Host Inflammatory Pathways in Malaria Infection: Potential Therapeutic Targets and Biomarkers of Disease Severity

by

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Abstract

Severe malaria infections cause almost 1 million deaths annually, mostly among non-immune African children. The pathogenesis of severe malaria is poorly understood. It is increasingly appreciated that while host innate immune responses such as inflammation and phagocytosis are critical for control of parasite replication, they can become dysregulated and contribute to severe disease. The goals of this work were: (1) to characterize inflammatory responses to malaria by defining their relationship to phagocytosis and identifying novel molecular mediators, and (2) to evaluate the utility of biomarkers of inflammation and other host responses for predicting outcome in severe malaria infection. Using an *in vitro* model of the malaria-macrophage interaction, inflammatory and phagocytic responses to *Plasmodium falciparum* were found to be partially coupled. Activation of Toll-like receptors (TLRs) by purified parasite components increased internalization of parasitized erythrocytes, but uptake of parasitized erythrocytes did not require TLRs, nor did it trigger cytokine production via TLRs or other receptors. Two candidate molecules – Triggering receptor expressed on myeloid cells-1 (TREM-1) and Chitinase-3 like-1 (CHI3L1) – did not appear to critically modulate inflammation to malaria *in vitro* or in murine models. However, exogenous TREM-1 activation enhanced the pro-

inflammatory nature of the response to *P. falciparum*, with potential implications for malarial-bacterial co-infection. CHI3L1-deficient mice showed a trend towards earlier death in experimental cerebral malaria, suggesting that CHI3L1 may protect against severe malaria; however, further investigation in more informative models is required. Admission levels of plasma TREM-1, CHI3L1, and other biomarkers of inflammation and endothelial activation were increased in Ugandan children with severe malaria. Simple combinations of these biomarkers predicted mortality among severe malaria patients with high accuracy, warranting larger validation studies. Taken together, these findings identify host responses as putative targets for adjunctive therapies, and suggest the utility of host biomarker combinations as prognostic tests for severe malaria.

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Abbreviations

ANG-1:	Angiopoietin-1
ANG-2:	Angiopoietin-2
Ad-Ctl:	Control (empty) adenoviral vector
Ad-TREM-1:	Adenoviral vector expressing Fc-TREM-1
AUC:	Area under the curve
BBB:	Blood-brain barrier
CFR:	Case fatality rate
CHI3L1:	Chitinase-3 like-1
CLR:	C-type lectin receptor
CM:	Cerebral malaria
CO:	Carbon monoxide
CRP:	C-reactive protein
CSF:	Cerebrospinal fluid
DAP-12:	DNAX activation protein 12
DC:	Dendritic cell
DIC:	Differential interference contrast microscopy
EBAB:	Erythrocytes coated with Biotin, Avidin, and Biotinylated antibody
ECM:	Experimental cerebral malaria
fAβ:	fibrillar β-amyloid
Fc-TREM-1:	Fusion protein of the Fc portion of IgG and the extracellular domain of TREM-1
Flt-1:	FMS-like tyrosine kinase-1
GPI:	Glycosylphosphatidylinositol
HMGB-1:	High-mobility group protein B1
HO-1:	Heme oxygenase-1
ICAM-1:	Intercellular adhesion molecule-1
IFN:	Interferon
IL:	Interleukin
IL-1R:	Interleukin-1 receptor
IP-10:	10 kDa interferon gamma-induced protein
IRAK-4:	IL-1R-associated kinase-4

ITAM: Immunoreceptor tyrosine-based activation motifs

LODS: Lambaréné Organ Dysfunction Score

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

LTα: Lymphotoxin-alpha

LT-HSC: Long-term hematopoietic stem cell

MAL: MyD88 adaptor-like

MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

MIF: Migration inhibitory factor

MP: Microparticle

mRNA: messenger RNA

MSU: Monosodium urate

Myd88: Myeloid differentiation factor 88

NLR: Nod-like receptor or negative likelihood ratio

NO: Nitric oxide

NPV: Negative predictive value

oxLDL: Oxidized low-density lipoprotein

PAMP: Pathogen-associated molecular pattern

PbA: *Plasmodium berghei* ANKA

PBMC: Peripheral blood mononuclear cell

PCT: Procalcitonin

PE: Parasitized erythrocyte

PfEMP-1: *Plasmodium falciparum* erythrocyte membrane protein-1

*Pf*GPI: *Plasmodium falciparum* glycosylphosphatidylinositol

*Pf*Hz: Hemozoin purified from *Plasmodium falciparum* cultures

PGN: Peptidoglycan

PI3K: Phosphoinositide-3 kinase

PLR: Positive likelihood ratio

PPAR: Peroxisome proliferator-activated receptor

PPV: Positive predictive value

PRR: Pattern recognition receptor

PTEN:	Phosphatase and tensin homologue deleted on chromosome Ten
R10G:	RPMI 1640 medium with 10% fetal bovine serum and gentamicin
RA:	Rheumatoid arthritis
RBC:	Red blood cell
RLR:	Rig-like receptor
RNAi:	RNA interference
ROC:	Receiver operating characteristic
ROS:	Reactive oxygen species
sHz:	Synthetic β-hematin crystals
SMA:	Severe malarial anemia
SNP:	Single nucleotide polymorphism
s Prefix:	soluble
sTREM-1:	soluble Triggering receptor expressed on myeloid cells-1
TGF-β:	Transforming growth factor-β
TH1:	T helper cell-1
TH2:	T helper cell-2
Tie-2:	TEK Tyrosine Kinase, Endothelial
TIR:	Toll/IL-1R homology
TLR:	Toll-like receptor
TNF:	Tumour necrosis factor
TNFR2:	TNF receptor 2
TRAM:	TRIF-related adaptor protein
Treg:	T regulatory cell
TREM-1:	Triggering receptor expressed on myeloid cells-1
TRIF:	TIR domain-containing adaptor inducing IFN-β
uRBC:	Uninfected red blood cell
VCAM-1:	Vascular cell adhesion molecule-1
vWF:	von Willebrand factor
vWFpp:	von Willebrand factor pro-peptide
WPB:	Weibel-Palade bodies

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LKE, AD, CM, SH, and NR performed the experiments
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Chapter 1

Literature review: Pathogenesis of severe malaria

1.1 Malaria-related morbidity and mortality

Plasmodium species, the causative agents of malaria, are single-celled eukaryotic protozoa that can infect a wide range of hosts, including birds, reptiles, and mammals. Five *Plasmodium* species are known to infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi*. While malaria transmission was once widespread globally, it is now mainly limited to tropical and sub-tropical regions. Malaria causes 300-500 million infections annually [1,2], an enormous disease burden with significant economic implications [3]. In 2009, an estimated 870,000 deaths were attributed to malaria, mostly due to *P. falciparum* infection among children under 5 years old in sub-Saharan Africa [1].

The parasite has a complex life cycle in which it undergoes several transformations (Fig. 1.1). Following sexual reproduction in the midgut of the female *Anopheles* mosquito, haploid sporozoites accumulate in the salivary glands of the vector and are transmitted to the human host during a bloodmeal. Sporozoites migrate to the liver and replicate within hepatocytes for approximately one week. Following this clinically silent liver stage, merozoite progeny exit into the bloodstream and initiate the erythrocytic replication cycle, during which all clinical manifestations of malaria infection occur. Merozoite invasion of red blood cells (RBCs) yields early ring forms, followed by growth and replication to produce mature schizont forms. Schizont rupture releases daughter merozoites that reinitiate the cycle. Some parasites develop into sex cells termed gametocytes, which can be acquired by the vector during feeding.

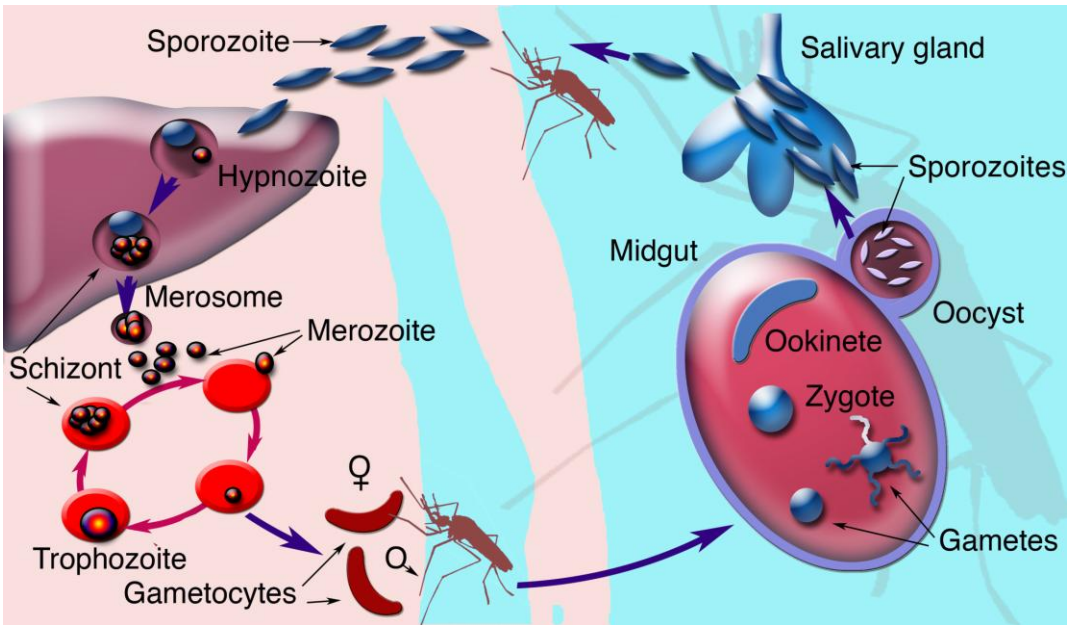


Fig. 1.1: The life cycle of the malaria parasite. (Reproduced with permission from ©Nature Publishing Group, ref. [4].)

1.1.1 Uncomplicated and severe malaria

Most malaria infections are uncomplicated and resolve with appropriate treatment. Symptoms of acute mild malaria are non-specific, such as fever, chills, and myalgia. Fevers are classically described as periodic – although this pattern is not always observed – and have been linked to spiking levels of pro-inflammatory cytokines such as tumour necrosis factor (TNF) [5]. In turn, cytokine responses of immune cells to *P. falciparum in vitro* are maximal following schizont rupture [6,7], indicating that release of components upon schizont rupture initiates an inflammatory response that produces the typical symptoms of acute infection. In addition to these symptoms, malaria infection is associated with increased susceptibility to pathogens such as non-typhoidal Salmonella [8] and exacerbation of existing infections such as HIV [9]. Moreover, chronic *P. falciparum* and *P. vivax* infection are important contributors to anemia in children and pregnant women [10-12].

Approximately 1-2% of infections progress to severe malaria, a heterogeneous group of clinically- and laboratory-defined syndromes that are associated with higher risk of mortality [13] (Table 1.1). Many of these syndromes cause rapid deterioration, and between 50-80% of patients die within 24 hours of admission to hospital [14-16], underscoring the critical importance of prompt triage and treatment. Severe malaria primarily afflicts children in highly endemic areas and adolescents and adults in regions of lower endemnicity. Presentations differ to some extent between these groups, possibly reflecting differences in age, ethnicity, or parasite strain. While the vast majority of malaria-related mortality is due to *P. falciparum*, there is increasing recognition that *P. vivax*, previously considered benign, and *P. knowlesi* can cause severe and even fatal infection [17-20]. Moreover, pregnancy-associated malaria can lead to miscarriage, pre-term delivery, and low birth weight infants (reviewed in [12]). Pediatric severe malaria caused by *P. falciparum* will be the focus of this work due to its immense public health importance.

Table 1.1: Major severe malaria syndromes (adapted from [13]).

Syndrome	Definition[a]	More common in:
Cerebral malaria	Blantyre Coma Score < 3 (children) or Glasgow Coma Score < 11 (adults) in the absence of hypoglycemia, recent seizures, recent administration of anticonvulsant medications, and another cause for neurological abnormalities	--
Severe malarial anemia	Hemoglobin < 5 g/dl or hematocrit < 15%	Children
Respiratory distress	Deep breathing, nasal flaring	Children
Pulmonary edema	Radiological evidence	Adults
Renal impairment	Serum creatinine > 265 μmol/L	Adults
Hypoglycemia	Blood glucose < 2.2 mmol/L	Children
Hyperlactatemia	Plasma lactate > 5 mmol/L	--
Metabolic acidosis	Plasma bicarbonate < 15 mmol/L	--
Hyperparasitemia	>2% in low intensity transmission areas >5% in areas of high stable transmission areas	--

[a]Diagnosis of all severe malaria syndromes requires presence of peripheral asexual parasitemia and no other obvious cause for illness.

The most striking severe malaria syndrome is cerebral malaria (CM). CM is characterized by profound neurological disturbances, namely coma, and has a 20% case-fatality rate (CFR) even with appropriate medical intervention [14,21]. Severe malarial anemia (SMA) is the most common syndrome in African children; it can occur acutely but is often associated with recurrent or chronic infection [22]. CFR estimates for SMA vary widely depending on co-morbidities (~1-30%) [14,21,23]. Respiratory distress, which reflects severe metabolic acidosis [24,25], significantly increases mortality risk in CM and SMA, and has been recognized as an independent severe malaria syndrome in children [14].

Treatment guidelines for severe malaria prescribe parenteral administration of anti-malarial drugs, supportive and/or intensive care (as appropriate and available), and blood transfusion for

SMA [13]. The anti-malarial agent artesunate is rapid-acting and highly effective, and has been shown to reduce severe malaria mortality compared to standard regimens [26,27]. However, artesunate does not significantly reduce CM mortality, and overall death rates for severe malaria remain high (10-20%). Novel adjunctive therapies are urgently needed.

1.1.2 Severe malaria pathogenesis: Host, parasite, and environmental factors

Development of adjunctive therapies for severe malaria requires understanding of its pathogenesis. This has proved a difficult challenge, in no small part due to the heterogeneity between and likely within the clinically-defined syndromes. However, it is known that progression to severe malaria can be influenced by parasite, host and socio-cultural factors.

A major host-related factor affecting disease progression is prior exposure to malaria and acquisition of a semi-immune state. Due to immune evasion tactics employed by the parasite such as antigenic variation and use of multiple erythrocyte invasion pathways (reviewed in [28]), the development of anti-malarial immunity requires repeated infections and is never fully protective. However, it protects against severe disease by controlling parasite growth and possibly virulence. While still under investigation, there is evidence for both cell-mediated [29] and antibody-mediated [30] adaptive immune responses that interrupt the parasite replication cycle and clear infected erythrocytes. Thus, non-immunes – young children in highly endemic areas and travellers to endemic areas – are at highest risk of severe malaria. Age itself, independent of exposure, has also been shown to affect susceptibility to severe malaria in humans and murine models, possibly due to age-related differences in immune system components [31,32].

Among individuals with little or no immunity to malaria, severe disease is often associated with high parasite burden or chronic infection [33,34]. Therefore, conditions that favour intense or prolonged parasite replication increase susceptibility to severe malaria. Socio-economic and cultural factors that delay access to medical treatment or increase inoculation rates have been associated with severe malaria, such as poverty, distance from health facilities, lack of access to accurate diagnostics and insecticide-treated nets, and prior treatment with traditional remedies or ineffective drugs [35-37]. Intrinsic parasite characteristics have also been investigated in this context. Inter-strain differences in the duration of the replication cycle and number of merozoite progeny per schizont have been reported for *P. falciparum* isolates and cloned lines [6,38]. *P. falciparum* isolates from Thai adults with severe malaria were shown to have increased *ex vivo* multiplication rates compared to those from uncomplicated cases [39], although these findings were not replicated in an African setting [40]. There is also variation between parasite strains in terms of erythrocyte invasion pathways and cytoadherence to endothelial cells (a virulence mechanism discussed below). These parasite phenotypes may contribute to malaria severity, although this is controversial [41,42].

While adaptive immunity to malaria has been a traditional research focus for the purposes of vaccine development, there is substantial evidence that other host factors can influence the course of infection. Co-infection with bacteria or HIV has been associated with severe malaria [43]. It is well-established that hemoglobinopathies such as sickle cell and thalassemia protect against severe malaria, and numerous other genetic loci have been associated with susceptibility or resistance to severe disease (reviewed in [44]). A pedigree-based study in Kenya estimated that approximately 25% of the variation in development of severe malaria was attributable to host genetics, with only a small percentage (2%) related to hemoglobinopathies [45]. Underlying some of these findings, and increasingly investigated, is the innate host response to

malaria. Innate responses are the major forces combating infection in non-immune individuals, and they can mediate both protection and pathology. Beneficial and detrimental host responses to malaria will be discussed in the following sections, with a focus on the erythrocytic stage due to its relevance to the development of novel adjunctive therapies for severe malaria.

1.2 Innate host responses to malaria

Different strategies have been employed to investigate innate host responses to malaria infection. Cross-sectional or longitudinal studies of malaria-endemic populations allow for correlation of genetic or immunological features with the presence and/or severity of infection. Experimental sporozoite challenges in non-immune volunteers have been useful for examining the early stages of infection, although the ethical necessity to treat upon development of symptoms precludes association of variables with disease severity. Autopsy studies comparing fatal malaria to other causes of death have yielded key insights, but studies are small, artifacts are a concern, and comparisons with mild malaria cases are clearly untenable.

In vitro model systems expose primary host cells, such as peripheral blood mononuclear cells (PBMCs) or endothelial cells, or cell lines to *Plasmodium* isolates or laboratory clones. These co-culture systems are by nature reductionist but are relatively easy to manipulate and permit controlled exposure of host cells to parasites. Murine models have also been used to investigate the host response to malaria, as a variety of *Plasmodium* strains naturally infect rodents. The host-parasite combination is critical for the course and outcome of infection (Table 1.2). While no single model fully recapitulates *P. falciparum* malaria, various aspects of human malaria infection can be probed using the appropriate model. Moreover, different host-parasite pairings are used to dissect host responses to infection [46-48].

Table 1.2: Commonly used murine models of malaria infection.

Plasmodium strain	Mouse strain	Disease phenotype (primary infection)
P. berghei ANKA	Susceptible: C57BL/6, CBA	~90-100% develop fatal CM-like neurological syndrome, die 5-12 days post-infection
	Resistant: BALB/c	~40% develop CM syndrome, others die of hyperparasitemia/anemia 2-3 weeks post-infection
P. berghei K173	C57BL/6	Low dose: CM syndrome High dose: Develop hyperparasitemia/anemia 2-3 weeks post-infection
P. chabaudi chabaudi	Susceptible: A/J, BALB/c	Rapid and uncontrolled parasite replication leading to death 7-10 days post-infection
	Resistant: C57BL/6	Self-resolving parasitemia, with minor recrudescences until ~40 days
P. yoelii 17XL (lethal)	Susceptible: C57BL/6	Rapid and uncontrolled parasite replication leading to death 7-10 days post-infection
P. yoelii 17XNL (non-lethal)	Resistant: C57BL/6, BALB/c	Self-resolving parasitemia
P. vinckei	Susceptible: C57BL/6, CBA	Rapid and uncontrolled parasite replication leading to death 7-10 days post-infection

1.3 Beneficial innate host responses to malaria

Innate immunity is an evolutionarily ancient and highly conserved system that provides a first line of defense against infection (reviewed in [49]). Immune cells such as monocyte/macrophages, natural killer (NK) cells, neutrophils, mast cells, dendritic cells (DCs), and certain T cell subsets, as well as epithelial and endothelial cells, sense the presence of pathogens or the tissue damage they cause, and respond by elaborating chemical signals (cytokines and chemokines). These pro-inflammatory mediators recruit and activate immune cells to destroy pathogens using various effector mechanisms, such as phagocytic clearance, production of reactive oxygen and nitrogen species, and secretion of anti-microbial peptides. Cytokines can also gird host tissue cells against invasion. Activation of platelets and the complement cascade further enhance inflammation and can directly kill microbes. Finally, innate immune responses initiate and shape the development of adaptive immunity [50], which is important for resolving primary infection and protecting against subsequent challenge. As

discussed below, innate host responses to malaria have been found to contribute to control and clearance of infection.

1.3.1 Phagocytic clearance of parasitized erythrocytes

Phagocytosis of parasitized erythrocytes (PEs) and merozoites by myeloid cells is thought to be a primary mechanism for parasite clearance in non-immune individuals. Monocyte/ macrophages and neutrophils have been shown to phagocytose PEs *in vitro* in the absence of malaria-specific antibodies [46,51]. Non-opsonic uptake of mature-stage PEs by macrophages is largely mediated by scavenger receptor CD36 [52,53], likely via engagement of parasite proteins exported to the RBC membrane such as *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) [54]. Despite immense sequence diversity in the PfEMP-1 family of immunogenic variant surface antigens, all *P. falciparum* isolates – except pregnancy-associated isolates – can bind CD36 [55] and thus are expected to be susceptible to clearance by this pathway. Ring-stage PEs can also be phagocytosed in a CD36-dependent manner, though less efficiently [56]. As a result of oxidative damage to RBC membrane proteins during parasitization, ring-stage PEs can be opsonized by complement and autologous natural IgG, and internalized via complement and Fc receptors [57,58].

PEs are effectively killed in the phagolysosome [59], but hemozoin – an insoluble heme polymer produced by parasites to detoxify heme released during hemoglobin utilization – cannot be degraded and persists in the cell. In infected humans and mice, hemozoin-laden myeloid cells are abundant in the blood, liver, and particularly the spleen, which is thought to be the primary site of PE clearance [60].

The importance of innate phagocytic clearance in controlling malaria infection has been suggested by studies of genetic loci associated with resistance to severe malaria. While hemoglobinopathies may exert their protective effects by several mechanisms, it has been shown that ring-parasitized RBCs from affected donors were more efficiently phagocytosed via the innate opsonic route compared to normal infected RBCs, presumably due to increased oxidative damage to the RBC membrane [57,61,62]. A loss of function mutation in Fc gamma receptor IIb, which normally inhibits IgG-mediated phagocytosis and inflammatory responses, was associated with protection from severe malaria in Kenyan children [63]. This variant enhanced opsonic PE internalization by macrophages *in vitro* and reduced parasitemia in the *Plasmodium chabaudi* murine model [64]. These data suggest that increased PE phagocytosis decreases the risk of severe malaria by controlling parasite burden.

Parasite clearance by macrophages has been further explored using the *P. chabaubi* model. Susceptible mouse strains develop fulminant parasitemia and succumb to infection, while resistant strains resolve infection due to a combination of innate immune and Th1 $CD4^+$ T cell responses. Peritoneal macrophages isolated from resistant mice at the peak of infection showed greater phagocytosis of PEs than those from susceptible mice, and this was dependent on macrophage activation by the cytokine interferon-gamma (IFNγ) [46]. Macrophage depletion in resistant mice using silica delayed parasite clearance, resulting in hyperparasitemia and increased mortality [65]. Blockade of scavenger receptors with polyinosinic acid in resistant mice reduced merozoite phagocytosis *in vitro* and increased parasitemia *in vivo* [46], and deletion of CD36 had similar effects, suggesting that impaired phagocytic capacity increases parasite burdens – although altered inflammatory responses may have also played a role in these models [66,67]. Recently, recruitment of a $CD11b^{hi}Ly6C^+$ monocyte population from bone marrow to spleen was found to be critical for resolution of *P. chabaudi* infection, and showed

extensive phagocytic activity towards PEs *in vitro* and *in vivo* [68]. Macrophages have also been found to control parasitemia during non-lethal *P. yoelii* infection [69].

Thus, innate clearance of PEs by phagocytes has been correlated with control of malaria infection in humans and mice. It is evident from the above discussion that there is a close relationship between phagocytosis and inflammation, and indeed inflammation has been found to play an important role in the innate immune response to malaria.

1.3.2 Innate inflammatory responses to malaria infection

Studies in semi-immune populations and experimental vaccine trials have demonstrated that a robust T-helper cell-1 (TH1) response is important for control of parasite burden. Canonical TH1 cytokine IFNγ, which is induced by interleukin-12 (IL-12) and regulates various immune pathways, appears to be a critical mediator. Protection from clinical malaria is correlated with IFNγ production upon *ex vivo* PBMC stimulation with *P. falciparum* [29,70-73]. While memory T cells are likely the major source of IFNγ in malaria-infected semi-immune individuals, IFNγ can also be produced by innate immune cells and exerts effector functions that are highly relevant for non-immune individuals, such as enhanced PE phagocytosis [46,74]. Exposure of malaria-naive PBMCs to *P. falciparum* induces early IFNγ production in NK cells, gamma delta T cells, and (cross-reactive) alpha beta T cells in an IL-12-dependent manner [75,76]. There is considerable inter-individual variation in this response [77], and a study of experimental sporozoite challenge in malaria-naive volunteers demonstrated that early and robust IFNγ responses correlated with better control of parasitemia [78]. In African children, severe malaria syndromes have been associated with low plasma IL-12 and reduced capacity for *ex vivo* IL-12 production [79,80], as well as promoter variants that reduce transcription of IFNγ or IL-12 p40 [81,82]. This suggests that robust TH1 responses control infection and limit disease severity.

Murine malaria studies have causally implicated innate inflammatory responses, and IFNγ in particular, in control of parasite burden. In the *P. chabaudi* model, deletion of IFNγ or its receptor in resistant mice led to impaired cytokine responses, uncontrolled parasitemia and increased mortality [83,84]. Deficiency in the IL-12 p40 subunit in resistant mice reduced IFNγ induction and increased mortality [85], while administration of recombinant IL-12 to susceptible mice enhanced IFNγ production, parasite control, and survival [86]. IFNγ has been implicated in control of parasitemia and survival in most [87,88] although not all [69] murine models.

Another cytokine implicated in control of parasite replication is TNF. Produced early in infections by monocyte/macrophages, TNF promotes macrophage growth/differentiation and initiates inflammatory cascades. PBMC TNF responses to *P. falciparum ex vivo* were shown to correlate with protection from clinical malaria in a prospective study of children in Papua New Guinea [89]. TNF enhanced PE killing by phagocytes *in vitro* [90], and administration of exogenous TNF reduced parasitemia in *P. chabaudi* and *P. yoelii* infections [88,91]. The role of endogenous TNF during *P. chabaudi* infection is controversial, likely due to incompletely back-crossed deletion strains and the proximity of the *TNF* gene to the major histocompatibility complex (MHC) locus (reviewed in [92]). Careful studies have shown that deletion of TNF or the closely related lymphotoxin-alpha (LTα), which also signals through the TNF receptors, did not affect peak parasitemia but prolonged the period of parasite recrudescence [93].

A role for inflammation in control of malaria infection is further supported by studies of immunoregulatory mechanisms. In self-resolving infections in mice, interleukin-10 (IL-10) and/or transforming growth factor-β (TGF-β) usually increase as parasitemia declines in order to prevent immunopathology [94,95]. However, the timing of this response is critical. Lethal

models of *P. yoelii* and *P. chabaudi* are characterized by early production of TGF-β, low IFNγ, and uncontrolled parasitemia. Inhibition of TGF-β in these models enhanced pro-inflammatory responses, reduced parasitemia, and improved survival, while continuous administration of TGF-β rendered resistant mice susceptible to infection [96,97]. During experimental sporozoite challenge in human subjects, a burst of plasma TGF-β was observed in some individuals at the time of parasite emergence into the blood. This response correlated with reduced plasma levels of pro-inflammatory cytokines, increased rates of parasite replication, and an elevated proportion of T regulatory cells (Tregs) [98]. Tregs can be induced by TGF-β and were found to be generated during co-culture of *P. falciparum* with malaria-naive PBMCs [99]. In some studies, increased Treg levels and activity have been associated with susceptibility to clinical and severe malaria in humans and murine models, although this is controversial (reviewed in [100]).

In summary, human and murine studies indicate that early and robust inflammatory responses are important for control of parasite replication and protection from severe malaria.

1.3.3 Other beneficial innate responses to malaria

Other innate immune components contribute to control of malaria infection, such as PE destruction by platelets [101]. Parasites are also sensitive to oxidative stress: administration of pro-oxidants reduced parasitemia in *Plasmodium vinckei* infection [102]. Whether phagocyte production of reactive oxygen species (ROS) and nitric oxide (NO) contributes to control of parasite replication is controversial. Phagocyte-derived ROS and NO were shown to kill parasites *in vitro* [103,104], and *ex vivo* neutrophil ROS production inversely correlated with parasite clearance time in Gabonese children [105]. While NO is induced during *P. chaubaudi* infection of resistant mice, most studies have reported that blockade of NO synthases did not

affect parasitemia [106,107]. Similarly, deficiency in NADPH oxidase activity combined with an NO synthase inhibitor did not affect blood-stage infection in a variety of murine models [108], suggesting that phagocyte-derived ROS is not critical for parasite control. Although NO does not appear to be parasiticidal *in vivo*, it has been shown to protect against severe malaria by maintaining normal endothelial function [109,110].

1.4 Pathological innate host responses to malaria

While innate host responses are critical for defense against pathogens in non-immune individuals, they also have the potential to mediate pathology. Strategies used to contain pathogens are by nature harsh and destructive, and some collateral damage to the host is to be expected. However, if anti-microbial responses are excessive, prolonged, or dysregulated, at inappropriate locations or without eventual downregulation, excessive morbidity or even mortality may result. Evidence for contribution of host responses to CM and SMA will be considered separately due to their distinct pathogeneses.

1.4.1 Cerebral malaria – The sequestration hypothesis

Early autopsies of *P. falciparum* CM fatalities revealed remarkable accumulation of PEs in brain capillaries and venules. Autopsy series of Southeast Asian adults reprised this finding, and demonstrated specific interaction between PEs and endothelial cells using electron microscopy [111-114]. PE accumulation was found to be more extensive in fatalities due to CM compared to other severe malaria syndromes, and in CM cases, PE density was greater in the brain than other organs [111,113,115,116]. Other noted features included petechial hemorrhages and “ring hemorrhages” consisting of a necrotic vessel core packed with PEs and surrounded by extravasating blood cells [113,114]. While frank cerebral edema is infrequently observed in CM

patients, focal alterations to the blood-brain barrier (BBB) and perivascular edema have been reported [115,117]. Neuronal injury has also been noted [118,119].

These studies engendered the "sequestration hypothesis" of CM pathophysiology. Mature forms of *P. falciparum* bind to endothelial cells in small vessels, a process called sequestration. This interaction is mediated by the binding of PfEMP-1 or other exported parasite proteins on the PE surface to endothelial host receptors, including CD36, intercellular adhesion molcule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin [120-122]. Sequestration occurs widely in the vasculature of the skin and other organs [123], and is believed to promote parasite fitness by preventing splenic clearance. However, heavy PE sequestration in the brain is thought to cause mechanical obstruction of blood flow and hypoxia, as well as metabolic competition between host cells and PEs. Stasis in cerebral vessels may be exacerbated by reduced deformability of PEs and RBCs [124]. Collectively, these events lead to endothelial cell damage, BBB disruption, vessel necrosis, local hemorrhaging and edema, and the neurological manifestations of CM.

In support of the sequestration hypothesis, adult CM patients were found to have increased vascular resistance and reduced oxygen consumption in the brain [125]. Lactate levels in blood and cerebrospinal fluid (CSF) are elevated in CM, indicating a switch to glycolysis, and decline upon emergence from coma [125-127]. Due to their common embryonic origin, the retina has been studied in CM patients as a surrogate for examining the brain. Hemorrhages and PE sequestration in retinal vessels are commonly observed, and fluorescein angiography has revealed regions of non-perfusion, ischemia, and vascular leak [128]. Orthogonal polarization spectral imaging of the rectal microcirculation in adult CM patients showed disturbed or blocked capillary flow, which correlated with disease severity and was not present in

uncomplicated malaria [129]. Expression of brain endothelial ICAM-1 was shown to be increased in fatal CM compared to deaths from other causes, and to be associated with sites of PE accumulation [130]. Furthermore, parasite isolates from CM patients had higher affinity for ICAM-1 *ex vivo* than those from mild malaria patients [55], suggesting that ICAM-1-mediated sequestration contributes to CM pathogenesis.

Nevertheless, sequestration has been challenged as the primary factor in CM pathogenesis. The presence of cerebral ICAM-1 upregulation and PE sequestration in fatal cases of non-CM severe malaria [113,131] suggests that mechanical obstruction alone is insufficient for development of the CM syndrome. The absence of persisting neurological deficits in 75% of recovering CM patients is more consistent with reversible metabolic encephalopathy than ischemia, and clinical similarities between coma in malaria and bacterial infections led to proposals for an inflammatory basis for CM pathophysiology [132]. Early autopsy studies in adult CM patients rejected a role for inflammation due to infrequent observation of leukocytes in the brain [111,116]. However, marginating monocytes as well as activated microglia cells have been reported in brain sections from adult [112,114,119,133] and to a greater extent, pediatric CM patients [131,134-136]. In addition, it is possible that systemically released inflammatory mediators can affect brain function (reviewed in [137]). This may explain the recent reports of CM caused by *P. vivax* [18,20], which is not thought to sequester in the brain. The evidence for a pathogenic role of inflammatory and other innate host responses are discussed below.

1.4.2 Dysregulated pro-inflammatory responses in CM pathogenesis

Inflammation was first associated with CM following the implication of TNF in the pathophysiology of endotoxemia and sepsis. Cross-sectional studies in African children showed that serum TNF levels were elevated in severe and cerebral malaria compared to uncomplicated

cases, and correlated with disease severity and risk of fatality independent of parasitemia [138,139]. Subsequent investigations have generally shown increases in other pro-inflammatory cytokines in the blood of CM patients, including interleukin-1β (IL-1β), IFNγ, and interleukin-6 (IL-6), or an increase in the ratio of pro- to anti-inflammatory cytokines [140-142]. Locally elevated TNF and IL-1β mRNA and protein were found in brain sections from fatal CM cases compared to deaths from SMA or non-cerebral pathologies [131,143].

While correlative, these studies suggested a plausible role for inflammation in CM pathogenesis. While cytokines such as TNF contribute to physiological processes in the central nervous system, excessive or dysregulated cytokine signalling has been associated with neurological disease (reviewed in [144]). Intracerebral overexpression of TNF in mice caused neurological symptoms similar to those of CM, with histopathological evidence of demyelination, macrophage accumulation, and microglial activation [145]. TNF promotes its own synthesis and that of other cytokines [146], suggesting that it could generate a cytokine storm. Of relevance to CM, treatment of endothelial cells with TNF increases permeability – which may permit entry of parasite products or cytokines into brain parenchyma – and enhances adhesion molecule expression [147]. Indeed, TNF stimulation of endothelium was shown to increase PE cytoadherence via adhesion receptor upregulation *in vitro* [120,122], indicating that excessive cytokines may exacerbate sequestration (thus allowing integration and augmentation of these competing hypotheses).

Further support for an inflammatory pathology in CM has come from genetic studies. In African children, single nucleotide polymorphisms (SNPs) in the *tnf* promoter that enhance constitutive and inducible TNF expression were found at higher frequency among CM compared to uncomplicated or SMA patients, with the highest frequency among fatal CM cases [148,149].

These findings have been replicated in studies in Southeast Asia [150,151] but not Papua New Guinea [152]. However, in the latter population, a SNP in the gene encoding Galectin-2, which promotes LTα secretion, was associated with CM versus mild malaria in children [153].

