the malaria shock like syndrome (5, 107, 153). Antibodies against GPI prevent the generation of proinflammatory cytokines against *P. falciparum in vitro* (156, 163-165). An extremely striking feature of GPI-host interactions becomes evident when examining the data from mice immunized

with synthetically produced GPI. Normally CM susceptible B6 mice were infected with *P. berghei* ANKA; the GPI-immunized group was protected against CM, as well as pulmonary edema and respiratory acidosis. The survival curve comparisons for these experiments showed that all unimmunized mice died before a single immunized mouse succumbed to infection. Overall, immunized mice enjoyed 80% survival after 12 days versus 0% survival of unimmunized mice at day 7 post infection, despite similar parasitemias (163). Unfortunately, the influence for Hz is not as clear as it is for GPI. As previously mentioned, Hz is capable of suppression of host immune machinery and induction of T_H2 cytokines. However, contradictory evidence exists, which purports that Hz is also an activator of DCs and inducer T_H1 cytokines (153, 166). The culpable characteristic of this discrepancy probably lies in the heterogeneous compositions of experimental Hz (5, 153).

Previously NK and NKT cells were shown to be important in IFN-γ production, communicating with the adaptive arm of immunity, and pre-erythrocytic malaria, however, protection is not the only role that they play in the response to malaria. Not all NK or NKT cells are created equal; both cell types have the ability to modulate host resistance by altering the T_H1/T_H2 cytokine balance depending on their genetic makeup. The balance between T_H1/T_H2 is mediated by early and sustained release of IL-12 (T_H1) vs. IL-4 (T_H2). The polarization to one response or the other and the susceptibility to severe malaria (particularly CM) depends on which genes are expressed within these cells. For example, in murine models of CM, the absence of CD1d-restricted NKT cells, (NKT cells without the ability to present lipid based antigens such as GPI) naturally resistant, T_H2-dominant BALB/c mice become more susceptible as their immune response becomes T_H1 polarized. In contrast, with B6 mice, which are naturally susceptible, the absence of CD1d-restricted NKT cells leads to a T_H2-like cytokine schism, and as a result, the

mice are more resistant to CM (167-169). In other experiments, a genetic region of susceptible B6 mice was inserted into resistant BALB/c background; the congenic mice were made more susceptible to *P. berghei* ANKA-mediated severe malaria than the BALB/c controls (170). The genetic region used to make the congenic mice is a region of highly linked genes encoding several receptors involved in the control of NK and NKT cell function called the natural killer complex or NKC (171). The NKC controls which NK and NKT receptors are present on their cell surface and thus their subsequent activity upon activation. Polymorphisms between humans as shown in different strains of inbred mice may genetically predispose some individuals to severe malaria. In the above experiments, significantly enhanced IFN-γ (T_H1) production was found to be detrimental to CM resistance and survival despite similar parasite burden as a result of receptors encoded by the NKC which dictated the response (170-171). It remains to be elucidated as to which receptor-ligand interaction determines the activities of these cells, however, the involvement of high circulating levels of IFN-γ in the development of CM has at least been confirmed in mice via IFN-γ Ab neutralization and IFN-γ and IFN-γR null mutants (153, 172-174).

The degree to which T cells contribute to pathogenesis is not completely understood. The majority of data is circumstantial and a direct mechanism to connect $CD8^+$ T cells to specific pathology has proven elusive. However, thymectomy and passive transfer of antithymocyte serum has been demonstrated to abrogate CM during *P. berghei* infection in hamsters (153, 175-176). Both nude mice and T cell receptor null mutants are resistant to cerebral seizures, ataxia, and neurological syndrome (post-infective encephalopathy) (153, 177). Increased numbers of cytotoxic $CD8^+$ T cells are found in the brain of mice with CM, where it is presumed that pathology is mediated by a perforin dependent BBB permeability change (153, 178-179).

Additionally, mice with *P. berghei*-induced CM display circulatory shock and respiratory distress brought on by $CD8^+$ T cells. Amelioration of these symptoms was accomplished by $CD8^+$ specific mAb treatment (180). Clinically, children who have intact or higher T cell responsiveness present with CM to a significantly greater degree than those with impaired T cell function (153, 181-183).

Thus, using CM as context, accumulation of parasites, platelets, microparticles, and exposure of malarial toxins drives a local acute phase response via the release of potent proinflammatory cytokines. A self-perpetuating feedback loop, as proposed by Schofield and Grau, is established wherein EC activation and cytokine/chemokine release are increased, resulting in the further accumulation of iRBCs within the microvasculature of target organs. Involvement of NK and NKT cells activated by GPI may drive $CD4^+$ T_H1 responses, which heighten the proinflammatory response (5). This inflammatory milieu is responsible for further chemokine release, which then recruits monocytes that differentiate into macrophages and become arrested in brain microvessels. Additionally, recognition of GPI can activate macrophages, a process that is amplified by interferon-γ. These locally activated macrophages release additional chemokines producing a systemic response that amplifies the infiltration of cells, sequestration of PRBCs, and release of microparticles. This may continue for days until $\gamma\delta$T T cells and $CD8^+$ T cells become involved, drastically augmenting local and systemic release of chemokines and cytokines with coincident BBB architecture alteration via perforin-mediated lesions in the endothelium. In concert with the aforementioned locally arrested macrophages, large numbers of platelets are sequestered by interfacing with macrophage CD40-CD40L interactions and participate in altering endothelial-cell functions. More microparticles of platelet, endothelial-cell and monocyte origin are released, which leads to the dissemination of

pro-inflammatory and pro-coagulant effects. Damage to the endothelium, with possible perivascular hemorrhage, axonal injury, and neurotransmitter and metabolic changes results (5). How is it then that proinflammatory responses are required for parasite clearance and proper education of the adaptive immune response? Evidence demonstrates that it is the timing, proportionality, and proper regulation of the response that dictates the outcome of primary infection in non-immune individuals (73, 152). It may be that the timing of proinflammatory responses are most critical in that they will drive proportionality and regulation (152).

Severe malaria anemia (SMA) is another syndrome of severe malaria, which occurs by way of similar cellular and chemical progressions but physically manifests itself quite distinctly. Two processes contribute to SMA: (i) increased destruction of uninfected RBCs, and (ii) decreased erythropoiesis (153). While clearance of RBCs is a normal homeostatic event, during malaria infection uninfected RBCs suffer a shortened life span. There are several mechanisms by which uninfected RBCs are targeted for destruction: (i) oxidation (ii); phosphatidylserine exposure on extracellular leaflet; (iii) reduced deformability; (iv) complement binding; (vi) autoantibodies; (vii) immune complexes; and (viii) recognition of IgGs to non-specifically bound parasite Ag (153, 184-190). RBCs are destroyed by either intravascular hemolysis or through reticuloendothelial mediated clearance. IFN-γ induced activation of splenic macrophages has been demonstrated to increase the destruction of uninfected RBCs (153, 191-192). Significantly, rodent experiments have been able to replicate what happens in humans during SMA and they have demonstrated that by depleting $CD4^+$ T cells or phagocytes, anemia is avoided (153, 193). Suppression of erythropoiesis is brought about by toxin-mediated and cellular inflammatory damage during infection (153). Again, evidence in both mouse and human studies support the concept that suppression is cytokine (IFN-γ and TNF-α) driven and tied to NK and NKT

responses to malaria toxins Hz and GPI, which may also explain its varied presentation in both mice and humans (107, 153, 165, 170, 194-198).

Placental Malaria

Analysis by Desai et al. has established that approximately 25% of women have placental malaria (PM) at delivery in Africa (199). The most critical feature of malaria infection during pregnancy in regards to neonate success is low birth weight (LBW), which is clinically defined as having a mass of < 2500g (47). In sub-Saharan Africa, some 600,000 neonates suffer LBW each year; up to a third of those will die with the cause of LBW found to be maternal malaria (200). As with most cases of severe malaria, placental malaria is most often caused by *P. falciparum*, however, *P. vivax* has been shown to lead to poor pregnancy outcomes such as LBW in India, Southeast Asia, and Oceania (47, 201-203). Falciparum malaria displays the only clinical manifestation of placental sequestration of the five species of *Plasmodium* (80, 204).

Sequestration of parasites within the maternal vascular space is fundamental to the observed PM pathology. Sequestration in the placenta, however, differs markedly from sequestration in other organs. For example, in the brain IE adhesion is principally mediated by PfEMP1 recognition of and binding to CD36 and ICAM-1 (143, 147). In the placenta, the major pregnancy-specific, host adhesion ligand appears to be CSA, not CD36 or ICAM-1, and the major parasite binding protein is VAR2CSA, which is a variant of PfEMP1 (80, 205-207). CSA is highly expressed by syncytiotrophoblasts (ST), fetal multinucleated cells, which are the fetal interface with maternal blood in the intervillous space of the placenta (208). Another dichotomy between CM and PM is the absence of rosetting or clumping within the placenta (209-210). Furthermore, because VAR2CSA preferentially binds CSA moieties, sequestering IEs may shift the balance of hemostasis, a critical mechanism in a developing and dynamic organ such as the

placenta. For example, thrombomodulin (TM), one of the principal regulators of coagulation, is highly decorated with CSA. The CSA moieties bind to thrombin, the protein responsible for fibrin generation and inhibit its coagulant activity; pRBCs binding to these CSA moieties may inhibit TM functionality (211-216). There are, as of yet, no studies that have investigated this aspect of malaria or TM biology, however, coagulation is known to be activated in placenta malaria as evidenced by significant perivillous fibrin deposition and initiation by tissue factor, the protein responsible for coagulation initiation, expressing macrophages on exposure to pRBCs and many others (discussed later) (217-221). The level of fibrin deposition has been positively correlated to prematurity induced LBW in placental malaria in high transmission areas (219).

The poor birth outcomes associated with placental malaria have been mainly attributed to trophozoite and schizont IE stage sequestration within the intervillous space, higher placental vs. peripheral parasitemia, substantial infiltrates of monocytes and macrophages, robust cytokine responses (INF-γ, TNF-α, IL-1, IL-2, and IL-8), and the presence of accumulated Hz; all of which have been associated with anemia, IUGR, preterm delivery, LBW, or even pregnancy failure (219-229). The mechanisms by which these characteristics of PM influence pathology are incompletely understood and represent a significant gap in knowledge. It is known, however, that pregnant women are more susceptible to malaria infection than non-pregnant women even in endemic areas and in individuals with established protective immunity, with susceptibility being inversely proportional to gravidity (199). Several features of pregnancy have been implicated in enhanced susceptibility. It has been demonstrated that pregnant women attract twice the number of mosquitoes than non-pregnant women, a phenomenon that was attributed to the fact that pregnant women present a larger host signature to the mosquito in terms of increased body heat, increased respiration and exhalation of CO2, increased release of volatile compounds from the

skin, and increased exposure due to frequent nightly extra-bed net excursions, such as the need to use the bathroom (230). Pregnancy is also supported by a T_H2 polarization; however, malaria induces and clearance is dependent on a T_H1 type response, a condition negatively associated with proper maintenance of pregnancy (231-232). Additionally, parasites that sequester in the placenta are seeing a new organ with new tissues and ligands. As a result, the parasites express proteins that are not well recognized by the host (VAR2CSA), even in already clinically immune individuals, which allows the parasites to evade the immune system (47, 233). The strong, inversely related, gravidity-dependent pathological responses in placental malaria are indicative of Ab mediated recognition of pregnancy specific Ags, VAR2CSA in particular (80, 223, 234-238).

The gaps in knowledge of PM may find closure in rodent models of malaria during pregnancy. Recent murine studies have shown that the features of pregnancy-related recrudescence of *P. berghei* infection in mice with pre-existing immunity mimic those of *P. falciparum* PM in humans including similarities between the pathological changes in the placenta, adhesion phenotype of the parasites, and immunological basis of susceptibility and acquired immunity (239-240). The significance of these data point to a central role that rodent malarias can have as models to aid in the study of the pathogenesis, pathology, and acquired immunity of PM (239). The previously mentioned pathological features observed in humans are also seen in the placentas of *P. berghei* infected mice and include: basal zone necrosis, syncytiotrophoblast hyperplasia, mononuclear infiltration, distension of perivascular spaces, sinusoid constriction, and accumulation of IEs and Hz (239, 241-243). What is critically important in the translation of observations from mouse to human are the complications presented in these models of PM that may match complications from those observed in high

areas of endemicity. Complications representative of clinical presentations in women who might otherwise be asymptomatic if they were not pregnant and infected with sequestering parasites. For example, *P. berghei* infections during early pregnancy result in abortion, stillbirth, fetal resorption, IUGR, prematurity, LBW and impaired growth of the surviving offspring (239, 241). Unfortunately, *P. berghei* falls short in closely recapitulating the complications in humans from low endemic areas, as *P. berghei* is universally fatal in non-immune mice (244). This shortcoming of the *P. berghei* model is compounded due to the paucity of studies of pregnant, *P. falciparum* infected women with limited or no pre-existing immunity (239, 245). The *P. chabaudi* model may be able to help bridge this gap. For example, previous studies in our lab using C57BL/6 mice have been able to demonstrate, similar to that of the majority of human PM cases: 1) course of parasitemia is predictable and maternally non-lethal; 2) pregnancy complications are due to selective accumulation of iRBCs within the placenta and robust cytokine responses relative to uninfected pregnant counterparts, including the elaboration of IL-β and IFN-γ particularly at time of abortion; 3) inflammatory control is impaired, if not systemically, at least at the level of the placenta due to a local lack of IL-10; and 4) there appears to a close connection between inflammation and coagulation as neutralization with anti-TNF-α antibodies significantly reduces the expression of the protein responsible for the initiation of coagulation, tissue factor (TF), two factors which protects against pregnancy loss in this model (221, 224, 246).

