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The Role of Histidine Rich Protein II in Cerebral Malaria

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The Role of Histidine Rich Protein II in Cerebral Malaria Pathogenesis

by

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Thanks to my mother and my two younger brothers and my sister for being champion enthusiasts, fabulous friends and reminding me that there is more to life than work.

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Dedicated to my family, for always being there and believing in me even when they probably shouldn't have.

Abstract of the Dissertation

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By

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Professor Daniel Goldberg, Chair

Human malaria is caused by five species of *Plasmodium*. Of these, *P. falciparum* is the deadliest and is the only species that causes cerebral malaria (CM). CM is a disease of the vascular endothelium characterized by parasite sequestration, increased inflammatory cytokine production, vascular leakage and leukocyte infiltration. A distinguishing feature of *P. falciparum* infection is the parasite's production and secretion of histidine-rich protein II (HRPII). HRPII accumulates to high concentrations (up to 100 µg/ml) in serum, which correlates with disease severity. Due to high serum levels of this protein, HRPII has classically been considered a biomarker for *P. falciparum* infection. Although many functions have been ascribed to HRPII, the function of this protein remains ambiguous. Our work provides a new framework for thinking of this protein from serum biomarker to parasite virulence factor. Using a cellular model of the blood-brain barrier, we demonstrate that HRPII activates the innate immune system in human cerebral microvascular endothelial cells, resulting in redistribution of tight junction proteins and compromise of barrier integrity. This process is Myd88-dependent, NFκB-mediated and requires inflammasome activation. Intravenous infusion of HRPII induced vascular leakage

in the cerebellum and cortex of mice and increased early mortality in a *P. berghei ANKA* experimental cerebral malaria model. Analogously, transgenic *P. berghei* expressing falciparum HRPII produced more severe disease than wild-type or control *P. berghei*. HRPII induced endothelial expression of adhesion receptors used by plasmodium parasites, suggesting that this protein also contributes to pathogenesis by enhancing parasite cytoadherence and thereby avoiding splenic destruction. This study establishes that HRPII is a *Plasmodium falciparum* virulence factor that triggers an innate immune inflammatory response in vascular endothelium and contributes to cerebral malaria by compromising the integrity of the blood-brain barrier.

Preface

The work presented in this thesis is one of several projects I have worked on during graduate

school; while this work progressed towards publication, most other projects did not. My favorite

of the failed projects was aimed at understanding how the parasite has adapted to living in such a

highly oxidative environment, and how it handles reactive oxygen species. Although the parasite

produces some proteins to handle oxidative stress, I wondered whether the parasite recruited

proteins from the host to complement the parasite arsenal. To try to understand this we initiated a

large scale immune-precipitation approach with various host proteins to identify parasite proteins

that may interact with them. Homologous parasite proteins complicated this approach so we next

went to a stem cell approach where we would tag host proteins in the red blood cell (prior to

differentiations, since RBCs eject their nucleus during development, this would not be possible

with more conventional approaches). While we were able to successfully differentiate RBCs

from human cord blood and modify with retroviruses, this was not a very large scale-able

approach and therefore not applicable for immuno-precipitation and mass spectrometry.

The project presented here focusing on HRPII also developed along a rather circuitous

path. Initial interest in HRPII was focused on its highly charged nature and its ability to bind to a

variety of glycosaminoglycans (GAGs). Glycosaminoglycans are present along all endothelial

cell surfaces and provide many functions including low affinity interactions between endothelial

cells and chemokines/ cytokines until they can bind to their receptors. This interaction mediates

directed migration of immune cells to areas of infection or damage. The rationale being if HRPII

could disrupt effective GAG: chemokine interaction, it could slow down the formation of an

effective immune response. A delayed effective immune response to the parasite is characteristic of patients displaying severe symptoms. Immunofluorescence assays revealed endothelial cells whose cell membrane looked unhealthy in the presence of HRPII, and thereby a side investigation became a new front focus. This new focus turned into a thesis project, and will be the main work presented herein

Chapter 1: Introduction

This chapter includes general background on malaria, the parasite and immunology that applies to all sections; more relevant background will be present in separate chapters as needed.

1.1 Epidemiology and Pathophysiology

Malaria is a mosquito transmitted parasite illness present in some of the most economically disadvantaged populations world-wide. According to recent WHO estimates roughly 3.3 billion people are at risk for infection. This results in roughly 600,000 deaths, 90% of which are in Africa- of these the majority are children under the age of five (World Health Organization, World Malria Report, 2014)(Bell et al., 2006). Disappointingly, this infection is both preventable and treatable. Although there has been a significant decline in transmission since 2000 due to insecticidal sprays, insecticide treated bed nets, rapid diagnostic tests as well as increased access to therapy and preventative therapy to pregnant women, there is also significant amount of work remaining with the increasing challenge of parasites resistant to current drugs and mosquitos resistant to insecticides.

Malaria in humans is caused by five parasite species, *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlsei.(Bell et al., 2006)* This obligate intracellular parasite is transmitted by the bite of an anopheles sp. mosquito; a single successfully transmitted sporozoite can result in roughly 10,000 blood stage parasites, see schematic of parasite life cycle in Figure 1 below borrowed from a review (Lycett and Kafatos,
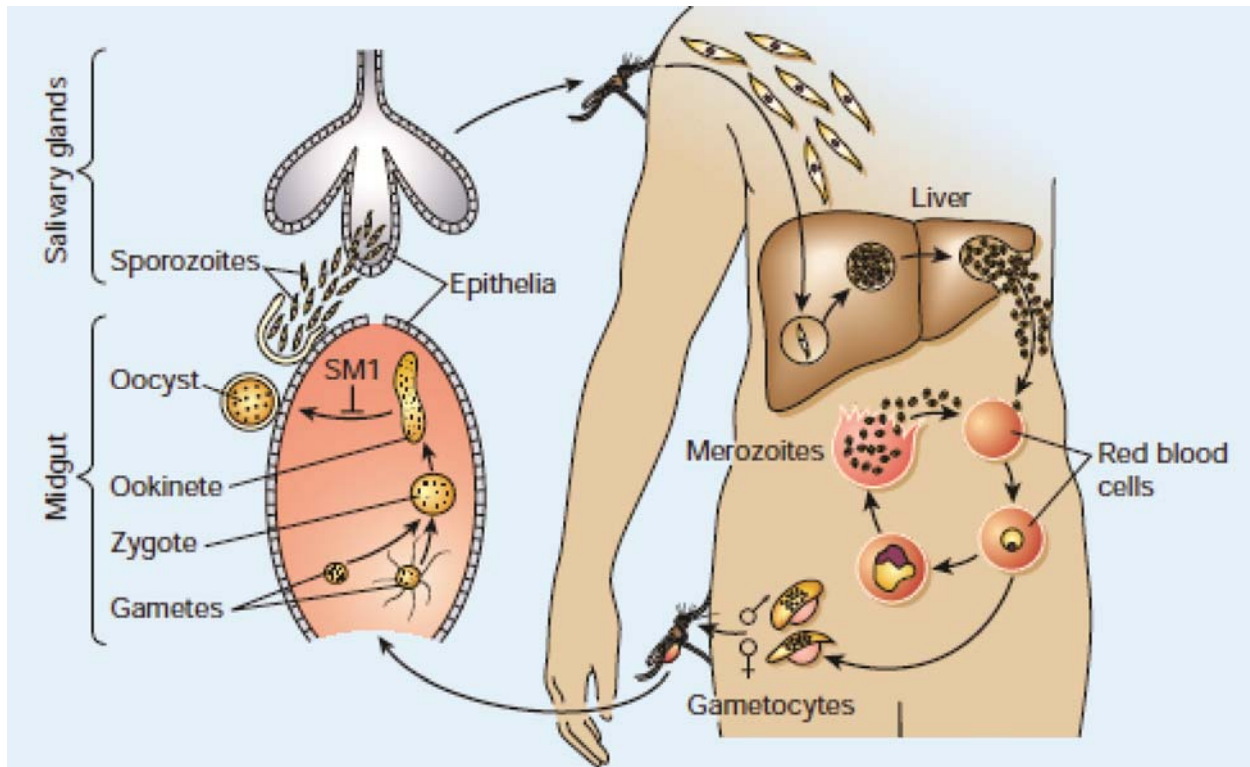
2002).

Figure 1.1] Parasite life cycle borrowed from (Lycett and Kafatos, 2002)

Progression of the parasite out of the liver stage, initiates the blood stage of infection which is associated with a majority of symptoms. The early symptoms are nonspecific and incudes "headache, lassitude, fatigue, abdominal discomfort, and muscle and joint aches, usually followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise" (Reyburn, 2010). Treatment of a patient at this stage results in full rapid recovery, thereby underscoring the importance of early detection and timely intervention. Treatment at a later stage, once organ dysfunction has ensued and parasite burden has increased, often results in rapid progression to severe malaria, particularly in the setting of a *P. falciparum* infection resulting in "coma (cerebral malaria), metabolic acidosis, severe anaemia, hypoglycaemia, acute renal failure or

acute pulmonary oedema" (Reyburn, 2010). Severe malaria is almost always fatal when left untreated, and about 20% fatal even with treatment.(Reyburn, 2010)

Cerebral Malaria

Cerebral malaria is the most severe manifestation of malaria infection and results in roughly 300,000 deaths annually. About 25% of survivors have lasting neurological complications (Christensen and Eslick, 2015; Fernando et al., 2010). In a group of Ugandan children the sequelae of cerebral malaria included spastic motor weakness, loss of speech, hearing deficits, behavioral problems, epilepsy, blindness, and severe cognitive impairment (Idro et al., 2010).

Extensive neurological damage has been evidenced in pathology from patients who died from cerebral malaria. Demyelination, damaged neurons, (Schluesener et al., 1998; Toro and Roman, 1978) parasitized capillaries, petechial hemorrhages are among the many changes observed on autopsy specimens along with malarial retinopathy (hemorrhages, whitening and vascular changes) (Taylor et al., 2004). Due to the variability and complexity of disease presentation, pathologists have defined three predominant form of cerebral malaria defined by pathology

"CM1—clinical cerebral malaria with sequestration of parasitized red blood cells (PRBCs) in the brain, no additional cerebral histopathological changes, and no other cause of death

CM2—clinical cerebral malaria with sequestration of PRBCs in the brain and the presence of cerebral microthrombi, ring hemorrhages and extra-erythrocytic malaria pigment, and no other cause of death

<u>CM3</u>—fulfilling the traditional definition of clinical cerebral malaria in life, but with no sequestration of PRBCs in the brain and another cause of death identified" (Dorovini-Zis et al., 2011; Milner et al., 2014; Taylor et al., 2004)

There are many models rationalizing the complex pathology present in cerebral malaria; the two prevailing hypotheses are 1.) sequestration/ mechanical and 2.) immunopathology. The mechanical notion suggests that multifocal lesions result from reduced blood flow from sequestered parasites. This then causes metabolic changes: acidosis, hypoglycemia, and hypoxemia which may conclude in a coma. However, this alone cannot account for the pathology since similar levels of sequestration have been seen in patients not suffering from cerebral malaria. The other dominant view suggests that an overactive immune response to the parasite results in endothelial damage and dysfunction which eventually results in a breakdown of the blood brain barrier and damage to the central nervous system as a byproduct of the immune system attempting to clear the parasite. Cerebral malaria is a complex pathological process and most likely results from a culmination of many factors.

1.2 Mouse Models for Cerebral Malaria

Plasmodium berghei ANKA infection of C57BL/6 mice is the most widely used murine model for cerebral malaria infection (mCM). This model replicates many of the clinical and histopathological features present in patients with human cerebral malaria (hCM). For example, mCM displays tissue edema, hemorrhages, presence of an inflammatory infiltrate, activation of microglia and a robust pro-inflammatory cytokine response. However, there are limited infected

red blood cells sequestered along the vascular endothelium and an a more robust inflammatory infiltrate than that present with hCM.

A very extensive debate on the validity of the model has been ensuing through articles and at conferences (Hunt et al., 2010; Renia et al., 2010; Riley et al., 2010; Stevenson et al., 2010; White et al., 2010). Amongst the most compelling arguments against using the murine cerebral malaria model is that 44 out of 48 therapies assessed in mCM model were successful in healing mice; however, of 17 assessed in human, only one has shown some efficacy. This underscores that the biology in these two distally related mammals may not be close enough to develop effective therapeutics. The main features differentiating murine cerebral malaria and hCM are highlighted below in a table borrowed from a recent review. (Medana et al., 2001)

Journal of Neuroparasitology

Observation	Human	Murine
Loss of vascular cell integrity/tissue oedema	+	+
Congestion of microvessels with infected erythrocytes	+	−/+
Haemorrhages	+	+
Mononuclear cell adherence to, or extravasation through, the vascular endothelium	−/+	+
Astrocyte response (redistribution, astrogliosis, activation, apoptosis)	?	+
Microglia and perivascular macrophage response (redistribution, morphological changes, activation)	+	+
Pro-inflammatory cytokine expression	+	+
Neurological complications, including convulsions, paralysis, coma	+	+

Table 1] Major differences between human cerebral malaria and the murine model, *P. berghei* ANKA

Despite the controversy present around the *berghei* mouse model, there is some advantage to studies that use it. At a first pass, it is important to remember that the *berghei* model is at the end of the day a model and therefore an approximation of what happens in the human cerebral malaria case and not a replicate. It provides a glance to how different factors will affect the infection in the presence of an immune system, other organs, a vasculature, and many other components. It is of particular ease of use when studying factors exclusive to *P. falciparum* and

not present in *P. berghei*. In this setting, the *berghei* model provides a background akin to a knock-out. As a consequence it is simple to compare infection in an isogenic background with one protein different. The studies we have performed using *P. berghei ANKA* added a protein present only in *P. falciparum*, HRPII. We were able to demonstrate that addition of HRPII into the *berghei* genome reduced mouse survival and was therefore important for the parasite outside of simple parasite growth as we suspected.