A causal role for pro-inflammatory cytokines has been explored using rodent models of experimental cerebral malaria (ECM), most commonly the *Plasmodium berghei* ANKA (PbA) model (Table 1.2). PbA infection in susceptible strains induces a neurological syndrome characterized by paralysis, ataxia, convulsions, coma, and death (reviewed in [154]). Other signs of severe malaria, such as hypoglycemia and pulmonary edema, are also present. Similar to human CM, ring hemorrhages and activated endothelial cells can be observed on brain histopathology. Cerebral edema is more global and severe in ECM, indicating a greater degree of BBB disruption than in humans. Early studies reported marked sequestration of monocytes and $CD8^+$ T cells in the brain in an ICAM-1 dependent manner [155] but no PE accumulation [156,157], leading some to discount this as a relevant model for human CM. However, as mentioned, monocyte accumulation does occur in the brains of pediatric CM patients. New techniques enabling visualization of parasites in live mice have revealed PE sequestration in the brain during ECM, though to a lesser extent than human CM [158,159]. Thus, while the PbA model does not perfectly recapitulate human CM, it provides a tractable system for identification of pathogenic factors that may be further investigated in humans.

Similar to human CM, ECM-susceptible strains exhibit higher serum levels of pro-inflammatory cytokines than resistant strains [160]. Brain expression of TNF and IFNγ was also elevated in susceptible mice [47], and TNF production was localized to intravascular monocytes, endothelial cells, and activated microglia [161]. Antibody blockade of TNF improved survival in susceptible mice, and correlated with reduced leukocyte accumulation, hemorrhages, and

edema in the brain [160]. TNF was shown to induce brain ICAM-1 upregulation and subsequent leukocyte sequestration via TNF receptor 2 (TNFR2), and TNFR2-deficient mice were protected from ECM [162]. Greater ICAM-1 upregulation on brain endothelium from susceptible versus resistant mice upon *ex vivo* TNF stimulation implied a host genetic component to this pathogenic mechanism [163]. Inhibition or deletion of IFNγ or its receptor also protected against ECM, possibly due to the ability of IFNγ to promote production of TNF and other cytokines [164-166].

These early findings required some revision when the use of knockout strains on clean genetic backgrounds demonstrated that deletion of LTα, but not TNF, improved survival in the PbA model [167]. (The polyclonal antibody used in the initial study likely inhibited both of these related molecules.) Interestingly, both $LT\alpha^{-/-}$ and $TNF^{-/-}$ mice showed reduced ICAM-1 expression and leukocyte accumulation in the brain, indicating the requirement for other events to lead to fatal outcome. In addition to signalling through TNFR2, LTα also activates the lymphotoxin-β receptor, and lymphotoxin-β receptor and TNFR2 were found to play non-redundant roles in ECM pathogenesis [168]. Notably, parasite biomass in the brain was decreased in $LT\alpha^{-/-}$ mice but increased in $TNF^{-/-}$ mice [158], suggesting the importance of PE sequestration in ECM pathology. These LTα-specific effects may ultimately induce detrimental metabolic changes in the brain. Magnetic resonance spectroscopy showed reduced Krebs cycle activity and decreased neurotransmitter levels in infected wild-type and $TNF^{-/-}$ mice, but not $LT\alpha^{-/-}$ or $IFN\gamma^{-/-}$ mice [169] – although it is possible that these alterations represent neuroprotective mechanisms.

Macrophages are thought to be a major cellular source of pro-inflammatory cytokines in ECM, and macrophage depletion early in infection prevented the development of the neurological

syndrome [170]. Depletion of either $CD4^+$ and $CD8^+$ T cells also reduced pro-inflammatory cytokine levels and improved survival [158,165]. In addition, $CD8^+$ T cells directly mediate pathology: malaria-specific αβ-$CD8^+$ cells are primed in the spleen during early infection [158,171], migrate to the brain under the influence of chemokines produced by neural cells [159,172], and damage brain endothelial cells via perforin secretion, leading to increased BBB permeability [173]. Interestingly, deletion of chemokine 10 kDa interferon gamma-induced protein (IP-10) led to retention of malaria-specific T cells in the spleen and better control of parasite burden [159], suggesting that T cell migration to the brain detracts from anti-parasitic activity. However, the relevance of these findings to human CM are unclear, since lymphocytes are not frequently seen in the brain on autopsy.

The balance of pro- to anti-inflammatory cytokines is important in PbA infection. Increased expression of TGFβ in the spleen and IL-10 in spleen and brain have been associated with ECM resistance, and treatment of susceptible mice with recombinant IL-10 reduced ECM incidence and improved survival [47,174]. The role of Tregs in PbA infection is controversial, likely due to the use of Treg depletion strategies that also target activated T cells. A recent study employing more specific approaches indicated that Tregs exert protection against ECM [175], consistent with the known immunopathology of the model.

It should be noted that, as with the blood-stage models discussed above, an early inflammatory response appears to be protective in PbA infection. Co-infection of ECM-susceptible mice with PbA and high-dose *P. berghei* K173 strain, which induces IFNγ on Day 1 post-infection, led to protection from ECM [87]. Administration of low dose TNF early in infection suppressed parasitemia and reduced the incidence of ECM, while a higher dose or later administration promoted progression to ECM [170]. Thus, ECM-susceptible mice seem to lack an early

balanced inflammatory response to control initial infection, and instead mount a delayed, excessive response that contributes to cerebral pathology via BBB disruption and neuronal damage. Despite some differences between human CM and the ECM model, the development of human CM may proceed similarly.

While much research has focused on canonical cytokines, a number of novel mediators have been shown to contribute to CM/ECM pathogenesis.

Complement system: C5a. Activation of the complement cascade can occur through several pathways. Most converge with the cleavage of factor C3, which initiates production of effectors with pro-inflammatory or cell-lytic properties. C5a is generated by cleavage of C5 and is a potent anaphylatoxin that has been implicated in the pathophysiology of sepsis (reviewed in [176]). Complement consumption and plasma levels of C5a are increased in CM compared to uncomplicated malaria ([177,178], and unpublished observations). Blockade of C5a or its receptor during PbA infection prolonged survival in ECM-susceptible mice [179]. *In vitro*, C5a synergized with stimulatory parasite components to amplify PBMC pro-inflammatory responses to *P. falciparum* [180], suggesting that it contributes to cytokine dysregulation in CM.

Histamine. Histamine is a biogenic amine produced by mast cells, basophils, and some neurons. Histamine modulates inflammation during infectious and allergic responses, and acts as a neurotransmitter regulating behaviour and memory [181]. Plasma histamine was shown to be elevated in uncomplicated and severe malaria in humans [182]. During PbA-induced ECM, plasma and brain histamine levels were increased, and mice deficient in Histamine Receptor-1 or -2 or the histamine biosynthetic enzyme were protected against ECM [183]. Protection was associated with reduced inflammation and decreased leukocyte and parasite sequestration in the

brain, suggesting that histamine is a critical node in inflammatory cascades in this model. Moreover, local dysregulation of histamine in the brain appears to mediate pathology: mice deficient in Histamine Receptor-3, which negatively regulates histamine expression by histaminergic neurons, showed earlier development of ECM and death [184]. Interestingly, *P. falciparum* secretes a homolog of human histamine-releasing factor [185]; this suggests that histamine release is of benefit to the parasite, but may cause host immunopathology if produced in excess.

Heme oxygenase-1 and carbon monoxide. Upon schizont rupture, free heme is released into the bloodstream, and can mediate damage through its potent oxidizing and inflammatory properties. The heme oxygenase-1 (HO-1) enzyme is induced by free heme and other factors, and degrades heme into iron, biliverdin, and carbon monoxide (CO). Both biliverdin and CO possess anti-inflammatory properties [186]. During PbA infection, HO-1 was found to be more highly expressed in the brains of ECM-resistant versus susceptible mice. HO-1 deletion in resistant mice increased inflammation and ECM incidence, while HO-1 induction or CO gas in susceptible mice was protective [187]. The anti-inflammatory effects of CO were attributed to its ability to bind free hemoglobin, thus preventing hemoglobin oxidation and degradation into heme. In humans, HO-1 was strongly induced in the brains of pediatric fatal CM cases compared to other causes of death [188], which may represent an attempt by the host to dampen cerebral inflammation. Seemingly at odds with the murine data, *Hmox1* promoter variants that enhance HO-1 expression were more prevalent in CM versus uncomplicated malaria [189]. This discrepancy may be explained by a report that HO-1 induction in the liver by murine *Plasmodium* strains facilitated establishment of liver-stage infection and increased parasite liver load, which has been correlated with disease severity [190]. Thus, HO-1 may confer protection or susceptibility to CM in a time- and tissue-dependent manner.

Reactive oxygen species. ROS can mediate direct neural damage and have been proposed to contribute to CM pathogenesis (reviewed in [191]). Lipid peroxidation products were found to be elevated in the blood of CM versus mild malaria patients [192]. In the PbA model, administration of anti-oxidants or anti-oxidant enzymes (e.g. catalase) were protective against ECM [193]. However, phagocytes did not appear to be the source of the ROS, as genetic deficiency in NADPH oxidase assembly did not alter brain pathology or survival [194].

1.4.3 Other host responses implicated in CM pathogenesis

1.4.3.1 Endothelial activation

As discussed, upregulation of cellular adhesion molecules on brain endothelial cells by pro-inflammatory cytokines is thought to promote CM via PE sequestration. Direct contact with PEs and parasite soluble factors also enhanced endothelial ICAM-1 expression [195], highlighting the self-amplifying nature of PE sequestration. *P. falciparum* was shown to profoundly affect transcription and protein production in primary brain microvascular endothelial cells, including NFκB activation and cytokine/chemokine secretion [196]. Thus, sequestration and inflammation fuel each other via activation of endothelial cells.

Endothelial activation may also contribute to CM pathogenesis via increased vascular permeability. *In vitro*, both cytokines [147] and *P. falciparum* PEs [197] reduce the integrity of monolayers of primary human brain endothelium. In brain sections of CM patients, endothelial tight junction proteins were decreased and/or redistributed, with detection of perivascular fibrinogen indicating BBB leak [117,134]. Similar changes were observed in the PbA model of ECM [198]. Whether increased permeability is a regulated response or secondary to endothelial

dysfunction and death is not clear. It is also unknown to what extent BBB leakage contributes to CM pathogenesis. However, it seems plausible that extravasation of plasma proteins and parasite components into brain parenchyma would contribute to glial cell activation, thus augmenting local inflammation.

Recently, the exocytosis of Weibel-Palade bodies (WPB) by endothelial cells has been investigated as a potential contributor to CM pathogenesis. WPB are intracellular vesicles that store proteins such as von Willebrand factor (vWF), Angiopoietin-2 (ANG-2), and P-selectin, and are exocytosed upon endothelial activation [199]. Plasma levels of vWF and ANG-2 correlate with malaria severity and mortality [200-202]. *In vitro*, vWF can tether PEs to endothelial cells via platelets [203], thus potentially promoting sequestration. ANG-2 inhibits endothelial quiescence by interfering with the interaction between Angiopoietin-1 (ANG-1) and endothelial TEK Tyrosine Kinase (Tie-2). ANG-2 has been shown to sensitize endothelium to TNF [204] and promote vascular leak [205], and plasma ANG-2 was associated with endothelial dysfunction in adults with CM [200].

1.4.3.2 Apoptosis

Apoptosis in the brain has been investigated as a host process underlying the neurological signs and BBB permeability in CM/ECM. *In vitro*, *P. falciparum* was shown to induce apoptosis in human endothelial cell lines [206,207]. However, these findings were not replicated with primary human brain endothelium [196], potentially reflecting differences in cell type or experimental conditions. In an autopsy study of Vietnamese adults, a greater proportion of CM cases (4/10) versus non-CM malaria cases (1/11) showed positive neuronal staining for activated Caspase-3 [133]. Consistent with the complete neurological recovery of most CM patients, apoptosis was not widespread, though presumably location may be more important than extent.

The role of apoptosis in PbA-induced ECM is better established. Apoptosis-related genes were upregulated in the brains of susceptible versus resistant mice [48]. Brain or retinal sections from mice with ECM showed increased apoptosis in neurons, endothelial cells, astrocytes, and/or intravascular monocytes by TUNEL or activated Caspase-3 staining [48,208-211]. In one study, apoptosis mostly occurred in the brainstem – consistent with ECM symptoms such as ataxia – and correlated with disease severity [208]. Perforin secretion by sequestering $CD8^+$ T cells was implicated in endothelial cell apoptosis and loss of BBB integrity [209]. Interestingly, mice deficient in Fas or Fas ligand developed neurological symptoms but subsequently recovered, and did not exhibit the neuronal apoptosis present in fatal ECM [210]. These studies suggest that endothelial cell apoptosis promotes ECM progression by disrupting the BBB, while neuronal apoptosis directly contributes to death [210].

1.4.3.3 Hemostasis and coagulation

Thrombocytopenia and activation of the coagulation cascade are common in *P. falciparum* infection and correlate with disease severity [212]. While not observed in adult CM [111], platelet accumulation and microthrombi in the brain are increased in pediatric CM fatalities compared to SMA or non-malarial encephalopathy [135]. Platelet sequestration in the brain is also present in the PbA model of ECM, and platelet depletion or interventions inhibiting platelet adhesion or function have been shown to protect against ECM [213-215]. Platelets may promote CM by tethering CD36-binding PEs to brain endothelium, which has low expression of CD36 [216]. Platelets also release pro-inflammatory mediators (e.g., Platelet Factor 4) that can contribute to ECM pathology [214], and platelets and cytokines synergistically induced endothelial monolayer permeability *in vitro* [217]. Thus, platelet sequestration in the brain may augment a number of pathogenic processes linked to CM progression. It has been suggested that

extensive coagulation occurs in CM and may contribute to pathology, but that this is not recognized clinically or histopathologically due to compensatory fibrinolysis [218]. *P. falciparum* PEs were recently shown to upregulate expression of tissue factor on endothelial cells *in vitro* and initiate coagulation, and brain endothelial tissue factor expression was elevated in fatal malaria versus non-malaria [219]. The significance of these finding requires further investigation.

In summary, innate host responses involving a variety of cell types and soluble mediators have been shown to contribute to the pathophysiology of CM/ECM.

1.4.4 Host responses contributing to severe malarial anemia

There has been less investigation into the pathogenesis of SMA due to its perceived lower CFR, the high frequency of co-morbidities that complicate disease classification, and the lack of appropriate murine models. Blood transfusions reduce mortality only among SMA patients with very low hemoglobin and/or respiratory distress, and are most effective when given shortly after admission [220,221]. In many African settings, the blood supply is unreliable and inadequately screened for blood-borne pathogens, leading to delays and substantial risk of infection and transfusion reactions. Safer, more easily administered therapies would be useful for treating very severe cases as well as preventing progression of clinically stable SMA [23].

SMA results from both increased RBC destruction and decreased RBC production. Parasitization causes RBC loss, but SMA can occur at relatively low parasite burden [34]. Destruction of uninfected RBCs (uRBCs) is increased in acute malaria [222], and is estimated to account for 90% of RBC loss [223,224]. In addition, dyserythropoeisis has been observed in bone marrow aspirates of SMA patients [22,225] despite adequate erythropoietin production

[226]. While hemozoin can directly cause hemolysis [227] and erythropoietic suppression [228], host pathways also appear to contribute to anemia, as discussed below.

Existing rodent models are not ideal for investigating SMA: anemia is acute and primarily due to parasitization, and compensatory erythropoiesis can occur in the spleen. However, erythropoietic defects in bone marrow do occur in murine malaria (reviewed in [10]). Recently, repeated *P. berghei* infection and cure was reported to induce severe anemia at low parasitemia [229], thus providing a better model of human SMA.

1.4.4.1 Phagocytosis of uRBCs

There is evidence that loss of uRBC in SMA occurs via phagocytic clearance. Myeloid cells in bone marrow containing uRBCs and erythroblasts have been reported in anemic malaria patients and *P. berghei* infected rats [22,225,230]. RBCs from children with SMA were more avidly phagocytosed *ex vivo* than those from children with mild or asymptomatic malaria, and this was associated with increased bound IgG and lower levels of complement regulatory proteins [231]. Immunologic opsonization of uRBCs may arise in a number of ways. Parasite proteins are deposited on the surface of uRBCs in *P. falciparum* culture, likely as a result of aborted invasions; these proteins can be recognized by antibodies in the sera of SMA patients, leading to complement deposition and erythrophagocytosis [232]. Hemozoin can also induce complement opsonization of uRBCs [233]. It is possible that uRBC clearance is promoted by oxidative damage to the cell membrane. Malarial anemia has been associated with increased leukocyte ROS production, greater oxidative damage to RBCs, and decreased RBC deformability [234-236], which may lead to increased retention and clearance in the spleen.

1.4.4.2 Inflammation

As in CM, cross-sectional studies have demonstrated dysregulated cytokines in children with SMA compared to mild malaria. Low plasma IL-10 or high TNF/IL-10 ratios are consistently observed, and IL-10 is further depressed with concomitant respiratory distress [37,80,141,237-239]. An IL-10 promoter haplotype was associated with higher IL-10 levels and protection from SMA in a cohort of Kenyan children [240]. Decreased IL-12 levels have also been linked to SMA in African children [241].

The ability of IL-10 to suppress pro-inflammatory cytokine production by PBMCs in response to *P. falciparum* [242] suggests that unrestrained inflammation contributes to SMA pathogenesis. TNF suppresses erythropoiesis *in vitro* [243] and in chronically-exposed mice [244]. Anti-serum blockade of TNF partially alleviated ineffective erythropoiesis in *P. berghei* infection [245] (although in hindsight it is possible that LTα mediated the pathology). The cytokine macrophage migration inhibitory factor (MIF) was recently shown to inhibit erythroid development and contribute to anemia in the *P. chabaudi* model [246]. Paradoxically, lower MIF levels are associated with human SMA [247,248], potentially due to confounding by production of MIF homologues by *Plasmodium* [249]. Consistent with observations in human studies, IL-12 promoted erythropoiesis *in vitro*, and in the *P. chabaudi* model, high IL-12 production correlated with resistance to severe anemia and its depletion impaired erythropoiesis [250].

In addition to suppressing RBC production, pro-inflammatory cytokines may enhance RBC destruction via macrophage activation, although evidence is indirect. Non-lethal *P. yoelii* infection reduced survival of labelled RBCs [251], and *P. vinckei*-infected mice injected with

TNF showed increased phagocytosis of RBCs and erythroblasts in bone marrow [252]. In the low-parasite burden SMA model, accelerated turnover of uninfected RBCs accounted for the majority of the anemia, and this was alleviated by depletion of macrophages or $CD4^+$ T cells [229].

1.4.4.3 Other host responses contributing to SMA

In addition to promoting erythrophagocytosis, complement deposition on uninfected RBCs may induce intravascular hemolysis by the membrane attack complex [231,232]. Malaria-induced apoptosis of uninfected RBCs and erythroid precursors has recently been demonstrated *in vitro* and in murine models [253,254], although careful validation of these studies is required due to risk of artifact [255].

1.5 Inflammatory pathways as targets for adjunctive therapy in severe malaria

Based on the implication of pro-inflammatory pathways in severe malaria pathogenesis, anti-inflammatory interventions have been tested in randomized controlled trials for their effectiveness in severe (usually cerebral) malaria. These include corticosteroids [256,257], anti-TNF antibodies [258,259], pentoxyfylline, which inhibits TNF secretion [260-263], and anti-oxidant N-acetylcysteine [264]. Despite success in preclinical studies [160,265], none have conclusively improved survival in humans, and some interventions actually worsened outcome [256,258]. While these findings could be interpreted as evidence against a role for inflammation in CM pathogenesis, it is critical to consider issues of timing, dose, underpowered study designs [266], or misleading results from murine models (i.e., TNF versus LTα in ECM [167]). It is also

possible that inhibition of a single cytokine in a complex network may be insufficient to alter outcome.

Efforts to develop more effective anti-inflammatory therapies have persisted. Modulation of recently implicated pathways – e.g. histamine, HO-1 – has been proposed [184,267]. There is increasing focus on identifying the molecular stimuli and sensors that induce inflammation, so that interventions can target initiating events.

1.6 Host pattern recognition receptors that initiate inflammatory responses

Detection of pathogens by the innate immune system relies on germline-encoded receptors that recognize highly conserved pathogen-associated molecular patterns (PAMPs) (reviewed in [268,269]). These pattern recognition receptors (PRRs) mediate critical phagocytic and inflammatory responses to infection. PRRs that participate in the clearance of pathogens and their products include membrane-bound receptors such as the mannose receptor and Dectin-1, as well as secreted proteins that opsonize microbes to promote complement deposition. The discovery of mammalian Toll-like receptors (TLRs) in the late 1990s confirmed theories that the innate immune system can mount tailored inflammatory responses upon sensing foreign invaders, or more broadly, danger signals [270]. Indeed, TLRs and other PRR families (Fig. 1.2) can recognize both pathogenic and endogenous ligands, and have been investigated for their roles in host defense, homeostasis, and pathobiology. These receptors are briefly discussed below with respect to the pro-inflammatory responses they mediate.

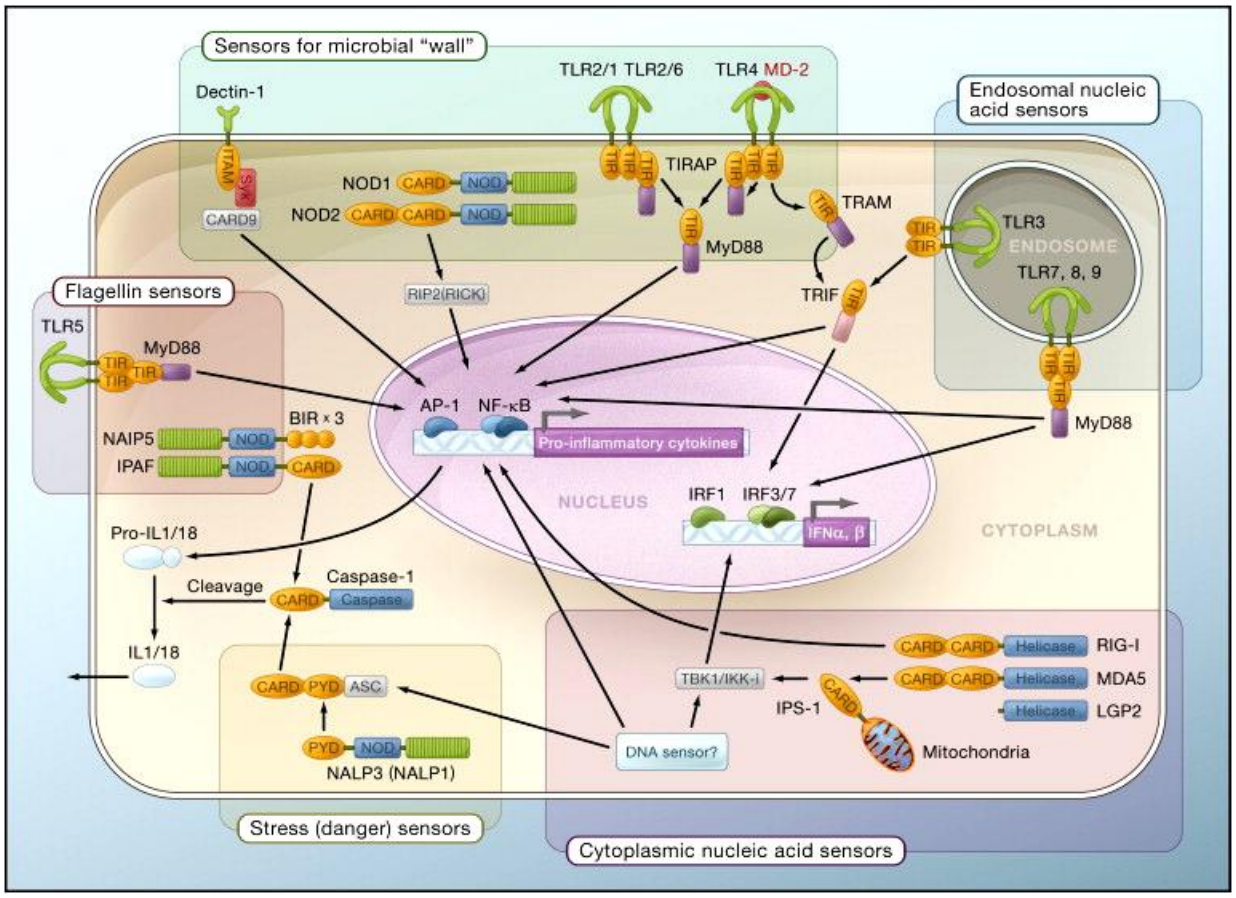


Fig. 1.2: Major families of host pattern recognition receptors contributing to innate inflammatory responses. Reproduced with permission from ©Elsvier, ref. [269].

1.6.1 Toll-like receptors (TLR)

To date, 10 TLRs have been identified in humans and 12 in mice (reviewed in [271]). These transmembrane receptors consist of an extracellular domain containing leucine-rich repeats and a cytoplasmic Toll/IL-1R homology (TIR) domain. TLRs are widely expressed in a cell type-specific manner, and each TLR recognizes a distinct set of ligands. TLR1, 2, 4, 5, and 6 can be detected on the cell surface and intracellular compartments. TLR4 senses lipopolysaccharide (LPS) and other microbial products, as well as "danger signals" released from damaged or dying cells, such as heat-shock proteins and High-mobility group protein B1 (HMGB1). TLR5 detects flagellin, while TLR2 forms heterodimers with TLR1 or TLR6 to recognize ligands such as lipoproteins, lipoteichoic acid (LTA), and yeast cell wall. TLR3, 7, 8, and 9 sense nucleic acids

in endosomal structures: TLR9 recognizes CpG DNA while the others detect RNA. TLRs have been shown to cooperate with other receptors for ligand recognition. For example, a complex of CD14, LBP, MD-2, and other receptors is required to present LPS to TLR4.

Ligand binding induces TLR dimerization and recruitment of adaptor proteins that initiate signalling pathways. The two major TLR adaptors are myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor inducing IFN-β (TRIF). MyD88 binds to all TLRs (except TLR3) via a homotypic TIR domain interaction, and activates IL-1R-associated kinase-4 (IRAK-4). An additional adapter, MyD88 adaptor-like (MAL), is required for MyD88-dependent signalling downstream of TLR2 and TLR4 [272]. The MyD88 signalling cascade leads to activation of MAPKs and NF-κB, transcription of pro-inflammatory cytokines and chemokines, and generation of anti-microbial effectors such as NO. The TRIF-dependent pathway is unique to TLR3 and TLR4, and fires from the endosomal compartment [273]. Assisted by TRIF-related adaptor protein (TRAM), TRIF activates IRF3 and transcription of Type I IFNs, which are important for control of viral infection. Notably, TLR7 and TLR9, which are critical viral sensors, can also activate Type I IFNs via the MyD88 pathway. This and other examples reflect how the TLR system is tailored to initiate pathogen-appropriate responses, both with respect to activating innate immunity for control of primary infection and shaping subsequent adaptive responses. For instance, specific combinations of TLR agonists synergistically regulate transcriptional responses in DCs, suggesting a "combinatorial code" that discriminates between microbes [274].

In numerous infectious disease models, TLRs have been shown to be important for innate and adaptive immune responses to variety of pathogens (reviewed in [50,275]). Not surprisingly, pathogen strategies to evade or inactivate TLR signalling have been described [276-278].

Although TLR stimulation activates regulatory mechanisms to limit excessive inflammation – e.g., production of negative regulators such as A20 and IL-10, induction of TLR tolerance, and Treg activation [279] – aberrant TLR signalling has been implicated in the pathogenesis of acute and chronic inflammatory conditions such as sepsis, ischemia-reperfusion injury, arthritis, and lupus [271]. Both foreign and endogenous TLR ligands can contribute to pathology. For example, in endotoxemia models, excessive TLR4 activation is effected by bacterial LPS and host protein HMGB1 [280-282].

1.6.2 Nod-like receptors (NLR)

NLRs are cytosolic PRRs and have been implicated in inflammatory responses to pathogens and environmental hazards. NLR structure consists of a C-terminal LRR domain implicated in ligand sensing, a central nucleotide-binding domain that mediates oligomerization, and a variable N-terminal domain. NLR proteins Nod1 and Nod2 recognize components of bacterial peptidoglycan, and interact with downstream adaptors to activate NF-κB and initiate transcription of pro-inflammatory cytokines [283,284]. Nod proteins can also induce Type 1 IFN responses to bacteria and viruses [285,286]. While intracellular pathogens are well-known to be recognized by Nod proteins, endocytosed peptidoglycan components can also be translocated into the cytosol for recognition, suggesting a mechanism by which these PRRs can sense extracellular bacteria [287].

Other NLRs, such as NLRP3 and IPAF, self-oligomerize when activated and drive assembly of the Caspase-1 inflammasome. This leads to Caspase-1 autoactivation, and maturation and secretion of IL-1β and IL-18 (reviewed in [288]). Other signals (e.g. TLR stimulation) are required for production of the pro-forms of IL-1β and IL-18. IPAF detects flagellin injected into the cytosol by bacterial secretion systems. NLRP3 has been shown to sense intracellular and

extracellular bacteria and viruses, and in some cases the molecular component has been identified. NLRP3 also recognizes host danger signals, including extracellular ATP, elevated glucose levels, fibrillar β-amyloid (fAb), and monosodium urate (MSU) crystals, as well as silica, asbestos, and environmental irritants. Ligand recognition by NLRP3 is not fully understood but appears to be indirect. Potassium efflux, ROS generation, and in the case of large indigestible particles, lysosomal rupture have been implicated [289,290].

Like TLRs, NLRs have been shown to contribute to innate and adaptive immune responses to a variety of pathogens, and dysregulated NLR signalling has been implicated in the pathogenesis of diseases such as atherosclerosis, inflammatory bowel disease, asthma, and gout (reviewed in [291,292]).

1.6.3 Other pattern recognition receptors mediating inflammation

Among other PRRs that initiate inflammatory responses are the C-type lectin receptors (CLRs), which recognize pathogen carbohydrate components, and Rig-like receptors (RLRs), which detect double-stranded RNA in the cytoplasm and induce Type I interferon responses (reviewed in [271]). RLRs have been implicated in host defense against RNA viruses; DNA viruses and intracellular bacteria (due to reverse transcription of microbial DNA) [293]; and extracellular bacteria by an unknown mechanism [294]. Moreover, the AIM2 inflammasome and DAI receptor detect cytoplasmic double-stranded DNA [295,296]. Thus, every pathogen is sensed by multiple host PRRs during infection. Different PRRs may be activated on different cell types, depending on the interaction of each cell type with the microbe (i.e. cell surface versus cytosol, or both). Moreover, numerous co-operative and synergistic interactions between PRRs activated on the same cell have been reported [297], further supporting the concept of a “combinatorial code” that is used by the innate immune system to mount appropriate responses to infection.

1.7 Stimuli and sensors that induce inflammation during malaria infection

Attempts to define the "malaria toxin" that induces inflammatory responses – the parasite equivalent of LPS – have been ongoing for decades. These efforts were complicated by a poor understanding of innate immunity and widespread mycoplasma contamination of *P. falciparum* cultures [298]. The recent explosion of PRR discovery has facilitated research in this field, although not without controversy.

1.7.1 *Plasmodium* glycosylphosphatidylinositols (GPIs)

Protozoa make extensive use of GPIs to anchor membrane proteins [299] (Fig. 1.3). *P. falciparum* GPIs (*Pf*GPIs) are structurally dissimilar to mammalian GPIs and 10-100 times more abundant at the cellular level [300]. Both free and membrane-associated *Pf*GPIs are thought to be released upon schizont rupture. Schofield and colleagues first isolated *Pf*GPIs from *P. falciparum* culture and showed that they stimulated TNF and NO secretion from murine macrophages [301,302]. Studies using highly purified, mycoplasma-free *Pf*GPIs confirmed these results [300]. Purified *Pf*GPIs were also found to promote PE cytoadhesion to endothelial cells via ICAM-1 upregulation [303]. Injection of *Pf*GPIs into mice recapitulated signs of malaria infection, including pyrexia, hypothermia and hypoglycemia. TNF blockade reversed many of these effects, thus linking GPIs, inflammation, and disease manifestations [301]. Notably, immunization with the *Pf*GPI glycan core prevented the cerebral, pulmonary, and systemic manifestations of PbA challenge. Anti-GPI antibodies from these mice inhibited macrophage TNF responses to *P. falciparum* extracts [304]. Thus, *Pf*GPIs appeared to be critical inducers of inflammatory pathology in malaria infection.

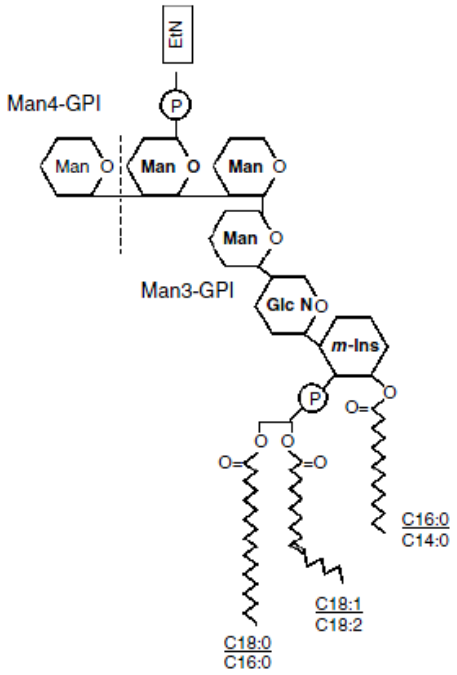


Fig. 1.3: Structure of *Plasmodium falciparum* glycosylphosphatidylinositols (GPIs). EtN, ethanolamine; P, phosphate; Man, mannose; GlcN, unacetylated glucosamine; m-Ins, myo-Inositol; Cn:n, number of saturated/unsaturated carbon molecules in the lipid chain. Fatty acid substituents are heterogeneous; the dominant form at each position is indicated above the line. The glycan core consists of the EtN-P group, 3 inner Man residues, and Glc N component. Reproduced with permission from ©Cambridge University Press, ref. [305].

Human studies subsequently showed that levels and persistence of serum anti-GPI antibodies positively correlated with age in malaria-endemic areas [300,306,307], suggesting that GPI neutralization may contribute to protection from severe malaria in semi-immune adults. In high transmission regions, anti-GPI titres were inversely associated with signs of malaria infection [300] and parasite density in asymptomatic infections [306]. Consistent with findings in mice [304], one study found lower levels of anti-GPI antibodies in CM compared to mild malaria [308]; however, others did not replicate these findings [307,309]. It should be noted that these studies evaluated titres during infection rather than prior to infection (the latter may be more relevant to protection from severe malaria), and did not assess the GPI neutralizing ability of the antibodies. Recently, it was shown that anti-GPI antibodies in the serum of Gambian adults strongly suppressed macrophage TNF responses to purified *Pf*GPI, but had a lesser effect on

responses to *P. falciparum* extracts, indicating the presence of other stimulatory parasite components in this model [310]. Thus, *Pf*GPIs likely induce inflammation in human malaria infection, but their relative importance in these responses and role in severe malaria pathogenesis are unclear.

Similar to GPIs from *Trypanosoma cruzi*, *Pf*GPIs were shown to induce pro-inflammatory cytokines via TLR2, with a lesser contribution from TLR4 [311]. Interestingly, an earlier report demonstrated that HEK293T cells transfected with TLR2 (but not other TLRs) responded to *P. falciparum* cultures [312]. Murine models have been used to investigate roles for TLR2 and TLR4 in protective inflammatory responses during blood-stage malaria. Deletion of TLR2 or TLR4 in resistant C57BL/6 mice did not alter disease parameters during *P. chabaudi* infection. *Myd88*$^{-/-}$ mice showed reduced symptoms, plasma IFNγ, and CD4^{+} T cell activation compared to wild-type mice, but this did not translate into differences in control of parasitemia [313,314]. Infection of *MyD88*$^{-/-}$ mice with non-lethal *P. yoelii* led to lower IL-12 levels, increased parasitemia and decreased survival. However, these effects were attributable to the IL-1 and IL-18 pathways, which also signal through MyD88, rather than TLRs [315].

