INVESTIGATING THE EXTRINSIC PATHWAY OF COAGULATION AS A THERAPEUTIC TARGET FOR SEVERE MALARIA

by

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(Under the Direction of Julie M. Moore)

ABSTRACT

Malaria poses a serious threat to global health, with 3.2 billion people in 95 countries at risk of infection. Pregnant women and young children in malaria-endemic areas bear the heaviest burden of malaria infection due to two severe clinical manifestations of malaria, placental (PM) and cerebral malaria (CM) resulting from sequestration of infected erythrocytes in the maternal blood space of the placenta or the brain. The use of adjunctive therapies to specifically target pathogenic processes known to occur during PM and CM hold great promise to prevent the more serious outcomes of malaria infection. However, to date, therapies targeting the best-understood contributor to malaria pathogenesis — inflammation — have so far been unsuccessful. Previous experiments in our lab and evidence in the clinical literature have shown that a potently procoagulant state exists during PM and CM, and that suppression of that state with a research-grade low molecular weight heparin could mitigate mid-gestational pregnancy loss during murine placental malaria infection. These studies indicated activated coagulation was an important contributor to malaria pathogenesis, but which components of the coagulation cascade were relevant to the observed pathologies remained unclear. This dissertation, therefore, sought to build upon that work by identifying members of the extrinsic pathway of coagulation contributing to malaria

pathogenesis and by assessing the potential of anticoagulant therapy to mitigate severe clinical outcomes of malaria. We began using Cre-lox technology to induce tissue-specific deletions of Tissue Factor, the initiator of the extrinsic pathway of coagulation, to identify tissue types contributing to this damaging procoagulant state. We then tested two anticoagulants currently approved for clinical use in humans — the low molecular weight heparin, Dalteparin, and the coagulation FXa inhibitor, Fondaparinux — to assess how blocking the coagulation cascade affects disease progression and outcome. This dissertation also includes an assessment of naturally acquired *Helicobacter* infection in laboratory mouse colonies and how such infections may interfere with mouse models of disease in pregnancy. The results here presented provide compelling evidence that anticoagulant drugs have the potential to serve as useful adjunctive therapies for the treatment of both placental and cerebral malaria.

INDEX WORDS: *Plasmodium falciparum*, placental malaria, cerebral malaria, coagulation, anticoagulant therapy, adjunctive therapy, Tissue Factor, Thrombin, coagulation Factor X, extrinsic pathway of coagulation

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DEDICATION

To my family, who insisted I sleep from time to time: Mom and Dad, Liam, Alana, Archibald, Mayzie, all my aunts and uncles and cousins, Father Peter, Sean, Garrett, Jenny, Megan, Brendan, Liam, Tara and Alana, too.

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TABLE OF CONTENTS

Page

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER

1 INTRODUCTION 1

Specific Aims 5

2 LITERATURE REVIEW 6

Placental Malaria Characterization and Epidemiology 9

Inflammatory Damage in Placental Malaria 11

Inflammation in Murine Models of Placental Malaria 13

Cerebral Malaria Characterization and Epidemiology 20

Parasite Sequestration in Cerebral Malaria 21

Histopathologic Characteristics of Cerebral Malaria 23

Inflammatory Responses to Cerebral Malaria 24

The *Plasmodium berghei* ANKA Model of Cerebral Malaria 27

An Overview of Hemostatic Mechanisms and Drugs 29

The Intersection of Inflammation, Coagulation and Disease 35

Activated Coagulation in Malaria 38

Summary 41

References 41

3 CELL-SPECIFIC DELETION OF TISSUE FACTOR AND TARGETED ANTICOAGULANT THERAPY ALTER THE IMPACT OF *PLASMODIUM CHABAUDI* AS INFECTION ON MURINE PREGNANCY OUTCOME....63

Abstract....64

Introduction....65

Materials and Methods....66

Results....69

Discussion....76

Figures....80

Tables....96

References....98

4 TISSUE FACTOR DEFICIENCY AND ANTICOAGULANT THERAPY REDUCE BLOOD BRAIN BARRIER PERMEABILITY AND SUSCEPTIBILITY TO NEUROLOGICAL SYMPTOMS IN EXPERIMENTAL CEREBRAL MALARIA 101

Abstract....102

Introduction....104

Materials and Methods....106

Results 109

Discussion 113

Figures 116

Tables 123

References 126

5 *HELICOBACTER* SPECIES SIGNIFICANTLY IMPACT PREGNANCY SUCCESS IN LABORATORY MICE....130

Abstract....131

Introduction....132

Materials and Methods....133

Case Report....138

Discussion....141

Acknowledgements....145

Figures....146

Tables....150

References....151

6 CONCLUSIONS AND FUTURE DIRECTIONS....162

APPENDICES

A A PROPOSED MODEL FOR CEREBRAL MALARIA-INDUCED BLOOD BRAIN BARRIER DISRUPTION....165

LIST OF TABLES

Page

Table 3.1: Pregnancy outcomes of $TF^{flox/flox}$,Tie2Cre mice 96

Table 3.2: Pregnancy outcomes of $TF^{flox/flox}$,LysMCre mice 97

Table 4.1: Cerebral malaria clinical scoring rubric 123

Table 4.2: C57BL/6J susceptibility to ECM 124

Table 4.3: LTF-/- and control susceptibility to ECM 125

Table 5.1: Characterization of colony-wide *Helicobacter* spp. infection 148

Table 5.2: Strain-specific fecundity rates in the presence and absence of *Helicobacter* spp. infection 149

Table 5.3: *Helicobacter* spp. significantly reduce implantation frequency and embryo viability in pregnant mice 150

LIST OF FIGURES

Page

Figure 3.1: Deletion of Tissue Factor from maternal endothelium or hematopoietic cells improve midgestational pregnancy-associated weight gain 80

Figure 3.2: $TF^{flox/flox}$, Tie2Cre+ mice are protected from malaria-induced pregnancy loss at mid-gestation 81

Figure 3.3: $TF^{flox/flox}$, Tie2Cre+ mice are protected from malaria-induced placental pathology at mid-gestation.....82

Figure 3.4: $TF^{flox/flox}$, Tie2Cre+ mice are protected from malaria-induced placental fibrin deposition at mid-gestation. 83

Figure 3.5: $TF^{flox/flox}$, Tie2Cre+ mated with homozygous-Cre male mice exhibit mixed embryo phenotypes at mid-gestation 84

Figure 3.6: Fibrin immunohistochemical analysis in $TF^{flox/flox}$, Tie2Cre female mice mated with homozygous-Cre expressing stud males at GD12 85

Figure 3.7: Deletion of Tissue Factor from maternal myeloid cells accelerates malaria-induced midgestational pregnancy loss. 86

Figure 3.8: $TF^{flox/flox}$, LysMCre+ mice exhibit exacerbated malaria-induced gross pathology at mid-gestation.....87

Figure 3.9: $TF^{flox/flox}$, LysMCre+ mice exhibit more severe malaria-induced placental pathology at mid-gestation relative to TF-intact mice 88

Figure 3.10: $TF^{flox/flox}$,LysMCre+ mice exhibit only slightly more severe malaria-induced fibrin deposition at mid-gestation 89

Figure 3.11: Anticoagulant treatment improves midgestational pregnancy-associated weight gain during murine placental malaria.....90

Figure 3.12: Anticoagulant-treated mice exhibit healthier conceptuses at mid-gestation .91

Figure 3.13: Dalteparin-treated infected, pregnant mice exhibit improved but not completely rescued malaria-induced placental pathology at mid-gestation 92

Figure 3.14: Dalteparin-treated, infected placentae exhibit reduced fibrin deposition at mid-gestation..... 93

Figure 3.15: Fondaparinux-treated, infected placentae are indistinguishable from uninfected placentae at mid-gestation..... 94

Figure 3.16: Fondaparinux-treated, infected placentae exhibit fibrin staining equivalent to that seen in UP mice at mid-gestation..... 95

Figure 4.1: Reduced Tissue Factor expression does not significantly alter the course of *P. berghei* ANKA infection..... 116

Figure 4.2: Course of *P. berghei* ANKA varies significantly between CM+ and CM- mice of the same strain 117

Figure 4.3: Tissue Factor deficiency improves survival but does not alter non-neurological clinical outcomes of ECM 118

Figure 4.4: Cerebral malaria-positive LTF-/- mice are protected from pathologic characteristics of human and ECM..... 119

Figure 4.5: Cerebral malaria-positive LTF-/- mice are protected from ECM-associated blood brain barrier disruption 120

Figure 4.6: Cerebral malaria-positive LTF-/- mice do not exhibit ECM-associated increases in proinflammatory cytokine expression 121
Figure 4.7: Dalteparin-treated mice are protected from ECM-associated blood brain barrier disruption 122
Figure 5.1: *Helicobacter* spp. infection significantly impacts pregnancy-associated weight gain but does not induce maternal anemia 146
Figure 5.2: *Helicobacter* spp. negatively impacts midgestational embryo health 147
Figure A.1: A proposed model for blood brain barrier disruption in the presence of a cerebral malaria-induced procoagulant state 166
Figure A.2: A proposed model for blood brain barrier protective mechanisms induced by low tissue factor expression in the presence of malaria infection 170

CHAPTER 1

INTRODUCTION

Malaria poses a serious threat to global health, with 3.2 billion people in 95 countries at risk of infection. The end of the era of the Millennium Development Goals in 2015 saw a 48% reduction of malaria deaths and an 18% reduction in cases globally since 2000. Despite this marked improvement, malaria was responsible for 438,000 deaths in 2015 alone, 90% of which occurred in Sub-Saharan Africa[1]. Though significant advances toward malaria elimination have been made over the last 15 years, much remains to be understood about this disease before the necessary tools can be developed to successfully remove this immense burden from half of the world's population.

Pregnant women and young children in malaria-endemic areas bear the heaviest burden of disease. Sequestration of infected erythrocytes in the maternal blood space of the placenta or the brain results in two severe clinical manifestations of malaria, placental (PM) and cerebral malaria (CM). Due to a state of immunological naïveté, children under the age of five in Sub-Saharan Africa are the most susceptible population to CM, contributing to 90% of CM-related fatalities[2]; for a similar reason, women living in endemic areas are most susceptible to malaria-associated poor pregnancy outcomes during that their first or second pregnancy. Despite the fact that effective antimalarial drugs exist to combat the parasite, cerebral malaria still carries a case fatality rate between 15-20%[3,4], and placental malaria induces an estimated 900,000 low birth weight (<2500 g) deliveries[5] and over 200,000 stillbirths annually[6].

The use of adjunctive therapies to specifically target pathogenic processes known to occur during severe malaria hold great promise to prevent the more serious outcomes of malaria infection[7]. However, to date, therapies aimed at decreasing the inflammation associated with malarial infection have been unsuccessful in mitigating the pathology associated with PM [8,7,9]. Thus, characterization of related pathogenic processes underlying CM and PM is critical in order to develop effective therapies to combat these diseases.

Evidence of a procoagulant state during PM and CM[10–12] prompted our lab's initial foray into investigating the coagulation cascade in the context of murine placental malaria. Those initial experiments revealed that treatment with a research-grade low-molecular weight heparin could mitigate mid-gestational pregnancy loss during murine placental malaria infection[13]. Additional analysis of clinical samples collected previously by investigators in our lab at a field site in Kisumu, Kenya showed histopathologic evidence of activated coagulation, most notably excessive fibrin deposition in the placenta, which correlated negatively with birth weight and positively with inflammatory infiltrates in the maternal blood space of infected placentae[13]. These studies clearly indicated that excessive activation of coagulation occurs in PM, but the components of the coagulation cascade that were relevant to the observed pathologies remained unclear. Thus, my project sought to elaborate on these studies to build our understanding of how coagulation functions during severe malaria.

The work presented in Chapter 3 assesses the role of tissue factor (TF)-induced coagulation in causing malaria-associated pregnancy loss. We took two different approaches to addressing that question. First, we used genetically-modified mice with tissue-specific deletions of TF, the initiator of the extrinsic pathway of coagulation, to identify cell types participating in PM-associated coagulopathy. As the initiator of the

extrinsic pathway of coagulation, TF expression can support the activation of the factors in the common coagulation pathway, notably coagulation factor X (FXa) and thrombin (FIIa), both of which are capable of inducing a proinflammatory response in addition to a procoagulant one. Through Cre-lox technology and creative mating strategies, we were able to modify TF expressed on specific cell types of interest, particularly maternal monocytes and the trophoblast. The second approach to interrogate the role of the coagulation cascade in the phenotype associated with PM was to use chemotherapeutic interventions, including dalteparin (a low molecular weight heparin) and fondaparinux (an indirect FXa inhibitor). Investigating these drugs in our murine model of PM allowed us to use another method to decrease FXa activity to understand the role of coagulation in the pathogenesis of PM. In addition, these experiments provided initial data for investigating the potential use of anticoagulant drugs as adjunctive therapies for PM (although in this case they were administered alone and not in conjunction with antimalarial drugs).

The experiments in Chapter 4 used a similar approach to that in Chapter 3 in order to assess the contribution of the activation of coagulation to neurological symptoms and brain pathology in experimental CM (ECM). Rather than using with tissue-specific deletions of TF, the genetically-modified mice used in these experiments expressed very low levels of TF — ranging from approximately 1-4% of normal expression levels — across all tissues. Fibrin deposition and petechial hemorrhaging in the brain are classic pathologic characteristics of fatal CM[14–16]. Additionally, malaria-infected erythrocytes have been found to be able to directly induce TF expression and activate thrombin on vascular endothelial cells *in vitro*[17]. Dalteparin was also administered to mice with ECM. By administering anticoagulant drugs to these mice or significantly reducing TF expression in the brain microvasculature, we aimed to investigate if these pathologic features are causes or effects of the negative outcomes of ECM, including blood brain barrier disruption, altered neurological function and death.

Early on during the PM experiments presented in Chapter 3, we observed poor pregnancy outcomes even in mice uninfected with *Plasmodium* spp. and a high incidence of rectal prolapse in stud males. Shortly thereafter, it was discovered our colony was infected with *Helicobacter spp.*, a gastrointestinal bacterial infection that is frequently endemic in research animal facilities. C57BL/6J mice, the strain that makes up the background of the majority of our colony, are widely considered "resistant" to the negative effects of *Helicobacter* infections, which can range from typhlocolitis to hepatitis. However, one species found in our colony, *Helicobacter hepaticus*, had previously been reported to affect pregnancy in certain strains of transgenic mice. Because we could not be sure this bacterial infection was not interfering with our studies, we undertook efforts to eradicate *Helicobacter* from our mouse colony, led by our highly skilled technician, Caitlin Cooper. To eliminate *Helicobacter*, Cesarean sections were performed on pregnant mice at term and pups were fostered with time-mated, uninfected foster mothers. Since very little information on the effect of *Helicobacter* spp. on pregnancy outcome and breeding colony efficiency existed in the literature, we analyzed the data generated in malaria-uninfected, *Helicobacter* spp.-infected pregnant mice of multiple transgenic and congenic strains for comparison with pregnancy outcomes in *Helicobacter* spp.-free mice. The results of that analysis are presented in Chapter 5, and will provide useful insight for other laboratories that use transgenic mouse models in the presence of endemic *Helicobacter* spp.

This dissertation has been formed around the Specific Aims outlined below. Following these aims, Chapter 2 provides readers with a review of the current literature in the malaria field, particularly as it pertains to inflammation and coagulation in the pathogenesis of severe malaria.

Specific Aims

Aim 1: To characterize the effect of Tissue Factor on pregnancy outcome and malaria pathogenesis in a murine model of PM.

Aim 1A: Characterize the effect of genetic modifications in TF expression on pregnancy outcome and placental pathology in a murine model of PM.

Aim 1B: Use pharmacological interventions to assess the relative roles of distinct thrombotic processes in PM-induced pregnancy loss and placental damage.

Aim 2: To assess the role of Tissue Factor in the development of neurological abnormalities and brain pathology in a murine model of Cerebral Malaria (ECM).

Aim 2A: Use genetically-modified mice and anticoagulant therapies to assess the effect of impaired coagulation on the development of clinical signs of ECM.

Aim 2B: Use genetically-modified mice and anticoagulant therapies to assess the effect of impaired coagulation on the development of the histopathologic lesions of ECM.

CHAPTER 2

LITERATURE REVIEW

Malaria is a mosquito-vectored, blood-borne disease caused by parasites of the genus *Plasmodium*. Of the five species that are known to cause disease in humans, including *P. vivax, P. malariae, P. ovale, P. falciparum,* and *P. knowlesi, P. falciparum* and *P. vivax* pose the most significant threat to humans. Malaria infection is initiated when a female mosquito of the genus *Anopheles* takes a blood meal from a human host, injecting 5-50 sporozoites into the skin. These sporozoites migrate to the liver, and develop into merozoites in the hepatocytes. Merozoites, now numbering in the thousands, are released into the bloodstream, where they infect red blood cells to begin the intraerythrocytic stage of the malaria lifecycle. Once inside an erythrocyte, merozoites develop first into a mononucleated ring stage, then into a multinucleated schizont. When the schizont ruptures, 4-16 merozoites are released into the bloodstream, continuing the intraerythrocytic cycle. In response to an unknown signal, some merozoites differentiate into gametocytes, which can be taken up by another female mosquito. The sexual stages of the parasite's lifecycle occur in the mosquito's gut and salivary glands, and the life cycle is continued upon her next blood meal.

The resulting malaria infection causes a febrile disease in which intermittent episodes of fever and spikes in parasitemia are caused by the intraerythrocytic stage of the life cycle. In some cases of *P. falciparum* infection, severe malaria can develop, characterized by severe anemia induced by infected erythrocyte (IE) lysis. Tissue hypoxia from anemia can be exacerbated by impaired erythropoiesis and by endothelial cell injury resulting from IE sequestration, leading to lactic acidosis[1]. Severe malarial

anemia is reported to affect a patient's neurologic function, even in the absence of cerebral malaria, and in infected children is associated with the equivalent of an 11 Intelligence Quotient point reduction relative to unaffected children in the same community[2].

Malaria poses a serious threat to global health, with 3.2 billion people in 95 countries at risk of infection. The end of the era of the UN-sponsored Millennium Development Goals in 2015 saw a 48% reduction of malaria deaths and an 18% reduction in cases globally since 2000. Further, malaria prevalence among children 2-10 years old dropped from 33% to just 16%, causing malaria to no longer be the leading cause of death among children in Sub-Saharan Africa. Despite these marked improvements, malaria was responsible for 438,000 deaths in 2015 alone, 90% of which occurred in Sub-Saharan Africa[3]. Cerebral malaria still carries a case fatality rate between 15-20%[4,5], and placental malaria induces an estimated 900,000 low birth weight (<2500 g) deliveries[6] and over 200,000 stillbirths annually[7]. Though significant advances toward malaria elimination have been made over the last 15 years, much remains to be understood about this disease before the necessary tools can be developed to successfully remove this immense burden from half of the world's population.

Effective antimalarial drugs, including chloroquine and artemisinin combination therapies, are regularly used for the treatment and prevention of malaria. However, the populations who bear the heaviest burden of disease often cannot afford the full course of antiparasitic drug treatment. Additionally, a rising incidence of drug resistance even in the most potent antimalarial drugs currently available make treatment of CM and PM complicated. Chloroquine and pyrimethamine/sulfadoxine resistance has spread throughout malaria endemic regions[8–13], and even artemisinin resistance was found in *Plasmodium* spp. in some areas of Southeast Asia[14–17]. For some drugs, resistance was very quick to develop; resistance to mefloquine is only seen in areas of widespread use,

but arose in only six years from the drug's introduction[18], and resistance to atovaquone emerged in as little as six months[19].

Insecticide treated nets (ITNs) have been shown to reduce malaria incidence by approximately 50% among groups most at risk for developing severe malaria, including pregnant women and children under five years old[20]. ITNs have been used to great effect in global malaria elimination efforts, and access to ITNs in Sub-Saharan Africa rose from <2% to an estimated 67% between 2000 and 2015[3]. However, provision and distribution of ITNs can be costly and logistically difficult, resulting in low coverage in many malaria-endemic countries[21]. Also, proper care of ITNs, including regular retreatment and hole repair, is often neglected, reducing the efficacy of this intervention over time[21].

P. falciparum Erythrocyte Membrane Protein-1 (PfEMP1) is a highly polymorphic protein expressed on the IE surface that is encoded by the multi-copy *var* gene family[22]. PfEMP1 possesses a large, variable external segment that contains binding sites for host endothelial cell receptors involved in cytoadherence, including Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), Cluster of Differentiation (CD) 36, E-selectin, P-selectin, endothelial protein C receptor (EPCR), thrombomodulin (TM), chondroitin sulfate A (CSA) and thrombospondin[1,23,24]. Binding to these receptors results in IE sequestration in the vital organs of infected patients. Such IE sequestration is typical of all malaria infections, but is particularly pervasive in severe falciparum malaria and is not seen to the same extent in other *Plasmodium* species. Indeed, during *P. falciparum* infection, mature IE forms are virtually absent from the peripheral circulation due to sequestration[23].

Sequestration of IE during malaria infection has been known since the late 19th century, and is associated with the pathologic features of severe malaria. In particular, IE

sequestration in the maternal blood space of the placenta or the brain results in two severe clinical manifestations of malaria, placental (PM) and cerebral malaria (CM).

Placental Malaria Characterization and Epidemiology

Placental malaria results from the sequestration of IE in the intervillous space of the placenta, where the maternal blood bathes the placental villi. IE expressing a specific *var* gene, VAR2CSA, bind chondroitin-sulfate A (CSA) on the syncytiotrophoblast (ST)[25–27] — a continuous, multinucleated, specialized epithelial layer lining the placental intervillous space. The ST exists as a physical barrier at the maternal-fetal interface, and thus is responsible for a number of important functions, including the facilitation of nutrient and gas exchange and the prevention of disease transmission between the mother and developing fetus. The maternal response to PM induces severe placental pathology, disrupting normal ST physiological function and interfering with the fetus's growth and development[28,29]. Resultant placental insufficiency, preterm delivery (before 37 weeks gestation), fetal growth restriction (FGR) and maternal anemia contribute significantly to the high prevalence of malaria-associated low birth weight (LBW, <2500g) in endemic regions (20% of live births in malaria-endemic areas between 1985 and 2000)[6]. Babies born with LBW, particularly in areas with poor medical infrastructure such as those commonly found in areas of high malaria transmission, are not only at a significantly increased risk of death shortly after delivery, but also have an increased risk for developing health problems during adulthood[30,31]. Additionally, fatality rates in neonates born with FGR are 10-20-fold higher than those in those who undergo normal development[32]. Interestingly, the prevalence and severity of these pathologies and birth outcomes are parity dependent; with each successive infected pregnancy, a mother acquires adaptive immunity that limits the extent of placental infections[28] by blocking parasite binding to CSA on the ST[33,34]. High levels of antibodies against CSA-binding

parasites have been associated with an increased birth weight and reduced maternal anemia[35].

Without pregnancy-specific antimalarial countermeasures, it is estimated that 11.4 million pregnant women (41.2% of all live births in malaria-endemic areas of Africa in 2010) would have had placental malaria infection, leading to 900,000 LBW deliveries per year[6]. While this estimation comes with the caveat that these risks were calculated under the circumstances that no malaria prevention services were provided to the populations in question, these numbers are useful considering only 17% of pregnant women in malaria-endemic regions currently receive the WHO-recommended 3 doses of sulfadoxine/pyrimethamine intermittent preventative treatment[3] (IPTp-SP).

Such prophylaxis has been shown to be successful in preventing PM, and can be administered safely and effectively in as few as three doses[36,37]; unfortunately, as evidenced by the low reported IPTp-SP coverage rates, the distribution and administration of prophylactics are not always practical where malaria is highly endemic, as even prenatal care may not be available in some of these areas. Widespread sulfadoxine/pyrimethamine (SP) resistance poses a further challenge to the effectiveness of IPTp-SP and the treatment of malaria during pregnancy. Though SP was until recently the drug most commonly used to treat malaria in pregnancy, the WHO now recommends pregnant women diagnosed with uncomplicated malaria be treated with artemisinin-based combination therapy (ACT). While ACT and SP are both more effective at preventing poor malaria-associated birth outcomes, neither is capable of complete cure or rescue of pregnancy. In one recent study conducted in Uganda[38], prevalence of adverse birth outcomes including stillbirth, LBW, preterm delivery, congenital anomaly and spontaneous abortion was 9.2% in ACT-treated and 18.6% in SP-treated pregnant women. In both groups, LBW was the most common adverse birth outcome, followed by preterm delivery. Another study[39] held at 7 sites across four Sub-

Saharan African countries — Burkina Faso, Ghana, Malawi and Zambia — found that of the babies born to mothers treated with one of four different ACTs, 14.1-17.2% were LBW, 3.4-10.2% were delivered preterm, and 2-2.8% were stillborn. While these are significant improvements relative to outcomes of untreated PM, these drugs are unable to completely mitigate malaria-associated adverse pregnancy outcomes despite high cure rates among the mothers.

Placental histopathologic changes characteristic of PM include intervillusitis, fibrinoid necrosis, deposition of hemozoin (the by-product of hemoglobin degradation by malaria parasites), ST degradation, destruction of villous integrity, decreased villous surface area and thickening of the trophoblast basement membrane. These pathologic changes can be used to group PM patients into the following infection statuses, first defined by Bulmer et al. in 1993[29], then refined by Rogerson et al. in 2007[28]: uninfected (no evidence of parasites or hemozoin), active-acute (parasites visible with absent or minimal hemozoin deposition within fibrin in the intervillous space), active-chronic (parasites visible with substantial amounts of pigment in fibrin or in cells), and past (presence of hemozoin in the absence of parasites). These infection statuses have been associated with birth weight; active-chronic infection correlates with decreased birth weight due to fetal growth restriction, as well as maternal anemia, while active-acute infections are associated with preterm birth, especially when in combination with high parasitemia[28].

Inflammatory Damage in Placental Malaria

These histopathologic changes and poor birth outcomes are the result of a series of responses initially triggered by the binding of IE to the ST; these responses are amplified as the disease progresses and the ST is continually exposed to IE, malarial components, immune mediators, microparticles, procoagulant molecules and damage-

associated molecular patterns (DAMPs). Inflammatory damage induced by maternal and placental immune responses to malaria infection known to play a major role in PM-induced placental dysfunction, though the precise mechanisms underlying this inflammatory response remain incompletely understood.

The existence of a proinflammatory state in the placentae of women with PM has long been established [79]. Inflammation in human PM is characterized by an influx of maternal phagocytic cells, particularly monocytes, into the placental intervillous space (IVS)[28,32,40–43], termed "intervillositis." A T helper type 1 (T_H1) cytokine bias is characteristic of human PM, with concentrations of proinflammatory cytokines Tumor Necrosis Factor (TNF)-α [40,42,44,45], TGF-β, Interleukin- (IL) 2[44], and IFN-γ[44,46,47] typically elevated in placental plasma. PM has also been correlated with increased placental plasma concentrations of IL-1β[41], IL-6, soluble CD163[42], and soluble TNF Receptor II (TNFRII)[48]. Elevated production of monocyte-attracting chemokines by macrophages and monocytes in the IVS, including CCL1, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL8 (IL-8) and CXCL10 (IP-10), has been correlated with the degree of infiltration of inflammatory monocytes cells[47,49].

High-density inflammatory cell infiltration into the IVS and elevated levels of TNF-α, IFN-γ, IL-1β, soluble TNFRII, CXCL9, and CXCL13 all negatively correlate with birth weight in primigravid, PM-positive women[32,40,41,44,46,48,49]. This is not unexpected; whereas healthy, infection-free pregnancies are T_H2 phenomenon, T_H1 cytokine expression is known to be incompatible with pregnancy success[50]. A maternal T_H2 immune bias during pregnancy functionally induces maternal tolerance and immune suppression, protecting the fetus from rejection. In contrast, a T_H1 bias has been reported to be associated with clinical cases of recurrent miscarriage and preeclampsia[45,51–53], and exogenous administration of T_H1 cytokines induces preterm labor and fetal loss in pregnant mice[50,54,55].

The proinflammatory environment in the IVS is due in large part to the ST response to stimulation by VAR2CSA-expressing IE, malarial components (primarily hemozoin and glycosylphosphotidylinositol (GPI)), immune cells, cytokines, microparticles from cell membranes and platelets, among others, initiating a series of signaling events in the trophoblast that induces the release of proinflammatory mediators from cells. In addition to CSA serving as a receptor for VAR2CSA-expressing IE, the ST expresses pattern recognition receptors (PRR) that allow it to respond to other Pathogen-Associated Molecular Patterns (PAMPs) and Danger-Associated Molecular Patterns (DAMPs) associated with malaria infection. Toll-like receptors (TLR) 1-10[56–59] and NOD-like Receptors (NLR), including inflammasomes[60], are expressed by the ST.

Most of what is directly known about the ST response to stimulation was gleaned from studies performed *in vitro*. Our lab has performed extensive work in an *in vitro* model of PM in which BeWo cells (a human placental choriocarcinoma cell line) or primary human trophoblast cells are stimulated with laboratory isolates of CSA-binding *P. falciparum* IE[61]. Using this model, we have shown that IE are able to directly stimulate ST to release immunologic factors through the activation of the C-Jun N-terminal kinase 1 (JNK) signaling pathway[62]. It was further shown that hemozoin stimulation of ST induces extracellular signal-regulated kinases (ERK) 1/2 phosphorylation, inducing the release of CXCL8, CCL3, CCL4, TNF-α, and soluble ICAM-1[63]. These cytokines and chemokines produced *in vitro* are also commonly seen in the placentae of malaria-infected women, indicating IE and malarial hemozoin are capable of directly inducing the proinflammatory state of the IVS seen during PM.

Inflammation in Murine Models of Placental Malaria

Several murine models also exist to study the inflammatory response to PM during different stages of gestation. A model to study the effects of malaria infection

during late pregnancy was developed by Neres et al. using the murine malaria parasite *Plasmodium berghei* ANKA[64]. In order to avoid complications and maternal mortality due to cerebral malaria (CM), this model is performed using CM-resistant BALB/c mice. Gestational day (GD) 1 is defined as the day on which a vaginal plug is observed in timed-mated virgin BALB-c female mice. Pregnancy is confirmed upon measurement of a subsequent 5 g weight gain between GD10-13, at which time mice are infected intravenously with 10^6 *P. berghei* ANKA. Infection at this late time point ensures pregnancy can be taken to term, though a clear phenotype still develops before the end of gestation; the authors report that infection earlier in gestation results in midgestational pregnancy loss.

This model is useful to study the effects of PM on late gestation and replicates several aspects of human infection. Infection-induced inflammation in the placenta and impairment of placental function are seen upon sacrifice between GD17 and GD 19, resulting in FGR, fetal resorption and stillbirth. The inflammation is characterized by significant inflammatory cell infiltration in the maternal blood space; as in human PM, immunohistochemical staining for CD11b reveals these cells are primarily monocytes and macrophages. Increased placental expression of the macrophage-attractant chemokines MIP-1α and MCP-1 also indicate a proinflammatory state in the maternal blood space of the placenta. Significant placental pathology is observed in this model; placentae isolated from infected pregnant (IP) mice show accumulation of IE and hemozoin in the placenta, basal zone fibrinoid necrosis, thickening of the placental labyrinth (where critical nutrient and gas exchange occurs), restriction of vascular space, and ST hyperplasia. When dams were allowed to deliver, infection resulted in decreased litter size, prematurity, low birth weight, increased perinatal mortality, and impaired growth of surviving pups. Notably, as seen in human PM-infected placentae,

cytoadherence assays show *P. berghei*-ANKA IE bind to CSA on murine placental tissue sections.

Later work in this lab elaborated on this model by using the same experimental paradigm with different strains of *P. berghei* to induce several distinct placental pathologies[65]. The authors infected pregnant C57BL/6 mice with 10^6 *P. berghei* ANKAΔpm4 at GD10, or with 10^6 *P. berghei* NK65 or K173 at GD13. ANKAΔpm4 lacks Plasmepsin IV, a *Plasmodium* protein involved in the degradation of hemoglobin, and has a reduced multiplication rate[66]; thus, infection with that strain was initiated at an earlier time point than was used for the others in order to ensure sufficient parasite expansion by late gestation. All three strains induced trophoblast thickening, placental tissue disorganization, labyrinth necrosis, and thrombosis formation, albeit to varying degrees. NK65 seemed to induce in be the most severe infection, resulting in the largest vascular restriction and occasionally in maternal death. Interestingly, ANKAΔpm4 induced a strong innate immune response characterized by increased expression of MCP-1, MIP-1α, Toll-like Receptor (TLR)-2, TLR-4, and TNF- α not seen in the placentae of mice infected with the other two strains. Despite exhibiting different pathologies, infections with these strains resulted in placental parasite burdens that were not significantly different as measured by RT-PCR. The results of this study could have interesting implications for the study of PM; the varying phenotypes resulting from infection with these different strains could be strategically implemented to study specific aspects of malaria-associated placental pathology seen in human disease.

Following the observation that *P. berghei* ANKA binds to murine placental CSA during late gestation, this model was used to assess the effects of infection with a VAR2CSA-expressing, CSA-binding *P. berghei* ANKA[67]. *In vitro* assays revealed that erythrocytes infected with these parasites bound better to placental tissue than unaltered *P. berghei* ANKA IE. In pregnant BALB/c mice infected with a relatively low inoculum

(10^4 IE administered intravenously), the CSA-binding parasites induced higher rates of stillbirth and LBW relative to non-CSA-expressing parasites. Higher inocula (10^5 or 10^6 IE administered intravenously) resulted in greater maternal mortality and LBW, respectively. All three inocula resulted in higher incidences of fetal loss compared to that induced by non-CSA-binding parasites. However, these results are questionable due to the very high maternal mortality rates observed in these studies. Untransfected *P. berghei* ANKA parasites resulted in maternal mortality of 83% at the 10^6 inoculum (compared to 63% in previous studies[64]), and the VAR2CSA-expressing *P. berghei* ANKA parasites induced mortality rates of 44.4% and 60% in mice infected with 10^5 and 10^6 parasites, respectively[67]. Interestingly, despite the effects on maternal mortality, parasite burdens did not vary significantly between parasite strains. Of particular concern was the observation that maternal mortality reached 20% even in the uninfected mice (compared to 0% in previous studies[64]), indicating some underlying factor was causing adverse outcomes in these mice. Nevertheless, moving forward, these parasites could be very useful in more accurately modeling the placental response to IE binding to CSA on the ST in an *in vivo* system.