1.3 HRPII

Histidine rich protein II (HRPII) is a protein produced exclusively by *Plasmodium falciparum* and exported out of the parasitophorous vacuole into the red blood cell. Upon RBC lysis HRPII is released into the blood stream where it is found at concentrations > 1000ng/mL (Dondorp et al., 2005) and can be detected at least 1 month post clearance of parasites. HRPII is a curious protein not only due to its biochemical properties, but because it is only present in the *P. falciparum* genome. HRPII is highly basic, composed 37% by histidines; two repeats (His- His-Ala-His-His-Ala-Ala-Asp-Ala and His-His-Ala-Ala-Asp) cover 85% of its sequence. The sequence shown below highlights the histidine rich nature of the sequence. Since its discovery in 1986 (Wellems and Howard, 1986), many functions have been ascribed to it including hemozoin crystallization, actin formation, T cell suppression, glycosaminoglycan binding and procoagulation (Benedetti et al., 2003; Choi et al., 1999; Das et al., 2006; Mashima et al., 2002; Ndonwi et al., 2011; Sullivan et al., 1996). It has been shown to interact selectively with heparin, heparan sulfate and dermatan sulfate with high affinity (Ndonwi et al., 2011). This particular interaction led to the discovery of its procoagulant property, and may explain the pro-coagulant state seen during *P. falciparum* infection. Heparin at low concentrations is known to

bind to the serpin antithrombin III and increase heparin's inhibition of Factor Xa or thrombin 2000-4000 fold. HRPII has been shown by our lab to neutralize this enhancement of antithrombin activity and thereby promote a pro-coagulative environment. (Ndonwi et al., 2011)

MVSFSKNKVLSAAVFASVLLLDNNNSAFNNN-
LCSKNAKGLNLNKRLLHETQAHVDDAHHAH-
HVADAHHAHHVADAHHAHHVADAHHAHH
AADAHHAHHAADAHHAHHAADAHHAHHA
HHAADAHHAHHAADAHHAHHAADAHHAH
HAADAHHAHHAADAHHAHHASDAHHAHH
AAYAHHAHHASDAHHAADAHHAAYAHHAH
HAADAHHAADAHHATDAHHAADAHHAAD
AHHAADAHHATDAHHAHHAADAHHATDAH
HAHHAADAHHAAAHHATDAHHAAAHHATD
AHHAAAHHEAATHCLRH

Figure 1.2] Amino Acid sequence of HRPII from *P. falciparum*

HRPII is produced by almost all natural isolates of *Plasmodium falciparum.* Due to its presence at high concentrations in infected individuals it has been used as a biomarker for infected individuals and forms the basis of the dipstick test (Chiodini et al., 2007; Dondorp et al., 2005; Moody, 2002; Parra et al., 1991). The asexual stages of infection by all other species occur exclusively in the blood stream and therefore blood parasitemia is a decent indicator of parasite burden. In contrast, *P falciparum* parasites in the trophozoite and schizont stages are sequestered

along endothelial walls and can rarely be seen in the blood stream; therefore blood parasitemia is a poor indicator of parasite burden. Since HRPII is released into the blood stream as schizonts rupture, and is cleared from the blood stream slowly; HRPII serves as a good measure of recent *P. falciparum* infection.

HRPII has been used as a biomarker for *P. falciparum* infection and forms the basis of current rapid diagnostic tests (Chiodini et al., 2007; Dondorp et al., 2005; Moody, 2002; Parra et al., 1991). On post-mortem analyses, HRPII has been observed to line the endothelial walls of blood vessels (Aikawa et al., 1990). Several correlative studies have shown an association between HRPII levels in acute serum and disease severity or development of CM (Dondorp et al., 2005; Fox et al., 2013; Hendriksen et al., 2012; Hendriksen et al., 2013; Kariuki et al., 2014; Seydel et al., 2012). Natural populations of HRPII-deficient *Plasmodium falciparum* parasites exist (Gamboa et al., 2010; Koita et al., 2012; Kumar et al., 2013), though these tend to be in areas of low CM incidence. We questioned whether HRPII might contribute to disease pathogenesis

1.4   Blood Brain Barrier and Vascular endothelium

The BBB regulates access of peripheral circulatory compounds and cells to the central nervous system. The BBB is formed by a complex network of intercellular junctional proteins. It is supported by many components a basement membranes, a complex extracellular matrix and various cells including astrocytes and microglia (Kawai and Akira, 2010). "Astrocytes, pericytes and extracellular matrix (ECM) components provide both structural and functional support to the BBB. The term 'neurovascular unit' (NVU) additionally refers to neurons, microglial cells and, optionally, peripheral immune cells that also contribute to this cellular interplay" (Obermeier et al., 2013). A schematic from a recent review is shown below   Disruption of this network results

[8]

in BBB compromise and has been linked to a various disease states.
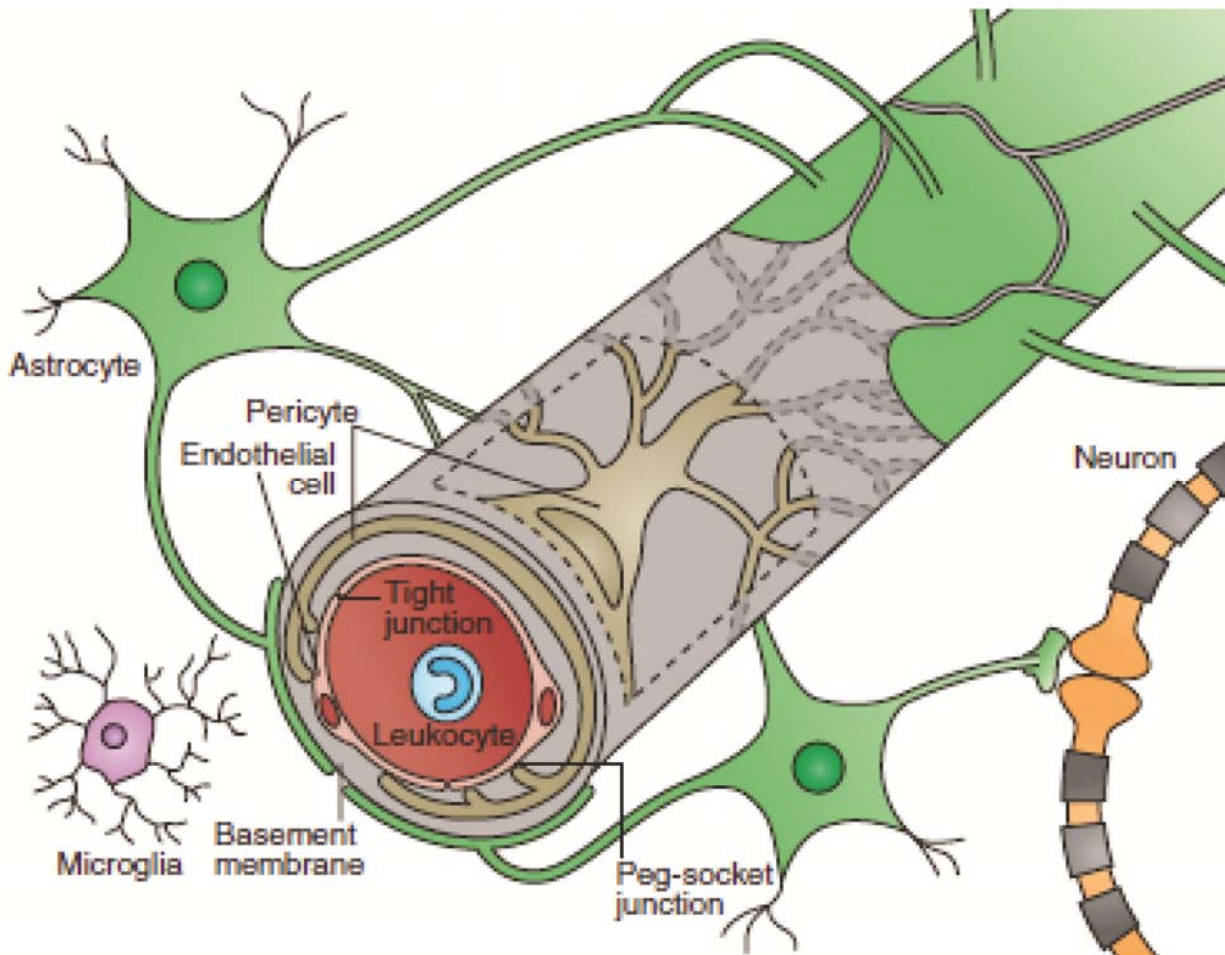
Fig 1.3] Neurovascular unit of the blood brain barrier. Figure borrowed from (Obermeier et al., 2013)

The endothelial cells that line the vasculature in the brain are distinct from those in other organs in part due to the stringency and selectivity guiding which compounds can permeate past this endothelial lining. This selective barrier between the peripheral circulatory components of the blood and the brain, the blood brain barrier proper "lies in the presence of tight junctions between the cerebral endothelial cells of the vasculature of the brain both within the parenchyma

and over the surface in the pia-arachnoid" (Stolp et al., 2013). Tight junctions prevent the paracellular transport of molecules into the parenchyma of the brain.

We used an *in vitro* model blood brain barrier to study how different components affect it. An illustration is shown below in figure 1.4. We culture a human brain cerebrovascular immortalized endothelial cell line hCMEC/D3 cells on collagen coated trasnwell inserts. The cells are allowed to grow to complete confluence and during this time they form appropriate tight junctions as previously described (Daniels et al., 2013; Weksler et al., 2005). We can measure the electrical resistance across this endothelial monolayer, high resistance values are indicative of an intact barrier while decreasing resistance values indicate a compromised model blood brain barrier.
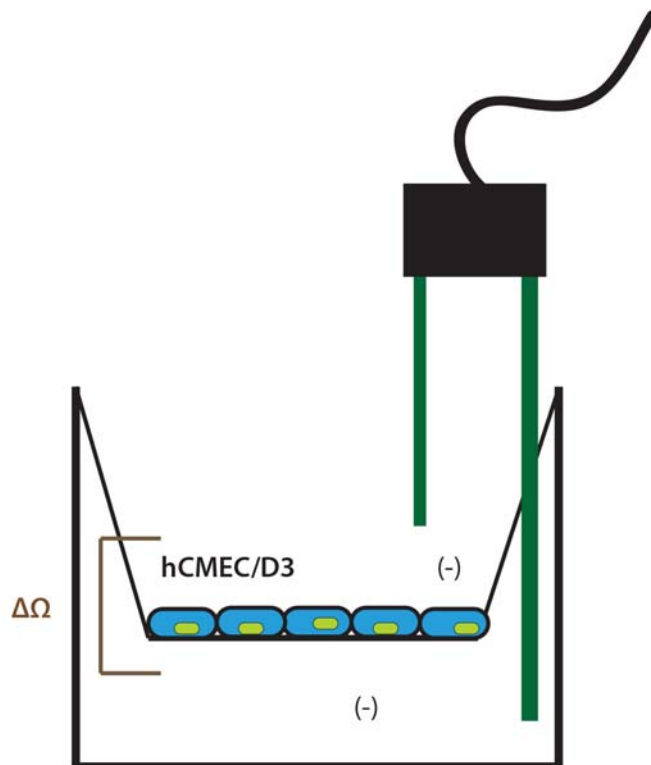
Figure 1.4] Model BBB

1.5   Inflammasome

The innate and adaptive immune system work together to alert the host of danger signals both foreign and inappropriate host responses. The innate system is activated first and responds to a wide range of pathogenic or host patterns that have evolutionarily been deemed dangerous, and activates the adaptive immune response which is able to mount a specific response via B and T cells. (Basset et al., 2003). The innate immune system operates through PRRs (pathogen recognition receptors) which recognize PAMPs (pathogen associated molecular patterns). The innate immune system can also be activated by components released by injured cells termed danger associated molecular patterns (DAMPs) such as mammalian double stranded DNA and uric acid crystals (Ishii et al., 2001; Martinon et al., 2006). So the innate immune system recognizes common patterns from invading bacteria viruses and fungi as well as damage resulting from the invasion to the host. PRRs can be cytoplasmic, membrane bound or even secreted and are present in specialized immune cells such as macrophages, monocytes, dendritic cells (DCs), neutrophils, in addition to normal mononuclear endothelial and epithelial cells. Toll-like receptors (TLRs) are one set of well-established PRRs (O'Neill and Bowie, 2007). More recently discovered PRRs are RIG-like helicases (RLH) and NOD- like receptors (NLRs) which are soluble cytoplasmic receptors unlike TLRs which are membrane bound (Martinon and Tschopp, 2005; Yoneyama et al., 2004). NLRs are a common class of sensor molecules for inflammasomes.

Inflammasomes are large molecular weight complexes that recognize pathogenic or sterile danger molecules and activate the pro inflammatory cytokines IL-1β (interleukin 1 beta) and IL-18 (interleukin 18) (Latz et al., 2013). The complex is formed from the association of a sensor

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molecule, the adaptor protein ASC which recruits caspase-1 and caspase-1. The adaptor protein ASC has two death- fold domains: a pyrin domain and a caspase activation and recruitment domain (CARD) (Vajjhala et al., 2012). The pyrin domain allows for association with the upstream sensor molecule and the CARD domain brings pro caspase-1 molecules in close proximity allowing cleavage and self-activation. Active caspase-1 molecules are able to cleave pro- IL-1β and pro IL-18 into their respective active forms IL- 1β and IL-18. Many sensor molecules have been identified such as NLRP1, NLRP3, NLRP12, NAIP1, NAIP2, NAIP5, or AIM2 and are collectively able to detect a diverse array of host and pathogenic danger signals. Activation of the inflammasome eventually results in a form of programmed inflammatory cell death known as pyroptosis. Although activation of the inflammasome is often thought of in the context of a bacterial infection or host danger molecules, it can also be activated from an intracellular protozoan parasite infection (Zamboni and Lima-Junior, 2015).
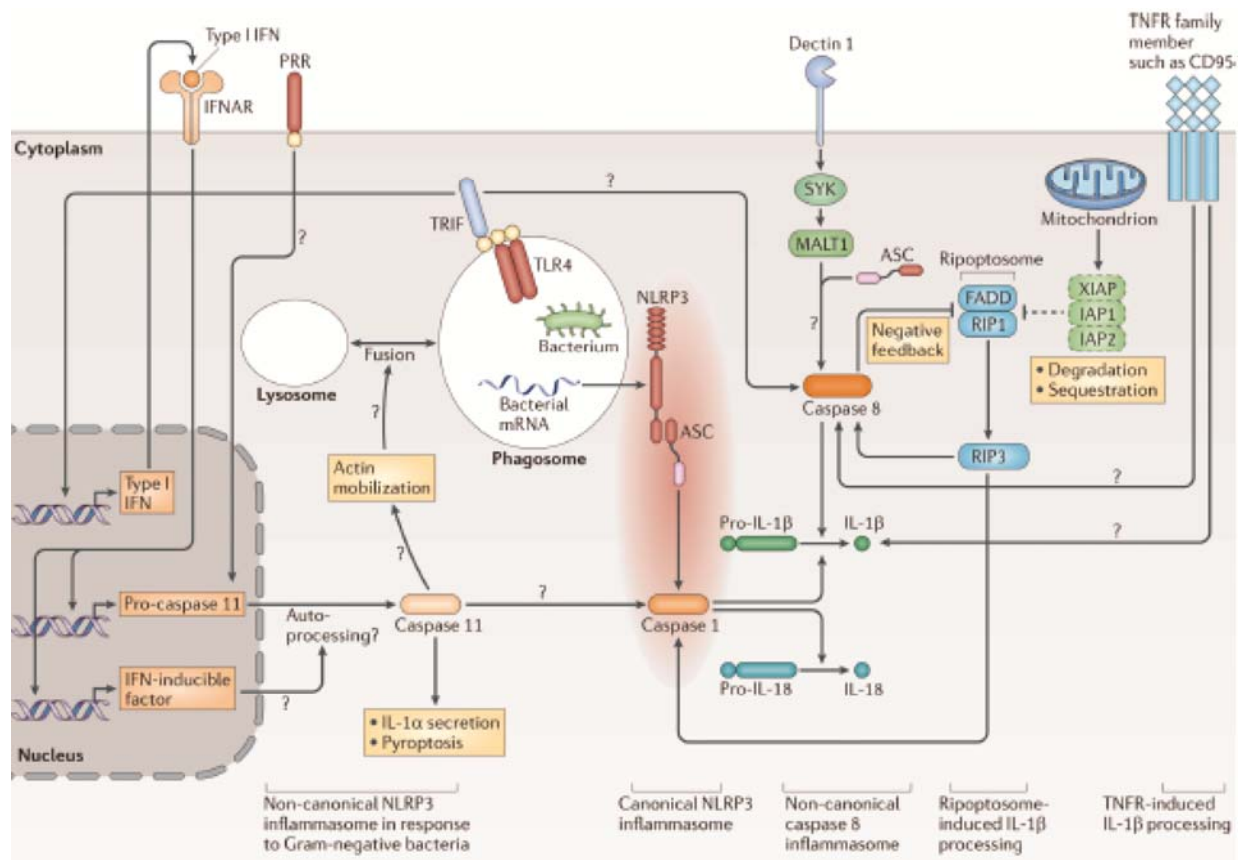
Fig 1.5 Inflammsome highlighting the many points of activation borrowed from (Latz et al., 2013)

The work that follows was pursued to understand the function of HRPII, a parasite protein that is biochemically very unique, produced at high levels and whose serum levels correlate with disease severity and cerebral malaria. We reasoned that a function should exist since the protein is produced to such high levels. In addition, early work had shown that parasites were viable in the presence of a genetic deletion of HRPII, in fact, natural HRPII null parasites exist. Therefore, we hypothesized that a function for HRPII, if existed, would be not be in parasite growth, but perhaps something important during the life cycle in the host. In consideration of the correlation between HRPII levels and cerebral malaria, we began studying HRPII on human brain

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endothelial cells. Interesting findings led to the work described in chapter 2.