Placental Biology

Any discussion of placental malaria would be incomplete without at least an elementary introduction to the anatomy and physiology of the placenta. As an exhaustive description of mammalian placentation would be superfluous, it will suffice to say that proper formation of the

placenta is the single most critical factor in predicting successful mammalian pregnancies; environmental insults or genetic abnormalities at any developmental stage can result in placental insufficiency, IUGR, and spontaneous abortion or stillbirth (247-248). In brief, the purpose of the placenta is to facilitate feto-maternal interaction to promote fetal growth and viability while concomitantly preserving maternal welfare (249). This functionality is accomplished through several broad mechanisms. The placenta provides adequate feto-maternal nutrient and gas exchange as well as fetal waste removal (249). Fetally derived cells are capable of modulating maternal vasculature to ensure an uninterrupted and amplified (as needed) volume of maternal blood (249). Additionally, fetal cells are also capable of synthesizing and secreting hormones into maternal circulation via the placenta that manipulate maternal metabolism in favor of the fetus (250-253). The placenta protects the fetus from the maternal immune system, which will, if permitted, mount a response to the semi-allogenic progeny (254). Finally, the placenta also maintains the integrity and function of the uterus in the timing of gestation (249, 255).

The placenta is composed of both embryonically- and maternally-derived cells; for all of its complexity, the most vital cell types of the placenta are those of the trophoblast lineage. Syncytiotrophoblast cells are multinucleated cells that offer a large surface area for exchange of nutrients and information between mother and progeny. Ontologically, the trophoblasts are the progenitors of the placenta and appear four days after fertilization as the outer layer of cells (trophoectoderm) of the blastocyst (248, 255-258). These blastocyst trophoblasts are the antecedents of all other placental cell types (248, 255-256, 258-259). Therefore, trophoblasts are critical as they mediate implantation, regulate pregnancy endocrine function, provide immune protection of the fetus, drive increases in maternal vascular blood flow into the placenta, and may dictate delivery (248-250, 252-256, 258-260).

The placenta function and development knowledge base is chiefly based on murine studies (247-248, 257). This fact is mirrored by the level of knowledge rodent malarias have given to the understanding of PM (244). Placental formation is initialized when embryonic metabolic requirements overcome the capacity of the embryonic yolk sac just after implantation (248, 257). Development of the placenta arises from the fusion of two separate tissues: (i) the embryonic mesoderm derived structure called the allantois, which materializes from the posterior end of the embryo and gives rise to placental blood vessels and the umbilical cord; (ii) the extra-embryonic membrane structure known as the chorion, which is composed mostly of the trophoectoderm. (248, 257). Upon fusion, fetal blood vessels emerge from the allantoic portion and invade the chorionic plate where trophoblast stem cells receive instruction to differentiate. Once the trophoblasts differentiate they fuse and form syncytiotrophoblasts, followed by the coalescence of the allantoic portion and chorion sandwiching the syncytium to form what is known as the hemotrichoral labyrinth (248, 257-258). The labyrinth becomes the main site of maternal and fetal interface via integration of both maternal and fetal blood spaces. Trophoblasts from the ectoderm continue to differentiate and develop into trophoblast giant cells which then anchor the placenta into the uterine wall (257). In the mouse placental development begins around day 6 post coitus, direct feto-maternal interactions are complete by day 10 and the placenta is fully mature around day 13-14 of their 19-21 day gestation (248, 258). The time period from gestational day 10 through delivery in the mouse corresponds to the final two trimesters of human development (249). Labyrinthine disruption has been shown to lead to placental insufficiency and poor birth outcomes; a feature that has also been shown during PM in mice. In mice infected with *P. chabaudi*, disruption of the labyrinthine architecture is mediated by TNF-α and possibly excessive coagulation. This architectural disruption is abrogated upon

TNF-α neutralization improving mid gestational pregnancy outcomes in this mouse model (221, 257, 261).

While the labyrinth is the most critical structure in the placenta, there are yet two other important placental structures. The first of these two structures lies between the labyrinth and maternal uterine tissue and is referred to as the junctional zone (basal plate in humans). This area is crossed by maternal blood vessels but there is an obvious absence of fetal blood vessels. The maternal blood channels are, however, lined with fetal trophoblasts which act as a conduit that funnel blood into the labyrinth (249). The second of these structures, known as the decidua or placental bed is composed of maternal uterine tissue and lies proximal to maternal endometrium and borders the trophoblast giant cell layer of the invading fetal ectoderm. Fetal trophoblasts invade this region from the junctional zone through the uterine tissue past the endometrium and into the myometrium (250, 262). The maternal vessels that feed into the junctional zone originate from the decidua/placental bed and serve to support the maintenance of the feto-placental unit (250, 262).

Hemostasis

Hemostasis is a dynamic process wherein a suite of cells and proteins within various vascular systems attempt to maintain the physiologic set point of plasma fluidity and vascular integrity. The basic function of hemostasis is to stop blood flow from a damaged blood vessel (263). Hemostasis can be separated into three functionally distinct but temporally overlapping elements: (i) primary hemostasis or platelet aggregation; (ii) secondary hemostasis or coagulation, and (iii) tertiary hemostasis or fibrinolysis (264). Overlapping processes permit proper initiation and regulation of the response to bleeding at the site of insult, limits the dissemination of the response beyond where it is needed, and restricts protracted lingering of

clots (263-265). In many disease states such as those that occur in sepsis, cancer, and chronic inflammatory syndromes, the hemostatic axis is skewed and dysregulated; the physiological result is pathological thrombus formation (266-276).

Primary hemostasis is mediated by platelets aggregating at the site of injury. Upon insult, proteins which would otherwise be kept cryptic within the subendothelium are exposed to plasma. Platelets do not adhere to normal, intact endothelium; however, when the endothelium is disrupted, the primary platelet binding protein, collagen, is exposed. Blood is a fluid which generates considerable shear stress and therefore platelets must slow down before they can efficiently interact with the exposed subendothelium. This slowing down is mediated by a rolling mechanism in which collagen interacts with an adhesive protein, von Willebrand factor (vWF), which is constitutively expressed and present in plasma in inactivated form. When collagen is exposed to the plasma vWF becomes activated and binds collagen. Platelets are able to bind to vWF via formation of a glycoprotein-vWF complex (GPIb-IX-V). Once the rolling platelets stop and become firmly adherent to the damaged subendothelium, they form a basal layer to which other platelets may bind through a different glycoprotein complex (GPIIb-IIIa). Eventually a mass of aggregating platelets forms The platelets are activated then bind to and support the activation of other blood proteins such as coagulation proteins and fibrinogen which will eventually form the scaffold structure that cellular infiltrate will adhere to and initiate wound repair. Platelet activation and aggregation are controlled by a number of compounds of which cyclic adenosine monophosphate (cAMP) is the most critical. Once platelets are activated and begin the aggregation process, inflammatory mediators derived from the arachidonic acid pathway signal the platelets to increase adenylate cyclase activity, thus converting ATP to cAMP. The physiologic consequence of increased platelet cAMP concentrations is multifaceted;

platelets are inhibited from aggregating, secreting any further activating compounds into the injury milieu, and from adhering to subendothelial surfaces (263-264, 277-283).

Secondary hemostasis, or coagulation proceeds in a stepwise fashion commonly referred to as a cascade that is driven by successive cleavage of serine protease zymogens into their active enzymes. The cascade is typically divided into two separate mechanisms, the intrinsic (activating components are "intrinsic" to the plasma) and the extrinsic (activating components are "extrinsic" to the plasma), however, this distinction has proven irrelevant outside of a laboratory setting as physiologically, the components of both systems interact and are capable of activating one another (263, 284-285). The physiologic way to treat coagulation is to describe it in terms of a cell based model. In this manner, the coagulative process occurs by activation of a trigger mechanism, which results in the cleavage of a serine protease; downstream procofactors are cleaved into active cofactors that permit the assembly of procoagulant complexes on cell surfaces. Assemblages such as these procoagulant complexes allow for rapid local increases of coagulant reactants as well as restricting activation to the site of injury. The final step in coagulation is the generation of thrombin from its zymogen prothrombin. Thrombin cleaves fibrinogen to release fibrin which is insoluble and precipitates out of plasma, forming the foundation of a blood clot. In response to tissue injury, sepsis, cancer, and many other disease states, the "extrinsic pathway" triggered by the exposure of TF is the dominant model to explain both normal and pathological coagulation and is therefore discussed in this thesis (274, 286-299). The overall process can be explained in phases: (i) initiation by TF, (ii) amplification by proper platelet accretion and initial thrombin generation, and (iii) propagation by the generation of procoagulant complexes on a plasma membrane, which drives the bulk of clot formation (265, 285, 300-301).

The extrinsic pathway of coagulation is initiated by the exposure of blood plasma to TF. TF is cryptic under normal conditions but may be revealed under conditions of insult. Insult in the vasculature is not necessarily solely defined by mechanical injury; biochemical insults are just as potent and are encountered much more frequently in models of infection and tumorogenesis (299, 302-307). These biochemical stimuli refer to cytokine release. Two principle cytokines that are capable of decrypting TF on a multitude of cell types are TNF-α and IL-1 (308-309). Decryption of TF results in the interaction with its endogenous ligand factor VII (FVII) (310-311). When FVII binds to TF, FVII is cleaved to form its active protease FVIIa (312-314). Cell surfaces decorated with TF/FVIIa complexes provide a substrate platform for generation of active factor ten (FXa) from FX (284). FXa is the protease responsible for the generation of thrombin from prothrombin. Thrombin is a multipotent molecule; one of its roles is to enhance platelet adhesion and fully activate responding platelets at site of insult (315-316). Although FXa is necessary to produce thrombin, it is hardly sufficient in producing physiologically potent amounts rapidly enough. In order to create the quantities needed, FXa requires the assistance of other cofactors: (i) FVa; (ii) a suitable phospholipid surface; and (iii) Ca^{2+} (301, 317-320). The FVa is produced by FXa and the slowly rising levels of thrombin. This serves as a regulating step ensuring that the indication to generate thrombin is a physiological necessity and not an errant signal. When FVa forms on the surface of a suitable cell, such as a platelet, in the presence of extracellular Ca^{2+} it serves as a receptor for FXa. When FXa binds to membrane bound FVa it forms what is referred to as the prothrombinase complex. The prothrombinase complex is capable of rapidly generating thrombin, with rates exceeding 300,000 fold increases over FXa alone (301, 317-321). The purpose of such rapid levels of thrombin generation is to drive fibrin formation. Before fibrin can create the scaffold of

the blood clot, monomers generated by fibrinogen cleavage must cross-link to form fibrin polymers. Thrombin also plays a role in this cross-linking event: fibrin, in the presence of thrombin and plasma transglutaminase (factor VIIIa, liberated from platelet bound vWF) undergoes intermolecular covalent cross-linking (322-326). The resulting fibrin mesh serves to bind platelets and facilitates their anchoring to the vasculature. The fibrin clot is constructed so rapidly due to early initiation that it is estimated that up to 95% of all thrombin generation occurs after clot formation has already begun (327).

It turns out that thrombin also plays a critical role in the structure and maintenance of the blood clot and serves as a potent signaling molecule bridging coagulation with inflammation. For example, the rate of thrombin generation serves to control the tightness and rigidity of fibrin cross-linking and therefore protects it against mechanical abruption (265, 328). Thrombin generated after initial clot formation also leads to the activation of thrombin activatable fibrinolysis inhibitor (TAFI). TAFI is a carboxypeptidase that removes terminal lysine residues from fibrin, which protects the clot from fibrinolytic enzymes, enhancing clot resistance to fibrinolysis (discussed later) (265, 329-330). ECs exposed to thrombin *in vitro* release vWF, express P-selectin (a potent adhesion molecule for leukocytes), and produce a myriad of chemokines, such as IL-1β, IL-6, MCP-1, TNF-α, IL-10, FGF-2, and VEGF (331-336). Thrombin carries out its role as a signaling molecule through a family of G-coupled protein receptors (GPCRs) termed protease activated receptors (PARs), which carry their own ligands that remain silent until activated by receptor cleavage via activated serine proteases (336-337). For example, thrombin can signal through PAR1 (there are four known PARs) on ECs, leukocytes, lymphocytes, and several other cell types to activate any number of GPCR α or β

subunits to drive changes in cell shape, secretion, integrin activation, metabolic responses, transcriptional responses, and cellular mobility (336-339).

Because the activation of coagulation is rapid and its consequences far reaching, regulation of this response is paramount. Several redundant mechanisms are in place to adequately handle the task of controlling coagulation. These mechanisms include dilution of factors, flow rate of blood, fibrinolysis, endogenous proteolytic inhibitors, and the thrombin molecule itself (263). Because the clot is exposed to shear forces, any weak interactions of platelets are removed and cleared in circulation. Soluble proteins that are not bound to membrane coagulation complexes are diluted by blood flow or they diffuse way from the clot to be inactivated and marked for destruction by inhibitory proteins. Moreover, thrombin itself generates its own inhibitor, antithrombin III, which is generated by scissile bond cleavage of the C-terminus of ATIII, forming a covalent, 1:1 acyl enzyme intermediate also known as a thrombin-antithrombin (TAT) complex (340). Antithrombin also inhibits FXa but not as tightly as thrombin (341-342). Thrombin's self regulating activity does not end there. Thrombomodulin (TM), a protein cofactor expressed on endothelial cell surfaces, binds to and modifies the substrate specificity of thrombin (343). By changing thrombin's substrate specificity, TM inhibits thrombin's ability to cleave FV and FVIII, drastically reducing the formation of the prothrombinase activity and the cross linking of fibrin (344). Additionally the thrombin-TM complex activates the zymogen protein C into activated protein C (APC) through complexation with the endothelial protein C receptor (345). APC has numerous effects on endothelium, acting as an anti-inflammatory molecule suppressing cytokine (TNF-α, IL-1β, IL-6, and IL-8) secretion by monocytes, down-regulating pro-inflammatory intracellular signaling pathways, and inducing a cytoprotective expression profile in ECs (346-347). APC enhances fibrinolysis, and inactivates

factors Va and VIIIa (347). The binding of APC to EPCR inhibits endotoxin-induced TF expression on mononuclear cells (345). Additionally, the activation of TAFI is amplified by the TM-thrombin complex, and is the key enzyme in charge of the inactivation of complement factors C3a and C5a, thereby providing protection against complement-mediated injury in the microvasculature (345, 348). Finally, the TM-thrombin complex inhibits proinflammatory thrombin signaling through PARs while APC-EPCR engaged signaling heralds anti-apoptotic and survival signals through these same PARs, a switch which has been shown to be critical in the maintenance of pregnancy and brain ischemia (349-355). As already discussed, thrombin activity is not limited to coagulation, it is a potent inflammatory mediator which potentiates its action through PARs. Additionally, however, thrombin also exerts a potent endothelial cell activating capacity, including a ten-fold upregulation of adhesion molecules such as E- selectin, CD36, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (356-359). In conjunction, thrombin also promotes the recruitment of monocytes through induction of monocyte chemotactic protein-1 (MCP-1) (357, 360). If the thrombin response is not too great, free thrombin binds to TM, which activates protein C. The protein C pathway is responsible for inhibiting further cleavage of FX and prothrombin by degrading FVa and FVIIIa thus limiting further thrombin generation (211, 361-362). However, if thrombin generation is too great or TM expression not commensurate with generated thrombin levels, both a hypercoagulant and hyperinflammatory environment can ensue.