As with any animal model of a human disease, there are limitations to this model's ability to effectively represent human PM. The high virulence of *P. berghei* ANKA, even in CM-resistant BALB/c mice, induced maternal pre-term mortality rates of 63% in mice receiving a 10^6 inoculum. In comparison, though malaria-specific maternal mortality rates are difficult to accurately estimate, maternal mortality due to all causes in Sub-Saharan Africa is 546 per 100,000 live births, or roughly 0.5%[68] – a number that is far too high, but that pales in comparison to the rates seen in this model. For this same reason, this model cannot be altered to investigate mechanisms of poor birth outcome resulting from infection in early gestation. As the placental is inaccessible until delivery, the pathogenic mechanisms occurring in early gestation generally cannot be studied in

humans due to logistical and ethical reasons. While models of PM in late gestation are useful for drawing comparisons to clinical data taken at term, it leaves a large gap in knowledge as to how PM affects the placenta and surrounding environment during early to mid gestation. This is significant, as an estimated 65% of PM infections occur near the end of the first trimester, the time at which the placenta first becomes susceptible to infection[6]. Early infection has been correlated with an increased risk for miscarriage compared to infection occurring later in pregnancy[69].

In order to address this gap in knowledge, our lab developed a model of murine placental malaria to study the effect of PM in early pregnancy[70]. In this model, detection of a vaginal plug in timed-mated 8-10 week-old C57BL/6J mice indicates GD0, on which day plugged mice are infected intravenously with 10^3 *Plasmodium chabaudi* AS. In order to avoid stress-induced failure of the blastocyst to implant on the uterine wall, the mice are not handled again until GD6. Pregnancy in this model is not taken to term; abortion is first seen in these mice at GD10, and no viable embryos remain by GD12. Mice are sacrificed either at GD12 or upon observation of a bloody, mucoid vaginal discharge, a sign of active abortion; mice exhibiting vaginal bleeding were shown to have open cervices upon necropsy, with embryos in the cervix or vagina indicating pups were being actively expelled.

P. chabaudi AS is relatively less virulent than *P. berghei* ANKA, and C57BL/6J mice are able to clear infection with a subsequent brief, mild recrudescence. In *P. chabaudi* AS-infected, pregnant C57BL/6J mice, maternal mortality is 0%. However, despite the mild maternal outcomes, this parasite has significant impacts on pregnancy. This model results in significantly more embryo resorptions in IP mice versus UP; an average of 37% and 100% of embryos were resorbed in IP mice on GD10 and GD12, respectively, compared to 0% GD10 and 3.3% on GD10 and GD12 in the UP group. Significant IE accumulation occurs in the placentae of *P. chabaudi* AS-infected pregnant

(IP) mice, with placental parasitemias more than 40% higher than that in peripheral blood on GD10 and 11[70]. Severe placental pathology develops, as well, consisting of widespread placental hemorrhage, thinning of the placental labyrinth, disruption of placental architecture, and thrombus formation in the maternal blood space. However, unlike human infection, while proinflammatory cytokines are significantly increased exists in response to infection in this model, there is very little accumulation of monocytes and macrophages in the placenta of these mice[42,70].

This model was later used to study PM in A/J mice[7172], which are believed to have a T_H2-biased immune response as opposed to the T_H1 bias characteristic of C57BL/6J mice. Non-pregnant A/J mice are also susceptible to lethal infection with *P. chabaudi* AS, whereas non-pregnant C57BL/6J mice are resistant[73,74]. Mature, non-pregnant A/J mice reach a peak parasitemia of approximately 60% and die 11 days post intravenous infection with 10^4 *P. chabaudi* AS parasites; non-pregnant C57BL/6J mice infected with the same number of parasites reached a much lower peak parasitemia of about 42% on day 9 post-infection, before clearing parasites with only a low level of recrudescence. Accordingly, 100% of the IP A/J mice used in this model of PM in early gestation do not survive past GD12; thus, serial sacrifices in both strains of mice are performed through GD11 in this model, rather than GD12 as in previous experiments. As in non-pregnant mice, A/J mice reach significantly higher peak parasitemia than C57BL/6J mice one day later in infection. IP A/J mice also exhibit more severe anemia than IP C57BL/6J, achieving a significantly lower hematocrit GD11.

Fetal loss is significantly higher in IP A/J mice on GD9 relative to both UP A/J and IP C57BL/6J mice (abortion begins in IP C57BL/6J mice at GD10). However, abortion rates observed in IP mice did not significantly differ between strains. IP C57BL/6J and A/J mice exhibited different systemic cytokine profiles at sacrifice; A/J mice showed higher levels of systemic TNF-α and IL-1β relative to C57BL/6J mice on

GD11. Notably, IP A/J mice also had significantly elevated levels of TNF-α, IFN-γ, IL-1β, and IL-6 relative to the UP A/J group on GD 9, 10, and 11. This cytokine profile is notable for two reasons: firstly, it is very similar to that characteristic of human PM infection[28,42,44,70–72]; secondly, it indicates that A/J mice do not, in fact, have a T_H2-biased immune system as was previously thought, but rather exhibit a delay in the onset of a proinflammatory T_H1 response to malaria infection. In all, A/J susceptibility to lethal *P. chabaudi* AS infection imposes some limits on what can be done in this model; however, the similarity of the proinflammatory cytokine profile to that seen in human clinical cases of PM renders it a useful model for the study of PM.

P. chabaudi AS infection has also been used to assess the roles of TNF-α and IFN-γ in the pregnancy loss and placental pathology characteristic of this model of PM[75] [8]. These two proinflammatory cytokines are notable, as they are known be incompatible with pregnancy success even in the absence of infection[54,55,50,52,51]. Additionally, elevated levels of these cytokines are associated with poor pregnancy outcomes in clinical cases of PM. Implementing the *P. chabaudi* AS model of PM in early gestation in IFN-γ-knockout mice mice induced a more severe course of maternal infection, but resulted in delayed fetal loss[76]. Additionally, antibody neutralization of TNF-α in IP C57BL/6J mice reduced embryo resorption on GD12 to 15% (compared to 100% in previous studies[70]), levels comparable to that normally seen in UP C57BL/6J mice, though no attempts were made to take pregnancy in these mice to term. TNF-α neutralization did not rescue pregnancy in IP A/J mice[76]. Further studies revealed that TNF-α is likely a central player in malaria-induced fetal loss in this model and in human PM, but that it does so in a mechanism independent of its ability to drive apoptotic programmed cell death[72].

Cerebral Malaria Characterization and Epidemiology

Cerebral Malaria (CM) is another clinical manifestation of severe malaria defined by the WHO as the presence of *P. falciparum* parasitemia and unrousable coma (Blantyre scale <3) with no other apparent etiology[77]. CM occurs in approximately 1% of malaria infections, and carries a 15-20% case fatality rate[78]. Children under the age of five in Sub-Saharan Africa are the most susceptible population, contributing to 90% of CM-related fatalities[79]. Children at this age in malaria-endemic areas are at high risk of severe outcomes, as during this time period they simultaneously are no longer protected by maternal immunity and have not yet developed their own state of partial immunity due to repeated exposure to and survival of malaria infection[78].

Neurologic symptoms associated with CM other than coma include retinopathy, agitation, psychosis, seizure and impaired consciousness[80,81]. Retinopathy is one of the best methods of diagnosing CM currently available. In one study of pediatric CM, 24% of patients who fulfilled the WHO-defined criteria for CM before death had evidence post-mortem for another cause of coma (e.g. meningitis); the presence of retinopathy was the only clinical feature that distinguished real CM cases from those with other, similar pathologies[82]. Seizures are most common preceding coma in pediatric CM and are associated with a rise in intracranial pressure; around 15-20% of adults diagnosed with CM have seizures, compared to 80% of children[83]. Seizures during pediatric CM may occur in the absence of fever and in many cases are repetitive or prolonged; status epilepticus, a condition in which epileptic seizures follow one after another without the patient regaining consciousness in between, is common in children[84]. Psychiatric manifestations including hallucinations and psychoses can occur during CM, though these can also be caused by antimalarial drugs administered to treat the disease. Patients with CM also often exhibit a change in diurnal rhythm, with difficulty in sleeping or excessive sleepiness[85]. Reports indicate that upon discharge from hospital care, 5-

28% of children have neurological deficits including epilepsy, acute hemiparesis, hypertonia, cortical blindness and ataxia[79,86,87].

One quarter of children develop long-term neurological sequelae following CM, including cognitive impairment, neurodisabilities and epilepsy[79,88]. CM has been associated with long-term adverse effects on general cognition, attention, working memory, visual spatial skills, somatosensory discrimination, speech and language. In fact, children who had recovered from CM exhibited a 13 Intelligence Quotient difference from non-affected children living in the same community one year after their recovery, and 26% showed similar impairment two years after recovery[87]. Malaria with multiple seizures has been particularly associated with long-term problems with speech and language, and malaria with impaired consciousness is associated with attention and language issues[87]. These long-term neurologic sequelae can have a serious impact on a child's development and education, leading to a huge loss in human potential in malaria-endemic countries.

Parasite Sequestration in Cerebral Malaria

Sequestration of *P. falciparum*-infected erythrocytes (IE) in the brain microvasculature is a characteristic feature of CM. This can occur through several different mechanisms. *P. falciparum* IE can adhere to the vascular endothelium via interaction of *P. falciparum* Erythrocyte Membrane Protein1 (PfEMP1) with cell adhesion molecules (CAM) including Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), CD 36, E-selectin, P-selectin, endothelial protein C receptor (EPCR), thrombomodulin (TM) and thrombospondin. Alternatively, this same protein on the IE surface can bind CD36 expressed on the surface of platelets that have been activated by thrombin or TNF-α; CD-36 binding IE can then use adherent molecules on platelets as an adaptor to bind to other endothelial receptors even if CD36

is absent from cerebral endothelial cells. Lastly, similar aggregation to platelets or uninfected red blood cells ("rosetting") can cause physical obstruction of microvessels and capillaries, allowing IE to sequester without the need for interaction with endothelial proteins[23].

Such obstruction was long thought to be the root cause of CM. This "mechanical" hypothesis, which dominated the thinking of the CM field for most of the 1900's, stated that reduced blood flow and tissue perfusion resulting from blood vessel obstruction would induce cerebral hypoxia and tissue death, leading to the neurological symptoms that are characteristic of CM. Key pieces of evidence cited in support of this hypothesis include the presence of lactic acidosis during CM[1,89], the decrease in functional capillary density in murine models of CM (experimental cerebral malaria, ECM), and the fact that the extent of IE deformability is an indicator of a poor CM prognosis[90].

However, this theory has fallen out of favor due to several observations. Firstly, high parasitemia is not a good predictor of death, though this would be expected if microvessel occlusion due to IE and inflammatory cell obstruction were responsible for disease. Secondly, such parasite sequestration occurs in all infections, but signs of CM only occur in 1% of infected individuals. Thirdly, the rapid improvement in patients following antimalarial treatment and supportive therapy does not support the stroke-like mechanism proposed for the onset of CM. Fourthly, lactic acidosis, which is present in CM and regularly cited as a key support for this theory, is not indicative solely of hypoperfusion and can be attributable to multiple causes during malaria infection including liver dysfunction, reduced oxygen delivery secondary to anemia, and mitochondrial dysfunction, among others[91]. Lastly, although neurologic symptoms and pathologic characteristics of CM can be replicated in (ECM), whether or not sequestration occurs during ECM has never been documented and remains a subject of debate[92–96].

Histopathologic Characteristics of Cerebral Malaria

Sequestration of IE in the brain microvasculature trigger a series of events that lead to the pathologies typically seen in autopsied brains of patients who succumbed to CM. Adherent IE induce endothelial activation, with several consequences following. Endothelial activation induces the upregulation of cellular adhesion molecules IE and infiltrating immune cells can use to adhere to the endothelium, notably ICAM-1, VCAM-1 and E-selectin[97–101]. Loss of endothelial cell tight junction proteins including occluding, zonular occludin-1 and vinculin are also associated with endothelial activation by sequestered IE[102]. Integrity of the blood-brain barrier (BBB), the highly selectively permeable structure responsible for regulating ion and nutrient transport between the blood and the central nervous system, is compromised in CM, likely due in part to this loss of tight junction proteins[102–104]. BBB disruption could contribute to hemorrhaging and edema during CM. Though the extent to which edema contributes to pathology, features of cerebral edema, including compression of cerebrospinal fluid spaces and an increase in brain weight, are reported in CM[105,106].

In brains of patients with high numbers of sequestered IE, brain tissue has been found to have a slate-gray discoloration due to extensive hemozoin deposition[106]. Demyelination and axonal damage are seen in areas of severe vascular damage, along with Dürck's granulomas, accumulations of microglia surrounding areas of demyelination[105–107]. Gliosis, a focal proliferation of glial cells in response to insult to the central nervous system, has been noted in CM patients[107]. Ring and petechial hemorrhages are visible throughout the brain during CM[105,108106], and have been associated with cases presenting with seizures prior to death[106]. Marked fibrin deposition occurs near areas of IE sequestration, and is associated with localized loss of Endothelial Protein C Receptor (EPCR) and thrombomodulin (TM), both of which can serve as receptors for IE binding[109,110]. Additionally, extravasation of fibrinogen, the

zymogen form of fibrin, into the brain parenchyma has been observed near areas containing thrombi, ring hemorrhages, capillary damage, and high IE density[108]. Focal accumulation of lymphocytes and macrophages with active phagocytosis of IE, hemozoin and ghost IE has been observed during CM[105,106,111].

Due to the inaccessible nature of the brain, information on the pathologic characteristics of human CM is limited to that measured in samples collected post-mortem. As such, all of the described histopathologic characteristics of human CM are seen in end-stage, fatal CM.

Inflammatory Responses to Cerebral Malaria

Release of pro-inflammatory cytokines and chemokines, leukocyte infiltration, and reactive oxygen species (ROS) production further mediate CM-induced damage, inducing severe brain pathology and disruption of the Blood-Brain Barrier (BBB)[102,112]. Hyperinflammation consisting of increased proinflammatory cytokine expression and inflammatory cell and platelet accumulation are commonly associated with CM. Malarial components, primarily hemozoin and glycosylphosphotidylinositol (GPI), bind PRR on endothelial and immune cells, stimulating the secretion of proinflammatory cytokines and production of superoxide and nitric oxide (NO). Hemozoin is a known TLR-9 agonist, though it is hypothesized that host DNA[113–115] or fibrinogen[116] bound to the malarial pigment are responsible for its associated PRR activation. Malarial GPI released during schizont rupture has been shown to activate TLR2 signaling in a MyD88-dependent fashion[117–119].

Systemic alterations in levels of circulating plasma cytokine levels are seen in severe malaria in general and CM in particular, including TNF-α, IFN-γ, IL-1β, IL-6, IL-4, IL-8, IL-12p70, MCP-1, MIP-1α, MIP-1β, TGF-β and IL-10[120–128]. Increased levels of proinflammatory cytokine levels have been linked to disease severity and complications

during CM, notably TNF-α and IL-6[102,126,129–133]. In pediatric CM, TNF-α levels in circulating plasma have been reported to be higher in CM patients than in children with uncomplicated malaria. Further, the highest plasma TNF-α levels have been found in samples from fatal CM cases, in one study reaching levels as much as ten times higher on average than in cases of non-fatal CM[127,129]. In those cases, TNF-α was a strong predictor of fatal outcome independent of parasitemia and hypoglycemia. Recent work also found a significant association between the development of CM and the TNF-promoter polymorphism TNF-238, suggesting a genetic component of the ability of TNF-α to induce CM. These observations led to a double-blind, placebo-controlled trial of a monoclonal TNF-α neutralizing antibody (B-C7) in pediatric CM patients[134]. B-C7 did not improve survival in these patients, and in fact was associated with a significant increase in the incidence of neurological sequelae in the treated group. The study authors postulated that this was because the antibody retained TNF-α in the brain vasculature rather than clearing it, prolonging its pathologic effects on the cerebral microvasculature. While this is possible, other clinical studies have shown that while TNF-α levels are elevated in malaria and positively correlate with parasite density, high TNF-α levels are associated with multiple manifestations of severe malaria and are not specific to CM[135]. Taken together, these studies seem to indicate that elevated TNF-α levels are necessary but not sufficient to induce CM.

Modulation of the inflammatory response in CM has been studied with differing results depending on the mechanism. Particular focus has been given to the multifunctional cytokines IL-10 and TGF-β. Circulating plasma IL-10 is increased during CM, and administration of anti-IL-10 antibody was shown to significantly increase monocyte production of proinflammatory cytokines TNF-α, IL-6 and IL-1β in response to *P. falciparum* IE *in vitro*[125]. TGF-β1 levels significantly decrease during CM and negatively correlate with IFN-γ and IL-6 expression. This drop in TGF-β1 correlated with

the onset of thrombocytopenia, leading to the hypothesis that malaria-induced low circulating platelet counts play a role in disrupted proinflammatory cytokine regulation in CM[128]. In ECM, combination treatment with VEGF and lovastatin or VEGF and low levels of bacterial lipopolysaccharide protected against the onset of ECM and prolonged survival by seven days by decreasing the inflammatory response to *Plasmodium berghei* ANKA. This effect was mediated by activation of the anti-inflammatory gene *Nrf-2*[136].

Inflammatory cytokines typically seen elevated during CM, particularly TNF-α, upregulate cell adhesion molecules on the vascular endothelium. In doing so, they modulate cytoadherence of IE and immune cells during CM and contribute in part to the expression of these molecules on the cerebral vascular endothelium seen by histopathology. Cerebral vascular endothelial cells constitutively express low levels of ICAM-1; significant, long-lasting increases in ICAM-1 expression on these cells is induced upon exposure to TNF-α, IFN-γ, IL-1β and IL-1α, and is particularly high with exposure to combination of those cytokines[137–139]. In ECM, increased ICAM-1 expression is primarily due to IFN-γ exposure[140], though TNF-α may still play a role[141]. VCAM-1 is not constitutively expressed on cerebral vascular endothelial cells, but is readily expressed in response to proinflammatory cytokine stimulation, including IL-1, TNF-α and IL-4[142,143]. *P. falciparum* IE have been shown to bind to vascular endothelial cells *in vitro* in response to this proinflammatory cytokine induction of cell adhesion molecule expression[97].

Accumulation of inflammatory cells in the brain microvasculature is a pathologic characteristic of CM, and likely occurs in response to increased proinflammatory cytokine and endothelial cell adhesion molecule expression. Fatal pediatric cerebral malaria has been associated with intra- and perivascular leukocytes — particularly monocytes — and platelets in the brain[102,108,144–146]. In one study, nine times more accumulation of monocytes and platelets were seen in the brain microvasculature of

autopsy-confirmed cases of CM than in brains from patients with non-malarial causes of coma[145]. Though neutrophils and evidence of Neutrophil Extracellular Traps (NETs) are rarely seen in autopsied brains of CM patients, fatal pediatric CM has been associated with increases in soluble neutrophil factors. Retinopathy-positive CM patients exhibited higher circulating plasma concentrations of human elastase, myeloperoxidase and proteinase 3. Neutrophil chemotaxis was found to be impaired when assessed *ex vivo*, possibly due to increased heme in the plasma[147].

The *Plasmodium berghei* ANKA Model of Cerebral Malaria

Accurately replicating CM in animal models has proven to be a challenge for researchers investigating the pathogenic mechanisms of the human disease. Logistical and ethical concerns have resulted in the preferential use of mice over non-human primates in CM studies[148]. Though the inoculum and routes of administration vary widely across the literature, murine-specific malaria species *Plasmodium berghei* ANKA is the standard parasite used to model cerebral malaria in mice, termed “Experimental Cerebral Malaria” (ECM)[136,141,149–152]. Differences in ECM to human CM have led some to question the appropriateness of this model in attempts to understand the human disease. The primary concern is the fact that despite the presence IE accumulation in the cerebral microvasculature, it is questionable whether actual sequestration of IE through interaction with endothelial cell adhesion molecules occurs in ECM. As such interactions are thought to play a critical role in the development of CM in humans, some have questioned the validity of extrapolating findings generated in the mouse model to the human disease[92]. Indeed, these concerns were the subject of such controversy that at the 2010 Keystone Symposium on Malaria, a special scientific session was convened at the request of participants to discuss animal models in malaria research[148].

Despite these concerns, ECM has served as a useful tool that has provided many insights into the unique processes underlying CM. *P. berghei* ANKA infection in susceptible mouse strains, primarily C57BL/6, mimics many key features of human CM. ECM results in severe neurological symptoms similar to those seen in human CM, including seizure, tremor, ataxia, impaired righting reflex, abnormal gait, impaired memory and reversible coma. Further, these mice are subject to the development of long-term neurological sequelae, as in human CM[149,150]. Theses long-term defects, including memory dysfunction and behavioral impairment, correlate with cerebral inflammation and hemorrhaging, microglial activation, and leukocyte migration to the brain microvessels. ECM also reliably mimics the Th1-biased immune response to malaria infection seen in human CM[149,153,154]. Notably, whereas TNF-α-neutralizing antibody was unsuccessful in preventing CM in human clinical trials, this treatment successfully prevented the onset of ECM[152]. However, exogenous administration of TNF to susceptible mice infected with non-ECM-inducing malaria species failed to induce a neurological phenotype, producing instead a terminal infection that more closely resembled severe malaria[155]. As in human CM, this indicates that TNF-α is necessary, but not sufficient, to induce ECM.

ECM also replicates many histopathologic features of human CM, including inflammatory cell and IE accumulation in the cerebral vasculature, petechial and perivascular hemorrhaging, and hemozoin deposition[151,156,157,112,158]. Endothelial dysfunction and activation also occur during ECM, along with upregulation of endothelial cell adhesion molecules[111,156,159,160]. Vascular damage in ECM reflects that seen in human CM, including narrowing of vascular lumen (albeit by adherent leukocytes instead of IE), reduced blood flow, loosening of tight junctions, vascular leakage, edema, occluded blood vessels and areas of low or no perfusion of tissues[156,157,161]. Breakdown

of the BBB is also typical of ECM, and Evan's Blue staining is regularly used to assess the extent of BBB disruption during ECM.

Despite its limitations, its many similarities to human CM render the *P. berghei* ANKA model of ECM a powerful tool in understanding the mechanisms underlying the human disease. While, as in any animal model of a human disease, interventions tested in this model cannot be guaranteed to work in a human clinical setting, they can provide valuable insight into the pathology of human CM.

While inflammation is clearly associated with the pathologic effects of severe malaria, administration of adjunctive therapies to reduce inflammation has repeatedly been insufficient to prevent negative outcomes of cerebral (CM). As previously discussed, a monoclonal TNF-α neutralizing antibody (B-C7) not only failed to prevent or ameliorate disease in pediatric CM patients, it actually exacerbated disease, significantly increasing the incidence of long-term neurological defects in treated children[134]. High-dosage treatment of pediatric CM patients with dexamethasone, a corticosteroid used to treat many inflammatory and autoimmune conditions[162], in combination with intravenous quinine treatment resulted in no significant difference in outcome compared to patients treated with quinine and a placebo[163]. More recently, though TNF-α levels were reduced in pediatric CM patients treated with intravenous quinine and pentoxifylline, a competitive nonselective phosphodiesterase inhibitor known to inhibit TNF-α synthesis and reduce inflammation[164], no significant difference in outcome or clinical parameters was observed compared to children treated with quinine and saline[165].

An Overview of Hemostatic Mechanisms and Drugs

The failure of promising anti-inflammatory drugs as adjunctive therapies led investigators to search for other mechanisms that could give rise to severe clinical manifestations of malaria. Given the extensive interactions between inflammation and

coagulation described in other inflammatory diseases, the malaria research community quickly gained interest in understanding how dysregulated hemostasis may affect the pathogenesis of severe malaria.

Hemostasis is maintained through the delicate balance of pro- and anti-coagulant mechanisms throughout tissues. Clotting is initiated and propagated by the coagulation cascade, which consists of the proteolytic cleavage of a series of serine proteases. Ultimately, this cascade ends with the activation of fibrin, an essential component of hemostatic clots, through the cleavage of its zymogen form, fibrinogen. The coagulation cascade is classically divided into the “extrinsic,” or “tissue factor,” and the “intrinsic,” or “contact activation,” pathways. However, increasing evidence of significant interactions between the components of these two pathways makes it difficult to separate the two in modern descriptions of the coagulation cascade.

Tissue Factor (TF) is the primary initiator of the coagulation cascade, and in particular of the extrinsic pathway of coagulation. TF often exists in nonfunctional form that can be activated through several mechanisms, including Tissue Factor Pathway Inhibitor inactivation, changes in the membrane surrounding TF such as translocation of phosphatidylserine to the outer leaflet of the cell membrane, protein disulfide isomerase-mediated disulphide bond formation between a cysteine pair in TF’s extracellular domain.

Clot formation is initiated when TF residing in the vascular subendothelial matrix is exposed to coagulation factors in the circulation through mechanical or inflammatory injury to the endothelium. TF cleaves and coagulation Factor VII (FVII) to its activated form (FVIIa), forming coagulation Factor X (FX)-ase[166]. The TF/FVIIa complex cleaves FX to generate FXa, which complexes with activated coagulation Factor V (FVa) to form prothrombinase, which cleaves prothrombin to its active form. The TF/FVIIa/FXa

complex and its procoagulant effect are antagonized by the Tissue Factor Pathway Inhibitor (TFPI), which is secreted by activated platelets.

Coagulation can also be initiated through the intrinsic pathway by a process called “contact activation,” in which coagulation Factor XII (FXII) is activated upon interaction with a negatively charged surface that either results in a conformational change and FXII autoactivation, or cleavage of bound FXII by plasma prekallikrein. This leads to the sequential proteolytic cleavage and activation of FXI, FIX, FXIII, and FX. The extrinsic and intrinsic pathways of coagulation merge at this final step, in which FX activation culminates in prothrombinase formation and thrombin generation.

Thrombin, also known as activated coagulation Factor II (FIIa) is a serine protease common to both pathways of the coagulation cascade. Its main function is the cleavage of fibrinogen to fibrin, and is formed from the proteolytic cleavage of its zymogen, prothrombin (coagulation Factor II, FII), by prothrombinase. It can also amplify the procoagulant response via activation of FVIII and FV, and by the activation of FXIII, which is responsible for clot stabilization through fibrin crosslinking.

Thrombin is a potent procoagulant molecule that central to clot formation, and so is carefully regulated via several different mechanisms. Thrombin can be inactivated by the actions of Antithrombin (AT), α1-antitrypsin, α2- macroglobulin, and activated Protein C (APC) in concert with several accessory molecules, including thrombomodulin and endothelial protein C receptors (EPCR). α1-antitrypsin and α2-macroglobulin are general antiproteases produced by the liver. They target many different proteases other than thrombin; for example, α2-macroglobulin also inhibits kallikrein and plasmin, two key molecules in fibrinolysis, the process by which fibrin clots are degraded. APC is a serine protease that circulates as a Vitamin-K dependent zymogen form, Protein C (PC). It is activated by binding to thrombin, particularly that which is bound to thrombomodulin (TM). EPCR is an endothelial transmembrane glycoprotein that binds PC and APC in a

calcium-dependent manner that is highly expressed in the large blood vessels and the trophoblast[167]. EPCR has important anticoagulant functions through promotion of the activation of protein C. Protein C is activated by thrombin, a process that is significantly enhanced when protein C is simultaneously bound to thrombomodulin and on endothelial cell surfaces. APC proteolytically inactivates coagulation FVa and FVIIIa, impeding amplification of a procoagulant response. EPCR is highly expressed in arteries and veins, but is only expressed at low levels in many microvascular beds, including those in the brain[168].

AT is a serpin that is the main plasma inhibitor of coagulation. It has the ability to inhibit FXa and thrombin activity via a reactive loop that mimics serine protease substrates, causing the proteases to be trapped in a covalent, inactive complex when the loop is cleaved. This ability is increased by conformational changes induced upon the binding of AT to naturally circulating heparins. A major class of anticoagulant, the heparins, activate AT, enhancing its activity several 1,000- fold. They are known to bind AT via a specific pentasaccharide sequence in heparin, without which they can still activate AT, though it requires much higher concentrations of heparin to do so. They occur naturally in the body in small amounts in order to maintain hemostasis, but are also administered exogenously for the treatment of thrombotic disorders. Among these drugs are low molecular weight heparins (LMWH) and unfractionated heparin (UFH). While both inhibit FXa and thrombin, LMWH is less efficient at thrombin inhibition. This is due to the fact that while the characteristic pentasaccharide sequence central to its activity is sufficient to block FXa activity, thrombin inhibition requires a bridging contribution from heparin for the formation of a thrombin/AT/heparin complex, and LMWH is not large enough to effectively provide this[169].

While these drugs are effective and widely used in clinical settings, several concerns surround the use of heparins. Both UFH and LMWH can cause heparin-

induced thrombocytopenia (HIT), resulting from the immunologic activation of platelets. Most heparins are isolated from animal sources, rendering them prone to contamination and raising concerns of anaphylaxis in some patients treated with these drugs. An alternative to these is fondaparinux, a selective but indirect FXa inhibitor composed solely of the heparin pentasaccharide sequence known to bind AT. This synthetic drug results in fewer clinical complications, but is costly and complicated to make, and has no effective antidote to combat excessive bleeding[170].

Another key player in thrombosis and hemostatic is the platelet. Platelets are tiny (~1-3 um), discoid, anuclear cells produced by megakaryocytes in the bone marrow [126]. In addition to mediating clot formation by initiating fibrin generation, particularly through calcium-dependent exposure of negatively charged phospholipids like phosphatidyserine[171], activated platelets serve as a major structural component of hemostatic plugs. Platelets can be activated by two distinct mechanisms. In the first, platelets are recruited to the site of injury upon exposure of subendothelial matrix proteins like collagen and von Willebrand Factor (vWF) not normally present in the intravascular space to circulating blood. These proteins interact with GPVI and GPIb-V-IX receptors, respectively, on the surface of the platelets, leading to the capture and tethering of platelets to the blood vessel wall and platelet activation[172]. Alternatively, platelets can be activated by thrombin independently of collagen, vWF, GPVI, and even endothelial disruption through the cleavage of PAR1 (PAR4 in mice) on the platelet surface[173]. One of these two methods may be favored over another depending on the disease or injury, but ultimately both result in platelet activation, conformational change, and degranulation, inducing platelet aggregation with leukocytes and other platelets mediated by GPIIb/IIIa (αIIbβ3) binding to fibrinogen and vWF and the release of platelet agonists like ADP and thromboxane A2 (TXA2)[173]. Most agonists that activate platelets do so through G-protein coupled receptors (GPCRs); for example, ADP release

stimulates platelet activation via the GPCRs P2Y1 and P2Y12, and TXA2 activates the thromboxane receptor (TP). Activation of GPIIb/IIIa and subsequent platelet aggregation are the ultimate result of receptor binding by any of the platelet.

Several anticoagulant drugs act by interfering with platelet activation and function. Notable among these are aspirin and Plavix. Aspirin, a non-steroidal anti-inflammatory drug (NSAID), interferes with platelet aggregation by irreversibly inhibiting COX-1; this prevents the generation of thromboxane, which as a result cannot go on to bind TP. Plavix (Clopidogrel) is a P2Y12 antagonist that interferes with the receptor's ability to react to ADP agonism. The downstream result of both of these drugs is failure to activate the GPIIb/IIIa receptor, which in turn will be unable induce further platelet activation and aggregation. Aspirin and Plavix both induce significant, but not complete, inhibition of platelet activation, and as such markedly reduce platelet aggregation in patients but do not completely ablate their function.

Novel oral anticoagulant drugs (NOACs) represent a major advance in modern anticoagulant treatment. The advantages these drugs have several over traditional anticoagulant therapies are primarily that they are safer and more convenient to use. Relative to tradition therapies such as LMWH, NOACs have an overall lower bleeding risk, no potential to induce HIT, fewer interactions with food or other drugs, a wide therapeutic window, fixed dosing without the need for routine monitoring by a physician and are provided in a convenient pill, rather than injectable, form[174–176,170,177,178]. Currently available NOACs include dabigatran etexilate, a prodrug of the oral direct thrombin inhibitor dabigatran, and four oral FXa inhibitors: apixaban, rivaroxaban, edoxaban and betrixaban.

The Intersection of Inflammation, Coagulation and Disease

Inflammation and coagulation have a shared evolutionary history. Though they have evolved into separate processes in vertebrates, significant interaction and communication between mammalian inflammation and coagulation serve as a modern reminder of their evolutionary link.

Such interactions have been well studied in the context of human inflammatory diseases and their model systems. The “inflammation-coagulation cycle,” in which inflammation activates a procoagulant response that in turn promotes further inflammation, has been well characterized in models of bacterial sepsis and inflammatory bowel disease. Acute inflammatory response to such inflammatory injury or disease can result in systemic activation of coagulation, called “disseminated intravascular coagulation,” leading to severe, life-threatening thrombosis[179].