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Chapter 2: A role for HRPII in cerebral malaria pathogenesis

2.1 Abstract

Human malaria is caused by five species of *Plasmodium*. Of these, *P. falciparum* is the deadliest and is the only species that causes cerebral malaria (CM). CM is a disease of the vascular endothelium characterized by parasite sequestration, increased inflammatory cytokine production, vascular leakage and leukocyte infiltration. A distinguishing feature of *P. falciparum* infection is the parasite's production and secretion of histidine-rich protein II (HRPII). HRPII accumulates to high concentrations (up to 100 ☐g/ml) in serum, which correlates with disease severity. Using a cellular model of the blood-brain barrier, we demonstrate that HRPII activates the innate immune system in human cerebral microvascular endothelial cells, resulting in redistribution of tight junction proteins and compromise of barrier integrity. This process is Myd88-dependent, NF☐B-mediated and requires inflammasome activation. Intravenous infusion of HRPII induced vascular leakage in the cerebellum and cortex of mice and increased early mortality in a *P. berghei ANKA* experimental cerebral malaria model. Analogously, transgenic *P. berghei* expressing falciparum HRPII produced more severe disease than wild-type or control *P. berghei*. HRPII induced endothelial expression of adhesion receptors used by plasmodium parasites, suggesting that this protein also contributes to pathogenesis by enhancing parasite cytoadherence and thereby avoiding splenic destruction. This study establishes that HRPII is a *Plasmodium falciparum* virulence factor that triggers an innate immune inflammatory response in vascular endothelium and contributes to cerebral malaria by compromising the integrity of the blood-brain barrier.

2.2 Introduction

Malaria infections cause an estimated 627,000 deaths per year, with 90% occurring in Sub-Saharan Africa, primarily in children under five (WHO World Malaria Report 2013). Of the five species of *Plasmodium* capable of infecting humans, *P. falciparum* causes the most morbidity and mortality (MacPherson et al., 1985). The clinical presentation of malaria ranges from a simple febrile illness (uncomplicated malaria) to various syndromes alone or in combination, including severe anemia, respiratory distress multi-organ failure, and cerebral malaria (CM) (Miller et al., 2002). CM is a deadly manifestation that is caused almost exclusively by *P. falciparum.* Patients present with decreased sensorium progressing to coma. This neurological syndrome is characterized by sequestration of infected RBCs in cerebrovascular beds, vascular occlusion, leukocyte infiltration, pro-inflammatory cytokines, perivascular edema, brain swelling and a diffuse encephalopathy (Engwerda et al., 2005; Menezes et al., 2012; Pongponratn et al., 2003; Seydel et al., 2015). CM results in about 300,000 deaths annually, has a 20-30% case fatality rate despite treatment (Brewster et al., 1990; Idro et al., 2005), and 25% of survivors have lasting neurological sequelae (Fernando et al., 2010), including cognitive impairment (Idro et al., 2010). Infection of mice with the rodent malaria parasite strain *P. berghei ANKA* serves as a model for cerebral malaria. The pathology present in experimental cerebral malaria (eCM) is similar to that in human cerebral malaria (CM) with notable exceptions of limited sequestration of infected RBCs, a more robust infiltration of leukocytes and a different quality of the immune response (Medana et al., 2001; White et al., 2010). The biological basis of these differences is poorly defined but the topic of spirited debate

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and an active area of research (Craig et al., 2012; de Souza et al., 2010; de Souza and Riley, 2002; Riley et al., 2010).

Histidine-rich protein II (HRPII) is a unique protein produced exclusively by *P. falciparum*. It is highly basic: 37% of its amino acid sequence is histidine and repeats of histidine plus alanine cover 85% of its sequence. HRPII is exported by the parasite into the RBC cytosol (Howard et al., 1986). Upon maturation of the parasite and rupture out of the host cell, the contents of the RBC cytosol including HRPII are released into the bloodstream (Parra et al., 1991). In serum, it can be detected at high concentrations (~1-100 □g/mL) and can be detected at least 1 month post clearance of parasites (Dondorp et al., 2005). Since its discovery in 1986 (Wellems and Howard, 1986), many functions have been ascribed to it including hemozoin crystallization, actin formation, T cell suppression, glycosaminoglycan binding and procoagulation (Benedetti et al., 2003; Choi et al., 1999; Das et al., 2006; Mashima et al., 2002; Ndonwi et al., 2011; Sullivan et al., 1996).

HRPII has been used as a biomarker for *P. falciparum* infection and forms the basis of current rapid diagnostic tests (Chiodini et al., 2007; Dondorp et al., 2005; Moody, 2002; Parra et al., 1991). On post-mortem analyses, HRPII has been observed to line the endothelial walls of blood vessels. Several correlative studies have shown an association between HRPII levels in acute serum and disease severity or development of CM (Dondorp et al., 2005; Fox et al., 2013; Hendriksen et al., 2012; Hendriksen et al., 2013; Seydel et al., 2012). Natural populations of HRPII-deficient *Plasmodium falciparum* parasites exist (Gamboa et al., 2010; Koita et al., 2012; Kumar et al., 2013), though these tend to be in areas of low CM incidence.

We questioned whether HRPII might contribute to disease pathogenesis. Herein, we provide evidence that HRPII is a *P. falciparum* virulence factor that triggers the inflammasome

in vascular endothelial cells. HRPII binding to brain endothelial cells results in rearrangement of tight junction proteins and a compromised blood-brain barrier (BBB). We propose that HRPII contributes to the pathogenesis of cerebral malaria.

2.3 Results

HRPII is both necessary and sufficient to compromise endothelial barrier integrity

Prior work has shown that addition of cultured *P. falciparum* parasites to brain endothelial cells compromises barrier integrity (Tripathi et al., 2007), and that soluble factors released during infection are adequate to mediate this phenotype. Due to the established correlation between HRPII and disease severity (Dondorp et al., 2005; Fox et al., 2013) we assessed the consequence of HRPII exposure using an *in vitro* BBB model of human cerebral microvascular endothelial cells (hCMEC/D3) grown on trans-well porous inserts (Daniels et al., 2013) The upper chamber of this cellular model represents the luminal face of a blood vessel; *P. falciparum* clone 3D7 parasitized erythrocytes were added to the upper chamber and trans-endothelial electrical resistance (TEER) was measured across the endothelial barrier. These parasites induced a time-dependent decrease in resistance (**Fig 2.1A)**. In contrast, clone Dd2, which contains a deletion of the HRPII gene, caused minimal change in barrier integrity. Wild-type Dd2 parasites were transfected to generate transgenic parasites that ectopically express HRPII. Integration of the gene for HRPII was confirmed by PCR and isolated clones demonstrated an ability to produce HRPII by western blot (**Fig 2.2**). Two clones expressing HRPII from independent transfections compromised barrier integrity (**Fig 2.1B**).
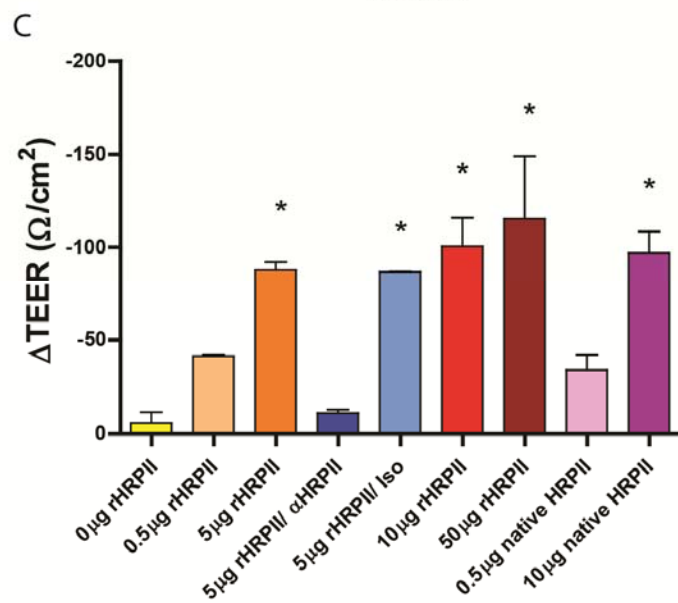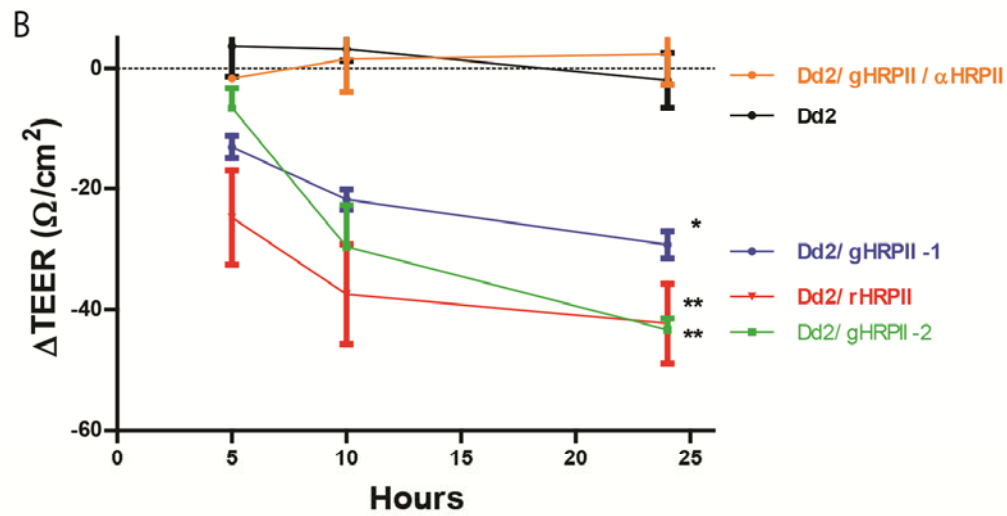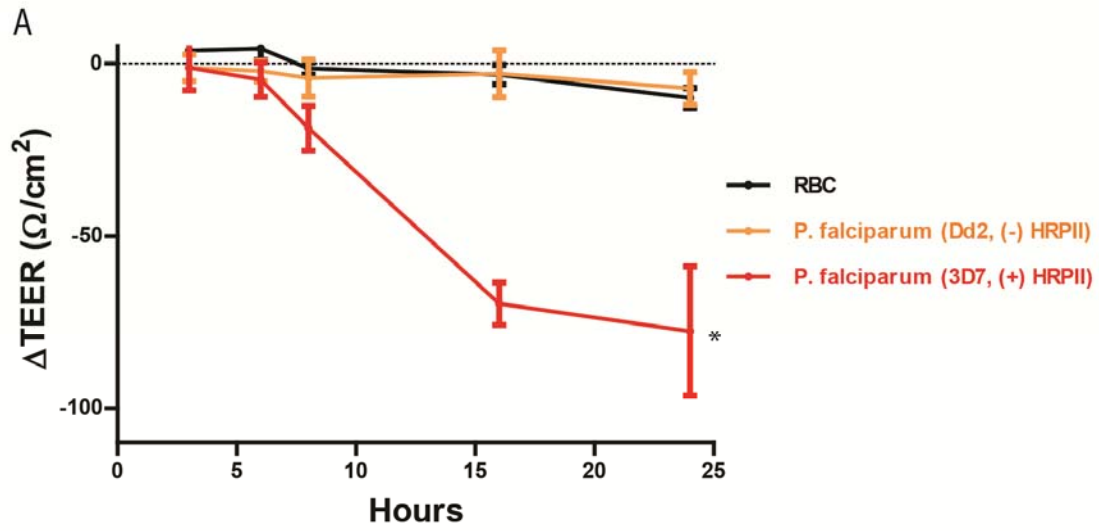
Figure2.1] HRPII is both necessary and sufficient to compromise the integrity of an in vitro BBB. Trans-endothelial electrical resistance (TEER) was measured across an hCMEC/d3 monolayer over time, and components for assessment were added to the upper chamber. (A) Addition of 10^8 uninfected RBCs, Plasmodium falciparum strain Dd2 (which does not produce HRPII) and strain 3D7 (which produces HRPII) were added to the BBB model. All values are relative to resistance measurements at time 0. Data are mean values +/- SEM for 2 replicates. Curves are significantly different from 3D7 chambers by one-way ANOVA. (B) Addition of 10^8 Dd2 parasites, parasites engineered to produce HRPII (Dd2/ gHRPII-1 and Dd2/gHRPII-2), Dd2 parasites with 10 µg of added recombinant HRPII (Dd2/ rHRPII), or in the presence of specific antibody (Dd2/ gHRPII/ αHRPII). Data are mean values +/- SEM for 6 replicates spread over 3 independent experiments. Difference from untreated *p < 0.0001 and ** p < 0.009 by one-way ANOVA. (C) Addition of recombinant purified HRPII (rHRPII), HRPII purified from 3D7 parasites (native HRPII) or in combination with monoclonal anti- HRPII antibody (αHRPII) or isotype control (Iso). Data are mean values +/- SEM from a 24 hour time point for 4-8 replicates spread over 5 independent experiments. Difference from untreated p < 0.0001, by one-way ANOVA.
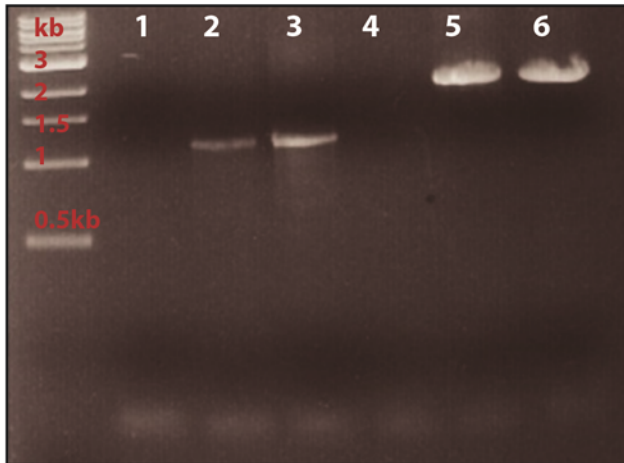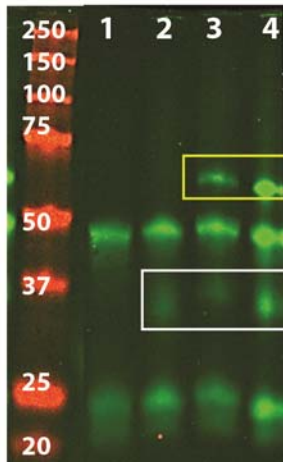
Figure 2.2] Dd2 transgenic parasites clones have been successfully transfected with the gene encoding HRPII-GFP and produce protein. (A) PCR of the HRPII gene from wild-type Dd2 (lane1,4) parasites as well as the transgenic clones C5 (lanes 2,5) and D3 (lanes 3, 6) amplifying for HRPII (lanes 1-3) and HRPII-GFP (lanes 4-6). (B) Western blots of parasite extracts from wild-type Dd2 (lane1), 3D7 (lane 2), clone C5 (lane 3), clone D3 (lane 4). WB using anti-HRPII (clone 2G12, 1:10,000). The yellow box highlights the band for HRPII-GFP, and the white box highlights native untagged HRPII. Both transgenic clones gave similar results in the TEER assay.