Data from the PbA model of ECM have been contentious. An early report demonstrated that *Tlr2*$^{-/-}$, *Tlr9*$^{-/-}$ (discussed below), and *MyD88*$^{-/-}$ mice, but not *Tlr4*$^{-/-}$ mice, were relatively protected from ECM and death [316]. Others did not replicate these findings [317,318]. Another study reported protection in *MyD88*$^{-/-}$ and *Tlr9*$^{-/-}$ but not *Tlr2*$^{-/-}$ mice (and excluded contributions of IL-1 and IL-18) [319]. This group also demonstrated that MyD88 deletion in resistant BALB/c mice rendered them susceptible to ECM. Infected *MyD88*$^{-/-}$ mice had reduced pro-inflammatory cytokines in plasma and brain, and in C57BL/6 mice, MyD88 deletion led to increased numbers of Tregs. Reasons for the discrepancies between studies are unclear, but may

relate to differences in PbA clone or dose [275,320]. On balance, however, TLRs appear to mediate inflammation during PbA infection. TLR2/4 activation by GPIs may not be a major contributor in this model (although it should be noted that GPIs from murine malaria strains have not been formally shown to activate these TLRs).

In support of a role for *Pf*GPIs in malaria-induced inflammation, SNPs related to TLR2 and TLR4 pathways have been associated with malaria manifestations in human populations. Some studies suggest that impairments in these pathways predispose to disease manifestations: TLR1 variants that decrease TLR2-mediated signalling were associated with clinical malaria in Brazil [321] and maternal anemia during pregnancy-associated malaria [322]. On the other hand, heterozygosity for a SNP in TLR2/4 adaptor protein MAL, shown to attenuate TLR2 signalling, was associated with protection from mild and severe malaria [323]. Similarly, TLR4 SNPs linked to enhanced cytokine production [324] were more common in severe malaria cases versus healthy children, and were associated with poor outcomes in pregnancy-associated malaria [325,326]. The effects of these TLR variants on malaria infection are not completely understood, especially considering the complex role of inflammation in severe disease. Overall, however, these data suggest that TLR2 and TLR4 – and thus potentially *Pf*GPI – may play a role in determining susceptibility to mild and severe malaria.

1.7.2 Hemozoin

Hemozoin, the crystalline heme polymer generated by the parasite during intraerythrocytic growth, has been investigated for its inflammatory properties. Hemozoin purified from *P. falciparum* cultures (*Pf*Hz) is avidly phagocytosed by myeloid cells and has various effects on monocytes, including impairment of repeated phagocytosis and long-term suppression of the oxidative burst [327]. *Pf*Hz has also been found to induce cytokines and chemokines by

monocytes and DCs, including TNF, IL-1β, and IL-10 [241,328-331]. Hemozoin can have cell type-specific effects: *Pf*Hz suppressed monocyte IL-12 production [241] but induced DC IL-12 production [332].

Some reports have attributed the immunostimulatory activity of *Pf*Hz to parasite components associated with the Hz crystal, such as proteins [329] or lipids that undergo oxidation in the phagosome [333,334]. Others have found that synthetic β-hematin crystals (sHz) can induce cytokines and chemokines *in vitro* and upon injection into mice [328,335-337], suggesting that the Hz crystal itself can promote inflammation. However, these results are controversial [329,338,339]. Discrepancies may be partly explained by the use of different cell types, experimental protocols, and read-outs. Moreover, different methods of sHz preparation lead to variability in size, shape and crystallinity, and this can affect sHz activity [336,340]. There are also some concerns regarding contaminants in sHz preparations [338].

Recently, Coban and colleagues reported that cytokine responses to purified *Pf*Hz *in vitro* and *in vivo* were mediated by TLR9 [341]. TLR9-stimulatory activity was attributed to the Hz crystal: activity was unaffected by heat or DNase treatment, and *in vivo* injection of sHz recapitulated findings with *Pf*Hz. sHz also appeared to directly bind to TLR9 [340]. However, Jaramillo *et. al.* could not detect TLR9 activation by sHz using transfected cell lines [336]. Parroche *et. al.* reported that their sHz preparation did not induce DC cytokine production, apparently due to absence of contaminants, and that the stimulatory activity of *Pf*Hz was attributable to associated parasite DNA. In this system, the Hz crystal was critical for trafficking the parasite DNA into the endosome for TLR9 recognition [338]. These results have subsequently been contested [339].

Regardless of the precise identity of the TLR9 ligand, TLR9 has been found to contribute to pathology in murine malaria models. As discussed above, *Tlr9*$^{-/-}$ mice were protected from PbA-induced ECM, and an inhibitor of endosomal TLRs (including TLR9) was shown to reduce inflammation and improve survival [342]. While *Tlr9*$^{-/-}$ mice did not have a phenotype in *P. chabaudi* infection [314,343], TLR9 was implicated in "immunological priming" in this model. *P. chabaudi* infection upregulated a variety of IFNγ-inducible genes in splenocytes, including TLRs and downstream signalling molecules, in a TLR9-, MyD88-, and IFNγ-dependent manner; this resulted in hyperresponsiveness to a sub-lethal LPS dose and death in wild-type but not *Tlr9*$^{-/-}$ mice [344]. TLR upregulation and hyperresponsiveness has also been observed in immune cells from *P. falciparum*-infected individuals [344,345]. Thus, TLR9 may contribute to excessive inflammation in severe malaria and/or increase susceptibility of malaria-infected individuals to the effects of bacterial co-infection. Finally, in the lethal *P. yoelii* model, *Tlr9*$^{-/-}$ mice showed increased survival due to reduced TLR9-mediated activation of Tregs by DCs, suggesting that in some contexts TLR9 activation by the parasite may constitute an immune evasion strategy [346].

Different cellular expression patterns of TLR9 in mice and humans does warrant some reservations regarding the above murine data [347]. In human genetic studies, a TLR9 promoter SNP of unknown functional significance was associated with high parasitemia in mild malaria in a Brazilian population [321] and increased risk of low birth weight infants in PAM [326]. Another TLR9 promoter SNP, shown to enhance TLR9 transcription *in vitro*, was linked to low parasitemia in the Brazilian population [321], but lower levels of IFNγ in Ugandan children with CM [348]. Large studies have not detected association of TLR9 variants with pediatric severe malaria, although distant *cis*-acting elements that affect TLR9 expression may be worthwhile to

investigate [349]. Overall, TLR9 appears to contribute to host responses to malaria in mice and in humans.

The similarity of Hz to other particulate NLRP3 agonists (e.g. MSU crystals) led to identification of Hz as a novel activator of NLRP3. *In vitro,* sHz stimulated macrophage IL-1β secretion via NLRP3 and Caspase-1 activation, and this was dependent on sHz phagocytosis, potassium efflux, ROS, and activation of Src kinase Lyn and tyrosine kinase Syk [350,351]. Similarly, neutrophil recruitment upon intraperitoneal injection of sHz into mice was dependent on NLRP3, Caspase-1, and the IL-1 receptor (IL-1R) [350,351]. In PbA infection of ECM-susceptible mice, NLRP3 mRNA was found to be upregulated in brain endothelial cells [352]. Unexpectedly, while *Nlrp3*$^{-/-}$ mice were partially protected in this model, Caspase-1- and IL-1R-deficient mice remained highly susceptible, and IL-1β was undetectable even in wild-type control mice [319,351,352]. In contrast, mice deficient in NLRP3 or IL-1β exhibited slightly lower parasitemia and increased survival in *P. chaubaudi adami* infection [350]. Thus, NRLP3 contributes to severe malaria pathogenesis in murine models, but in some cases this is not critically dependent on the canonical Caspase-1 inflammasome.

1.7.3 *Plasmodium* DNA

As discussed above, Parroche et. al. identified parasite DNA, rather than Hz, as the malaria TLR9 agonist that activates immune cells *in vitro* [338]. An earlier report demonstrated that blood-stage schizonts activated plasmacytoid DCs via TLR9 [329]. The active component appeared to be a protein, but it was speculated that a protein-DNA complex may be required for effective TLR9 activation. A recent study systematically evaluated parasite material released upon *P. falciparum* schizont rupture. It was found that only protein-DNA complexes induced DC cytokine production and upregulation of co-stimulation molecules, and this occurred in a

TLR9- and MyD88-dependent manner [339]. The protein component appeared to be required for entry of parasite DNA into endosomes. The authors found no role for *Pf*Hz in their system, and argued that previous findings were artefactual due to contamination of purified *Pf*Hz with mature PEs. Thus, despite controversy regarding its agonist, TLR9 is relatively consistently implicated in the host response to malaria.

1.7.4 Microparticles

Microparticles (MPs) are small (0.1-1 μm) vesicles generated by blebbing of plasma membranes. This occurs physiologically, but is upregulated during cell activation by cytokines or cell death. MPs are characterized by phosphatidylserine exposure and markers from the cell of origin, and can have pro-thrombotic and pro-inflammatory activity. Plasma levels of MPs derived from platelets, RBCs, endothelium, and leukocytes were increased in African children with CM compared to UM or SMA, and declined upon recovery from CM [353,354]. Similar findings have been reported in adult *P. falciparum* infection [355]. PbA-infected mice had elevated plasma MPs, which were shown to promote coagulation and inflammation *ex vivo* [356]. Genetic and pharmacologic manipulations that reduced membrane vesiculation (among other effects) reduced MP generation and inflammation, and protected mice from ECM, [356,357], providing indirect evidence that MPs contribute to ECM pathogenesis.

Purified MPs from PbA-infected mice – but not endotoxemic mice – were found to stimulate macrophage TNF production in a manner dependent on TLR4, MyD88, and to a lesser extent, TLR2 and TLR9 (endotoxin levels in MP preparations were reportedly negligible) [358]. Interestingly, the majority of MPs from heavily PbA-infected mice were found to react with anti-PbA antibodies, indicating their derivation from PEs. Given the implicated TLRs, parasite components in the MPs likely contributed to the observed inflammation (although a strongly

TLR4-activating malaria ligand has yet to be identified). *P. falciparum* PEs were also found to generate MPs, and this increased with parasite maturation [355]. The role of MPs in pro-inflammatory responses in human and murine malaria infection requires further clarification.

1.7.5 Uric acid

Uric acid was recently found to stimulate inflammation via a *Plasmodium*-host collaborative mechanism. *Plasmodium* species scavenge precursors hypoxanthine and xanthine from host plasma for purine synthesis, and these precursors are released in large amounts during schizont rupture. Host enzyme xanthine oxidase rapidly converts hypoxanthine and xanthine into uric acid, which then stimulates immune cells to secrete TNF [359,360]. This response was MyD88-independent and no IL-1 was produced, excluding inflammasome activation. The authors acknowledged that substantial uric acid release in a microenvironment (e.g. brain capillary) may create conditions for formation of MSU crystals [359], which activate NLRP3. It was reported that intraperitoneal injection of sHz into mice stimulated uric acid production, and neutrophil recruitment was partly dependent on activation of the NLRP3 inflammasome in this system [337]. However, the relevance of this finding to *in vivo* malaria infection remains unknown.

It is unclear whether uric acid contributes to inflammation during malaria infection. Serum uric acid is correlated with disease severity in *P. falciparum* infection [361,362]. A study evaluating allopurinol, a xanthine oxidase inhibitor, as adjunctive therapy for complicated malaria showed that allopurinol treatment was associated with more rapid resolution of fever and splenomegaly, but also more rapid parasite clearance [363], complicating conclusions about any anti-inflammatory effects. Paradoxically, allopurinol exacerbated parasite burden in murine malaria models [364]. This may be due to differences in parasite species or host uric acid metabolism, and may impair further analysis of this inflammatory stimulus.

1.8 Links between innate inflammatory and phagocytic pathways in malaria infection

Many questions remain regarding the initiation and propagation of inflammation during human malaria infection. What is the relative importance of different stimuli and sensors, how do they interact, and which cells types are involved? Do these pathways contribute to protection from and/or development of severe malaria, and does this hinge on host and/or parasite factors? Are there other PRRs and mediators that participate in the inflammatory response to malaria?

Another issue – particularly if considering therapeutic modulation of innate immunity – is to what extent inflammatory pathways are linked to other important responses such as phagocytosis. As discussed above, phagocytosis of PEs by myeloid cells is thought to be critical for control of malaria infection in non-immune individuals. How do inflammatory PRRs affect parasite clearance? Conversely, does PE phagocytosis stimulate macrophage cytokine secretion via TLRs or other PRRs?

1.8.1 Phagocytosis: A highly regulated and diverse process

Phagocytosis of most particles is governed by an orderly set of core events (reviewed in [365]). Cross-linking of phagocytic receptors by a target particle leads to actin assembly at the site of binding, delivery of additional membrane from endosomes to permit extension of pseudopodia, and eventual closure of the phagocytic cup to form a phagosome within the cell. Variations of "zippering" phagocytosis exist: in macropinocytosis, receptor stimulation induces membrane ruffling that can capture large particles. Phagosomes then undergo a series of maturation events involving fusion with endosomes and lysosomes, which results in acidification and release of

ROS and hydrolytic enzymes to degrade phagosomal contents. Key signalling molecules include the Rho family of GTPases (Rac1, Cdc42, ARF6), which mediate actin assembly; phosphoinositide-3 kinase (PI3K), which contributes to pseudopodial extension; and SNAREs and the Rab family of GTPases, which mediate membrane fusion events.

Despite these basic commonalities, phagocytosis is a diverse process that is highly dependent on the target particle and the receptors it engages (reviewed in [268]). Major receptors include the Fc and complement receptors, scavenger receptors such as MARCO and CD36, and the mannose receptor. Receptors can activate different signalling pathways leading to variation in phagosomal fate and other outcomes. Microbes generally activate combinations of receptors, leading to crosstalk and synergies, and pathogens can interfere with the phagosomal maturation process to promote their own survival (reviewed in [366]).

The diverse nature of phagocytosis is well-illustrated by its relationship with inflammation. Macrophage phagocytic activity is enhanced by inflammatory stimuli, and internalization of many particles – particularly pathogens – is often accompanied by secretion of inflammatory mediators. However, in some host-pathogen interactions, inflammation and phagocytosis are uncoupled. Inhibition of a phagocytic receptor can impair phagocytosis but not cytokine production, and/or inhibition of inflammatory PRRs can reduce inflammation but not the rate of phagocytosis [367-369]. Additionally, phagocytosis of apoptotic cells is accompanied by release of anti-inflammatory cytokines such as IL-10 and TGFβ [370,371]. Thus, the relationship between inflammation and phagocytosis at the cellular level is highly complex and context-dependent. Identification of inflammatory PRRs has increased our understanding of the mechanisms underlying these linkages, as discussed below.

1.8.2 Contribution of phagocytic receptors to inflammatory responses

Some phagocytic receptors have been shown to activate pro-inflammatory responses via direct signalling. For example, TNF and ROS are induced upon crosslinking of Fc receptors by IgG-opsonized particles, but not during complement-mediated phagocytosis [372]. Dectin-1, a C-type lectin receptor that mediates binding and internalization of yeast, triggers production of ROS and cytokines during this process [369,373]. Dectin-1-mediated Syk activation was also shown to enhance TLR2-dependent cytokine responses to yeast, indicating that synergistic signalling between phagocytic receptors and TLRs can promote inflammation [369,374].

Phagocytic receptors also appear to facilitate ligand recognition by inflammatory PRRs. Hoebe *et. al.* showed that CD36 participated in the cytokine response to TLR2/6 agonists such as LTA or synthetic diacylated lipoprotein FSL-1 [375]. CD36 was found to cluster with TLR2/6 upon LTA stimulation [376], suggesting that CD36 concentrates ligands for presentation to TLR2 similarly to CD14/MD2-mediated presentation of LPS to TLR4. A recent report indicated that CD36 can enhance inflammation via mobilization of inflammatory PRRs. Two known CD36 ligands, fAb and oxidized low-density lipoprotein (oxLDL), were shown to be recognized through co-operation of CD36, TLR4, and TLR6; in this system, CD36 was found to be required for TLR4/TLR6 heterodimerization [377].

Another mechanism by which phagocytic receptors appear to contribute to inflammation is delivery of ligands to the phagosome for PRR recognition. TLRs and TLR adaptors are recruited to phagosomes where they are thought to "sample" the contents for danger signals [378,379]. Deletion or inactivation of CD36, the major phagocytic receptor for *Staphylococcus aureus* and purified LTA, abrogated TLR2-mediated cytokine responses to both of these ligands [380,381]. Treatment with cytochalasin D, which inhibits phagocytosis by blocking actin polymerization,

had a similar effect, leading to the suggestion that CD36 contributed to inflammation by transporting ligands to phagosomal TLR2 (although effects of cytochalasin D on TLR trafficking along cytoskeletal elements were not considered [382]). Supportive of this model, liberation of PAMPs in the phagosome has been found to be important for PRR recognition of some pathogens. For *S. aureus*, LTA release from cell wall peptidoglycan (PGN) by phagosomal acidification and lysozyme was essential for phagosomal TLR recognition and TNF production [380]. Phagosomal PGN degradation was also important for NLRP3 inflammasome activation by *S. aureus* [383]. Moreover, phagosomal degradation of *S. aureus* or *Listeria monocytogenes* PGN led to transport of Nod2 ligands into the cytosol for recognition [285,384]. Thus, by delivering pathogens to the phago-lysosomal compartment, phagocytic receptors can facilitate recognition by membrane-bound and cytosolic PRRs. As mentioned, the dependence of inflammatory responses on internalization is pathogen-specific, and appears to be related to the spatial arrangement and accessibility of PAMPs to their cognate receptors. For instance, inhibiting phagocytosis of *E. coli* did not diminish cytokine responses, likely because LPS is exposed on the surface of the bacteria [380].

1.8.3 Modulation of phagocytic processes by inflammatory PRRs

While phagocytosis can promote inflammation, activation of inflammatory PRRs can also regulate phagocytosis. Stimulation of macrophages with purified TLR agonists has been shown to activate phagocytic signalling effectors such as PI3K, Rac1, Pyk2 and paxillin [385-387], and to upregulate transcription of phagocytic receptors and signalling molecules [388]. TLR stimulation was found to increase phagocytosis of bacteria by macrophages and fluid-phase macropinocytosis by DCs. MyD88, IRAK4, p38 MAPK, and increased expression of scavenger receptors have been implicated in TLR-enhanced uptake [388,389], and a MyD88-independent, RIG-I-dependent pathway leading to increased Rho GTPase activation was recently shown to

contribute to LPS-induced phagocytosis [294,386]. Enhancement of phagocytosis by microbial as well as endogenous TLR agonists has been confirmed in other systems [390-392].

The above data suggest that TLR stimulation by free microbial components, perhaps shed during growth, or secreted host-derived danger signals can increase phagocytic activity during infection. However, a role for TLRs during internalization of intact pathogens varies between studies. Blander *et. al.* first reported that murine macrophages deficient in TLR2/4 or MyD88 showed reduced internalization and delayed phagosomal maturation for *E. coli* and *S. aureus* targets, but not latex beads or apoptotic cells [393]. Others have reported similar findings [394-396]. Interestingly, intracellular pathogens have been shown to promote their own uptake by stimulating TLR2, which in turn activates Rab5 [397] or phagocytic receptor β2 integrin [398].

In contrast, others have found that TLR or MyD88 deletion did not affect phagocytosis of the aforementioned or other microbes [380,399], or phagosomal maturation [400]. Differences between experimental systems (e.g. type of macrophages, bacterial strain, timepoints) may contribute to these discrepancies. There is also concern that TLR- or MyD88-deficient macrophages may exhibit defects in phagosomal maturation even in the absence of TLR stimulation [400]. Overall, however, there is consensus that TLR stimulation can regulate phagocytosis in some contexts.

1.8.4 CD36-mediated PE uptake – linked to inflammation?

CD36 is a major binding and phagocytic receptor for non-opsonized *P. falciparum* PEs [52]. Might the interaction of CD36 with PEs induce inflammatory responses? As discussed, CD36 has been shown to promote inflammation by delivering ligands to TLRs in the phagosome and

potentially at the cell surface [375,377,380,381]. This could conceivably occur upon CD36-mediated phagocytosis of PEs, which contain TLR agonists.

CD36 engagement may also directly induce pro-inflammatory mediators. Despite its small intracellular tails, antibody cross-linking of CD36 leads to activation of Src and Syk kinases, JNK and p38 MAPK, and Vav [401-403] (although it is unclear whether co-receptors are involved). A CD36-mediated signalling pathway resulting in cytoskeletal mobilization has been mapped [404], consistent with the role of CD36 in regulating phagocytosis and migration. While CD36 signalling has been proposed to directly initiate ROS and chemokine production in response to oxLDL or fAb [405-407], the recent implication of TLR4/6 heterodimers in these systems [377] reinforces the model that CD36 simply facilitates TLR recognition of complex ligands. In support of this, CD36 participates in internalization of apoptotic cells [408], which is anti-inflammatory, and in fact contributes to IL-10 production during this process [409]. Nevertheless, cross-linking of monocyte CD36 was found to induce ROS generation [410], implying that CD36 can directly initiate pro-inflammatory responses. CD36-mediated production of cytokines and chemokines have not been assessed using this model.

The inflammatory consequences of CD36-mediated PE binding and internalization have been investigated in a small number of studies [52,411]. We sought to better characterize the pro-inflammatory cytokine response to CD36 activation and CD36-mediated PE internalization. Moreover, since the effects of TLR activation on CD36-mediated internalization are unknown, we addressed this question from a general standpoint and in the context of PE uptake.

1.9 Novel molecular candidates in the inflammatory response to malaria

While a variety of host sensors and mediators have been implicated in the inflammatory response to malaria and severe malaria pathogenesis, it is highly likely that other key players have not yet been identified and may represent suitable therapeutic targets. We assessed two novel candidate proteins for a role in malaria-induced inflammation and more generally host responses to malaria.

1.9.1 Triggering receptor expressed on myeloid cells-1 (TREM-1)

TREM-1 is a 30 kD transmembrane protein encoded on human chromosome 6p21.1 within a relatively ancient gene cluster of Ig superfamily proteins [412]. The structure of TREM-1 consists of a single extracellular Ig domain, a transmembrane domain, and a 5-residue intracellular tail [413]. Murine TREM-1 is located on chromosome 17C3 and has 46% sequence identity to its human ortholog [413]. TREM-1 is expressed on monocytes, macrophages, and neutrophils [414,415]. More recently, TREM-1 expression has been demonstrated in human gastric epithelium [416], human monocyte-derived DCs exposed to hypoxic conditions [417], and murine hepatic sinusoidal cells [418].

In all cell types assessed, TREM-1 activation has been associated with pro-inflammatory responses. In monocytes, antibody-induced cross-linking of TREM-1 induces recruitment of DNAX activation protein 12 (DAP-12) [414], an adaptor protein containing immunoreceptor tyrosine-based activation motifs (ITAM). DAP-12 associates with TREM-1 through a charge-based interaction, leading to phosphorylation of its ITAMs. This results in activation of PI3K, phospholipase C-γ, ERK MAPK, and NF-κB, as well as calcium mobilization [414,419,420].

Ultimately, these signalling events lead to production of pro-inflammatory cytokines and chemokines, such as TNF, IL-1β, IL-8, and MCP-1 [414,420,421]. A moderate increase in macropinocytosis is also observed, as well as upregulation of costimulatory and adhesion molecules [414,421]. Over time, TREM-1-activated monocytes differentiate into DCs [421]. Neutrophil responses to TREM-1 activation include IL-8 and myeloperoxidase production, phagocytosis, and the respiratory burst [422].

Natural ligands for TREM-1 are poorly characterized, but for the most part appear to be endogenous danger signals. A fusion protein composed of the Fc portion of IgG and the extracellular domain of TREM-1 (Fc-TREM-1) was shown to bind to resting human platelets (but not other hematopoietic cells), indicating the presence of a TREM-1 ligand on platelets [423]. This ligand appeared functional, as a TREM-1-dependent interaction between platelets and neutrophils augmented the neutrophil respiratory burst response to LPS [423]. Inflammation induced by necrotic THP-1 cell lysates was partially TREM-1-dependent, but the TREM-1 ligand was not further defined [424]. In murine systems, Fc-TREM-1 did not bind platelets, but bound neutrophils and monocytes stimulated with LPS *in vitro* or isolated from septic mice [415,425], suggesting that the TREM-1 ligand is inducible. One group has reported a pathogen-derived ligand for TREM-1: the GP protein of the Marburg virus bound to Fc-TREM-1, and the inflammatory response of neutrophils to the virus was attenuated by TREM-1 blockade [426].

Despite the uncertain identity of its ligands, the role of TREM-1 in innate immunity has been investigated in a number of models. *In vitro* data support a role for TREM-1 as an amplifier of inflammatory responses, particularly in the context of TLR activation. Stimulation of monocytes with TLR2, TLR4, or TLR5 induced upregulation of TREM-1 mRNA and surface protein [427,428]; this occurred in a PI3K- and NF-κB-dependent and mainly cytokine-

independent manner [427,429]. Concomitant TREM-1 crosslinking and TLR4 stimulation synergistically increased monocyte production of pro-inflammatory cytokines such as TNF and IL-1β [419-421,430], although other pro-inflammatory cytokines (e.g. IL-12) appeared to be antagonized [420]. The observed synergy was PI3K-dependent [420] and may occur via TREM-1-mediated transcription of TLR signalling components [431]. Similar TREM-1 upregulation and synergy was observed for other TLR and NLR agonists [430]. TREM-1 may also contribute to a pro-inflammatory state by suppressing LPS-induced IL-10 [420,421].

Further studies in the sepsis field have confirmed that TREM-1 is upregulated and promotes pro-inflammatory responses *in vivo*. Sepsis patients and volunteers with experimental endotoxemia had elevated TREM-1 levels on peripheral blood monocytes compared to healthy controls [427,432,433], as did blood monocytes and tissue macrophages in the cecal-ligation puncture murine model of sepsis [432]. Notably, TREM-1 blockade using competitive inhibitors (Fc-TREM-1 or synthetic peptide LP17) reduced pro-inflammatory cytokine production, improved pathophysiological parameters, and prolonged survival in multiple murine sepsis models [419,425,428,433]. These results have raised the possibility of therapeutically inhibiting TREM-1 in sepsis [427,434].

TREM-1 has been implicated in other diseases characterized by inflammatory pathology. In patients with inflammatory bowel disease and murine models of this condition, TREM-1 levels on intestinal monocytes were increased, and inhibition of TREM-1 using the LP17 peptide ameliorated disease pathology in mice [435]. Monocytes in synovial samples from patients with rheumatoid arthritis displayed higher levels of surface TREM-1 than those from controls [436], and Fc-TREM-1 and LP17 administration reduced inflammation and paw thickness in a murine model of autoimmune arthritis [437]. Similarly, monocyte TREM-1 was upregulated in

pneumonia patients [438] and TREM-1 mediated pathology in a rat pneumonia model [425]. Thus, TREM-1 expression is increased in a number of inflammatory conditions and appears to contribute to poor outcomes in the corresponding animal model.

Patients with the above diseases also have increased levels of a soluble form of TREM-1 in blood or other compartments. Membrane TREM-1 can be cleaved by metalloproteinases, leading to release of the extracellular domain [439]. Soluble TREM-1 (sTREM-1) generation occurs following upregulation of membrane TREM-1 [439], and is thought to regulate TREM-1 signalling by competitively binding ligand and/or decreasing levels of the membrane receptor. Serum sTREM-1 is increased in sepsis, and was found to discriminate well between sepsis and non-infectious systemic inflammatory response syndrome [419,432], although its accuracy as a sepsis biomarker has been contested [440,441]. sTREM-1 was increased in serum and synovial fluid of patients with arthritis [417,436], and plasma levels of sTREM-1 were correlated with disease activity in inflammatory bowel disease [436]. Higher sTREM-1 concentrations in bronchoalveolar fluid were associated with pneumonia in mechanically-ventilated patients [419,432,442]. A variety of other infectious and non-infectious conditions have been associated with elevated sTREM-1 levels, leading to speculation about the role of TREM-1 in disease pathology [443,444].

Little is known regarding the regulation and role of TREM-1 in malaria infection. A recent study conducted at the Thai-Burmese border found elevated surface levels of TREM-1 on monocytes of malaria patients compared to healthy controls [445]. Given this finding, and the excessive pro-inflammatory responses implicated in severe malaria pathogenesis, we investigated TREM-1 as a potential mediator of inflammation in malaria infection and contributor to the immunopathology of severe malaria.

1.9.2 Chitinase-3 like-1 (CHI3L1)

CHI3L1 (also known as YKL-40 and HCgp-39) is a 40 kDa secreted glycoprotein encoded on human chromosome 1q32.1. It belongs to the 18 glycosyl hydrolase family, a group of chitinases and chitinase-like proteins from across phylogenetic kingdoms with high conservation among mammalian members [446]. Structurally similar to true chitinases, chitinase-like proteins can bind chitin but cannot degrade it. CHI3L1 lacks chitinase activity due to substitution of two key catalytic residues in the chitinase active site [447,448]. In addition to chitin, CHI3L1 can also bind heparin and collagen [449,450].

CHI3L1 is secreted by chondrocytes, synovial fibroblasts, vascular smooth muscle cells, epithelial cells, astrocytes, macrophages and activated monocytes, neutrophils, and many types of tumour cells (reviewed in [451]). Expression is constitutive in some cell types (e.g. macrophages [452]) and can be induced in others in a cell type-specific manner. TH1 cytokines such as TNF and IL-1β upregulated CHI3L1 in chondrocytes, osteoblasts, and macrophages, but not fibroblasts [453]. Both TH1 and T-helper cell-2 (TH2) cytokines (e.g. IL-4, IL-13) induced CHI3L1 in intestinal epithelial cells, and this was dependent on NF-κB activation [454]. PMA, LPS, and oxidized LDL increased expression of monocyte/macrophage CHI3L1 [455-457]. A variety of insults (e.g. ionizing radiation, hypoxia, etoposide and serum starvation) were found to upregulate CHI3L1 in a glioblastoma cell line [458]. Factors suppressing CHI3L1 expression include TGF-β1 for chondrocytes [447] and p53 for glioblastoma cell lines [458].

Consistent with CHI3L1 regulation by inflammatory and injurious stimuli, tissue and serum/plasma levels of CHI3L1 are elevated in numerous disease states (reviewed in [451]). Many of these conditions are characterized by chronic inflammation and/or tissue remodelling,

such as inflammatory bowel disease, rheumatoid arthritis (RA), osteoarthritis, systemic lupus erythematosus, atherosclerosis, asthma, diabetes, Alzheimer's disease, pre-eclampsia, and multiple types of cancer. Infectious processes can also stimulate CHI3L1 production: serum levels were elevated in community-acquired pneumonia [459], sepsis [460,461], and experimental endotoxemia [462]. CSF levels of CHI3L1 were increased in bacterial meningitis [463] and HIV encephalopathy [464]. CHI3L1 levels were correlated with disease activity, severity, and/or prognosis in bacterial and viral infections [461,463,464], RA [465], cancer [466], pre-eclampsia [467], and Alzheimer's disease [468].

An obvious starting point when considering CHI3L1 function is its ability to bind chitin. Chitin binding induces a major conformational shift in CHI3L1, potentially regulating its activity [469]. Mammals are not thought to produce chitin, but this polysaccharide is abundant in the cell walls of bacteria and fungi and in helminth eggs. Thus, CHI3L1 may participate in the immune response to pathogens. CHI3L1 was produced in the spleen during helminth infection in mice and mediated chemoattraction and extravasation of eosinophils and T cells [448], suggesting that it may recruit cells to sites of infection. However, it is unclear whether the observed activity was strictly chitin-dependent. In a murine model of *Salmonella typhimurium* infection, CHI3L1 expression was induced in colonic epithelial cells [454], and promoted bacterial adhesion and invasion in a chitin-dependent manner [470]. Treatment with anti-CHI3L1 antibody reduced bacterial burden and death [454].

Other, presumably chitin-independent functions have been determined for CHI3L1, including roles in pathological inflammation and tissue remodelling. Intra-articular injection of CHI3L1 induced chronic relapsing arthritis in mice, characterized by inflammatory infiltrates and synovial fibroblast proliferation [471]. CHI3L1 is presented as an autoantigen in the joint space,

leading to the generation of autoreactive T cells that contribute to the pro-inflammatory milieu [471,472]. In addition, CHI3L1 has been shown to directly modulate connective tissue components *in vitro*. Exogenous CHI3L1 stimulated proliferation of fibroblasts and chondrocytes via activation of MAPKs and PI3K [473,474]. CHI3L1 can also regulate collagen fibrillogenesis [450] and the production of MMPs [453,475], which are important mediators of matrix degradation in arthritis. Interestingly, CHI3L1 reduced MMP-1 and MMP-13 production by TNF-stimulated chondrocytes and skin fibroblasts [476], suggesting that although CHI3L1 can promote inflammation, in certain contexts it may counter the effects of pro-inflammatory cytokines.

Consistent with its role in tissue remodelling, CHI3L1 can stimulate angiogenesis, with implications for tumorigenesis. The pig ortholog of CHI3L1 was found to promote migration and tubulogenesis by human umbilical vein endothelial cells [477], as well as migration and adherence of porcine vascular smooth muscle cells [478]. Shao and colleagues showed that transfection of cancer cell lines with a CHI3L1 transgene led to larger, more vascularized tumours when injected into mice, and CHI3L1 silencing reduced angiogenesis and tumour growth [479]. This was also the first (and presently only) report to delineate a mechanism for CHI3L1 signal transduction. CHI3L1 treatment of endothelial cells specifically induced association of $a_v b_3$ integrin with membrane protein S1; this interaction resulted in phosphorylation of FAK and ERK, which mediated changes in endothelial cell organization [479]. The authors proposed that CHI3L1 binds to heparan sulfate decorations on the S1 protein to induce signalling via integrins.

CHI3L1 production by cancer cells may also contribute to tumorigenesis by promoting cell survival in the face of ionizing radiation, serum starvation, and chemotherapeutic drugs

[480,481]. Moreover, CHI3L1 enhanced glioma cell invasiveness by regulating matrix adhesion and cytoskeletal organization, and augmented anchorage-independent growth [480].

The recent generation of a *Chi3l1*$^{-/-}$ mouse strain has shed new light on CHI3L1 function, particularly with regards to inflammation. A role for CHI3L1 in asthma pathogenesis in humans was previously suggested by the association of asthma severity with a *Chi3l1* promoter polymorphism that enhanced CHI3L1 expression [482]. In a murine model of asthma based on antigen sensitization and challenge, CHI3L1 was induced in the lung during challenge [483]. *Chi3l1*$^{-/-}$ mice had decreased cellular infiltrates and TGF-β1 levels in lung, as well as reduced DC activation and IgE production. Selective induction of human CHI3L1 in the lung epithelium of *Chi3l1*$^{-/-}$ mice restored pathology. The role of CHI3L1 in these TH2 inflammatory responses was partially attributed to its pro-survival effects on infiltrating leukocytes: recombinant CHI3L1 protected macrophages against apoptotic stimuli *in vitro*, and immune cells in the lungs of CHI3L1-deficient mice showed higher Fas levels and greater apoptosis than those in controls.

Interestingly, the ability of CHI3L1 to promote cell survival may also reduce inflammation. In a model of hyperoxic lung injury, *Chi3l1*$^{-/-}$ mice had enhanced epithelial cell apoptosis, resulting in increased immune cell infiltrates, alveolar capillary leak, and death [484]. In yet other models, the pro-inflammatory and pro-survival effects of CHI3L1 appear to be separable: *Chi3l1*$^{-/-}$ mice exposed to cigarette smoke showed less lung inflammation, but increased epithelial apoptosis, overall leading to greater alveolar destruction [485]. Thus, the role of CHI3L1 is model-specific and likely depends on the relative contributions and interactions of different pathological processes.

CHI3L1 has not yet been examined in malaria. Considering the implication of inflammation in malaria pathogenesis, and other processes regulated by CHI3L1 (e.g. leukocyte migration, angiogenesis, apoptosis), we investigated the regulation and role of CHI3L1 in malaria infection.