TF itself plays a significant role in the intersection of inflammation and coagulation; in addition to being the initiator of the extrinsic pathway of coagulation, it is also a member of the cytokine receptor superfamily of proteins, and so can function as a signaling receptor[166]. TF on many different cell types has shown to signal a transient increase in intracellular calcium ion concentration upon binding with FVIIa, the only ligand found to induce TF signaling. Further work on the subject has shown it signals through the Src signaling pathway, with downstream effects on p38 and ERK1/2. Proinflammatory cytokines TNF-α, IL-6 and IL-1β were shown to induce the expression of TF in endothelial cells, an effect that can be modulated by IFN-γ or IL-4[180,181]. The effect of TNF-α and IL-6 on TF expression can be so strong that treatment with local thrombin inhibitors are insufficient to prevent fibrin deposition in areas of TF upregulation[182]. Monocytes are the dominant TF-expressing cell type, though neither they nor endothelial cells constitutively express TF; LPS, some cytokines, including TNF-α, IL-1, and IL-6, and platelets can all stimulate TF expression on monocytes and

endothelial cells. Neutrophil Extracellular Traps (NETs) are capable of enhancing TF procoagulant activity. Neutrophil elastase on NETs indirectly enhance TF activity through cleavage and inactivation of TFPI[183]. Extracellular histones, a known proinflammatory mediator released by neutrophils on NETs or by dying cells in response to inflammatory damage, contribute to intravascular thrombin formation through the induction of TF expression on vascular endothelial cells and monocytes in an NF-κB and AP-1-dependent mechanism via TLR2 and TLR4 activation[184]. Importantly, blocking the TF/FVIIa pathway reduces coagulopathy and inflammation in models of septic shock, and even reduces mortality[185186,187].

Platelets further facilitate communication between the inflammatory and hemostatic responses. Upon activation, platelets not only aggregate with each other, but also with leukocytes via P-selectin/ P-selectin glycoprotein ligand-1 (PSGL-1) interactions. Both proteins are expressed on both cell types, though P-selectin is more abundant on platelets, and PSGL-1 is more abundant on leukocytes. P-selectin/PSGL-1 binding on monocytes leads to monocyte activation, TF expression on the monocyte surface, and the release of PSGL- 1/TF/Phosphotidylserine (PS)+ microparticles. PS is also increased on the surface of the monocyte, and its negative charge serves to amplify contact activation of FXII. Interestingly, platelets have also been shown to have bactericidal activity resulting from expression of the pattern recognition receptor Toll-like Receptor (TLR) 4 on their surfaces. When activated, TLR4-expressing platelets migrate to and bind activated neutrophils, which form Neutrophil Extracellular Traps. Platelets also release numerous factors that recruit and augment the microbicidal activity of leukocytes, including CXCL1, CXCL4, CXCL5, CXCL7, CCL3, CCL5, CCL7, CD154 and the triggering receptor expressed on myeloid cells 1 (TREM1)[173,188–190].

Thrombin has also proven to be an important linkage protein between inflammation and coagulation due to its broad stimulatory capacity. Thrombin has been

shown to induce a procoagulant state by stimulating the production of inflammatory cytokines such as IL-1β, IL-6, and TNF-α through NF-κB signaling, and of platelet-activating factor (PAF), a neutrophil chemoattractant[183]. IL-6 production resulting from thrombin-stimulated NF-κB signaling is then able to upregulate TF as mentioned previously, continuing in a TF-Thrombin-IL-6 positive feedback loop. Thrombin also induces upregulated expression of ICAM-1 and vascular CAM-1 (VCAM-1) on monocytes, contributing to their adherence to vessel walls[191].

Thrombin and FXa are significantly linked to inflammatory mediators through their action on Protease Activated Receptors (PARs), GPCRs that are activated by cleavage of a part of their extracellular domain. There are four PAR types, PAR1-4. PARs are particularly highly expressed on platelets, but also to a lesser degree on many other cell types, including endothelial cells, mononuclear cells, fibroblasts, and smooth muscle cells. Thrombin cleaves PAR-1 or PAR-4 on human platelets, and resulting activation of the receptor continues the procoagulant response by inducing platelet aggregation and can cause endothelial cell damage by stimulating an influx of calcium ions, nitric oxide release, and endothelial cell retraction. Thrombin or FXa cleavage can activate the PAR1-sphingosine-1-phosphate receptor 3 axis on dendritic cells, activating a massively proinflammatory inside-out response[192]. PAR-2 can signal to promote inflammation through the activation of the MAPK signaling pathway and the transcription factor Nuclear Factor-κB (NF- κB). PAR-2 is activated by the TF/FVIIa/FXa ternary complex or by trypsin to recruit leukocytes and mediate aspects of eosinophil function, including the NF-κB-dependent production of proinflammatory cytokines, superoxide generation, and degranulation. APC can also cleave PAR-1, leading to a transactivation of PAR-2 and a suppression of inflammation; however, thrombin is 1,000-10,000-fold more efficient at cleaving PAR-1, so this does not happen as readily as thrombin PAR-1 cleavage[193].

Natural anticoagulant molecules also interact with inflammation, primarily in a negative regulatory capacity. AT has an indirect anti-inflammatory action resulting from decreased thrombin generation with increased AT activity. Interestingly, it can also directly interfere with the production of pro-inflammatory cytokines through transcription factor NF-κB inhibition by preventing the phosphorylation and proteolytic degradation of IκBα, the inhibitor of NF-κB, resulting in decreased levels of IL-6, TNF-α, and TF[183]. TM is also dampens inflammation by interfering with complement activation, binding LPS in the context of a bacterial infection, and by binding high mobility group box 1 (HMGB-1), a danger-associated molecular pattern that promotes inflammation.

This interaction between inflammation and coagulation in response to disease, in particular infectious disease, inspired the concept of "immunothrombosis." Immunothrombosis is the idea that in addition to its procoagulant function, thrombosis serves as an intravascular component of the innate immune response[183].

Activated Coagulation in Malaria

Coagulation may play a significant pathologic role in severe falciparum malaria. Clinical evidence suggests the presence of a hypercoagulable state in both cerebral (CM) and placental malaria (PM) patients. The most common indicators of coagulation in malaria patients are consumption-induced thrombocytopenia[128,175,194,195] and fibrin deposition[5,28,105,108]. Several studies have shown that the degree of coagulation activation correlates with the severity of disease, though not all[196–198]. Overt Disseminated Intravascular Coagulation (DIC) occurs in 5-10% of severe malaria cases; however, it is seen at a higher frequency (~20%) and is correlated with fatal outcome in retinopathy-positive cases of pediatric CM[179].

Procoagulant activity in malaria has been characterized best in CM. Autopsy examination of brains from fatal pediatric CM revealed fibrin deposition and

hemorrhages occur near sites of IE adherence to the microvascular endothelium[82]. Fibrin deposition is commonly seen in brain tissue in fatal CM[108,199]. IE have also been found to have direct procoagulant activity, inducing TF expression and activate thrombin on the apical surface of endothelial cells and supporting assembly of the extrinsic Xase (TF-FVIIa) complex *in vitro*[200]. Microvascular obstruction due to hemostatic dysregulation is also a feature of CM; long von Willebrand Factor (vWF) multimers resulting from ADAMST13 deficiency, a vWF protease, tether IE and platelets to the endothelium, occluding microvessels[201].

Endothelial Protein C Receptor (EPCR) plays a particularly important role in CM pathogenesis. IE expressing PfEMP1 variants linked to severe malaria bind to EPCR on the brain microvasculature, likely directly interfering with protein C binding and activation by EPCR[109]. Depletion of EPCR in the brain microvasculature was observed in autopsied brains from fatal pediatric CM cases, which was negatively correlated with increasing IE sequestration in the brain[110]. This same study revealed significantly reduced thrombomodulin (TM) expression in subcutaneous microvessels, where IE sequestration also occurs. Loss of endothelial EPCR and TM were associated with increased concentrations of soluble EPCR and TM, respectively, in the patients' cerebrospinal fluid, suggesting these receptors are shed in response to malaria infection, though this occurrence has yet to be directly observed[110]. IE binding to EPCR and subsequent loss of EPCR and TM from the brain endothelium likely contribute to significant disruptions to the anticoagulant protein C system observed in CM, exacerbating the procoagulant state in CM-affected brains[82,202]. EPCR is also known to bind to Mac-1 (CD11b/CD18), contributing to monocyte adhesion to the brain microvasculature, another common pathologic feature of CM[203].

Evidence of a procoagulant state also exists in both human and murine models of PM. A common notable feature of human PM is significant fibrin deposition in the

placentae of infected individuals relative to uninfected placentae, often with Hz trapped inside it[29,43]. Analysis of clinical samples from a public referral hospital in Kisumu, Kenya showed malaria infection induces enhanced perivillous fibrin deposition, and an increase plasminogen activator inhibitor-1 (PAI-1) and of D-dimers, markers of activated coagulation and fibrinolysis, in intervillous blood plasma. PAI-1 levels were significantly increased in placentae exhibiting high, but not low fibrin deposition; conversely, a significant increase in D-dimer concentration was associated with low amounts of fibrin in the placenta, but not high. In this study, birth weight trended downward and density of inflammatory infiltrates in the intervillous blood space correlated positively with high fibrin score[42]. Significantly increased infiltration of TF-expressing monocytes has been reported in *P. falciparum*-infected human placentae[204].

A similar pathogenic procoagulant environment has been observed in murine models of PM. As in human PM, significant placental fibrin deposition is commonly seen in murine models of PM[42,64]. In a model of malaria infection in early gestation, malaria-infected, pregnant mice treated with research-grade low molecular weight heparin showed reduced placental pathology and successfully maintain pregnancy through midgestation[42]. Placental TF has been shown to contribute to poor pregnancy outcome and vascular dysregulation in a murine model of recurrent spontaneous miscarriage, and may contribute to placental pathology during PM; placental thin-sections from *P. chabaudi* AS-infected mice aborting mid-gestation showed elevated levels of TF in the ST, but not in the cytotrophoblast or trophoblast giant cells, which are not in contact with maternal blood. TF upregulation on the ST was ablated in this model following treatment with a TNF-α neutralizing antibody[76].

Summary

The multifaceted host responses to placental and cerebral malaria pose a significant challenge to developing effective therapies against these diseases. While much evidence exists to implicate the interaction of inflammation and coagulation in the pathogenesis of these severe clinical manifestations of malaria, large gaps in knowledge will need to be filled before a complete understanding of the pathogenic mechanisms underlying these diseases can be formed. The experiments in this dissertation explore how the extrinsic pathway of coagulation contributes to CM and PM through the use of genetic modification and targeted anticoagulant drugs. The following work provides valuable insight into the procoagulant response to severe malaria and the potential of the extrinsic pathway of coagulation as a target for adjunctive therapy to mitigate poor disease outcomes.

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CHAPTER 3

CELL-SPECIFIC DELETION OF TISSUE FACTOR AND TARGETED ANTICOAGULANT THERAPY ALTER THE IMPACT OF *PLASMODIUM CHABAUDI* AS INFECTION ON MURINE PREGNANCY OUTCOME[1]

[1] Bracken TC, Cooper CA, Moore JM. To be submitted to *Plos Medicine*.

Abstract

Sequestration of *Plasmodium falciparum*-infected erythrocytes in the maternal blood space of the placenta results in a severe clinical manifestations of this disease, placental malaria (PM). PM results in disruption of placental function, leading to low birth weight, or in extreme cases, fetal loss. Recent evidence indicates the existence of a procoagulant state in both human and experimental murine PM, including extensive fibrin deposition and Tissue Factor (TF) expression in affected tissues, indicating dysregulated coagulation contributes to malaria pathogenesis. However, the molecular basis for these pathologies remains incompletely understood. Timed pregnancy experiments were conducted using 8-10 week old female mice with floxed TF expressing Cre-recombinase under the Tie2 promoter (Tie2Cre+) or under the Lysozyme M promoter (LysMCre+) and their phenotypically normal Cre-negative littermates (Tie2Cre- and LysMCre-, respectively). Clinical parameters were measured between gestational days 0-12, embryo viability and health were assessed at sacrifice and whole conceptuses were preserved in paraformaldehyde and processed for histology or flash frozen for gene expression analyses. Malaria-infected, pregnant (IP) Tie2Cre+ mice exhibit improved embryo health and reduced placental pathology at G12 relative to IP Tie2Cre- littermates; however, embryos are not rescued to an uninfected phenotype, and may not survive to term. Additionally, IP Tie2Cre+ mice exhibit significantly increased pregnancy-associated weight gain at GD12 relative to IP Tie2Cre- mice ($p=0.0338$). Though there is no significant difference in the magnitude of peak percent weight gain or parasitemia between the two strains, IP LysMCre+ mice abort and reach peak parasitemia two days earlier than IP LysMCre- mice. These results indicate that TF on both hematopoetic cells and either maternal endothelium or fetal-derived trophoblast play a significant role in determining pregnancy outcome during malaria infection.

Introduction

Sequestration of *Plasmodium falciparum*-infected erythrocytes in the maternal blood space of the placenta results in a severe clinical manifestation of malaria, termed placental malaria (PM). The maternal response to PM induces severe placental pathology, disrupting normal ST physiological function and interfering with the fetus's growth and development[1]. Placental insufficiency, preterm delivery (before 37 weeks gestation), fetal growth restriction (FGR) and maternal anemia contribute significantly to the high prevalence of malaria-associated low birth weight (LBW, <2500g) in endemic regions (20% of live births in malaria-endemic areas between 1985 and 2000)[2].

Recent evidence indicates the existence of a procoagulant state in both murine models of PM and the human clinical disease[3–6]. A common notable feature of human PM is significant fibrin deposition in the placentae of infected individuals relative to uninfected placentae, often with hemozoin, the by-product of malarial hemoglobin digestion, trapped inside it [1,3,6,7]. Placental malaria infection has been shown to induce enhanced perivillous fibrin deposition and increased plasminogen activator inhibitor-1 (PAI-1) and D-dimer levels, markers of activated coagulation and fibrinolysis, in intervillous blood plasma. Histological fibrin score has been shown to positively correlate with the density of inflammatory cell infiltration in the intervillous blood space during human PM, and is negatively associated with birth weight[6].

Significant placental fibrin deposition is also commonly seen in murine models of PM [6,8]. Previous work in our lab has shown that infected mice treated with research-grade low molecular weight heparin exhibit reduced placental pathology and successfully maintain pregnancy through midgestation[6]. Together, these results indicate dysregulated coagulation contributes to malaria pathogenesis, but the molecular basis for these pathologies remains incompletely understood.

In these studies, we attempted to further characterize the role of the extrinsic pathway of coagulation in mediating malaria-associated midgestational pregnancy loss by interfering with that pathway through genetic modification and chemotherapeutic interventions. Cre-lox technology is a useful tool for the tissue-specific deletion of a gene of interest[9]; here, we take advantage of that technology to identify relevant tissue sources of Tissue Factor (TF), the initiator of the extrinsic pathway of coagulation, that may be contributing to malaria pathogenesis. These tissue sources include the maternal endothelium, maternal hematopoietic cells[10], maternal myeloid cells[11,10], and the fetal-derived trophoblast[12], the placental cell type in contact with the maternal blood and, therefore, malaria-infected red blood cells.

The chemotherapeutic interventions applied in these studies include dalteparin, a low molecular weight heparin with slightly different activity than the research-grade low molecular weight heparin previously described, and fondaparinux, a selective but indirect FXa inhibitor composed solely of the heparin pentasaccharide sequence known to bind antithrombin. In addition to its procoagulant function as a component of the prothrombinase complex, FXa is known to play an important role in mediating proinflammatory signaling, particularly through its ability to cleave Protease Activated Receptors[13]. Thus, inhibiting FXa function could be beneficial during PM due to a direct anticoagulant and indirect anti-inflammatory effect. The results of these studies indicate that coagulation is an important mediator of PM pathogenesis, and thus may present an attractive target for adjunctive therapy to mitigate poor birth outcomes associated with malaria infection during pregnancy.

Materials and Methods

Parasites and Mice

Plasmodium chabaudi AS was routinely passaged from frozen stocks in female

A/J mice as previously described[14].

C57BL/6J mice were originally purchased from The Jackson Laboratory and used to generate breeding stock and experimental animals in the University of Georgia Coverdell Vivarium. Transgenic mice used in these experiments include mice with floxed Tissue Factor (TF, *F3*), the initiator of the extrinsic pathway of coagulation, expressing Cre-recombinase under the control of the Tek (Tie2) or Lyz2 (LysM) promoters.

Founder *F3*$^{flox/flox}$LysMCre breeder pairs were generously donated by Dr. Nigel Mackman, University of North Carolina, Chapel Hill, NC, USA, and were maintained by mating hemizygous *F3*$^{flox/flox}$LysMCre^{+} (TF$^{flox/flox}$,LysMCre+) studs with *F3*$^{flox/flox}$LysMCre^{-} (TF$^{flox/flox}$,LysMCre-) dams. *F3*$^{flox/flox}$Tie2Cre mice were initially generated by mating unfloxed hemizygous Tie2Cre^{+} studs sourced from the Jackson Laboratory with *F3*$^{flox/flox}$, LysMCre^{-} dams bred in-house from the line established by the founders describes above. Once a *F3*$^{flox/flox}$Tie2Cre line was established, it was maintained by breeding hemizygous *F3*$^{flox/flox}$Tie2Cre^{+} (TF$^{flox/flox}$, Tie2Cre+) studs with *F3*$^{flox/flox}$Tie2Cre^{-} (TF$^{flox/flox}$,Tie2Cre-) dams.

TF$^{flox/flox}$, Tie2Cre+ mice have tissue specific deletions of TF in vascular endothelial cells and TF$^{flox/flox}$, Tie2Cre+ fetal-derived trophoblast. Phenotypically normal TF$^{flox/flox}$,Tie2Cre- littermates are used as controls in experiments that included TF$^{flox/flox}$, Tie2Cre+ mice. TF$^{flox/flox}$,LysMCre+ mice have a specific deletion of TF from myeloid cells. Phenotypically normal TF$^{flox/flox}$,LysMCre- littermates were used as controls in these experiments. The transgenic strains tested in these experiments are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities compared to C57BL/6J mice.

All experimental mice were virgin females between the ages of 8-10 weeks old. Stud males derived from the same breeders as the experimental virgin females were

used until approximately 6 months of age, then replaced by younger males of the same lineage.

Mouse experiments were performed in accordance with the guidelines and with the approval of the University of Georgia Institutional Animal Care and Use Committee (Animal Use Protocol number A2015-03-005-Y1-A1).

Initiation of Pregnancy, Infection and Clinical Assessment

Timed pregnancy experiments and monitoring of experimental mice was performed using a previously established protocol[14]. Briefly, the day on which a vaginal plug was observed in time-mated 8- to 12-week old female mice was gestational day zero (GD0). Mice were infected via tail vein injection on GD0 with 10^3 malaria-infected red blood cells. Mice were monitored daily from GD6 to GD12. Each mouse was weighed, and blood collected from the tail vein into a heparinized capillary tube to measure parasitemia and hematocrit. Parasitemia was measured by flow cytometric analysis as previously described[15]. Remaining blood was centrifuged in a microhematocrit centrifuge and percent hematocrit was calculated according to the following: (volume of packed erythrocytes)/(total blood volume)x100%.

On GD12, mice of all strains were anaesthetized using 2.5% Avertin (2,2,2-Tribromoethanol) administered via intraperitoneal injection and euthanized by exsanguination through the caudal vena cava into acid citrate dextrose (final concentration, 0.76%). Embryo viability was assessed at necropsy as previously described[6]. Briefly, embryos exhibiting extensive intrauterine and/or intraembryonic hemorrhaging by gross pathology or lacking fetal heartbeat were scored as nonviable. Gross pathologic images were captured using a Canon EOS 70D Digital SLR Camera.

Mouse Conceptus Histology

Uteri were fixed in neutral-buffed formalin for overnight and paraffin-embedded for histological analysis. H&E-stained and unstained thin sections (5 μm thick) were generated from paraffin-embedded tissues. Indirect immunolocalization of fibrin(ogen) was performed on unstained sections using a polyclonal rabbit anti-human fibrin/fibrinogen/fibrin fragment D- and E-reactive antibody (A0080, Dako North America, Inc.), which has cross-reactivity with mouse fibrin and fibrinogen.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software package (version 6.0). Correlation analysis was done using Spearman's test and 2x2 contingency tables were used for testing differences between proportions. The significance of difference of group means in the case of normally distributed data were compared via t-tests for pair-wise comparisons. Non-normally distributed data were analyzed by a non-parametric, Mann-Whitney test for pairwise comparisons. Differences with $p \leq 0.05$ were considered significant unless corrections were required for multiple comparisons.

Results

Tissue Factor-floxed Tie2Cre+ female mice mated with hemizygous Cre-expressing males are protected from malaria-induced mid-gestational pregnancy loss.

P. chabaudi AS-infected pregnant $TF^{flox/flox}$,Tie2Cre- female mice exhibit malaria-associated pregnancy loss beginning GD10, whereas $TF^{flox/flox}$,Tie2Cre+ mice maintained pregnancy through the duration of the experiment. Pregnancy-associated weight gain was significantly decreased in $TF^{flox/flox}$,Tie2Cre- on GD11-12 relative to both uninfected pregnant mice of the same strain (Figure 3.1a) and the infected pregnant $TF^{flox/flox}$,Tie2Cre+ group (Figure 3.1e). However, infected pregnant $TF^{flox/flox}$,Tie2Cre+

mice continued gaining weight through GD12 in a manner indistinguishable from that seen in the uninfected pregnant group (Figure 3.1c). No significant differences were seen between infected pregnant groups in the course of parasitemia (Figure 3.1f) or malaria-induced change in hematocrit (Figure 3.1g).

Maternal endothelial or hematopoietic cell-derived Tissue Factor contributes to murine placental malaria pathogenesis.

At GD12, no significant difference was seen between uninfected pregnant $TF^{flox/flox}$,Tie2Cre- and $TF^{flox/flox}$,Tie2Cre+ littermates in indicators of poor pregnancy outcome, including average uterus weight (Figure 3.2a), total number of embryos per uterus (Figure 3.2b), proportion of resorbing embryos per total number of embryos, or prevalence of intrauterine hemorrhaging (Table 3.1). Uninfected mice of these strains were indistinguishable at a gross macroscopic level (Figure 3.2e, 3.2f).

Infected pregnant $TF^{flox/flox}$,Tie2Cre- mice exhibited significantly worse pregnancy outcome relative to infected pregnant $TF^{flox/flox}$,Tie2Cre+ mice. At GD12, $TF^{flox/flox}$,Tie2Cre+ exhibit significantly greater average number of embryos per uterus ($p=0.032$, Figure 3.2d) and a significantly smaller proportion of resorbing embryos ($p=<0.0001$, Table 3.1), with a corresponding significant increase in average uterine weight ($p=0.041$, Figure 3.2c). Though prevalence of hemorrhaging was similar between groups (Table 3.1), intrauterine bleeding appeared relatively less severe in $TF^{flox/flox}$,Tie2Cre+ mice upon gross macroscopic evaluation (Figure 3.2g, 3.2h).

$TF^{flox/flox}$,Tie2Cre+ mice are protected from malaria-induced placental pathology at mid-gestation.

Histological examination revealed reduced malaria-associated pathology in whole conceptuses from infected pregnant $TF^{flox/flox}$,Tie2Cre+ mice relative to

$TF^{flox/flox}$,Tie2Cre- conceptuses. H&E stained thin sections from uninfected pregnant mice of both strains showed that the three primary anatomical zones of the placenta, the decidua, junctional zone and labyrinth, were intact with no evidence of tissue degradation, necrosis or fibrin deposition (Figure 3.3a-c,g-i). Light fibrin(ogen) staining was visible by immunohistochemical analysis, expressed diffusely throughout the decidua and junctional zone and localized to tissues surrounding blood vessels in the labyrinthine zone (Figure 3.4a-c,g-i). Extensive fibrin deposition was observed in infected $TF^{flox/flox}$,Tie2Cre- placentae; large deposits of fibrin were visible in the labyrinth by immunohistochemistry (Figure 3.4d-f) and on H&E stained sections surrounding degraded trophoblast, pyknotic nuclei, fetal red blood cells, parasitized red blood cells, and hemozoin (Figure 3.3d-f). Hemozoin and erythrocytes engulfed by trophoblast giant cells were also seen on H&E-stained thin sections. In contrast, placentae from $TF^{flox/flox}$,Tie2Cre+ mice closely resembled uninfected placentae, showing little placental pathology, though hemozoin deposition was commonly visible in trophoblast giant cells(Figure 3.3j-l).

Employment of an alternative mating scheme involving homozygous Cre-expressing ($TF^{flox/flox}$,Tie2Cre+/+) stud males resulted in a similar, but altered phenotype. Infected pregnant $TF^{flox/flox}$,Tie2Cre- females abort early such that no material remains in uteri at GD12, though uteri are highly vascularized (data not shown). At GD10, $TF^{flox/flox}$,Tie2Cre- uteri exhibit a phenotype similar to that seen in infected GD12 $TF^{flox/flox}$,Tie2Cre- mated with hemizygous Cre-expressing males, though less developed (Figure 3.5a-d, Figure 3.6a-c). However, infected $TF^{flox/flox}$,Tie2Cre+ females mated with $TF^{flox/flox}$, Tie2Cre+/+ results in uteri containing individual healthy embryos surrounded by a majority hemorrhagic, necrotic, resorbing embryos at an average ratio of approximately 1:3 (Figure 3.5m-t).

H&E-stained thin histological sections of paraffin-embedded whole conceptuses

show engulfed red blood cells in the trophoblast giant cells of both infected and uninfected placentae of both strains. Uninfected placentae from $TF^{flox/flox}$,Tie2Cre- and $TF^{flox/flox}$,Tie2Cre+ look otherwise normal, with no disruption of placental tissue architecture. Infected $TF^{flox/flox}$,Tie2Cre- exhibit extensive placental pathology, including trophoblast degradation and extensive fibrin deposition and hemorrhaging throughout the labyrinth and thickened junctional zone. Hemozoin, infected red blood cells, and pyknotic nuclei are visible in the deposited fibrin. As would be expected, healthy and resorbing conceptuses from $TF^{flox/flox}$,Tie2Cre+ mice show significantly different phenotypes. Healthy conceptuses retain intact placental architecture, with no difference in fibrin(ogen) staining visible by immunohistochemistry or fibrin deposition assessed on H&E stained sections. However, hemozoin engulfed by trophoblast giant cells is prevalent in these conceptuses. Unhealthy conceptuses from $TF^{flox/flox}$,Tie2Cre+ females have a thickened junctional zone, hemorrhaging and fibrin deposition throughout the junctional zone and labyrinth, though not to the extent seen in $TF^{flox/flox}$,Tie2Cre- conceptuses, and hemozoin and infected red blood cells trapped in fibrin and engulfed by trophoblast giant cells.

Tissue Factor-floxed, LysMCre+ mice exhibit accelerated malaria-associated pregnancy loss.

Similar to pregnancy outcomes exhibited by infected $TF^{flox/flox}$,Tie2Cre- mice, *P. chabaudi* AS-infected pregnant $TF^{flox/flox}$,LysMCre- mice begin aborting at GD10, as evidenced by significantly decreased pregnancy-associated weight gain in infected mice relative to the uninfected group on GD11-12 (Figure 3.7a). Infected pregnant $TF^{flox/flox}$,LysMCre+ appear to abort one day earlier than the TF-intact group, with significantly decreased weight gain relative to uninfected mice and the infected pregnant $TF^{flox/flox}$,LysMCre- group becoming evident on GD10 (Figure 3.7c,e). Following

pregnancy loss in $TF^{flox/flox}$,LysMCre- mice on GD10, reductions in pregnancy associated weight gain did not significantly differ between infected pregnant groups (Figure 3.7e).

Though reductions in hematocrit were similar between infected groups (Figure 3.7f), the course of parasitemia varied significantly in infected pregnant $TF^{flox/flox}$,LysMCre+ mice relative to $TF^{flox/flox}$,LysMCre- mice. Parasitemia in $TF^{flox/flox}$,LysMCre+ followed a similar course to that seen in $TF^{flox/flox}$,LysMCre- mice until GD10; at this timepoint, parasitemia began to decline in the $TF^{flox/flox}$,LysMCre+ group, whereas it rose to peak parasitemia on this day in $TF^{flox/flox}$,LysMCre- mice (Figure 3.7g).

Myeloid cell-specific deletion of Tissue Factor worsens murine placental malaria outcome.

Both infected pregnant groups failed to maintain pregnancy, with a significant proportion of embryos resorbing at GD10. Poor pregnancy outcomes were exacerbated in $TF^{flox/flox}$,LysMCre+ mice compared to in $TF^{flox/flox}$,LysMCre- mice, with significantly smaller uteri ($p=0.0023$, Figure 3.8c) and significantly greater numbers of resorbing embryos ($p<0.0001$, Table 3.2) observed in in $TF^{flox/flox}$,LysMCre+ mice at GD10. Gross macroscopic evaluation at GD10 revealed infected $TF^{flox/flox}$,LysMCre+ uteri contained small, necrotic embryos (Figure 3.8h), whereas blood pools and resorbing embryos but little necrotic tissue were visible in $TF^{flox/flox}$,LysMCre- uteri (Figure 3.8g). No significant difference was seen in the average number of embryos or prevalence of intrauterine hemorrhaging in infected uteri from either strain (Table 3.2). No significant difference was seen in pregnancy outcome between uninfected pregnant $TF^{flox/flox}$,LysMCre- and $TF^{flox/flox}$,LysMCre+ mice (Table 3.2, Figure 3.8a-b,e-f).

Myeloid cell-derived Tissue Factor deletion results in reduced malaria-associated fibrin deposition in the murine placenta.

In spite of accelerated pregnancy loss in $TF^{flox/flox}$,LysMCre+ mice, histological analysis of infected $TF^{flox/flox}$,LysMCre+ conceptuses showed placental pathology was not as severe in these mice as in $TF^{flox/flox}$,LysMCre- controls. Infected pregnant $TF^{flox/flox}$,LysMCre- placentae exhibited severe placental pathology, with thickening of the junctional zone, degraded spongiotrophoblast, enlarged giant trophoblast cells containing engulfed hemozoin and infected red blood cells, and extensive fibrin deposition with trapped hemozoin, infected red blood cells, fetal red blood cells and pyknotic nuclei throughout. Immunohistochemical staining for fibrin deposition shows corresponding intense fibrin(ogen) staining in the thickened junctional zone and, to a lesser extent, in the labyrinth. While pockets of fibrin containing malarial components and degraded trophoblast are visible by both H&E and immunohistochemical fibrin(ogen) staining, placental tissue architecture degradation and fibrin deposition are less evident in infected $TF^{flox/flox}$,LysMCre+ placentae compared to infected $TF^{flox/flox}$,LysMCre- placentae.

Placental architecture was undisturbed in samples from uninfected pregnant mice of both genotypes, with the decidua, junctional zone and labyrinth clearly intact. Immunohistochemical analysis revealed similar fibrin(ogen) staining patterns in uninfected $TF^{flox/flox}$,LysMCre+ and Cre- placentae as those seen in uninfected pregnant $TF^{flox/flox}$,Tie2Cre+ and Cre- mice; samples stained positive for fibrin(ogen) throughout the decidua and junctional zone, but was localized to maternal and fetal blood vessel walls in the labyrinth.

Anticoagulant drug treatment mitigates malaria-associated midgestational pregnancy loss.

Whereas sham-treated infected pregnant mice aborted on GD10, *P. chabaudi* AS-infected, pregnant C57BL/6J mice treated with the low molecular weight heparin, dalteparin, or with the specific coagulation factor Xa inhibitor, fondaparinux, successfully maintained pregnancy through GD12. Through the entire course of infection, infected pregnant dalteparin- and fondaparinux-treated mice were indistinguishable from their respective uninfected control mice, exhibiting comparable weight gain throughout the experiment (Figure 3.11a,c). Sham-treated mice exhibited significant reductions in pregnancy-associated weight gain on GD11-12 compared to the anticoagulant-treated infected and uninfected pregnant groups (dalteparin, Figure 3.11a; fondaparinux, Figure 3.11c). No significant difference in course of parasitemia (Figure 3.11b,d) or hematocrit (data not shown) was seen between groups.

At GD12 sacrifice, infected dalteparin- and fondaparinux-treated mice exhibited significantly improved pregnancy outcome relative to sham-treated controls. Infected, pregnant dalteparin-treated mice yielded significantly lower proportions of resorbing embryos compared to sham-treated mice ($p<0.0001$, Table 3.3), and uteri from infected dalteparin-treated mice weighed significantly more on average than those from sham treated mice ($p=0.0159$, Figure 3.12a) though uteri from both groups contained the same average number of embryos (Figure 3.12b). Similarly, infected fondaparinux-treated mice had a significantly lower proportion of resorbing embryos ($p<0.0001$, Table 3.3) and significantly greater average uterine weight ($p=0.0167$, Figure 3.12c) than sham-treated control mice. Infected dalteparin- and fondaparinux-treated embryos were round, pink and healthy-looking (Figure 3.12d,f), compared to resorbing, necrotic and hemorrhagic embryos from infected sham-treated uteri (Figure 3.12a,b).

Infected, sham-treated mice exhibited significant placental pathologies typical of murine placental malaria, including thickening of the junctional zone, degradation of the trophoblast, and hemozoin, malaria-infected red blood cells and fetal red blood cells trapped within fibrin (Figure 3.13d-f). Immunohistochemical analysis of fibrin(ogen) deposition showed extensive fibrin deposition in the junctional and labyrinthine zones of sham-treated placentae (Figure 3.14d-f). With the exception of rare, small fibrin deposits and corresponding localized trophoblast degradation, the tissue architecture of infected, dalteparin-treated placentae was almost entirely intact (Figure 3.13g-k, Figure 3.14g-i). Infected, fondaparinux-treated placentae were virtually indistinguishable from uninfected treated placentae by both immunohistochemical analysis for fibrin(ogen) (UP Fondaparinux:Figure 3.14a-c, IP Fondaparinux:Figure 3.14g-i) and on H&E-stained thin histological sections (Figure 3.13a-c,g-i); the decidua, junctional zone and labyrinth of treated placentae remained entirely intact, and no fibrin deposition or hemorrhaging were observed (Figure 3.13g). However, hemozoin and infected red blood cells were commonly found engulfed by giant trophoblast cells in both treated groups (Figure 3.13b,h).