Addition of a neutralizing anti-HRPII monoclonal antibody to the upper chamber confirmed the specific effect of HRPII as it abolished the barrier compromise observed using the transfected parasites. Addition of recombinant HRPII to wells containing wild-type Dd2 parasites also resulted in barrier compromise. These experiments demonstrate that HRPII is required for parasites to disrupt endothelial barrier integrity. Subsequent studies revealed that purified HRPII alone (recombinant or isolated from *P. falciparum 3D7* parasites) similarly disturbed barrier integrity (**Fig 2.1C**). This activity was specific, as antibody blockade of HRPII abolished the effect. These experiments establish that HRPII is both necessary and sufficient to compromise endothelial barrier integrity.

HRPII results in redistribution of tight junction and adherens junction proteins.

The BBB regulates access of peripheral circulatory compounds and cells to the central nervous system. The BBB is formed by a complex network of intercellular junctional proteins, basement membranes, and various cells including astrocytes and endothelial cells (Kawai and Akira, 2010). Disruption of this network results in BBB compromise and has been linked to a various disease states.

We queried whether HRPII caused altered localization of junctional proteins contributing to compromised barrier integrity. Immunohistochemical detection within hCMEC/d3 cells for several junctional proteins and their intracellular adaptors, revealed redistribution of the tight junction protein claudin-5 (Cld-5) and the adherens junction protein VE-cadherin (**Fig 2.3A**). Zona-occludens-1 (ZO-1), the intracellular adaptor for claudin-5, stained poorly in the hCMEC/D3 cell line. Therefore, we stained for ZO-1 on Caco-2 cells and observed clear

alteration of its distribution from cell borders to a diffuse cytoplasmic staining in the presence of

HRPII (**Fig 2.3B**), similar to what was observed with LPS.
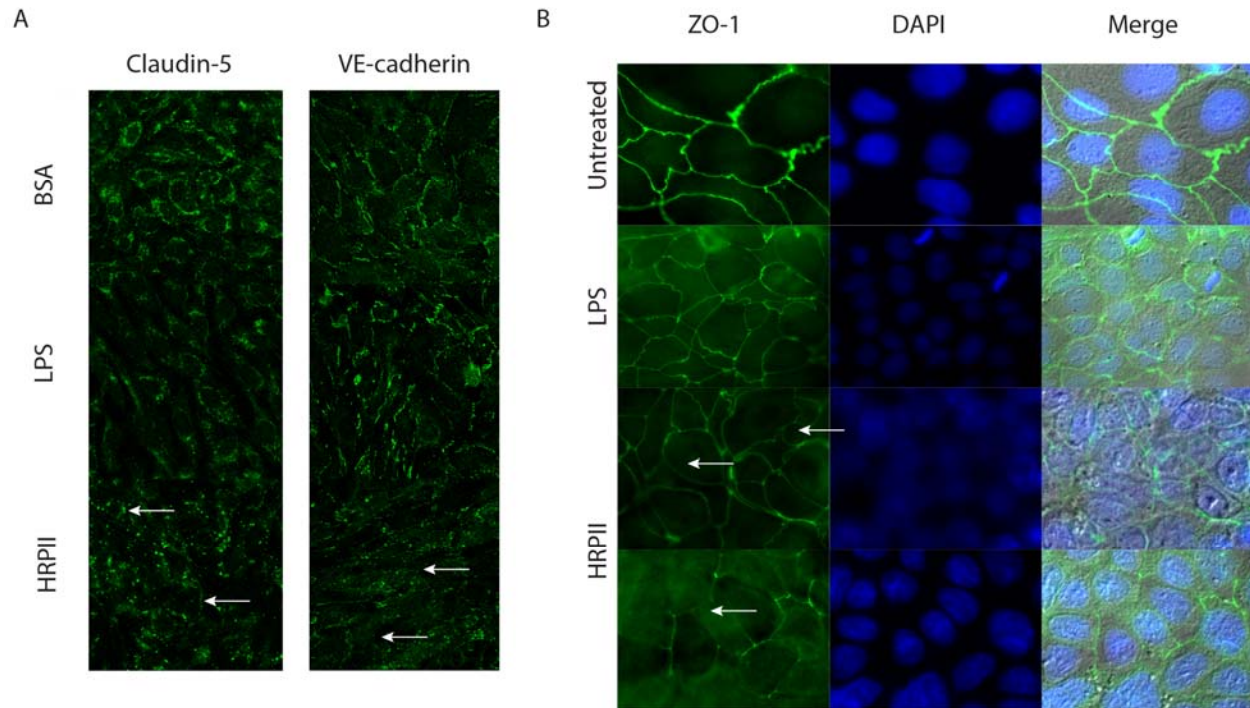
Figure 2.3] HRPII exposure to human cerebral microvascular endothelial cells results in redistribution of junctional proteins. (A) hCMEC/d3 monolayer cultures were incubated 24 hours with 25 µg of BSA or HRPII, or 3 µg of LPS, staining for tight junction protein claudin-5 and the adherens junction protein VE-cadherin. (B) Caco-2 cultures were treated as in (A) and stained for Zona occludens-1 (ZO-1), the intracellular adaptor for claudin-5 and nuclei (DAPI). Representative images of 4 replicates from 2 independent experiments. All images taken using the same settings. Arrows highlight areas of incomplete staining.

HRPII activates an innate immune responses in endothelial cells

The disruption of BBB integrity by HRPII indicated that endothelial cells respond to the

protein. We next questioned whether this was an immunological response. Quantitative RT-PCR

analysis of chemokine and cytokine transcripts in hCMEC/D3 cells showed that several were up-

regulated within 8 hours post exposure to recombinant HRPII (**Fig 2.4A**), and the response was kinetically different from that observed with LPS (**Fig 2.5**). As transcription of chemokines and cytokines can be induced by NFκB activation (Lawrence, 2009), we tested whether the HRPII effects were mediated via NFκB signaling.
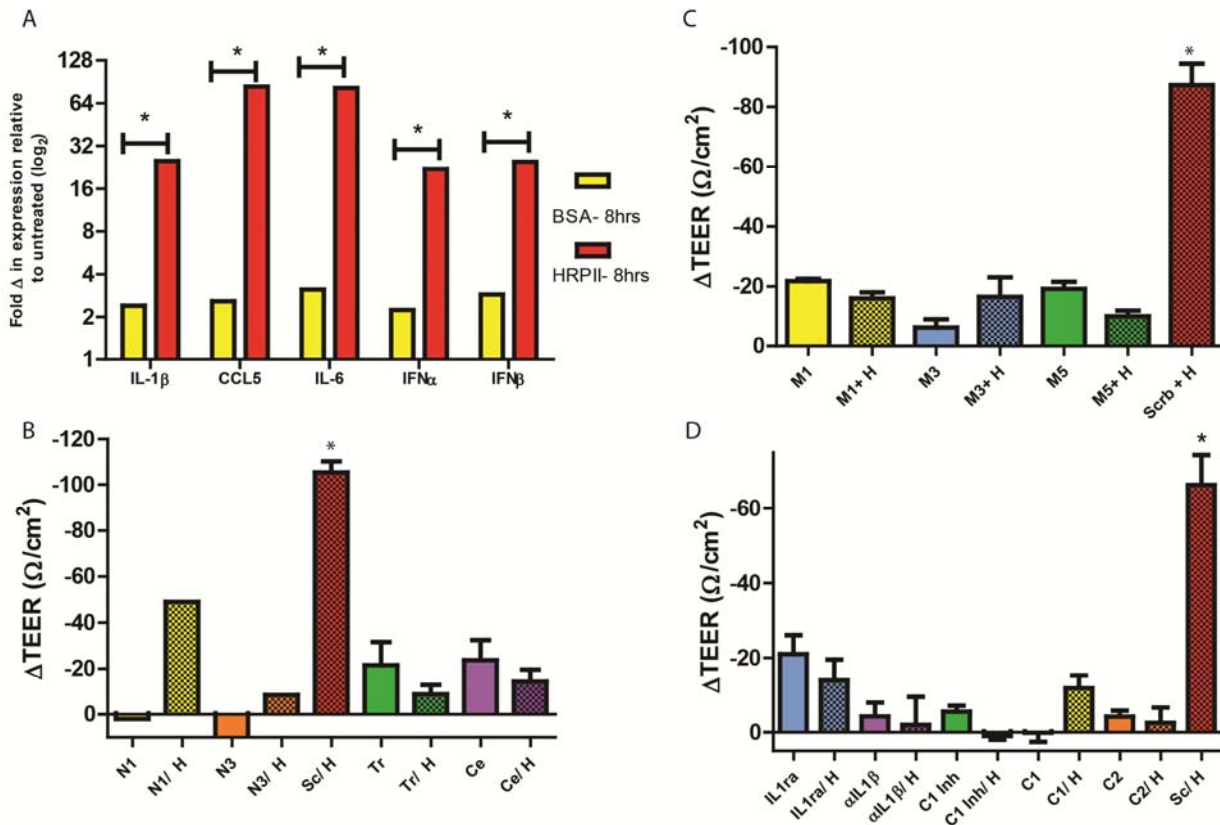
Figure 2.4] HRPII activates an inflammatory pathway in human cerebral microvascular endothelial cells. (A) qRT-PCR of chemokine/ cytokine mRNA levels of hCMEC/d3 cells treated with 25 µg HRPII or BSA for 8 hours. *$p = 0.0079$, by two tailed t- test. (B) TEER measurements for in vitro BBB barriers transfected with shRNAs for NFκB (N1 and N3) or a scrambled control (Sc) for 36 hours or incubated with inhibitors for NFκB, Celastrol (Ce) and Triplotide (Tr) for 2 hours prior to addition of HRPII (H, 10 ug). Data are mean values +/- SEM for 6-8 replicates spread over 3 independent experiments. All curves are significantly different from scrambled, HRPII-treated control chambers $p < 0.0001$, by one-way ANOVA. (C) TEER measurements for in vitro barriers transfected with shRNAs to MyD88 (M1, M3 and M5) or a scrambled control (Sc) for 36 hours prior to addition of recombinant purified HRPII (10 µg). Data are mean +/- SEM from 6-8 replicates over 3 independent experiments. All treatment groups are different from scrambled, HRPII-treated control chambers $p<0.0001$, by one-way ANOVA. Assessment of knockdown levels shown in figure 2.6. (D) TEER measurements for in

vitro BBB barriers transfected with shRNAs for caspase 1 (C1 and C2) or a scrambled control (Sc) for 36 hours or with IL1Ra (500 ng), αIL-1β (25 ng) or the caspase-1 inhibitor YVAD-CMK (80 µM) (C1 Inh) for one hour prior to treatment with recombinant purified HRPII (10 µg, H). Data are mean values +/- SEM for 6-8 replicates spread over 4 independent experiments. All bars are significantly different from HRPII treated, $p < 0.05$ by one-way ANOVA.

Two different chemical inhibitors of NFκB (targeting subunit p65), Triplotide (Zeng et al., 2011; Zhu et al., 2009) and Celastrol (Ni et al., 2014; Yang et al., 2006), ablated the TEER changes induced by recombinant HRPII and resulted in normalized barrier integrity (**Fig 2.4B**). To confirm independently the role of NFκB in HRPII-mediated effects on barrier integrity, we silenced the p105 subunit of NFκB (**Fig 2.6**). Again, a decrease in TEER was prevented (**Fig 2.4B**). These experiments suggest that the HRPII-mediated drop in TEER requires an NFκB-dependent signal.
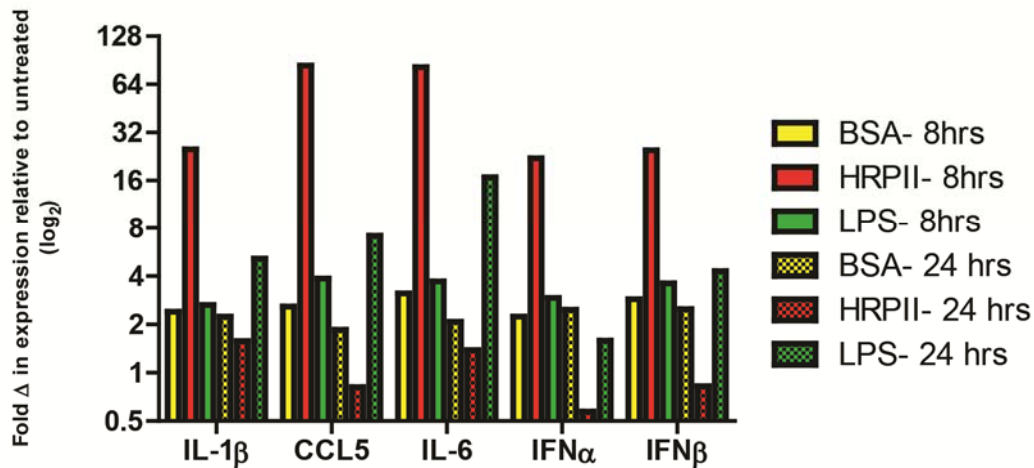
Figure 2.5] HRPII and LPS induce chemokines and cytokines with distinct kinetic profiles. Up-regulation of chemokine and cytokine mRNA levels seen in response to 25 µg HRPII or LPS at 8, and 24 hours post exposure. Fold mRNA induction relative to untreated cells.