1.10 Predictive biomarkers for severe malaria in African children

In addition to the urgent need for adjunctive therapies for severe malaria, there is also a lack of tools to accurately triage children with potentially life-threatening infection at clinical presentation, and to determine the prognosis of children with severe malaria. In resource-poor health care settings, identifying patients at increased risk of morality would allow for efficient allocation of limited intensive care resources and/or closer monitoring by staff for complications. In the context of clinical trials of adjunctive therapies for severe malaria, prognostic information could be used to stratify patients into risk categories, since treatment efficacy can vary depending on risk of mortality [486]. Moreover, it would facilitate enrolment of the intended target group (i.e. those at high risk of death), and spare low-risk patients from unnecessary exposure to experimental treatments. Ideally, a prognostic test for severe malaria would be highly accurate, rapid, easy to use and interpret, objective, inexpensive and available at point-of-care [487].

Malaria-associated mortality has been associated with a variety of demographic, clinical and laboratory parameters. In African children, these include age, level of consciousness, convulsions, respiratory distress, hypoglycemia, jaundice, dehydration, prolonged capillary refill time, metabolic acidosis, elevated blood lactate levels, parasite density, stage of parasite development, retinopathy, malnutrition, concomitant bacteremia, leukocytosis, and detection of

hemozoin in circulating myeloid cells [14-16,23,25,43,126,488-497]). There is some discordance between studies, likely due to variation in malaria endemnicity, inclusion criteria, and/or testing protocols. Predictors of mortality in adults include age, level of consciousness, metabolic acidosis, hyperlactatemia, elevated plasma creatinine or bilirubin, and hemodynamic shock [498,499], thus reflecting the differences in severe malaria manifestations between adults and children.

Considering the clinical heterogeneity of severe malaria and its complex pathophysiology, it is not surprising that combinations of factors have performed better than individual factors for prognosis. Molyneux and colleagues were the first to propose a "prognostic index" based on data from pediatric CM patients in Malawi: one point was assigned to each of 8 risk factors that could be rapidly assessed at the bedside. A score ≥ 4 more accurately predicted poor outcome (death or neurological sequelae) than any factor alone, with 66% sensitivity and 96% specificity [126]. In a large study of Kenyan children with severe malaria (n=1844), the presence of one or more of prostration, respiratory distress, hypoglycemia, and jaundice predicted death with 92.2% sensitivity and 62.4% specificity [14]. The Lambaréné Organ Dysfunction Score (LODS) was systematically developed from a large database comprising six research sites across sub-Saharan Africa, and assigns one point for each of coma, prostration, and deep breathing. A LODS >0 had 85% sensitivity and 63% specificity for predicting death, while a LODS <3 had 25% sensitivity and 98% specificity [500]. The simplicity, rapidity, and negligible costs of these tests are attractive. However, assessment of clinical signs is subjective, determinations may vary between observers, and a requirement for trained health care providers limits implementation at the community level where most severe malaria cases present. Moreover, a single criterion with both high sensitivity and specificity would enable more efficient resource

allocation by identifying children with the most severe infections while limiting the rate of false positives.

There has been considerable interest in elevated blood lactate levels (≥5 mmol/L) as a prognostic indicator for severe malaria, since this parameter can be objectively measured and reflects disease severity and pathogenesis. Hyperlactatemia in severe malaria is thought to arise from a combination of parasite metabolic activity [501], glycolytic activity in host tissues due to impaired oxygen delivery or/and consumption [125,127,498], convulsions [488], and decreased hepatic/renal clearance [498]. Upon initiation of treatment, lactate levels fall rapidly in survivors but decline more slowly or increase in fatal cases [488]. Hyperlactatemia has been consistently associated with malaria mortality across studies in children and adults, and can be rapidly and objectively determined using a "near-patient" hand-held analyzer. However, this test is expensive and predictive accuracy is not exceptionally high (70-75% sensitivity and specificity [488,502], or 91% sensitivity and 52% specificity when combined with impaired consciousness [490]). It was recently reported that the addition of hyperlactatemia into a prognostic model based on clinical parameters and hypoglycemia barely improved prediction of mortality [502].

While the classification performance of hyperlactatemia appears inadequate, the concept of deriving prognostic information from a quantifiable biomarker of disease severity at point-of-care is appealing. A number of groups have examined serum/plasma/CSF biomarkers of host pathways thought to contribute to severe malaria pathogenesis, and assessed differences in admission levels between patients who subsequently died of infection versus survivors. Inflammatory biomarkers found to be significantly altered in fatal cases include cytokines (TNF [138,139,488], IL-6, IL-10 [142,503]); chemokines (IP-10 [504], RANTES, MIP-1β [142], MCP-1 [505]), and cytokine antagonists (IL-1Rα [505], soluble TNF receptors [506,507]).

Increased levels of apoptosis-related molecules, such as soluble Fas, were associated with CM mortality [504], as were markers of endothelial activation, such as soluble ICAM-1 and ELAM-1 [508]. Recently, our group and others have examined angiogenic factors implicated in CM pathogenesis: serum/plasma levels of VEGF and Ang-1 decreased in fatal cases [200,201,504], while Ang-2 levels increased [200]. Some reports have used ratios of biomarkers to improve discrimination between survivors and fatalities [201,503,504], but combinatorial approaches have not been adequately studied.

Further work in this area is warranted, since advances in microfluidics, nanoparticles, and multiplexing technologies may enable the development of portable and affordable platforms for rapid quantification of multiple analytes from a small blood sample [509,510]. Plasma markers associated with malaria mortality have rarely been formally evaluated for classification performance (e.g. sensitivity, specificity, predictive values). Moreover, most work has focused on CM to the exclusion of other severe malaria syndromes. We obtained admission plasma samples from Ugandan children with UM, CM, and SMA, and measured biomarkers of endothelial activation and inflammation, including novel candidates sTREM-1 and CHI3L1. We assessed their ability to predict mortality, either individually or in combination.

1.11 Research aims and hypotheses

While the vast majority of malaria infections resolve without complication, millions of individuals – most often young African children with minimal immunity to malaria – develop severe disease and are at high risk of death. Adjunctive therapies to complement anti-parasitic agents are sorely needed, as are prognostic tools to allow triage of severely ill children to receive limited supportive or intensive care resources. Given the importance of innate host responses – particularly inflammation – in determining progression to severe malaria, achieving a more nuanced understanding of these responses may help define molecular targets for intervention and useful prognostic biomarkers. The aim of this work was to investigate host inflammatory responses to malaria from a number of angles. This included an exploration of interactions between innate inflammatory and phagocytic pathways under consideration as therapeutic targets in severe malaria; examination of novel pathways that may modulate inflammatory responses to malaria; and evaluation of biomarkers of inflammation and other host pathways as prognostic factors in severe malaria.

In **Chapter 2**, we assessed potential crosstalk between CD36-mediated PE phagocytosis and TLR-mediated inflammatory responses to malaria. Since the malaria-infected erythrocyte is a complex and dynamic entity, we first used parsimonious model systems to isolate the effects of CD36 and TLR stimulation and determine how they interact. We then extended our investigations to an *in vitro* model of the malaria-macrophage interaction. Specifically, we inquired:

1. Does CD36-mediated phagocytosis lead to production of pro-inflammatory cytokines?
2. Does TLR stimulation modulate CD36-mediated phagocytosis?

3. Does CD36-mediated uptake of PEs lead to production of pro-inflammatory cytokines via TLR stimulation?
4. Does TLR stimulation modulate CD36-mediated phagocytosis of PEs?

We found that targeted activation and internalization of CD36 do not by themselves stimulate pro-inflammatory cytokine production. Despite the presence of malaria TLR agonists in PEs, CD36-mediated PE internalization was also found to be non-inflammatory. In addition, we show that CD36 and TLRs can co-operate to promote internalization of particles, including PEs.

In the next two chapters, we investigated two host molecules as novel candidates for participation in the host response to malaria. In **Chapter 3**, we examined whether TREM-1 contributes to the inflammatory response to malaria and the immunopathology of severe malaria. The following questions were posed:

1. How is TREM-1 regulated upon exposure to malaria *in vitro* and *in vivo*?
2. Does TREM-1 contribute to inflammatory responses to malaria *in vitro* and *in vivo*, and does it affect survival in murine models of severe malaria?
3. Does concomitant TREM-1 activation alter the inflammatory response to malaria?

We found that sTREM-1 was generated upon exposure of human PBMCs to malaria. Plasma sTREM-1 was elevated in the plasma of children with severe malaria syndromes compared to those with uncomplicated malaria, and highest in children who died of severe malaria. In the PbA model of ECM, plasma sTREM-1 and monocyte surface TREM-1 levels were increased. Inhibition of TREM-1 *in vitro* and *in vivo* did not modulate inflammatory responses to the malaria parasite. However, the ratio of pro- to anti- inflammatory cytokine responses of PBMCs

to malaria was increased by concomitant TREM-1 activation, potentially providing insight into the increased disease severity associated with malarial-bacterial co-infections.

In **Chapter 4**, we investigate the hypothesis that CHI3L1 may modulate inflammatory or other responses to malaria and ultimately affect outcome of infection. We asked the following questions:

1. Is plasma CHI3L1 increased in patients with severe malaria compared to those with uncomplicated malaria?
2. Is CHI3L1 regulated by malaria *in vitro* and what are its effects on inflammatory responses to malaria?
3. Does genetic deletion of CHI3L1 modulate inflammation and/or affect survival in experimental cerebral malaria?

We found that plasma levels of CHI3L1 were significantly elevated in children with severe and fatal malaria compared to those with uncomplicated disease. CHI3L1 expression was induced by *P. falciparum* in PBMCs, and plasma and brain levels were increased in murine models of malaria. We did not observe modulation by CHI3L1 of the pro-inflammatory response to malaria either *in vitro* or *in vivo*. However, CHI3L1 was differentially regulated in ECM-susceptible and ECM-resistant mice. Mice with genetic deletion of CHI3L1 showed a trend towards earlier death during PbA infection, warranting further study of this molecule in a more informative model.

In **Chapter 5** we address the hypothesis that our novel candidates, regardless of whether they modulate or simply reflect malaria-induced inflammation, have utility as prognostic biomarkers in pediatric severe malaria. We asked the following questions:

1. How accurately do plasma levels of CHI3L1 or sTREM-1 at presentation predict mortality among severe malaria patients?
2. Does combining CHI3L1 and/or sTREM-1 with other inflammatory markers and markers of endothelial activation improve prognostic accuracy?

Using receiver operating curve characteristic analysis, we found that CHI3L1 and sTREM-1 discriminated well between severe malaria survivors and fatalities, as did five other biomarkers of inflammation and endothelial activation. A score based on dichotomization and combination of these 7 markers predicted death with ~90% sensitivity and specificity. Using this approach as well as classification tree analysis, 3-marker models were generated with 100% sensitivity and >80% specificity.

Chapter 2

CD36 and TLR interactions in inflammation and phagocytosis: Implications for malaria

2.1 ABSTRACT

CD36 participates in macrophage internalization of a variety of particles, and has been implicated in inflammatory responses to many of these ligands. To what extent CD36 co-operates with other receptors in mediating these processes remains unclear. Because CD36 can co-operate with TLR2, we investigated the roles and interactions of CD36 and TLRs in inflammation and phagocytosis. Using antibody-induced endocytosis of CD36 and phagocytosis of erythrocytes displaying antibodies to CD36, we show that selective engagement and internalization of this receptor did not lead to pro-inflammatory cytokine production by primary human and murine macrophages. In addition, CD36-mediated phagocytosis of *Plasmodium falciparum* malaria-parasitized erythrocytes (PEs), which contain parasite components that activate TLRs, also failed to induce cytokine secretion from primary macrophages. Furthermore, we demonstrate that CD36-mediated internalization did not require TLR2 or the TLR-signalling molecule IRAK4. However, macrophage pre-treatment with TLR agonists markedly stimulated particle uptake via CD36. Similarly, PE uptake was unaffected by TLR deficiency, but in wild-type cells was increased by pre-treatment with *P. falciparum* glycosylphosphatidylinositols, which activate TLR2. Our findings indicate that CD36 must co-operate with other receptors such as TLRs to participate in cytokine responses. Although purified *P. falciparum* components activate TLRs, CD36-mediated internalization of intact PEs is not inflammatory. Further, CD36 mediates internalization of particles, including PEs, independently of TLR signalling, but can functionally co-operate with TLRs to enhance internalization.

2.2 INTRODUCTION

CD36, a member of the class B scavenger receptor family, is expressed in a variety of cell types and binds a diverse array of ligands [511]. CD36 has been shown to participate in the internalization of a number of particles, including fAβ [512], oxLDL [513], non-opsonized bacteria [418], apoptotic cells [408], and *Plasmodium falciparum* malaria-infected erythrocytes [52]. Binding and uptake of fAβ, oxLDL, and bacteria is accompanied by release of pro-inflammatory mediators, and CD36 has also been implicated in these responses [381,405,418,514,515].

In contrast, CD36 has been shown to contribute to production of immunoregulatory cytokines during internalization of apoptotic cells [370,409]. It may be that the pro-inflammatory effects of CD36 engagement during apoptotic cell clearance are suppressed by the IL-10 and TGFβ secreted during this process. An alternative possibility is that CD36 does not directly mediate pro-inflammatory signalling, but rather presents ligands for recognition by other signalling receptors. In support of this hypothesis, CD36 has been shown to augment cytokine responses by delivering ligands to TLRs [375,377,380,381,516]. Thus, it remains unclear whether CD36 can independently mediate inflammation, or whether it must partner with other receptors such as TLRs to contribute to inflammatory responses.

While recent studies have focused on CD36-TLR interactions in the context of inflammation, these receptors could also conceivably co-operate in mediating phagocytosis. Although TLRs do not mediate phagocytosis directly [517], they have been shown to enhance macrophage uptake of bacterial pathogens [388,393,398], and in one report this was dependent on upregulation of

scavenger receptors [388]. However, it is not known if TLRs can modulate CD36-mediated internalization.

In this study, we dissected the roles and interactions of CD36 and TLRs in inflammation and phagocytosis. The existing uncertainty regarding the interaction between CD36 and TLRs in mediating inflammatory responses results, at least in part, from the complexity of the experimental models used. To simplify the analysis, we devised model systems that selectively activate CD36 to investigate whether this receptor can mediate inflammation independently of TLRs. We also employed selective, receptor-targeted strategies to assess whether CD36 and TLRs can co-operatively mediate phagocytosis.

Furthermore, we explored potential CD36-TLR collaborations in an *in vitro* malaria-macrophage model, as CD36 mediates non-opsonic internalization of *P. falciparum* PEs [52] and *P. falciparum* components have been shown to stimulate TLRs [311,338,339,358]. Although PE binding and internalization is often assumed to induce pro-inflammatory responses [518], few studies have examined this issue experimentally. Binding of PEs to human monocytes was reported to stimulate the respiratory burst, and this was inhibited by CD36 blockade [411]. It is unclear whether CD36 signalling directly mediated this response or tethered the PE for recognition by another receptor; notably, CD36 was recently shown to collaborate with TLR2 in the inflammatory response to *Pf*GPI [67]. Moreover, the relevance of phagocyte-derived ROS to control of malaria infection and severe malaria pathogenesis is unclear [108,194]. Of greater interest, our group has demonstrated that CD36-mediated PE uptake did not induce TNF secretion by human or murine monocyte/macrophages [52,53]. However, these studies used cells in the resting state, and it is increasingly appreciated that monocyte activation by IFNγ can significantly alter phagocyte responses [285,384,519,520].

Thus, we revisited this question, and furthermore investigated whether TLR stimulation by malaria parasites can regulate CD36-mediated PE internalization.

Here, we demonstrate that targeted activation and internalization of CD36 do not by themselves stimulate pro-inflammatory cytokine production. Despite the presence of malaria TLR agonists in PEs, CD36-mediated PE internalization was also found to be non-inflammatory. In addition, we show that CD36 and TLRs can co-operate to promote internalization of particles, including PEs.

2.3 METHODS

2.3.1 Parasites. *P. falciparum* (ITG strain) was cultured as previously described [53]. Cultures were treated with Mycoplasma-Removal Agent (MP Biochemicals), confirmed to be mycoplasma-free (MycoAlert Mycoplasma Detection Kit, Lonza), and synchronized by alanine treatment [521]. Mature-stage cultures were washed and used at a 20:1 PE:macrophage ratio.

2.3.2 Mice. C57BL/6 mice were purchased from Charles River. *Cd36*$^{-/-}$, *Tlr2*$^{-/-}$, and *Irak4*$^{-/-}$ mice on the C57BL/6 background were bred in the University of Toronto animal facility. Animal protocols were approved by the Animal Care Committee of the University of Toronto and all animal work was performed in compliance with university institutional guidelines.

2.3.3 Macrophage isolation. Human peripheral blood mononuclear cells were isolated from blood of healthy volunteers by gradient centrifugation using Ficoll-Paque (GE Healthcare). Monocytes were purified by adherence to glass coverslips in 24-well plates ($1.5x10^6$

PBMCs/well), and cultured for 3-5 days in RPMI 1640 with 10% fetal bovine serum and gentamicin (Gibco-Invitrogen; medium R10G) to allow for differentiation into macrophages. Peritoneal murine macrophages were isolated [53], adhered to coverslips in 24-well plates ($0.125x10^6$/well) in R10G, and used within 5 days.

2.3.4 CD36 cross-linking and endocytosis assay. Human macrophages were incubated on ice for 15 min with mouse-α-human CD36 IgG clone 131.2 (a kind gift from Dr. Narendra N. Tandon, Thrombosis Research Laboratory, Otsuka Maryland Medicinal Laboratories; 3.3 μg/ml) or mouse non-immune matched IgG1 isotype (Ebioscience). For murine macrophages, mouse-α-mouse CD36 IgA clone 63 (Cascade Biosciences; 2 μg/mL) or isotype control antibody (Ebioscience) were used. Cells were washed twice with cold PBS and incubated on ice for 10 min with a secondary antibody to cross-link CD36: goat-α-mouse IgG-Cy2 antibody (Jackson ImmunoResearch Laboratories) and goat-α-mouse IgA-FITC antibody (Sigma) for human and mouse macrophages, respectively. For imaging of cell surface CD36, cells were fixed immediately with 4% PFA. For imaging of endocytosed CD36-antibody complexes, cells were incubated at 37^{o}C for 30 min before fixation, and fluorescent staining of remaining surface CD36 was performed with donkey-α-goat IgG-Cy3 tertiary antibody (Jackson ImmunoResearch Laboratories). Images were acquired with a spinning disk confocal microscope and Volocity acquisition software. To assess cytokine production, macrophages were incubated at 37^{o}C for 24 hrs following cross-linking; supernatants were collected and assayed by ELISA for TNF and IL-6 (Ebioscience, BD Bioscience). LPS (Sigma; 100 ng/mL) was used as a positive control.

2.3.5 Preparation of erythrocytes coated with anti-CD36 antibody. Erythrocytes coated with Biotin, Avidin, and Biotinylated antibody (EBABs) were generated as previously reported [522,523]. Briefly, human erythrocytes were sequentially treated with biotin and avidin; then

incubated with a biotinylated mouse-α-human CD36, clone SMO (ID Labs) to target EBABs to human CD36, or with a biotinylated goat-α-mouse Ig $F(ab')_2$ (Abcam) followed by a mouse-α-mouse CD36 (clone 63, Cascade Bioscience) to target EBABs to murine CD36. Control EBABs for human and murine systems were prepared using a biotinylated mouse IgM isotype-matched antibody (ID Labs) or a mouse IgA isotype-matched antibody (Ebioscience), respectively. Antibody conjugation was confirmed using Cy3-labeled secondary antibodies (Jackson Immunoresearch Laboratories). EBABs were added to macrophages at a 20:1 ratio.

2.3.6 Internalization assays. PE internalization assays were performed as described [53] for 2 hours. Images were acquired with an Olympus BX41 microscope and an Infinity2 camera. EBAB internalization assays were performed at 37^{o}C; macrophages were then washed with PBS, subjected to a hypotonic lysis to remove bound but not internalized EBABs, and fixed with 0.75% glutaraldehyde (Sigma). EBAB binding assays were performed at 4^{o}C and fixed without hypotonic lysis. EBABs were stained with *o*-dianisidine as described [524], and macrophages were stained with 1% eosin. Differential interference contrast (DIC) microscopy was used for imaging of EBABs. To demonstrate EBAB uptake, murine macrophages were incubated with EBABs for 30 min at 37^{o}C, then incubated for 10 min on ice with donkey-α-goat IgG-Cy3 antibody to stain bound EBABs. A hypotonic lysis was performed, and DIC and fluorescence microscopy were used to identify unstained (i.e. internalized) EBABs. Phagocytic indices were determined as reported [53].

2.3.7 Cytokine release upon CD36-mediated phagocytosis. α-CD36 EBABs or *P. falciparum* cultures were added to macrophages in R10G. Control EBABs or uninfected red blood cells (uRBC) were used as negative controls, respectively, and LPS (100 ng/mL) was used as a positive control. In some experiments, macrophages were pre-incubated for 12 hrs with

recombinant IFN-γ from the corresponding species (Ebioscience; 100 U/mL). After 4 and 8 hrs at 37°C, supernatants were collected and assayed by ELISA.

2.3.8 Treatment with TLR2 agonists. HPLC-purified *Pf*GPI was isolated and coated onto gold beads as previously described [67,311] to enhance stability and facilitate quantification. For cytokine assays, macrophages were incubated with *Pf*GPI, or gold beads alone as a control, in R10G (200 ng/mL) for 24 hrs at 37°C; supernatants were collected and assayed by ELISA. For internalization assays, macrophages were pre-incubated at 37°C with *Pf*GPI or gold beads (400 ng/mL), FSL-1 (Invivogen; 20 ng/mL), or Pam_3CSK_4 (Invivogen; 100 ng/mL) in RPMI, followed by addition of EBABs or PEs in a 50 μL volume.

2.3.9 Flow cytometry. Following treatment with media or TLR2 agonists, human or murine macrophages were washed with cold PBS with 2% fetal bovine serum and gently scraped. Human macrophages were blocked with 10% mouse serum for 10 min, then stained with mouse-α-human CD36 conjugated to FITC (clone FA6.152, IOTest; 20 μL/test) for 20 min on ice. A rat IgG isotype-matched antibody conjugated to FITC (Ebioscience; 20 μL/test) was used as a control. Murine macrophages were treated with anti-mouse CD16/32 (Ebioscience) for 10 min on ice to block Fc receptors, then incubated with mouse-α-mouse CD36 IgA (2 μg/mL) for 20 min, followed by rat-α-mouse IgA-PE (Ebioscience; 1.25 μg/mL) for 20 min on ice. A mouse IgA isotype-matched antibody plus secondary antibody was used as a control. Cells were analyzed using the FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software.

2.3.10 Statistical analysis. Using GraphPad Prism software, differences between groups were analyzed using the Student's t-test or one- or two-way ANOVA with Bonferroni post-tests. Each type of experiment was performed in duplicate or triplicate. Data are presented as means ± SD.

2.4 RESULTS

2.4.1 Targeted activation of CD36 and CD36-mediated internalization do not induce pro-inflammatory cytokine secretion.

We initially examined whether CD36 could induce cytokine production, independently of TLRs or other pattern recognition receptors, by selectively activating CD36 by antibody-mediated cross-linking, leading to receptor endocytosis (Fig. 2.1A). Surface CD36 on primary human and murine macrophages was cross-linked on ice with an α-CD36 antibody and a fluorophore-conjugated secondary antibody (Fig. 2.1B, upper panels), and these antibody-induced complexes were internalized upon incubation at 37^{o}C (Fig. 2.1B, middle panels). The system was CD36-specific, as no complexes were observed when using an isotype-matched antibody or *Cd36*$^{-/-}$ murine macrophages (Fig. 2.1B, lower panels).

Cytokine production was assessed 24 hrs following CD36 cross-linking and endocytosis. While LPS stimulated strong cytokine production, there was no secretion of TNF (Fig. 2.1C) or IL-6 (Suppl. Fig. 2.1A) upon cross-linking with α-CD36 antibody. These data suggest that CD36 activation and endocytosis do not induce pro-inflammatory cytokine release.

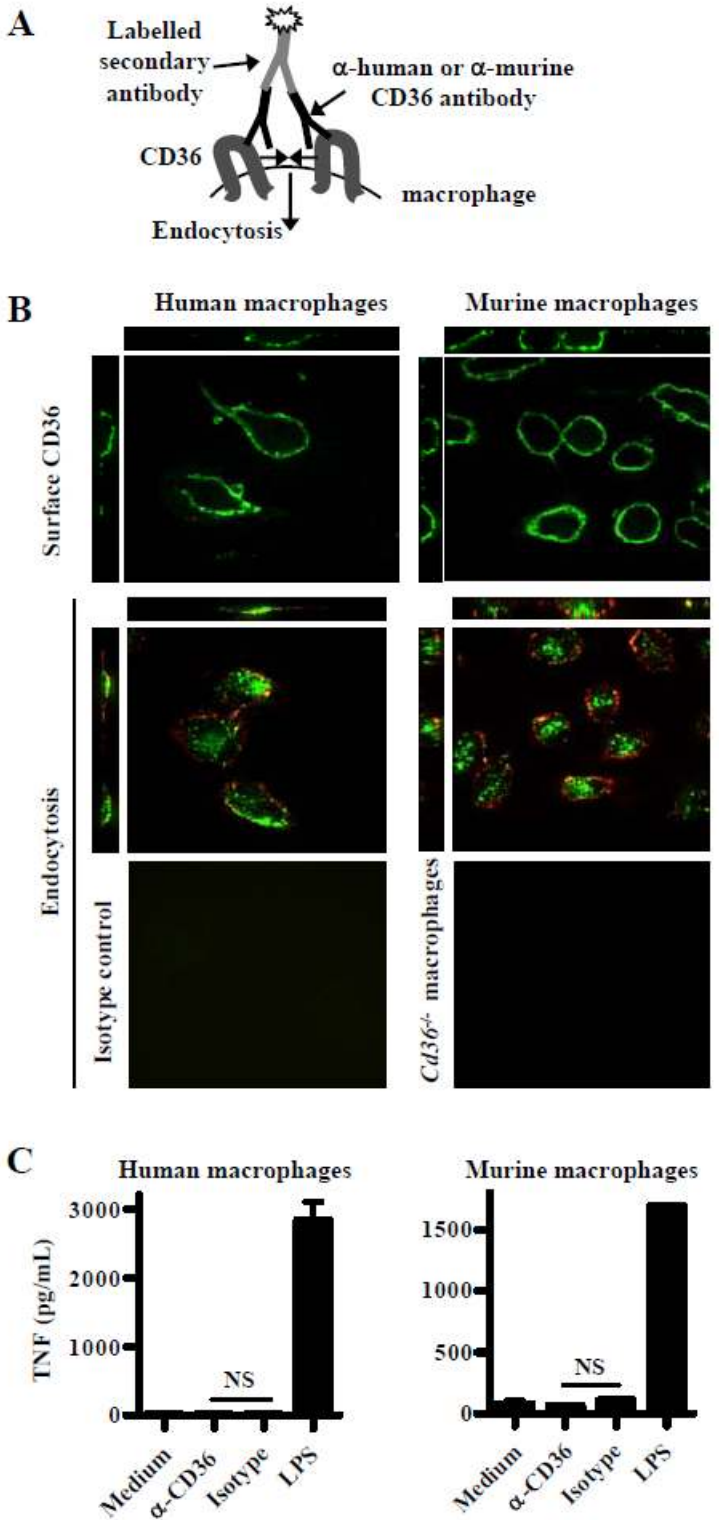


Fig. 2.1: Antibody-induced CD36 cross-linking and endocytosis do not stimulate secretion of pro-inflammatory cytokines. **(A)** Diagrammatic representation of antibody-induced CD36 cross-linking and endocytosis model. **(B)** *Upper panels:* Surface CD36 was cross-linked on primary human and murine macrophages by incubation at 4°C with α-CD36 antibody, followed by a FITC- or Cy2-conjugated secondary antibody. *Middle panels*: Following transfer of macrophages to 37°C, CD36-antibody complexes were internalized. Internalization of the

complexes was confirmed by labeling the remaining surface CD36 with a tertiary Cy3-coupled antibody and examining vertical sections of cells constructed from Z-stacks (above and to left of individual images). Green signal within the macrophage represents internalized CD36-antibody complexes. *Lower panels*: The CD36-specific nature of the system was demonstrated by treating human macrophages with an isotype-matched antibody (*left*) and using *Cd36*$^{-/-}$ murine macrophages (*right*). **(C)** Macrophages were incubated at 37^{o}C for 24 hr following CD36 cross-linking, and TNF levels in supernatants were assessed by ELISA. ND, not detectable. NS, not significant by Student's t-test. Results are representative of three independent experiments.

Signalling can differ when receptors are clustered into small vs. large aggregates [525,526]. We therefore investigated whether CD36-mediated phagocytosis of large particles stimulates inflammation. We employed erythrocytes conjugated to α-CD36 antibodies by an Erythrocyte-Biotin-Avidin-Biotinylated antibody (EBAB) coupling procedure ([522], Fig. 2.2A). α-CD36 EBABs bound to wild-type, but not *Cd36*$^{-/-}$, macrophages (Fig. 2.2B, upper panels). α-CD36 EBAB internalization, confirmed by selective fluorescent labeling (Fig. 2.2B, lower panels), was CD36-specific. α-CD36 EBABs were poorly internalized by *Cd36*$^{-/-}$ macrophages, and EBABs prepared with an isotype-matched control antibody were poorly internalized by wild-type macrophages (Fig. 2.2C, bottom; $p<0.01$). Similarly, human macrophages bound (data not shown) and internalized α-CD36 EBABs but not control EBABs (Fig. 2.2C, top; $p=0.004$).
To assess the inflammatory outcomes of CD36-mediated phagocytosis, α-CD36 and control EBABs were incubated with macrophages for 4 and 8 hrs. There was no difference in TNF (Fig. 2.2D) or IL-6 (Suppl. Fig. 2.1B) secretion between α-CD36 and control EBABs . Thus, like CD36-mediated endocytosis, CD36-mediated phagocytosis of large particles did not stimulate pro-inflammatory cytokine release.

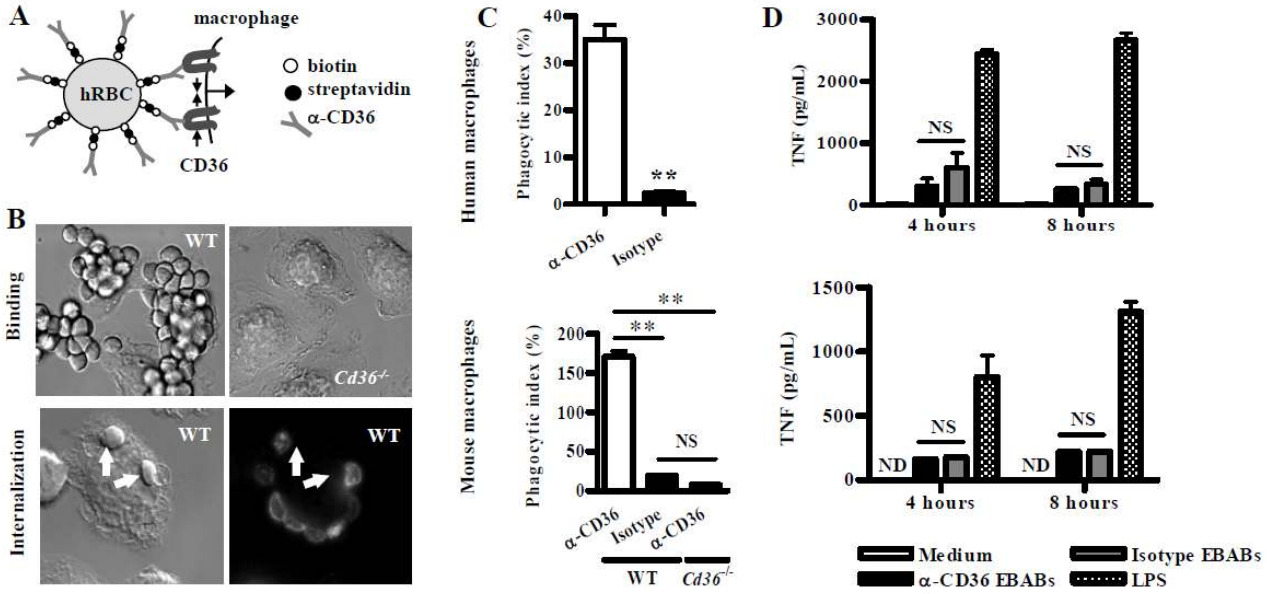


Fig. 2.2: CD36-mediated phagocytosis of large particles does not result in secretion of pro-inflammatory cytokines. (A) Schematic diagram of human and mouse α-CD36 EBABs. α-CD36 antibodies conjugated to human red blood cells (RBC) via biotin and streptavidin cross-link CD36 on the macrophage surface, resulting in internalization. **(B)** Differential interference contrast images of α-CD36 EBABs binding to wild-type and *Cd36*$^{-/-}$ murine macrophages (*upper panels*) and internalization by wild-type macrophages (*lower panels*). After an internalization assay, EBAB uptake was confirmed by staining bound EBABs with a fluorophore-conjugated antibody; following a hypotonic lysis, the ghosts of lysed bound EBABs appeared stained, whereas internalized EBABs (indicated by arrows) did not. **(C)** Human macrophages (*top*) and wild-type and *Cd36*$^{-/-}$ murine macrophages (*bottom*) were incubated with α-CD36 EBABs and EBABs prepared with an isotype-matched control antibody, and phagocytosis was assessed. ** indicates $p<0.01$ by Student's t-test (human data) or one-way ANOVA with Bonferroni post-tests (murine data). **(D)** α-CD36 EBABs or isotype control EBABs were incubated with human (*top*) or murine (*bottom*) macrophages at 37^{o}C for 4 and 8 hr, and TNF in supernatants was measured by ELISA. NS, not significant by Student's t-test. Results are representative of three independent experiments.

2.4.2 CD36-mediated PE internalization does not induce pro-inflammatory cytokine secretion.

We next investigated the inflammatory outcomes of non-opsonic internalization of *P. falciparum* PEs, which is largely mediated by CD36 ([52,53], Suppl. Fig. 2A,B). While selective CD36 activation and internalization did not stimulate cytokine production (Fig. 2.1, 2.2), CD36 can participate in inflammatory responses to bacterial and fungal pathogens via collaboration with TLR2 [375,376,516]. A number of purified *Plasmodium* components have

been characterized as TLR agonists. Of note, TLR2 mediates macrophage TNF and IL-6 responses to purified *Pf*GPI (Suppl. Fig. 2.2C), which function as membrane anchors for parasite proteins [311]. IRAK4, a signalling molecule downstream of most TLRs, is also required for these cytokine responses (Fig. Suppl. 2.2C). Interestingly, *Pf*GPI-induced TNF production was facilitated by CD36 ([67], Suppl. Fig. 2.2C), indicating some degree of co-operation between CD36 and TLR2 in the macrophage response to malaria. However, it is unclear whether, similar to other pathogens [380,381], CD36-mediated internalization of whole PEs induces inflammation in co-operation with TLRs.