Discussion

Though evidence of a procoagulant state during severe malaria has been long known, very little work has been performed to elucidate how coagulation contributes to the pathogenesis of the disease. In particular, most research that has been performed on the subject has been within the scope of cerebral malaria, a manifestation of severe falciparum malaria related to, but separate from, placental malaria (PM). However, the observation that extensive fibrin deposition in the placenta is both a consistent pathologic feature of human PM and is correlated with negative malaria-associated birth outcomes indicate that the coagulation cascade likely plays a critical role in mediating

PM pathogenesis[3,5,6,8,16–19]. In these studies, we sought to characterize how the extrinsic pathway of coagulation may be initiating malaria-induced placental pathology and pregnancy loss.

This investigation revealed that interfering with the coagulation cascade can be an effective way to mitigate poor pregnancy outcome due to malaria. In particular, Tissue Factor (TF), the initiator of the extrinsic pathway of coagulation, appears to play a critical role in determining pregnancy outcome during PM; however, this role could be beneficial or detrimental depending on where TF is expressed. The experiments performed in $TF^{flox/flox}$,Tie2Cre+ mice indicate that TF derived from maternal endothelial cells contribute significantly to PM pathogenesis. When mated with hemizygous $TF^{flox/flox}$,Tie2Cre+/0 stud males, malaria-infected $TF^{flox/flox}$,Tie2Cre+ female mice were able to maintain successful pregnancy through mid-gestation, at which timepoint TF-intact mice typically abort in this model of PM. Additionally, placental pathology, particularly fibrin deposition was entirely absent in these mice.

These results alone could implicate a number of cell types in which the Tie2 promoter is active, including maternal endothelial and hematopoietic cells and the fetal-derived trophoblast. However, when females were mated with a homozygous $TF^{flox/flox}$,Tie2Cre+/+ stud male, $TF^{flox/flox}$,Tie2Cre+ females exhibited a different phenotype, in which individual embryos within the uterus experienced different outcomes; in this specific mating scheme, a completely healthy embryo could be positioned next to a hemorrhagic, resorbing embryo. In this second mating scheme, all embryos receive at least one Cre allele from the stud male, which is sufficient to delete a flox gene of interest from a cell; thus, TF will have been deleted from the fetal-derived trophoblast cells of all embryos in these mice. These results indicate that trophoblast-derived TF is unlikely to be contributing to poor outcomes in murine PM, as pups resorb with or without TF on their trophoblast. However, the only instances in which healthy

pups resulted from an infected dam were ones in which the dam was $TF^{flox/flox}$,Tie2Cre+, which is to say had an endothelial and hematopoietic cell-specific TF deletion. Though whether maternal endothelial or hematopoietic cell TF induces these outcomes is cannot be confirmed or denied through these studies, maternal endothelial-derived TF is the more likely relevant source of TF due to the relatively greater prevalence of endothelial versus hematopoietic cells in the placenta.

Myeloid cell-derived TF was also shown to be an important mediator of PM pathogenesis in this model, though in an opposite way. Deleting TF from maternal myeloid cells exacerbated disease, resulting in abortion one day earlier than observed in wild-type mice. Imamura et. al previously showed a positive correlation between excessive fibrin deposition and upregulation of TF on monocytes infiltrating the intervillous space of malaria-infected placentae. In human PM or *Plasmodium berghei* ANKA murine models of PM, these infiltrating monocytes would likely be the source of TF responsible for the relative, albeit dubious, protection TF-intact mice experience relative to LysMCre+ mice.

This work has particularly important implications for the potential of anticoagulant drug treatment to serve as an effective adjunctive therapy to prevent malaria-associated poor birth outcomes. Previous work in our lab showed that treatment with research-grade low molecular weight heparin prevented malaria-induced mid-gestational pregnancy loss in this model[6]. These experiments not only replicate those results with a different low molecular weight heparin, but also show the efficacy of a different anticoagulant drug, fondaparinux, in protecting against placental malaria. Treatment with both low molecular weight heparins resulted in significant improvements in mid-gestational embryonic health mid-gestation during PM, but did not completely rescue the embryos to the levels of health seen in uninfected pregnant mice; hemorrhaging was still common despite treatment with research-grade low molecular weight heparin, and focal

points of fibrin deposition was visible in dalteparin treated, infected pregnant mice. However, fondaparinux mice were virtually indistinguishable from uninfected treated controls. Despite hemozoin deposition and the presence of infected red blood cells in the maternal blood space of the placenta, placental tissue architecture remained intact and no fibrin deposition or hemorrhaging occurred in fondaparinux-treated infected pregnant mice.

The success of fondaparinux treatment in this model has several important implications. Fondaparinux treatment is preferable to that with low molecular weight heparin due to the reduced risk of excessive bleeding, selectivity for FXa and absent risk for heparin-induced thrombocytopenia. Though it is structurally similar to low molecular weight heparin, fondaparinux treatment is likely less disruptive to normal hemostatic and immune signaling processes requiring thrombin[20]. Theoretically, interfering with the procoagulant and proinflammatory activities of FXa would be sufficient to slow the hyperactivation of coagulation occurring in severe malaria without causing significant disruptions in the other direction, inducing bleeding. Though many questions remain to be answered, these results warrant continued investigation into the coagulation cascade in general, and the extrinsic pathway of coagulation in particular, as a potential therapeutic target for PM.

Figures

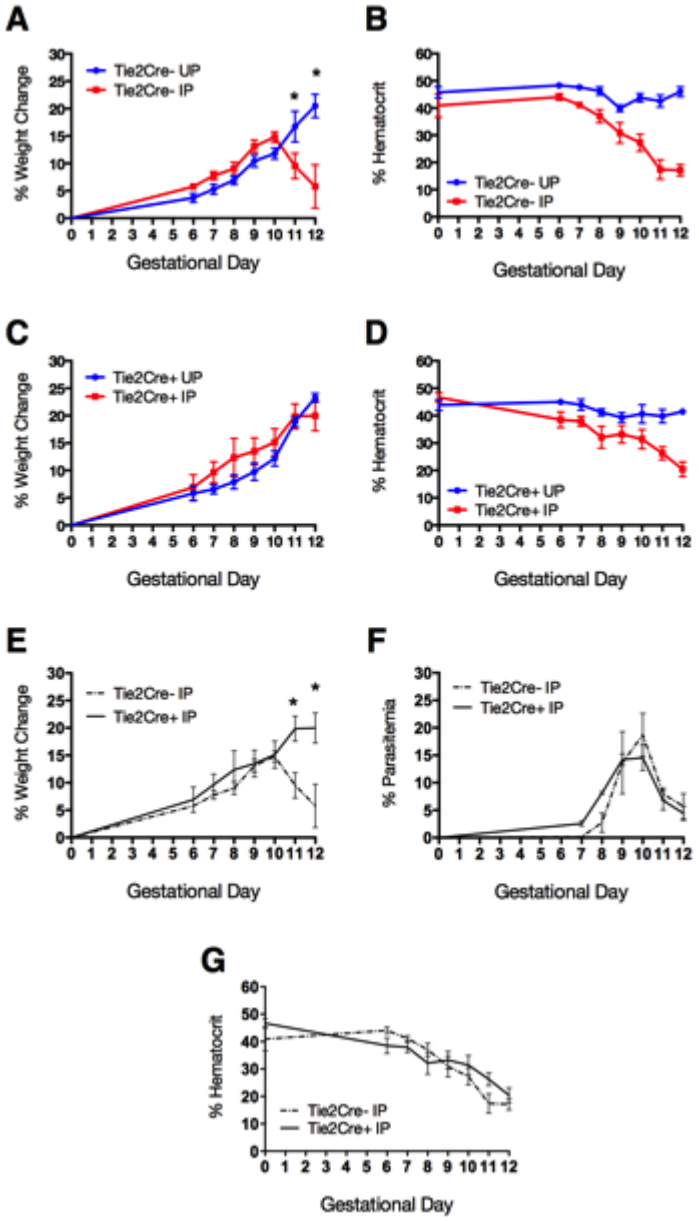

Figure 3.1. ***Deletion of Tissue Factor from maternal endothelium or hematopoietic cells improve midgestational pregnancy-associated weight gain.*** (A-D, F) Percent parasitemia, hematocrit and change in body weight of UP $TF^{flox/flox}$,Tie2Cre-, IP $TF^{flox/flox}$,Tie2Cre-, UP $TF^{flox/flox}$,Tie2Cre+ and IP $TF^{flox/flox}$,Tie2Cre+ are shown. (E, G) Pregnancy-associated weight gain and % hematocrit are compared between IP groups of both strains. Clinical parameters were measured on GD 0 and between GD 6-12. Data represent mean ± SEM.*$p<0.05$

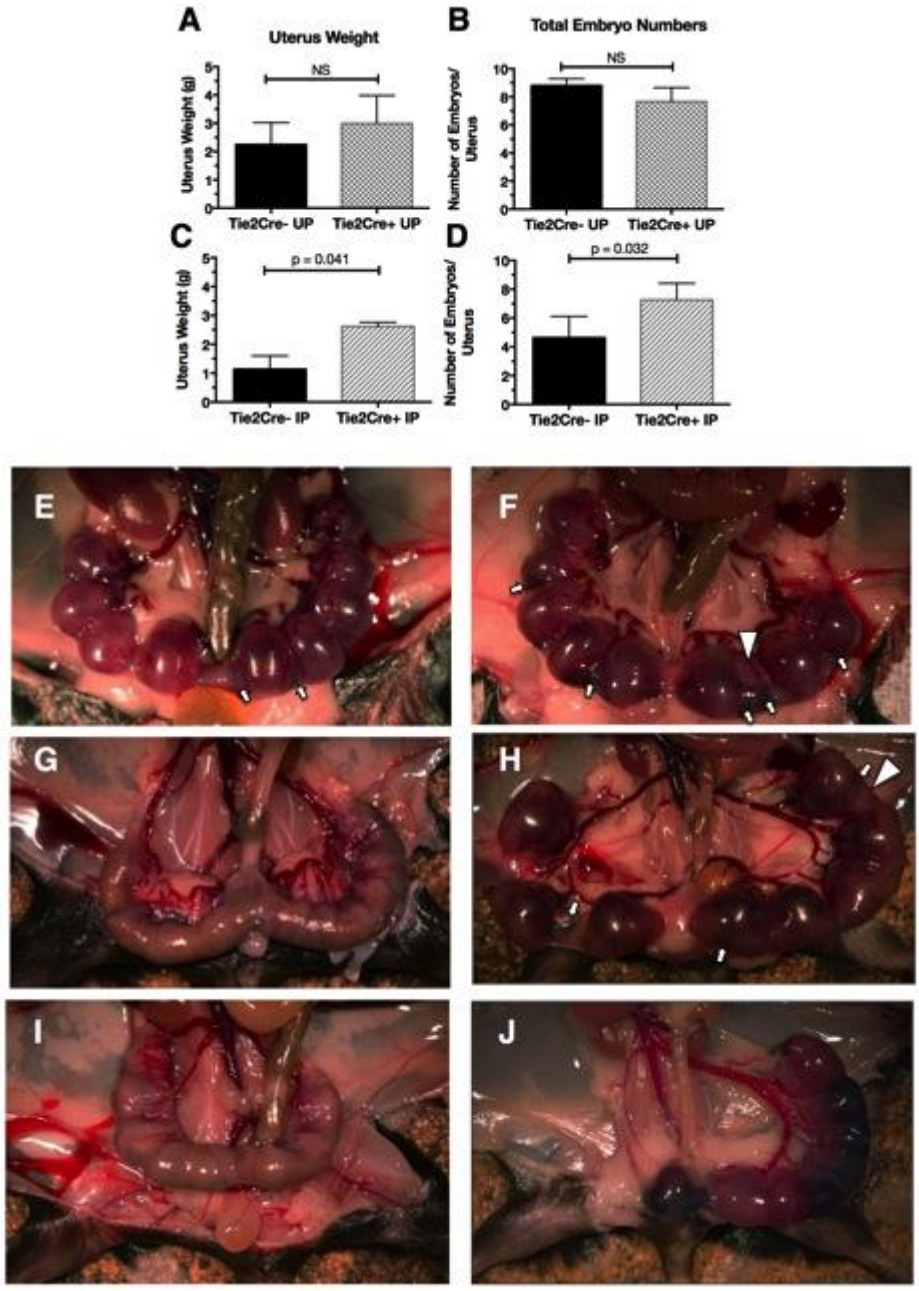

Figure 3.2. ***TF$^{flox/flox}$,Tie2Cre+ mice are protected from malaria-induced pregnancy loss at mid-gestation.*** Average uterine weight from (A) TF$^{flox/flox}$,Tie2Cre- UP and IP-, and (C) TF$^{flox/flox}$,Tie2Cre+ UP and IP mice are shown. Gross pathological images were taken at GD12 for UP (E) TF$^{flox/flox}$,Tie2Cre- and (F) TF$^{flox/flox}$,Tie2Cre+ mice, IP (G) TF$^{flox/flox}$,Tie2Cre- and (H) TF$^{flox/flox}$,Tie2Cre+ mice, and IP (I) TF$^{flox/flox}$,Tie2Cre- and (J) TF$^{flox/flox}$,Tie2Cre+ mice mated with a homozygous-Cre expressing stud male (TF$^{flox/flox}$,Tie2Cre+/0).

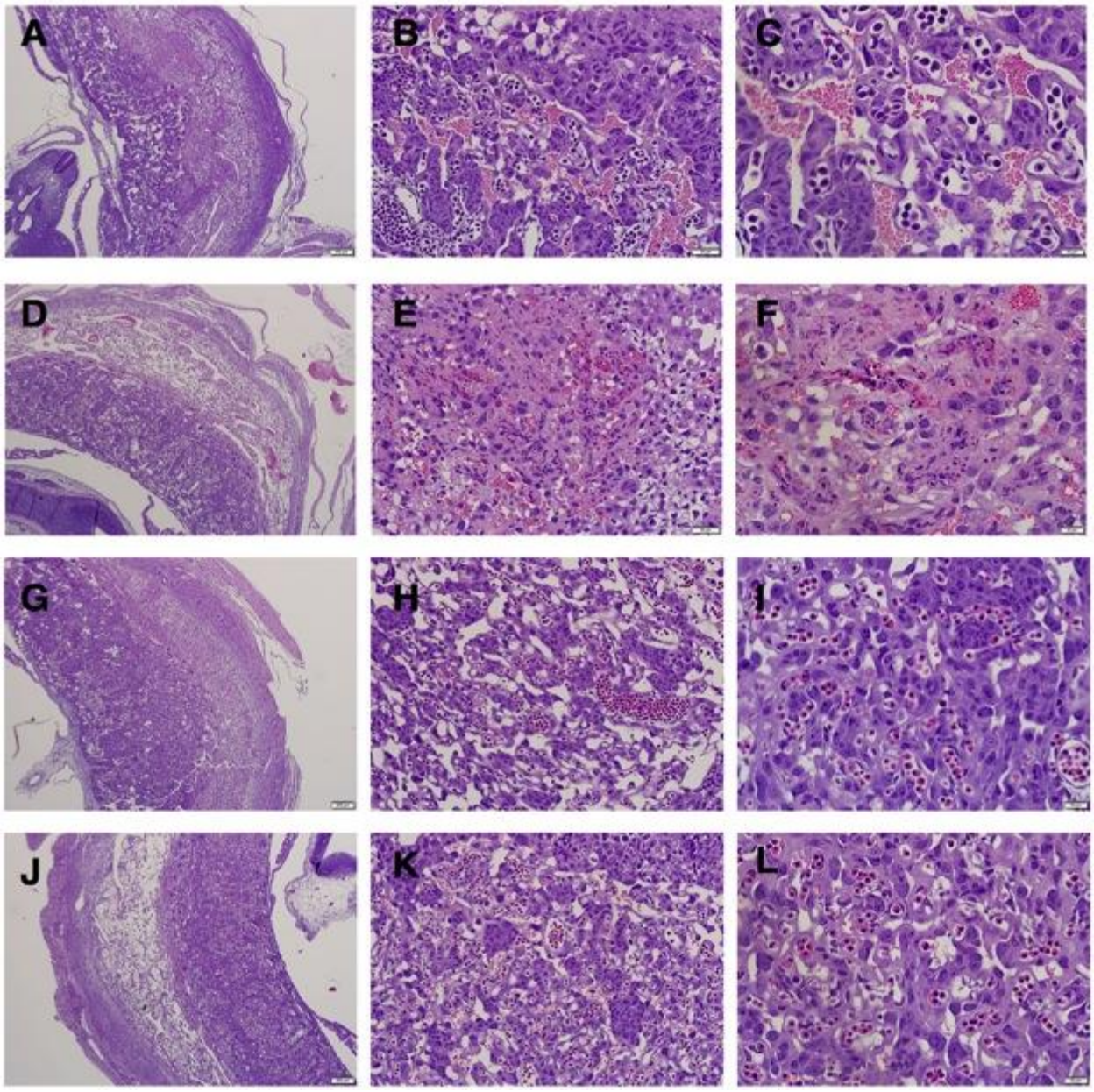

Figure 3.3. ***TF$^{flox/flox}$,Tie2Cre+ mice are protected from malaria-induced placental pathology at mid-gestation.*** H&E stained thin histological sections for TF$^{flox/flox}$,Tie2Cre- UP (A-C) and IP (D-F), TF$^{flox/flox}$,Tie2Cre+ UP (G-I) and IP (J-L) mice are shown. Images were taken using a 4x (A, D, G, J), 20x (B, E, H, K) and 40x (C, F, I, L) objective.

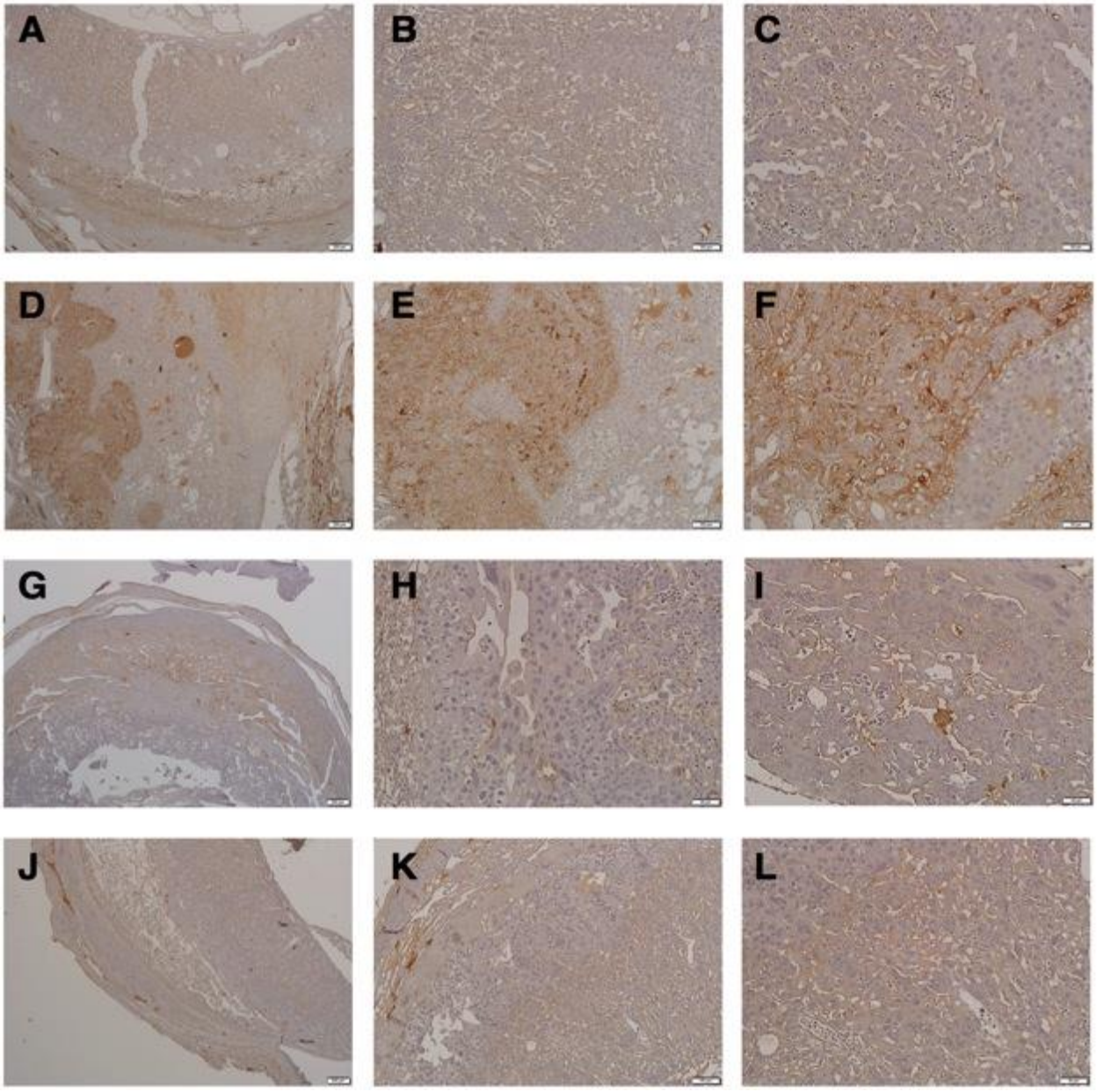

Figure 3.4. ***TF$^{flox/flox}$,Tie2Cre+ mice are protected from malaria-induced placental fibrin deposition at mid-gestation.*** Sections were assayed for presence of fibrin(ogen) using a polyclonal antibody against fibrin and fibrinogen. Stained histological sections for TF$^{flox/flox}$,Tie2Cre- UP (A-C) and IP (D-F), TF$^{flox/flox}$,Tie2Cre+ UP (G-I) and IP (J-L) mice are shown. Images were taken using a 4x (A, D, G, J), 20x (B, E, H, K) and 40x (C, F, I, L) objective.

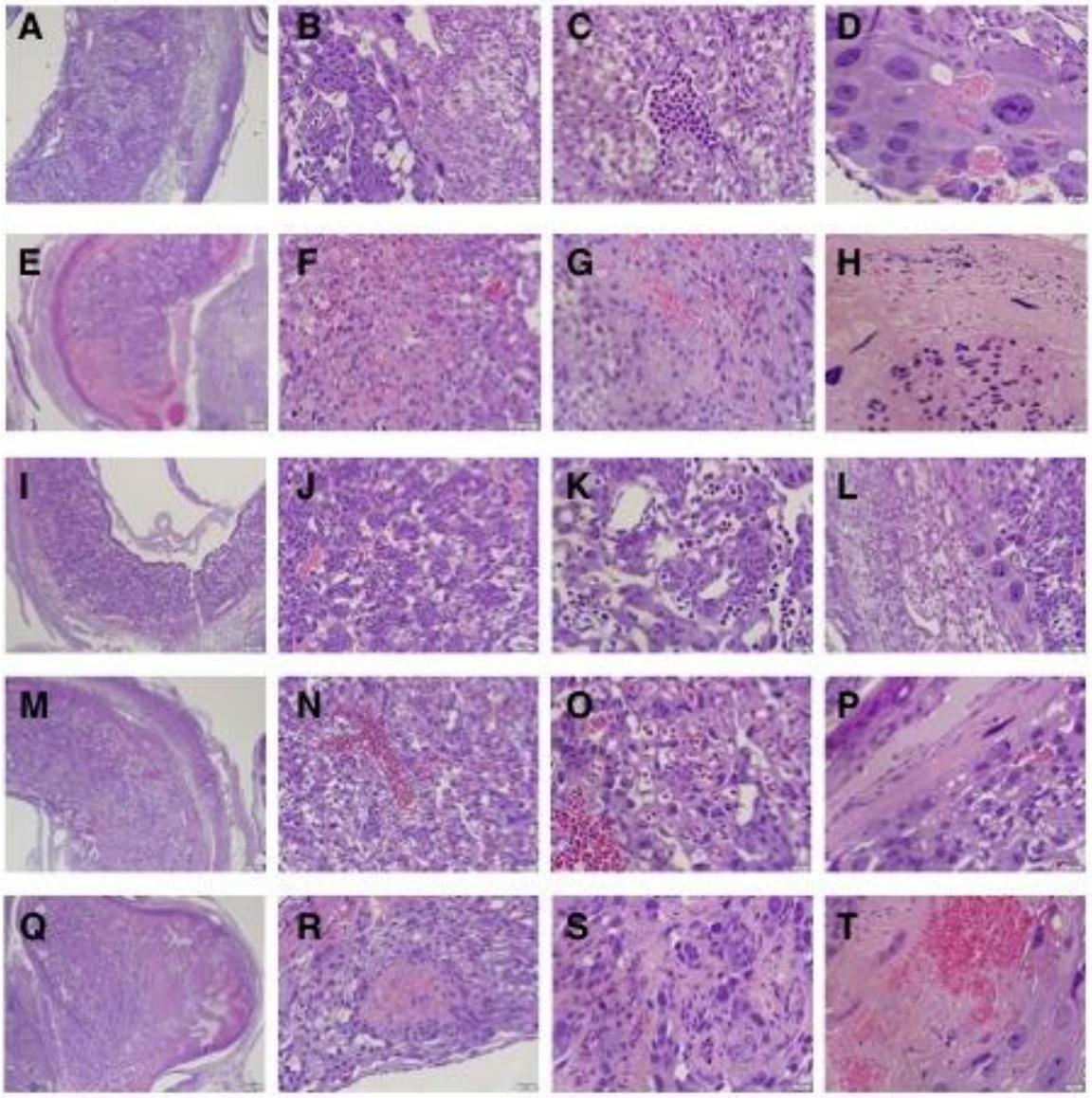

Figure 3.5. ***TF$^{flox/flox}$,Tie2Cre+ mated with homozygous-Cre male mice exhibit mixed phenotypes at mid-gestation.*** H&E stained thin histological sections for TF$^{flox/flox}$,Tie2Cre- UP (A-D) and IP (E-H), TF$^{flox/flox}$,Tie2Cre+ UP (I-L) and healthy (M-P) or resorbing (Q-T) embryos from TF$^{flox/flox}$,Tie2Cre+ IP mice mated with homozygous Cre (TF$^{flox/flox}$,Tie2Cre+/+) stud males are shown. Images were taken using a 4x (A, E, I , M, Q), 20x (B, F, J, N, R) and 40x (C-D, G-H, K-L, O-P, S-T) objective.

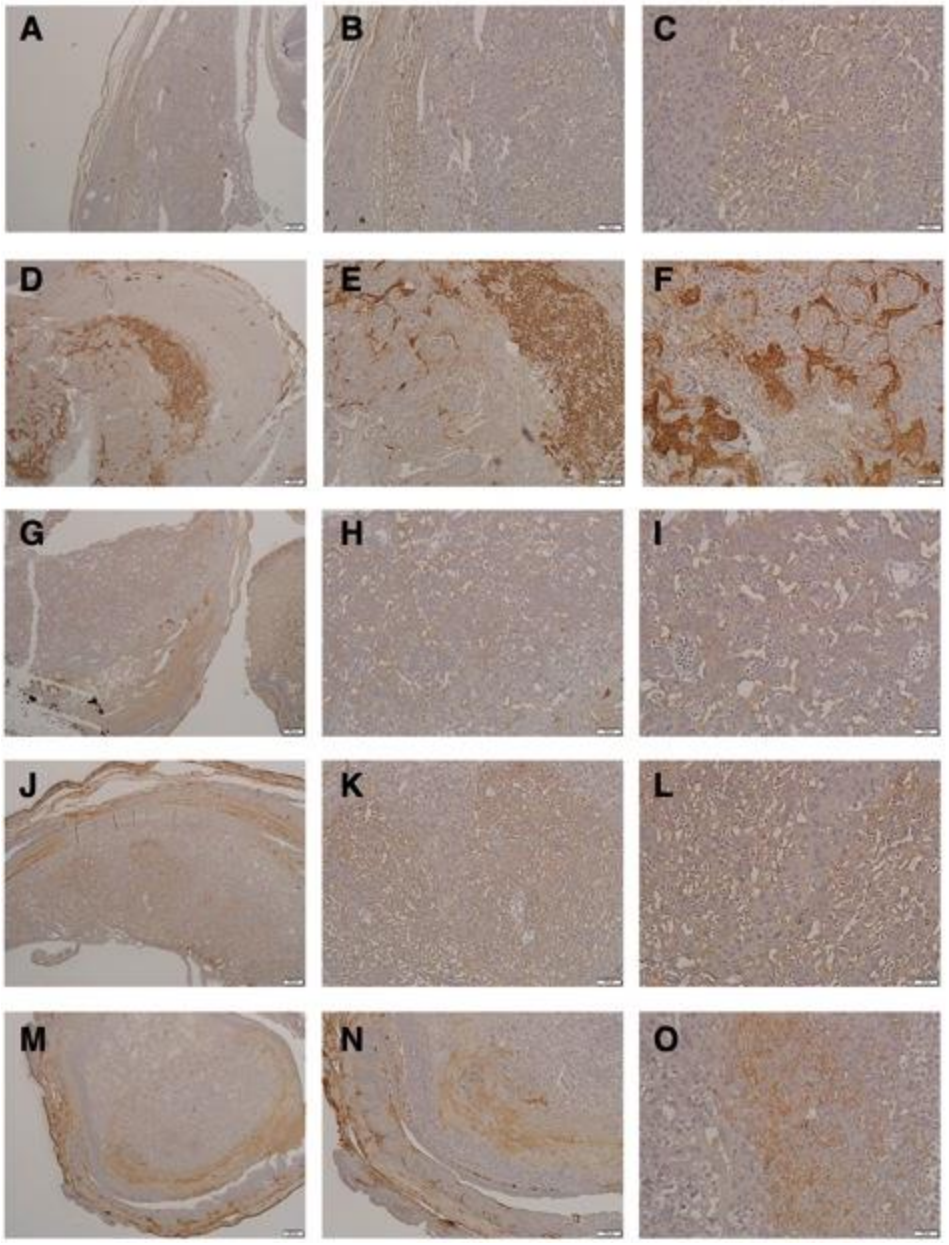

Figure 3.6. ***Fibrin immunohistochemical analysis in TF$^{flox/flox}$,Tie2Cre female mice mated with homozygous-Cre expressing stud males at GD12.*** Sections were assayed for presence of fibrin(ogen) using a polyclonal antibody against fibrin and fibrinogen. Stained histological sections for TF$^{flox/flox}$,Tie2Cre- UP (A-C) and IP (D-F), TF$^{flox/flox}$,Tie2Cre+ UP (G-I) and healthy (J-L) or resorbing (M-O) embryos from TF$^{flox/flox}$,Tie2Cre+ IP mice mated with homozygous Cre (TF$^{flox/flox}$,Tie2Cre+/+) stud males are shown. Images were taken using a 4x (A, D, G, J), 20x (B, E, H, K) and 40x (C, F, I, L) objective.

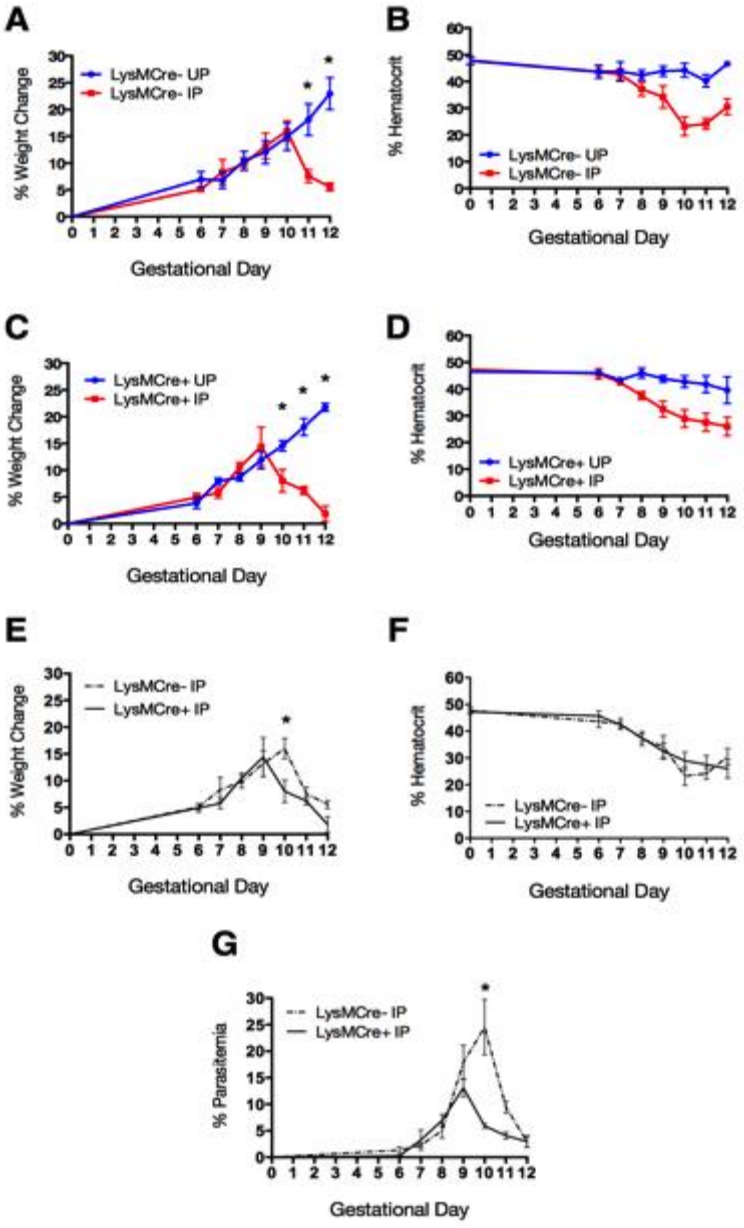

Figure 3.7. Deletion of Tissue Factor from maternal myeloid cells accelerates malaria-induced midgestational pregnancy loss. (A-D, F) Percent parasitemia, hematocrit and change in body weight of UP $TF^{flox/flox}$,LysMCre- (n = 8), IP $TF^{flox/flox}$,LysMCre- (n = 10), UP $TF^{flox/flox}$,LysMCre+ (n=9) and IP $TF^{flox/flox}$,LysMCre+ (n=10) are shown. (E, G) Pregnancy-associated weight gain and % hematocrit are compared between IP groups of both strains. Clinical parameters were measured on GD 0 and between GD 6-12. Data represent mean ± SEM.*$p<0.05$

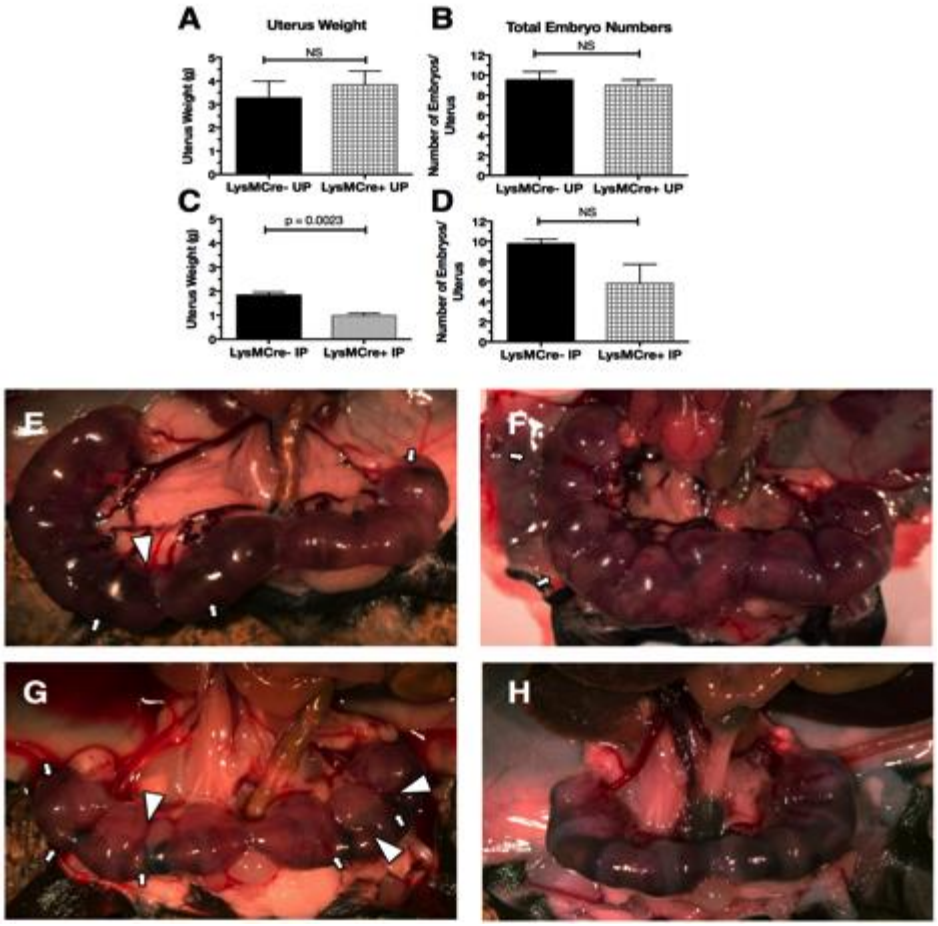

Figure 3.8: *TF$^{flox/flox}$,LysMCre+ mice exhibit exacerbated malaria-induced gross pathology at mid-gestation.* Average uterine weight from (A) TF$^{flox/flox}$,LysMCre- UP and IP-, and (C) TF$^{flox/flox}$,LysMCre+ UP and IP mice are shown. Gross pathological images were taken at GD12 for UP (E) TF$^{flox/flox}$,LysMCre- and (F) TF$^{flox/flox}$,LysMCre+ mice, IP (G) TF$^{flox/flox}$,LysMCre- and (H) TF$^{flox/flox}$,LysMCre+ mice.