Many cellular pathways can activate NFκB signaling. To begin to define the HRPII-dependent pathway we silenced the most common intracellular adaptor, MyD88 (**Fig 2.6**). Knocking down MyD88, significantly reduced the drop in TEER present from HRPII by three different shRNAs (**Fig 2.4C**). These data highlight that HRPII-mediated inflammation is NFκB and MyD88- dependent.
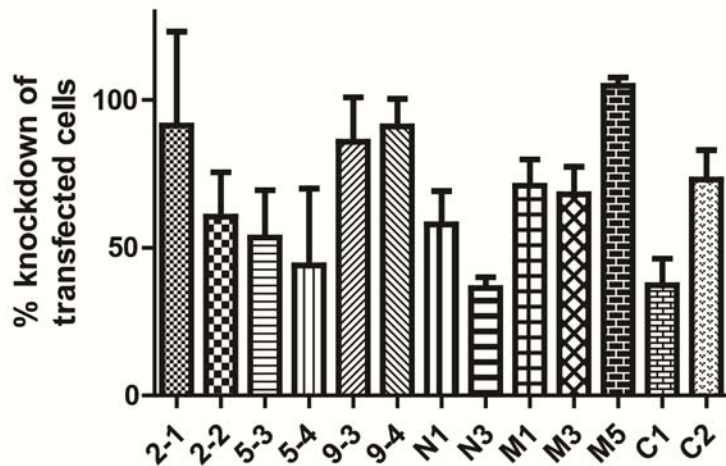
Figure 2.6] Knockdown levels of the various shRNAs used. shRNAs to TLR2 (2-1 and 2-2), TLR5 (5-3, 5-4), TLR9 (9-3, 9-4), NFkB (N1 and N3), to Myd88 (M1, M3 and M5), to caspase 1 (C1 and C2). hCMEC/D3 cells were incubated with shRNAs as in Figure 3. mRNA levels were quantified by qRTPCR. Shown are data from triplicate determinations. Values are normalized for percent of cells transfected, as determined from visualization of GFP-expressing shRNA.

HRPII activates the inflammasome

MyD88 is an intracellular adaptor for a number of innate immune receptors, and several of these proteins use MyD88 as an exclusive intracellular adaptor: TLR1/2, TLR2/6, TLR5, TLR7, TLR9, IL-1R and IL-18R (Kawai and Akira, 2005, 2010). Silencing of TLR2, 5, and 9 (**Fig 2.6**) did not impact HRPII-mediated endothelial cell barrier disruption (**Fig 2.7**).
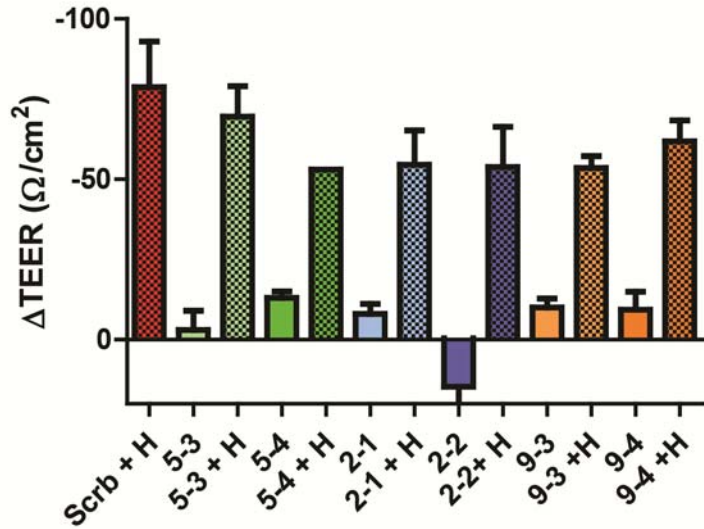
Figure 2.7] HRPII-mediated BBB compromise does not require TLR 2, 5 or 9. TEER measurements for in vitro BBB models transfected with scrambled control (Scrb) or shRNAs to TLR2 (2-1, 2-2), TLR5 (5-3, 5-4), and TLR9 (9-3, 9-4).

However, using a neutralizing polyclonal antibody against IL-1β or using IL1Ra, a natural antagonist of IL-1R, we demonstrated that HRPII-mediated change in TEER requires IL-1β activation and signaling (**Fig 2.4D**). These data point to involvement of the inflammasome. A requirement for caspase-1 was confirmed using two distinct shRNAs for caspase-1 as well as the caspase-1 specific inhibitor YVAD-CMK. Endothelial barriers treated with these reagents did not display a change in TEER in the presence of HRPII (**Fig 2.4D**). These data indicate that activation of the inflammasome is required for HRPII-mediated BBB disruption.

HRPII-induced cell death and loss of barrier integrity are independent and kinetically distinct phenotypes

Activation of the inflammasome can cause cell death. To determine whether endothelial cells lose viability in response to HRPII exposure, we monitored cell death at various time

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points. Cells undergoing programmed cell death display nicked DNA which can be visualized with a TUNEL stain. HRPII-treated cells showed no TUNEL staining at 6 hours (when barrier disruption is evident) although nicking of cellular DNA was evident at 24 hours post exposure (**Fig 2.8A**).
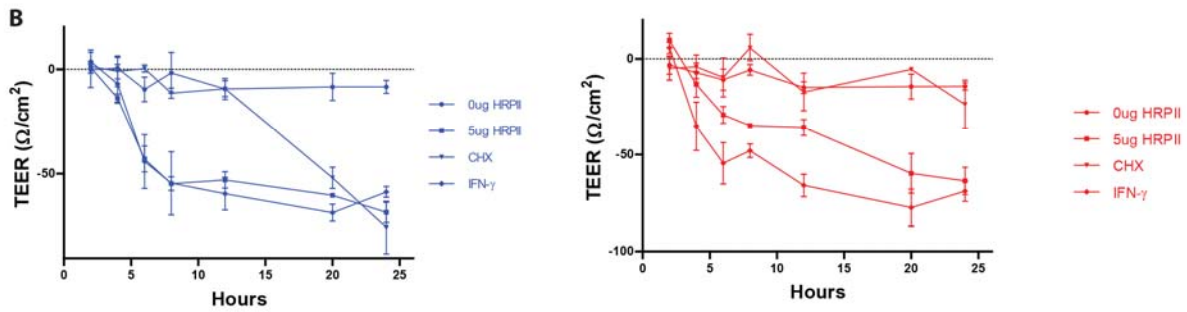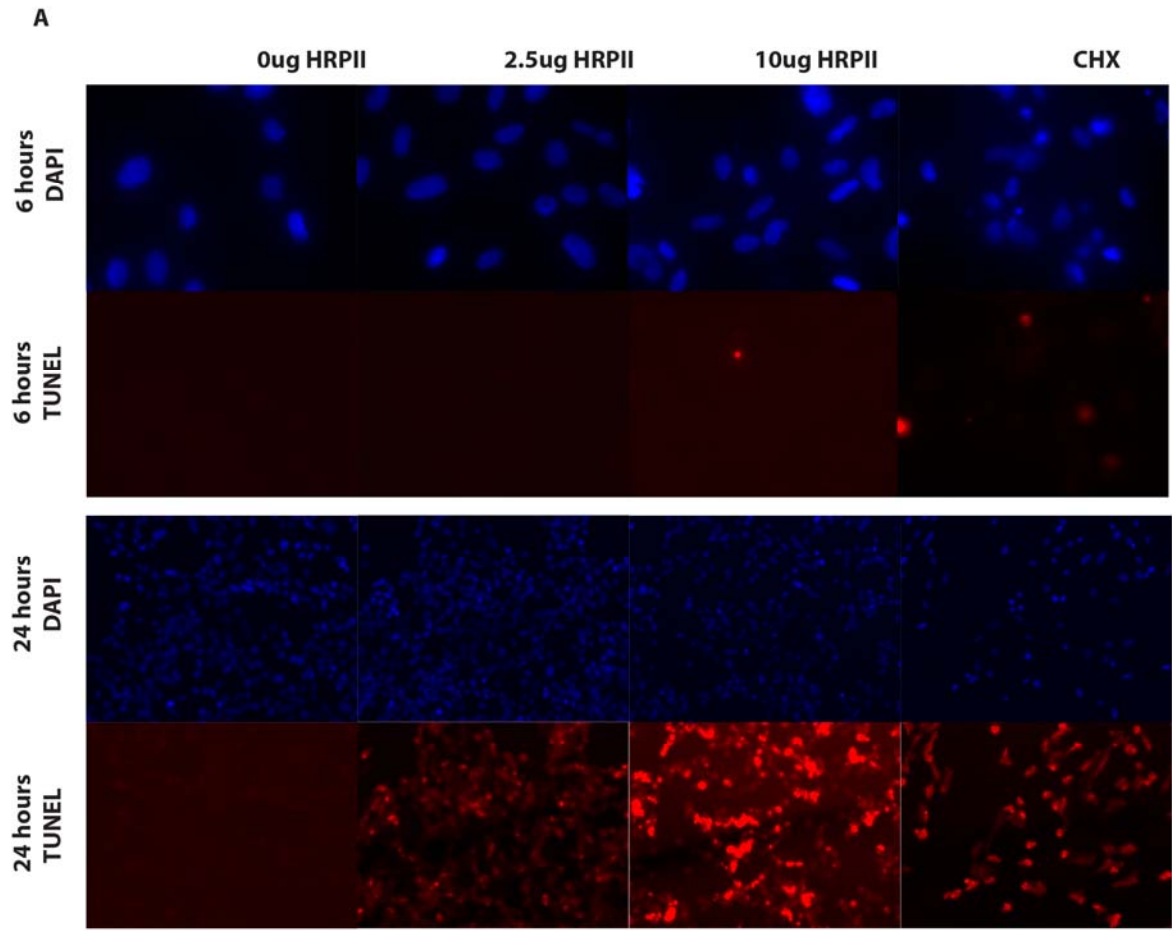
Figure 2.8] HRPII mediated BBB compromise is independent of cell death. (A) HRPII results in cell death, evidenced by nicked DNA in this TUNEL stain at 24 hours post exposure to protein, but not at 6 hours. Cyclohexamide (CHX) is a positive control for cell death (B) TEER measurements for in vitro BBB models untreated, treated with IFN-y (10 ng), or HRPII, or cyclohexamide (CHX, 10 ng/mL) in the absence (blue) or presence (red) of an apoptosis inhibitor (Z-VAD-FMK, 10 µg/mL).

To rule out the possibility that HRPII-mediated loss of barrier integrity was a consequence of cell death, we reassessed TEER changes in the presence of an apoptosis inhibitor, Z-VAD-FMK. In this experiment, cycloheximide (CHX) serves as a positive control for endothelial cell disruption via apoptosis. In the presence of the Z-VAD-FMK, cell death and barrier leakage by CHX was prevented. IFN-γ serves as a second control for TEER changes via a mechanism (rearrangement of junctional proteins) that is distinct from cell death. IFN-γ compromised barrier integrity even in the presence of the apoptosis inhibitor. Similar to the IFN-γ control, HRPII-mediated TEER changes were sustained when cells were treated with Z-VAD-FMK (**Fig 2.8B**). These data indicate that the loss of barrier integrity induced by HRPII is not a byproduct of cell death.

HRPII promotes BBB permeability *in vivo*

To determine whether the HRPII could induce a compromise in barrier integrity of brain endothelial cell monolayers *in vivo*, four week-old female C57BL/6 mice were administered two 200 µg doses of HRPII 24 hours apart by intravenous injection. Forty-eight hours after the first dose, mice were injected via an intraperitoneal route with sodium fluorescein. 45 minutes later, animals were perfused and levels of fluorescein were measured in the brain parenchyma. We observed an increase in vascular leakage of fluorescein into the cortex and cerebellum of mice infused with HRPII compared to control animals (**Fig 2.9A, B**). Peak serum HRPII levels at 1 hour post infusion were found to be around 300-400 ng/mL. At the time of harvest, HRPII levels

were 150-200 ng/mL, substantially lower than levels observed in patients with cerebral malaria, where 1 to 100 µg/mL has been reported (Dondorp et al., 2005). Presumably much of the HRPII redistributes to the vascular wall, where it can be seen lining the endothelial surface in mice (**Fig 2.9C**) as it is in humans.
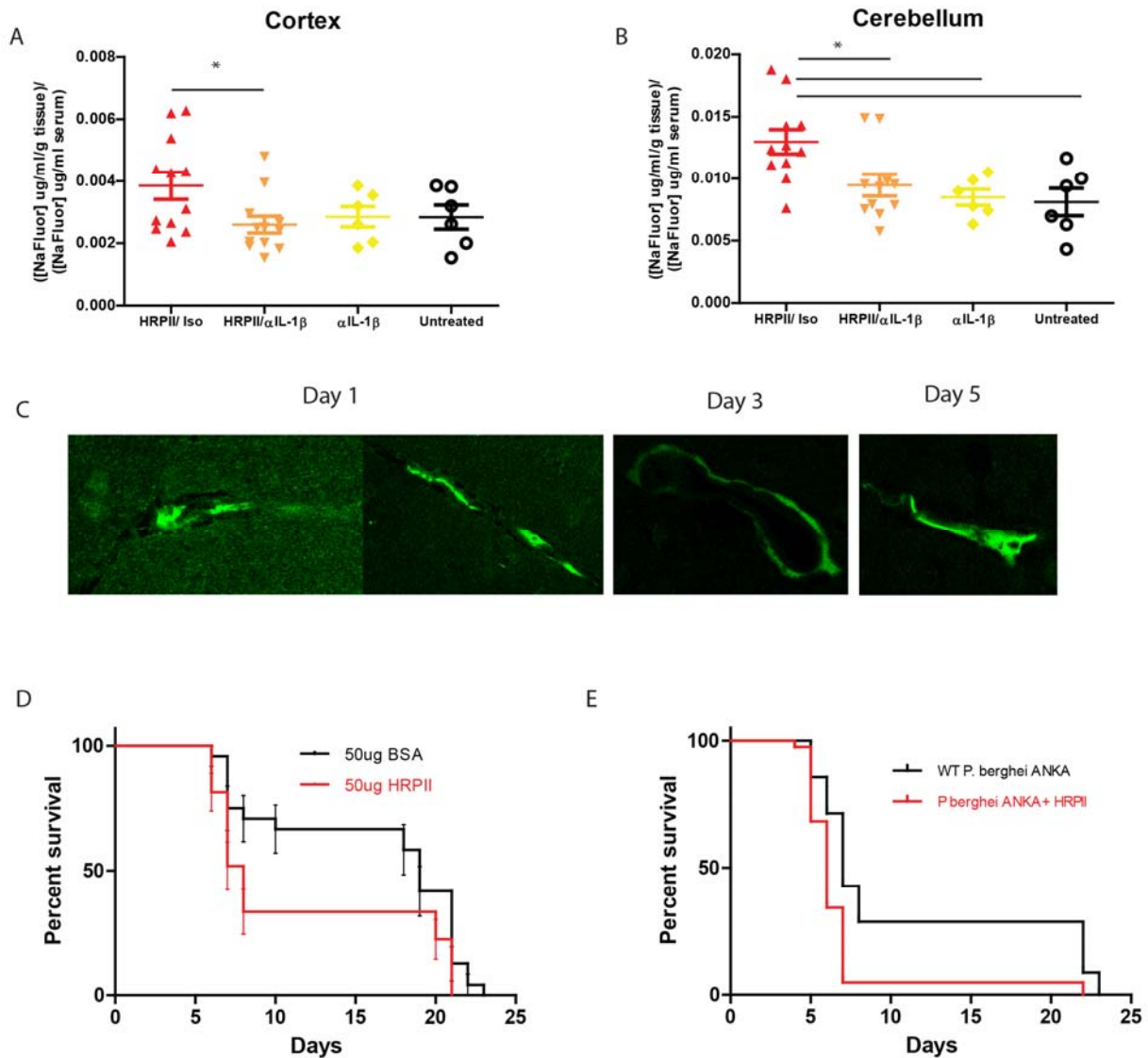
Figure 2.9] HRPII causes IL-1β mediated vascular leakage in vivo. HRPII (200 µg) was injected into 4 week old female mice. After 48 hours, fluorescein was injected and levels

measured in the cortex (A) and cerebellum (B). HRPII treatment was significantly different from control. Fluorescein levels in the cortex (A) and cerebellum (B) of mice infused with HRPII along with an iso-type antibody control (Iso), or a αIL-1β antibody, compared to just αIL-1β treated or untreated mice. HRPII-iso is significantly different from HRPII/ αIL-1β, p= 0.004 (cerebellum) and p= 0.06 (cortex), by one way ANOVA. (C) 4 week old female mice infused with 50ug HRPII. Representative immunohistochemistry sections showing persistent vascular staining with HRPII over five days. (D) 4 week old female mice infused once with 50 μg of BSA or HRPII prior to infection with P. berghei ANKA (10^5 parasites). Data are mean +/- SEM for 24-27 mice over 4 independent experiments. Curves are sig different, p = 0.03, by Log-Rank (Mantel-Cox test) (E) 4 week old female mice infected with P. berghei ANKA wild-type parasites or a transgenic line created to express HRPII (10^5 parasites). Data are mean +/- SEM for 35-41 mice over 4 independent experiments. Curves are sig different, p < 0.0001, by Log-Rank (Mantel-Cox test).