To assess the inflammatory outcomes of PE phagocytosis, we incubated human and murine macrophages with PEs or uRBCs as a control, then collected supernatants after 4 hrs. Since mature PEs rupture to release parasite progeny as well as inflammatory parasite components, we used washed, synchronized mid-mature-stage PEs to focus on the effects of intact PE internalization. While LPS stimulated TNF and IL-6 production, neither cytokine was produced by macrophages internalizing PEs or macrophages incubated with uRBCs (Fig. 2.3A; Suppl. Fig. 2.1C), consistent with our previous observations (6, 35). Because macrophage priming by other immune cells is necessary for robust cytokine responses to *P. falciparum* [527], we repeated these experiments using macrophages primed with IFN-γ. This cytokine is produced early in malaria infection and enhances cytokine responses to *Pf*GPI and other TLR agonists [311,520,528]. Priming did not alter the CD36-dependence of PE uptake (data not shown). Although IFN-γ-primed macrophages internalizing PEs did not produce IL-6 (Suppl. Fig. 2.1C), they produced more TNF compared to macrophages incubated with uRBC (Fig. 2.3B). However, this increased TNF production was not a result of CD36-mediated PE internalization, as the same response was observed upon PE incubation with *Cd36*$^{-/-}$ macrophages (Fig. 2.3B). Thus, CD36-mediated PE uptake did not induce pro-inflammatory cytokine secretion despite the

presence of TLR agonists in PEs, indicating a lack of co-operation between CD36 and TLRs in this context.

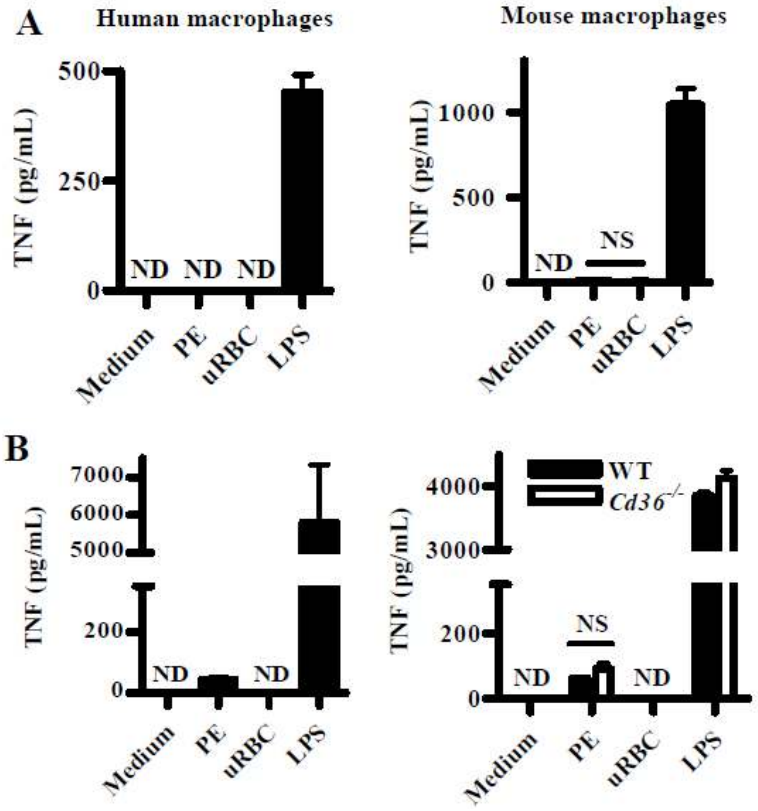


Fig. 2.3: CD36-mediated internalization of *P. falciparum* PEs by macrophages does not stimulate pro-inflammatory cytokine secretion. (A) Human macrophages (*left*) and wild-type murine macrophages (*right*) were incubated for 4 hr at 37°C with carefully synchronized and washed mature-stage PEs, uninfected red blood cells (uRBC), or LPS. Supernatants were collected and analyzed by ELISA for TNF. NS, not significant by Student's t-test. **(B)** To ensure that lack of priming was not obscuring inflammatory consequences of CD36-mediated PE internalization, macrophages were pre-incubated for 12 hr with IFN-γ (100 U/mL) prior to the internalization assay. *Cd36*$^{-/-}$ murine macrophages were included as a control to assess the contribution of CD36-mediated PE internalization to the low levels of TNF production observed. NS, not significant by Student's t-test (human data); *** indicates $p<0.001$ and NS indicates not significant by one-way ANOVA with Bonferroni post-tests (murine data). ND, not detectable. Results are representative of three independent experiments.

2.4.3 CD36-mediated internalization is not impaired by the absence of TLRs, but is enhanced by macrophage pre-treatment with TLR agonists. We next examined whether TLR2 or other TLRs co-operate with CD36 in mediating phagocytosis. Macrophage deficiency

in TLR2 or IRAK4 did not impair internalization of α-CD36 EBABs by murine macrophages (Fig. 2.4A), indicating that TLRs are not essential for CD36-mediated internalization.

As macrophage pre-stimulation with exogenous TLR agonists has been shown to enhance phagocytosis [388], we next treated macrophages with synthetic TLR2 agonists for 1 hr prior to α-CD36 EBAB internalization. Pre-treatment with Pam_3CSK_4 increased EBAB internalization by human (Fig. 2.4B; $p<0.05$) and wild-type murine macrophages (Fig. 2.4C; $p<0.001$) in a TLR2- and IRAK4-dependent manner (Fig. 2.4C). Similar increases were observed upon pre-treatment of human (Fig. 2.4D; $p<0.001$) and murine macrophages (Fig. 2.4E; $p<0.001$) with FSL-1, which stimulates TLR2-mediated inflammation in a CD36-dependent manner [376]. The enhanced uptake was largely CD36-dependent, as TLR2 pre-stimulation of *Cd36*$^{-/-}$ macrophages induced only a small increase in EBAB internalization over baseline compared to wild-type macrophages (Fig. 2.4F). This increase was not statistically significant, although we cannot exclude the minor contribution of other receptors to the observed enhancement of phagocytosis.

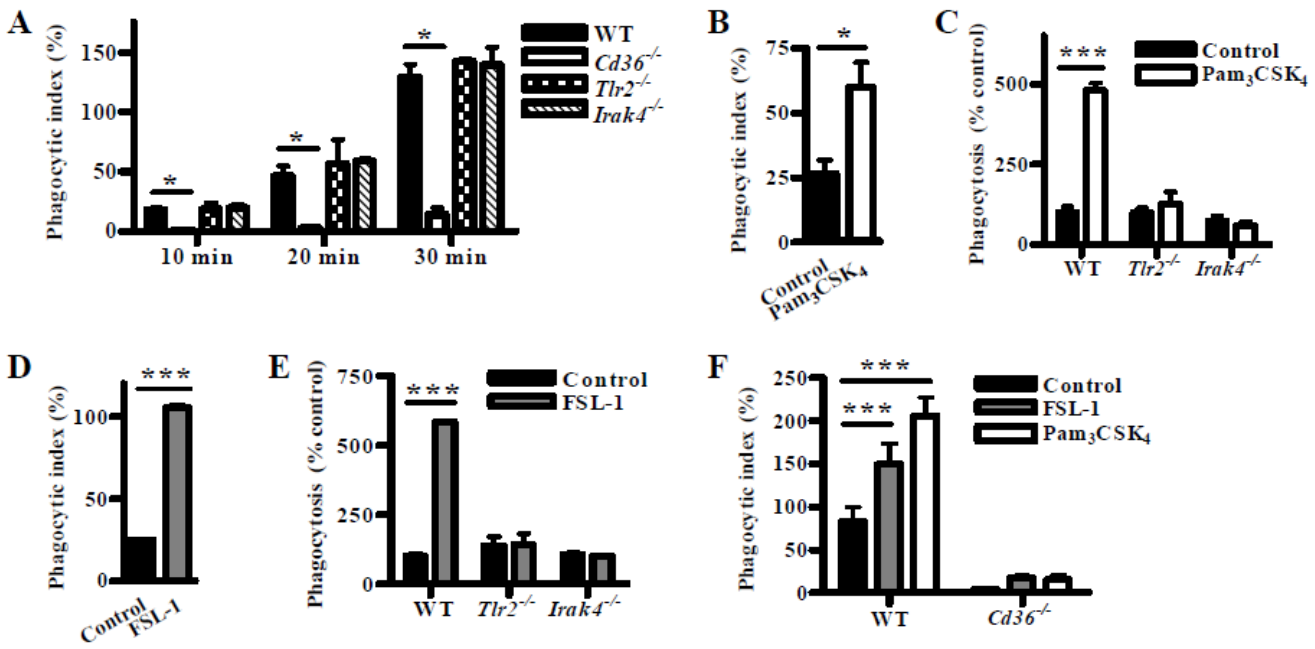


Fig. 2.4: Macrophage pre-stimulation by TLR2 agonists enhances CD36-mediated internalization in a TLR2-, IRAK4-, and CD36-dependent manner. **(A)** Wild-type, *Tlr2*$^{-/-}$, *Irak4*$^{-/-}$, and *Cd36*$^{-/-}$ murine macrophages were incubated with α-CD36 EBABs over a 30 min time course, and phagocytic indices were determined. * indicates $p<0.05$ by one-way ANOVA with Bonferroni post-tests. **(B)** Human and **(C)** murine macrophages were treated with medium alone or TLR2 agonist Pam_3CSK_4 (100 ng/mL) for 1 hr, followed by a 30 min α-CD36 EBAB internalization assay. **(D)** Human and **(E)** murine macrophages were treated with medium alone or CD36-dependent TLR2 agonist FSL-1 (20 ng/mL) for 1 hour prior to α-CD36 EBAB internalization. **(F)** Wild-type and *Cd36*$^{-/-}$ murine macrophages were pre-stimulated with medium alone, Pam_3CSK_4, or FSL-1 for 1 hr prior to α-CD36 EBAB internalization. * indicates $p<0.05$, ** $p<0.01$, and *** $p<0.001$ by Student's t-test (human data) or two-way ANOVA with Bonferroni post-tests (murine data). Data are representative of three independent experiments.

Other reports have demonstrated that TLR stimulation can upregulate expression of scavenger receptors, including CD36 [388,390]. We investigated the possibility that TLR2 activation enhanced CD36-mediated internalization in our system by augmenting surface CD36 levels. However, increased gene expression of CD36 seemed unlikely to play a role, as EBAB uptake was enhanced by FSL-1 and Pam_3CSK_4 pre-treatments as brief as 5 min (Fig. 2.5A). Further, we did not detect any change in surface CD36 levels after incubation with these ligands for 15 min, 1 hr, and 2hr (Fig. 2.5B). Thus, TLR2 stimulation does not enhance CD36-mediated internalization by increasing the number of CD36 receptors on the macrophage surface.

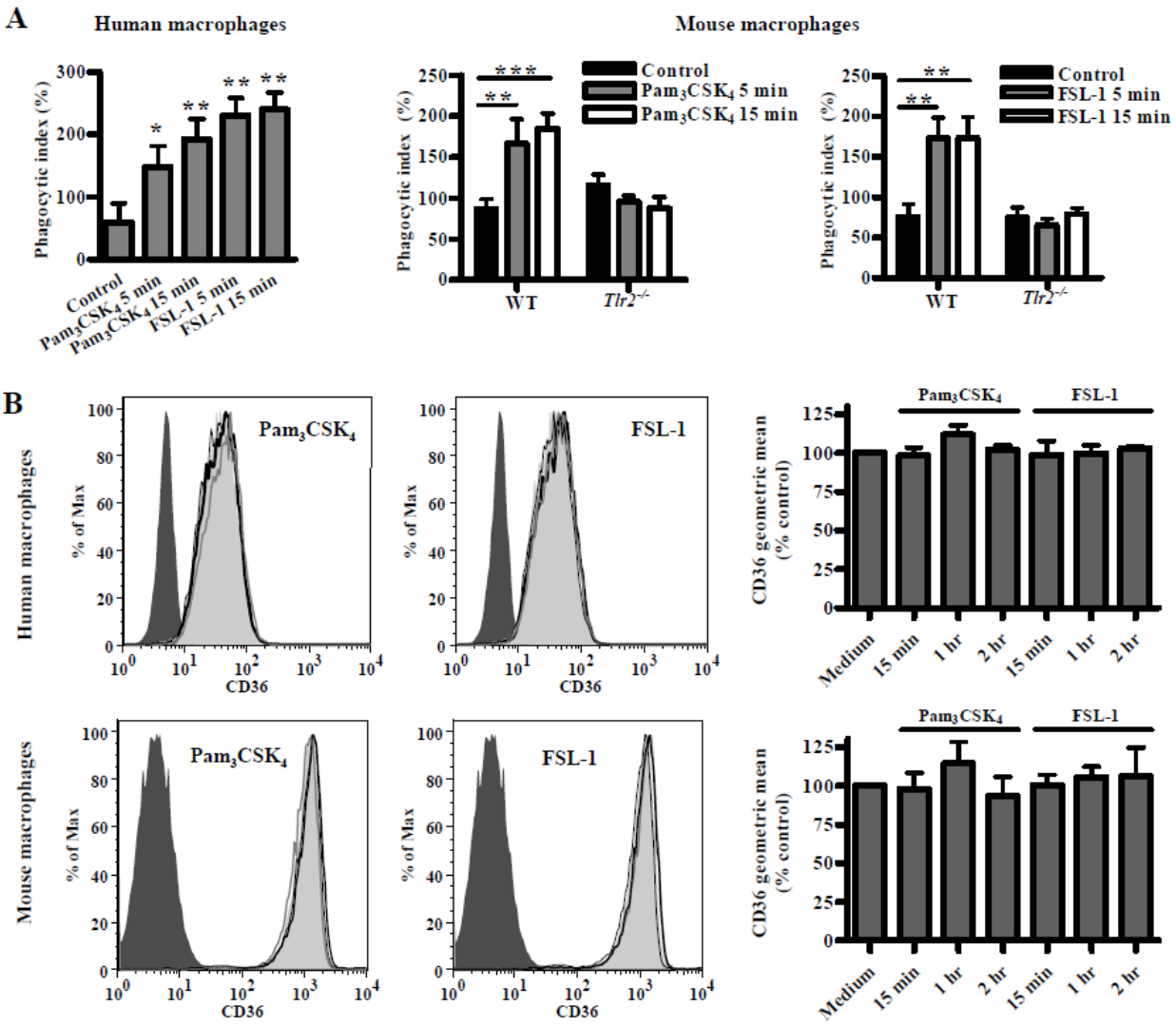


Fig. 2.5: Enhancement of CD36-mediated internalization by TLR2 stimulation occurs with brief pre-treatment periods and is not related to increased surface levels of CD36. (A) Pre-stimulation with FSL-1 and Pam_3CSK_4 for 5 min and 15 min significantly increased uptake of α-CD36 EBABs by human macrophages (*left*) and wild-type murine macrophages, but not *Tlr2*$^{-/-}$ macrophages (*right*). * indicates $p<0.05$, ** $p<0.01$, and *** $p<0.001$ by one-way ANOVA (human data) or two-way ANOVA with Bonferroni post-tests (murine data). **(B)** Human and wild-type murine macrophages were incubated with medium alone (light gray shaded histogram) or with FSL-1 or Pam_3CSK_4 for 15 min (thick black line), 1 hr (gray line), or 2hr (thin black line). Cells were stained for surface CD36 and analyzed by flow cytometry. The dark gray shaded histogram indicates macrophages incubated with the appropriate isotype-matched antibody control. Histogram plots (*left*) show data from a representative experiment. For at least three independent experiments, the geometric means of CD36 levels were normalized to medium alone and pooled (*right*). None of the treatments induced a significant change in CD36 levels as analyzed by one-way ANOVA.

We next examined whether the enhancement of CD36-mediated internalization is specific to TLR2 or if other TLRs can exert a similar effect. Pre-stimulation of human and murine macrophages for 1 hr with LPS, CpG oligonucleotides, and polyI:C (which activate TLR4, TLR9, and TLR3, respectively) significantly enhanced phagocytosis of α-CD36 EBABs (Suppl. Fig. 2.3). Collectively, these data demonstrate that multiple TLRs can co-operate with CD36 to promote phagocytosis.

2.4.4 CD36-mediated PE internalization is not dependent on TLRs, but is enhanced by pre-treatment with TLR2 agonist *Pf*GPI. We next investigated whether TLRs regulate CD36-mediated uptake of *P. falciparum* PEs. Unlike EBABs, PEs contain malaria TLR agonists that may stimulate TLRs and thus modulate uptake. However, uptake of PEs was similar in wild-type, *Tlr2*$^{-/-}$ and *Irak4*$^{-/-}$ macrophages (Fig. 2.6A), even upon IFN-γ pre-treatment for 12 hrs to enhance macrophage TLR responses (data not shown). Thus, TLR2 and other IRAK4-dependent TLRs were not essential for CD36-mediated internalization of PEs.

Although TLRs were not required for PE uptake, we hypothesized that macrophage pre-treatment with the malaria TLR2 agonist *Pf*GPI may modulate PE internalization, similar to TLR2-enhanced α-CD36 EBAB uptake. In support of this premise, human and wild-type murine macrophages pre-treated with purified *Pf*GPI for 1 hr exhibited increased α-CD36 EBAB internalization compared to controls (Fig. 2.6B,C; $p=0.011$ and $p<0.001$, respectively), while uptake by *Tlr2*$^{-/-}$ and *Irak4*$^{-/-}$ macrophages was unaffected (Fig. 2.6C). Similarly, *Pf*GPI pre-treatment for 2 hrs enhanced non-opsonic PE internalization by human (Fig. 2.6D, $p=0.005$) and murine (Fig. 2.6E, $p<0.001$) macrophages in a TLR2- and IRAK4-dependent manner (Fig. 2.6E). Using *Cd36*$^{-/-}$ macrophages, we confirmed that the increased uptake was largely dependent on CD36 (Fig. 2.6C, E), but was not due to a *Pf*GPI-induced increase in surface

levels of CD36 (Suppl. Fig. 4). These data indicate a functional co-operation between CD36 and TLR2 in promoting innate PE internalization by macrophages.

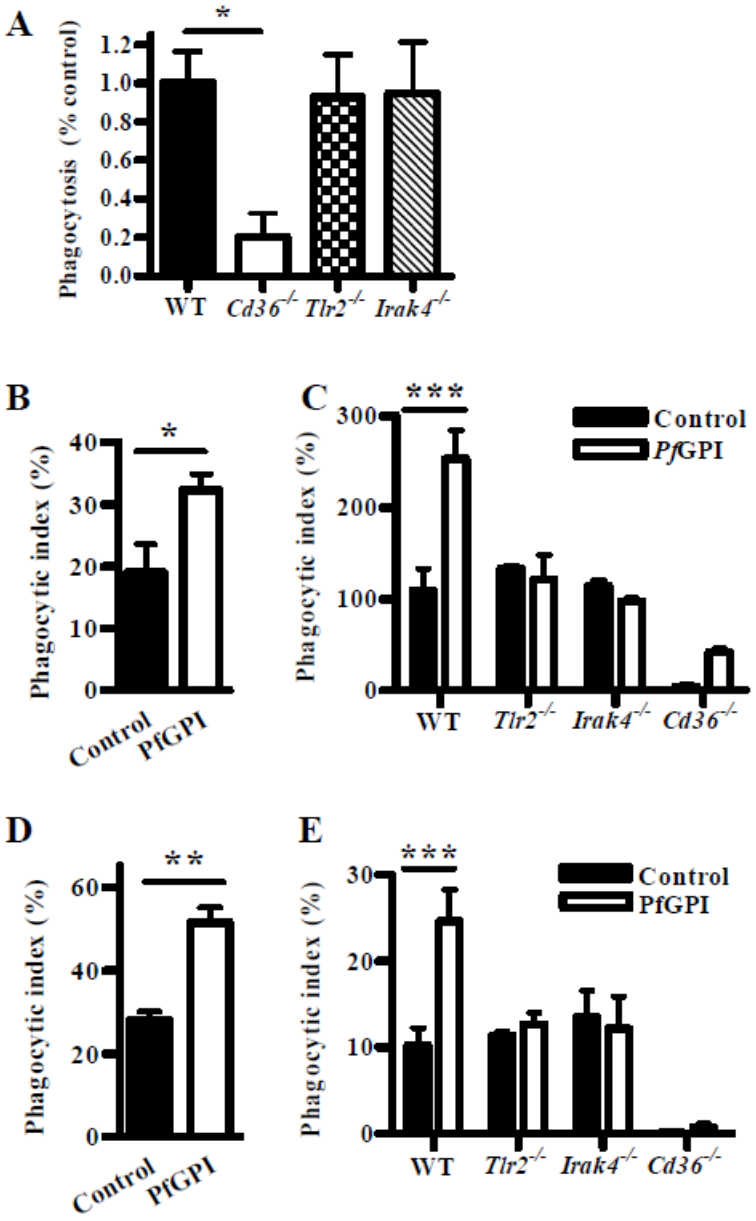


Fig. 2.6: TLRs are not required for CD36-mediated PE internalization, but macrophage pre-stimulation with a malaria TLR2 agonist enhances internalization of α-CD36 EBABs and PEs. **(A)** Wild-type, *Tlr2*$^{-/-}$, *Irak4*$^{-/-}$, and *Cd36*$^{-/-}$ murine macrophages were incubated with *P. falciparum* PEs for 2 hr. Data represent the results of at least 5 independent experiments expressed as a percentage of the phagocytic index of wild-type macrophages and pooled together. * indicates $p<0.05$ by one-way ANOVA with Bonferroni post-tests. **(B)** Human and **(C)** murine macrophages were pre-treated with gold beads alone (control) or HPLC-purified *Pf*GPI (400 ng/mL) for 1 hr, followed by a 30 min α-CD36 EBAB internalization assay. **(D)** Human and **(E)** murine macrophages were pre-treated with *Pf*GPI or control beads for 2 hr, followed by 2 hr PE internalization assay. * indicates $p<0.05$, ** $p<0.01$, and *** $p<0.001$ by Student's t-test (human data) or two-way ANOVA with Bonferroni post-tests (murine data). Results are representative of at least two independent experiments.

2.5 DISCUSSION

2.5.1 CD36 and TLRs: Co-operative roles in inflammation and phagocytosis

The role of CD36 in the regulation of inflammatory responses has been controversial, with some reports implicating direct CD36-mediated pro-inflammatory signalling [405,418] and other reports suggesting a more passive role for CD36 in presenting ligands to TLRs [375]. To address this issue, we used robust model systems to examine selective activation of CD36-induced endocytosis and phagocytosis. We did not observe pro-inflammatory cytokine production in either model (Fig. 2.1, 2.2), indicating that CD36 activation can generate signals to induce internalization without inducing cytokine secretion. Our data support the hypothesis that CD36 participates in inflammation by presenting ligands to other receptors such as TLRs, rather than independently initiating pro-inflammatory signals. This model is consistent with the diverse binding capacities of CD36 [511] and the induction of a physical association between CD36 and TLR2 upon FSL-1 treatment [376]. Notably, pro-inflammatory responses to ligands (e.g. oxLDL, fAβ) that were thought to be directly mediated by CD36 have since been found to be TLR-dependent, with a collaborative role for CD36 [377,529,530].

It was recently reported that transfection of CD36 into TLR2/4-deficient cell lines enhanced IL-8 responses to LPS and Gram negative bacteria, suggesting a role for CD36-mediated TLR-independent pro-inflammatory signalling [418]. However, it is possible that in this system CD36 delivers ligands to other innate sensing receptors present in the cell. We cannot formally exclude a direct contribution of CD36-mediated signalling to cytokine responses initiated by other receptors, and CD36 may be able to independently induce production of specific inflammatory mediators, such as ROS [531]. Nevertheless, in the models we examined, CD36 activation alone

did not produce pro-inflammatory cytokines, suggesting that these responses depend on the ability of CD36 ligands to also activate pro-inflammatory signalling receptors such as TLRs.

While CD36 and TLR2 have been shown to co-operatively induce inflammation [375,376,381,516], we demonstrate here a novel functional co-operation between these receptors in mediating phagocytosis. Although CD36-mediated internalization did not require TLRs, exogenous TLR2 activation enhanced CD36-mediated particle uptake (Fig. 2.4). In our experiments it is unlikely that TLR-mediated transcription of scavenger receptors [388,390] is the basis for the increased phagocytosis, as surface CD36 levels did not change upon TLR2 stimulation (Fig. 2.5), nor did the extent of EBAB binding (data not shown). Cytokines induced by TLRs (e.g. TNF) can promote phagocytosis, but the brief duration of TLR agonist stimulation argues that this is not the major mechanism. Early signalling events via the MyD88-IRAK4 complex appear to be critical for TLR2-mediated enhancement of internalization, as *Irak4*$^{-/-}$ macrophages did not exhibit increased phagocytosis after stimulation with TLR2 agonists. The observation that TLR3 stimulation also promoted uptake indicates that MyD88-IRAK4-independent pathways can mediate this effect, and suggests the involvement of downstream events common to all TLRs.

A number of signalling molecules activated by TLRs participate in phagocytosis, raising the possibility that synergy between TLR- and CD36-mediated signals underlies TLR enhancement of CD36-mediated internalization. For example, MAP kinases p38 and ERK have been implicated in TLR-enhanced internalization [389,393], are also activated by CD36 cross-linking and play a role in CD36-mediated PE internalization [52]. In addition, TLR stimulation activates Rho GTPases [385], which are critical for actin remodelling during phagocytosis. Although some groups have not detected increases in activated Rho GTPases during TLR-enhanced

internalization [389,393], another report using RNAi implicated Rho GTPases and RIG-I in TLR-induced phagocytosis [294,386].

While future studies are required to address the precise underlying mechanism, our findings may have relevance for various biological systems. TLR2 regulation of *S. aureus* phagocytosis [393] may occur via co-operation with CD36, which functions as a phagocytic receptor for this pathogen [381]. Moreover, TLR2 recognizes fAβ [529] and has been implicated in fAβ clearance in a murine model of Alzheimer's disease [532], suggesting that TLR2 and CD36 may collaboratively enhance fAβ phagocytosis.

2.5.2 Co-operation between CD36 and TLRs: Implications for malaria

We extended our studies of CD36-TLR interactions to an *in vitro* malaria-macrophage model in which both CD36 and TLRs are engaged. We found that CD36-mediated PE internalization *per se* did not induce pro-inflammatory cytokine production (Fig. 2.3). On the other hand, our group previously implicated CD36 in TNF production induced by *Pf*GPI [67]. Given the non-inflammatory nature of targeted CD36 activation, we propose that these distinct inflammatory outcomes of CD36 engagement in our malaria-macrophage model are attributable to differential activation of TLRs by distinct CD36 ligands. TLR2 is an essential sensor for *Pf*GPI, and the affinity of CD36 for lipid moieties [511] suggests that it may present this ligand to TLR2. In contrast, CD36-mediated PE binding is non-inflammatory, suggesting that components exposed on the PE surface do not activate TLRs or other pro-inflammatory receptors. Notably, all known malaria TLR agonists are thought to be confined inside the intact PE, and only released upon rupture of mature PEs at the end of the parasite's intraerythrocytic life cycle [533]. Thus, these products are likely inaccessible to cell surface TLRs upon PE binding, and hence would not stimulate inflammation. This is similar to what has been observed for *S. aureus*: since the major

TLR ligand, LTA, is inaccessible in intact PGN, phagocytosis of *S. aureus* and degradation in the phagosome is required for cytokine responses [380]. Additionally, our data suggest that the PE surface components that directly bind macrophage CD36 – likely parasite-encoded variable surface antigens such as PfEMP-1 [52,534] – do not activate TLRs or other pro-inflammatory receptors.

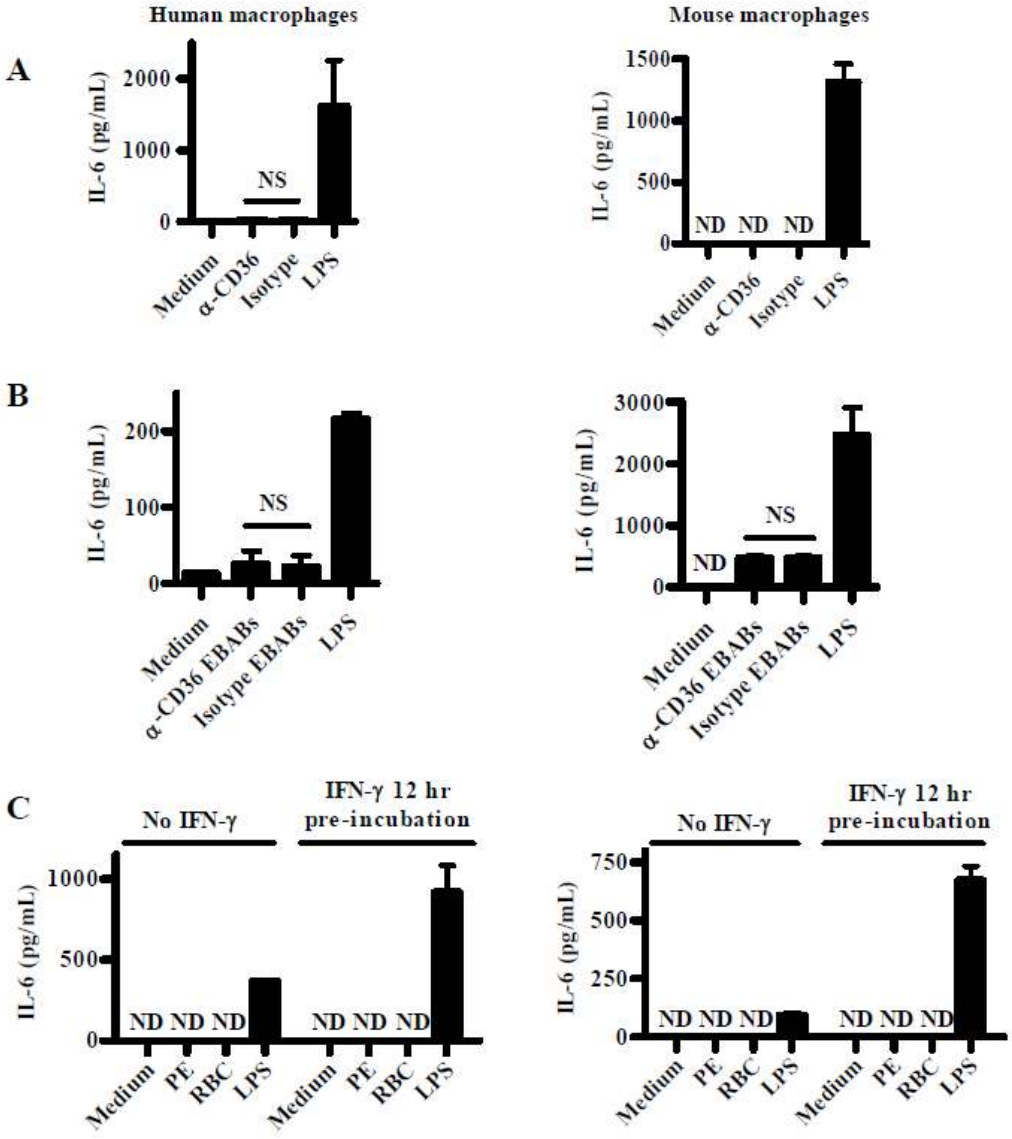
TLRs have been reported to be recruited to phagosomes, where they sample the contents for cognate agonists [378,379]. Thus, one might expect that PE degradation within the phagosome would release malaria TLR agonists and trigger TLR-mediated inflammation, similarly to the *S. aureus* model [380]. If TLRs are present in PE-containing phagosomes, they may not be effectively activated in our model due to rapid degradation of parasite TLR agonists or PE activation of other pathways that dampen TLR signalling. For example, PfEMP-1 was shown to suppress PBMC IFNγ responses to *P. falciparum* in a CD36-independent manner [535], and may have similar immunoregulatory effects on macrophages during PE uptake. Alternatively, TLRs may not be recruited to PE-containing phagosomes. In other systems, TLR2 was found to be recruited to all phagosomes, even in the absence of relevant ligands [378]. However, we were unable to assess the localization of TLR2 during PE uptake due to the low fidelity of available reagents. A recent study reported increased recruitment of TLR9 to phagosomes containing *Aspergillus fumigatus* compared to those containing latex beads, suggesting that signals from phagocytic cargo may actively recruit this TLR [536]. Assessing the presence of TLR9 on PE-containing phagosomes may be worthwhile given the quite consistent implication of this PRR in the inflammatory response to malaria.

We cannot exclude the possibility that our *in vitro* model fails to replicate the immunological environment encountered by macrophages during *in vivo* malaria infection, and that signals

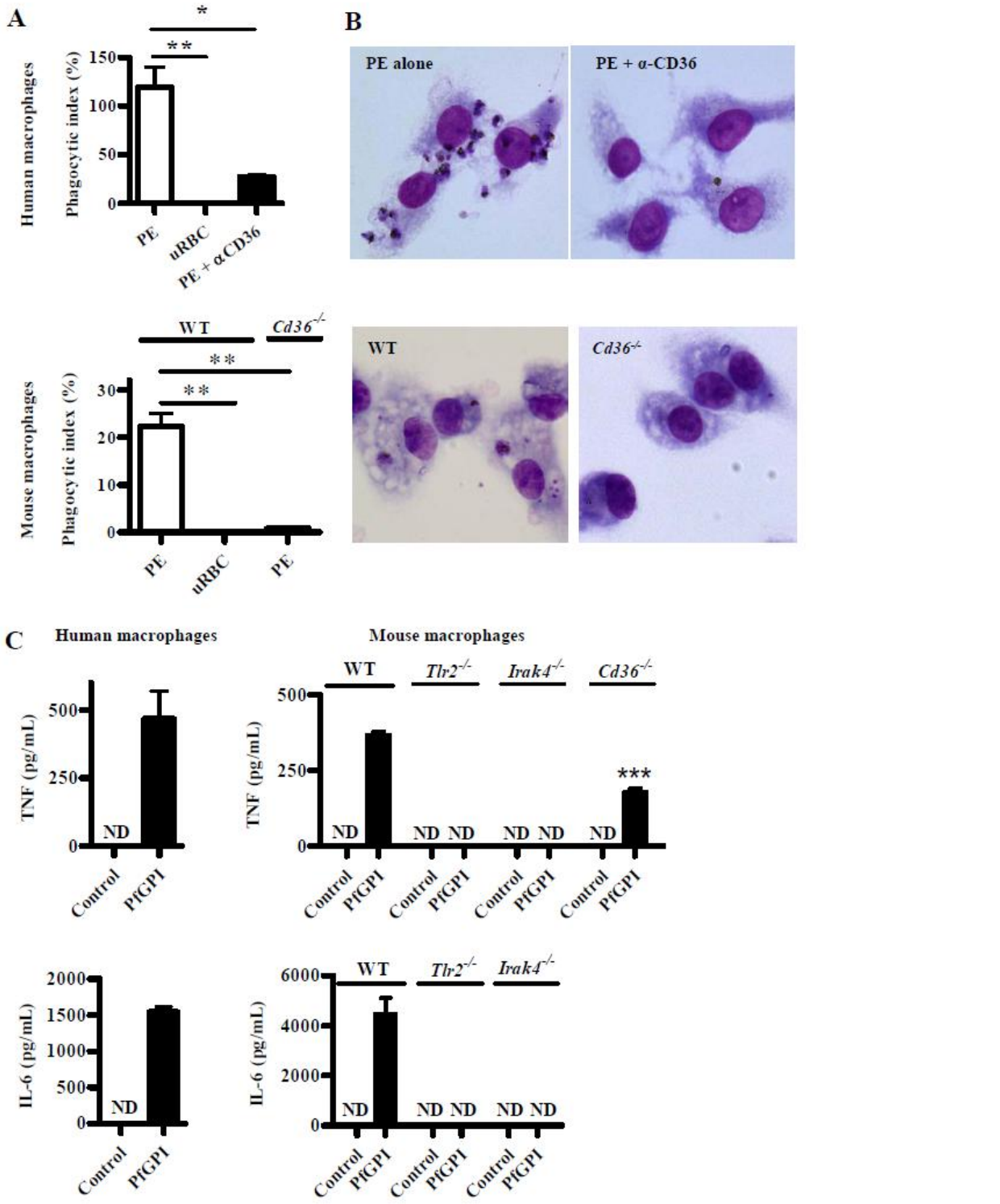
from other cells may increase responsiveness to internalized PEs. However, we did activate macrophages with a high dose of IFNγ. Moreover, similar *in vitro* systems used to investigate other pathogens have shown that microbe binding and/or uptake have pro-inflammatory consequences. This coupling of phagocytosis to inflammation would be expected to strengthen immune defenses by activating additional cells [537]. It is tempting to speculate that *P. falciparum* evolved mechanisms to avoid TLR activation upon innate PE clearance in order to more closely resemble uptake of apoptotic cells. Precedents for such pathogen strategies are common: of relevance, both encapsulation and O-acetylation of PGN were identified as strategies employed by *S. aureus* to inhibit PGN degradation in the phagosome and thus reduce recognition by inflammatory PRRs [380,383]. In the case of malaria, evading TLR activation during phagocytosis would reduce host inflammatory responses and prevent accelerated PE clearance. As excessive production of pro-inflammatory cytokines has been implicated in the pathophysiology of severe malaria [538], non-inflammatory PE uptake may in fact represent a host-pathogen co-evolution to limit immunopathology.