Redundancy is a key feature of hemostatic control. It is of no surprise then that TM has an additional role in modulating inflammation; TM can inactivate HMGB-1 induced inflammation which has been shown to be important in sepsis and malaria alike (363-364). HMGB-1 is a molecule with potent cytokine-like activity that contributes to late-stage

inflammatory disease (365). HMGB-1 is released from necrotic cells late in the host response to infection after many of the major proinflammatory cytokines like TNF-α and IL-1 have come and gone (365-366). Critically, HMGB-1, in proportion to degree of illness, can be predicted to exert more proinflammatory influence in brain and placenta, where more of it is functionally available due to a relative lack of thrombomodulin (357).

Hemostatic regulation can occur further upstream by proteolysis, which serves to shut the entire cascade down. A class of protein known as serpins (serine protease inhibitor) plays a major role in regulating coagulation and fibrinolysis. The serpin that regulates the initiation of coagulation is called tissue factor pathway inhibitor (TFPI) (367). TFPI is constitutively expressed principally by the endothelium and megakaryocytes but is also expressed in smaller amounts by monocytes, macrophages, lung fibroblasts, vascular smooth muscle cells, laryngeal squamous epithelial cells, astrocytes, mesothelial cells, and mesangial cells (368-377). TFPI expression is critical to the maintenance of fluid flow within the placenta and is therefore abundantly expressed by syncytiotrophoblasts and cytotrophoblasts during development but wanes during delivery to protect the mother from hemorrhage (263, 378-381). The mechanism of inhibition by TFPI proceeds in two steps: first, TFPI binds to and inhibits FXa in a 1:1 stoichiometric manner; second, the TFPI–FXa complex then binds to and inhibits the TF–FVII complex and then to the VIIa/TF complex forming a quaternary complex of TFPI-FXa-TF-FVII or TFPI-FXa-TF-FVIIa (367, 382-383). In this way TFPI is able first to reduce thrombin generation by FXa and then to reduce the activation of FX by inhibiting the generation of FVIIa from FVII. A homolog of TFPI, originally discovered in the placenta and referred to as placental protein 5 (PP5), was found to have inhibitory activity towards plasmin and fibrinogen cleavage by thrombin (384-389). It was later discovered that the PP5 protein also demonstrated

TF-FVIIa complex inhibition and was therefore renamed TFPI-2; this newly named TFPI-2 was also found outside of the placenta in the liver, skeletal muscle, heart, kidney and pancreas (389-392). Besides their functionality in inhibiting coagulation, TFPI-1,2 have also demonstrated roles in supporting angiogenesis, modulating inflammatory cytokines, wound repair, remodeling of the extracellular matrix, and may also support metastasis of certain cancers (367, 370, 389, 393-400).

Tertiary hemostasis or fibrinolysis is the process that results in the degradation of fibrin polymers during the removal of blood clots and is the main mechanism to cope with clot formation (401-402). If clotting were allowed to continue or clots remain indefinitely, every vascular bed would quickly occlude (264). Fibrinolysis is also a critical component of wound repair (401). The process of fibrinolysis is similar to the cascade of proteolytic enzyme activation found in the coagulative process and has a single main effector enzyme, plasmin, which cleaves fibrin (401). The process can be deconstructed into two stages. The first stage is the activation of plasminogen to plasmin by a plasminogen activator (PA) and the second stage is the degradation fibrin (402). The triggers of fibrinolysis are the formation of the clot itself and signaling from thrombin; however, during initial clot formation, activated platelets and endothelial cells produce factors that inhibit plasmin formation in order to support the generation of the hemostatic plug (403-404). Plasmin is generated through activation of its liver-produced zymogen plasminogen by two enzymes: (i) tissue-type plasminogen activator (tPA) and/or (ii) urokinase-type plasminogen activator (uPA) (401-402, 405). While plasminogen is primarily produced in the liver, reports also indicate that plasmin is generated in the kidneys, brain, testis, heart, lungs, uterus, spleen, thymus and intestine (406). Endothelial cells are responsible for tPA production while uPA is produced by connective tissue cells, epithelial cells, macrophages, and

endothelial cells (402, 405, 407). Like coagulation, fibrinolysis uses an amplifying feedback mechanism to magnify the effectiveness of clot degradation. Mechanistically the process commences once tPA binds to the cross-linked fibrin within the blood clot, which results in activation and enhanced cleavage of plasminogen to yield plasmin. Plasmin then reciprocally cleaves tPA, into an even more active two chain tPA, which results in further plasminogen cleavage. In contrast, uPA does not directly interact with fibrin but rather uPA binds to the cellular receptor urokinase-type plasminogen activator receptor (u-PAR) resulting in enhanced activation of cell-bound plasminogen (401, 406, 408-410).

The action of these activators is tightly controlled by three serpins, which form a covalent complex with their targets and irreversibly modify the normal function of these proteases (406, 411). The three inhibitors are known as plasminogen activator inhibitor (PAI)- 1, 2, and 3. PAI-1 is synthesized by several types of cells and tissues including endothelium, megakaryocytes, human endometrium, peritoneum, macrophages, mesothelial cells and adipocytes, but it is principally stored in platelets (406, 412-420). Inhibition of fibrinolytic activation is not the only function of PAI-1, it also plays a vital role in cell signaling, adherence, and migration (406, 421-422). PAI-2 expression was originally believed to be limited to the placenta and monocytes/macrophages; however, it is now known that PAI-2 is expressed in many different tissues but is undetectable in men or non-pregnant women (406, 423-425). PAI-1 and PAI-2 expression increase throughout pregnancy, and they have both been ascribed a role in the maintenance of hemostasis during both pregnancy and delivery. PAI-2 has been further implicated in fetal growth regulation and may also be a marker of placental function during pregnancy (406, 426-429). Interestingly, it has been suggested that intracellular PAI-2 can defend placental cells from apoptosis in normal pregnancies by abrogating TNF-α-induced

cytolysis (406, 430). The consequence of the diminution of its local concentration within the fetoplacental unit may decrease the PAI-2 cytoprotective effect, impair placental nutrient transport and result in intrauterine growth restriction (431). PAI-3 is synthesized in the liver and steroid responsive organs and is responsible for the inhibition of several hemostatic proteases such as uPA, tPA, APC, and thrombin (406, 432-436). Finally, plasmin and plasminogen activators have also been associated with tissue proliferation and cellular adhesion, as they are both capable of proteolytically degrading the extracellular matrix (ECM) and regulating the activation of growth factors and matrix metalloproteinases (MMPs); uPA has also been shown to control cell migration processes during both normal and pathological conditions, such as those that occur in angiogenesis, embryo implantation, and inflammation (406, 437-441).

Implications of Coagulopathy in Malaria

In addition to accumulation of iRBCs and HZ, PM can be characterized by trophoblast basement membrane thickening, excess perivillous fibrin deposition, and fibrinoid necrosis of fetal villi, which house the fetal placental circulation (217-220, 227-228, 442-450). Excessive perivillous fibrin deposition is an indication that placental malaria either leads to excessive coagulation or that tertiary hemostasis is impaired in some way. Each of these perturbations of the hemostatic axis, however, is able to support a hypercoagulable state (379, 451-452). In addition, substantial maternal inflammatory cell infiltrate, predominantly monocytes, is frequently found in the maternal blood space (218, 220, 227-228, 446, 449, 453-455). Many studies have demonstrated significant correlation between proinflammatory cellular infiltrate to pre-term delivery and LBW (219-220, 227-228, 456-458). However, the underlying mechanism(s) which result in these poor birth outcomes have been incompletely characterized (459).

Aside from placental malaria, the other major outcome of *P. falciparum* infection that is associated with cytoadherence and significant morbidity and mortality is cerebral malaria. This disease is characterized by seizures and coma, frequently culminating in death. While the precise pathological mechanisms are not fully understood, current findings provide support for contributions from parasite PfEMP1 binding to ICAM-1 and CD36, the inflammatory response elicited by iRBC cytoadherence to these molecules, and local coagulation supported by activation of the endothelium and the ability of iRBCs to support the formation of multimolecular coagulation complexes, all of which have important parallels with placental malaria (460-461). Sequestration of parasites within the brain is thought to culminate in mechanical obstruction of blood flow through the cerebral microvasculature with concomitant decreases in perfusion and waste removal (462-463). Postmortem histological examinations of brain tissue from CM victims show not only sequestration of iRBCs, but also large quantities of accumulated platelets, fibrin, monocytes, and evidence of endothelial activation and damage by the significant expression of TF (145, 460, 464-466). Massive proinflammatory cytokine elaboration, such as occurs with TNF-α in CM, not only plays a direct role in pathogenesis, but has also demonstrated profound influences on coagulopathic damage as well in models of sepsis (345, 467-469). All together, these findings have recently led to a new theory of cerebral malaria pathogenesis in which an inflammation-coagulation cycle is propagated by sequestration of *P. falciparum* in the brain microvasculature, ultimately leading to organ dysfunction and death (470).

The inflammation-coagulation cycle in malaria, recently proposed by Ivo Francischetti (see references (470-471) for excellent reviews), is based on recent observations, as well as a substantial history of coagulation abnormalities in malaria. In general, coagulation is

dysregulated in malaria (444, 460, 470-475). A seminal study in 1967 of patients with severe falciparum malaria displayed clinical markers for increased coagulation and possible DIC: prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT); thrombocytopenia; decreased Factors V, VII, VIII, and X, as well as decreased plasminogen activation; and accumulation of fibrin degradation products (476). PT and aPTT are global clotting assays, which estimate the time to the formation of thrombin. Both PT and aPTT measure the lag time of thrombin formation and fibrinogen derived clotting, prolonged times may indicate exhaustion of clotting mechanisms due to hypercoagulation, e.g. DIC. Subsequent studies revealed high fibrin degradation product levels in CM patients (471, 477-487). In many cases the degree of hemostatic aberration was related to the clinical severity of infection (471, 488-489). Varying reports of DIC also appeared in the literature, with typical presentations of thrombocytopenia, elevated fibrin degradation products, and increased VWF levels, but with very few reports of hemorrhage (471, 487, 490-493). In fact, as many as 10% of malaria cases result in DIC, but the majority of uncomplicated malaria cases result in compensated DIC, where the clinical metrics indicative of a coagulation disorder are present without overt bleeding (462, 471, 474, 489, 494-501). DIC is characterized by systemic activation of coagulation, leading to intravascular fibrin formation and thrombi preceding impaired organ perfusion with concurrent depression of anticoagulant mechanisms and diminished fibrin degradation due to inhibition of fibrinolysis (502-503). According to the International Society on Thrombosis and Haemostasis (ISTH), overt DIC is established using a cumulative score of ≥ 5 using the metrics from global coagulation tests: platelet count (<100= 1; 50 = 2); prolonged prothrombin time (>3sec but <6sec = 1; >6sec =2); fibrinogen(>1.0gL^{-1} = 1) and fibrin associated markers such as D-dimers and fibrin monomers (moderate increase = 2; strong increase = 3) (504). In contrast, compensated

(non-overt) DIC, represented by subtle hemostatic changes, can be established with a continued cumulative score < 5 using the same clinical metrics (504). To date, these metrics, or their predictive value, have not been explicitly described in malaria. In another study, 22 patients with severe falciparum malaria were found to have evidence of complement activation subsequent to activation of the intrinsic coagulation pathway, with reduced Factor XII, kallikrein (an intrinsic system protein), and protein C (PC) activity levels, as well as increased thrombin-antithrombin (TAT) complex levels. Nine of the patients presented with multi-organ dysfunction, four developed bleeding, and two died (473). In fact, the intrinsic pathway is directly supported *in vitro* by iRBCs, which expose phosphatidylserine on their surface (460, 505). Indeed, iRBCs from infected patients had reduced recalcification and clotting times relative to normal RBCs (506-507). Recalcification assays involve the return of physiologic concentrations of Ca^{2+}, which is typically removed during blood collection. Recalcification assays have a broader span than PT or aPTT tests in disease states that involve non-genetic disorder driven coagulopathy, namely infectious diseases and cancers; recalcification time assays include whole blood not plasma, as in other coagulation tests, and therefore take into account contributions to coagulation from all circulating cellular and chemical mediators. A reduction in recalcification time reflects hypercoagulability due to TF generation. Various disease states are associated with hypercoagulability related to the generation of monocyte TF activity, which is a pathological feature found in malaria infection both in CM and PM (508).