Our *in vitro* BBB model indicated that HRPII-mediated permeability was inflammasome dependent. To assess this effect *in vivo*, we infused HRPII-treated mice with a neutralizing antibody to IL-1β or an isotype control (**Fig 2.9A, B**). IL-1β specific antibody blocked HRPII-induced sodium fluorescein leakage. These data confirm the *in vivo* relevance of IL-1β-mediated signaling for the actions of HRPII on BBB permeability.

HRPII reduces survival in an experimental cerebral malaria model
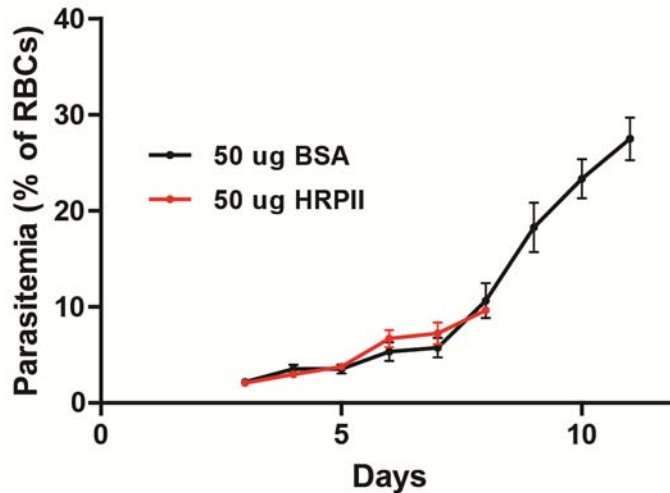
Figure 2.10] Parasitemia of mice dying from cerebral malaria-like symptoms is low, and the parasitemia of mice infused with HRPII or control protein is similar. Representative parasitemia from one full experiment. Mice displaying cerebral malaria-like symptoms died at low parasitemia by day 10, and parasitemia between both groups were closely matched on each day. Representative data from one experiment, 10 mice per group. Experiment was repeated three times.

We next determined whether the compromise in vascular integrity observed with purified HRPII had functional consequences during a malaria infection. We infused 6 week old female C57BL/6J mice with 50 μg of BSA or HRPII prior to infection with 2 x10^5 *P. berghei ANKA*. The experimental cerebral malaria model has variable penetrance, with a 40 to 100% lethality rate from cerebral malaria reported (de Oca et al., 2013; de Souza and Riley, 2002), defined as neurological symptoms and death at or below 10% parasitemia, by day 10 post infection. Mice infused with HRPII had a statistically significant increased incidence of neurological symptoms and early lethality compared to control mice (**Fig 2.9D)**. The parasitemia of mice that died from

cerebral malaria-like symptoms was low, as expected (**Fig 2.10**), and importantly no differences were observed between mice infused with HRPII or control protein.

As a further test of the role of HRPII in the cerebral malaria model, we generated transgenic *P. berghei ANKA* parasites that express HRPII. Insertion of the gene was confirmed by PCR, and sequence confirmed by TOPO-TA cloning and sequencing of the insert (**Fig 2.11**). Expression of HRPII was confirmed by quantitative ELISA. Levels of HRPII in the serum of mice at day 8 after infection were 100 to 200ng/mL (data not shown). Mice infected with transgenic parasites also had a more rapid time to death compared to those infected with the parental line (**Fig 2.9E**).
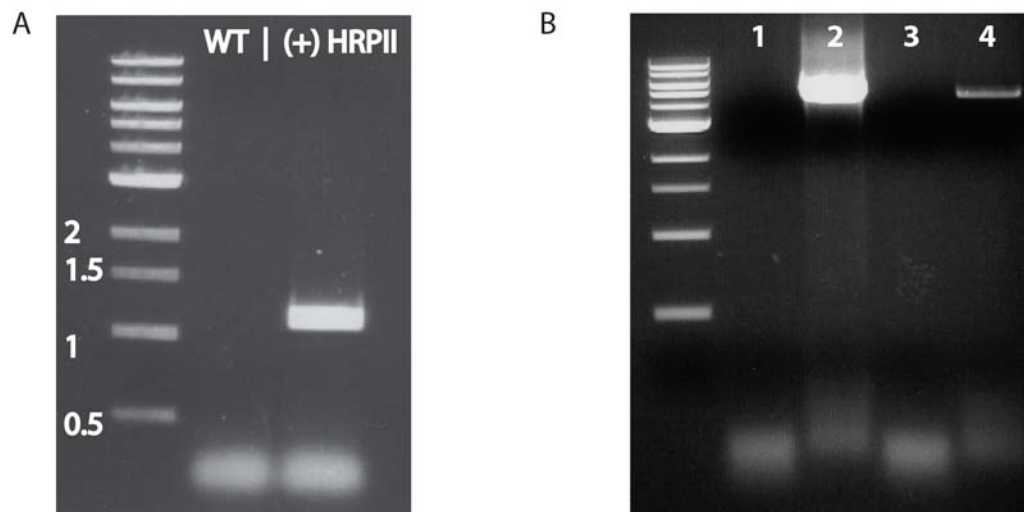
Figure 2.11] Transgenic P. berghei ANKA parasites carry the gene for HRPII (A) PCR of HRPII from genomic DNA purified from whole blood of mice infected with wild -type P. berghei ANKA parasites (lane 1) or those engineered to produce HRPII (lane 2) using primers at the 5' and 3' ends of the HRPII-GFP. (B) PCR to illustrate integration of HRPII from genomic DNA purified from whole blood of mice infected with wild -type P. berghei ANKA parasites (lane 1, 3) or those engineered to produce HRPII (lane 2, 4) using primers resting upstream of the 5' 230p integration site (forward) and the end of GFP (reverse), lanes 1 and 2. Lanes 3 and 4 use primers resting at the start of HRPII (forward) and post the 3' integration site of 230p (rev).

HRPII treatment upregulates cytoadherence molecules on endothelial cells

Cerebral malaria is accompanied by upregulation of cytoadherence molecules on the vascular endothelium (Hawkes et al., 2013; Madkhali et al., 2014; Storm and Craig, 2014). We assessed the surface expression of several relevant adhesion receptors on brain microvascular endothelial cells after treatment with HRPII. The expression of ICAM-1 and VCAM-1 was increased upon HRPII treatment (**Fig 2.12**). In contrast, E-selectin was not increased. ICAM-1 expression levels and binding by parasites is known to be associated with severity of disease (Madkhali et al., 2014).
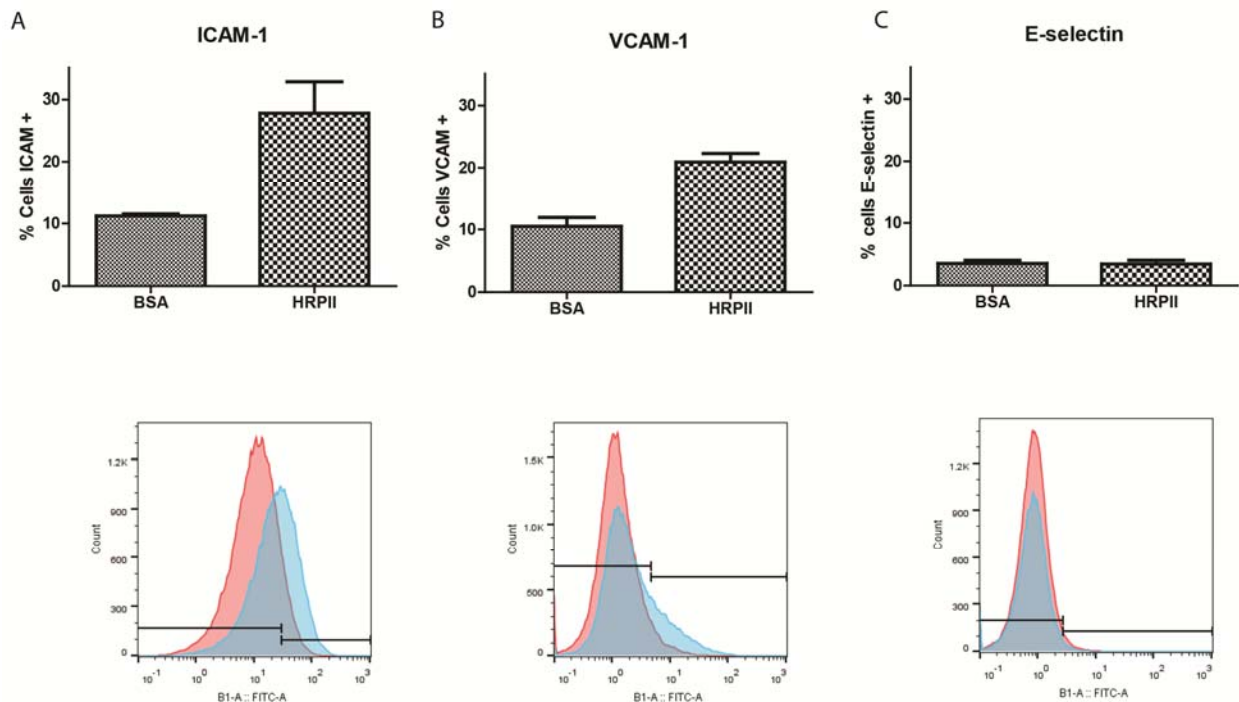
Figure 2.12] Human brain microvascular endothelial cells exposed to HRPII display an increase in surface adhesion receptors. Human brain endothelial cells were exposed to BSA or HRPII (10 μg) for 24 hours. Surface expression of cell surface adhesion receptor is measured by flow cytometry. Using a two tailed t-test, p< 0.03 for ICAM-1 and VCAM-1 expression, E-

Selectin expression is not significantly different between BSA and HRPII treatment. Representative flow plots shown below each bar graph.

2.4 Discussion

BBB leakage during *Plasmodium falciparum* infection is a hallmark of CM. The pathophysiology underlying this effect, however, is poorly understood. Our study has identified HRPII as a parasite virulence factor that is recognized by the host innate immune system, activates the inflammasome and promotes redistribution of endothelial junctional proteins, which results in increased BBB permeability.

P. falciparum parasites produce two highly homologous histidine-rich proteins: HRPII and HRPIII. Of the two, HRPII is produced abundantly whereas HRPIII is minor (Rock et al., 1987). These proteins are exported by the parasite into the host erythrocyte and then gain access to the host bloodstream, largely after parasite rupture (Desakorn et al., 2005; Howard et al., 1986; Rock et al., 1987). The Dd2 *P. falciparum* parasite strain produces only HRPIII and has a deletion in HRPII. Using this background strain, we generated transgenic parasites that express HRPII, and isolated two clones from independent transfections. Whereas parental Dd2 parasites caused minimal change in TEER in an *in vitro* human BBB model, the HRPII-expressing clones caused a substantial decrease in TEER, similar to that seen with the 3D7 *P. falciparum* isolate that endogenously expresses HRPII. Evidently, the small amount of HRPIII expressed in Dd2 is not sufficient to effect barrier disruption. HRPII, the major histidine-rich protein, appears to be responsible for this action on the endothelium.

Several lines of experimental evidence suggest that a direct effect of HRPII is responsible for BBB leakage: (1) *P. falciparum* strains that naturally carry the HRPII gene or have been

genetically modified to carry it can compromise the integrity of an *in vitro* BBB model; (2) purified native and recombinant HRPII can mediate decreased TEER directly; (3) a monoclonal antibody to HRPII neutralizes both the infected RBC-mediated and the isolated protein-mediated effects on TEER; (4) HRPII infusion at clinically relevant levels causes leakage of sodium fluorescein into the brain parenchyma of mice. This last statement is complicated by the pharmacokinetics of HRPII in mice and humans. While HRPII in humans can be detected roughly one month post clearance of parasites, in mice the half-life is roughly 24 hours. Despite this, vascular leakage was observed with infusion of HRPII that gave peak concentrations lower than those in human cerebral malaria.

Assessment of transcriptional responses to HRPII suggested activation of the NF☐B pathway. Gene silencing and antagonist experiments confirmed a role for MyD88 and NF☐B in mediating the HRPII effect. Triplotide has been show to decrease expression of NFκB/p65 and increase expression of the cytosolic inhibitor IκB-α (Zeng et al., 2011; Zhu et al., 2009) Celastrol has been shown to decrease expression and translocation of NFκB/p65 to the nucleus as well as to diminish cleavage and activation of IκB-α (Ni et al., 2014; Yang et al., 2006). In the presence of these inhibitors we observed a significantly decreased drop in TEER from HRPII. However, we did not identify an upstream pathogen recognition receptor. Rather, additional studies suggested involvement of the IL-1 receptor, as we could block HRPII-mediated barrier disruption with IL-1Ra, a natural antagonist, or with antibody to IL-1β. IL-1β is a cytokine that is activated from its pro-form by caspase-1. Caspase-1 is auto-catalytically processed when molecules are brought in proximity by the inflammasome. Consistent with a key role for the inflammasome the HRPII effect on TEER also was abolished by silencing of caspase-1 or treatment of brain microvascular endothelial cells with a caspase-1 inhibitor.

Inflammasome activation has been implicated in malaria infections previously. Opsonization of parasitized red blood cells as well as pooled patient sera from *P. falciparum* infections was shown to activate the inflammasome in macrophages (Zhou et al., 2012). IL-1β has also been seen in histopathological sections from patients who died from cerebral malaria (Armah et al., 2005; Udomsangpetch et al., 1997).