Despite this potential co-evolution to restrict inflammation, the obligate rupture of PEs to disperse parasite progeny concomitantly releases parasite products such as *Pf*GPI that stimulate TLRs and induce inflammatory responses. TLR-mediated inflammation has been linked to severe malaria pathogenesis, leading to proposals for inhibition of TLR pathways as an anti-inflammatory adjunctive therapy [342,343,539]. Here we demonstrate that macrophage TLR2 stimulation with *Pf*GPI enhances PE uptake (Fig. 2.6), indicating that TLRs can co-operate with CD36 to integrate inflammatory and phagocytic responses to malaria. Moreover, our finding suggests a novel and potentially beneficial role for TLRs in the innate immune response to malaria: promotion of PE clearance by macrophages, which has been implicated in the control of acute blood stage parasite replication [46,540-543].

SUPPLEMENTAL FIGURES

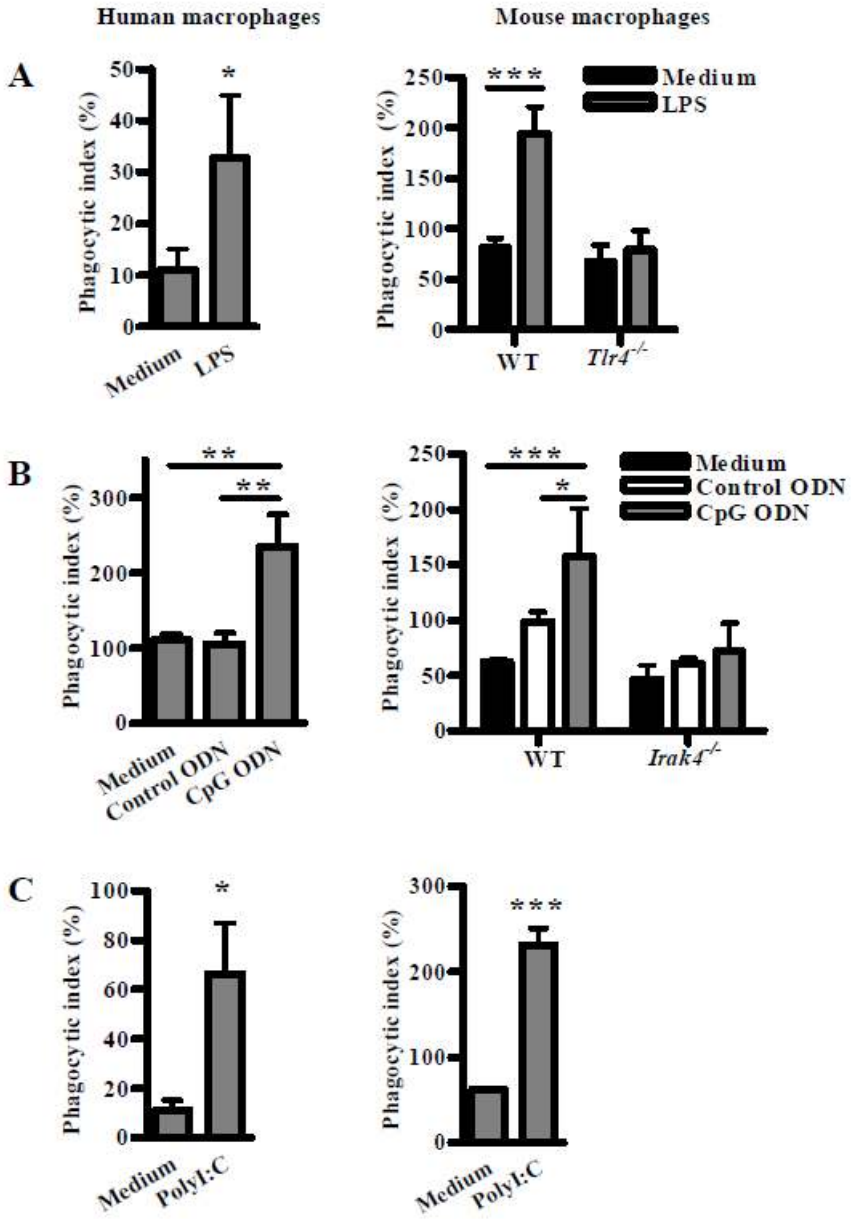


Supplemental Fig. 2.1: Pro-inflammatory cytokine IL-6 is not secreted upon CD36-mediated internalization. Cell-free supernatants were collected from the following studies on primary human and murine macrophages and assayed by ELISA for IL-6 levels: **(A)** Macrophages were incubated with α-CD36 antibody or an isotype-matched control, cross-linked by a secondary antibody, and incubated at 37°C for 24 hrs to assess the inflammatory outcomes of CD36-mediated endocytosis. **(B)** α-CD36 EBABs or EBABs prepared with an isotype-matched control antibody were incubated with human and wild-type murine macrophages for 6 hrs. **(C)** To evaluate the inflammatory consequences of CD36-mediated PE internalization, human and wild-type murine macrophages were incubated for 4 hrs with synchronized mature-stage PEs, uninfected red blood cells (uRBC), or LPS, with or without prior priming with IFN-γ (100 U/mL, 12 hrs). ND, not detectable. NS, not significant by Student's t-test. Results are representative of at least two independent experiments.

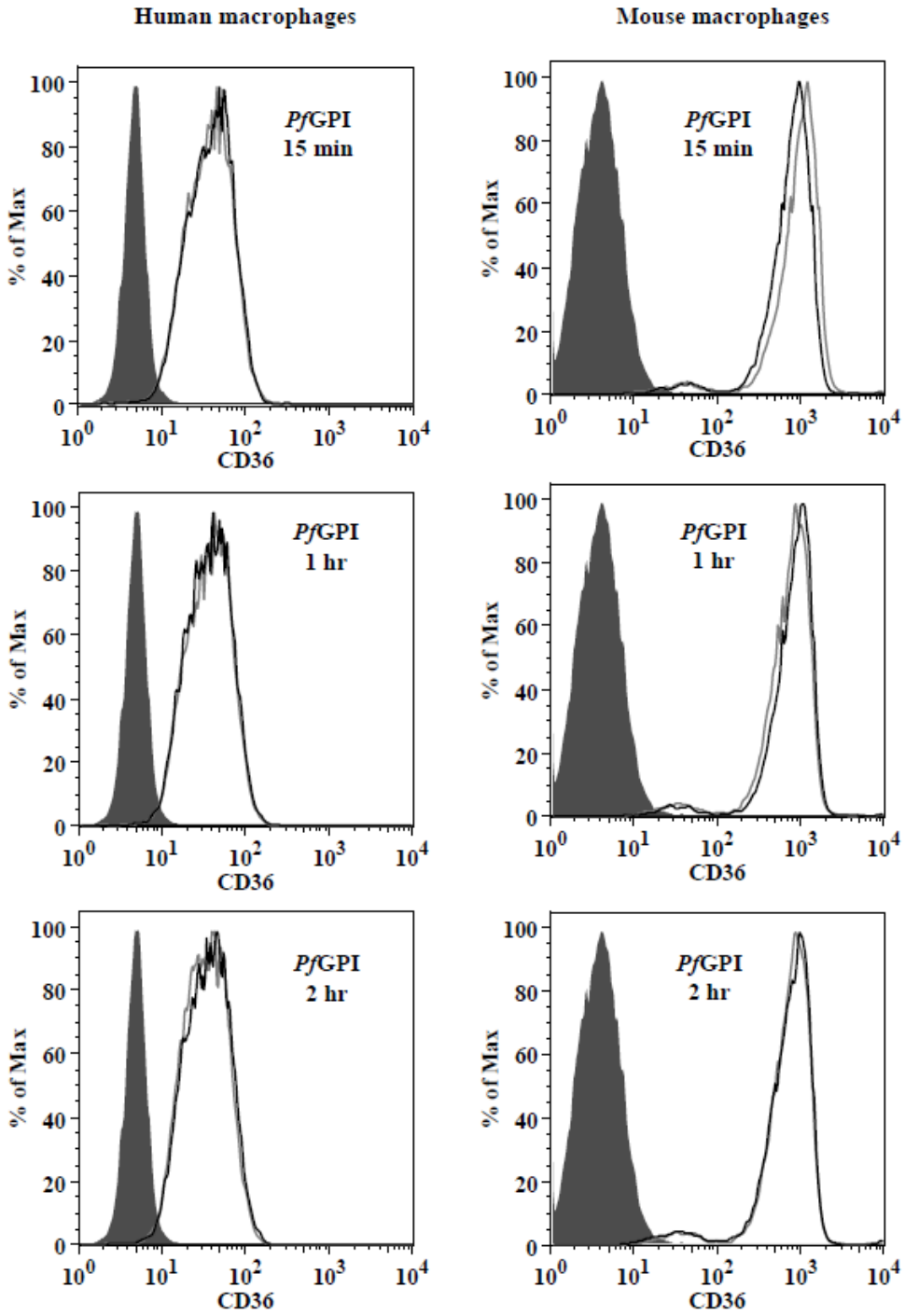


Supplemental Fig. 2.2: CD36 mediates non-opsonic PE internalization, and co-operates with TLR2 in the inflammatory response to *Pf*GPI. (A) Primary human and murine macrophages were incubated with mature-stage *P. falciparum* PEs (ITG strain, PE:macrophage ratio of 20:1) or uninfected red blood cells (uRBC) as a negative control in a 2 hr internalization assay. Human macrophages pre-treated with CD36-blocking antibody (FA6.152 clone) as previously described [52] showed impaired internalization of PEs (*top*), while incubation with an isotype-matched control antibody had no effect (data not shown). PEs were poorly

internalized by *Cd36*$^{-/-}$ murine macrophages compared to their wild-type counterparts (*bottom*). ND, not detectable. * $p<0.05$ and ** $p<0.01$ by one-way ANOVA with Bonferroni post-tests. **(B)** CD36 dependence of PE internalization was apparent in light micrographs of PE internalization by human macrophages incubated with and without α-CD36 antibody (*top*), and by wild-type and *Cd36*$^{-/-}$ murine macrophages (*bottom*). **(C)** HPLC-purified *Pf*GPI (200 ng/mL) was used to stimulate human macrophages and wild-type, *Tlr2*$^{-/-}$, *Irak4*$^{-/-}$ and *Cd36*$^{-/-}$ murine macrophages. Gold particles alone were used as a negative control. After 24 hrs, cell-free supernatants were collected and assayed for TNF and IL-6 by ELISA. Wild-type, *Cd36*$^{-/-}$ and *Tlr2*$^{-/-}$ macrophages responded to LPS to a similar degree, while as expected, *Irak4*$^{-/-}$ macrophages had a reduced response (data not shown). *** indicates $p<0.001$ by two-way ANOVA with Bonferroni post-tests. Results are representative of at least two independent experiments.



Supplemental Fig. 2.3: Pre-stimulation of macrophages with other TLR agonists enhances CD36-mediated internalization. Human and murine macrophages with pre-treated for 1 hour with the following TLR ligands prior to a 30 min α-CD36 EBAB internalization assay: **(A)** TLR4 agonist LPS (100 ng/mL); **(B)** TLR9 agonist CpG oligonucleotides (ODN) (#2006 for human macrophages and #1826 for murine macrophages) and the respective control ODNs (all 10 ug/mL; Invivogen); and **(C)** TLR3 agonist polyI:C (10 ug/mL; Invivogen). Murine macrophages deficient in TLR4 and IRAK4 were included as controls where appropriate. * indicates $p<0.05$, ** $p<0.01$, and *** $p<0.001$ by Student's t-test, or one- or two-way ANOVA with Bonferroni post-tests.



Supplemental Fig. 2.4: Pre-treatment of macrophages with *Pf*GPI does not alter CD36 levels on the macrophage surface. Human and murine macrophages were incubated with *Pf*GPI (black line) or control gold particles (gray line) for 15 min, 1 hr, or 2 hr. Cells were stained for surface CD36 and analyzed by flow cytometry. The shaded histogram indicates macrophages treated with gold particles alone and incubated with the appropriate isotype-matched antibody control.

Chapter 3

Investigating a role for TREM-1 in the inflammatory response to malaria

3.1 ABSTRACT

Dysregulated host pro-inflammatory responses to malaria infection have been implicated in the pathogenesis of severe disease. Toll-like receptors (TLRs) and other innate immune components contribute to these responses, but the full complement of pathways involved remains to be characterized. Triggering receptor expressed on myeloid cells-1 (TREM-1) is a germline receptor on monocytes and neutrophils that is upregulated by TLR stimulation. TREM-1 synergizes with TLRs to induce inflammation, and plays a role in sepsis pathophysiology. We hypothesized that TREM-1 expression is modulated during malaria infection, and that TREM-1 signalling contributes to inflammation and the immunopathology of severe malaria. TREM-1 transcription and release of soluble TREM-1 (sTREM-1; generated by cleavage of membrane TREM-1) were increased following exposure of human peripheral blood mononuclear cells (PBMCs) to *Plasmodium falciparum* PEs. In Ugandan children presenting to hospital with malaria, admission plasma sTREM-1 levels were significantly increased in severe malaria compared to uncomplicated cases, with the highest levels among children who subsequently died from infection. Monocyte surface TREM-1 and plasma TREM-1 were also elevated in a murine model of experimental cerebral malaria. Blockade of TREM-1 by competitive inhibition or genetic deletion did not alter inflammatory responses to malaria *in vitro* or *in vivo*, or affect survival in the murine model. However, activation of TREM-1 by antibody cross-linking significantly enhanced the pro-inflammatory nature of the PBMC response to *P. falciparum* PEs. Thus, TREM-1 expression is modulated during malaria infection but TREM-1 signalling does not appear to critically contribute to inflammatory responses to malaria in the models

examined. The increased pro-inflammatory skew of the response to *P. falciparum* during concomitant TREM-1 activation may have implications for malarial-bacterial co-infection.

3.2 INTRODUCTION

TREM-1, an Ig superfamily member expressed on myeloid cells, is upregulated by TLR stimulation [427,428] and can synergize with TLRs in the production of pro-inflammatory cytokines [420,421,430]. Investigations into a number of inflammatory conditions (sepsis, rheumatoid arthritis, inflammatory bowel disease) appear to link elevated membrane TREM-1 on monocytes, increased soluble TREM-1 (sTREM-1) in serum or plasma, and a causal role for TREM-1 in murine models of disease. In some conditions, sTREM-1 levels are correlated with disease severity [436,443].

Since severe malaria is characterized by dysregulated inflammation, and parasite components activate a number of TLRs [311,338,544], it was not surprising that monocyte TREM-1 levels were found to be elevated in uncomplicated malaria patients compared to healthy controls [445]. However, it is unknown if plasma sTREM-1 is elevated in severe malaria, and whether TREM-1 directly contributes to pro-inflammatory responses and immunopathology in malaria infection. If so, TREM-1 may be a potential target for therapeutic immunomodulation.

To investigate the regulation of TREM-1 upon malaria exposure, we used a PBMC-malaria co-culture system and plasma samples obtained from Ugandan children with either uncomplicated or severe malaria. We further explored the role of TREM-1 in mediating inflammatory responses to malaria *in vitro* and in two models of murine malaria (infection of C57BL/6 mice with *P. chabaudi* and PbA). We found that sTREM-1 was released by human monocytes exposed to *P. falciparum in vitro*, and plasma sTREM-1 levels positively correlated with disease severity in children with malaria. TREM-1 inhibition did not impair inflammatory responses to malaria *in vitro*, and while TREM-1 levels were modulated in murine malaria infection, TREM-

1 blockade did not appreciably alter cytokine production or survival. However, exogenous stimulation of TREM-1 skewed the host response to *P. falciparum* towards a more pro-inflammatory profile.

3.3 METHODS

3.3.1 *P. falciparum* culture. *P. falciparum* (ITG strain) was cultured as previously described [53]. Cultures were treated with Mycoplasma-Removal Agent (MP Biochemicals), confirmed to be mycoplasma-free using the LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich), and synchronized by alanine treatment [521]. Mature-stage cultures were used in co-culture studies.

3.3.2 PBMC-malaria co-culture. Human PBMCs were isolated from blood of healthy volunteers (both male and female) by gradient centrifugation using Ficoll-Paque (GE Healthcare). PBMCs were washed, resuspended in RPMI 1640 with 10% fetal bovine serum and gentamicin (Gibco-Invitrogen; medium R10G), and added to 24 well plates ($1.5x10^6$ PBMCs/well). PBMCs were then stimulated with medium alone, LPS (10 ng/mL), *P. falciparum*-infected RBCs (3: 1 infected RBCs:PBMCs), or uninfected RBCs (0.5 mL/well final volume). All conditions were performed in duplicate or triplicate. After 8 or 24 hours at 37^{o}C, cell-free supernatants were collected and stored at -80^{o}C until analyzed by ELISA (TNF, IL-1β, IL-10, Ebioscience; sTREM-1, R&D). Cells were placed on ice for 15 min, removed gently with a cell scraper, washed twice with cold PBS, and analyzed for TREM-1 mRNA or protein levels. In some experiments, whole blood was diluted 1:5 with R10G and 0.5 mL of the resulting solution was added to each well of a 24-well plate.

3.3.3 Quantification of TREM-1 mRNA. Total RNA was purified from PBMCs using the RNeasy kit (Qiagen). Following DNase treatment of samples (Fermentas), mRNA was converted to cDNA (iScript, Bio-Rad Laboratories) and subjected to quantitative real-time PCR analysis for TREM-1 mRNA levels (cycling protocol: 10 min 95°C, 40 cycles of 95°C 1 sec/60°C 15 sec/72°C 15 sec). Copy number was interpolated from a standard curve of human genomic DNA and normalized to the geometric mean of 3 housekeeping genes (*Hbms*, *Ywhaz*, *B2m*). Primer sequences were as follows:

Trem1-For	5'-TGGTCTTCTCTGTCCTGTTTG-3'
Trem1-Rev	5'-ACTCCCTGCCTTTTACCTC-3'
Hbms-For	5'-ATGACCCACAGTTGGTAGGCATCA-3'
Hbms-Rev	5'-ATCGTTAAGCTGCCGTGCAACATC-3'
Ywhaz-For	5'-ACTTTTGGTACATTGTGGCTTCAA-3'
Ywhaz-Rev	5'-CCGCCAGGACAAACCAGTAT-3'
B2m-For	5'-TGCTGTCTCCATGTTTGATGTATCT-3'
B2m-Rev	5'-TCTCTGCTCCCCACCTCTAAG-3'

3.3.4 TREM-1 flow cytometry. For TREM-1 staining of human monocytes, cells were gently scraped from co-culture wells, washed with flow buffer (PBS, 2% FBS, 5 uM EDTA), and blocked with 10% mouse serum for 15 min at 4°C. Cells were stained for 20 min at 4°C with PE-conjugated mouse-α-human TREM-1 antibody (clone 193015, R&D Systems) or PE-conjugated mouse IgG1 isotype control (Ebioscience), and APC-conjugated mouse-α-human CD14 antibody (clone 61D3, Ebioscience). Cells were washed twice, resuspended in 200 uL of FACS Lysing solution (Becton Dickinson) to ensure RBC lysis. For TREM-1 staining of murine monocytes, mice were euthanized by CO_2 exposure and blood was collected by cardiac puncture into heparinized syringes. Samples were subjected to three rounds of RBC lysis with 1 mL Tris-ammonium chloride solution (pH 7.2) at 37°C for 10 min, followed by 2 PBS washes. $4x10^5$ cells per sample were blocked with mouse serum (as above) and stained for 20 min at 4°C with the following antibodies: rat-α-mouse CD11b-FITC (clone M1/70, Ebioscience), rat-α-mouse

TREM-1-PE (clone 174031, R&D Systems), rat-α-mouse Gr-1-PerCP (clone RB6-8C5, Biolegend), rat-α-mouse F4/80-Alexa 647 (clone BM8, Biolegend). Another set of the samples was stained with all antibodies but α-TREM-1 (TREM-1 FMO). Following washes, cells were stained with Live-Dead Aqua (Molecular Probes) according to the manufacturer's instructions, washed, and fixed in 1% paraformaldehyde. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson), and data analysis was performed using FlowJo software. Human monocytes were defined as CD14-positive cells, and TREM-1 mean fluorescence intensity (MFI) ratios were calculated by dividing the MFI of the TREM-1-stained sample by the MFI of the same sample incubated with isotype control antibody. For murine cells, all dead ($Aqua^+$) cells were excluded. Murine monocyte subsets were defined by their Gr-1 and F4/80 staining properties, and were confirmed to be $CD11b^+$. TREM-1 MFI ratios were calculated using the TREM-1 FMO.

3.3.5 TREM-1 activation by antibody crosslinking. Wells of 24 well plates were coated overnight at room temperature with monoclonal mouse-α-human TREM-1 antibody (R&D Systems) or mouse IgG isotype antibody (5 ug/mL, 0.25 mL/well). The following day, wells were washed twice with PBS, and freshly isolated PBMCs were added. Cells were allowed to settle for 2 hours at 37^{o}C, and then stimulated with malaria or controls as outlined above. *P. falciparum*-infected RBCs were purified using an 80% Percoll gradient (to minimize steric interference with TREM-1 crosslinking of monocytes) and added at a 3:1 RBC:PBMC ratio.

3.3.6 Measurement of soluble TREM-1 in pediatric malaria samples. As part of a large case-control study, admission pre-treatment plasma samples were obtained from children with malaria infection presenting to Mulago hospital in Kampala, Uganda between October 2007 and October 2009. Mulago Hospital is a national referral hospital that serves Kampala and

surrounding districts. Malaria transmission in this region and the patient population at Mulago Hospital have been previously described [545]. Children presenting to hospital were eligible for enrollment if they were between 6 months and 12 years old and had microscopy-confirmed *P. falciparum* infection (asexual parasitemia with clinical signs or symptoms of malaria). Participants were categorized as having severe malaria if they fulfilled at least one of a number of WHO criteria, including CM and SMA [13]. CM was defined as an unrousable coma (not attributable to any other cause) in a child with asexual *P. falciparum* parasitemia (i.e. Blantyre Coma Scale score <3, either before or >6 h after seizures or anticonvulsant medication (if applicable), or repeated (>3) seizures witnessed within a 24 h period, in the absence of hypoglycaemia (<40 mg/dL or 2.2 mM) or any known alternative neurologic abnormalities). SMA was defined as hematocrit <15% or hemoglobin <5.0 g/dL in the presence of asexual parasitemia. Six children had concurrent CM and SMA and were categorized as "CM", and five children with SMA exhibited decreased consciousness but did not meet study criteria for CM. Children were excluded if they had sickle cell trait/disease, HIV co-infection, or severe malnutrition.

Clinical and demographic data were collected upon enrollment, and venous blood samples were collected for routine measurement of hemoglobin and platelet count (EDTA anticoagulant), and for plasma banking (sodium citrate anticoagulant, stored at -20^{o}C prior to testing). Thin blood smears were obtained at presentation for determination of parasitemia, which is reported as the arithmetic mean of two independent readings by expert microscopists. Treatment was in accordance with Ugandan national guidelines: artemether/lumefantrine was administered to children with uncomplicated malaria, and parenteral quinine was used in severe malaria cases [546]. All SMA cases received blood transfusions. Children were followed for recovery or death.

For sTREM-1 analysis, a sub-group (n=156) of UM outpatients, CM inpatients, and SMA inpatients in roughly equal numbers was selected from the larger study based on availability of an adequate volume of previously unthawed plasma. A commercially available sTREM-1 ELISA (Quantikine kit, R&D Systems) was used to quantify sTREM-1 levels. Testing was performed according to the manufacturer's instructions and blinded to all associated clinical data. Background signal was determined from blank wells included on each plate (assay buffer added instead of sample), and background optical density was subtracted from all samples and standards prior to analysis. Samples with optical densities below the lowest detectable standard were assigned the value of that standard.

Ethical approval for the study was obtained from the Mulago Hospital Research Ethics Committee, Makerere University Faculty of Medicine Research Ethics Committee, Uganda National Council for Science & Technology, and the University Health Network. Written informed consent was obtained from parents/guardians before enrollment.

3.3.7 Murine models of malaria. Female C57BL/6 mice were purchased from Charles River. *Trem-1*$^{+/-}$ mice were obtained from Wyeth/Pfizer and bred in the University of Toronto animal facility to generate *Trem-1*$^{-/-}$ mice and wild-type littermate controls. Animal protocols were approved by the Animal Care Committee of the University of Toronto and all animal work was performed in compliance with university institutional guidelines. Frozen stocks of *P. chabaudi chabaudi* AS (*P. chabaudi*) or PbA were passaged through a C57BL/6 mouse to obtain infected blood. Experimental mice (6-10 weeks old) were administered $1x10^6$ infected RBCs by intraperitoneal (i.p.) injection. Daily tail vein blood smears were made and stained with Hema-3 Stain Set (Fisher Scientific) to determine parasitemia. Saphenous vein bleeds were performed on

Day 0 and Day 5 of infection to obtain plasma for cytokine analysis by ELISA (IFNγ, Ebioscience) or Cytometric Bead Array (Mouse Inflammation Kit, Becton Dickinson). Mice were closely monitored and humanely euthanized if moribund, as defined in approved animal use protocols. Where indicated, replication-deficient human type 5 adenoviral vectors coding for murine Fc-TREM-1 or Fc alone were injected intramuscularly on the day prior to infection ($1x10^9$ p.f.u. in 60 uL sterile PBS, 30 uL injected into each leg; PBS alone used as a further control). In other experiments, LP17 (LQVTDSGLYRCVIYHPP) or scrambled control peptide (TDSRCVIGLYHPPLQVY; Pepscan) were resuspended in sterile PBS, and 100 ug of peptide was administered by i.p injection daily starting on the day of infection (4 hours prior to infection).

3.3.8 Statistical analysis. Analysis was performed using GraphPad Prism software. Differences between groups with non-normal distributions were analyzed using the Mann-Whitney U test or the Kruskal-Wallis test with Dunn's post-tests. Spearman's rho was used to assess non-parametric correlations. Synergy in cytokine production was determined by a significant interaction term upon performing a two-way ANOVA test. For *in vitro* experiments, each condition was performed in duplicate or triplicate. Survival curves were compared using the log-rank test.

3.4 RESULTS

3.4.1 Modulation of monocyte TREM-1 upon stimulation with *Plasmodium falciparum*.

To gain insight into regulation of TREM-1 during malaria infection, we exposed human PBMCs to *P. falciparum* PEs and measured levels of TREM-1 mRNA, surface protein on monocytes, and soluble protein in supernatants after 24 hrs. This system specifically evaluated modulation of monocyte TREM-1 since neutrophils are excluded during PBMC preparation. As a control, PBMCs were stimulated with LPS, which led to increased TREM-1 transcription, monocyte surface expression of TREM-1, and sTREM-1 in the culture supernatant (Fig. 3.1A,B,C, respectively). Upon incubation with *P. falciparum*, TREM-1 transcription was upregulated compared to incubation with uninfected RBCs ($p<0.05$; Fig. 3.1A), although to a lesser extent than LPS. Malaria exposure did not alter surface TREM-1 levels (Fig. 3.1B), but caused an increase in sTREM-1 at 24 hours ($p<0.05$; Fig. 3.1C).

The absence of an increase in surface levels of monocyte TREM-1 after *P. falciparum* exposure was unexpected, in light of upregulated transcription under the same conditions and elevated monocyte TREM-1 in human malaria infection [445]. We evaluated TREM-1 levels at 8 and 48 hours, but did not observe any changes at these timepoints either (data not shown). Nevertheless, in this *in vitro* system, *P. falciparum* modulated monocyte TREM-1 at the level of transcription and receptor cleavage.

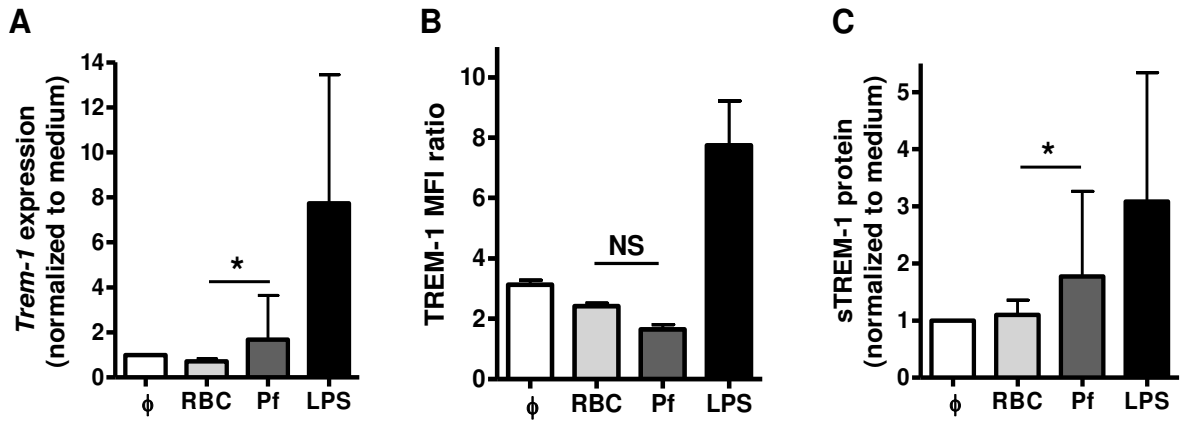


Fig. 3.1: *P. falciparum* modulation of human monocyte TREM-1. Human PBMCs were exposed to medium alone (Φ), uninfected red blood cells (RBCs; negative control), *P. falciparum* infected RBCs (Pf), or LPS (positive control) for 24 hrs. (A) TREM-1 mRNA was assessed by quantitative real-time PCR, (B) TREM-1 surface levels on $CD14^+$ monocytes were examined by flow cytometry, and (C) sTREM-1 in supernatants was measured by ELISA. Graphs represent pooled data from at least 3 independent experiments using different PBMC donors. Data are presented as medians with interquartile ranges. * $p<0.05$, Kruskal-Wallis test with Dunn's post-tests.

3.4.2 Plasma levels of soluble TREM-1 in children with uncomplicated and severe malaria.

To examine whether our *in vitro* data correlated with human malaria infection, we investigated TREM-1 regulation in Ugandan children with uncomplicated malaria (UM) or severe malaria (CM or SMA). Although we were unable to obtain peripheral blood cells for assessment of monocyte TREM-1, we quantified sTREM-1 by ELISA in plasma samples taken upon presentation to hospital. We hypothesized that sTREM-1 would be increased in severe malaria since *P. falciparum* stimulates sTREM-1 release and severe malaria is associated with higher and/or more chronic parasite burdens [10,33].

Table 1 presents the demographic and clinical characteristics of study participants (UM, n=53; CM, n=44; SMA, n=59). As expected, children with severe malaria had lower hemoglobin

levels and platelet counts than children with UM. Children with SMA were younger than children with UM and CM ($p<0.001$) and presented significantly later than the other groups ($p<0.001$, approximately one day later). We found that plasma sTREM-1 levels were elevated in children with CM and SMA compared to those with UM ($p<0.001$; Fig. 3.2A).

Table 3.1: Demographic and clinical characteristics of study participants presenting with uncomplicated and severe malaria.[a]

Characteristic	UM[b] (n=53)	CM[c] (n=44)	SMA (n=59)
Gender (% female)	45.3	52.3	49.2
Age (years)	4.4 (2.1, 8.1)	3.0 (1.5, 4.3)	1.3 (0.9, 2.0)$^{***\#\#\#}$
Days reported ill prior to presentation	3 (2, 4)	3 (2, 4)	4 (3, 5)$^{***\ \#}$
Parasitemia (parasites/uL)	$3.8x10^4$ ($1.6x10^4$, $1.2x10^5$)	$9.8x10^4$ ($1.5x10^4$, $2.7x10^5$)	$2.6x10^4$ ($7.4x10^3$, $1.2x10^5$)$^{\#}$
Hemoglobin (g/dL)	10.1 (9.4, 11.3)	6.3 (5. 3, 8.4)***	3.8 (3.2, 4.4)$^{***\#\#\#}$
Platelet count ($x10^9$/L)	166 (107, 219)	73 (47, 128)***	116 (71, 165)*
Fatal cases	0	14	9

[a]All variables except gender are presented as median (interquartile range). Groups were compared using Kruskal-Wallis test with Dunn's post-hoc tests (continuous variables) or Chi-square test (categorical variables). [b]UM, uncomplicated malaria; CM, cerebral malaria; SMA, severe malaria anemia. [c]6 children with concurrent CM and SMA were included in the CM group. 5 children with SMA exhibited decreased consciousness but did not meet criteria for CM. * $p<0.05$, *** $p<0.001$ CM or SMA vs. UM. $^{\#}$ $p<0.05$, $^{\#\#\#}$ $p<0.001$ SMA vs. CM.

Table 3.2: Demographic and clinical characteristics of severe malaria patients segregated by survival.[a]

	CM[b]		SMA	
Characteristic	Survivors (n=30)	Deaths (n=14)	Survivors (n=50)	Deaths (n=9)
Gender (% female)	43.3	71.4***	48.0	55.6
Age (years)	3.1 (1.3, 4.9)	2.8 (1.4, 3.7)	1.3 (0.9, 2.2)	1.3 (1.0, 1.8)
Days reported ill prior to presentation	3 (3, 4)	2 (2, 4)	4 (3, 5)	5 (3, 7)
Parasitemia (parasites/uL)	$6.3x10^4$ ($1.1x10^4$, $2.5x10^5$)	$2.4x10^5$ ($2.4x10^4$, $5.7x10^5$)	$2.3x10^4$ ($5.9x10^3$, $9.2x10^4$)	$1.2x10^5$ ($1.5x10^4$, $2.7x10^5$)
Hemoglobin (g/dL)	6.2 (5.2, 8.1)	7.0 (5.4, 9.9)	3.8 (3.2, 4.4)	4.2 (3.4, 4.6)
Platelet count ($x10^9$/L)	73 (46, 121)	70 (40, 156)	124 (82, 177)	86 (38, 136)*

[a]All variables except gender are presented as median (interquartile range). Groups were compared using the Mann Whitney U test (continuous variables) or Chi-square test (categorical variables). [b]CM, cerebral malaria; SMA, severe malaria anemia. * $p<0.05$, *** $p<0.01$ survivors vs. fatalities within each severe malaria syndrome.

We next examined children with severe malaria to determine whether sTREM-1 levels were altered in fatal cases. Patient characteristics are shown in Table 2. Median plasma sTREM-1 levels at presentation were increased in those who subsequently died of infection compared to those who survived (Fig. 3.2B; $p<0.05$ for CM, $p<0.01$ for SMA). These results indicate that TREM-1 is differentially regulated in uncomplicated and severe malaria, and that plasma sTREM-1 correlates with infection severity in this population.