At the tissue level, fibrin deposition typically coincides with parasite sequestration, with evidence from the heart, lung, brain, liver, kidney, subcutaneous tissues and placenta (5, 142-143, 146, 454, 460, 471, 509-518). That fibrin deposition and/or thrombi are often found in concert with *P. falciparum* infection is not surprising. Models of sepsis have long reported

activation of coagulation and coincident depression of fibrinolytic mechanisms, firmly establishing a committed interplay between inflammation and coagulation (519-521). Primary among these interactions is TF whose expression is the primary trigger for the extrinsic pathway of coagulation (74, 337, 351, 460, 470-471, 522-533). Importantly, administration of exogenous TNF-α to human volunteers yielded a state of hypercoagulation and reduced fibrinolysis, a state linked to upregulation of TF on endothelial cells and monocytes (519, 534-548). *P. falciparum* infection indirectly induces TF expression by increasing levels of circulating and local concentrations of TNF-α and directly through iRBC cytoadherence to endothelial cells (301, 357, 460, 470-471, 506, 549-550). In one study, patients with severe malaria displayed increased TNF-α and decreased PC levels, concurrent with increased concentrations of TAT complexes, all of which correlated with parasite burden and organ impairment. Significantly, exposure of cultured endothelial cells to sera from these patients increased the procoagulant activity of the cells (measured as a function of recalcification time) and TF transcript levels. Upon treatment, inflammatory and coagulant indicators in these patients returned to normal (499). TF upregulation on endothelial cells stimulated by patient serum was reduced with anti-TNF-α antibodies, confirming the link between this inflammatory cytokine and endothelial pro-coagulant activity in malaria (551). Perhaps the most compelling data for the involvement of TF and coagulopathy in malaria to date was the demonstration by Francischetti et al. that cerebral endothelial cells in patients who died from cerebral malaria expressed high levels of TF, while no TF expression was found in those patients who died from other causes (460).

While endothelial pro-coagulant activity is well established in malaria, other cell types are also critical. For example, it has been shown that TF expression increases on monocytes following exposure to malaria-infected patient plasma (268). TF expression was reported to be

high on monocytes in the infected placenta, and was also increased on syncytiotrophoblasts (217). Importantly, in keeping with a connection between malaria-induced inflammation and coagulation, TF expression on monocytes and endothelial cells is associated with increased production of proinflammatory cytokines IL-1, IL-8, TNF-α, as well as release of reactive oxygen species (531, 552-554). Platelet activation during malaria infection upregulates both TF and TNF-α, and has been shown to play a role in thrombosis associated with CM (460, 470-471, 555-570). It is particularly noteworthy that both cerebral endothelium and syncytiotrophoblast present low levels of thrombomodulin (TM) and thus reduced levels of APC, characteristics which encourage coagulation (354, 357, 471, 571-574). Moreover *P.falciparum* infected erythrocytes bind to the same side chain of TM that binds to thrombin. It may be that greater concentrations of free thrombin are allowed to persist within the placenta during placental malaria as a result of direct competition for binding TM. The increase in free thrombin concentration may lead to runaway coagulation/inflammatory signaling and cascade amplifications, which culminate in occlusion of the microvasculature with excess fibrin, impaired architecture, decreased proliferation, and apoptosis of placental cells leading to poor pregnancy outcomes.

In addition to immunologic function, syncytiotrophoblasts are also critical for normal hemostasis in the placental (maternal) vascular compartment. The intervillous space of the placenta represents a high volume, low resistance, low shear flow system, and is thus subject to coagulation due to hemostasis, dictating that the syncytiotrophoblast express anti-coagulants such as TM, PC receptor, and annexin V , as well as TFPI-1, 2 (575). In addition, these cells also produce tissue and urokinase plasminogen activators (t-PA and u-PA), which promote fibrinolysis (576). On the other hand, these cells must also ensure that the separation between

the maternal and fetal circulatory systems is not breached, or, if damaged, is rapidly sealed with fibrin. Thus, syncytiotrophoblast also expresses high levels of TF, and PAI-1, 2 that can block fibrinolysis (577-578) . Thus, together with other essential components of the coagulation cascade that are maternally-contributed, the syncytiotrophoblast can take an active part in the hemostasis of the villous placenta. If considered together, the evidence of dysregulated coagulation in *P. falciparum* malaria and the observation of excessive fibrin deposition in the infected placenta, which was originally described in 1938, indicate that along with inflammatory contributed pathology, there is a concomitant hypercoagulation that may occur in the malaria-infected placenta that has vital implications for birth outcome (579). Unfortunately, the possibility that coagulation may play a role as a contributor of PM pathogenesis has been largely ignored.(218). In fact, one study that observed a significant independent association between perivillous fibrin deposition and prematurity, did not even make mention of the finding in the report's discussion (219). Our lab's preliminary data, together with that report, as well as the increased TF expression on monocytes in association with significant fibrin deposition in the malaria-infected placenta, obliges us to propose that hypercoagulation is a central determinant in the pathogenesis of placental malaria (217) . This is especially important given that pregnancy is known to be a hypercoagulable state, necessitated by the need for rapid coagulation during the puerperium (the time period after childbirth, when the mother's body returns to its normal physiological state), especially during placental separation, but which predisposes women to thrombosis (580).

Summary and Gaps in Knowledge

Certainly, substantial efforts and strides have been made in understanding of the immunopathogenesis of falciparum malaria, particularly in the context of pregnancy; however,

the vast majority of this focus has been committed to host and parasite receptor/ligand interactions in cytoadherence and in anti-malarial immune responses. With the current knowledge of the complex interplay between inflammation and coagulation in many diseases states but especially as it has been demonstrated in CM, it would be prudent to approach PM pathogenesis from a similar mechanistic point of view (470-471, 520). While maternal inflammatory responses in response to placental malaria as well as disruption of normal placental architecture are likely to be involved in poor birth outcomes associated with this infection, the specific mechanisms that retard fetal development and/or contribute to pre-term labor in the context of placental malaria have not been determined (218-220, 227-228, 443-444, 448-450, 456-458). The lack of understanding of the dynamics of coagulation and inflammation during PM coupled with the lack of research focus in coagulopathic influence in PM represents a significant gap in knowledge and an opportunity to further characterize this disease. With the availability of an increasing array of drugs that can simultaneously target inflammation and coagulation, including statins, which have been shown to rescue pregnancy in mice prone to coagulation-induced recurrent abortion, it is crucial that we gain a better understanding of the pathogenesis of malarial infection so that new therapeutic strategies can be developed, assessed, and implemented (581). Ultimately, appropriate intervention and management of malaria during pregnancy will rely heavily on detailed knowledge about how the human host and malarial parasite interact and respond to each other. Of particular importance are how dysregulation of immune responses and coagulation contribute to the complications and poor birth outcomes observed in PM. Focusing on the junction between inflammation and coagulation in the context of PM, we can garner exceptional insights into the pathogenesis of malaria during pregnancy. Insights that will more appropriately arm us to combat such a devastating global health problem.

CHAPTER 3

MATERIALS AND METHODS

Mice, parasites, anticoagulant therapy, viability, and histology

C57BL/6 mice originally purchased from The Jackson Laboratory were maintained and bred by brother/sister pairing 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 twelve-week-old, female B6 mice were used in all of the experiments. Mice were maintained on a 10-hour dark and 14-hour light cycle with feed and water *ad libitum*. To improve breeding efficiency pregnant mice were fed a breeder diet (*LabDiet* 5008) purchased from LabDiet, Richmond, IN. To avoid bias in weight gain, all control mice were also fed the same diet. The day on which a vaginal plug was observed in timed mated mice was considered day zero of pregnancy, experiment day 0 (ED 0). All murine infection experiments used *P. chabaudi* AS, originally obtained from Dr. Mary M. Stevenson (McGill University and the Montreal General Hospital Research Institute, Quebec, Canada). These parasites were maintained as frozen stock and were passaged through susceptible A/J females (infections were initiated by intraperitoneal injection of 10^6 iRBC) prior to use in B6 females.

Virgin female C57BL/6 mice were crossed with C57BL/6 males and either infected with 10^3 *Plasmodium chabaudi* AS parasites in 200 µl of phosphate-buffered saline on ED 0 (infected pregnant, IP) or not (uninfected pregnant, UP) via intravenous tail vein injection. Control, infected, non-pregnant (INP) mice were similarly infected. The UP mice were intravenously sham injected with 200 µl of phosphate-buffered saline . After recording clinical parameters such as body weight, and hematocrit on ED 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. IP mice were administered indicated doses of subcutaneous injections, by tenting the scruff of the neck, of low molecular weight heparin (LMWH) (Calbiochem, San Diego, CA, USA), Ixolaris (a tick anticoagulant protein), graciously provided by Ivo Francischetti (Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases), or enoxaparin (Lovenox, Sanofi-Aventis, Bridgewater, NJ, USA) in 500 µL of phosphate-buffered saline or normal saline beginning on ED 6 days through ED11, ED 12 or until cull criteria were met. Because two low molecular weight heparins were used, LMWH refers to the research grade sample, while enoxaparin refers to the FDA approved sample in this text. The usage of "low molecular weight heparin" will refer to the drug class. Both IP and INP control mice were given sham subcutaneous injections of 500 µL of phosphate-buffered saline. Mice were observed during ED 6-12; parasitemia was monitored by counting 10^3 erythrocytes in four high-power fields on Giemsa stained tail blood thin smears. Mice with parasitemias which varied outside of the established infection kinetics for this model (<0.5% or >4% on ED 8 and <12.5% or >25% on ED10), indicative of improper infection at ED0 or parasite slippage, were eliminated from the study (246). 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 following: (volume of packed erythrocytes)/(total blood volume) x 100. Body weight was recorded in grams. The presence of discolored urine or bedding was indicative of renal crisis due to excessive hemolysis.

Euthanasia was accomplished via CO_2 asphyxiation followed by cardiac puncture. Blood was collected into Microtainer K_2EDTA tubes (Becton Dickson, Franklin Lakes, NJ, USA).

Scoring for embryonic viability began ante mortem; pregnant mice were evaluated for the presence of vaginal bleeding, evidence of active abortion. If evidence of embryonic expulsion was found prior to euthanasia, all of the embryos were scored as non-viable, regardless of the state of the embryos upon necropsy. Similarly, if upon necropsy, evidence of active expulsion, that is, presence of embryos within the cervix or exiting uterine horns with open cervix, was observed, all embryos were scored non-viable despite the condition of the remaining embryos. Only embryos of females that did not demonstrate active abortion were assessed for viability. Resorbing embryos were scored as passive abortion as were embryos exhibiting intra-embryonic hemorrhage. Embryos exhibiting passive abortion were scored non-viable but were scored independently of the remaining embryos within a given uterus. Uteri from UP, IP, and IP LMWH treated mice were harvested on ED12 and fixed in 4% paraformaldehyde overnight. Tissues were washed and subsequently paraffin embedded and processed for histology via hematoxylin and eosin staining of 5µm sections. Sections were evaluated independently by two authors (J.A. and T.N.), scoring for necrosis and loss of architecture of the placental layers and embryo, assigning values 0-3, where 0 designates normal; 1, slight; 2, moderate; and 3,diffuse necrosis.

Cell Culture and parasite stimulation

An outbred mouse cell line was used to investigate the ability of infected erythrocytes to support a hypercoagulant environment during PM. Dr. Joan Hunt (University of Kansas Medical Center, Kansas City, KS) generously provided the Swiss Webster mouse trophoblast SM9-1 cell line. SM9-1 cells were cultured in RPMI 1640 complete (10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 1mM sodium pyruvate, 1.75 µM 2-mercaptoethanol). Cells were thawed and passaged twice before stimulation experiments began

to reduce influence of stress. *P. chabaudi* AS infected erythrocytes were removed from *P. chabaudi*-infected A/J mice with 25-40% parasitemia via cardiac puncture into a 1:9 ACD (sodium citrate) solution and washed twice with phosphate buffered saline (PBS). Captured blood was diluted with PBS (1 ml), loaded onto a 74% Percoll (Sigma-Aldrich, St. Louis, MO, USA) density gradient, and centrifuged at 1500 x *g* for 20 min at 4°C. The top interface, containing >85% parasitized RBCs as determined by staining with Giemsa, was collected and contained mature trophozoite and schizont stages. Uninfected erythrocytes (nRBCs) were collected in the same fashion from uninfected A/J mice and loaded onto a 90% Percoll gradient, centrifuged at 1500 x *g* for 20 min at 4°C, and the top interface collected. All collected cells were washed twice with PBS and resuspended in complete RPMI 1640. 3x 10^6 SM9-1 cells were plated (10^6 cells/mL RPMI) in Corning Costar® 6 well culture plates (Sigma-Aldrich, St. Louis, MO, USA). Medium was replaced every 24 hours until cells reached greater than 80% confluence. SM9-1 cells were co-cultured with either iRBCs or nRBCs at a cell to RBC ratio of 10:1and placed in a 37°C incubator with an atmosphere of 5% CO2. SM9-1 cells were harvested at times 0 (without iRBC or nRBC stimulation), 2, 4, 6, and 8 hours post exposure via trypsinization. Collected cells were placed in 15 mL RPMI 1640 complete and centrifuged once at 500 x g for 5mins,the cells were then resuspended and washed with PBS three times at 500 x g for 5mins. The collected cells were then used to isolate RNA.