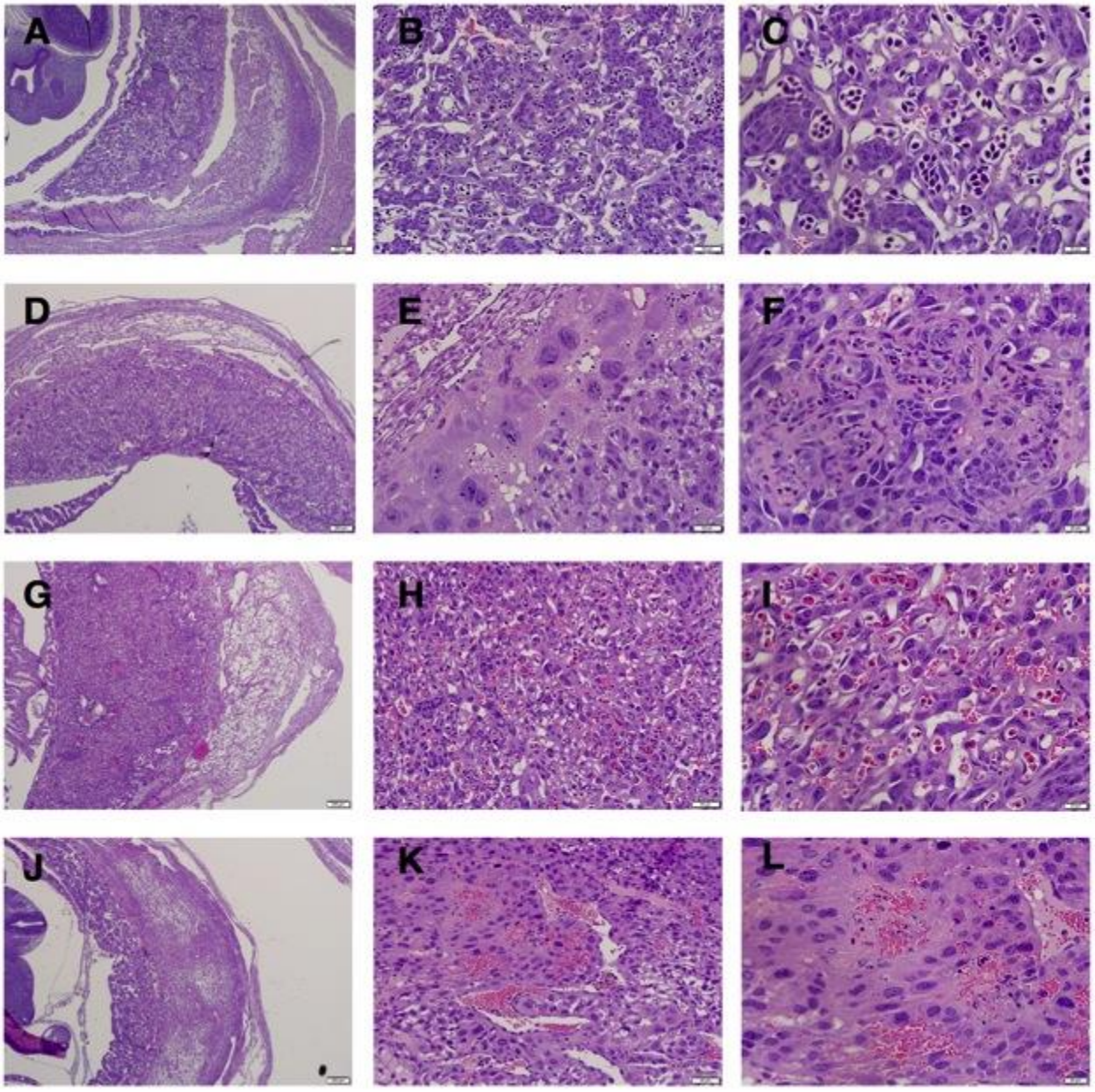

Figure 3.9: ***TF$^{flox/flox}$,LysMCre+ mice exhibit more severe malaria-induced placental pathology at mid-gestation relative to TF-intact mice.*** H&E stained thin histological sections for TF$^{flox/flox}$,LysMCre- UP (A-C) and IP (D-F), TF$^{flox/flox}$,LysMCre+ UP (G-I) and IP (J-L) mice are shown. Images were taken using a 4x (A, D, G, J), 20x (B, E, H, K) and 40x (C, F, I, L) objective.

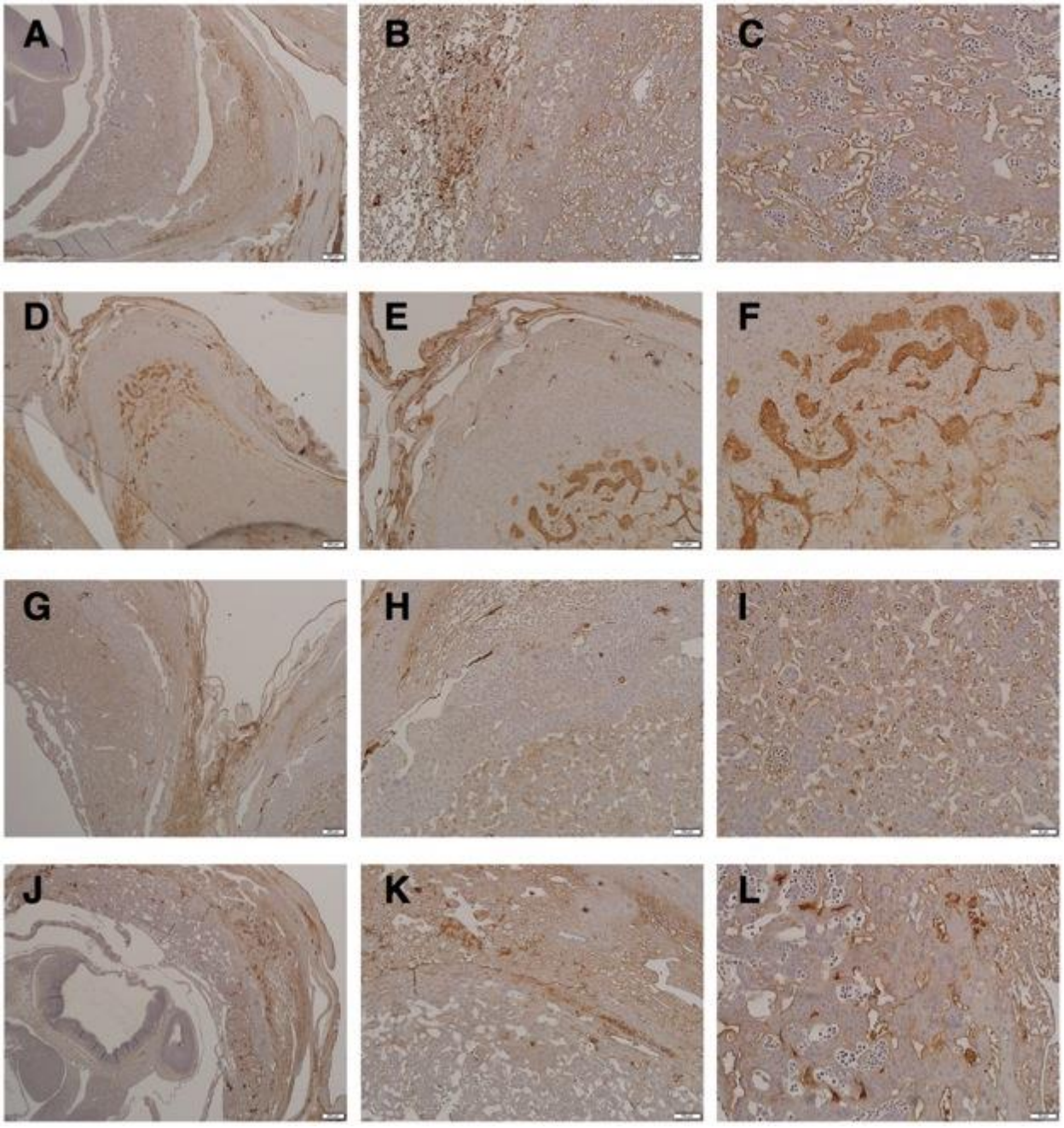

Figure 3.10: ***TF$^{flox/flox}$,LysMCre+ mice exhibit only slightly more severe malaria-induced fibrin depositionat mid-gestation.*** Sections were assayed for presence of fibrin(ogen) using a polyclonal antibody against fibrin and fibrinogen. Stained histological sections for TF$^{flox/flox}$,Tie2Cre- UP (A-C) and IP (D-F), TF$^{flox/flox}$,Tie2Cre+ UP (G-I) and IP (J-L) mice are shown. Images were taken using a 4x (A, D, G, J), 20x (B, E, H, K) and 40x (C, F, I, L) objective.

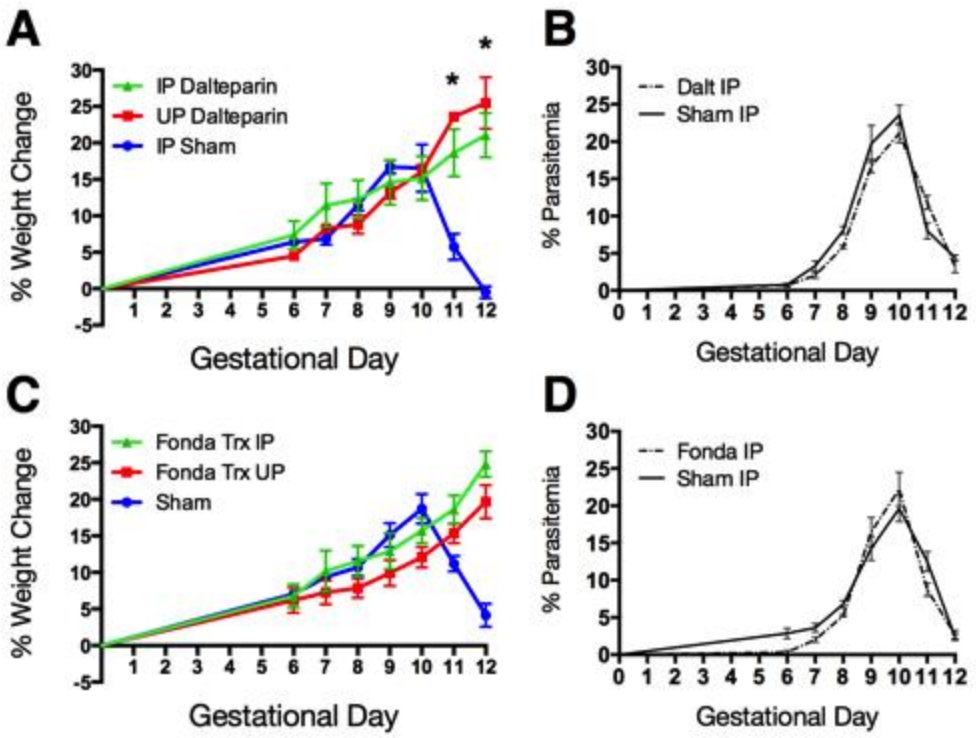

Figure 3.11: ***Anticoagulant treatment improves midgestational pregnancy-associated weight gain during murine placental malaria.*** (A-B) Percent change in body weight and parasitemia, of UP and IP dalteparin-treated mice and IP sham-treated mice. (C-D) Percent change in body weight and parasitemia, of UP and IP fondaparinux-treated mice and IP sham-treated mice. Clinical parameters were measured on GD 0 and between GD 6-12. Data represent mean ± SEM.*$p<0.05$

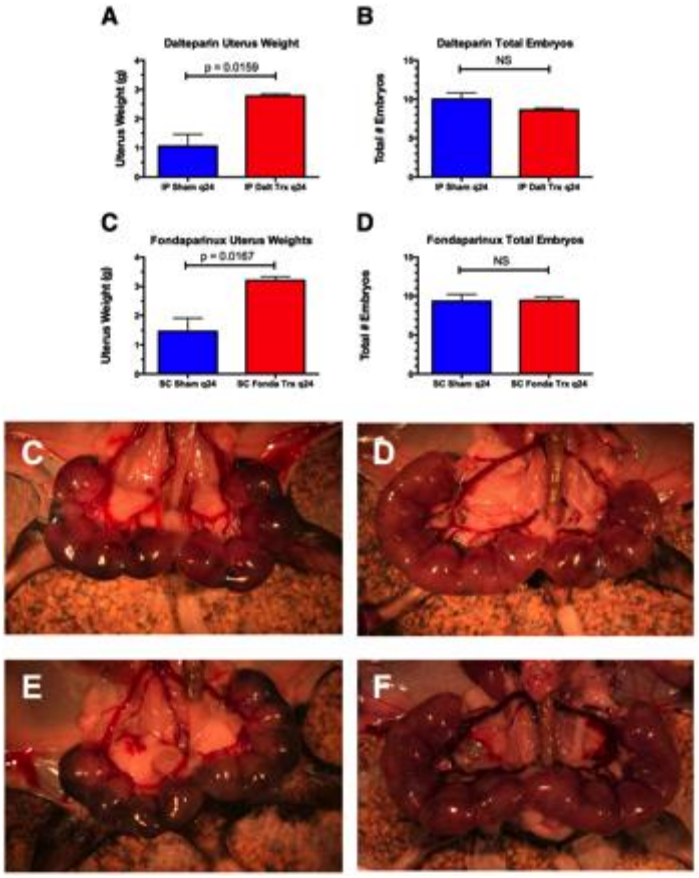

Figure 3.12: ***Anticoagulant-treated mice exhibit healthier conceptuses at mid-gestation.*** Average uterine weight and total number of embryos from (A-B) sham-treated IP and dalteparin-treated IP mice, and (C-D) sham-treated IP mice and fondaparinux-treated IP mice are shown. Gross pathological images were taken at GD12 for sham- (C) and dalteparin-treated (D) mice and sham- (E) and fondaparinux-treated (F) mice.

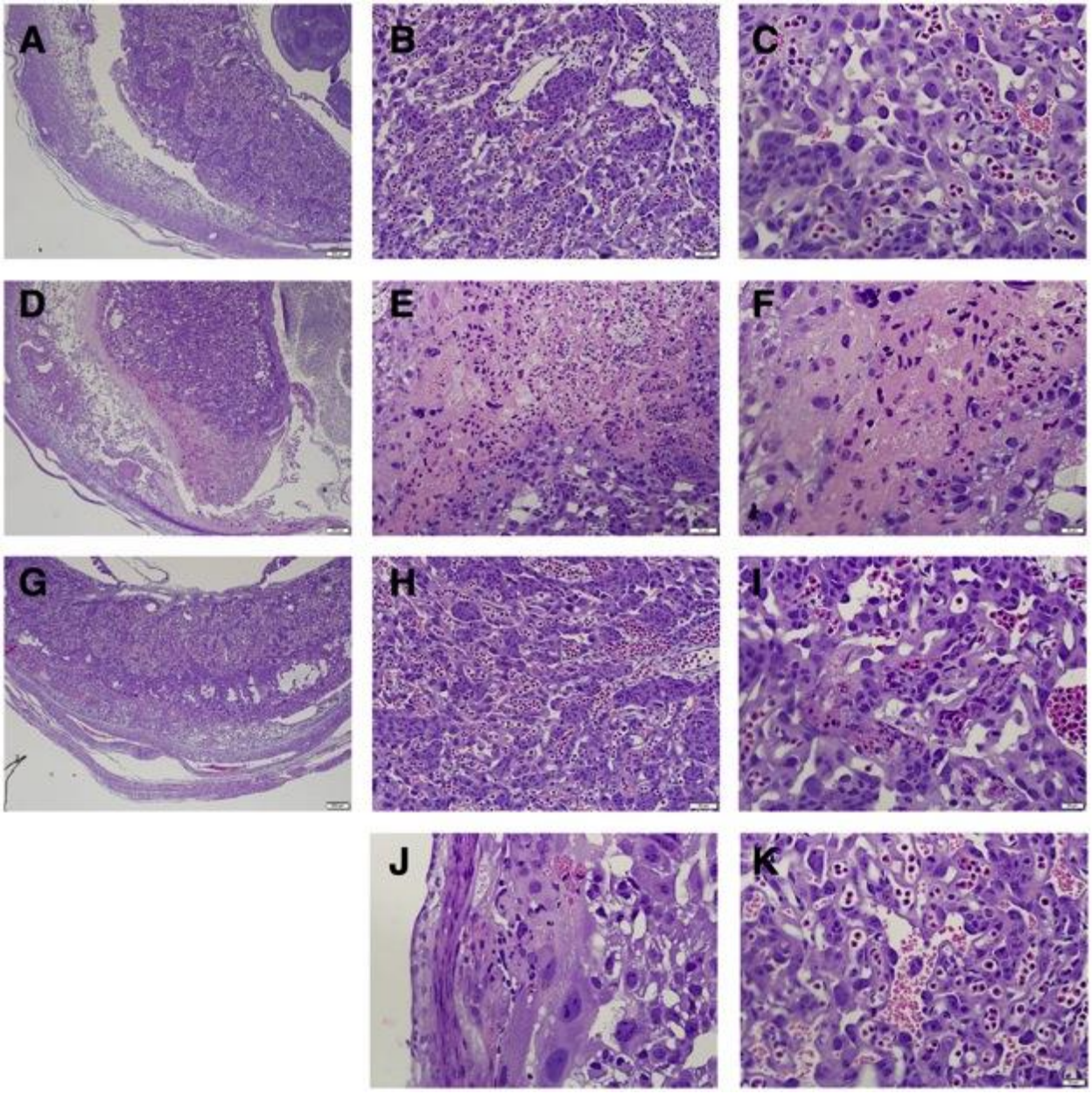

Figure 3.13: ***Dalteparin-treated, infected placentae exhibit improved but not completely rescued malaria-induced placental pathology at mid-gestation.*** H&E stained thin histological sections for dalteparin-treated UP (A-C), sham-treated IP (D-F), and dalteparin-treated IP (G-K) mice are shown. Images were taken using a 4x (A, D, G), 20x (B, E, H) and 40x (C, F, I, J,K) objective.

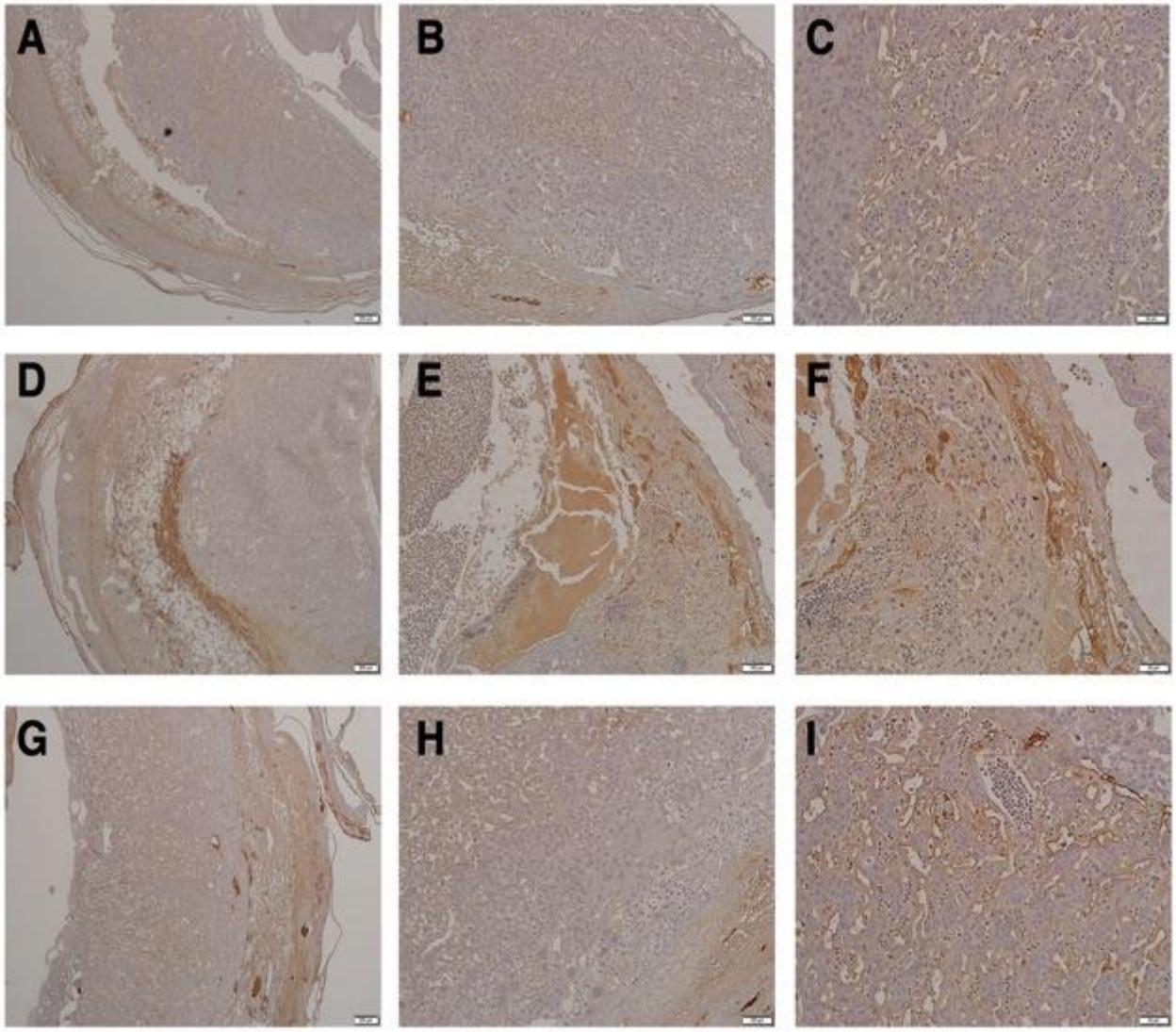

Figure 3.14: ***Dalteparin-treated, infected placentae exhibit reduced fibrin deposition at mid-gestation.*** Sections were assayed for presence of fibrin(ogen) using a polyclonal antibody against fibrin and fibrinogen. Stained histological sections for dalteparin-treated UP (A-C) and IP (G-I), and sham-treated UP (D-F) mice are shown. Images were taken using a 4x (A, D, G), 20x (B, E, H) and 40x (C, F, I) objective.

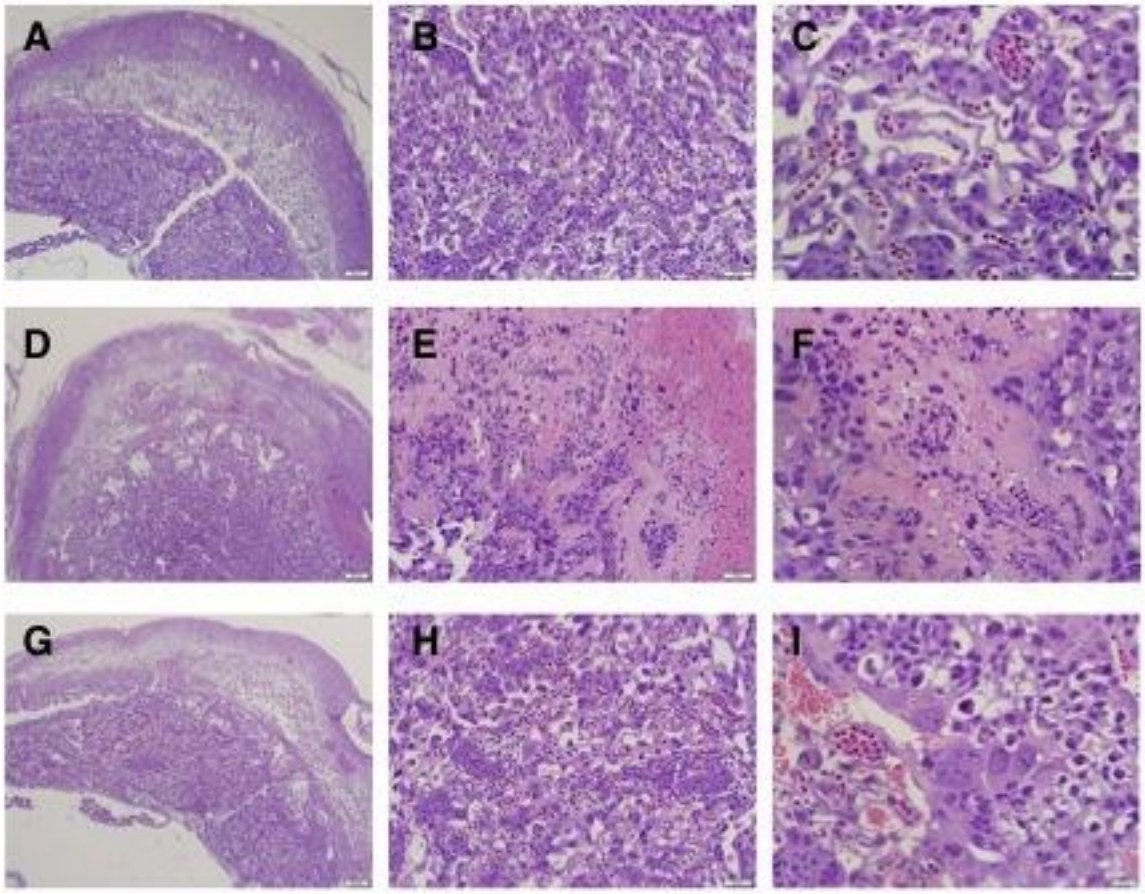

Figure 3.15: ***Fondaparinux-treated, infected placentae are indistinguishable from uninfected placentae at mid-gestation.*** H&E stained thin histological sections for dalteparin-treated UP (A-C), sham-treated IP (D-F), and dalteparin-treated IP (G-K) mice are shown. Images were taken using a 4x (A, D, G), 20x (B, E, H) and 40x (C, F, I, J,K) objective.

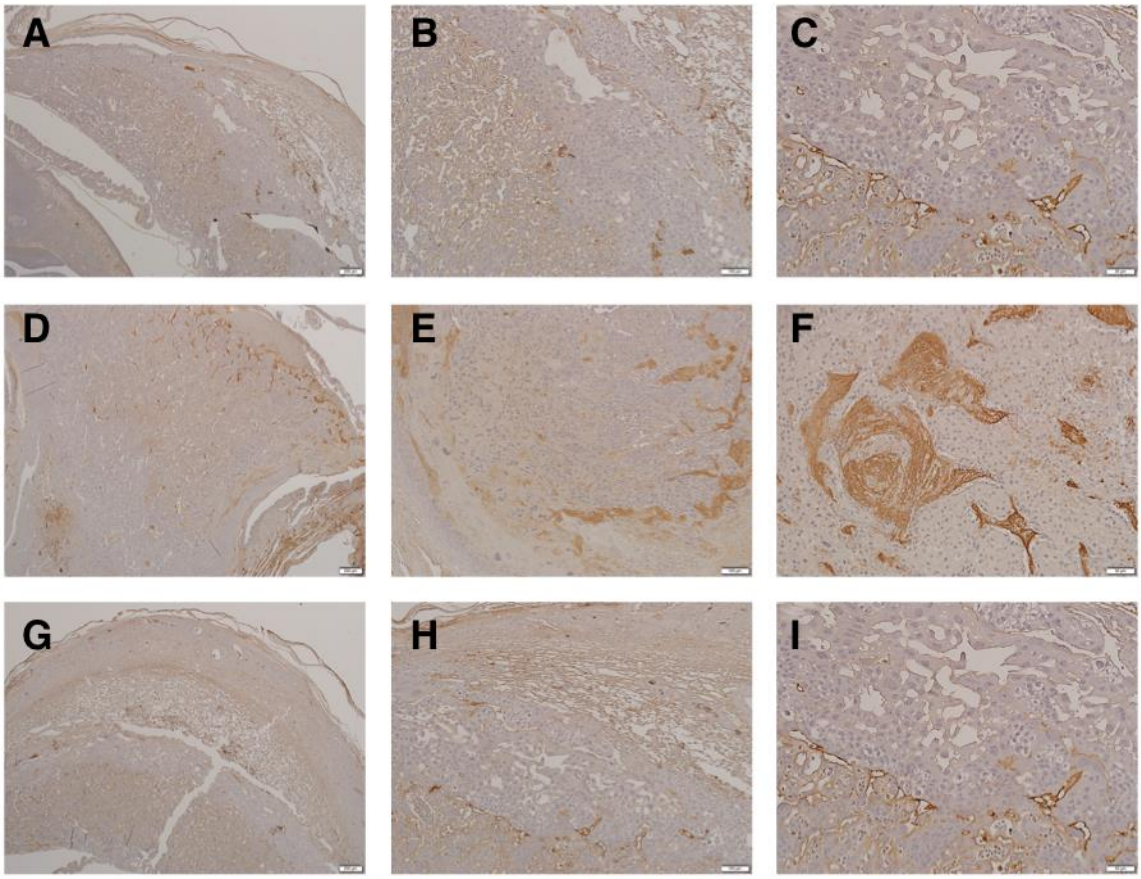

Figure 3.16: ***Fondaparinux-treated, infected placentae exhibit fibrin staining equivalent to that seen in uninfected placentae at mid-gestation.*** Sections were assayed for presence of fibrin(ogen) using a polyclonal antibody against fibrin and fibrinogen. Stained histological sections for fondaparinux-treated UP (A-C) and IP (G-I), and sham-treated UP (D-F) mice are shown. Images were taken using a 4x (A, D, G), 20x (B, E, H) and 40x (C, F, I) objective.

Table 3.1: ***Pregnancy outcomes of TF$^{flox/flox}$,Tie2Cre mice.*** The average number of embryos per uterus, total number of resorbing embryos and frequency of intrauterine hemorrhaging are shown for each Tie2Cre group. p-values calculated in a 2x2 contingency table with Fisher's exact test.

	Tie2Cre- UP	Tie2Cre+ UP	p-value	Tie2Cre- IP	Tie2Cre+ IP	p-value
Average #/ Uterus	8.80	7.67	0.362	4.11	8.50	**0.0320**
Resorbing/ Total	1/44	2/46	1.000	37/37	4/51	**<0.0001**
Intrauterine Hemorrhage	4/5 *(minor)*	3/6	0.545	5/5	6/7	1.0000

Table 3.2: ***Pregnancy outcomes of TF$^{flox/flox}$,LysMCre mice.*** The average number of embryos per uterus, total number of resorbing embryos and frequency of intrauterine hemorrhaging are shown for each Tie2Cre group. p-values calculated in a 2x2 contingency table with Fisher's exact test.