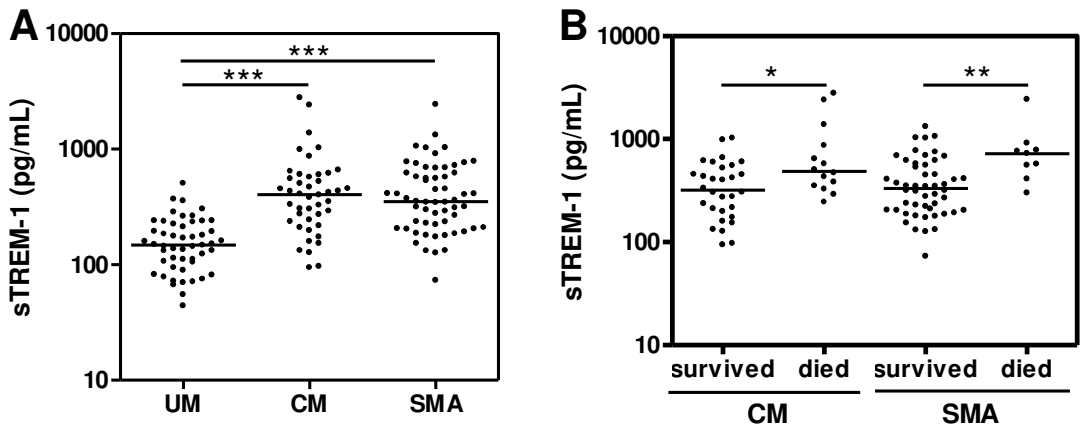


Fig. 3.2: Soluble TREM-1 is elevated in plasma of children with severe and fatal malaria at time of presentation. (A) sTREM-1 was measured by ELISA in children with uncomplicated malaria (UM), cerebral malaria (CM), and severe malarial anemia (SMA). (B) sTREM-1 levels are shown segregated by survival for children with CM and SMA groups. Data are presented as dot plots with medians. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ by Kruskal-Wallis test with Dunn's post-hoc tests.

3.4.3 TREM-1 inhibition *in vitro:* effects on malaria-induced inflammation. We next tested whether TREM-1 contributes to the inflammatory response to malaria *in vitro*. Prior to co-culture with *P. falciparum*, PBMCs were incubated for 1 hour with competitive inhibitor Fc-TREM-1. The Fc domain alone was used as a control. Cytokine levels in the supernatant after 8 hours were measured by ELISA. Fc-TREM-1 did not alter production of TNF (Fig. 3.3A) or IL-1β (Fig. 3.3B) in response to *P. falciparum*, but did block TNF responses to antibody-mediated crosslinking of TREM-1 as expected (Fig. 3.3D).

A TREM-1 ligand has been identified on human platelets [423], but PBMC preparations do not contain platelets. Therefore, we repeated the above assay using diluted whole blood in order to ensure the presence of this TREM-1 ligand in our system. However, we did not observe an effect of Fc-TREM-1 on the cytokine response to *P. falciparum* in whole blood (Fig. 3.3C).

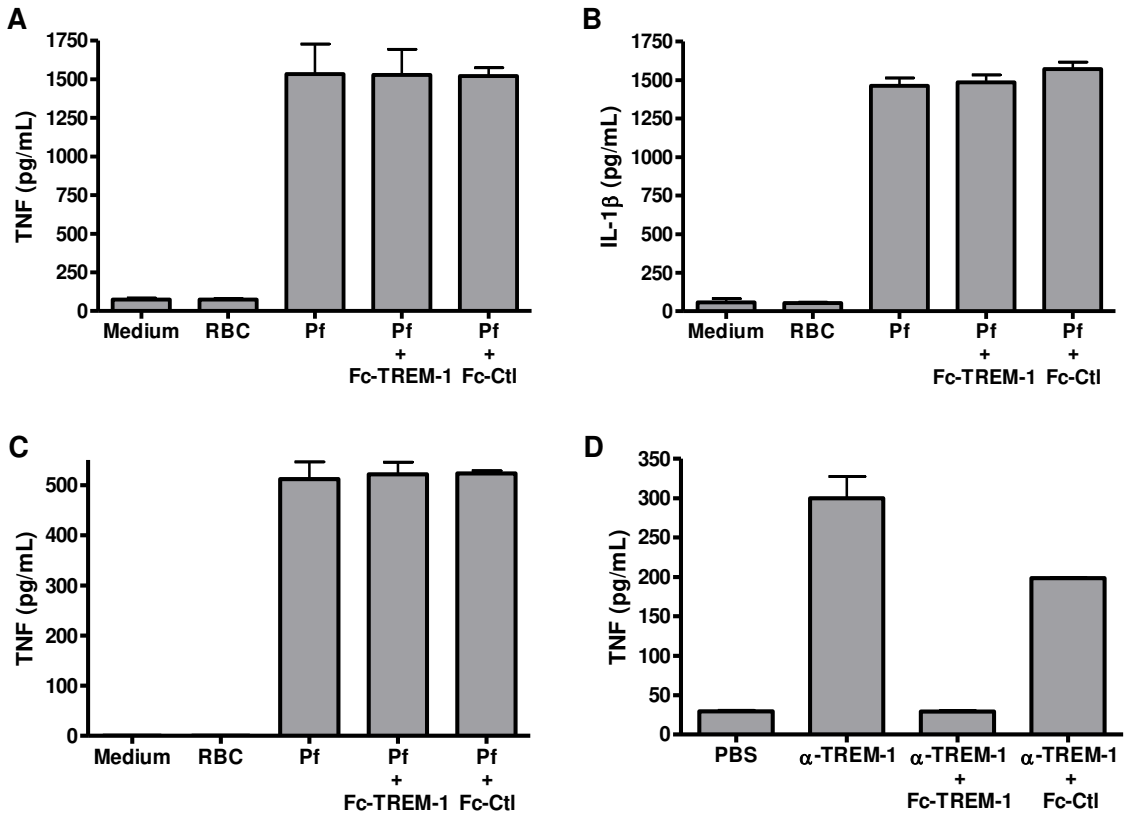


Fig. 3.3: Competitive inhibitor of TREM-1 (Fc-TREM-1) does not affect the pro-inflammatory response of PBMCs to *P. falciparum*. (A, B) PBMCs were incubated with Fc-TREM-1 or Fc alone (Fc-Ctl) 1 hour prior to co-culture with malaria. Supernatants were collected at 8 hrs and cytokine levels were assessed by ELISA. (C) The experiment was repeated using diluted whole blood as described in Materials and Methods. (D) The Fc-TREM-1 reagent effectively inhibits TNF secretion in response to TREM-1 crosslinking (achieved by pre-coating wells with α-TREM-1 antibody). Data are representative of at least 2 independent experiments.

3.4.4 TREM-1 modulation in PbA-induced experimental cerebral malaria. Although TREM-1 blockade did not decrease malaria-induced inflammation *in vitro*, this model may not fully recapitulate the role of TREM-1 *in vivo*. TREM-1 ligands could potentially originate from or require induction by the non-hematopoietic compartment. Therefore, we undertook studies in the PbA model of ECM to further investigate whether TREM-1 contributes to inflammatory responses to malaria.

We first investigated TREM-1 regulation in PbA infection of C57BL/6 mice, which are susceptible to ECM. We measured monocyte TREM-1 levels on Day 5 post-infection just prior to development of neurological signs. A previous report demonstrated that TREM-1 is differently expressed on the two murine monocyte subsets at baseline and during systemic endotoxemia [415]. The inflammatory monocyte subset ($F4/80^{+}Gr\text{-}1^{hi}$), which rapidly accumulates in the blood during inflammation and is recruited to inflamed tissues, expressed very little TREM-1 at baseline, but levels increased upon endotoxemia induction. The other subset, termed resident monocytes ($F4/80^{+}Gr\text{-}1^{lo}$), patrols blood vessels and extravasates into tissues early in inflammation. These cells were found to express TREM-1 at baseline, but mostly disappeared from the blood during endotoxemia.

We confirmed higher expression of TREM-1 on resident versus inflammatory monocytes in uninfected mice (Fig. 3.4A). Similar to the endotoxin model, resident monocytes decreased in the blood during PbA infection (Fig. 3.4B). For inflammatory monocytes, both TREM-1 mean fluorescence intensity (MFI) (Fig. 3.4C) and percent of TREM-1-expressing cells (Fig. 3.4D) were increased in infected versus uninfected mice. Correlation analysis demonstrated a strong

relationship between both of these parameters and parasitemia at time of euthanasia ($r = \sim 0.75$, Fig.3.4E and data not shown), indicating that surface levels of monocyte TREM-1 increase as PbA infection progresses.

We also measured plasma sTREM-1 levels on Day 5 post-infection and found that they were significantly elevated in infected versus uninfected mice ($p<0.01$; Fig. 3.4F). Thus, both membrane and soluble TREM-1 levels appear to be modulated in PbA infection, with parallels to observations in malaria-infected humans (Fig. 3.2, [445]).

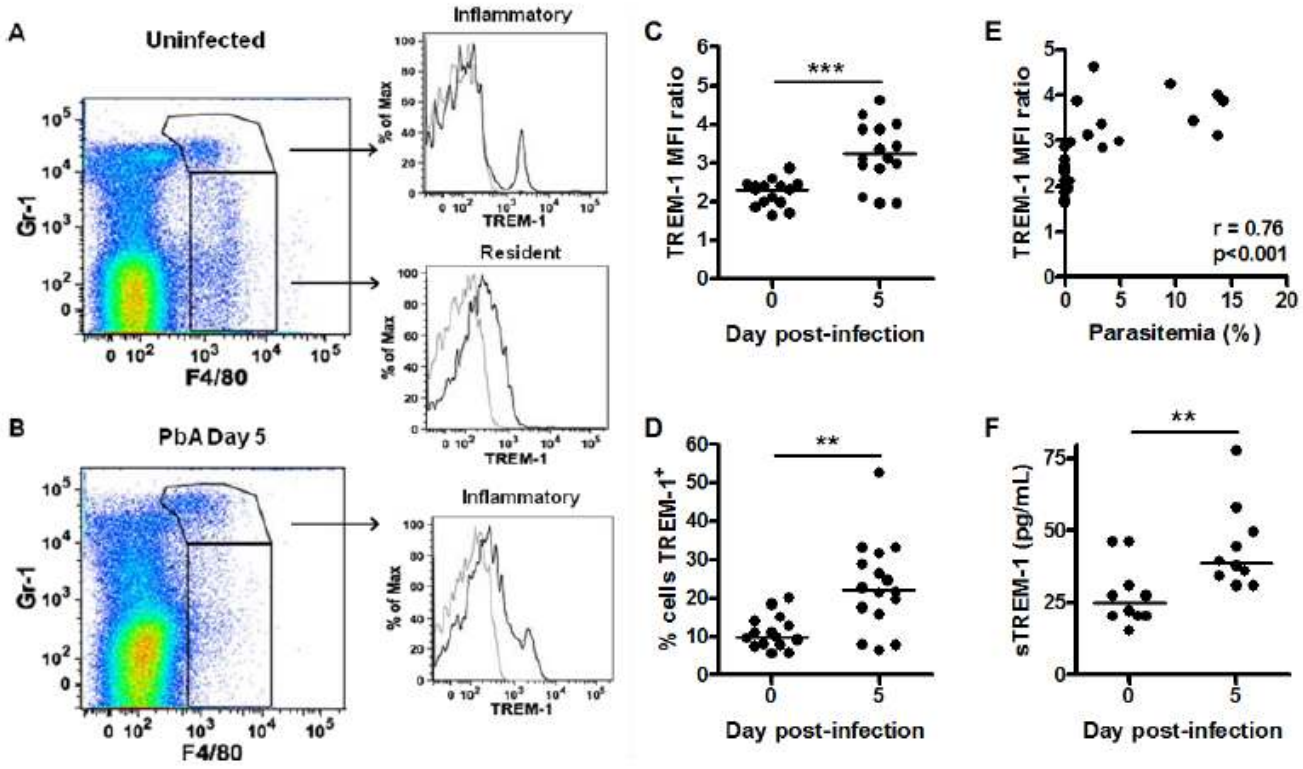


Fig. 3.4: TREM-1 levels are modulated during PbA infection of C57BL/6 mice. Representative plots of viable cells are shown for (A) an uninfected and (B) a PbA-infected mouse 5 days post-infection. Gates for inflammatory ($F4/80^+Gr\text{-}1^+$) and resident ($F4/80^+Gr\text{-}1^-$) monocyte subsets are displayed, as well as histograms of TREM-1 expression on each subset. For inflammatory monocytes, (C) TREM-1 MFI ratio and (D) percent of TREM-1 positive cells are compared between uninfected and infected mice. (E) TREM-1 MFI ratio is correlated to the percent parasitemia (assessed using Spearman's rho). (F) Plasma sTREM-1 is elevated 5 days post-PbA infection. Data from two independent experiments were pooled. ** $p<0.01$, *** $p<0.001$ by Mann-Whitney U test.

3.4.5 TREM-1 inhibition in murine models of malaria. We proceeded to determine whether TREM-1 contributes to the inflammatory response in the PbA model. Since inflammation is a key component of ECM pathogenesis, we hypothesized that TREM-1 inhibition would decrease pro-inflammatory cytokines and potentially improve survival. To block TREM-1, we administered Fc-TREM-1 using an adenoviral expression vector (Ad-TREM-1). Intramuscular injection of Ad-TREM-1 has been shown to sustain detectable blood levels of Fc-TREM-1 for 10

days [547]. One day prior to infection with PbA, C57Bl/6 mice were injected with Ad-TREM-1, a control adenovirus encoding Fc alone (Ad-Ctl), or PBS. We confirmed high plasma levels of Fc-TREM-1 the day following injection of Ad-TREM-1 by ELISA (data not shown), then infected mice with PbA.

There was no difference in survival (Fig. 3.5A), parasitemia (data not shown), or Day 5 plasma IFNγ levels (Fig. 3.5D) between mice injected with PBS, Ad-Ctl, or Ad-TREM-1. Similar results were obtained upon daily injection of the TREM-1 inhibitory peptide LP17 or a scrambled peptide control (Fig. 3.5B,E). Mice with genetic deficiency in TREM-1 showed comparable survival (Fig. 3.5C) and production of IFNγ (Fig. 3.5F), TNF, IL-6, and IL-10 (data not shown) to wild-type littermate controls.

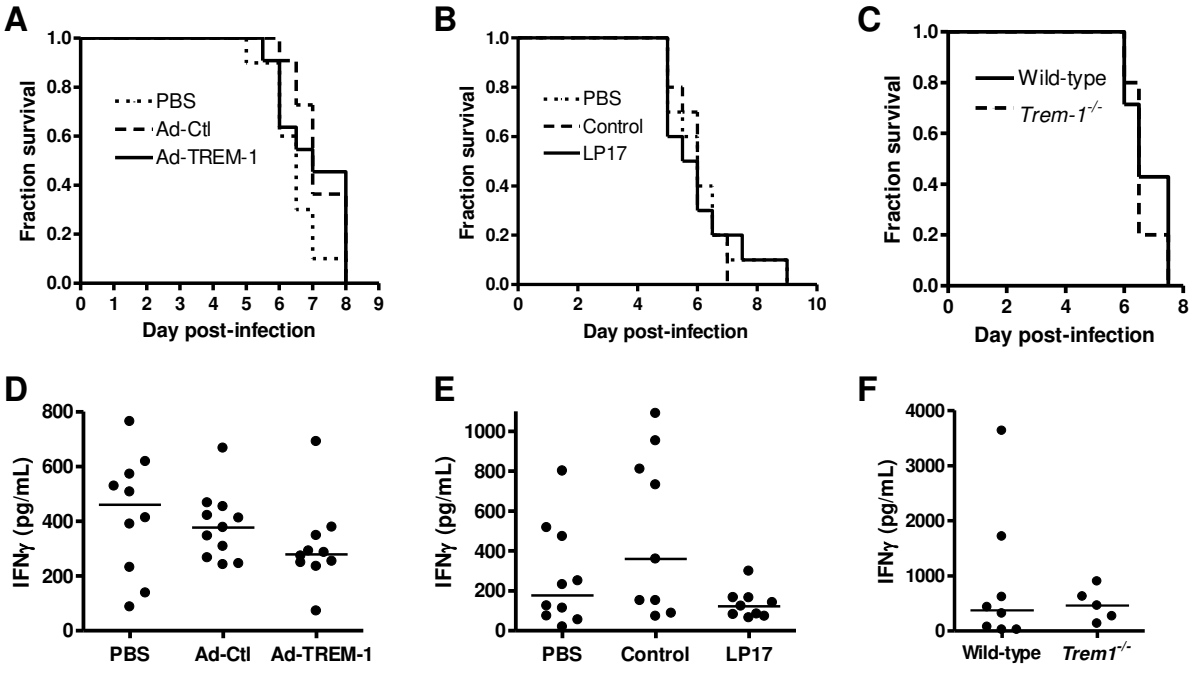


Fig. 3.5: TREM-1 blockade does not protect against experimental cerebral malaria. TREM-1 activation was inhibited using different strategies, and survival and plasma IFNγ levels were assessed compared to controls: (A,D) Ad-TREM-1 or Ad-Ctl were injected i.m. ($1x10^9$ virions) 1 day prior to infection (n=10 per group); (B,E) LP17 peptide or a control peptide were injected i.p. daily (100 ug/dose) beginning on the day of infection (n=10 per group); and (C,F) TREM-1-deficient mice (n=5) and wild-type littermates (n=7) were infected with PbA. For LP17 treatment and TREM-1-deficient mice, data are representative of 2 independent experiments.

We also investigated the role of TREM-1 in *P. chabaudi* infection of C57BL/6 mice using the Ad-Fc-TREM-1 reagent. Since early control of *P. chabaudi* infection is dependent on a robust pro-inflammatory response, we hypothesized that TREM-1 blockade may reduce inflammation, exacerbate parasitemia, and possibly impair survival. As expected, mice injected with PBS developed a peak parasitemia around Day 10, which subsequently declined to nearly zero, and only 9.1% of mice (1/11) died (Fig. 3.6A,B). Parasitemia and survival curves of mice injected with Ad-TREM-1 and Ad-Ctl were similar to those of the PBS group (Fig. 3.6A,B). Since TREM-1 inhibition may have reduced inflammatory responses but not strongly enough to alter other disease parameters, we measured plasma levels of IFNγ and TNF over a timecourse. IFNγ

levels on Day 5 were lower in the Ad-TREM-1 group than the PBS group, but this was likely a vector effect as IFNγ was similarly depressed in the Ad-Ctl group (Fig. 3.6C). There was a difference in TNF levels between the Ad-TREM-1 and Ad-Ctl groups on Day 15 (Fig. 3.6D); however, TNF was increased, not decreased in the Ad-TREM-1 group and by this point the infection in essentially resolved. Thus, TREM-1 does not appear to critically mediate systemic inflammation or affect key disease parameters in the PbA and *P. chabaudi* models.

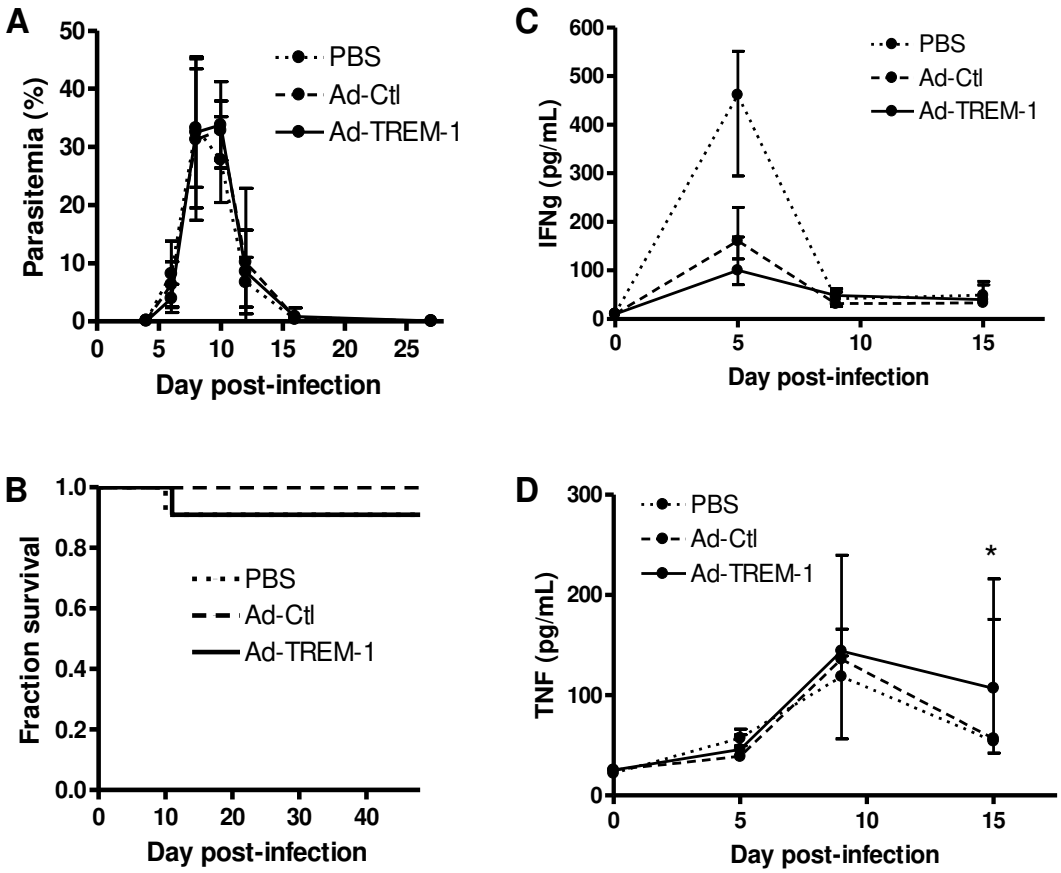


Fig. 3.6: Competitive inhibition of TREM-1 does not significantly alter the course of *P. chabaudi* infection. Mice were injected i.m. with adenoviral vector ($1x10^9$ virions) expressing Fc-TREM-1 (Ad-TREM-1) or Fc alone (Ad-Ctl) one day prior to *P. chabaudi* infection. PBS was included as a further control. Mice were monitored for parasitemia (A) and survival (B). Plasma IFNγ (C) and IL-12 (D) were measured by ELISA. Cytokine levels are presented as median with interquartile range and significance was tested using a Kruskal Wallis test with Dunn's post-test. * indicates $p<0.05$, Ad-TREM-1 versus Ad-Ctl.

3.4.6 Antibody-mediated activation of TREM-1 alters the inflammatory response to *P. falciparum.*

While TREM-1 does not appear to be a critical mediator of inflammation in malaria infection, it has been shown to contribute to inflammatory pathology in murine sepsis models [419,428]. Notably, concomitant bacteremia is present in a considerable proportion of children with severe malaria [548]. Thus, we investigated the effect of TREM-1 activation on malaria-induced inflammation in the PBMC-malaria co-culture model. TREM-1 was activated by antibody-crosslinking, followed by addition of purified *P. falciparum* PEs. After 24 hrs, supernatants were collected and assayed for TNF and IL-10, since these cytokines are synergistically and antagonistically affected by TLR-TREM-1 co-stimulation, respectively [414,421,430] and their ratio is dysregulated in severe malaria [238,239].

Results for 2 different PBMC donors are shown in Fig. 6. As previously shown, TREM-1 activation synergistically increased LPS-induced TNF production (Fig. 3.7A,B), and essentially abolished IL-10 production (Fig. 3.7C,D). The effects of TREM-1 activation on malaria-induced TNF secretion were variable: among 5 PBMC donors tested, 2 showed a negative synergy between TREM-1 crosslinking and *P. falciparum* stimulation (representative data in Fig. 3.7A), while there was no synergy for the other 3 donors (Fig. 3.7B). Consistently, however, IL-10 secretion in response to *P. falciparum* was abolished upon TREM-1 activation (Fig. 3.7C,D). As seen in Fig. 7E, crosslinking with α-TREM-1 antibody significantly increased the ratio of TNF/IL-10 responses to *P. falciparum* compared to isotype control antibody. Thus, while malaria-induced inflammation is not mediated by TREM-1, it can be skewed towards a more pro-inflammatory profile by TREM-1 activation.

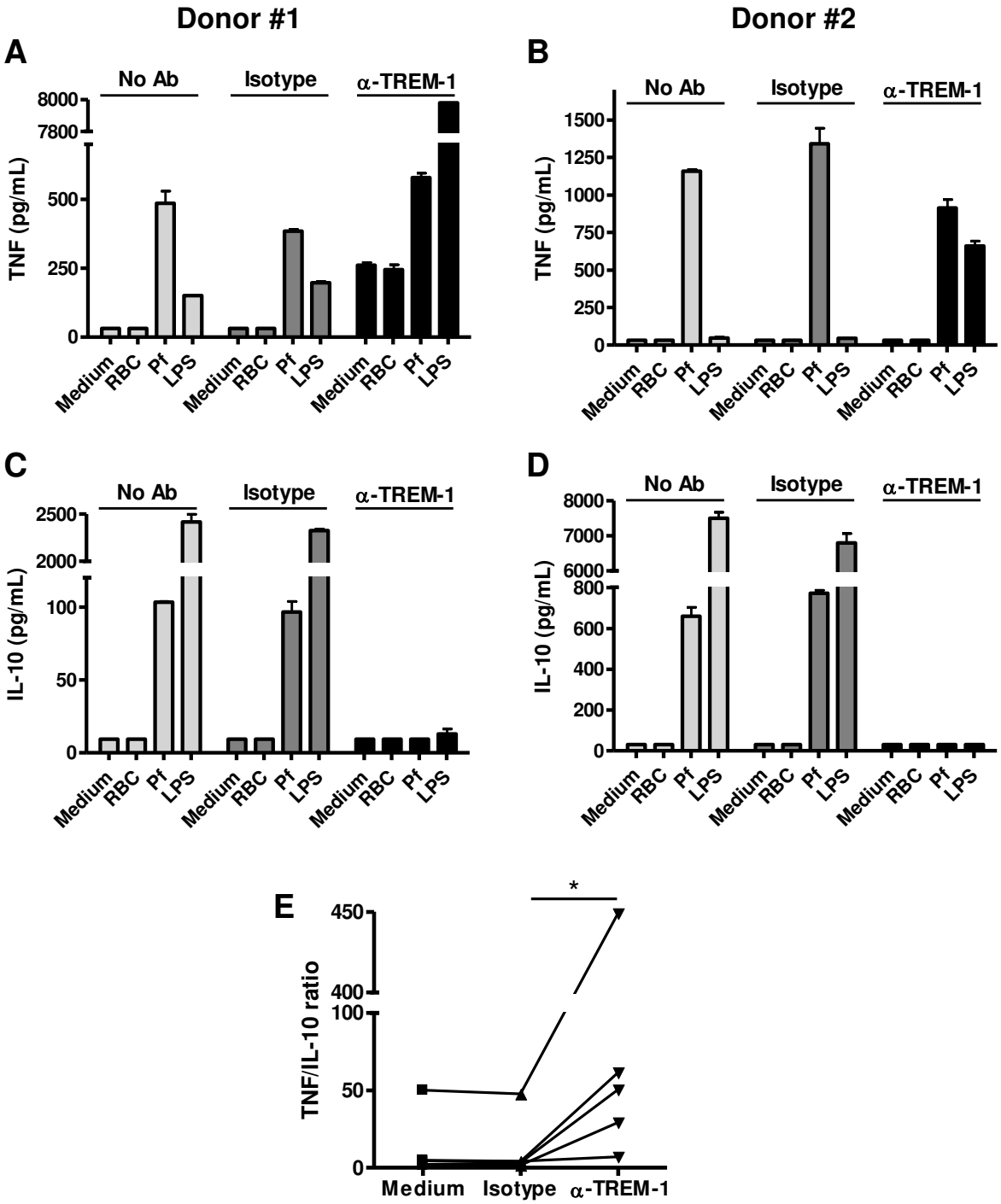


Fig. 3.7: TREM1 activation by antibody-mediated crosslinking modulates malaria-induced inflammation. 48-well plates were coated overnight with monoclonal α-TREM-1 antibody, isotype antibody, or PBS alone. PBMCs were added and allowed to settle for 1.5 hours, then medium, uninfected RBCs, purified *P. falciparum*-infected RBCs (Pf), or LPS were added. Supernatants were collected at 24 hours and assayed for (A,C) TNF and (B,D) IL-10. Results are shown for 2 of 5 PBMC donors tested. (E) Ratios of TNF/IL-10 responses to *P. falciparum* are shown for the 5 PBMC donors tested. * $p<0.05$, Kruskal-Wallis test with Dunn's post-test.

DISCUSSION

In this report, we investigated the regulation of TREM-1 by malaria and a potential contribution of this receptor to malaria-induced inflammation. We found that *P. falciparum* and murine malaria strain *P. berghei* ANKA modulated TREM-1 levels, but that inhibition of TREM-1 did not affect inflammatory responses to malaria *in vitro* or *in vivo*. We further demonstrated that TREM-1 activation increased the ratio of pro- to anti-inflammatory cytokine production in response to *P. falciparum*, a finding which may have relevance to malarial-bacterial co-infections.

Surface levels of monocyte TREM-1 can be upregulated by TLR stimulation [427,428], and this is typically followed by receptor cleavage to generate sTREM-1 [439]. Since parasite components have been shown to activate TLRs, we hypothesized that *P. falciparum* would increase membrane TREM-1 and sTREM-1 production. Incubation of human PBMCs with *P. falciparum* did increase sTREM-1 levels in supernatants. Consistent with this *in vitro* finding, plasma sTREM-1 correlated with disease severity in Ugandan children with malaria. Compared to uncomplicated cases, children with CM and SMA typically have higher or more chronic parasite burdens, respectively [10,33]; therefore, one might expect greater monocyte-parasite interaction and more TREM-1 shedding in severe malaria syndromes. Alternatively, elevated sTREM-1 in severe malaria patients may be related to their heightened inflammatory response, since the activity of monocyte metalloproteinases responsible for TREM-1 cleavage can be regulated by cytokines and chemokines [549].

Our results are in contrast to a recent report that serum sTREM-1 levels at presentation did not discriminate between uncomplicated and severe cases of imported *P. falciparum* malaria in The Netherlands [542]. However, this study lacked sufficient power to exclude a relationship. It is also possible that our findings are confounded by concomitant bacterial or other infections, which were not assessed in all children.

Despite increased TREM-1 transcription and sTREM-1 release upon PBMC-malaria co-culture, we did not detect elevated surface TREM-1 on monocytes. This was unexpected, as monocyte TREM-1 levels were previously reported to be higher in patients with mild malaria compared to uninfected individuals [445]. This difference may be related to our use of an *in vitro* model system. Synchronized PEs undergo schizont rupture and release stimulatory parasite components in a fairly unified manner. Surface TREM-1 may have been upregulated following this period, but only briefly due to subsequent TLR agonist inactivation combined with initiation of TREM-1 shedding and/or receptor internalization. Alternatively, the TLR agonists in our system may have been too weak to increase TREM-1 surface levels; *in vivo*, prolonged exposure to these components or additional host factors may have contributed to increased TREM-1 levels. It is also possible that monocyte contact with the culture dish, which initiates differentiation into macrophages, alters the effects of *P. falciparum* on TREM-1 surface levels, since TREM-1 is downregulated upon monocyte differentiation into macrophages or dendritic cells [414].

The observed increase in TREM-1 expression on inflammatory monocytes in PbA-infected mice suggests that the lack of surface TREM-1 modulation in our *in vitro* system is an artifact. Murine inflammatory monocytes are considered equivalent to the classical $CD14^{hi}CD16^{-}$ monocyte

subset in humans [550], which accounts for approximately 90% of monocytes in the blood of healthy individuals. Other human subsets are pro-inflammatory $CD14^{dim}CD16^{+}$ monocytes, which are analogous to murine resident monocytes, and intermediate $CD14^{hi}CD16^{+}$ monocytes, which are major IL-10 secretors [551]. TREM-1 regulation on these subsets has not been characterized, other than a report that $CD14^{hi}$ monocytes express TREM-1 at baseline, while $CD14^{dim}$ monocytes do not [414]. We observed this same pattern in unstimulated human monocytes, and found that LPS stimulation increased TREM-1 expression on $CD14^{hi}$ but not $CD14^{dim}$ monocytes (data not shown). This suggests that, similar to what occurs in mice, human TREM-1 expression is increased on the dominant classical subset in response to (sustained) inflammatory stimuli. Further analysis of monocytes isolated from malaria-infected patients would be required to determine subset-specific TREM-1 regulation in humans.

We found that TREM-1 inhibition did not reduce the pro-inflammatory response to malaria *in vitro*, at least for the cytokines assessed. Similar findings were obtained in murine models using various TREM-1-inhibitory strategies. It is possible that TREM-1 signalling makes a small but redundant contribution to malaria-induced inflammation. Alternatively, TREM-1 modulation may be a non-specific response to innate immune stimulation and does not necessarily imply TREM-1 activation and signalling. It is possible that surface TREM-1 is not sufficiently upregulated for effective ligand stimulation. TLR9, the TLR most consistently implicated in malaria-induced inflammation, was found to be a poor inducer of human monocyte TREM-1 compared to other TLR agonists [427]. Alternatively, unlike bacterial infection [415,425], murine malaria infection may not upregulate the TREM-1 ligand on granulocytes, perhaps due to

differences in combinations of TLR agonists or interfering factors produced by the parasite. We were unable to assess TREM-1 ligand expression in PbA infection due to reagent unavailability.

Although our studies did not reveal a direct role for TREM-1 in malaria pathogenesis, disease course may still be affected by co-infecting pathogens that activate TREM-1. Up to 4-6% of children with severe malaria can have concomitant bacteremia [495,548,552], and in some settings, co-infections have been associated with increased mortality [43,495,496]. Increased gastrointestinal permeability [553], TLR hyperresponsiveness [344], or reduced immune function [327] have been proposed as mechanisms by which malaria may predispose to invasive bacterial infection. Conversely, bacterial infection could aggravate a low-level malaria infection. We hypothesized that this could occur via pro-inflammatory synergy between malaria-activated TLRs and TREM-1, as bacterial species commonly isolated from severe malaria patients (e.g. *Streptococcus pneumoniae*, Gram negatives such as non-typhoidal *Salmonella*) have been found to upregulate TREM-1 and/or its ligand [427,428]. While we did not observe positive synergy between TREM-1 activation and *P. falciparum* stimulation for TNF secretion, TREM-1 crosslinking completely abolished malaria-induced IL-10 levels and increased the TNF/IL-10 ratio. Low IL-10 and high TNF/IL-10 ratios have been associated with malaria severity [141,237-240], suggesting that TREM-1 activation by concomitant bacterial infection could exacerbate inflammatory responses to malaria and promote progression to severe disease. Further experiments using whole bacteria and *in vivo* models are warranted. It also remains to be investigated whether the observed interaction between TREM-1 and *P. falciparum* is related to parasite stimulation of TLRs or other receptors.

Finally, although TREM-1 does not appear to be a suitable target for therapeutic immunomodulation in severe malaria, plasma sTREM-1 may have potential use as a prognostic biomarker in severe malaria infection.