RNA Isolation, cDNA generation, and quantitative polymerase chain reaction

Fetoplacental units of ED 10 infected pregnant, uninfected pregnant mice were homogenized in a TissueLyser II (Qiagen, Valencia, CA, USA). Total RNA from homogenates or SM9-1 cells were reverse transcribed using High-Capacity cDNA Reverse Transcription and DNA-free Kits (Applied Biosystems, Carlsbad, CA, USA) or RNeasy® Plus Mini Kit (Qiagen,

Valencia, CA, USA) as described by the manufacturers' protocols. Real-time PCR was performed on and analyzed with an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using Maxima® SYBR Green with passive reference (Fermentas, Glen Burnie, MD, USA). Reaction mixtures included 1x final concentration of Maxima® Sybr Green/Rox Master Mix, 300 nM of forward and reverse primers, 50ng of template cDNA, and water *quantum sufficit* to 20 µl. Target gene expression levels were normalized to the internal 18S signal and represented as relative expression calculated by the ΔΔCT method. Specific PCR primer pairs (Table 3.1) were used for the following mouse genes of interest: Tissue Factor (TF); Tissue Factor Pathway Inhibitor (TFPI); Thrombomodulin (TM); Protease Activated Receptor-1 and 2 (PAR-1, 2); Endothelial Protein C Receptor (EPCR), Plasminogen Activator Inhibitor-1 (PAI-1). Primers were created using Primer Express (Applied Biosystems, Carlsbad, CA, USA) or from previous literature as indicated and the oligonucleotides generated by (Eurofins MWG Operon, Huntsville, AL, USA) (582-583). Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 minutes; then 40 cycles PCR with denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute (total run time was approximately 90 minutes).

Statistical analysis

Data analysis was performed using GraphPad Prism 5 Software (La Jolla, CA, USA). The significance of difference of group means in the case of normally distributed data were compared via parametric, one-way ANOVA with Tukey's Multiple Comparison Test. Non-normally distributed data were analyzed by non-parametric, Kruskal-Wallis test with Dunn's Multiple Comparison post test. Proportional analysis was accomplished via two-sided Fisher's exact test. Values of $p \leq 0.05$ were considered to be significant.

Table 3.1 Primers used in quantitative real time PCR expression analysis

Target		Primer Sequences	Accession Number
TF	FWD	5'-CCA CCA TCT TTA TCA TCC TCC T-3'	NC_000069.5
	REV	5'-AGC CTT TCC TCT ATG CCA AGC-3'	
TFPI-1	FWD	5'-CCA GAG AAC CAC AGC ACC AC-3'	NC_000068.6
	REV	5'-CAA GGG CAA GAG GCA GAT-3'	
TM	FWD	5'-TAG GGA AGA CAC CAA GGA AGA G-3'	NC_000068.6
	REV	5'-GAG AGA GAGA GGA GAG GAG AGG-3'	
PAR-1	FWD	5'-TAC ATA ACA CCC CTT CGG CTA T-3'	NC_000079.5
	REV	5'-AAC ACA CCT TTCTCC TCT CGT C-3'	
PAR-2*	FWD	5'-CAC CTG GCA AGA AGC CTA AG-3'	NC_000079.5
	REV	5'-CCC AGG GTT ACT GAC GCT AA-3'	
EPCR†	FWD	5'-CAT CGG AGT TAC AAA GGG CG-3'	NC_000068.6
	REV	5'-CCC AGA ACT CCA GGA TGT TGA-3'	
PAI-1	FWD	5'-GGC ACA ACA CTT TCA TTC AGC-3'	NC_000071.5
	REV	5'-CGA CTT TTC TTA CAC CCT TTC C-3'	
18s	FWD	5'-CCA TCC AAT CGG TAG TAG CG-3'	NR_003278
	REV	5'-GTA ACC CGT TGA ACC CCA TT-3'	

*Li et al 2005; †Zhang et al. 2010

CHAPTER 4

RESULTS

Plasmodium chabaudi infection supports the upregulation of coagulation proteins

Previous studies in our mouse model of malaria during pregnancy showed that mice infected in early pregnancy fail to maintain viable embryos with conceptus failure beginning at experimental day (ED) 10 with complete pregnancy loss occurring before ED 12 (246). Inflammatory mediators of pathogenesis in this model have been well described, however, the contribution of coagulation proteins to the associated pathology have yet to be determined (221, 224, 246). To assess the role *P. chabuadi* infection has in the upregulation of coagulation proteins during pregnacy, pregnant B6 mice were infected with 10^3 *P. chabaudi* AS infected erythrocytes on ED 0 and allowed to progress until ED 10 when the mice were euthanized. Control UP mice were also euthanized at ED 10. Sacrifice at ED 10 was chosen due to previous evidence of high circulating proinflammatory cytokines and low anti inflammaotry IL-10 within the placenta at this time point as well as being the first day of fetal loss in this model (224, 246).

Both IP and UP mice were necropsied and both uterine horns removed. The fetoplacental units were quickly separated from the maternal uterine tissue and the conceptuses from each mouse pooled in RNAlater (Qiagen, Valencia, CA, USA). The pooled samples (UP n=5, IP n=6) were then homogenized, RNA isolated, cDNA generated, and expression analysis accomplished by quantitative PCR using coagulation specific primers as described in Materials and Methods. All of the investigated proteins demonstrated upregulation in IP mice relative to UP mice (Figure 4.1). The proteins thrombomodulin (TM), tissue factor (TF) and plasminogen activator inhibitor (PAI)-1 showed the most increase in fold expression, 3.9, 3.6, and 8.3, respectively. Both

protease activated receptors (PARs)-1 and -2 showed similar patterns of upregulation to each other and well above double that of UP mice (2.3 and 2.5, respectively). Furthermore, the thrombomodulin-thrombin complex controlled endothelial protein C receptor (EPCR) exhibited a three fold increase (3.0) over that of UP mice. The tissue factor protein inhibitor (TFPI)-1 message represented essentially no increase (1.2) with wide relative variability (SEM = 0.5).

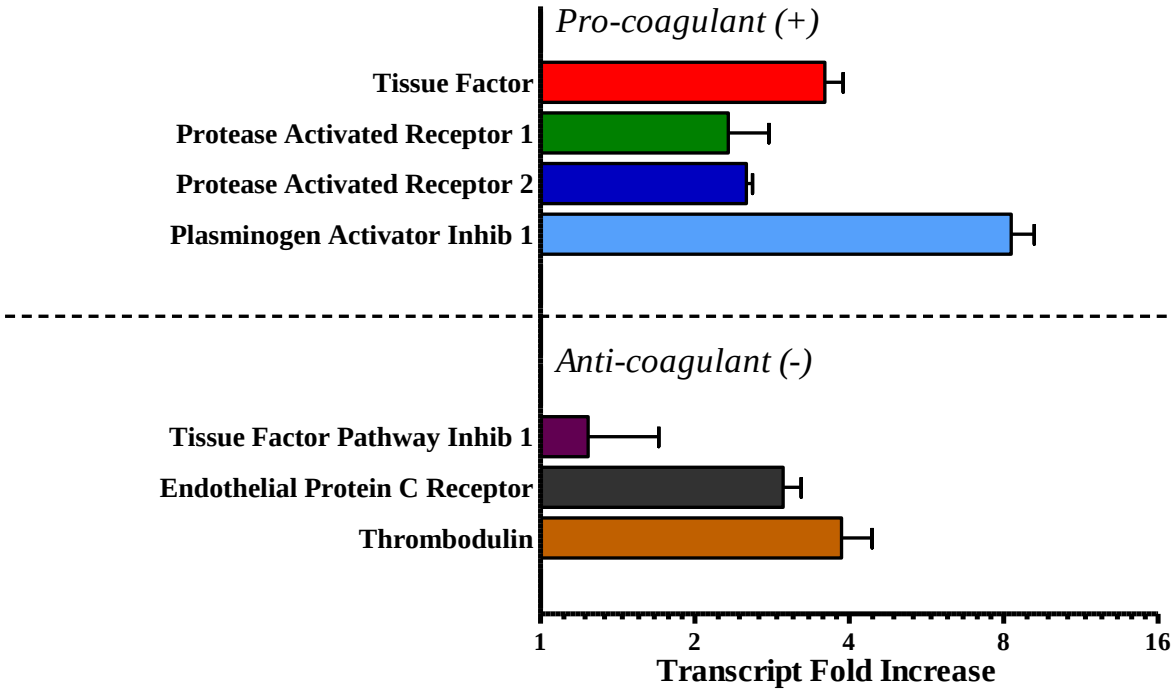

Figure 4.1 Coagulation protein gene expression analysis of infected pregnant B6 mice at ED 10. Feto-placental units were removed from the uteri of ED 10 UP (n =5) and IP (n=6) mice and homogenized. RNA was isolated to generate cDNA from message. Primers specific for TF, PAR-1&2, PAI-1, TFPI, EPCR, and TM normalized against murine 18S RNA were utilized to measure expression levels of pro/anticoagulants of IP mice relative to the expression levels of UP mice. Data are expressed as the ratio of fold increase in IP mice to that of UP mice ±SEM.

The data from pooled fetoplacental units of ED 10 mice demonstrated increased transcripts for key coagulation and coagulation-inflammation crosstalk proteins. However, these data did not reveal which cells might be responsible for the production of such proteins. To investigate this question, SM9-1 trophoblasts, an outbred mouse cell line, were stimulated *in vitro* with *P. chabaudi* infected or uninfected control RBCs and harvested over five time points

(0, 2, 4, 6 , and 8 hours). The message from three critical proteins was assayed: TF, the initiator of coagulation, TFPI, the principal inhibitor of coagulation initiation, and TM, the chief mediator of downstream coagulation inhibition.

Similar to the evidence from infected pregnant mice, SM9-1 trophoblast displayed the ability to support coagulation by marked increases of *TF* transcription (Figure 4.2), which steadily increased and was highest after 8 hours, displaying a mean fold increase of 2.54 (±0.10). *TM* transcripts were also increased at early time points but were supressed at 8hrs. While these data do not agree with those observed in the data collected from murine fetoplacental units, they do agree with TM expression levels observed upon human primary trophoblasts exposure to *P. falciparum* (Owino and Moore, unpublished data). SM9-1 trophoblasts aslo appear unable to mount a rapid response for control of coagulation at the gene expression level as *TFPI* transcripts, while slightly elevated, remained relatively unchanged, especially in light of the rapid increase in *TF* transcription.

Agressive LMWH therapy improves mid-gestational embryonic survival

While proteins involved in the coagulation cascade and their contribution to the pathology observed in sepsis are well described in the literature, the impact of parasite induced dysregulated coagulation has been relatively unexplored in the context of PM (274, 287-288, 302-305, 521, 533, 571, 584-586). Previously, our lab showed that anti-TNF-α neutralizing antibody treatment in infected pregnant B6 mice lead to a dimunition of placental TF and rescue of mid-gestational pregnancy in this model (221). Together with the data generated in ED 10 IP mice and the SM9-1 trophoblasts, it appears likely that coagulation initiation driven by TF occurs in PM in the context of this model. To determine the role coagulation plays in the pathology of this model we administered various anticoagulants to IP B6 mice and assessed the

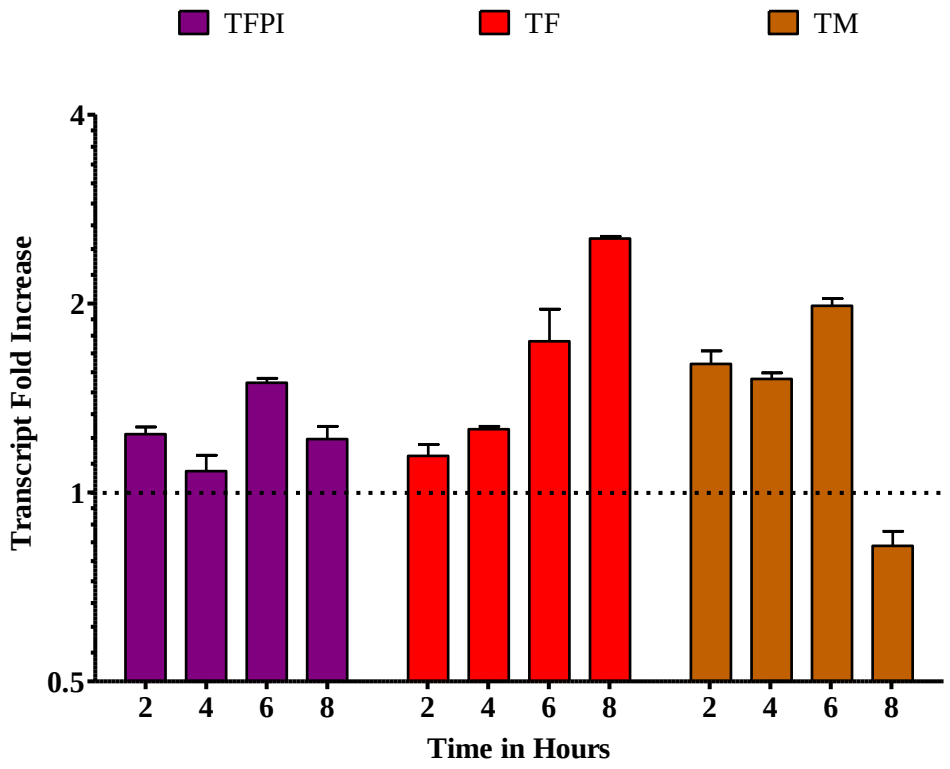

Figure 4.2 Murine trophoblasts support coagulation upon exposure to iRBCs. Time course expression analysis from *P. chabaudi* stimulated SM9-1 trophoblasts. RNA was isolated to generate cDNA from message. Primers specific for TF, TFPI, and TM were normalized against murine 18S RNA. Data are expressed as the ratio of fold increase relative to time matched SM9-1 trophoblasts stimulated with nRBC ±SEM and are representative of four separate experiments.

midgestational survival advantage among treated groups compared to non-treated IP B6 mice. The rationale for this approach was two fold: 1) several lines of evidence from prior research support the use of anticoagulants and their positive patient outcomes in models of sepsis; 2) anticoagulation therapy is safe during pregnancy and beneficial in some instances of recurrent abortion (587-606).