	LysMCre- UP	LysMCre+ UP	p-value	LysMCre- IP D10	LysMCre+ IP D10	p-value
Average #/ Uterus	9.50	9.00	0.626	9.75	8.75	0.4881
Resorbing/ Total	2/38	3/45	1.000	20/39	35/35	**<0.0001**
Intrauterine Hemorrhage	2/4	1/5	0.524	4/4	4/4	1.000

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CHAPTER 4

TISSUE FACTOR DEFICIENCY AND ANTICOAGULANT THERAPY REDUCE BLOOD BRAIN BARRIER PERMEABILITY AND SUSCEPTIBILITY TO NEUROLOGICAL SYMPTOMS IN EXPERIMENTAL CEREBRAL MALARIA[2]

[2] Bracken TC, Jenkinson TG, Cooper CA, Moore JM. To be submitted to *Plos Medicine*.

Abstract

Sequestration of *Plasmodium falciparum*-infected erythrocytes in the brain results in a severe clinical manifestation of this disease, cerebral malaria (CM). It is well established in other models of disease that inflammatory damage leads to systemic activation of coagulation and severe thrombosis. Recent evidence showing the existence of a procoagulant state in patients suffering from CM, including accumulation of platelets and fibrin in the brain, indicates the inflammation-coagulation cycle may play a significant role in malaria pathogenesis, and may provide useful diagnostic and therapeutic targets. However, the extent to which coagulation is responsible for the pathogenesis of these diseases is incompletely understood. In order to assess the extent to which Tissue Factor (TF) contributes to blood brain barrier disruption and the onset of neurological symptoms in a murine model of cerebral malaria, mice with a null mutation in TF that are transgenic for human TF expressed at 1% of the normal level ("low TF," LTF-/-), their mTF heterozygous littermates (LTF+/-), and TF-intact C57BL/6J mice were infected with *Plasmodium berghei* ANKA, a murine malaria strain capable of inducing cerebral malaria. A subset of C57BL/6J mice were treated with the low molecular weight heparin, Dalteparin (1000 IU/kg i.p., q24 hour). Mice were serially sacrificed between days 4 and 6 post-infection (ED4-6), monitored daily for parasitemia, weight, hematocrit, and exhibition of neurological symptoms, and brain pathology was assessed in H&E-stained histological sections. In order to determine the extent and location of blood brain permeability, a subset of mice was injected with 2% Evan's blue dye and blue staining of brain tissue was assessed. The different strains exhibited varying susceptibility to CM, with significantly reduced proportions of LTF-/- mice succumbing relative to control or C57BL/6J mice. Intense, extensive Evan's blue staining was seen in brains of B6 and LTF+/- mice that had succumbed to CM; however, LTF-/- mice that exhibited neurological symptoms showed only minimal, focal Evan's blue

staining in the cerebrum. Though extensive hemorrhage was seen in histological sections from CM-positive B6 and LTF+/- mice, minimal to no hemorrhaging was seen in histological sections from LTF-/- mice that had succumbed to CM. Similar effects were seen in Dalteparin-treated mice. Significant reductions in the incidence of CM in LTF-/- mice coupled with a marked decrease in blood brain barrier permeability in CM-positive mice of this strain suggest that TF is playing a significant role pathogenesis of CM. It is currently unclear if these effects are mediated by thrombosis or alterations to host immune signaling.

Introduction

Cerebral Malaria (CM) is a clinical manifestation of severe malaria defined as the presence of *P. falciparum* parasitemia and unrousable coma (Blantyre scale <3) with no other apparent etiology[1]. CM occurs in approximately 1% of malaria infections, and carries a 15-20% case fatality rate[2]. Children under the age of five in Sub-Saharan Africa are the most susceptible population, contributing to 90% of CM-related fatalities[3].

The precise mechanisms underlying the onset of the neurological symptoms that define CM remain to be elucidated; however, decades of clinical and laboratory evidence have shown that the host response to infection plays a critical role in the onset of disease. The release of pro-inflammatory cytokines and chemokines, leukocyte infiltration, and reactive oxygen species production mediate CM-induced neurological damage, inducing severe brain pathology and disruption of the Blood-Brain Barrier (BBB)[4–9]. Hyperinflammation in CM triggered by malarial components, primarily infected red blood cells, hemozoin and glycosylphosphotidylinositol (GPI), consists of increased proinflammatory cytokine expression and inflammatory cell and platelet accumulation in the brain microvasculature. While a potent proinflammatory response is known to be necessary for CM, the failure of anti-inflammatory treatments to prevent the onset of CM in clinical settings has led researchers to investigate alternative components of the host response that may mediate the induction of severe pathologies characteristic of CM.

One such host process that may play a significant role in severe falciparum malaria is the coagulation cascade. Clinical evidence suggests the presence of a hypercoagulable state in both cerebral (CM) and placental malaria (PM) patients. The most common indicators of coagulation in malaria patients are consumption-induced thrombocytopenia[10–14] and fibrin deposition[15–19], and several studies have shown that the degree of coagulation activation correlates with the severity of disease[14,20–22]. Overt Disseminated Intravascular Coagulation (DIC) occurs in 5-10% of severe malaria

cases[23]; however, it is seen at a higher frequency (~20%) and is correlated with fatal outcome in retinopathy-positive cases of pediatric CM[23].

Procoagulant activity in severe malaria has been characterized best in CM. Autopsy examination of brains from fatal pediatric CM revealed thrombin deposition and hemorrhages occur near sites of infected erythrocyte (IE) adherence to the microvascular endothelium[24]. Fibrin deposition is commonly seen in brain tissue in fatal CM[16,15]. IE have also been found to have direct procoagulant activity, inducing TF expression and activate thrombin on the apical surface of endothelial cells and supporting assembly of the extrinsic Xase (TF-FVIIa) complex *in vitro*[25]. The anticoagulant Endothelial Protein C Receptor (EPCR) plays a particularly important role in CM pathogenesis, as IE expressing PfEMP1 variants linked to severe malaria bind to EPCR on the brain microvasculature[26]. Depletion of EPCR in the brain microvasculature was observed in autopsied brains from fatal pediatric CM cases, which was negatively correlated with increasing IE sequestration in the brain[27]; this likely contributes to the significant disruptions to the anticoagulant protein C system observed in CM, exacerbating the procoagulant state in CM-affected brains[24,28].

The procoagulant response to malaria is an intriguing potential target for adjunctive therapy to mitigate the severe outcomes of CM due to its well-understood interactions with inflammation. The “inflammation-coagulation cycle,” in which inflammation activates a procoagulant response that in turn promotes further inflammation, has been well characterized in models of bacterial sepsis and inflammatory bowel disease[29]. Evidence suggests this cycle contributes to the pathogenesis of CM; however, before it can be targeted with anticoagulant drugs in a clinical setting, much remains to be understood about how procoagulant mechanisms are initiated during malaria infection and what are the downstream ramifications of such activation.

This study investigates how the extrinsic pathway of coagulation participates in the onset of neurological dysfunction in a murine model of CM by interfering with Tissue Factor (TF), the primary initiator of the coagulation cascade, and thrombin, a serine protease that is central to the procoagulant and proinflammatory effects of the coagulation cascade. The results provided herein are compelling evidence that the coagulation cascade contributes significantly to blood brain barrier disruption and induction of neurological symptoms during CM, and may be a promising target for future adjunctive therapies to mitigate severe outcomes of this disease.

Materials and Methods

Mice

C57BL6/J mice were originally purchased from The Jackson Laboratories, and were used to generate breeding stock and experimental animals in the University of Georgia Coverdell Vivarium. Mice with a null mutation in TF that are transgenic for human TF expressed at 1% of the normal level ("Low Tissue Factor," LTF-/-) and mTF heterozygous ("Control," LTF+/-) were bred from founders generously donated by Dr. Nigel Mackman, University of North Carolina, Chapel Hill, NC, USA. Mouse experiments were performed in accordance with the guidelines and with the approval of the University of Georgia Institutional Animal Care and Use Committee (AUP number A2014 04-002-Y3-A4).

Murine Model of Experimental Cerebral Malaria

Plasmodium berghei ANKA parasites constitutively expressing green fluorescent protein (*P. berghei* ANKA-GFP) were obtained from Dr. Donald Champagne, University of Georgia, Athens, GA, USA, and maintained by passage in naïve male C57BL6/J mice. Infection was initiated in female mice aged 6-7 weeks old via tail vein injection with

$1x10^6$ *P. berghei* ANKA-GFP-infected erythrocytes. Mice were monitored daily for up to six days post-infection; each mouse was weighed, and blood collected from the tail vein into a heparinized capillary tube to measure parasitemia and hematocrit. Parasitemia was measured by flow cytometric analysis. Briefly, 2 µL of infected whole blood was diluted in phosphate-buffered saline (PBS), pH 7.4, and acquired in a Beckman Coulter CyAn ADP Analyzer (Beckman Coulter, Indianapolis, IN, USA). Erythrocytes were gated by light scatter, and parasitemia was defined as the percentage of GFP-positive events occurring in the erythrocyte gate. 100,000 events in the erythrocyte gate were acquired per sample. Remaining blood was centrifuged in a microhematocrit centrifuge and percent hematocrit was calculated according to the following: (volume of packed erythrocytes)/(total blood volume)x100%. Development of disease was assessed at 12-hour intervals beginning day 3 post-infection (experimental day 3, ED 3) using a rubric that considers mouse body position, activity level, coat appearance, and eye position (Table 4.1).

In experiments where mice were given anticoagulant treatment, mice were treated with either 1000 IU/kg dalteparin sodium or sham treated with 400 µL PBS intraperitoneally every 24 hours.

Beginning ED 5, mice were monitored at 3-hour intervals for signs of CM, including tremor, ataxia, impaired righting reflex, limb paralysis, and seizure. Mice were euthanized upon exhibition of neurological symptoms or upon achieving the maximum score for any of the parameters outlined in Table 1. All non-moribund mice were sacrificed upon termination of the experiment at the end of ED 6. On GD12, mice of all strains were anaesthetized using 2.5% Avertin (2,2,2-Tribromoethanol) administered via intraperitoneal injection and euthanized by exsanguination through the caudal vena cava into acid citrate dextrose (final concentration, 0.76%).

Mouse Tissue Sample Collection

Mice were serially sacrificed between ED 4-6. At sacrifice, whole blood was collected from the caudal vena cava into acid citrate dextrose (final concentration, 0.05%). Brains were removed from the mouse and hemisected. In some animals, one hemisphere was flash frozen in liquid nitrogen, and the other was preserved in 4% neutral-buffered formalin and paraffin-embedded for histology. Alternatively, one hemisphere was frozen for RNA extraction or prepared for use in frozen sections. Platelet-poor plasma was generated by centrifugation of citrated whole blood and preserved in liquid nitrogen.

Histology

Paraffin-embedded brains were processed for histology. H&E-stained and unstained brain sections (5 μm thick) from infected and uninfected mice were prepared, and Masson's trichrome staining was performed per manufacturer's instructions using unstained sections (Sigma-Aldrich, St. Louis, MO, USA). Briefly, deparaffinized sections were mordanted in Bouin's Solution, then stained in sequence with Weigert's Irons Hematoxylin Solution and Biebrich Scarlet-Acid Fucshin. Slides were then incubated first in Phosphotungstic/Phosphomolybdic Acid, then in Aniline Blue followed by 1% Acetic Acid.

Assessment of Blood Brain Barrier Disruption

A 2% solution of Evans Blue dye was prepared using Evans Blue Dye powder (Sigma-Aldrich, St. Louis, MO, USA) and PBS, pH 7.4. The solution was sterile filtered through a 0.2 μm-pore filter. On ED4, ED5, or ED6, 200 μL of 2% Evans Blue dye solution was injected intravenously and allowed to circulate for 1 hour. Mice were

euthanized with Avertin and perfused with 15 mL 10 IU/mL heparin/PBS. Brains were removed and photographed.

RNA Extraction, cDNA generation, and Quantitative Real-time PCR

Total RNA was extracted from frozen brain tissue using the RNeasy kit (Qiagen, Germantown, MD) according to manufacturer's instructions. cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA, USA). Primer sets for the following genes were designed using Geneious Bioinformatics Software (Biomatters Limited, Auckland, NZ): *Il1b*, *Il6*, *Tnf*, *Ccl2*, *Serpine1*, *Procr*, *Thbd*, *Tfpi*, *F2r*, *F2rl1,* and *Gapdh*. Quantitative Real-time PCR analyses were performed by use of the iQ5 Real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The Power SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY, USA) was used for all reactions, and each sample was assayed in triplicate for all target and housekeeping genes. Average Ct values were normalized to the average of GAPDH Ct values and relative expression for each target was calculated by the $2^{\Delta\Delta CT}$ method.

Results

Reduced Tissue Factor expression does not significantly alter the course of P. berghei *ANKA infection.*

Weight, hematocrit, and parasitemia were measured daily beginning day 2 post-infection (experimental day 2, ED2). LTF+/- mice exhibited significantly greater weight loss at ED2 than LTF-/- mice at this timepoint (p=0.0060, Figure 4.1d). Percent parasitemia peaked at ED5 for each strain. Parasitemias for each strain did not vary significantly at any day post-infection. Percent hematocrit decreased slowly but steadily beginning on ED4. Hematocrits did not vary significantly between strains.

Course of P. berghei ANKA varies significantly between CM+ and CM- mice of the same strain.

Although the average course of *P. berghei* ANKA infection did not vary between mice of different strains, when groups were separated based on the presence (CM+) or absence (CM-) of neurological symptoms, CM+ C57BL/6J and CM+ LTF-/- mice showed significantly more profound weight loss than their CM- counterparts beginning on ED5 (4.12a, c). Parasitemia did not vary between mice of the same strain exhibiting different outcomes (4.12d-f).

Tissue Factor Deficiency Reduces Susceptibility to Experimental Cerebral Malaria.

Following infection, mice were serially sacrificed between ED4-6. CM+ mice were defined as those who were both infected and exhibited neurological symptoms, including ataxia, tremor, paralysis, seizures, impaired righting reflex or coma. No mice exhibited neurological symptoms on ED4 (Table 2.2, 2.3). Nearly half of all C57BL/6J mice exhibited neurological symptoms on ED5 (Table 2.3), corresponding with an 80% drop in survival (4.13a). While 100% of LTF+/- mice succumbed to CM on ED5 with none progressing to ED6, significantly fewer LTF-/- mice succumbed at that same time point (Table 2.3, p=0.0002 by Pearson's 2x2 contingency table). Of the 10 LTF-/- mice that progressed to ED6, only 10% exhibited neurological symptoms. LTF-/- mice additionally exhibited significantly increased survival at ED5, with a 100% drop in survival of LTF+/- mice at that time point (Figure 3b). Clinical scores were measured at 12-hour intervals beginning ED0 and continuing until sacrifice; however, no significant difference in morbidity was evident between outcomes or strains (4.13c-e). The only obvious clinical parameter distinguishing CM+ and CM- mice was the presence or absence of neurological symptoms.

CM+ mice exhibit pathologic characteristics of human CM in a strain-specific manner.

Hematoxylin- and eosin-stained brain histological thin sections were assessed for the presence of histopathologic features characteristic of human CM, including accumulation of inflammatory cells and infected red blood cells in the brain microvessels, deposition of the malaria pigment hemozoin (Hz), and hemorrhage. CM+ mice displayed one or more of each of these features, but at varying levels of intensity based on strain. The endothelium surrounding cerebral blood vessels of uninfected C57BL/6J mice were intact and inflammatory cells were absent (data not shown). CM- C57BL/6 mice exhibited no hemorrhaging, and some accumulation of inflammatory cells in cerebral blood vessels (4.14a). CM+ C57BL/6J mice exhibited large hemorrhages (4.14b) in the brain parenchyma and intense accumulation of inflammatory cells in the cerebral microvasculature, so much so that many vessels were visibly occluded by infiltrating immune cells (4.14c). While little hemorrhaging was found in CM- LTF+/- mice, CM+ LTF+/- mice showed significant hemorrhage comparable to that seen in C57BL/6J mice (4.14d) and significant deposition of hemozoin in the blood vessels (4.14f). Some infiltration of inflammatory cells was seen in both CM- and CM+ LTF+/- mice (4.14e). Contrary to expectations, LTF-/- CM+ mice displayed large numbers of inflammatory cells present in cerebral blood vessels, but no detectable hemorrhaging (4.14g).

Tissue Factor deficiency reduces the extent of blood brain barrier disruption in CM+ mice.

P. berghei ANKA-infected LTF-/- and LTF+/- mice injected with 1% Evan's Blue dye/PBS at ED5 revealed differential blood brain barrier permeability between strains and disease outcomes. As expected, mice that did not exhibit neurological symptoms did

not exhibit Evan's Blue staining in the brain post-mortem (4.15a-b,e-f). While the LTF+/- mice that succumbed on ED5 showed dark, diffuse staining throughout the brain tissue, indicating extensive blood brain barrier disruption, LTF-/- mice exhibited only minimal, focal Evan's blue staining. LTF-/- mice that survived to ED6 exhibited staining comparable to what is expected in CM- mice (4.15i-j).

Tissue Factor deficiency eliminates Cerebral Malaria-induced changes to proinflammatory cytokine expression.

RNA was isolated from homogenized brain samples from CM+ and CM- mice at ED5. RNA was assayed for proinflammatory cytokine transcripts by qRT-PCR. TNF-α was significantly increased in CM+ C57BL/6J brains on ED5 relative to CM- mice of the same strain (4.16e). At ED5, CM+ LTF+/- brains also exhibited a marked increase in gene expression of the proinflammatory cytokines TNF-α ($p<0.05$, 4.16f) and MCP-1 ($p<0.01$, 4.16b). However, no such alteration in proinflammatory cytokine expression was visible in homogenized samples from LTF-/- mice. Additionally, MCP-1 transcript levels were significantly increased in CM- LTF-/- brains relative to CM- LTF+/-. Other targets measured includes IL-1β (4.16c,d), though this gene was expressed at very low levels across all strains and outcomes.

Dalteparin treatment reduces blood brain barrier permeability in CM+ C57BL/6J mice.

C57BL/6J mice were treated once daily with the low molecular weight heparin, dalteparin, then assayed for blood brain barrier permeability with Evan's Blue dye. No Evan's Blue staining was visible in brain tissue from CM+ dalteparin-treated mice, indicating the blood brain barriers of these mice remained intact despite infection (4.17a). Conversely, sham-treated mice exhibited dark, extensive Evan's Blue staining throughout the brain, as expected in previous experiments (4.17b). No hemorrhaging

was visible in the cerebral parenchyma of dalteparin-treated mice by H&E-stained histological sections (4.17c), though 1-2 small points of hemorrhage were seen in the cerebellar parenchyma of 67% of these mice (4.17e,f). Extensive inflammatory cell infiltration was seen in the brain microvessels of dalteparin-treated mice (4.17d).

Discussion

Though the procoagulant state characteristic of severe malaria has been recognized for several decades[30,10], attempts to understand the implications of and etiologies underlying this host response have until recently been minimal. This long-standing lack of interest is likely due to early failures of unfractionated heparin treatment to improve disease outcome in pediatric CM patients[20]. However, increasing evidence suggesting that critical components and regulators of the coagulation cascade play a role in malaria pathogenesis[27,31,32] has reopened the door for investigations into anticoagulant drugs as potential adjunctive therapies for severe malaria.

In these studies, we sought to use genetic manipulation to identify a role for TF, the initiator of the extrinsic pathway of coagulation, in mediating the onset of severe disease during CM. Mice with a null mutation in TF that are transgenic for human TF expressed at 1% of the normal level ("Low Tissue Factor," LTF-/-) exhibited significantly reduced blood brain barrier disruption relative to their heterozygous littermates, which produce TF at 50% of the normal levels seen in wild-type mice. They also were found to have a corresponding absence of cerebral hemorrhaging and a 70% reduction in the incidence of neurological symptoms.

The precise mechanism by which TF deficiency confers protection in this model is as-yet unclear; however, it is reasonable to assume that the deletion of a key initiator of the coagulation cascade would have a profound effect on endothelial cell activation and proinflammatory signaling indirectly through significant reduction of coagulation

factor X (FX) activation and thrombin generation downstream of TF. However, TF deletion could also directly affect proinflammatory signaling by eliminating TF's function as a member of the cytokine receptor superfamily of proteins. Indeed, the gene expression analyses performed in this study would seem to support this hypothesis; though CM+ C57BL/6J and LTF+/- mice, which exhibited extensive blood brain barrier disruption, showed significant increases in the potent proinflammatory cytokine TNF-α relative to CM- mice of the same strain, no such elevation in TNF-α transcript levels was seen in either CM- or CM+ LTF-/- mice.

Treatment with the low molecular weight heparin, dalteparin, similarly yielded a barrier protective phenotype in *P. berghei* ANKA-infected mice. Dalteparin, like other heparins, does not directly bind to a procoagulant molecule, but rather enhances the binding of antithrombin, a regulator of the procoagulant response, to its targets, activated FX (FXa) and, to a lesser extent, thrombin. FXa is a central component of the prothrombinase complex, which cleaves thrombin from its zymogen, prothrombin. Thus, dalteparin is able to reduce thrombin procoagulant activity indirectly both through antithrombin binding to thrombin and inhibition of prothrombinase complex formation.

Reduced FX activation and thrombin generation resulting from decreased TF expression or dalteparin treatment could have important implications for blood brain barrier protection or disruption in the presence of an inflammatory insult like CM via their downstream effects on FXa- or thrombin-mediated cleavage of Protease Activated Receptors (PARs). PARs are G-protein coupled receptors that, when activated by cleavage of a part of their extracellular domain, initiate intracellular signaling that can generate either a proinflammatory, barrier disruptive response or anti-inflammatory, barrier protective response depending on the surrounding environment and activating protease.

Of particular importance to coagulation factor-mediated proinflammatory signaling are PAR1, which modulates this barrier protective/barrier disruptive dichotomy, and the protein C pathway. In the presence of excess thrombin generation, protein C (PC) is cleaved to Activated Protein C (APC) by thrombin bound to thrombomodulin; this process is greatly enhanced by Endothelial Protein C Receptor, which binds PC and presents it to thrombin:thrombomodulin. Activated Protein C (APC) is responsible for proteolytically inactivating coagulation factor VIIIa, which comprises part of the FXase complex, and factor V, which forms a prothrombinase complex with FXa. Thus, APC prevents the formation of both thrombin and FXa.

When ECPR is not associated with PC/Activated Protein C (APC) and not colocalized with PAR1 in Caveolin-1 (Cav-1)-enriched lipid rafts, thrombin cleaves PAR1 at its conventional cleavage site, Arg41, leading downstream to RhoA and PKC activation and endothelial disruption[33,34]. However, when ECPR is associated with PC/APC and colocalized with PAR1 in Caveolin-1 (Cav-1)-enriched lipid rafts, thrombin, FVIIa, and APC cleave PAR1 at the Arg46. This leads to Dvl-2 and Rac-1 activated barrier protective signaling. This second response is difficult to achieve in the context of malaria infection, however, as endothelial activation due to parasite binding and contact with a proinflammatory environment trigger the shedding and a subsequent loss of EPCR from the cerebral microvascular endothelium[27]. Thus, the initiation of a barrier disruptive response during CM may, in fact, be a self-propagating problem in which excessive thrombin generation leads to endothelial activation and shedding of EPCR, which leads to further proinflammatory and barrier disruptive signaling due to thrombin cleavage of PAR1 and subsequent RhoA activation. However, a substantial TF deficiency or treatment with a low molecular weight heparin like dalteparin could sufficiently slow FXa and thrombin generation to dampen these effects, leading to less blood brain barrier disruption even in the presence of *P. berghei* ANKA.

Figures

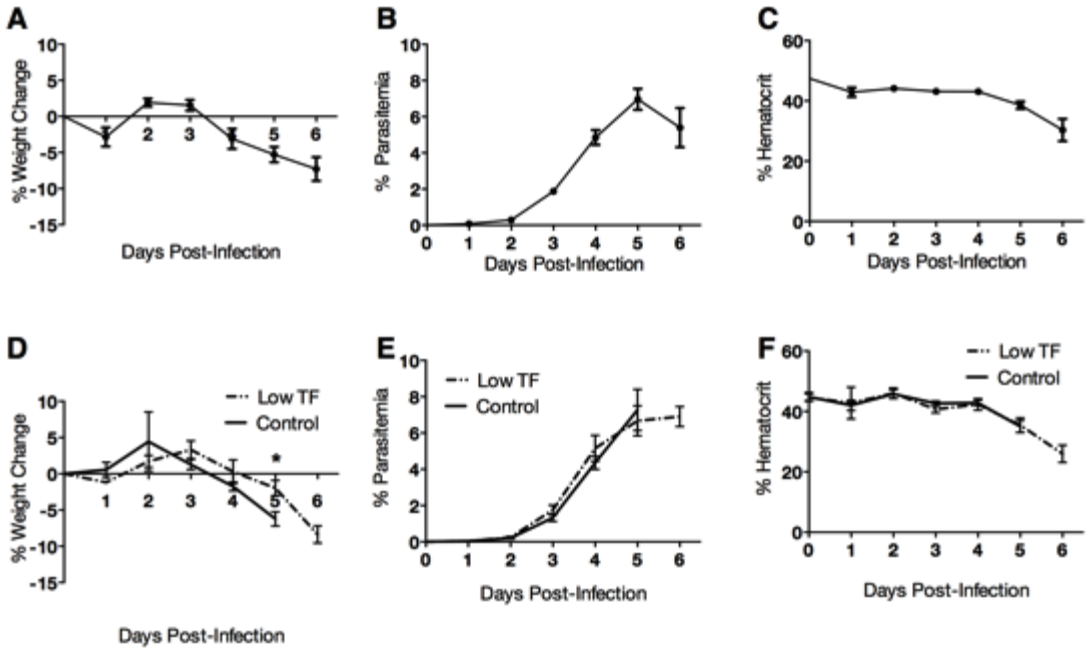

Figure 4.1: ***Reduced Tissue Factor expression does not significantly alter the course of*** **P. berghei** ***ANKA infection*****.** Weight, hematocrit, and parasitemia measurements are presented for infected C57BL/6J (A-C) and LTF and control strains (D-F). Clinical parameters were measured between ED 0-6. Data represent mean ± SEM.*p<0.05 by AOC analysis.

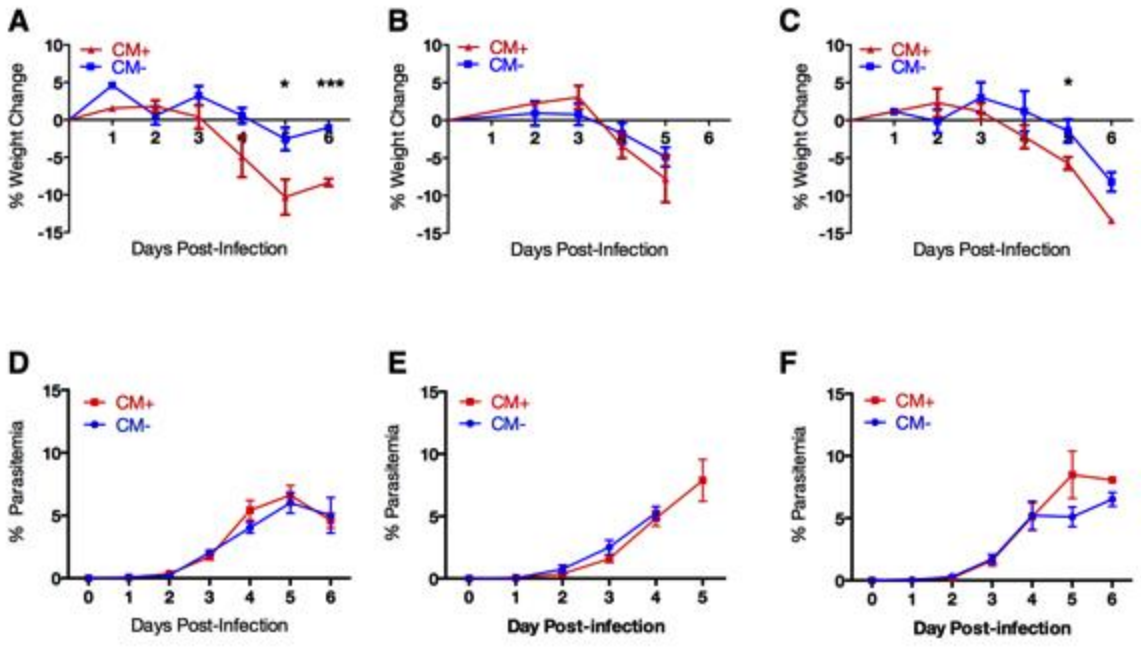

Figure 4.2: *Course of* P. berghei *ANKA varies significantly between CM+ and CM- mice of the same strain.* Though course of *P. berghei* ANKA infection did not vary on average between mice of different strains, significant differences in course of infection appeared when C57BL/6J (A,D), control (B,E) and LTF-/- (C,F) data were separated based on the presence (CM+) or absence (CM-) of neurological symptoms,Clinical parameters were measured between ED 0-6. Data represent mean ± SEM.*p<0.05, **p<0.001 by AOC analysis.

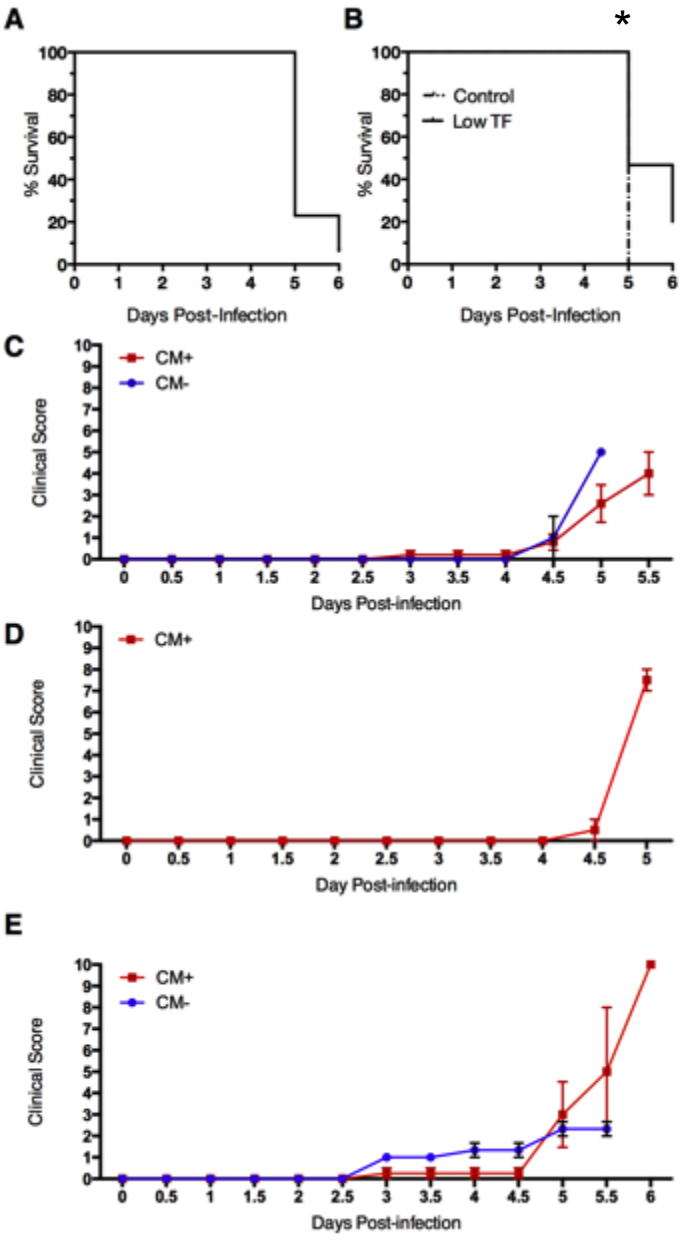

Figure 4.3: ***Tissue Factor deficiency improves survival but does not alter non-neurological clinical outcomes of ECM*.** Mice were assessed for clinical indicators of disease (outlined in Table 4.1) every 12 hours between ED0-6 (C57BL/6J, panel c; control, panel d; LTF-/- panel e). Survival rates were calculated for each strain through the duration of the experiment (C57BL/6J, panel A; control and LTF-/- panel B). *p<0.05; clinical scoring data represent mean ± SEM

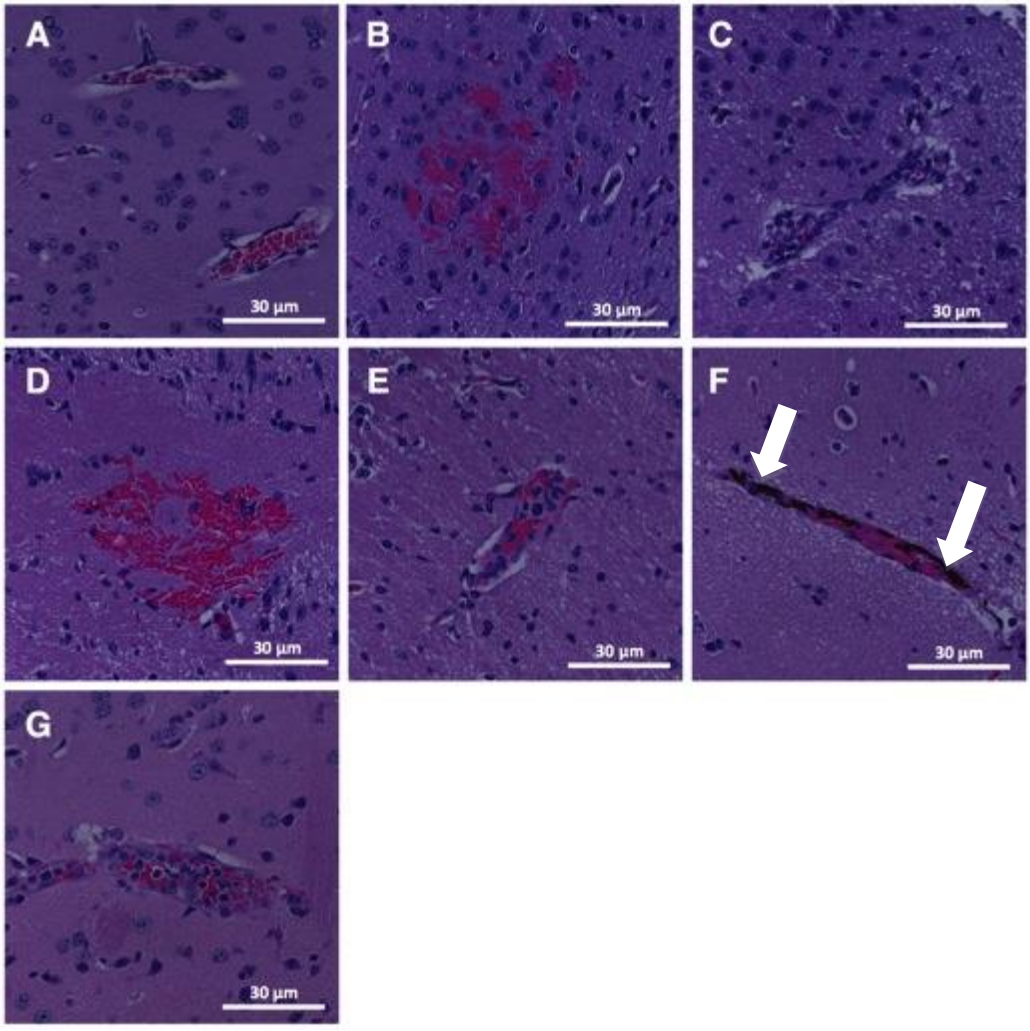

Figure 4.4: ***Cerebral malaria-positive LTF-/- mice are protected from pathological characteristics of human and ECM.*** H&E stained brain histological sections for uninfected C57BL/6J (A), CM+ C57BL/6J (B-C), CM+ control (D-F) and CM+ LTF-/- (G) are shown. Images were taken under a 40x objective. White arrows indicate hemozoin deposition.