The data suggest a model for HRPII action on endothelial cells (**Fig 2.13**). HRPII accumulates in the bloodstream and binds to vascular endothelium via an unknown receptor. Downstream signaling allows for recruitment of inflammasome components, which activate caspase-1 resulting in cleavage of substrates including pro-IL-1β, yielding mature IL-1β. Active IL-1β is secreted, where it can bind to cell surface receptor IL-1R. IL-1R ligation transmits a MyD88-dependent signal that activates the transcription factor NF□B. NFκB translocates to the nucleus and induces transcription of many genes including cytoskeletal components, which can redistribute tight and adherens junction proteins (Al-Sadi et al., 2009; Al-Sadi et al., 2012; Al-Sadi et al., 2010; Al-Sadi and Ma, 2007; Baumgartner et al., 2003; de Rivero Vaccari et al., 2014; Kimura et al., 2009; Sollberger et al., 2014). Although there have been reports of tight junction rearrangement via an NFκB-independent pathway (Zhu et al., 2012), we believe HRPII does not trigger this route as shRNA-mediated knockdown of NFκB significantly inhibited HRPII-mediated barrier compromise.
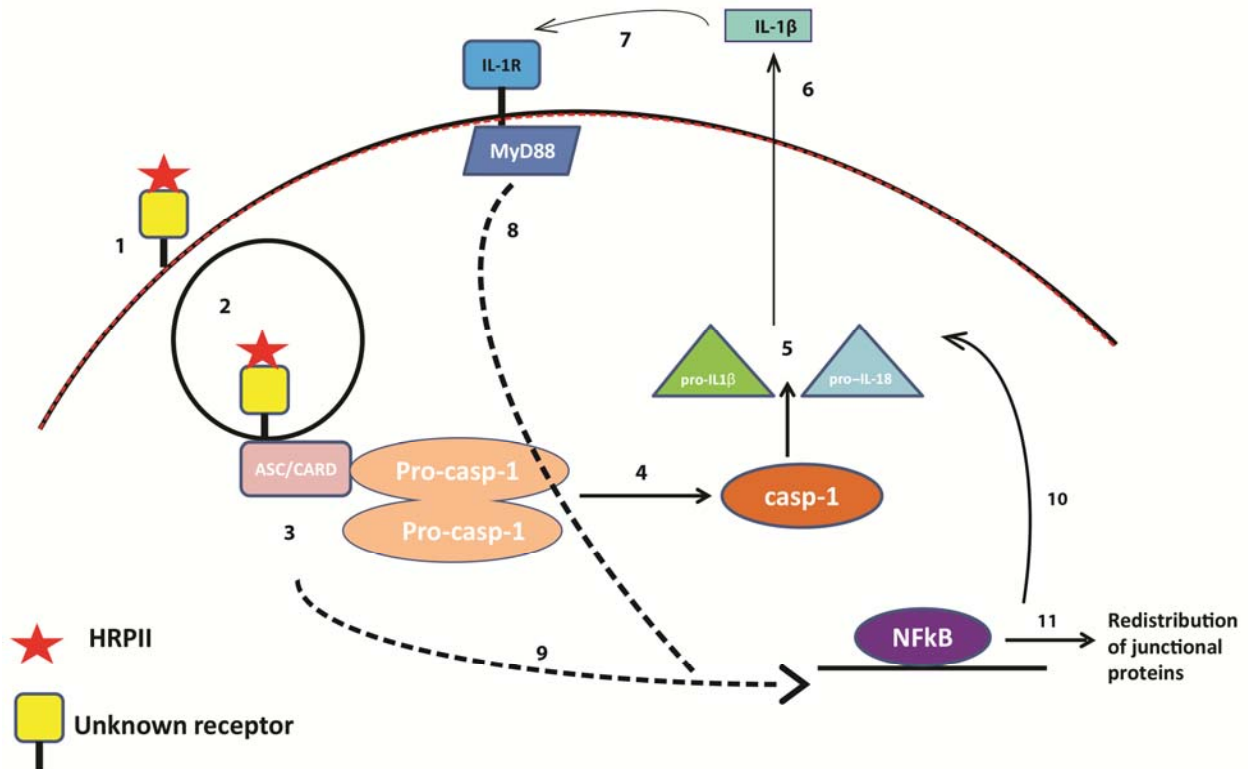
Figure 2.13] Model for HRPII recognition by human brain endothelial cells and the intracellular pathway that leads to BBB leakage. (1) HRPII binds to a yet unidentified receptor (1) and is internalized (2). Inflammasome adaptor proteins associate with this endosome (3) and recruit procaspase-1, which is auto-catalytically activated (4). Active caspase-1 can cleave pro-IL-1 and pro-IL-18 into their mature forms (5). Mature IL-1β is secreted (6), such that it can now bind to the IL-1 receptor, IL-1R (7). Signaling through MyD88, the IL-1R activates NFκB (8) as does downstream signaling from the inflammasome (9). NFκB mediates transcription of inflammatory genes (10), including those effecting redistribution of tight and adherens junction proteins (11), resulting in a compromised blood brain barrier.

HRPII was active *in* vivo, resulting in BBB leakage as well as an exacerbation of experimental CM when the protein was infused prior to infection or produced by transgenic rodent malaria parasites. Aspects of HRPII action help explain some differences between the pathophysiology seen in human CM and the experimental CM model. For example, tight junction protein redistribution is not observed in eCM, suggesting that vascular leakage in the rodent model occurs via an alternate mechanism than that observed in human pathology

(Mukhopadhyay and Gordon, 2004; Nacer et al., 2014). HRPII-mediated barrier compromise depends on caspase-1 activation of IL-1β, and IL-1β levels in the cerebrospinal fluid of patients correlates with disease severity (John et al., 2008) while it has been shown to be dispensable for the experimental CM model (Kordes et al., 2011). We propose that HRPII makes the *P. berghei* system more *falciparum*-like; our data suggests that this protein may be a significant contributor to human cerebral malaria. Directly targeting HRPII may prove to be therapeutic. Attempts in the 1980s to use HRPII in Aotus monkey vaccination trials were encouraging, but follow up studies were equivocal (Enders et al., 1992; Knapp et al., 1992; Knapp et al., 1988; Kocken et al., 1998). Perhaps new studies informed by the proposed role in cerebral malaria could lead to development of a vaccine that prevents CM. It may also be practical to prevent the outcomes of HRPII by targeting upstream components of the pathway. Drugs targeting caspase-1 and IL-1 β are already in clinical use, and may be efficacious in cerebral malaria.

Since causing CM is not likely to benefit the parasite, the question of why *P. falciparum* has evolved and maintained the HRPII gene is germane. One possible advantage for the parasite is that triggering an inflammatory pathway leads to upregulation of cytoadherence molecules on the endothelial surface. Cytoadherence allows the parasite to avoid clearance in the spleen and reside in a low-oxygen, high carbon dioxide environment. Parasite sequestration in the cerebral vasculature is hallmark feature of human CM and one that is largely absent in the murine experimental CM model. Other cytokines that are elevated in malaria such as TNF-□ also upregulate surface adhesion molecules that are receptors for malaria (Tchinda et al., 2007; Turner et al., 1998).

Several new questions arise from this work: What does HRPII bind to initiate the inflammatory process? Is HRPII synergistic with other *P. falciparum* virulence factors such as

glycosylphosphatidylinositol (Schofield et al., 2002). Does HRPII affect endothelium in other vascular beds in a similar manner? While our data strongly suggest that HRPII contributes to malaria pathogenesis by modulating the BBB, further mechanistic insight is needed to develop novel HRPII-dependent pharmacological strategies for disease control.

2.5 Materials and Methods

Reagents

Bovine serum albumin (BSA), reagent grade was purchased from Sigma. Lipopolysaccharide (LPS) from Escherichia coli O111:B4 was purchased from List Biologicals (catalog #201).

Antibodies

For confocal microscopy the following antibodies were used. Mouse αHRPII (2G12), a generous gift from Diane Taylor, was used at 1:100 dilution. Rabbit α-zona-occludin-1 from Invitrogen (40-2200) was used at 1:500 dilution. Goat anti-claudin-5 from Santa Cruz (sc-17667) was used at 1:100. VE-cadherin (Santa Cruz, sc-52751) was used at 1:100. 5 µg of anti-HRPII for neutralization for the TEER assays was purchased from Thermo Scientific (MA1-27094) while the isotype control was generated as described (Pal et al., 2013). Rabbit anti-IL-1β for neutralization in the TEER assay was from Rockland Immunochemical (209-401-301). Rabbit anti-caspase-1 for western blotting (Novus, NBP1-45433) was used at a 1:333 dilution. Rabbit anti-GAPDH (Abcam, ab37168) was used at 1:1000. Mouse anti-ICAM-1, -VCAM-1 and -E-selectin (BD Biosciences, 555510, 555645, and 555648) were used at 1:500, 1:100 and 1:500 dilutions, respectively. Armenian hamster anti-IL-1β for neutralization *in vivo* was purchased from Leinco and used at 300 µg/mouse one day before infusion of protein.

Inhibitors

Triplotide from Invivogen was resuspended at 10 mM in DMSO and used at a final concentration of 100 nM. Celastrol from Invivogen was resuspended at 4.4 mM in DMSO and

used at a final concentration of 8.8 µM. IL1Ra from Sigma was resuspended in water and used at a final concentration of 500 ng/mL. Caspase-1 inhibitor from Sigma (SML0429) was used at a final concentration of 80 µM.

HRPII Purification

The coding sequence for the mature form of HRPII (Chang et al., 2008) was PCR amplified from *P. falciparum* reverse-transcribed cellular RNA. The gene was cloned into a pET-15b vector (Novagen) without a tag. The vector was transformed into BL21(DE3) cells and HRPII production was induced using 0.4 mM IPTG at 16°C when the OD_{600} was between 0.6 and 0.8. HRPII was purified from *E. coli* lysate using batch nickel bead (Pierce, 88222) purification with a 250 mM imidazole wash and 1M imidazole elution in 20mM Tris, 500mM NaCl, pH 8.0. Following this, HRPII was exchanged into nickel column loading buffer (20 mM Tris, 500 mM NaCl and 50 mM imidazole) and loaded on a 5 ml nickel FPLC column (GE Healthcare, 17-5248-02). Column-bound HRPII was then washed with 60 column volumes of Triton X-114 wash buffer (20 mM Tris, 10 mM NaCl and 0.1% Triton X-114) to remove any residual LPS. Protein was washed subsequently on the column with 20 column volumes of loading buffer and then eluted with 1M imidazole buffer. All preparations of HRPII were tested for residual LPS levels using the commercially available LAL endotoxin test (Charles Rivers, R1708K); levels administered to mice were always less than 5EU/kg. Clean, fully active preparations of the protein were used for *in vitro* and *in vivo* experiments. Activity was measured using the Factor Xa assay developed previously (Ndonwi et al., 2011). Protein concentration was determined by BCA assay (Fisher, P123227).

P. falciparum culture and transfection

HRPII was PCR amplified from genomic DNA from 3D7 parasites and cloned into the TOPO vector (Life Technologies). HRPII was inserted upstream of GFP in the tEOE vector under control of the Hsp86 promoter. This vector is a modified form of the tyEOE vector with the selectable marker human dihydrofolate reductase replacing the yeast dihydroorotate dehydrogenase selection cassette (Beck et al., 2014). For transfection, 160 µl of a 50% hematocrit RBC stock was electroporated with 100 µg of purified vector DNA and 50 µg of the transposon vector MRA912 and then infected with 3D7 schizonts (Balu et al., 2005). After 72–90 h, 10 nM WR99210 was added to the medium for drug selection and plasmid maintainence. Parasites were grown under selective pressure for four weeks and then cloned out in a 96 well plate at 0.5 parasites/ well. Clones were screened by PCR.

In vitro BBB Cultures and TEER recordings

In vitro BBB cultures were prepared as previously described (Daniels et al., 2013). Briefly, 10^5 hCMEC/D3 cells (Weksler et al., 2005) were cultured on the apical side of a 0.9 cm^2 fibronectin-coated polyethylene terephthalate filter insert with 3.0 µm porosity (BD Falcon) for 4 to 6 days in supplemented endothelial basal medium with 1 ml in the upper chamber and 1.5 ml in the lower chamber. Components for assessment (protein, chemical inhibitors, and parasitized erythrocytes) were added to the apical chamber immediately after determining baseline values for each well. Resistance recordings were measured via chopstick electrode with an EVOM voltmeter (World Precision Instruments). Resistance values are reported as Ω/cm^2 (recorded values minus values for cell-free inserts).

shRNA knockdown and TEER

10^5 hCMEC/D3 cells were cultured on the apical side of a 0.9 cm^2 fibronectin-coated polyethylene terephthalate filter insert with 3.0 μm porosity (BD Falcon). Twenty-four hours later cells were transfected with 500 ng of shRNA with Lipofectamine 3000 at a 1.5:1 ratio of Lipofectamine to DNA. Cells were then incubated for 36 hours. At that time, fresh warm medium was added and then allowed to equilibrate for 30 minutes prior to taking baseline TEER measurements. HRPII was then added and TEER measurements recorded over 24 hours. shRNAs for each gene were purchased from Origene : Myd88 (TG311320), NFκB (TR318700), Caspase-1 (TG305640), TLR9 (TR301076), TLR5 (TR308792), TLR2 (TR320553); of the four shRNAs received from the vendor, 2-3 were used based on silencing efficiency in pilot studies. One additional shRNA was used for MyD88 from Invivogen (ksirna42-hmyd88). All gene silencing was performed using Lipofectamine 3000 at a ratio of 1.5: 1. Silencing efficiency for all assays was determined by qRT-PCR.

Quantitative RT-PCR

Total RNA was isolated from treated or untreated cultured hCMEC/d3 cells by using the RNeasy kit according to the manufacturer's instructions (Qiagen, http://www.qiagen.com/). During the isolation, to remove any contaminating DNA, samples were treated with RNAse-free DNAse (Qiagen). mRNA was quantified from total RNA by qRT-PCR as previously described (Samuel and Diamond, 2005). PrimeTime qPCR primers and probes were purchased from IDT and used to amplify human IL-1β, IL-6, CCL5, IFNα, IFN-β, Myd88, TLR-2, TLR-5, TLR-9, NFκB, and caspase-1 mRNA using the following assay IDs: IL-1β, Hs.PT.58.1518186; IL-6, Hs.PT.58.39866843.g; CCL5, Hs.PT.58.1724551; IFN-β, Hs.PT.58.39481063.g; IFN-α, Hs.PT.58.46311748.g; GAPDH, Hs.PT.39a.22214836; Myd88, Hs.PT.58.40601199.gs; TLR-2, Hs.PT.58.21312907; TLR-5, Hs.PT.58.38446229; TLR-9, Hs.PT.58.40576968; NFκB,

Hs.PT.58.892624; caspase-1, Hs.PT.56a.39122258.g. To analyze the relative fold induction of amplified mRNA, GAPDH mRNA expression levels also were determined and normalization was performed using the Ct method as described previously (Samuel and Diamond, 2005).