Initial experiments with low dose administrations of two anticoagulants, LMWH and Ixolaris, provided no mid-gestational survival advantage (Table 4.1). All treated mice succumbed to infection-induced pregnancy loss by ED 11 or 12. Initially, treatments were adminstered once daily (QD) but were then given once every 12 hours (Q12h). Initial low dose regimens were based on treatment in microvenous thrombosis in rat models and treatment of

spontaneous abortion in murine models (606-607). Ixolaris dosing was chosen based on *in vitro* evidence of activity from a collaborator, I.M.B. Francischetti, and tested for safety in UP mice prior to use (data not shown) (608-609). Although all mice from early treatment groups displayed 100% fetal loss, Ixolaris treatment resulted in lower histological scores for necrosis in placental layers and within the embryos compared to fetoplacental units from untreated IP mice (data not shown). These data suggested increased dosing regimens might yield improved pregnancy outcomes. However, based on the lack of commercially available Ixolaris and difficulties in obtaining purified protein, Ixolaris treatment was abandoned.

Table 4.1 Initial anticoagulant treatment regimens and outcomes

Treatment	**Administration**	**Day of Euthanasia**	**Viable Embryos/Total**
LMWH	70 IU/Kg s.c. QD ED 6-10	ED 12	0/28
LMWH	120 IU/Kg s.c. QD ED 6-11	ED 12	0/21
LMWH	120 IU/Kg s.c. QD ED 6-10	ED 11	0/26
LMWH	120 IU/Kg s.c. Q12h ED 6-11	ED 12	0/68
LMWH	220 IU/Kg s.c. Q12h ED 6-10	ED 11	0/24
Ixolaris	350 μg/Kg s.c. QD ED 8-11	ED 11	0/39

Aggressive therapy with LMWH was chosen based on clinical evidence and safety in pregnant humans (610). Mice were given 2000IU/Kg/day, (1000IU/kg/q12h) beginning ED 6-12. Before treatment in IP mice, this dosing regimen was tested for adverse affects in UP mice in spite of indications of safety within literature. Of 5 UP, LMWH treated mice no adverse effects were observed (data not shown).

To assess the health of treated mice during therapy, clinical metrics from UP, IP untreated, IP LMWH, and IP enoxaparin treated mice were compared from ED 0 and ED6-12 (Figure 4.3). As in all other mouse experiments, mice were infected after positive vaginal plug identification (considered ED 0). The mice were left undisturbed until ED 6, at which time treatment began. Treated mice exhibited the same infection kinetics as compared to untreated IP mice, indicating that survival advantages were not brought about by changes in parasitemia (Figure 4.3 A). Similarly, hematocrit values for the IP or IP treated groups did not differ during the course of infection (Figure 4.3B). Although the hematocrit of ED 10 LMWH treated mice appears to differ from that of the other IP groups, according to the nonparametric Kruskal-Wallis test and Dunn's multiple comparison post test, significant differences were only observed between the UP and the IP groups beginning ED 10 and after, but not between the IP groups. When weight change was examined, the treated groups followed the course of UP mice more so than that of their untreated IP counterparts . Untreated IP mice failed to maintain positive weight change after ED 6 and were the only group to fall below their starting weight at any point during the experiment. This weight loss was directly correlated with their inability to maintain viable pregnancies (Figure 4.4 A and B). LMWH treated IP mice demonstrated the most therapeutic effect in relation to positive weight gain. However, enoxaparin treated mice enjoyed a similar effect although they were unable to maintain viable pregnancies to the level of LMWH treated mice as judged by positive pregnancy outcomes as described in the Materials and Methods (Figure 4.4 A and B). Mean viable embryos did not differ between the enoxaparin treated IP mice and untreated IP mice (1 vs. 0.14, respectively). It follows, that the enoxaparin treated IP mice differed significantly from the UP group in terms of their number of viable embryos ($p < 0.0001$). In contrast, LWMH IP treated mice experienced a significant increase in the number of

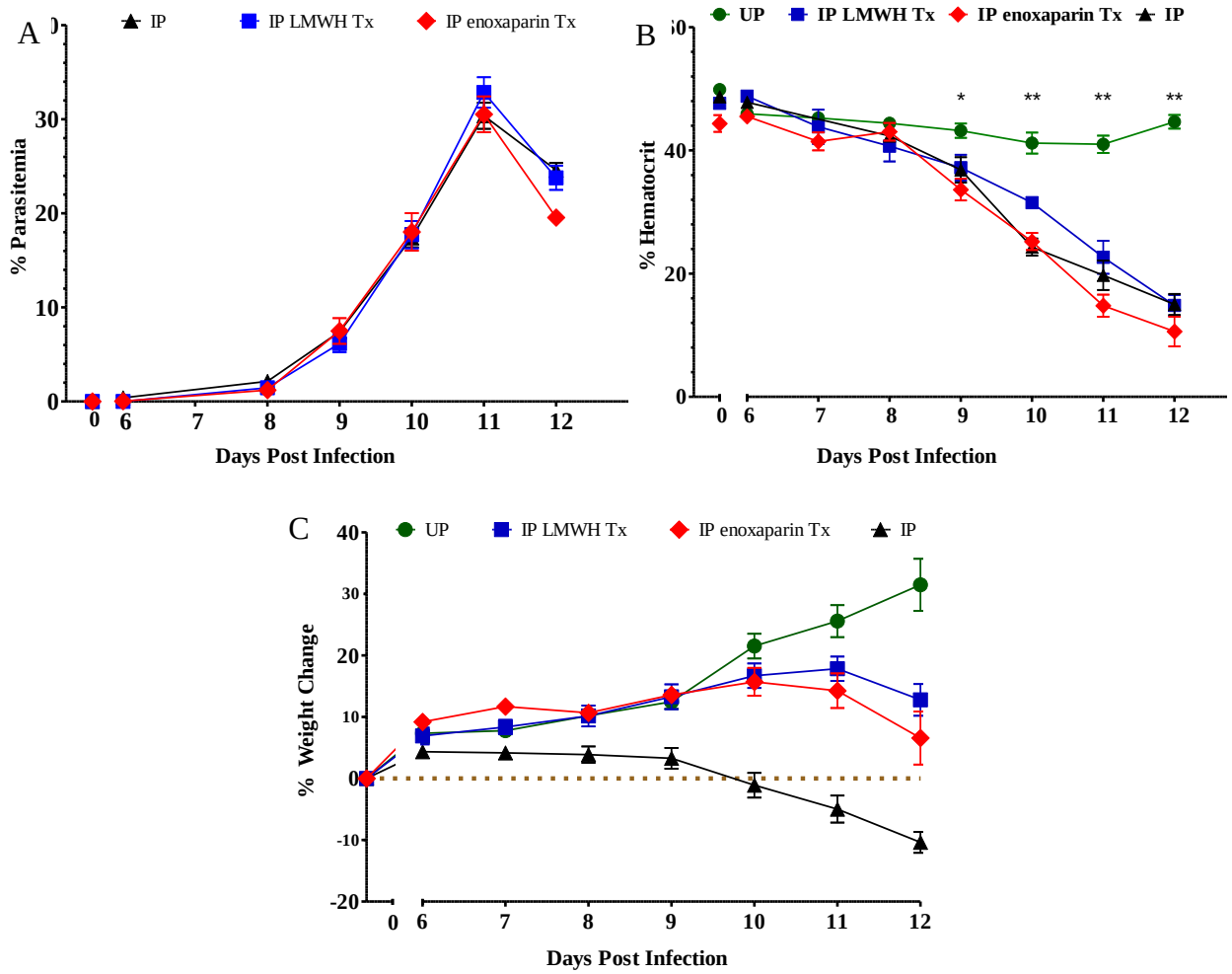

Figure 4.3 LMWH or enoxaparin therapy improve mid-gestational weight. Clinical metrics during the course of *P. chabaudi* AS infection in pregnant B6 mice. Percent parasitemia *(A)*, hematocrit *(B)* and change in body weight *(C)* of uninfected (n=19), infected untreated (n=14), infected low molecular weight heparin treated 1000IU/Kg/q12h (n=11), and infected enoxaparin 1000IU/Kg/q12h (n=5) treated mice are shown. Clinical metrics were taken on ED 0, 6-12 after which mice were euthanized. Data represent mean ± SEM; *, p< 0.0033; **, p <0.0001.

viable mid-gestational embryos over that of their untreated IP counterparts ($p < 0.0001$). The difference in viable embryos was not significantly different from the UP mice. Enumerating only the viable embryos is necessary but not sufficient to determine treatment success, however. As a result, Figure 4.4 B illustrates the number of viable embryos as a proportion of the total number of embryos for each group. In this case both the IP treated groups differed significantly from IP untreated mice with 61.3% of LMWH treated embryos surviving and 14.3% of

enoxaparin embryos surviving vs. 2.9% of the untreated IP group (LMWH $p < 0.0001$, enoxaparin $p = 0.0270$). However, neither treatment group afforded a return to the viability rate of the UP mice.

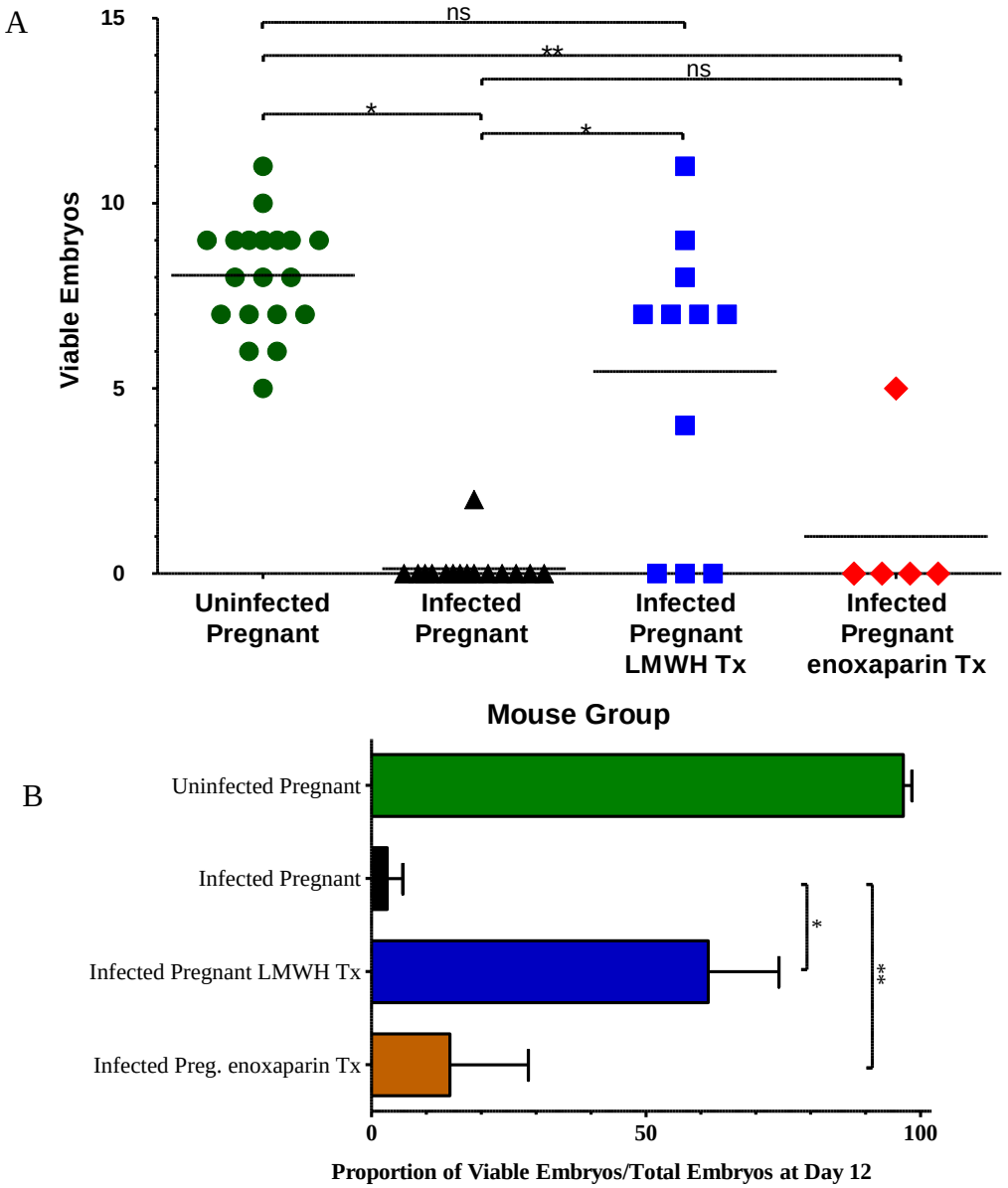

Figure 4.4 Comparison of mid-gestational embryonic survival. Comparisons were made between uninfected (n=18), infected untreated (n=15), infected low molecular weight heparin treated (n=11), and infected enoxaparin (n=5) treated mice after euthanasia on ED 12. Viable ED 12 embryos were compared via Kruskal-Wallis analysis utilizing Dunn's Multiple Comparison Test, means are displayed. *, $p < 0.0001$, **, $p = 0.0008$ *(A)*. Viable embryos as a proportion of total embryos were enumerated for each group and significance of treatment vs. non-treatment were analyzed via two tailed Fisher's exact test ($\alpha < 0.05$), *, $p < 0.0001$ **, $p = 0.0270$. Mean ± SEM are shown *(B)*.

Differences in mid-gestational embryonic survival were made clear during gross pathological and histological examinations (Figure 4.5). Viability assessments were made upon examination of the live pregnant female mice. Mice were first scored for the presence of vaginal bleeding and/or mucoidal vaginal discharge indicating active abortion. Mice positive for vaginal bleeding muccoidal vaginal discharge were automatically given a viability of zero regardless of the state of the embryos upon necropsy. If live females did not show signs of active abortion, they were further scored during necropsy. Females with embryos being expelled or open cervixes were considered to be actively aborting and given a viability of zero regardless of the state of the embryos (Figure 4.5 D black arrow). Only embryos of females that did not demonstrate active abortion were assessed for viability. Resorbing embryos were scored as passive abortion as were embryos exhibiting intra-embryonic hemorrhage (Figure 4.5 D purple arrow). Vascularization of the uterine arteries and veins were scored as poor, good, and extensive, but were not used as a criterion for viability, although causally, all actively aborting females and those females with large numbers of resorbing and/or hemorrhagic embryos had poor vascularization. After gross pathological examination, embryos were fixed and examined histologically to confirm viability (Figure 4.5 D-I).