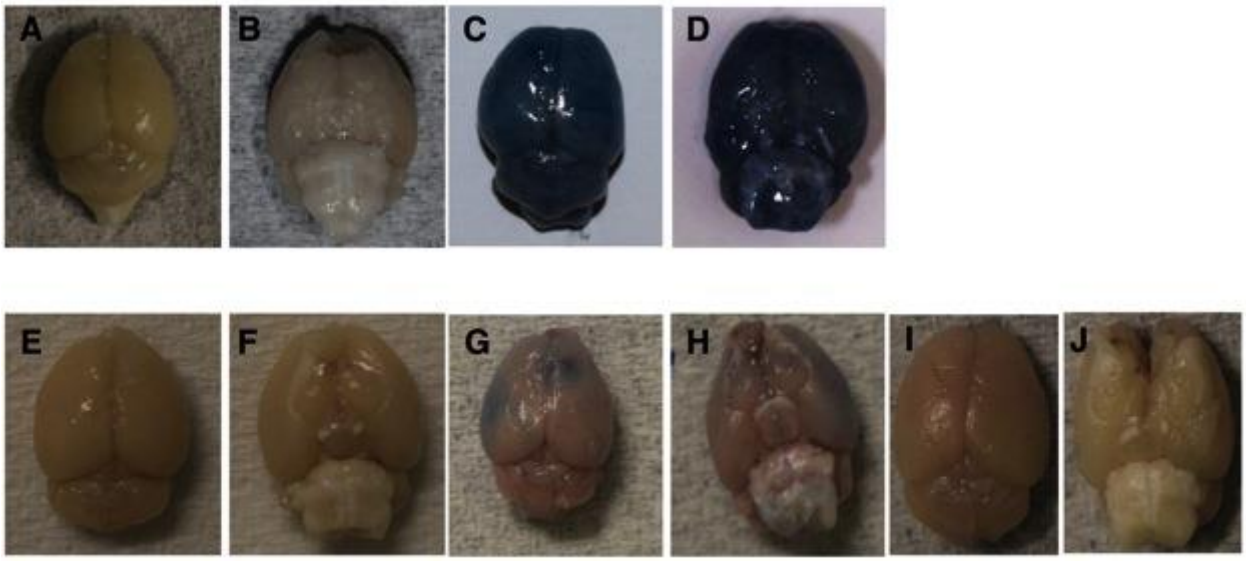

Figure 4.5: ***CM+ LTF-/- mice are protected from ECM-associated blood brain barrier disruption.*** Blood brain barrier disruption was assessed in CM- (A,B) and CM+ (C,D) control mice and CM- (E,F) and CM+ (G,H) LTF-/- mice on ED5. CM- LTF-/- mice were also observed on ED6 (I,J).

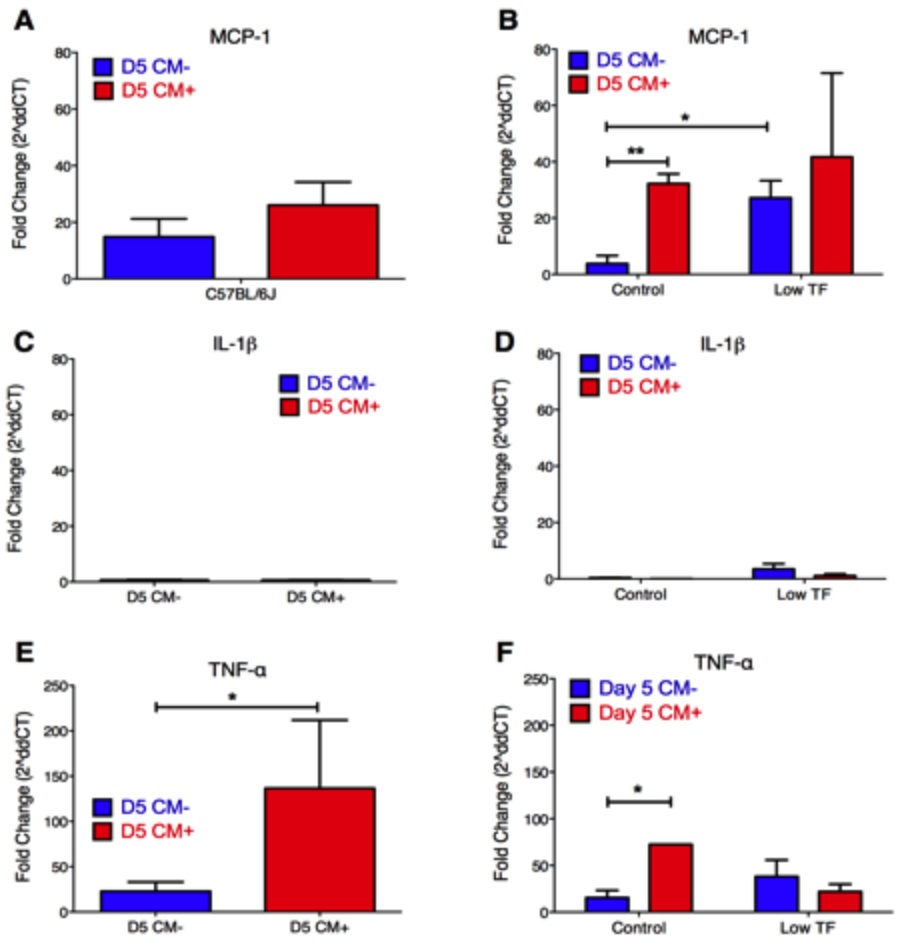

Figure 4.6: ***CM+ LTF-/- mice do not exhibit ECM-associated increases in proinflammatory cytokine expression.*** RNA isolated from CM+ and CM- C57BL/6J (A,C,E) and control and LTF-/- brains (B,D,F) and assessed for gene transcript levels of proinflammatory cytokines, including MCP-1, IL-1b, and TNF-a. *p<0.05, **p<0.01

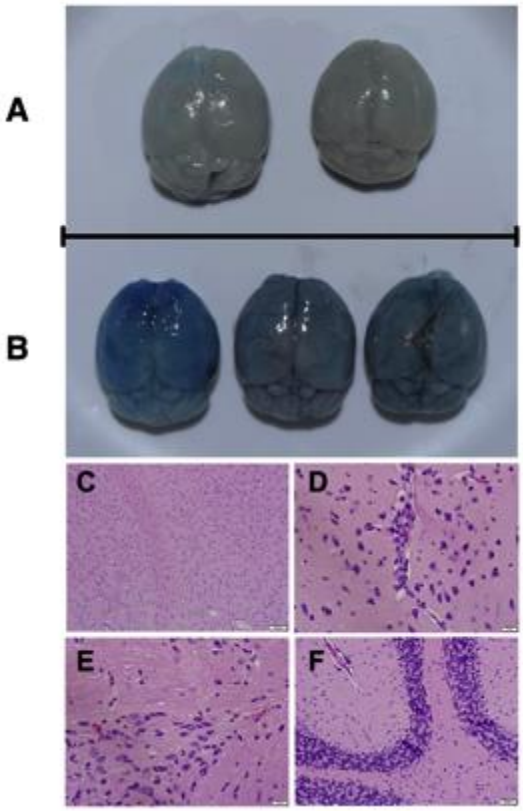

Figure 4.7: ***Dalteparin-treated mice are protected from ECM-associated blood brain barrier disruption.*** Blood brain barrier disruption was assessed in dalteparin-treated (A) and sham-treated (B) CM+ mice. H&E stained brain histological sections from CM+ dalteparin-treated mice were assessed for characteristics of CM, including inflammation, hemozoin deposition, and perivascular hemorrhaging. Images were taken under 10x (C, F) and 40x (D,E) objectives.

Tables

Table 4.1: ***Cerebral malaria clinical scoring rubric.*** Mice were assessed every 12 hours between ED0-ED6 for indicators of disease-related morbidity. Each mouse was assigned the appropriate score for each category, then those four scores were added to provide a single score at each time point. The presence of a neurological symptom (regardless of severity or number of symptoms evident) added 5 points to the total score.

	CLINICAL SCORE			
	0	**1**	**2**	**3**
Body Position	Normal	Curved	Hunched	Lying Flat
Activity Level	Active	Slow	Moves if Provoked	Unresponsive
Coat Appearance	Normal	Ruffled	Piloerected	Matted
Eye Position	Open	Half-closed	Closed	N/A

Table 4.2: ***C57BL/6J susceptibility to ECM.*** Between ED4-6, mice were assessed for the presence or absence of neurological symptoms. Any mouse exhibiting neurological symptoms was considered CM+ and sacrificed. The proportion of C57BL/6J mice designated CM+ on each experimental day is provided below.

Genotype	Day 4 CM+	Day 5 CM+	Day 6 CM+
C57BL6/J	0/42	16/34 (47%)	6/8 (75%)

Table 4.3: ***LTF-/- and control susceptibility to ECM.*** Between ED4-6, mice were assessed for the presence or absence of neurological symptoms. Any mouse exhibiting neurological symptoms was considered CM+ and sacrificed. The proportions of control and LTF-/- mice designated CM+ on each experimental day are provided below.

Genotype	Day 4 CM+	Day 5 CM+	Day 6 CM+
Control	0/15	11/11 (100%)	N/A
LTF	0/24	6/20 (30%)	1/10 (10%)

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CHAPTER 5

HELICOBACTER SPECIES SIGNIFICANTLY IMPACT PREGNANCY SUCCESS IN LABORATORY MICE[3]

[3] Bracken TC, Cooper CA, Ali Z, Truong H, Moore JM. Submitted to the *Journal of the American Association for Laboratory Animal Science*, 3/22/16.

Abstract

Helicobacter spp. are gram-negative, helical-shaped bacteria known to cause gastric and enterohepatic infections in mammalian species. While it is often noted that Helicobacter spp. infection may interfere with reproductive success, there is a dearth of empirical data to support these claims. The effect of Helicobacter spp. infection on murine pregnancy outcome was therefore investigated upon identification of endemic Helicobacter spp. infection in an animal research facility. Multiplex conventional PCR analysis was used to characterize Helicobacter spp. infection status in one inbred and two transgenic strains of mice. Outcomes of timed-mating experiments were compared among Helicobacter spp.-infected and uninfected mice of the same strain; Helicobacter spp. infection was eradicated from the colony through fostering with uninfected dams. Though fecundity rates were only affected by Helicobacter spp. infection in one strain of transgenic mouse Helicobacter spp. infection significantly reduced the total number of embryos per gravid uterus in C57BL/6J mice. Helicobacter spp. infection was also associated with a significant increase in the number of resorbing embryos per uterus and significant decreases in pregnancy-associated weight gain relative to uninfected mice in C57BL6/J mice and one transgenic strain. Helicobacter spp.-infected mice of all tested strains exhibited higher frequency of intrauterine hemorrhaging relative to uninfected mice. These results indicate that naturally-acquired Helicobacter spp. infection not only reduces the productivity of a research animal breeding colony, but also negatively impacts embryo health. Animal research facilities should therefore implement Helicobacter spp. surveillance and control practices in order to avoid confounding experimental results and to improve breeding colony efficiency.

Introduction

Helicobacter spp. are gram-negative, helical-shaped bacteria known to cause gastric and enterohepatic infections in mammalian species. Naturally acquired *Helicobacter* spp. infections have been reported in all rodent species commonly used for biomedical research[4,6,9,15,17,18,39,40,50].

Of the many *Helicobacter* spp., nine are known to infect mice: *H. bilis*, *H. hepaticus, H. typhlonius, H. muridarum, H. rodentium, H. mastomyrinus, H. rappini, H. pullorum* and *H. ganmani*[1,9,17,18,28,54]. Though *Helicobacter* spp. infections are typically subclinical in immunocompetent mice, these bacteria have been reported to be pathogenic and immunogenic in multiple inbred, transgenic, or immunodeficient mouse strains. Notably, *Helicobacter* spp. have been shown to induce liver,[45,53] colon[26,32,34] and prostate[38] carcinomas, typhlitis/typhocolitis[13,23,26,29,32,34,55], hepatitis[14,32,33,48,53], proctitis[13] and rectal prolapse[13,29] in certain mouse strains used as animal models for biomedical research. Further, *Helicobacter* spp. infection is known to induce a proinflammatory, Th1-biased immune response in infected mice that could confound the results of assays measuring an immune response to or assessing the phenotype resulting from an infectious or inflammatory disease[1,3,10,25,30,31,41,42,51,55].

A 2007 study investigating the incidence and prevalence of *Helicobacter spp.* infections in animal research facilities found that 84% of mice shipped from academic institutions worldwide tested positive for *Helicobacter* spp. by PCR[50]. Further, 64% of infected mice tested positive for *H. hepaticus*, which, along with *H. typhlonius,* is among the *Helicobacter* spp. most commonly reported to be pathogenic in mice[50].

While it is often noted that *Helicobacter* spp. infection may interfere with reproductive success, there is a dearth of empirical data to support these claims. Intentional infection with *H. typhlonius*, *H. rodentium* or both reduced reproductive performance of mice deficient in interleukin-10, a cytokine that normally limits

proinflammatory responses such as those induced by *Helicobacter* spp. infection. This effect was mitigated by antibiotic treatment[46]. Another report showed experimental *Helicobacter felis* infection of pregnant mice results in maternal iron deficiency, though no significant difference was seen in the number of pups born to infected versus uninfected mice[16]. *H. typhlonius* was also found in the reproductive organs of naturally infected C57BL/6J, C3H/HeJ, and athymic nude-*nu* immunodeficient mice[43]; however, the effect this bacterium had on organ function was not assessed. Lastly, experimental infection with *Helicobacter pylori*, a species known to colonize the human upper gastrointestinal tract, was reported to induce an increase in the number of fetal resorptions and resulted in lower average fetal weights compared to uninfected control mice[42].

These four studies comprise the full extent of the available published literature on how *Helicobacter* spp. infection affects murine pregnancy. Importantly, two of these four studies were performed using *Helicobacter* spp. not known to naturally infect mice, and therefore may not be relevant from the perspective of breeding colonies. To fill this significant gap in knowledge, we investigated the effect of *Helicobacter* spp. infection on pregnancy outcome upon identification of *Helicobacter* spp. infection via routine sentinel testing in experimental mice derived from our breeding colony.

Materials and Methods

Mice

C57BL/6J mice were originally purchased from The Jackson Laboratory and used to generate breeding stock and experimental animals in the University of Georgia Coverdell Vivarium. C57BL/6J were sourced from the Jackson Laboratory; at the time of this study, the C57BL/6J breeders had been housed in our facility for 6 months and were second generation. Transgenic mice used in these experiments include mice with floxed

Tissue Factor (TF, *F3*), the initiator of the extrinsic pathway of coagulation, expressing Cre-recombinase under the control of the Tek (Tie2) or Lyz2 (LysM) promoters.

Founder *F3*$^{flox/flox}$LysMCre breeder pairs were generously donated by Dr. Nigel Mackman, University of North Carolina, Chapel Hill, NC, USA, and were maintained by mating hemizygous *F3*$^{flox/flox}$LysMCre^{+} (LysMCre+) studs with *F3*$^{flox/flox}$LysMCre^{-} (LysMCre-) dams. *F3*$^{flox/flox}$Tie2Cre mice were initially generated by mating unfloxed hemizygous Tie2Cre^{+} studs sourced from the Jackson Laboratory with *F3*$^{flox/flox}$, LysMCre^{-} dams bred in-house from the line established by the founders describes above. Once a *F3*$^{flox/flox}$Tie2Cre line was established, it was maintained by breeding hemizygous *F3*$^{flox/flox}$Tie2Cre^{+} (Tie2Cre+) studs with *F3*$^{flox/flox}$Tie2Cre^{-} (Tie2Cre-) dams.

Tie2Cre+ mice have tissue specific deletions of TF in vascular endothelial cells and Tie2Cre+ fetal-derived trophoblast. Phenotypically normal Tie2Cre- littermates ere used as controls in experiments that included Tie2Cre+ mice in the absence of *Helicobacter* infection; *Helicobacter*-infected of this strain were not tested, as experiments were discontinued due to identification of endemic *Helicobacter* infection prior to the initiation of trials that included these animals. LysMCre+ mice have a specific deletion of TF from myeloid cells. Phenotypically normal LysMCre- littermates were used as controls in these experiments.

In the absence of *Helicobacter* infection, transgenic strains tested in these experiments are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities compared to C57BL/6J mice.

All experimental mice were virgin females between the ages of 8-10 weeks old. Stud males derived from the same breeders as the experimental virgin females were used until approximately 6 months of age, then replaced by younger males of the same lineage. Mice were initially intended for experiments studying the impact of malaria

infection on mid-gestational pregnancy outcome that required a gestational day 12 endpoint. These studies were temporarily discontinued once *Helicobacter* was identified.

Mouse experiments were performed in accordance with the guidelines and with the approval of the University of Georgia Institutional Animal Care and Use Committee (Animal Use Protocol number A2015 03-005-Y1-A1).

Husbandry

Mice were fed an irradiated diet and kept under specific pathogen free (SPF) conditions in the Coverdell animal facility. Routine facility-wide sentinel testing was performed monthly to monitor the presence of common mouse pathogens. Fecal flotation was performed, as well as PCR of fecal pellet DNA to detect mouse parvovirus, the Minute Virus of Mice (MVM), mouse hepatitis virus, *Clostridium piliforme*, mouse encephalomyelitis virus (strain GDVII), Epizootic Diarrhea of Infant Mice (EDIM) and Sendai virus. Mice were maintained in two rooms in a larger facility serving more than 20 investigators in 15 total rooms. At the time of this study, sentinels from seven other rooms in the Coverdell Vivarium tested positive for *Helicobacter* spp. infection by PCR. Strict protocols to restrict traffic between rooms within the facility and maintain clean cages and bedding were implemented to prevent further spread of infection within the facility.

Helicobacter Detection and Species Identification

Initial observation of *Helicobacter* occurring during standard sentinel testing, which identified genus-specific *Helicobacter* infection. Species-specific *Helicobacter* identification was performed in our laboratory; fresh fecal pellets were collected from each cage in the colony and pooled by strain. DNA was extracted from pooled samples using the DNeasy kit (Qiagen, Germantown, MD) according to manufacturer's

instructions. DNA was screened by multiplex conventional PCR as previously described[12] for the presence of DNA of five species of *Helicobacter — Helicobacter bilis, Helicobacter typhlonius, Helicobacter hepaticus, Helicobacter muridarum, and Helicobacter rodentium*. These species were selected based on evidence in the literature that they are commonly found in mouse colonies and have been associated with pathogenesis in other mouse models.

Initiation of Pregnancy and Clinical Assessment

Timed pregnancy experiments and monitoring of experimental mice was performed using a previously established protocol[37]. Briefly, the day on which a vaginal plug was observed in time-mated 8- to 12-week old female mice was gestational day zero (GD0). Mice were monitored daily from GD6 to GD12. Each mouse was weighed and hematocrit was used as a measure of anemia. Blood collected from the tail vein into a heparinized capillary tube was centrifuged in a microhematocrit centrifuge and percent hematocrit was calculated according to the following: (volume of packed erythrocytes)/(total blood volume)x100%.

On GD12, mice of all strains were anaesthetized using 2.5% Avertin (2,2,2-Tribromoethanol) administered via intraperitoneal injection and euthanized by exsanguination through the caudal vena cava. Embryo viability was assessed at necropsy as previously described[2]. Briefly, embryos exhibiting extensive intrauterine and/or intraembryonic hemorrhaging by gross pathology or lacking fetal heartbeat were scored as nonviable.

Fostering

Helicobacter-free Swiss Webster outbred mice (Charles River Laboratories) were utilized as foster mothers. A timed mating scheme was implemented in order to ensure

the presence of lactating foster females on the optimal day for Caesarean section. Swiss Webster females were paired with stud males 2-3 days before pairing the colony mice to undergo Caesarean section. Successful mating of mice was indicated by the presence of a vaginal plug, GD0. At GD19, colony mice were anaesthetized using 2.5% Avertin (2,2,2-Tribromoethanol). Under a laminar flow hood, the abdomen was opened and 70% ethanol was liberally applied to the peritoneum. The peritoneum was then opened and the whole uterus and pups excised with sterile scissors and forceps. The uterus was placed in doubled chlorhexidine-sprayed re-sealable zipper storage bags and immediately moved to a clean facility. The uterus was opened longitudinally and pups were removed. Sterile cotton-tipped applicators were used to remove amniotic membrane and to clear mucus from the neonates' noses and mouths. Each neonate was gently massaged until it was moving independently and breathing. The umbilicus was removed and neonates were placed on sterile paper towels laid over a hot water bottle containing 37°C water. Pups were covered to avoid loss of body heat and were massaged gently until sustained breathing without stimulation was achieved, approximately 10-15 minutes. Pups were rubbed with fresh fecal pellets from the foster mother's cage in order to give them a scent the foster mother would accept.

The foster mother was then transferred to a clean holding cage and her pups removed. Pups to be fostered were transferred to the foster mother's original cage, and intimate contact between pups and bedding was established in order to further coat the pups with the appropriate scent. In our experience, it was imperative that the numbers of pups in the endogenous and foster litters were identical; otherwise, the foster female rejected the new litter. If necessary, endogenous pups were added back to the foster litter in order to achieve the correct litter size. The foster mother was then returned to her original cage and monitored for approximately 15 minutes to ensure she accepted the foster pups.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software package (version 6.0). Correlation analysis was done using Spearman's test and 2x2 contingency tables were used for testing differences between proportions. The significance of difference of group means in the case of normally distributed data were compared via t-tests for pair-wise comparisons. Non-normally distributed data were analyzed by a non-parametric, Mann-Whitney test for pairwise comparisons. Differences with $p \leq 0.05$ were considered significant unless corrections were required for multiple comparisons.

Case Report

Detection and characterization of Helicobacter spp. infection.

During an ongoing study of the impact of malaria infection on mid-gestational pregnancy outcome, *Helicobacter* was detected by monthly sentinel testing in our colony. This prompted a cage by cage assessment for these bacteria using an in-house multiplex conventional PCR analysis (Table 1).14 mouse strains housed in two separate rooms within the same facility tested positive for *Helicobacter* infection, though pregnancy outcome was only assessed in the three strains described below. *Helicobacter* infection results in the additional 11 strains are presented in Supplemental Figure 1. DNA extracted from fecal pellets revealed that of the three mouse strains assessed for pregnancy outcomes, all were infected with *H. typhlonius.* All strains except the C57BL/6J mice were infected with *H. rodentium*, while only the $mTF^{flox/flox}LysMCre^{+}$ (LysMCre+), $mTF^{flox/flox}LysMCre^{-}$ (LysMCre-) mice and the $mTF^{flox/flox}Tie2Cre^{+}$ (Tie2Cre+) mice were infected with *H. hepaticus*. None of the strains tested returned a positive result for the presence of either *H. muridarum* or *H. bilis*.

Helicobacter spp. infection is associated with reduced fecundity rates in some transgenic mice.

In order to obtain a *Helicobacter* spp.-free colony, pups from infected mothers were fostered with lactating, uninfected dams and kept in a separate facility until no infected mice remained. *Helicobacter* spp. surveillance of breeder pairs was performed in-house on a monthly basis to ensure our colony remained *Helicobacter*-free.

Strain fecundity rates, defined as (number of pregnancies successfully initiated/number of observed plugs)x100%, were assessed for each strain of mouse in the presence and absence of *Helicobacter* spp. infection (Table 2). No significant difference was observed in fecundity of *Helicobacter*-infected and uninfected C57BL/6, LysMCre+, or LysMCre- mice. However, fecundity of *Helicobacter*-infected Tie2Cre+ mice was significantly impacted, resulting in 90% lower fecundity rates than those observed for uninfected mice of the same strain (p=0.002). Pregnancy outcome data for TF-intact, *Helicobacter* spp.-infected $mTF^{flox/flox}Tie2Cre^{-}$ (Tie2Cre-) littermates of Tie2Cre+ mice are not shown, as no *Helicobacter* spp.-infected mice of this strain yielded gravid uteri despite observation of a vaginal plug, indicating mating had occurred. However, this strain's fecundity rate in the absence of *Helicobacter* spp. is presented for comparison.

Helicobacter spp. *infection is associated with impaired weight gain in pregnant mice.*

C57BL/6J mice infected with *H. typhlonius* exhibited significantly reduced weight gain between GD6-12, resulting in a final average weight gain 23.4% lower in *H. typhlonius*-infected C57BL/6J mice relative to uninfected mice of the same strain (Figure 5.1). LysMCre+ mice were similarly affected by co-infection with *H. typhlonius*, *H. hepaticus*, and *H. rodentium*, exhibiting significantly reduced pregnancy-associated weight gain between GD7-10, with the trend continuing between GD11-12. Weights of

pregnant LysMCre- mice were unaffected by *Helicobacter* spp. infection. Maternal hemocrit remained unchanged by infection status relative to uninfected mice.

Helicobacter spp. infection negatively impacts midgestational embryo health.

Post-mortem gross pathologic assessment of uteri from *Helicobacter* spp.-infected mice revealed an increased frequency of intrauterine hemorrhaging and resorbing embryos relative to uninfected mice (Figure 5.2). Additionally, LysMCre+ uteri from *Helicobacter* spp.-infected mice were discolored compared to healthy uteri, exhibiting a notable grey-brown color on the embryo side of each horn. Vascularization was not obviously impaired in infected mice.

At GD12, numbers of viable and resorbing embryos per uterus were counted and the presence or absence of intrauterine bleeding was assessed in *Helicobacter* spp.-infected and –uninfected mice (Table 3). *Helicobacter* spp.-infected C57BL/6J mice had significantly small litter sizes (1.3-fold, p=0.0002) than uninfected mice of the same strain; however, *Helicobacter* spp.-infection did not significantly influence litter size of LysMCre+ mice. C57BL/6J (p=0.0108) and LysMCre+ (p=0.0006) mice infected with *Helicobacter* spp. had significantly more resorptions per uterus than their uninfected counterparts (p=0.0006). *Helicobacter* spp. infection had no significant impact on embryo viability or litter size in LysMCre- mice. *Helicobacter* spp. induced a significant increase in the incidence of intrauterine bleeding in all three strains.

Pregnancy outcome data are shown for *Helicobacter* spp.-uninfected Tie2Cre+ mice; however, post-mortem data were not collected for *Helicobacter spp.*-infected Tie2Cre+ mice. Despite the presence of vaginal plugs, no embryos were ever found in the uteri of infected mice of this strain. Thus, the gross pathologic effects of *Helicobacter* spp. infection on pregnancy in this mouse strain could not be assessed.

Discussion

Despite limited reports that presence of *Helicobacter* spp. infection impacts pregnancy in certain transgenic mouse strains, little work has been done to assess the effects of natural *Helicobacter* spp. infection on murine pregnancy. Here, we show that *Helicobacter* spp. infection has negative impacts on pregnancy not only in immunodeficient or transgenic mice, but also in C57BL/6J mice. C57BL/6 mice are often referred to as "*Helicobacter*-resistant[1,43]" due to the lack of obvious clinical signs of disease in *Helicobacter*-infected, non-pregnant C57BL/6 mice. However, these data show that while *Helicobacter*-infected C57BL/6J mice are able to carry pregnancy to term, endemic *Helicobacter typhlonius* infection in a mouse colony reduces reproductive success and embryo health *in utero* as early as six days post-mating.

In these studies, infection with a single *Helicobacter* species, *H. typhlonius*, had a more severe impact on C57BL6/J mice than infection with three *Helicobacter* species in the transgenic strains. No significant difference was seen in fecundity rate, litter size, uterine weight, or incidence of hemorrhaging between these strains in the absence of *Helicobacter* infection, indicating that observed heterogeneity among phenotypes in *Helicobacter*-infected strains is not due to inherent differences in fecundity, but rather in their respective responses to *Helicobacter* infection. Insufficient data were collected from this retrospective study to positively conclude why C57BL/6J mice fared worse in these experiments than their transgenic counterparts; however, while these mice are all either C57BL/6 or backcrossed to a C57BL/6 background, they originate from different substrains. C57BL/6J were sourced from the Jackson Laboratory, founder LysMCre breeder pairs were provided by a collaborator at UNC-Chapel Hill, and Tie2Cre mice were initially generated by mating unfloxed Tie2Cre+ breeders sourced from the Jackson Laboratory with LysMCre- breeders. Reports indicate C57BL/6 substrains acquired from different commercial sources and university animal facilities have genetic differences[21,27]

and different gut microbiome composition[19,24,52] that can have a significant effect on these substrains' immune responses to infection[8,20]. It is possible these effects were seen in the differently sourced strains used in these experiments. This hypothesis is in line with the observation that both strains of mice where at least one member of the founder breeder pair was sourced from the Jackson Laboratory exhibited similar phenotypes, while the outlier strain was obtained from an entirely different source. Additionally, mice with altered TF expression exhibit differential host responses to disease compared to TF-intact mice[7,11,36,44,49]. This worsened phenotype despite infection with fewer *Helicobacter* species could be a by-product of intact TF activity inducing a more robust inflammatory response in these mice than that produced by TF-altered mice.

Procoagulant activity and the extent of TF deletion in the *F3*$^{flox/flox}$, LysMCre+ and *F3*$^{flox/flox}$, Tie2Cre+ strains used in these studies have been assessed in detail in a mouse model of endotoxemia. LPS-induced procoagulant activity of peritoneal macrophages isolated from LysMCre+ mice and plasma Thrombin-Anti-Thrombin (TAT) complex levels, a marker of activated coagulation, were significantly reduced compared to LysMCre- littermates[35]. Using this same model, it was found that LPS-induced TF expression and activity are significantly reduced in both endothelial and hematopoietic cells isolated from Tie2Cre+ mice[35]. Effects of TF deletion on the immune response to infection in pregnant LysMCre+ and Tie2Cre+ mice have yet to be reported. However, as the initiator of the extrinsic pathway of coagulation, TF plays an important role in inducing thrombin-mediated proinflammatory responses; reduced production of proinflammatory cytokines due to ablation or impairment of TF activity has been seen in animal models of endotoxemia[7,11,44,49], sickle cell disease[36] and arthritis[56]. Thus, alterations to this gene's expression patterns likely would affect immune signaling following infection.

It is reasonable to assume that *Helicobacter* infection is somehow initiating a mechanism that, directly or indirectly, induces intrauterine hemorrhaging, and that the tissue-specific deletions of Tissue Factor differentially affect the host response to infection. Significant differences in pregnancy outcome existed among strains in the presence of *Helicobacter* spp. infection in only two cases: *Helicobacter* spp.-infected Tie2Cre+ and LysMCre+ mice exhibited a significantly reduced fecundity rate and a significantly greater proportion of resorbing embryos, respectively, compared to C57BL/6J mice. As the fecundity rate was drastically reduced in the Tie2Cre+ mice in the presence of infection, these data implicate endothelial or trophoblast-derived TF as playing a significant role in protecting against *Helicobacter* during the earliest stages of pregnancy. Additionally, the fact that fecundity rate was unchanged but embryo health was significantly reduced in *Helicobacter* spp.-infected LysMCre+ mice indicates that myeloid cell-derived TF may play a role in preventing *Helicobacter*-induced pathology during mid-gestation. However, since more detailed analyses could not be performed on these mice, precise determination of the mechanisms underlying these processes cannot be made using the data generated in these experiments. The different outcomes observed among *Helicobacter*-infected strains are possibly due to different host responses to infection. As the initiator of the extrinsic pathway of coagulation, TF plays an important role in inducing thrombin-mediated proinflammatory responses; reduced production of proinflammatory cytokines due to ablation or impairment of TF activity has been seen in animal models of endotoxemia[7,11,44,49], sickle cell disease[36] and arthritis[56]. Thus, alterations to the expression patterns of this gene likely would affect immune signaling following infection.

This study is a retrospective analysis of experiments that were discontinued upon detection of *Helicobacter* spp. infection, and tissue samples generated from infected mice were discarded to avoid further contamination of our facility before they could be

analyzed. As such, retroactive histologic assessment and culture of uteri lumen to positively identify the presence of *Helicobacter* spp. in the reproductive tract were not performed on samples generated from these mice. Thus, it is possible other common mouse pathogens that could have been eliminated through fostering are responsible for the phenotypes observed in these experiments; however, this is unlikely. Sentinel testing records from six months before, during, and six months after these experiments were performed indicate that the following parasites were absent from the colony during this time period: mouse parvovirus, the Minute Virus of Mice (MVM), mouse hepatitis virus, *Clostridium piliforme*, mouse encephalomyelitis virus (strain GDVII), Epizootic Diarrhea of Infant Mice (EDIM), Sendai virus and any parasites whose eggs or cysts are detectable by fecal flotation. These results rule out those specific common mouse pathogens as possible causes for the observed phenotypes.