Mouse Model for Cerebral Malaria

Four week-old female mice C57BL6 were purchased from Taconic. Animals were housed under pathogen-free conditions. All experiments were approved by and performed in compliance with Animal Studies guidelines at Washington University in St Louis. Mice were given intravenous retro-orbital injections of 50 µg of HRPII or BSA, in 100 µl of PBS approximately 12 hours prior to infection while under anesthesia. The mice were then anesthetized again for intravenous inoculation of *P. berghei* ANKA parasites (10^5 parasites in 100 µl) via retro-orbital injection Parasitemia of stock mice for making the infection cocktail was determined by blood smear by manually counting at least 2,000 cells; stock mice were used with parasitemias below 3%.

Transgenic *P. berghei*

Passage 2 *P. berghei ANKA* parasites were purified from 5 mice at close to 1% parasitemia by collecting blood via intracardiac puncture. *In vitro* schizonts were generated by incubating in a shaking (65 x rpm) gassed chamber at 5% CO_2, 5% O_2 and 90% N_2 at 37^0C in RPMI, 20% FCS with gentamicin. Next day maturation of schizonts was confirmed by blood smear and schizonts were purified across four 50 mL tubes filled with 35 mL of culture. This was done by placing a 10 mL 55% Histodenz column under each and spinning tubes at 200 g with no brake for 25 minutes; the schizont layers were pooled. 5 x10^7 purified schizonts were transfected with 10 µg of pL1694 (Annoura et al., 2014) + HRPII-GFP linearized with Bcl1 and Sap1 in 100 µl of

Nucleofector solution using an Amaxa Nucleofector on program U-033. Transfected parasites were injected immediately via tail vein injection into 6-8 week old female Swiss Webster mice. Mice were treated with pyrimethamine in their drinking water at 7 mg/mL starting the next day for 1 week to select for integrants. Blood was collected from mice via cardiac puncture and frozen. Single clones of transfected parasites were generated by infection of 20 mice at 1 parasite/mouse, and mice were screened by PCR for correct genetic manipulation.

TOPT-TA Cloning and sequence confirmation of transgenic *P. berghei* clone

Genomic DNA was purified from whole blood from an infected Swiss Webster Mouse at ~5% parasitemia using the Quiagen Genomic DNA kits (51106) as described. The fragment between 230p 1455 and 5527 was PCR amplified using the Pfu Polymerase (Agilent Tech, 600380) as described in the manufacturer's instructions. The PCR-amplified product was TA cloned into the TOPO vector as described in the manufacturer's instructions (Life Technologies, 450030) and the sequence verified.

***In vivo* assessment of BBB permeability**

Two intravenous injections of HRPII (200 µg in 100 ul) were given 24 hours apart by retro orbital injection to 4 week old female C57BL/6J mice from Jackson Labs. 48 hours post initial injection, the mice were injected IP with 100 ul of 100 mg/ml of sodium fluorescein salt (Sigma-Aldrich) in sterile PBS. After 45 minutes, mice underwent extensive cardiac perfusion with PBS, followed by collection of blood and harvesting of CNS tissues. Tissue homogenates and serum were incubated overnight at 4°C at 1:1 dilution in 2% trichloroacetic acid (Sigma-Aldrich) to precipitate protein, which was pelleted by 10 minute centrifugation at 16,100 x g at 4°C. Supernatants were diluted in equal volumes of borate buffer, pH 11 (Sigma-Aldrich).

Fluorescence emission at 538 nm was determined via a microplate reader using Synergy™ H1 and Gen5™ software (BioTek Instruments, Inc.). Tissue fluorescence values were standardized against plasma values for individual mice. In some mice, anti-IL-1β antibody or isotype control (300 ug each) were administered on day one before HRPII infusion.

Immunocytochemical and immunohistochemical analysis

Immunocytochemical analysis on hCMEC/d3 or Caco-2 cells was performed after 10 minute fixation in ice-cold methanol, followed by blocking for 1 hour in 3% BSA in PBS at RT. Cells were then incubated with primary antibodies to ZO-1, claudin-5, and VE-cadherin in blocking buffer for 1 hour at RT, washed 3x in PBS, then incubated for one hour in secondary AlexaFluor® antibodies in blocking buffer at RT. Slides were washed extensively, followed by staining with TOPRO at 1:500. Sections were sealed with ProLong Gold antifade and then images acquired by confocal microscopy (Carl Zeiss USA).

TUNEL staining

Terminal deoxynucleotidyl transferase–mediated BrdUTP nick end labeling using TUNEL assay kit (Roche, 12156792910) was performed on paraformaldehyde-fixed hCMEC/d3 cells that had been treated with 25 μg HRPII, control protein or 3 μg LPS for 24 hours, as indicated in the manufacturer's instructions.

HRPII ELISA for quantification

HRPII was quantified in mouse sera using an ELISA as previously described (Gitau et al., 2013).

Acknowledgements

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Chapter 3: Conclusions and Future Directions

Cerebral malaria (CM) is a disease of the vascular endothelium caused by *Plasmodium falciparum.* It is characterized by parasite sequestration, inflammatory cytokine production and vascular leakage. A distinguishing feature of *P. falciparum* infection is parasite production and secretion of histidine-rich protein II (HRPII). Plasma HRPII is a diagnostic and prognostic marker for falciparum malaria. Using a human cerebral microvascular endothelial blood-brain barrier model, we demonstrate that HRPII activates the inflammasome, resulting in redistribution of tight junction proteins and compromise of barrier integrity. Intravenous administration of HRPII induces vascular leakage in the brains of mice and increased early mortality in *P. berghei* experimental cerebral malaria. Transgenic *P. berghei* expressing *falciparum* HRPII display enhanced disease severity. HRPII induces endothelial expression of adhesion receptors, suggesting that this protein also contributes to pathogenesis by enhancing parasite cytoadherence. We propose that HRPII is a virulence factor that contributes to cerebral malaria by compromising blood-brain barrier integrity and promoting parasite sequestration in the brain microvasculature.

Toxic mediators have been known to be released during a *P. falciparum* infection (Clark and Cowden, 2003; Schofield et al., 2002). Furthermore, addition of cultured *P. falciparum* parasites to brain endothelial cells compromises barrier integrity, with parasites as well as with soluble released components (Tripathi et al., 2007). Although many factors have been suspected as a toxic mediator in *P. falciparum* infection, none have been convincingly shown. The studies compromising this thesis provide a new framework for thinking about HRPII, a protein formerly considered simply a diagnostic marker for *Plasmodium sp.* infection, to a toxic virulence factor. We have worked backwards up the pathway and found that HRPII activates an inflammatory

response in brain endothelial cells that is mediated by NFκB and dependent on MyD88. The pathway activates the inflammasome as it produces IL-1β and is caspase-1 dependent.

Having identified HRPII as a protein of interest there is a considerable amount of work to be done if we want to target it therapeutically in the context of a malarial infection. First, it would be enlightening to use the transgenic *P. berghei ANKA* lines to pursue further pathophysiological studies in mice. Do the parasites that produce HRPII also cause a rearrangement of tight junction proteins in the mouse brain, similar to that observed in human pathology or the human brain endothelial cell *in vitro* studies? Do we see increased blood brain barrier leakage using fluorescein as a vascular tracer when mice are infected with the transgenic parasites compared to wild- type parasites? Will we observe an altered immune response with the transgenic parasites, both in terms of the kind of cells activated as well as the kinetics of the response? Our *in* vitro studies showed that there was an up-regulation of surface adhesion markers such as ICAM-1 and VCAM-1 on the brain endothelial cells by flow cytometry. Does this increase in surface display have functional relevance in terms of parasite adhesion to endothelial cell monolayers? This can be tested by lateral flow of *P. falciparum* parasites on an HRPII activated endothelial cell monolayers. Furthermore, does HRPII also cause increased surface display of ICAM-1 and VCAM-1 on murine cerebral vasculatures? If so, do we see an increase in sequestered parasites in the infected mouse vasculature?

In preliminary studies, we visualized small vesicles in the human brain endothelial cells staining for HRPII, indicating that HRPII is internalized into the cell from the cell surface. How is the protein internalized? Is internalization receptor-mediated? A schematic of where the receptor would fit in our model is shown below in figure 3.1. If so, does this receptor also serve as the molecular sensor for inflammasome activation? The molecular sensor that HRPII binds to

allowing association with the inflammasome is also not identified. Identification of the receptor or the molecular sensor could be initiated with immunoprecipitation (IP). An IP done with the correct conditions could identify such a protein. This interaction could be corroborated with reciprocal IPs as well as knockdowns of the protein to see if functional consequence of HRPII could be abrogated. If HRPII activity could be halted in the absence or reduction (from knockdown) of the sensor/ receptor then a point of intervention could be identified. Early evidence, via co-immunofluorescence, lead us to suspect that the thrombin receptor PAR-1 may be this mystery receptor. In addition, knocking down the PAR-1 receptor with shRNAs also reduced the drop in electrical resistance seen from HRPII. However, qPCRs of various pro-inflammatory cytokines and chemokines in the presence of a synthetic inhibitor of the PAR-1receptor showed that suppression of the receptor is actually anti-inflammatory. Since our work has identified that NFκB is activated in the presence of HRPII, a nonspecific anti-inflammatory event such as an inhibitor of PAR-1or knocking down PAR-1 mRNA by shRNA could inadvertently prevent the blood brain barrier compromise we observe from HRPII, rather than the effect being mediated by a specific event. However, further follow-up studies are needed to make a conclusive call. If the PAR-1 receptor is not the receptor or molecular sensor, we will continue to pursue optimizing conditions for immunoprecipitation to identify the molecule in question.
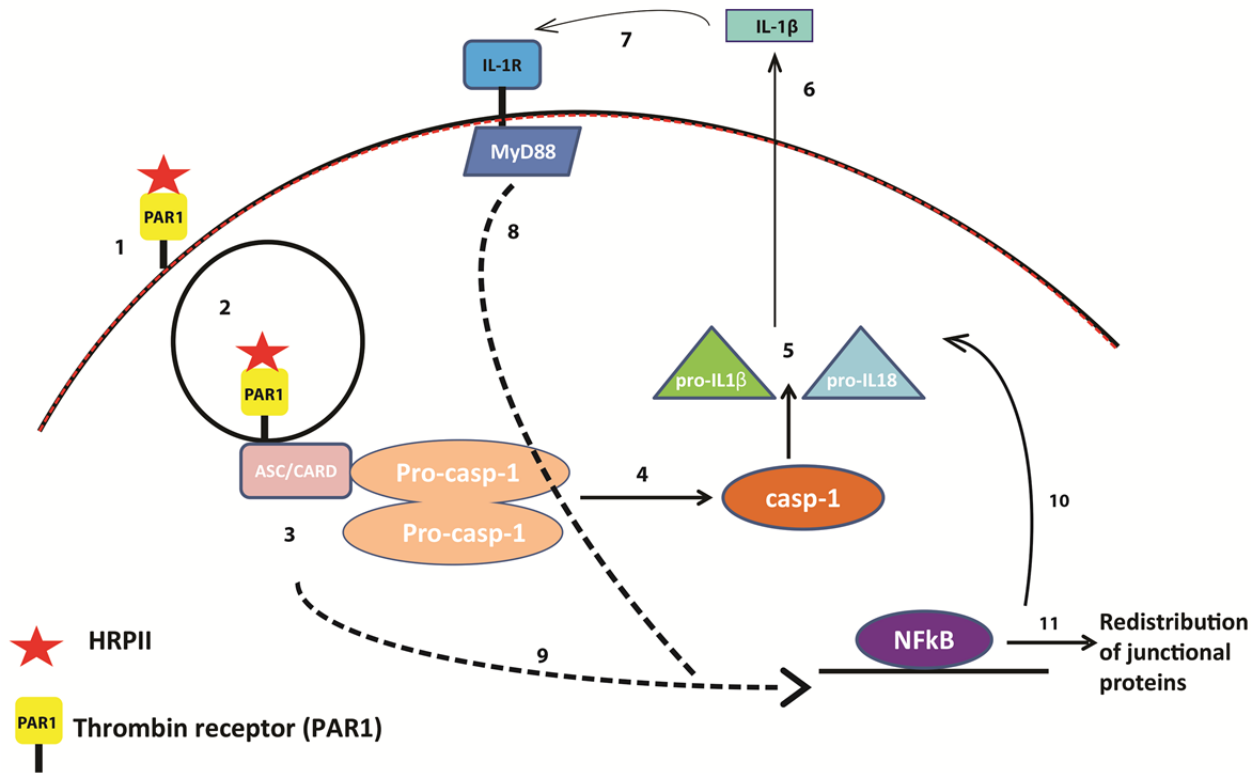
Figure 3.1] Model for HRPII recognition by human brain endothelial cells and the intracellular pathway that leads to BBB leakage. (1) HRPII binds to a yet unconfirmed receptor that may be the thrombin receptor PAR-1 (1) and is internalized (2). Inflammasome adaptor proteins associate with this endosome (3) and recruit procaspase-1, which is auto-catalytically activated (4). Active caspase-1 can cleave pro-IL-1 and pro-IL-18 into their mature forms (5). Mature IL-1β is secreted (6), such that it can now bind to the IL-1 receptor, IL-1R (7). Signaling through MyD88, the IL-1R activates NFκB (8) as does downstream signaling from the inflammasome (9). NFκB mediates transcription of inflammatory genes (10), including those effecting redistribution of tight and adherens junction proteins (11), resulting in a compromised blood brain barrier.

It is of evolutionary intrigue to consider why a parasite would carry a protein that is toxic to its host. Amongst the parasites that infect humans, HRPII is only present in *P. falciparum,* with no homologous proteins in the other four parasite species. Is this because it was never in the genome of other species, or was it lost because it was a disadvantageous to the parasite? Does HRPII make *P. falciparum* the most virulent of the species? Some insight into these questions may be

provided by studying the homologous histidine rich proteins in *Plasmodium* species that infect primates: *P. reichenowi* and *P. gaboni.* It would be fascinating to explore if these evolutionary ancestors are functionally similar to the *P. falciparum* protein, particularly considering that these are the only known homologs in any organism. To study this we have cloned these genes, and have transfected them into a *P. falciparum* parasite line that does not have HRPII. Preliminary TEER experiments with recombinant protein showed a small compromise in barrier integrity; however these preparations were not clean from LPS. Protein preparations clean from LPS may reveal limited compromise in barrier integrity considering that the impure batches shown small differences. It will also be exciting to see what similar experiments with the parasite lines containing primate HRPII will reveal. In addition we will perform growth curves with these are the parasite lines producing HRPII in isogenic backgrounds to determine whether HRPII protein production by the parasite shows any change in growth- either an advantage or disadvantage.

Pursuing these studies is sure to yield more burning questions. It will be exciting to see if the future holds a place for therapeutics targeted at HRPII either the protein directly or a step in the pathway of its activity. Directly targeting HRPII may prove to be therapeutic as it would reduce many of the consequences of infection BBB leakage and parasite sequestration or eliminate them if HRPII is the only protein that causes these effects. Attempts in the 1980s to use HRPII in Aotus monkey vaccination trials were encouraging, but follow up studies were equivocal (Enders et al., 1992; Knapp et al., 1992; Knapp et al., 1988; Kocken et al., 1998). Perhaps new studies informed by the proposed role in cerebral malaria could lead to development of a vaccine that prevents CM. It may also be practical to prevent the outcomes of HRPII by targeting upstream components of the pathway. Drugs targeting caspase-1 and IL-1 β are already in clinical use, and may be efficacious in cerebral malaria.

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