Viable embryos from UP mice at ED 12 showed no hemorrhage, no active expulsion and only 5 resorptions out of the 146 embryos examined (Figure 4.4 B and Figure 4.5 A). It is important to note that a nominal number of normal resorption events may occur with any B6 mouse pregnancy. Furthermore, histological examination demonstrated that the three principal layers of the placenta, the decidua (d), junctional zone (j), and labyrinth (l) exhibited normal and intact architecture with no evidence of necrosis of the embryo (e) (Figure 4.5 D and G). In contrast, untreated IP mice demonstrated massive intra-uterine hemorrhage, a large number of

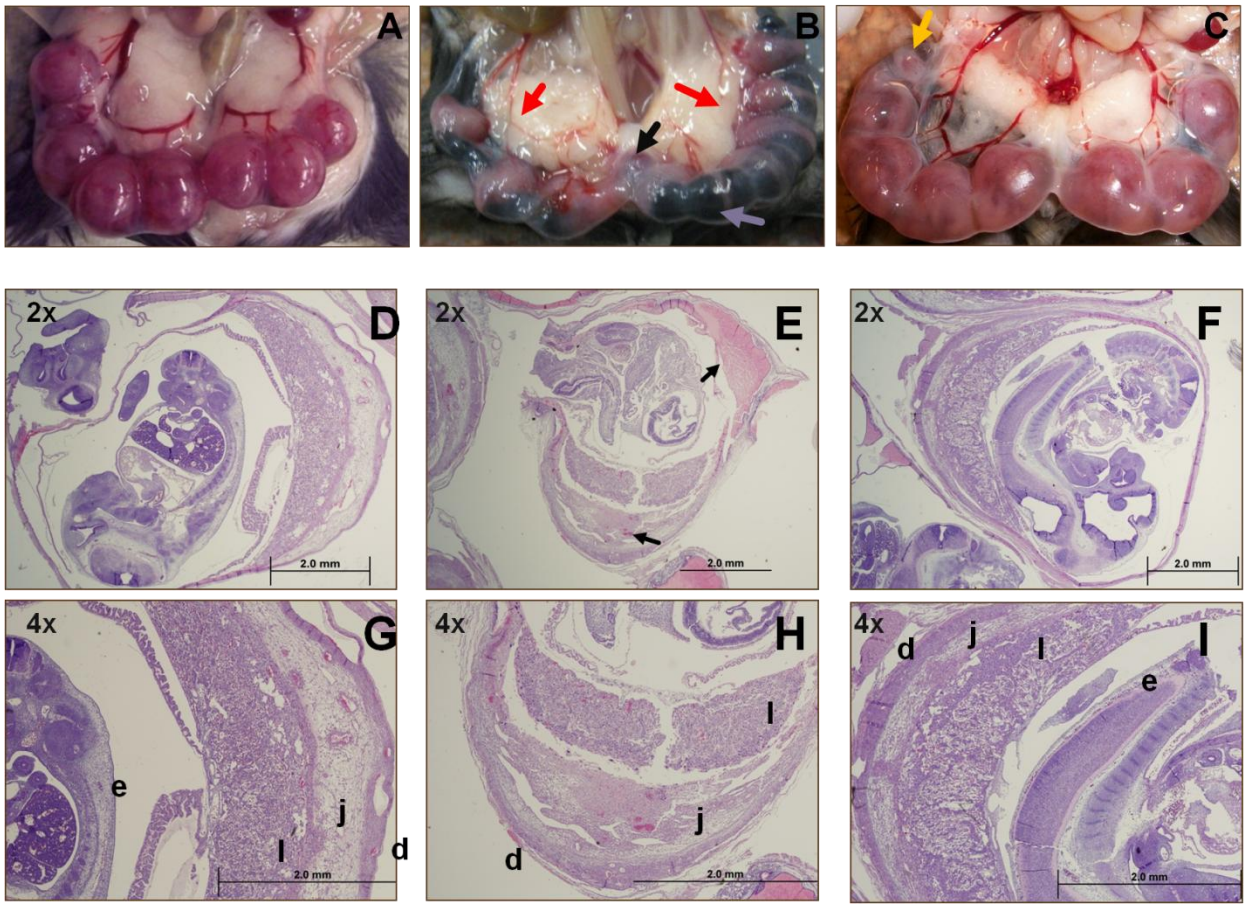

Figure 4.5 Gross pathological results from ED 12 uninfected pregnant *(A)*, infected pregnant *(B)*, and infected pregnant low molecular weight heparin treated *(C)* mice. Active embryonic expulsion (*B*, black arrow), diminished vascularization (*B*, red arrow), intra-uterine hemorrhage (*B*, purple arrow) and resorption (*C*, yellow arrow). Histological comparison of ED 12 uninfected pregnant (*D, G*), infected pregnant (*E, H*), and infected pregnant low molecular weight heparin treated (*F, I*) mice. Black arrows indicate fibrin deposition (*E*); enlargements including the 3 principle regions of the murine placenta (*G-I*): decidua (d), junctional zone (j), labyrinth (l), and the embryo (e) are shown.

resorptions, active expulsion and a loss of placental architecture (Figure 4.5 B, E, and H). Importantly, untreated IP embryos had considerable necrosis and large deposits of fibrin (Figure 4.5 E arrow) within the fetoplacental unit. These features were absent in IP LMWH treated mice. IP LMWH- treated mice showed similar gross pathological features to that of UP mice. However, few intra-embryonic hemorrhages were observed and resorption events were drastically reduced. Moreover, histological examination revealed a preservation of placental architecture and embryonic stability (Figure 4.5 C, F, and I).

An observed consequence of treatment with LWMH or enoxaparin was a diminution in the occurrence of hemolysis-induced renal crisis experienced by infected pregnant mice (Figure 4.6). Although the proportion of treated mice appears similar, only the LMWH group had a sufficient number of mice to be significant in comparison to untreated IP mice ($p = 0.0089$).

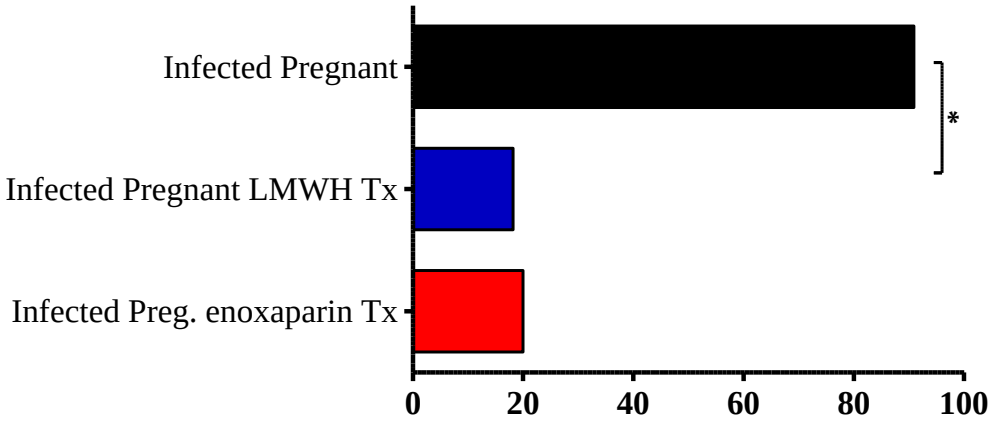

Figure 4.6 Comparison of infected pregnant mice experiencing renal crisis. Data represent proportion of mice without crisis at anytime during pregnancy. IP n = 11, IP LMWH n = 11, IP enoxaparin n = 5. Significance was determined using two tailed Fisher's Exact test ($\alpha < 0.05$), *, $p = 0.0089$.

Although unexpected, these data are consistent with other reports of LMWH therapy leading to amelioration of renal impairment in other clinical instances of severe hemolysis (611-613).

CHAPTER 5

DISCUSSION

Malaria infection during pregnancy in nonimmune or individuals from low transmission areas is associated with poor birth outcomes such as preterm labor, abortion and stillbirth, with the more severe outcomes occurring as a result of infection during the first or second trimesters (199, 614). To date, the consensus among immunologists and pathologists alike is that the pathogenesis associated with malaria infection is attributed to immunopathological mechanisms such as high cellular infiltrate of immune effector cells, excessive proinflammatory cytokine release, and sequestration of parasites within microvascular beds. This viewpoint, while not incorrect, may be insufficient to fully describe the pathology associated with PM. This reductionist perspective fails to consider the impact of homeostatic systems overlap and crosstalk in both CM and PM, such as that occurs between inflammation and coagulation. For instance, infection with *P. falciparum* in naive or nonimmune individuals is associated with systemic endothelial activation, irregular damage of the endothelium, thrombocytopenia, depressed or impaired levels of endogenous anticoagulants and antifibrinolytics, significant production and exposure of TF through various cells types, and high plasma levels of thrombin and procoagulant microparticles such as those derived from activated platelets (143-144, 473, 615-619). These data strongly indicate a dysregulated hemostatic system which is polarized towards a hypercoagulable state. While frank thrombi and hemorrhage are not well described in adult infections, children with CM present with hemorrhages that are seen in the microvessels of the retina (620). These hemorrhages have been shown to have significant prognostic value in

disease severity as the presence of hemorrhagic retinopathy correlates with multiple microthrombi and hemorrhages in the microvessels of the brain in children who have died as a consequence of severe disease (475, 620). Similarly, evidence for the involvement of a inflammatory linked dysregulated coagulation state in PM has been demonstrated in several studies. Poor birth outcomes during PM in humans is associated with massive monocyte infiltrate within the placental intervillous space (220). Imamura et al. demonstrated that TF was highly expressed on the monocytic infiltrate in *P. falciparum* infected placentas (217). The authors also found significant perivillous fibrin deposition associated with the high TF expressing monocytes (217). Excessive perivillous fibrin formation during PM has been found to be strongly correlated to pre-term delivery (219). Excessive fibrin deposition and frank thrombi are not always observed in PM and this has been a major obstacle for the acceptance of coagulopathy as a contributor in the poor birth outcomes of PM. Part of the reason for this discrepancy may lie in the fact that fibrin deposition during pregnancy is somewhat normal. Normal pregnancy is associated with alterations in both the coagulation and fibrinolytic systems, tending the overall hemostatic balance to shift towards a hypercoagulable state. These physiological procoagulant changes are suspected to minimize intrapartum blood loss, but they simultaneously increase the risk of thrombus formation, excessive fibrin and fibrin degradative products, as well as other complications such as preeclampsia during pregnancy (621). Therefore, pathologists may fail to make the distinction between normal and excessive fibrin deposition and the observation may go underappreciated. Detractors have failed to realize that hypercoagulation can be compensated even with impaired fibrinolysis, sometimes leading to an exhaustion of both clotting and fibrinolytic factors (DIC). However, Isermann et al. have shown that fibrin degradation products, not fibrin itself, damage and lead to trophoblast cell death both

in vivo and *in vitro* (354). Therefore, even if fibrin levels appear normal, compensated fibrinolysis may have contributed to poor fetal outcomes. Additionally, our previous mouse data provided evidence of hemorrhage and fibrin deposition within maternal placental blood sinusoids and revealed significant TF expression in the trophoblasts surrounding these sinusoids (221). TNF-α is known to upregulate TF expression and our lab has demonstrated, in a mouse model of PM, that ablation of TNF-α via treatment with neutralizing antibody is sufficient to reduce placental TF expression and rescue mid-gestational pregnancy (221, 622-624). Similar to the association of excessive fibrin deposition in poor birth outcomes during malaria in pregnancy, high placental TNF-α expression has been strongly correlated to pre-term delivery in placental malaria (222, 458). The convergence of these data point not to a singular cause of pathology, but to an interplay between dysregulated homeostatic mechanisms that serve to reciprocally agonize one another. Thus determining the contribution of coagulation in placental malaria is critical to understanding disease pathogenesis and developing appropriate treatment strategies.

To directly assess the importance of coagulation in PM-induced fetal compromise, we utilized anticoagulant therapy in our established model of PM. This model is characterized by intra-uterine hemorrhage, fetal resorption, and abortion during mid-gestation in naive B6 mice infected with *P. chabaudi* AS parasites (246). Because models are just approximate analogs of systems of interest, we first established that upon infection with malarial parasites, pregnant B6 mice do indeed provide the necessary environment for establishing a procoagulant or hypercoagulant state. In this model infected pregnant mice experience pregnancy failure beginning ED10 with complete pregnancy loss by the end of ED 11. We measured transcript levels of several coagulation or inflammation-coagulation crosstalk proteins at ED 10 to assess these levels at the onset of embryonic loss. Consistent with expectations, these proteins were

upregulated relative to time matched uninfected pregnant B6 mice and all but TM were upregulated in iRBC stimulated SM9-1 trophoblasts.

The importance of increased TF expression has already been discussed, however, the remaining proteins also play critical roles in coagulation and possibly in malaria pathogenesis. Protease activated receptors (PARs)-1 and -2 are G-protein coupled receptors (GPCRs) that play a pivotal role in bridging inflammation and coagulation (336, 625). PARs are signaled through their own tethered ligand that must be proteolytic cleaved by serine proteases such as the TF-FVIIa complex, FXa, thrombin, or even activated protein C (336, 353). PAR-1 can be activated by thrombin in response to TF-induced coagulation initiation. Thrombin initiated PAR-1 signaling results in activating endothelial cells to express adhesion molecules promoting the rolling of platelets and leukocytes, initiating platelet activation, inducing the expression of IL-1 and IL-8, TNF-α, and supporting proapoptotic signaling (288, 336, 626). PAR-2 activation is brought about by proteolytic cleavage by the TF-FVIIa complex with similar results including upregulation of IL-6 and IL-8, and TNF-α (288, 336, 626). PAR-2 signaling may serve to amplify its own expression; PAR-2 expression is increased upon exposure to proinflammatory cytokines such as TNF-α, TGF, and IL-1β (288). The data presented here demonstrate, at least at the transcript level, that PARs-1,2 message is significantly increased in IP mice vs. that of time matched UP mice. The upregulation of coagulation factors results in over expression of PARs, which may lead to a proinflammatory/procoagulation amplification feedback loop which could exacerbate pathology in PM.