Although the *Helicobacter* spp.-infected C57BL/6J mice were presumably acquired *Helicobacter*-free from the Jackson Laboratory, this cannot be confirmed as direct testing was not performed upon acquisition of these mice. Mice from other sources, including from investigators at other institutions, were also maintained in our colony (referring to two rooms assigned to our program in a much larger facility that currently serves more than 20 investigators); it is possible that *Helicobacter* was thus imported, but at this point that cannot be proven.

A decrease in breeding colony efficiency resulting from the *Helicobacter*-induced reductions in pregnancy success seen in this study could lead to costly increases in time and labor required to generate sufficient numbers of experimental mice. However, the impacts of these results may also extend beyond a simple increase in lost time and funds. Evidence in the literature indicates that *Helicobacter* spp. infection has the potential to confound the results of certain types of experiments, particularly those studying inflammatory diseases and the gut. However, if *Helicobacter* spp. infection

slows, stunts, or otherwise interferes with embryo development, the resulting reduction in embryo health could have lasting consequences for the embryos as they grow to mature mice used in an experimental setting. In both mice and humans, evidence suggests that many major chronic diseases, including coronary heart disease, hypertension, and type II diabetes, may originate in impaired intrauterine growth and development, a phenomenon commonly referred to as “programming” or the “Fetal Origins” hypothesis[5,22,47]. Thus, negative effects *Helicobacter* spp. infection has *in utero* may also have long-term repercussions that could impact future experiments using those mice. These results indicate that animal research facilities should implement *Helicobacter* spp. surveillance and control practices in order to avoid confounding experimental results and improve breeding colony efficiency, regardless of the ultimate intended use for the resulting pups.

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Figures

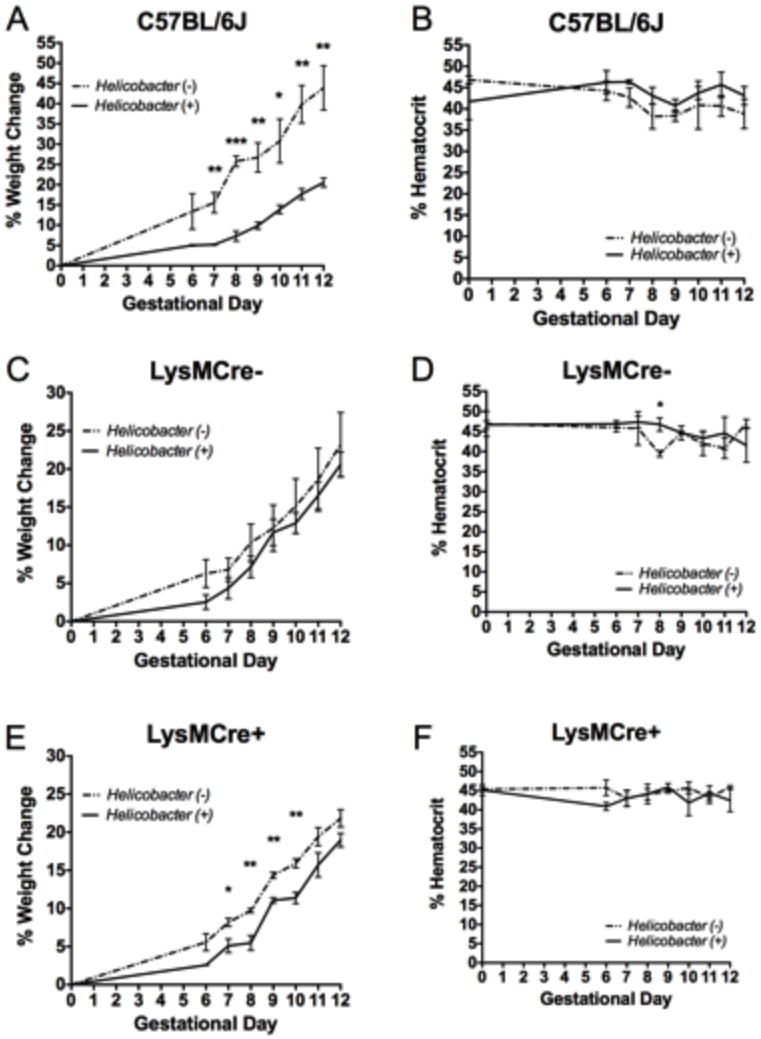

Figure 5.1: Helicobacter *spp. infection significantly impacts pregnancy-associated weight gain but does not induce maternal anemia.* Pregnant mice were weighed on GD0 and from GD6-12. *Helicobacter* spp. infection resulted in significantly reduced weight gain in C57BL/6 (A) and LysMCre+ (C) mice. Weight gain in LysMCre- mice was unaffected (B). Hematocrit was largely unaffected by the presence or absence of *Helicobacter* spp. infection in these strains. The data represent the mean ± SEM and n=4 mice per group. *p<0.05, **p<0.01, ***p<0.001.

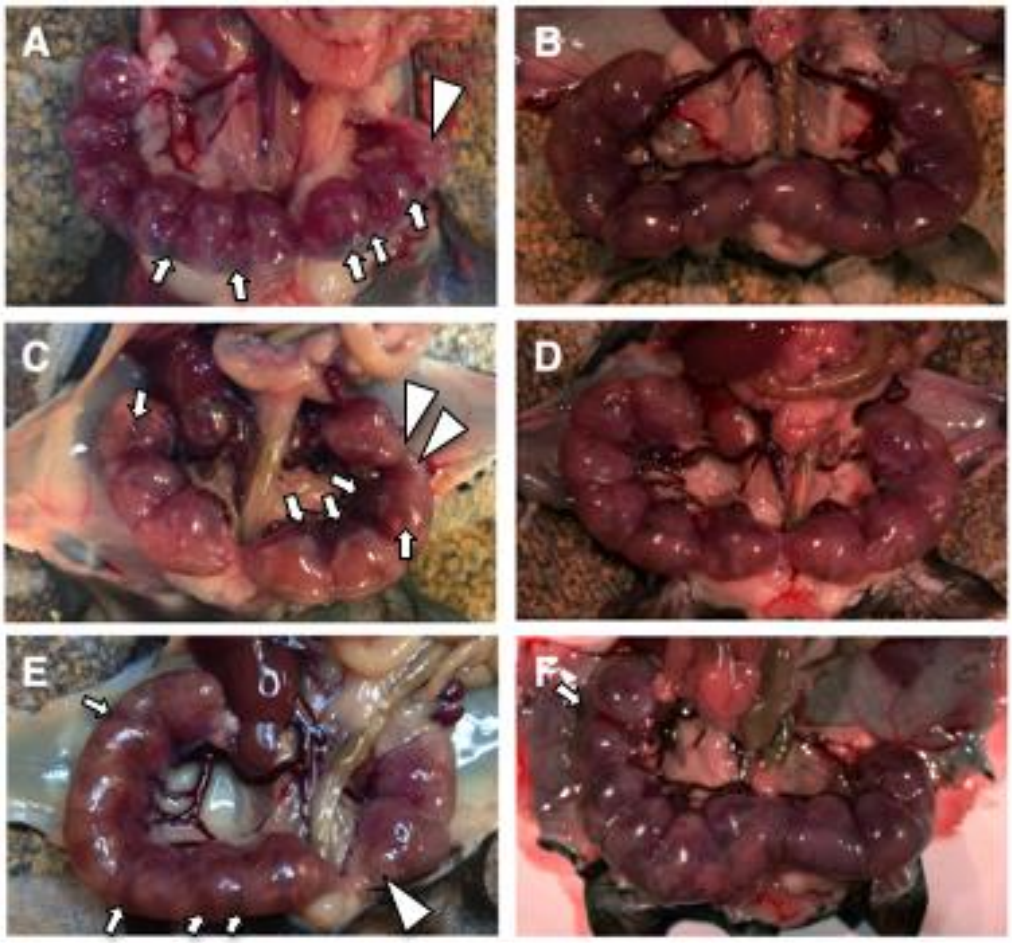

Figure 5.2: Helicobacter *spp. negatively impacts midgestational embryo health.* Gross pathological view of C57BL/6J (A, B), LysMCre- (C, D), and LysMCre+ (E, F) uteri in *Helicobacter* spp.-infected (A, C, E) and to uninfected mice (B, D, F). Macroscopic evaluation of uteri revealed a significant increase in intrauterine hemorrhage (white arrows) in *Helicobacter* spp.-infected C57BL/6J, LysMCre- and LysMCre+ mice relative to uninfected mice of the same strain (quantified in Table 3). *Helicobacter* spp.-infected C57BL/6J and LysMCre+ mice exhibited significantly greater numbers of resorbing embryos (blunt white arrows) than uninfected mice of these strains (Table 3).

Tables

Table 5.1: *Characterization of colony-wide* Helicobacter *spp. infection.* Fecal pellets were collected from each strain and assessed for presence of *Helicobacter* spp. DNA by species-specific multiplex PCR. *Helicobacter typhlonius* was present in all three strains. The TF-floxed, LysMCre and TF-floxed, Tie2Cre mice were additionally infected with *Helicobacter hepaticus* and *Helicobacter rodentium*. *p<0.001 compared to *Helicobacter* spp.-infected C57BL/6J.

Mouse Strain	*Helicobacter* Species Present				
	H. typhlonius	***H. hepaticus***	***H. muridarum***	***H. bilis***	***H. rodentium***
C57BL6/J	**+**	**-**	**-**	**-**	**-**
mTF$^{flox/flox}$LysMCre	**+**	**+**	**-**	**-**	**+**
mTF$^{flox/flox}$Tie2Cre	**+**	**+**	**-**	**-**	**+**

Table 5.2: *Strain-specific fecundity rates in the presence and absence of* Helicobacter *spp. infection.* At gestational day 12, mice were euthanized and the number of gravid uteri per observed vaginal plug was assessed. *Helicobacter* spp. infection only had a significant effect on the fecundity rate of Tie2Cre+ mice relative to uninfected mice of the same strain. C57BL6/J *Helicobacter* spp.-infected, n=22; *Helicobacter* spp.-uninfected, n=6. LysMCre- *Helicobacter* spp.-infected, n=12; *Helicobacter* spp.-uninfected, n=5. LysMCre+ *Helicobacter* spp.-infected, n=11; *Helicobacter* spp.-uninfected, n=6. Tie2Cre- *Helicobacter* spp.-infected, NT; *Helicobacter* spp.-uninfected, n=8. Tie2Cre+ *Helicobacter* spp.-infected, n=5; *Helicobacter* spp.-uninfected, n=10.

Mouse Strain	**Gravid Uteri/ Observed Plugs**		**p-value**
	***Helicobacter spp.* (+)**	***Helicobacter spp.* (-)**	
C57BL6/J	20/22 (90%)	5/6 (83%)	0.5
LysMCre-	9/12 (75%)	4/5 (80%)	1.00
LysMCre+	7/11 (64%)	5/6 (83%)	0.60
Tie2Cre-	NT	7/8 (88%)	N/A
Tie2Cre+	0/5 (0%)*****	9/10 (90%)	0.002

Table 3: *Helicobacter* spp. significantly reduces implantation frequency and embryo viability in pregnant mice. At GD12, numbers of viable and resorbing embryos per uterus were counted and the presence or absence of intrauterine bleeding was assessed in *Helicobacter* spp.-infected and –uninfected C57BL/6J, LysMCre-, LysMCre+ and Tie2Cre+ pregnant mice. *N/A* indicates that pregnancy outcome data are not available for *Helicobacter* spp.-infected Tie2Cre+ mice, as no mice of this strain successfully implanted while infected. C57BL6/J *Helicobacter* spp.-infected, n=12; *Helicobacter* spp.-uninfected, n=4. LysMCre- *Helicobacter* spp.-infected, n=12; *Helicobacter* spp.-uninfected, n=3. LysMCre+ *Helicobacter* spp.-infected, n=11; *Helicobacter* spp.-uninfected, n=5. Tie2Cre+ *Helicobacter* spp.-infected, N/A; *Helicobacter* spp.-uninfected, n=6. *p<0.001 compared to *Helicobacter* spp.-infected C57BL/6J.

Mouse Strain	Average Number of Embryos/ Uterus			Total Number of Resorptions/ Total Number of Embryos			Number of Uteri Exhibiting Intrauterine Hemorrhaging		
	***Helicobacter spp.* (+)**	***Helicobacter spp.* (-)**	**p-value**	***Helicobacter spp.* (+)**	***Helicobacter spp.* (-)**	**p-value**	***Helicobacter spp.* (+)**	***Helicobacter spp.* (-)**	**p-value**
C57BL6/J	9 [6,10]	11.00 [10,12]	0.0002	14/102 (13.73%)	0/44 (0.00%)	0.01	11/12 (91.67%)	0/4 (0.00%)	0.003
LysMCre-	8 [6,10]	9 [9,10]	0.2	22/96 (22.92%)	2/28 (7.14%)	0.1	12/12 (100%)	0/3 (0.00%)	0.002
LysMCre+	8 [7,11]	9 [8,11]	0.4	34/93* (36.56%)	1/45 (4.00%)	0.0001	11/11 (100%)	1/5 (20.00%)	0.003
Tie2Cre+	N/A	8.67 [6,11]	N/A	N/A	3/52 (5.77%)	N/A	N/A	2/6 (33.33%)	N/A

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Chapters 3 and 4 of this dissertation assessed how alterations to the coagulation cascade affect the outcome of placental (PM) and cerebral malaria (CM). The experiments outlined in Chapter 3 revealed that coagulation can ameliorate or exacerbate pathology depending on where it is expressed. Deletion of Tissue Factor (TF) from myeloid cells accelerated pregnancy loss by one day and induced significant alterations to the course of parasitemia in these mice; however, deletion of TF from either the maternal endothelium or hematopoietic cells significantly improved pregnancy outcome in the face of malaria infection, resulting in litters of normal weight and size at mid-gestation. Chapter 4 showed that global TF deficiency can prevent the onset of neurological symptoms and reduce blood brain barrier permeability in those few cases where neurological symptoms manifest. Both models showed that targeting FXa and, to a much lesser extent, thrombin, with anti-coagulant drugs mitigates severe pathologies associated with murine PM and experimental CM (ECM).

These studies provide compelling evidence that anticoagulant therapies may be an effective tool to consider for use in avoiding negative outcomes of severe malaria infection in human patients. However, many questions will need to be addressed before such therapies can be used in a clinical setting. It is still unclear how interfering with coagulation affects the outcome of CM and PM. Is TF on maternal monocytes assisting with parasite control in the maternal blood space? Could TF on the maternal endothelium allowing for hyperactivation of coagulation, leading to thrombin-mediated activation of inflammation and subsequent inflammatory damage to the placenta?

In CM, the lack of increased expression of cytokines typically seen elevated during ECM indicate that in the absence of a procoagulant environment, signaling directing the production of these

cytokines is not as potent. However, from where do those signals derive? Are immune cells that have migrated to the site of infection being activated and serving as the major source of proinflammatory cytokines? Are they the result of endothelial activation, whether by parasites, infiltrating immune cells, thrombin, APC, fibrin degradation products, malarial components, or any number of other factors present in the brain microvasculature during ECM?

How does the absence of TF provide protection for the blood brain barrier during ECM? Is the slowed rate of thrombin generation allowing for barrier-protective PAR1 signaling and Rac1 activation (as described in Figures A.1 and A.2)? Are the relatively lower levels of proinflammatory cytokines resulting in less inflammatory damage in the brain microvasculature? Could reduced blood vessel occlusion due to impaired fibrin production be preventing hypoxic/ischemic injury to the brain tissue, resulting in improved outcome?

The future directions of this dissertation aim to answer these questions several ways. First, a more complete analysis of cytokine levels in placental samples derived from the PM model and peripheral plasma from both models of infection are essential to provide context for the alterations seen in these experiments. Without such context, it is difficult to draw conclusions about how the host response is being altered. Additionally, given the ability of parasite-infected erythrocytes to bind the Endothelial Protein C Receptor (EPCR), enabling sequestration while interfering with natural anticoagulant processes, EPCR expression in the brain and on the placenta should be assessed in the context of disease. The difficulty experienced in successfully measuring endothelial targets in cerebral tissue by immunohistochemistry and qPCR over the course of the experiments described in Chapter 4 indicate this may not be feasible in the CM model. However, measuring soluble EPCR in infected mouse plasma may serve as a suitable substitute to provide insight into whether or not EPCR is being shed from the cerebral vascular endothelium in response to ECM. Ongoing experiments are also underway to assess the impact of these interventions in a model of placental malaria infection during late pregnancy; as human placental data is exclusively generated in term placentae due to the

inaccessibility of this organ prior to that time point, a late gestation model should better enable us to draw connections between human and mouse PM.

These data should provide a jumping off point from which to build a more complete understanding of how coagulation contributes to the pathogenesis of CM and PM. By showing that interfering with the coagulation cascade drastically improves disease outcomes in mice, hopefully this research will encourage other labs to consider coagulation when interpreting their results and planning new investigations. In the end, the better we understand which components of these complex systems contribute to pathogenesis, the better we'll be able to develop and implement more precise — and therefore more effective and safer — therapies to help relieve the burden of PM and CM on malaria-endemic populations.

APPENDIX A

A PROPOSED MODEL FOR CEREBRAL MALARIA-INDUCED BLOOD BRAIN BARRIER DISRUPTION

Outlined below is a proposed model for how a procoagulant state in Cerebral Malaria (CM) may induce blood brain barrier disruption (Figure A), and another for how Tissue Factor deficiency may result in a different, barrier protective series of events.

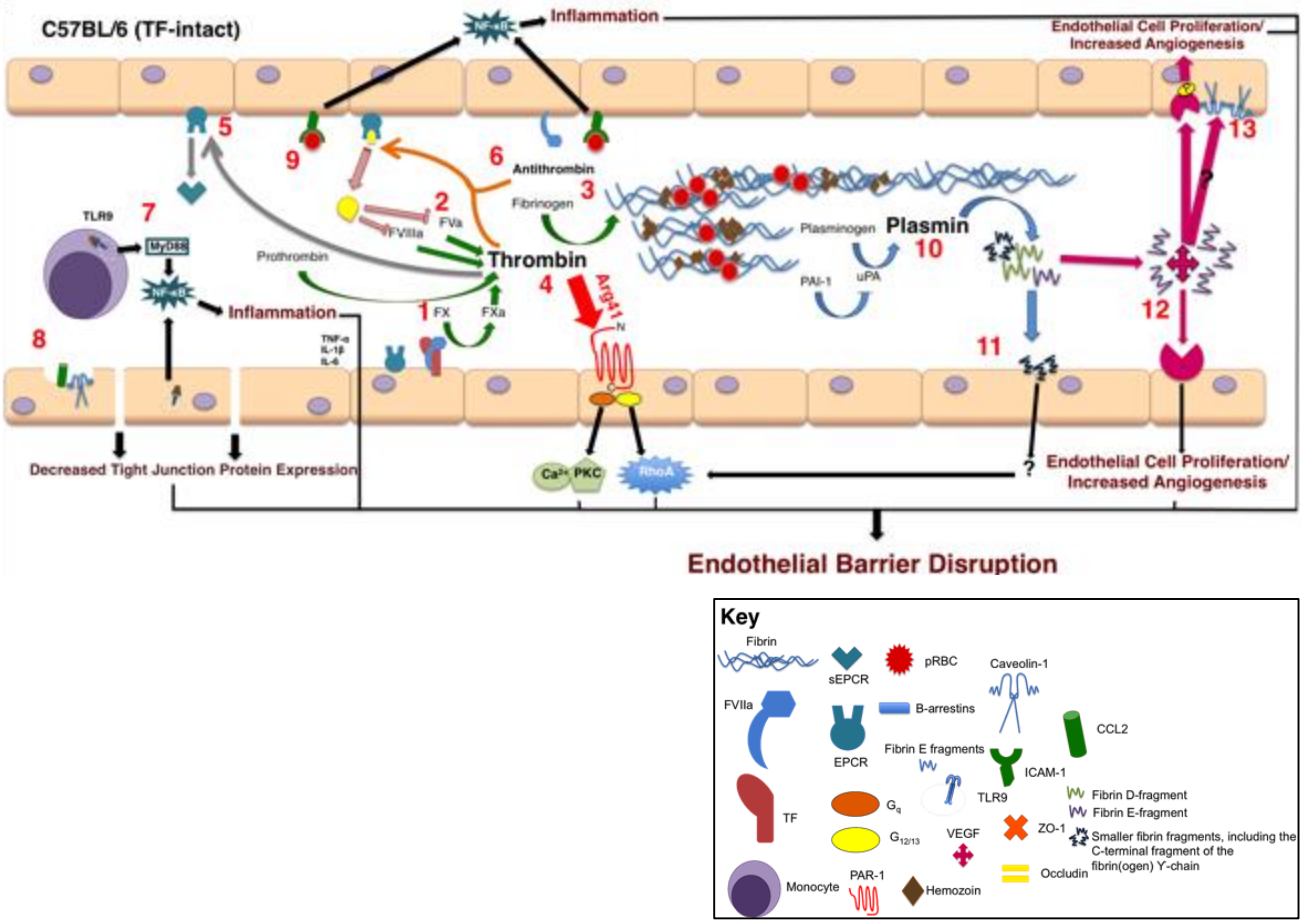

Figure A.1: A proposed model for blood brain barrier disruption in the presence of a cerebral malaria-induced procoagulant state.

The Tissue Factor/FVIIa complex cleaves FX to FXa to form the procoagulant TF-FVIIa-FXa ternary complex. The ternary complex cleaves the zymogen prothrombin to the active serine protease, thrombin. Active thrombin is cleaves other members of the coagulation cascade to generate FVIIIa and FVa, which by cleaving and complexing with FXa, respectively, lead to further thrombin production. Thrombin cleaves fibrinogen to fibrin, the main structural component of clots. In human CM, fibrin is deposited primarily in the vessel lumen, rather than along the endothelial lining. The fibrin deposited captures IE and the crystalline malarial by-product of hemoglobin digestion, hemozoin. When ECPR is not associated with PC/APC and

not colocalized with PAR1 in Caveolin-1 (Cav-1)-enriched lipid rafts, thrombin cleaves PAR-1 at the conventional cleavage site, Arg41, leading downstream to RhoA and PKC activation and endothelial disruption (Soh et al., PNAS Dec 2011; PMID: 22106258) (Russo et al., PNAS Apr 2009; PMID: 19332793)

At high concentrations of thrombin, the soluble extracellular domain of EPCR (sEPCR) is cleaved by thrombin and shed into the vessel lumen. (Gu et al., Blood Mar 2000; PMID: 10688825) (Bouwens et al, J. Thromb. Haemostat. Jun 2013; PMID: 23809128). This creates less endothelial-bound EPCR available for protective PAR-1 signaling. High plasma levels of sEPCR have been associated with mortality in human pediatric CM. (Moussiliou et al., JID Nov 2014; PMID: 25425698).
An increased procoagulant response activates a corresponding anticoagulant response to counteract excessive thrombin generation. Antithrombin irreversibly binds thrombin, inactivating it. Thrombin-Antithrombin (TAT) complexes cleave Protein C (PC) bound to Endothelial Protein C Receptor (EPCR), generating Activated Protein C (APC). APC, with the help of its cofactor, Protein S, degrades FVIIIa and FVa.

Hemozoin that comes into contact with immune cells (in this case, a monocyte) rather than being trapped in fibrin is recognized by TLR9, leading to MyD88 and NF-κB proinflammatory signaling. (Coban et al., Host Cell Microbe. Jan 2010, PMID: 20114028) (Jaramillo et al., J Immunol Mar 2004; PMID: 14978116) (Coban et al., Int Immunol Jan 2007; PMID: 17135446) (Griffith et al., JID Nov 2007; PMID: 18008236). A proinflammatory response leads to increased blood brain barrier permeability. (Huber et al, Am J Physiol Heart Circ Physiol. Mar 2001; PMID: 11179069).

Endothelial cells from patients with cerebral malaria were found to produce higher levels of CCL2 in response to TNF relative to those from patients with

uncomplicated malaria, and malarial components were found to be able to induce increased CCL2 production by murine macrophages in vitro (Jaramillo et al., J Immunol Jan 2005; PMID: 15611273). Additionally, reduced levels of CCL2 have been associated with improved phenotypes in experimental cerebral malaria (Francischetti et al., PLoS One Feb 2014; PMID: 24586264) (Kim et al., Infect Imm. Jan 2014; PMID: 24191300). Exposure to CCL2 has been reported to reduce expression of Cav-1, mediating loss of tight junctional proteins occludin and zonula occludens-1 (ZO-1), creating a decrease in tight junctional integrity, and increasing blood brain barrier permeability (Song et al., Blood Feb 2007; PMID: 17023578). It should be noted that APC signaling and blood-brain barrier protective effects were found to be abolished in cells lacking cav-1, though conventional thrombin cleavage of and signaling through PAR-1 remained intact (Russo et al., PNAS Apr 2009; PMID: 19332793).

IE sequester in the cerebral vasculature by binding to a number of receptors, including ICAM-1, VCAM-1, CD36, and EPCR. IE binding to the endothelium has been shown to activate NF-κB proinflammatory signaling, further exacerbating endothelial barrier disruption (Rowe et al., Expert Rev Mol Med May 2009; PMID: 19467172).

Urokinase-type plasminogen activator (uPA) cleaves plasminogen to plasmin, which cleaves fibrin into its degradation products. Plasmin cleavage of each fibrinogen molecule gives rise to two D fragments, several smaller fragments including β 1-42 (the amino terminal of the β chain), and one E fragment consisting of the amino terminal regions of the α-, β-, and γ-chains chains held together by disulfide bonds. The uPA activation of Plasminogen is inhibited by Plasminogen Activator Inhibitor-1 (PAI-1).

The C-terminal fragment of the fibrin(ogen) ϒ-chain directly interacts with the endothelium, inducing barrier disruption via an integrin-mediated, RhoA-dependent mechanism (Guo et al, ATVB Jan 2009; PMID: 19122172).

Fibrin E-fragment enhances the effects of proangiogenic factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF, not shown) . Significantly increased endothelial cell proliferation leads to further loss of endothelial barrier integrity (Bootle-Wilbraham et al, Angiogenesis 2001; PMID: 12197472).

Caveolin-1 significantly accelerates VEGF receptor 2 (VEGFR2) autophosphorylation, inducing proangiogenic signaling and increased endothelial barrier permeability (Tahir et al., Cancer Biol. Ther. Dec 2008; PMID: 19923922).

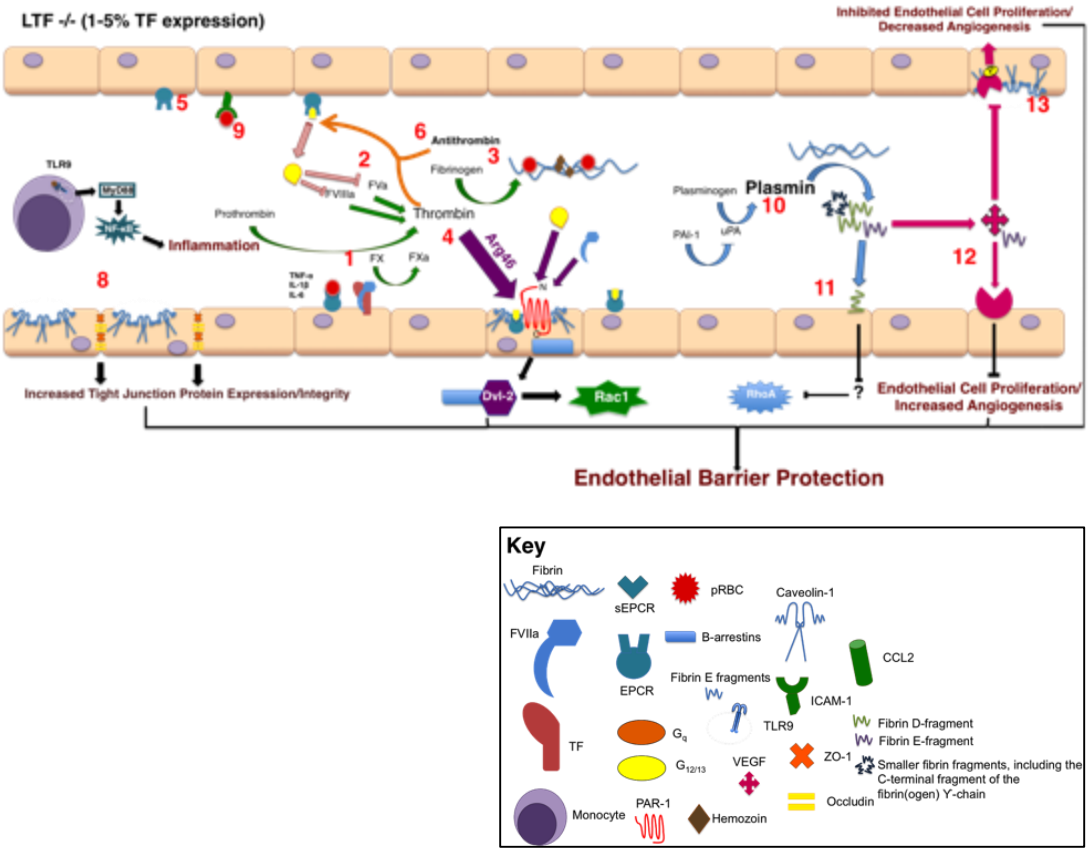

Figure A.2: A proposed model for blood brain barrier protective mechanisms induced by low tissue factor expression in the presence of malaria infection.

The Tissue Factor/FVIIa complex cleaves FX to FXa to form the procoagulant TF-FVIIa-FXa ternary complex. The ternary complex cleaves the zymogen prothrombin to the active serine protease, thrombin. Active thrombin is able to cleave other members of the coagulation cascade to generate FVIIIa and FVa, which by cleaving and complexing with FXa, respectively, lead to further thrombin production. This would be happening a lower levels than in TF-intact mice.

Thrombin cleaves fibrinogen to fibrin, the main structural component of clots. In human CM, fibrin is deposited primarily in the vessel lumen, rather than along the endothelial lining. The little fibrin deposited captures some parasitized red blood cells (pRBCs) and the crystalline malarial by-product of hemoglobin digestion, hemozoin.

When ECPR is associated with PC/APC and colocalized with PAR1 in Caveolin-1 (Cav-1)-enriched lipid rafts, thrombin, FVIIa, and APC cleave PAR-1 at the Arg 46. This leads to Dvl-2 and Rac-1 activated barrier protective signaling (Russo et al., PNAS Apr 2009; PMID: 19332793) (Soh et al., PNAS Dec 2011; PMID: 22106258). As not much thrombin is present, less sEPCR will be released into the vessel lumen.

An increased procoagulant response activates a corresponding anticoagulant response to counteract thrombin generation. Antithrombin irreversibly binds thrombin, inactivating it. Thrombin-Antithrombin (TAT) complexes cleave Protein C (PC) bound to Endothelial Protein C Receptor (EPCR), generating Activated Protein C (APC). APC, with the help of its cofactor, Protein S, degrades FVIIIa and FVa. Again, this will be happening at a lower level than in TF-intact mice.

Hemozoin that comes into contact with immune cells (in this case, a monocyte) rather than being trapped in fibrin is recognized by TLR9, leading to MyD88 and NF-κB proinflammatory signaling. (Coban et al., Host Cell Microbe. Jan 2010, PMID: 20114028) (Jaramillo et al., J Immunol Mar 2004; PMID: 14978116) (Coban et al., Int Immunol Jan 2007; PMID: 17135446) (Griffith et al., JID Nov 2007; PMID: 18008236).

Reduced CCL2 relative to TF-intact CM+ mice reduces loss of Cav-1, preventing the loss of tight junctional proteins occludin and zonula occludens-1 (ZO-1), maintaining tight junctional integrity, and decreasing blood brain barrier permeability (Song et al., Blood Feb 2007; PMID: 17023578).

pRBCs sequester in the cerebral vasculature by binding to a number of receptors, including ICAM-1, VCAM-1, CD36, and EPCR. pRBC binding to the endothelium has been shown to activate NF-κB proinflammatory signaling (Rowe et al., Expert Rev Mol Med May 2009; PMID: 19467172).

Urokinase-type plasminogen activator (uPA) cleaves plasminogen to plasmin, which cleaves fibrin into its degradation products. Plasmin cleavage of each fibrinogen molecule gives rise to two D fragments, several smaller fragments including β 1-42 (the amino terminal of the β chain), and one E fragment consisting of the amino terminal regions of the α-, β-, and γ-chains chains held together by disulfide bonds. The uPA activation of Plasminogen is inhibited by Plasminogen Activator Inhibitor-1 (PAI-1). As less fibrin generation is occuring in these mice, it will occur at a lower rate than in TF-intact mice.

Fewer C-terminal fragments of the fibrin(ogen) ϒ-chain interact with the endothelium, reducing barrier disruption (Guo et al, ATVB Jan 2009; PMID: 19122172). Fibrin E-fragment enhances the effects of proangiogenic factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF, not shown). As reduced amounts of fibrin are being cleaved into its degradation products, fewer Fibrin E-fragments are available to induce loss of endothelial barrier integrity (Bootle-Wilbraham et al, Angiogenesis 2001; PMID: 12197472). Further, Fibrinogen E-fragments cleaved by plasmin have been found to be potent inhibitors of angiogenesis; as much more fibrinogen is available in LTF-/- mice, fibrinogen E-fragments could be blocking angiogenesis in this system (Bootle-Wilbraham et al., Cancer Res Sept 2000; PMID: 10987275).

As few fibrin fragments are available to enhance the effects of VEGF, it is possible that the effects of caveolin-1 accelerating VEGF receptor 2 (VEGFR2) autophosphorylation, may not have as large an impact (Tahir et al., Cancer Biol. Ther. Dec 2008; PMID: 19923922).