Fibrinolytic mechanisms are also influenced by inflammatory signals. Proinflammatory cytokines such TNF-α, IL-,1 and IL-6 are potent stimulators of PAI-1 expression (627). Because proinflammatory cytokines are blamed for much of the pathogenesis in malaria infection, it is no

surprise that ED10 mice were found to over express PAI-1 transcripts. However, the effects of increased PAI-1 expression have become evident over the last several years in many disease states that occur exclusive of pregnancy but may also complicate pregnancies including thrombophilia, atherosclerosis, septicemia, and metabolic syndrome (627). Normal placental development depends on proper trophoblast invasion, vascular remodeling, and maintenance of intervillous blood flow (628). All of these processes entail tightly regulated degradation of the extracellular matrix by proteolytic enzymes (629). The fibrinolytic enzyme, plasmin, is chief among these enzyme critical for remodeling during pregnancy. As mentioned earlier, plasmin is the proteolytic cleavage product of plasminogen by tPA or uPA proteases that are controlled by PAI-1. If PAI-1 levels are too high and inactivation of uPA or tPA suppressed, then trophoblast invasion is impaired (629-630). Increased PAI-1 levels have also been implicated in preeclampsia, a condition marked by abnormal vascular response to placentation associated with increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation system, and endothelial-cell dysfunction (631). Preeclampsia is a leading cause of fetomaternal morbidity and mortality in both the developed and developing worlds (631). Interestingly, the pathology of preeclampsia is considered to be extremely similar to that of PM, and in some clinical studies, the severity of PM was increased in conjunction with preeclampsia (629, 631-633). The high level of PAI-1 transcripts observed in ED10 IP mice may result in PAI-1 over expression and subsequent significant depression of fibrinolysis.

Importantly, the similarities between PM and preeclampsia may also be able to resolve the apparent dichotomy between the TM expression data generated in the fetoplacental units of infected mice and the SM9-1 trophoblasts. In severe cases of preeclampsia, TM expression was diminished in the endothelium of the stem villi arteries and increased in the perivascular and

stromal myofibroblasts (634). The stem villi arteries represent the central branches of the villous trees. Stem villi are characterized by a condensed fibrous stroma in which the fetal arteries, veins, arterioles, and venules are rooted (635). Functionally they act as the mechanical support structures of the villous trees, and they control fetal blood flow into the maternofetal exchange area of the terminal villi (635). The perivascular and stromal myofibroblasts are components of an extravascular contractile sheath surrounding the stem villi that help maintain vessel tone and blood flow between the placenta and fetus and also mediate crosstalk between the villi and the extracellular matrix (636). Evidence presented by Bosco et al. suggests that in severe preeclampsia, decreased TM expression and a concurrent inability to control local thrombosis leads to a loss of structural maintenance and repair of these developing structures, which results in a loss of fluidity and diminished placental blood flow (634). Because our mouse transcript data include all of the structures within the placenta and not just trophoblasts, as in the SM9-1 data, portions of the placenta may express higher levels of certain transcripts and possibly proteins than the trophoblasts. We may be able to determine precisely which cells are responsible for these transcripts by various methods such as *in situ* hybridization or immunohistochemistry. The disparity in transcript production may also explain the loss in vascular perfusion seen in untreated IP mice, although murine placental structures are somewhat different than human structures at this level. However, coagulation-driven fatal arrest of placental morphogenesis has been shown to occur in murine placentas whose trophoblasts lack TM (637-638). Trophoblast expression of TM has also been shown to be diminished in recurrent abortion in human clinical samples (637). The implication for malarial induced dysregulated coagulation is further supported by an observed diminution of TM expression in cultured human trophoblasts upon exposure to *P. falciparum* iRBCs (Owino and Moore unpublished).

If malaria infection leads to dysregulated coagulation, how does this occur and can it be therapeutically managed? The observation of complications such as DIC in sepsis have been described for nearly fifty years; however, the scientific and medical communities alike have only confirmed that coagulation and inflammation are intimately linked and imbalanced in states of endotoxemia and sepsis in the last two decades. Anticoagulant therapeutic strategies in sepsis have had mixed successes and failures. Meta analyses show that the majority of promising strategies come from therapeutics which target FXa, thrombin, or activated protein C (APC) (639-640). In the current study we utilized two low molecular weight heparins: a heparin derivative for research purposes only, designated LMWH, and a commercially available product, enoxaparin, FDA approved for use in humans. Low molecular weight heparins are derivatives of unfractionated heparin (UH). UH is a heterogeneous mixture of highly sulfated polysaccharide chains ranging in MW from 3000 to 30,000 Daltons; depolymerization of UH releases low molecular weight fractions with a typical MW of 5000 Daltons. UH exerts the majority of its pharmacological effect on thrombin by stabilizing the thrombin antithrombin complex (TAT) , with a relatively small inhibitory effect on FXa. Low molecular weight heparins, in contrast, exert their activity against FXa and have low anti-thrombin activities. Low molecular weight heparins have received greater attention in therapeutic implications due to their more predictable anticoagulant response vs. UH, they have increased bioavailability, longer half-life, and dose independent clearance (641). Moreover, bleeding complications and thrombocytopenia are rarely encountered with low molecular weight heparin treatment, whereas UH therapy requires clinical monitoring to decrease the chances for bleeding complications (642). As previously mentioned, low molecular weight heparins are also the preferred anticoagulants during

coagulopathic complications in pregnancy. As a result, we investigated the ability of low molecular weight heparin therapy to improve pregnancy outcomes in our mouse model of PM.

Using 2000IU/Kg/day (1000IU/Kg/q12h) by subcutaneous administration of LMWH or enoxaparin our data demonstrate that LMWH was successful in enhancing mid-gestational embryonic viability in IP B6 mice by 61.3% (Figure 4.5 B) over that of non-treated IP control. In contrast, enoxaparin was successful in enhancing mid-gestational embryonic viability in IP B6 mice by only 14.3% (Figure 4.5 B). This disparity in pregnancy success may be attributable to the method of preparation of each compound. Low molecular weight heparins are generated through up to six different depolymerization reactions of UH (643). Enoxaparin, for example, is generated by benzylation followed by alkaline hydrolysis, whereas the research grade LMWH used in this study was manufactured by oxidative depolymerization with Cu^{2+} and hydrogen peroxide. The same method used to create the low molecular weight heparin parnaparin (644-645). As such, low molecular weight heparins cannot be used interchangeably, even when they have similar anti-FXa activities (enoxaparin anti-FXa activity = 100IU/mg; LMWH anti-FXa activity = 156 IU/mg). Because different manufacturing processes yield different structural fractions, the specificities are quite distinct for each low molecular weight heparin. Thus, low molecular weight heparins are a group of similar but different drug agents (643). In comparing enoxaparin and parnaparin (analog to the LMWH used in this experiment), enoxaparin has an anti-Xa/anti-thrombin ration of 2.7:1, whereas parnaparin has a 3:1 ratio; however, enoxaparin has a slightly longer half life of 4.5 hours vs. 4 hours to that of parnaparin (645).

We did observe a similar weight change pattern in IP mice between the two drug administrations. Additionally, and similar to the observations using Ixolaris, there were very few intra-uterine hemorrhages and fewer resorption events observed in the enoxaparin grouprelative

to untreated IP mice (data not shown). Taken together with the weight change data, it may be necessary to reexamine the strict criteria for viability and pregnancy loss that we employed in our analyses. It may be the case that while one embryo is aborted, not all embryos are compromised. It also may be the case that increasing doses of enoxaparin would result in increased mid-gestational survival; however, 2000IU/Kg/day is the recommended maximum for enoxaparin dosing in pregnant women (Sanofi-Aventis prescribing information, (610)).

Unexpectedly, we observed a significant difference in the occurrence of renal crisis in the mice treated with LMWH. Only 18% of the IP LMWH treated mice demonstrated hemolysis-induced renal crisis vs. 92% of IP untreated mice. The reasons for this phenomenon are not yet known. We speculate that the decreased load on glomerular filtration in the LMWH treat mice due to diminution of coagulation amplification and concomitant lack of fibrinolysis breakdown products preserves renal function even in the face of severe hemolysis. Histological examination of the kidneys and measurement of hemoglobin and levels of fibrin degradation products in the urine should provide us with a better clinical picture as to why treated IP mice do not develop renal impairment as readily as untreated mice.

It should be noted that anticoagulation therapy is not a new development in malaria therapies. Early studies attempted to control severe malaria using UH with mixed results. In two out of three rhesus macaque experiments, heparin treatment did not change the morbidity associated with *Plasmodium* infection (646-648). However, data in children with CM treated with heparin in two separate studies in Indonesia have demonstrated that treatment provided a reduction in morbidity and subsequent lowering of mortality (649-650). From the literature it appears that heparin's lack of favor in the treatment of severe malaria and therefore a lack of research into anticoagulant therapies in malaria stems from two sources. First, there is a risk of

severe bleeding associated with heparin use, a few cases of which were considered to be the cause of death in treated patients (477, 484, 651). Second, a report from the lead author of one of the simian studies twice decreed in the journal Lancet that heparin should not be used in malaria therapy, a statement that was reiterated in the British Medical Journal in 1976 (646, 652-653). It should also be noted that low molecular weight heparins, unlike UH, are second generation anticoagulants that provide more predictable clinical parameters. We propose, based on our data and the possibility of the use of a new generation of oral low molecular weight heparins, that anticoagulant therapy in malaria should be reexamined, especially in regards to PM.

Parasite sequestration and inflammatory cytokines may lie at the heart of coagulation induced pathology associated with malaria infection particularly as it relates to severe disorders such as CM and PM. The literature focused on sequestration induced occlusions mainly spotlight complications seen in the brain and placenta (144, 357, 654). However, sequestration does not occur in the absence of robust elaboration of proinflammatory cytokines (357). Proinflammatory cytokines such as TNF-α and IL-1 are known to activate the endothelium causing it to increase expression of adhesion molecules as well as to increase expression and exposure of TF. Similarly, these proinflammatory cytokines also induce TF expression and exposure on monocytes (345, 531, 539, 544, 655-657). The elaboration of proinflammatory cytokines can drive TF-dependent thrombin generation (658).

Hemostatic regulation must be robust in the instance of hyper proinflammatory /procoagulant environments. Thus, the importance of TM expression and activity cannot be underestimated as it relates to malarial complications. It seems more than just coincidence that sites of sequestration, hemorrhage, and monocyte infiltration that lead to severe complication in

malaria are the two sites with the lowest expression of TM, the brain and placenta.(357, 372). The thrombomodulin-EPCR-protein C pathway also plays a critical role in dampening inflammation and combating pro-apoptotic signals through thrombin-PAR1 signaling in placental tissues and the brain vasculature (349). When TM is activated by thrombin it complexes with EPCR to activate protein C; in addition to APC's role in limiting thrombin formation, APC bound to EPCR is able to cleave PAR1, but in contrast to cleavage of PAR1 by thrombin, APC cleavage leads to pro-survival signals suggested to promote growth, differentiation, and proliferation of trophoblast stem cells (349, 353, 659). This TM-APC pathway may be able to overcome signal from PAR-2 activation by the TF-FVIIa complex, which decreases proliferation and is proapoptotic (349). In light of the varied and powerful functions of TM, as well as its relatively weak expression in the placenta, any diminution in expression or activity could be extremely deleterious. The data reported here suggest that TM is down regulated at the trophoblast level in outbred mouse trophoblast cells upon stimulation *with P. chabaudi* iRBCs. Thus, it may be that an inability to properly control coagulation and inflammation in the microenvironment of the feto-maternal interface contributes to PM observed pathologies.

The paucity of TFPI transcripts reported here, even in response to increased TF message, may result in an early initiation system that is unable to be controlled, which together with PAI-1 increases at the end of the system results in massive coagulation and fibrinolytic dysregulation. Although EPCR transcripts are upregulated in ED10 IP mice, EPCR itself does not activate protein C, merely it serves as a receptor to hold APC long enough to initiate PAR signaling and activate protein S, another protein involved in regulating thrombin generation. Even if EPCR protein expression were increased, down regulated trophoblast TM transcription and possible

iRBC inhibition of TM-thrombin interaction may result in low levels of APC, rendering EPCR useless.

The data reported here directly implicate the coagulation cascade and its proteins in the pathogenesis of this mouse model of PM. Infection with *P. chabaudi* AS induces a proinflammatory state in B6 mice that may support the generation of a hypercoagulable condition relative to uninfected pregnant mice as measured by increased coagulation and anti-fibrinolytic transcript. LMWH treatment of mice infected with *P. chabaudi* AS enhanced mid-gestational embryonic survival relative to untreated IP mice. Previous data from our lab also support the link between inflammation and coagulation leading to pathogenesis, as TF expression was drastically diminished upon TNF-α neutralization (221). Whether direct thrombus generation, signaling from activated coagulation proteins, or coagulation degradation products can be singled out for pathology culpability is not yet known. However, these data suggest that research into anticoagulants as possible therapeutics for PM is warranted. Considering the similarities between coagulation proteins in the vasculature of the brain and placenta, many of the lessons we glean from one organ may be applicable to help combat malarial complications in the other. In conclusion, these data coupled to what has been demonstrated with the interplay between coagulation and inflammation in models of sepsis and CM suggest that coagulation at least plays an accompanying role in the pathology associated with severe malaria and one of its conditions, PM. It remains to be determined which comes first, immunological induction of hypercoagulation or vice versa. The exact molecular mechanism(s) need to be determined in order for viable therapeutic targets to be elucidated.

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