Chapter 4
Investigating a role for CHI3L1 in severe malaria pathogenesis

4.1 ABSTRACT

Chitinase-3 like-1 (CHI3L1) is a secreted glycoprotein that is produced by a variety of cell types and has been found to be elevated in diseases associated with inflammatory pathology. CHI3L1 has been implicated in physiological and pathological processes, including inflammation, tissue remodelling, and angiogenesis, and promotes survival of normal and cancerous cells. A number of these functions are relevant to the pathogenesis of severe forms of malaria, which claim almost 1 million lives annually. We hypothesized that CHI3L1 modulates inflammation or other host responses in malaria infection and therefore may affect outcome. We found that plasma CHI3L1 levels were associated with disease severity in Ugandan children presenting to hospital with malaria, and that human peripheral blood mononuclear cells (PBMCs) exposed to *Plasmodium falciparum* upregulated CHI3L1 transcription and secretion. CHI3L1 was increased in plasma and brain tissue in a murine model of experimental cerebral malaria (ECM). Interestingly, a mouse strain that has resistance to ECM showed an early spike of plasma CHI3L1 compared to ECM-susceptible mice, suggesting that timely expression of this protein may protect against severe malaria. Consistent with this hypothesis, genetic deletion of CHI3L1 on the ECM-susceptible background led to earlier death, although this difference only trended towards significance ($p=0.10$, log-rank test). This study demonstrates that CHI3L1 expression is increased in malaria infection and may play a role in resistance to severe malaria, although confirmatory studies using more informative models are required.

4.2 INTRODUCTION

Plasma levels of the chitinase-like protein CHI3L1 are elevated in numerous acute and chronic inflammatory conditions, including bacterial and viral infections [459-461,463,464]. Given that CHI3L1 is a biomarker of inflammation, and excessive inflammatory responses are observed in severe malaria, we hypothesized that plasma CHI3L1 would correlate with malaria severity.

We also investigated whether CHI3L1 plays a role in ECM pathogenesis. Conceivably, CHI3L1 may participate in pathogenesis by promoting inflammation in the brain. As discussed, $CD8^+$ T cell recruitment to the brain is critical for the development ECM [173,554], and monocyte accumulation in ECM and pediatric CM is thought to contribute to local cytokine production and consequently PE sequestration. Notably, CHI3L1 has been shown to promote migration [448] and survival [483] of immune cells. Additionally, CHI3L1 may enhance vascular leak in the brain via endothelial cell activation [477].

Local CHI3L1 production may also have direct neuropathic effects. In hippocampal cultures, CHI3L1 disrupted interactions between neurons and extracellular matrix-bound trophic factors [464]. This was proposed to contribute to the cerebral pathology of simian immunodeficiency virus encephalitis, in which CHI3L1 is highly expressed in microglia, perivascular macrophages, and astrocytes, and rises sharply in cerebrospinal fluid prior to death [464,555]. Increased CHI3L1 expression in the brain has been associated with other neuropathological conditions including multiple sclerosis [555], schizophrenia [556,557], and Alzheimer's disease [468]. If

CHI3L1 is induced in the brain during severe malaria, it could inhibit repair of the axonal injury reported in human CM and murine ECM [118,198,558] and thus contribute to disease progression.

On the other hand, CHI3L1 upregulation in the brain may be a neuroprotective response rather than a cause of pathology. A SNP leading to decreased CHI3L1 expression was found to be associated with increased risk of schizophrenia in Chinese populations [559], although this result has not been replicated in other populations [560,561]. CHI3L1 has anti-apoptotic effects in astrocytes exposed to harmful stimuli [480,481]. Neuronal apoptosis has been observed on autopsy of patients with CM [133], and apoptosis of neurons, astrocytes, and endothelial cells has been implicated in ECM pathogenesis [208-210,562]. By promoting survival of these cells, CHI3L1 may restrict damage to the BBB – thus limiting amplification of inflammation – and brain parenchyma.

We show here that plasma levels of CHI3L1 were elevated in children with severe malaria compared to those with uncomplicated infection, and were further elevated in fatal cases. Incubation of human PBMCs with *P. falciparum* induced CHI3L1 transcription and secretion. Resistance and susceptibility to PbA-induced ECM were associated with different patterns of CHI3L1 expression over time. Plasma levels of pro-inflammatory mediators were similar in CHI3L1-deficient mice and wild-type littermate controls, but there was a trend towards earlier death in the CHI3L1-deficient mice.

4.3 METHODS

All materials and protocols are as detailed in the Methods section of Chapter 3, with the following additions:

4.3.1 CHI3L1 ELISA. An ELISA kit measuring human CHI3L1 (CHI3L1 Duoset, R&D Systems) was used to determine CHI3L1 levels in the plasma of malaria-infected Ugandan children (dilution factor: 1:500) and in supernatants from PBMC-*P. falciparum* co-culture experiments (dilution factor: 1:2). ELISAs on patient samples were performed according to the manufacturer's instructions with the following changes: the assay was performed in a volume of 50 µL/well; plasma samples were incubated overnight at 4^{o}C; and ELISAs were developed using Extravidin®-Alkaline Phosphatase (Sigma, 1:1000 dilution, 45 min incubation) followed by addition of p-Nitrophenyl phosphate substrate (Sigma) and optical density readings at 405 nm.

4.3.2 PBMC stimulation with exogenous CHI3L1. Recombinant human CHI3L1 (R&D Systems) was added to PBMCs at a concentration of 0.2 or 1 ug/mL. These concentrations were chosen based the literature and on the plasma levels we observed in the Ugandan children.

4.3.3. Quantitation of human CHI3L1 mRNA. cDNA was generated from PBMCs and qRT-PCR was performed as described in Chapter 3. The primer sequences for amplification of human *Chi3l1* were as follows: forward primer: 5'-TGCCCTTGACCGCTCCTCTGTACC-3' and reverse primer: 5'-GAGCGTCACATCATTCCACTC-3'.

4.3.4 Mouse strains. Female C57BL/6, BALB/c, and 129sv/J mice were obtained from Charles River or Jackson Laboratories. *Chi3l1*$^{-/-}$ mice (backcrossed >10 times onto the C57BL/6 background) were obtained from the laboratory of Dr. Jack Elias, and mated with C57BL/6 mice to generate heterozygotes, which were then bred in the animal facility to generate *Chi3l1*$^{-/-}$ mice and wild-type littermate controls. Animal protocols were approved by the Animal Care Committee of the University of Toronto and all animal work was performed in compliance with university institutional guidelines.

4.3.5 Total brain RNA isolation and qRT-PCR for mouse CHI3L1. Mice were euthanized by inhaled CO_2 at various timepoints during PbA infection. Brains were removed and bisected; half of the brain was snap-frozen in liquid nitrogen and stored at -80^{o}C until processing. Briefly, RNA extraction was performed as follows: samples were thawed slightly on ice, homogenized in 200 µL Trizol (Invitrogen), and topped up to 1 mL with Trizol. After 5 min incubation at room temperature, 200 µL chloroform was added to samples, which were mixed and incubated at room temperature for 3 min prior to centrifugation (30 min, 2500xg, 4^{o}C). The aqueous phase was collected and RNA was precipitated with isopropanol (10 min room temperature). RNA pellets were washed 3 times with 75% ethanol, dried, and dissolved in 30 uL water. DNase treatment, conversion to cDNA, and qRT-PCR were performed as indicated in Chapter 3 Methods. Copy number was interpolated from a standard curve of mouse genomic DNA and normalized to the geometric mean of 3 housekeeping genes (*Gapdh*, *Ywhaz*, *Hrpt*). Primer sequences were as follows:

Chi3l1-For	5'-GTACAAGCTGGTCTGCTACTTC-3'
Chi3l1-Rev	5'-ATGTGCTAAGCATGTTGTCGC-3'
Tnf-For	5'-GACAGACATGTTTTCTGTGAAAACG-3'
Tnf-Rev	5'-AAAAGAGGAGGCAACAAGGTAGAG-3'
Icam1-For	5'-TGGCTGAAAGATGAGCTCGAGAGT-3'
Icam1-Rev	5'-GCTCAGCTCAAACAGCTTCCAGTT-3'
Gapdh-For	5'-TCAACAGCAACTCCCACTCTTCCA-3'
Gapdh-Rev	5'-TTGTCATTGAGAGCAATGCCAGCC-3'
Ywhaz-For	5'-AGCAGGCAGAGCGATATGATGACA-3'
Ywhaz-Rev	5'-TCCCTGCTCAGTGACAGACTTCAT-3'
Hprt-For	5'-GGAGTCCTGTTGATGTTGCCAGTA-3'
Hprt-Rev	5'-GGGACGCAGCAACTGACATTTCTA-3'

4.3.6 Statistical analysis. Analysis was performed using GraphPad Prism software. Differences between groups were analyzed using the Mann-Whitney U test, the Kruskal-Wallis test with Dunn's post-tests, or two-way ANOVA with Bonferroni post-tests For *in vitro* experiments, each condition was performed in duplicate or triplicate. Survival curves were compared using the log-rank test, and survival proportions were compared using Fisher's exact test.

4.4 RESULTS

4.4.1 CHI3L1 is elevated in the plasma of Ugandan children with severe and fatal malaria infection. We first assessed plasma levels of CHI3L1 in children presenting to hospital with malaria infection. Patient characteristics are described in Table 1 of Chapter 3. ELISA analysis of samples showed that plasma CHI3L1 at time of presentation was higher in children with CM and SMA compared to uncomplicated malaria ($p<0.001$; Fig. 4.1A). Furthermore, children with

severe malaria who subsequently died from their infection had higher CHI3L1 levels than those who survived ($p<0.01$ for CM, $p<0.001$ for SMA; Fig. 4.1B).

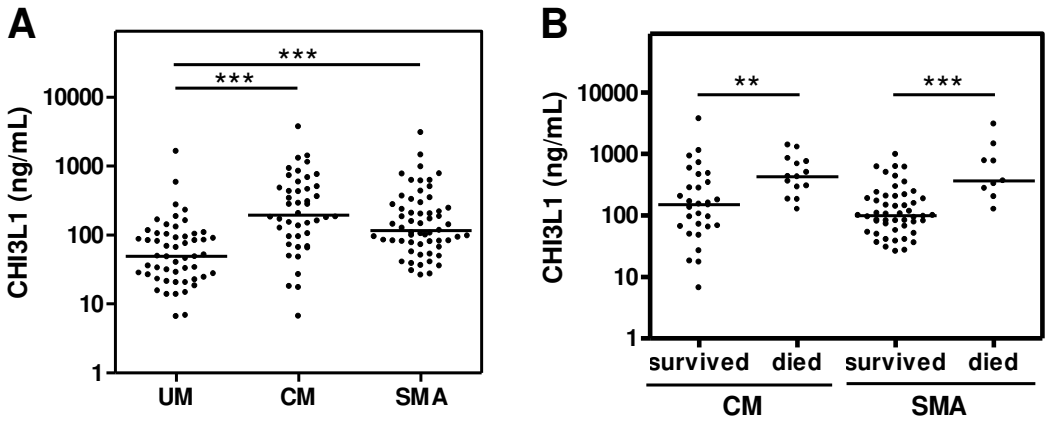


Fig. 4.1: Plasma CHI3L1 in children with uncomplicated and severe malaria at time of presentation to hospital. (A) CHI3L1 was measured by ELISA in children with uncomplicated malaria (UM), cerebral malaria (CM), and severe malarial anemia (SMA). (B) CHI3L1 levels are elevated in children with severe malaria who died from infection compared to those who survived. Data are presented as dot plots with medians. ** $p<0.01$, *** $p<0.001$ by Kruskal-Wallis test with Dunn's post-hoc tests.

4.4.2 CHI3L1 is produced upon PBMC exposure to *P. falciparum* but does not affect PBMC cytokine responses to malaria.

To characterize a potential source of CHI3L1 during malaria infection, human PBMCs were co-cultured with *P. falciparum* for 8 and 24 hours, and mRNA and protein levels of CHI3L1 were assessed. As a positive control, LPS was used to stimulate CHI3L1 transcription (Fig. 4.2A) and protein production (Fig. 4.2B) [455]. Compared to incubation with RBCs alone, exposure to malaria for 24 hours induced, on average, a 2-fold increase in CHI3L1 transcription (Fig. 4.2A), and this corresponded to increased secretion of CHI3L1 protein (Fig. 4.2B).

Next, we sought to determine whether CHI3L1 affects PBMC cytokine responses to malaria *in vitro*. This could occur in a number of ways; for instance, CHI3L1 stimulation of PBMCs and alveolar macrophages has been shown to induce release of MMPs [453,475], which can regulate TNF production [563]. PBMCs were incubated with recombinant human CHI3L1 alone or in combination with *P. falciparum* for 24 hours, and TNF and IL-10 levels were measured in supernatants. As seen in Fig. 2C and D, recombinant CHI3L1 alone did not induce TNF or IL-10 secretion, nor did it alter their production in response to malaria. Thus, *P. falciparum* stimulates CHI3L1 production by PBMCs, but CHI3L1 does not modulate the inflammatory response to *P. falciparum in vitro*.

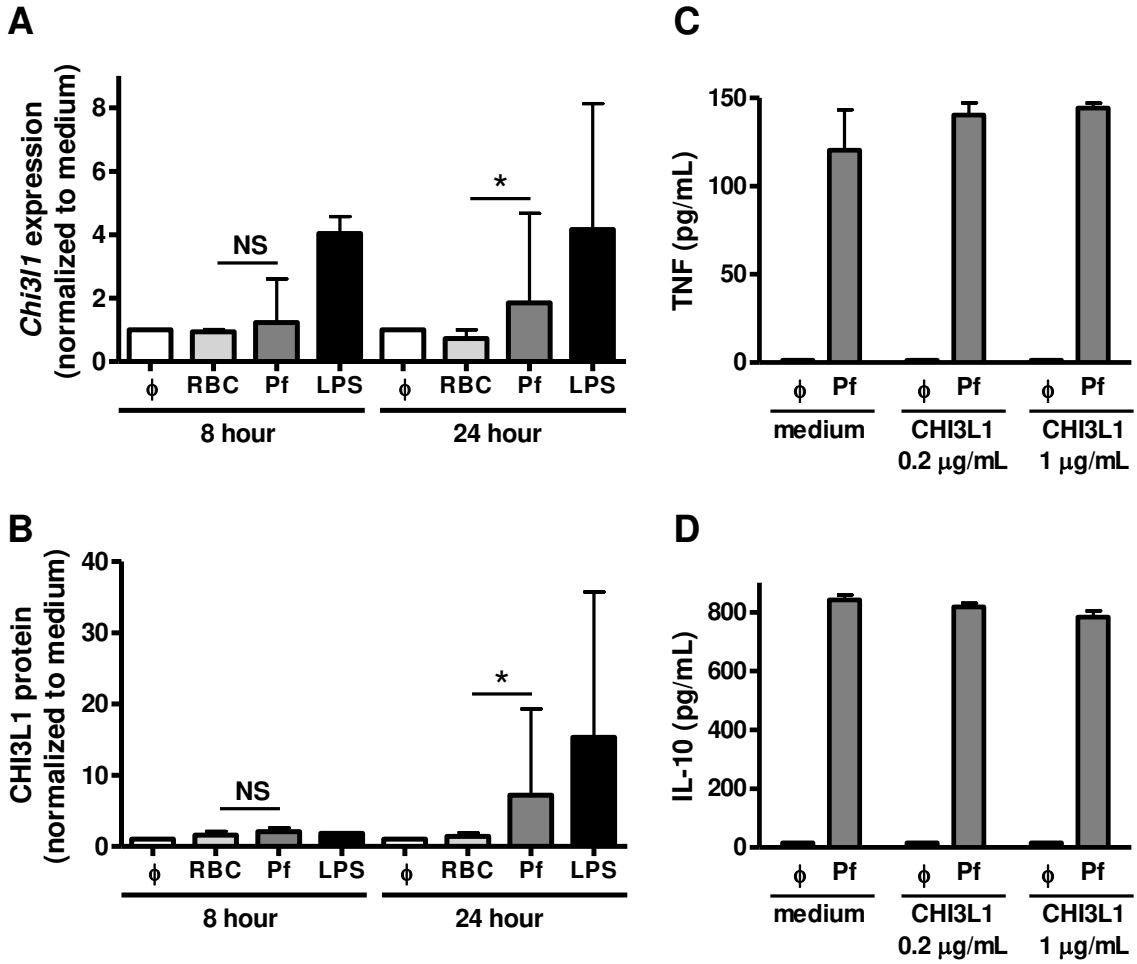


Fig. 4.2: PBMCs produce CHI3L1 upon stimulation with *P. falciparum* but exogenous CHI3L1 does not directly affect PBMC cytokine responses to malaria. Human PBMCs were exposed to medium alone, uninfected red blood cells (RBCs; negative control), *P. falciparum* infected RBCs (Pf), or LPS (positive control) for 8 and 24 hrs. (A) CHI3L1 mRNA was assessed by quantitative real-time PCR, and (B) CHI3L1 concentration in supernatants was measured by ELISA. Graphs represent pooled data from at least 3 independent experiments using different PBMC donors. Data are presented as medians with interquartile ranges. * $p<0.05$, Kruskal-Wallis test with Dunn's post-tests. (C,D) PBMCs were stimulated with recombinant human CHI3L1 alone or in combination with *P. falciparum*-infected RBCs, and TNF and IL-10 were assessed after 24 hours. Data are representative of at least 2 independent experiments. No comparisons were significant by Kruskal-Wallis test with Dunn's post-tests.

4.4.3 CHI3L1 levels in *Plasmodium berghei* ANKA infection of mouse strains with different susceptibilities to ECM. We turned to murine models of malaria to investigate whether

CHI3L1 plays a role in malaria infection. First, we characterized CHI3L1 expression in the *P. chabaudi* and PbA models. As shown in Fig. 3A, plasma CHI3L1 levels were significantly elevated on Day 9 of *P. chabaudi* infection in C57BL/6 mice, the timepoint corresponding to the peak of parasitemia and high cytokine production (data not shown). CHI3L1 levels had partially declined by Day 15, during the recovery phase. In PbA infection of C57BL/6 mice, plasma CHI3L1 was increased on Days 5 and 7 post-infection (Fig. 4.3B). These results are consistent with the induction of CHI3L1 by *P. falciparum* malaria *in vitro* and in human malaria infection, and with the characterization of CHI3L1 as a marker of inflammation.

Since increased CHI3L1 expression in the brain has been associated with other neuropathological states such as Alzheimer's disease, multiple sclerosis, and simian immunodeficiency virus encephalitis [464,468,555], we examined CHI3L1 mRNA expression in the brain during PbA infection by quantitative real-time PCR. CHI3L1 transcription increased over the course of infection, and was significantly elevated on Day 5 compared to uninfected mice (Fig. 4.3C, $p<0.01$). Therefore, both local and systemic expression of CHI3L1 is induced in the ECM model.

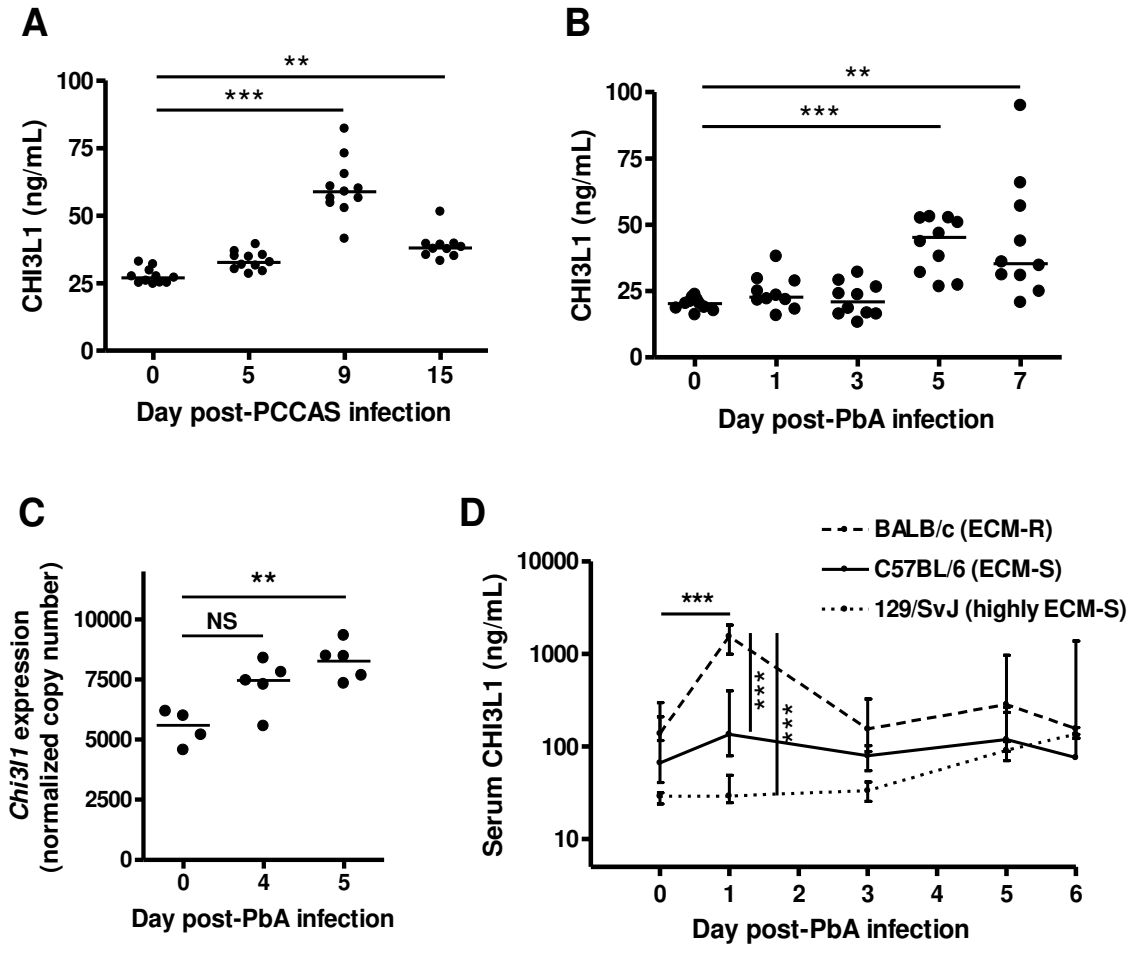


Fig. 4.3: Regulation of CHI3L1 in murine models of malaria infection. Plasma was collected over a timecourse for (A) *P. chabaudi* or (B) PbA infection of C57BL/6 mice, and assayed for CHI3L1 by ELISA. (C) On Day 5 of PbA infection, *Chi3l1* transcripts were upregulated transcription in brain tissue as measured by quantitative real-time PCR. ** $p<0.01$, *** $p<0.001$ by Kruskal-Wallis test with Dunn's post-test. (D) Mouse strains with different susceptibilities to ECM (BALB/c, CM-resistant (ECM-R); C57BL/6, CM-susceptible (ECM-S); 129sv/J, highly ECM-S) were infected with PbA. At each timepoint, 5 mice of each strain were euthanized, and their serum was collected and assayed for CHI3L1 by ELISA. *** $p<0.001$ by two-way ANOVA with Bonferroni post-tests.

The temporal linkage of increased plasma and brain CHI3L1 with the onset of neurological signs suggests that this protein may be mediating pathology in the ECM model. To further explore whether elevated CHI3L1 is associated with disease severity, PbA was used to infect three mouse strains with different susceptibilities to ECM, and plasma CHI3L1 was assessed over a timecourse. As with C57BL/6 mice, 100% of 129sv/J mice succumb to ECM, but they develop

neurological signs and die earlier in the course of infection than C57BL/6 mice [179]. In contrast, only 30-40% of BALB/c mice exhibit neurological symptoms and die during the cerebral window.

In uninfected animals, BALB/c mice appeared to have higher plasma CHI3L1 levels than the other strains (Fig. 4.3D), and the difference between 129sv/J and BALB/c mice was significant ($p<0.05$, Kruskal-Wallis with Dunn's post test). On day 1 of infection, BALB/c mice showed a 10-fold spike of CHI3L1 over baseline ($p<0.001$ compared to BALB/c uninfected and to other strains on Day 1, two-way ANOVA). By Day 3 post-infection, plasma CHI3L1 in BALB/c had returned to baseline, and remained near this level for the rest of the timecourse. C57BL/6 mice did not exhibit significant changes in CHI3L1, even on Day 6, which may reflect a slower course of infection during this experiment than occurred in Fig. 3B. However, the more susceptible 129sv/J mice showed increasing plasma CHI3L1 by Day 6 ($p<0.01$, Kruskal-Wallis with Dunn's post test). Thus, higher baseline levels and an early peak of CHI3L1 are associated with resistance to ECM, while a late rise of CHI3L1 is associated with susceptibility. These findings raise the possibility that CHI3L1 may in fact exert protective effects during PbA infection, if produced at the appropriate time.

4.4.4. Effects of CHI3L1 deletion in the PbA model of ECM. To more definitively determine a role for CHI3L1 in malaria infection, C57BL/6 mice genetically deficient in CHI3L1 were infected with PbA and compared to wild-type littermate controls with respect to parasitemia, pro-inflammatory mediator production, and survival. Parasitemia did not differ between CHI3L1-deficient and wild-type mice (Fig. 4.4A). *Chi3l1*$^{-/-}$ mice appeared to succumb to infection earlier

than wild-type mice (median survival: 6 days and 6.5 days, respectively), with a trend towards significance ($p=0.10$; Fig. 4.4B). A survival difference was consistently observed at earlier timepoints; for example, at day 6 post-infection, there was a significant difference between groups in the proportion of surviving mice ($p<0.01$; Fig 4.4B).

To determine whether CHI3L1 deficiency affected the inflammatory response to PbA, plasma levels of TNF, IFNγ, and IL-10 were measured at day 4 post-infection. There were no differences in these cytokines between wild-type and CHI3L1-deficient mice (Fig. 4.4D). There were also no significant differences in TNF or ICAM-1 mRNA expression in brain homogenates on day 4 or 5 post-infection (Fig. 4.4E).

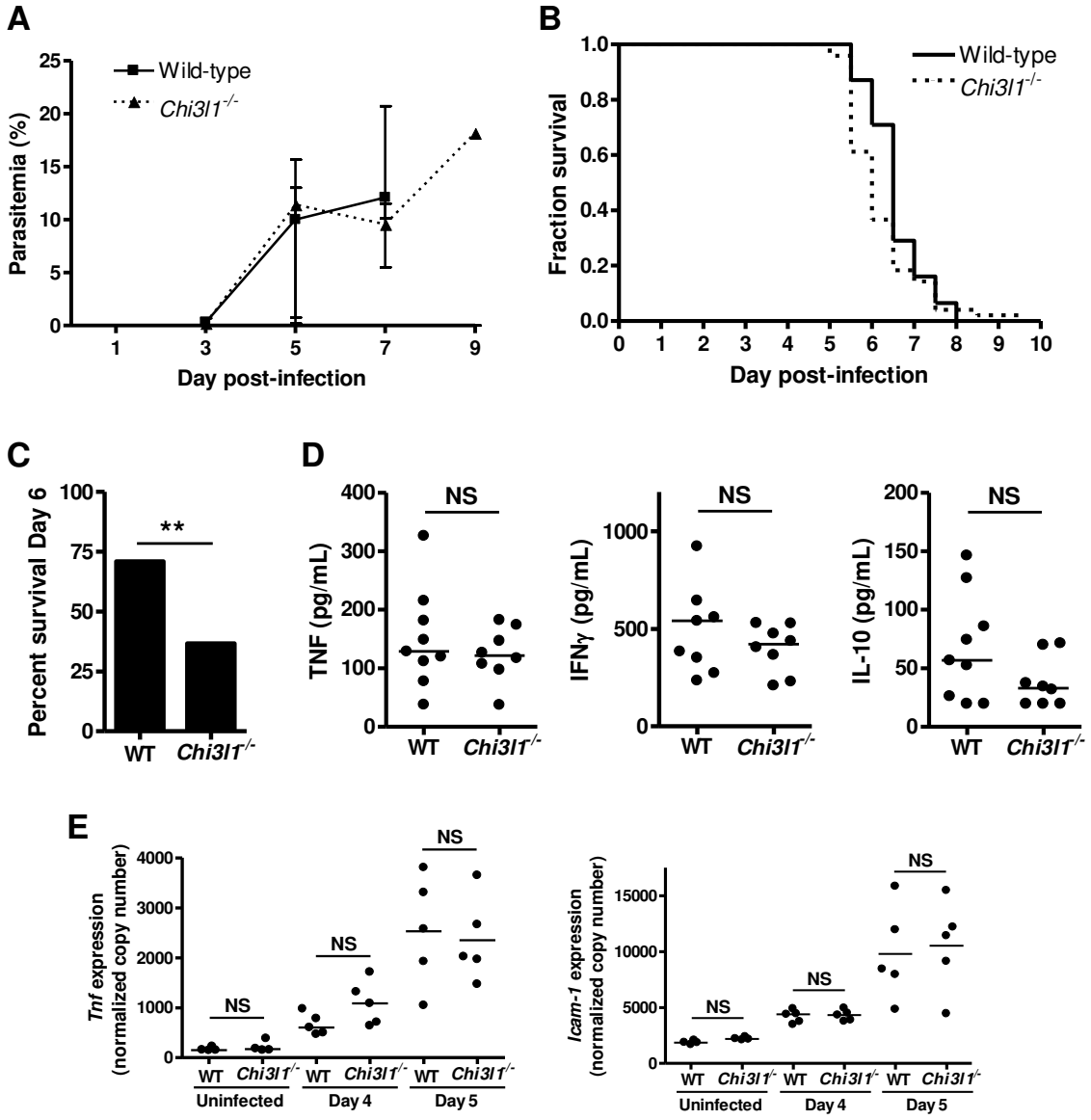


Fig. 4.4: CHI3L1-deficient mice do not show differences in inflammatory responses to PbA but tend to succumb to infection earlier than wild-type littermate controls. (A) Parasitemia is represented as medians and interquartile range. (B) Three independent experiments were pooled to generate the survival curves shown (WT, n=31; *Chi3l1*$^{-/-}$, n=49). The log-rank test was used to assess survival differences (p=0.10). (C) Survival of wild-type (WT) and *Chi3l1*$^{-/-}$ mice tabulated for Day 6 post-infection. Groups were compared using Fisher's exact test. (D) Plasma cytokine levels at day 4 post-infection were compared using the Mann Whitney U test. NS, not significant. All cytokines were undetectable in uninfected mice in both groups. (E) mRNA expression of TNF and ICAM-1 in the brains of WT and *Chi3l1*$^{-/-}$ mice at day 4 post-infection. NS, non-significant by Kruskal-Wallis test with Dunn's post tests.

4.5 DISCUSSION

In this study, we characterized the expression of chitinase-like protein CHI3L1 in malaria infection and examined its function in a murine model of experimental cerebral malaria. Plasma CHI3L1 was elevated in children with severe and fatal malaria, and malaria parasites stimulated CHI3L1 transcription and secretion both *in vitro* and *in vivo*. In the PbA model of ECM, we found that ECM-susceptible mouse strains exhibited distinct expression patterns of CHI3L1 in plasma compared to ECM-resistant strains, suggesting that CHI3L1 may play a role in PbA infection. While we did not observe a significant difference in disease parameters or inflammation between wild-type and *Chi3l1*$^{-/-}$ mice infected with PbA, the trend towards earlier death in *Chi3l1*$^{-/-}$ mice warrants further investigation in a more suitable model.

CHI3L1 production by PBMCs in response to *P. falciparum* may be a result of TLR2 and/or TLR4 activation by parasite components, as agonists for these receptors have been shown to stimulate CHI3L1 expression in monocytic cell lines [455,475]. As a variety of inflammatory stimuli induce CHI3L1, it is possible that other parasite-activated pathways (e.g. NLR, complement) are involved – although these have yet to be formally linked to CHI3L1 regulation. It is unclear whether CHI3L1 transcription is directly induced by parasites, or this response is mediated through autocrine/paracrine cytokine stimulation [453]. To investigate this, we treated *P. falciparum*-PBMC co-cultures with cycloheximide and assessed whether CHI3L1 transcription was reduced. We were unable to draw any conclusions, however, since cycloheximide inhibited parasite development and schizont rupture, and thus abolished PBMC

cytokine responses (data not shown). To circumvent this technical issue, cytokine-blocking antibodies could be used to address this question in future.

Whatever the precise mechanism of CHI3L1 induction, we see a similar upregulation in murine models of malaria infection. Both *P. chabaudi* and PbA infection in C57BL/6 mice increased plasma levels of CHI3L1, with kinetics that mirrored those of parasite burden and pro-inflammatory responses. Presumably, a major contributor to elevated CHI3L1 levels were PBMCs encountering parasitized erythrocytes in the blood, as demonstrated *in vitro*. Another potential source is neutrophils, which have been shown to be activated in malaria infection [564,565]. It is also possible that CHI3L1 production in tissues may contribute to increased levels in blood. While a comprehensive analysis of key organs remains to be done, we showed that CHI3L1 expression in the brain is elevated in C57BL/6 mice during PbA infection. This may represent upregulated transcription in resident brain cells such as astrocytes in response to cytokines or hypoxia [458,555], or reflect intravascular monocyte sequestration in the brain.

Increases in plasma and brain CHI3L1 levels in ECM-susceptible mice suggest that CHI3L1 may mediate pathology in this model. An alternative interpretation is that CHI3L1 exerts protective effects, and expression late in the course of infection is a delayed and inadequate host response to prevent the development of ECM. Consistent with the latter possibility, ECM-resistant BALB/c mice generally expressed higher levels of plasma CHI3L1, and showed a robust peak of plasma CHI3L1 on day 1 post-infection. Moreover, CHI3L1-deficient mice infected with PbA died earlier than wild-type controls (median survival: 6 versus 6.5 days, respectively), although this difference was not statistically significant.

It should be noted that it is challenging to detect worsening of PbA-induced ECM due to the severe nature and rapid progression of pathology in this fatal model. While 129sv/J mice exhibit greater susceptibility than C57BL/6 mice, there are numerous genetic differences between these strains, as opposed to a single gene deletion in the *Chi3l1*$^{-/-}$ mice. We were able to find only a few examples in the literature of a single gene alteration mediating earlier death in this model. One of these is deletion of Histamine Receptor-3 [184]. The deletion strain developed significantly higher parasitemia than wild-type controls, suggesting that the survival difference could be related to more rapid parasite replication and consequently earlier triggering of the inflammatory response [6]. Moreover, this group purchased wild-type control mice from a supplier, rather than using littermate controls; based on our experience, this may have exaggerated the difference between groups. Mice deficient in Caspase-12, an inhibitor of NK-κB and the Caspase-1 inflammasome, died earlier from ECM than wild-type controls [566]. In this study, data are displayed as escape from ECM at Day 10. At this point only 50% of the wild-type mice had died, suggesting that the full survival curve may not have been significant.

If CHI3L1 can in fact protect against ECM, this may be more readily observed by comparing wild-type and CHI3L1-deficient BALB/c mice during PbA infection. Of course, this depends on how CHI3L1 might confer protection and whether this pathway critically contributes to BALB/c resistance to ECM. For example, the apparent greater susceptibility of *Chi3l1*$^{-/-}$ C57BL/6 mice could be due to enhanced brain cell apoptosis in the absence of CHI3L1. Brain sections from BALB/c mice show considerably less apoptosis than those from C57BL/6 mice [48]. It is possible that the anti-apoptotic effects of CHI3L1 contributes to resistance in BALB/c mice. On

the other hand, brain cell apoptosis is a relatively late event in PbA infection and appears to be downstream of inflammatory insults such as perforin secretion by $CD8^+$ T cells [209]. PbA-infected BALB/c mice have lower levels of pro-inflammatory cytokine transcripts and immune cell infiltrates in the brain [47,48], suggesting that, in the absence of a strong apoptotic stimulus, CHI3L1 deficiency may not affect BALB/c survival.

CHI3L1 deficiency could conceivably increase susceptibility of BALB/c mice to PbA infection by modulating the inflammatory response. In *Chi3l1*$^{-/-}$ C57BL/6 mice infected with PbA, we did not observe any changes in plasma or brain levels of pro-inflammatory cytokines, nor did addition of recombinant CHI3L1 affect TNF or IL-1β production by *P. falciparum*-stimulated PBMCs. However, CHI3L1 may play a greater role in TH2 compared to TH1 inflammatory responses. In a murine model of asthma induced by IL-13 overexpression, CHI3L1 deficiency led to reduced levels of key pathological mediators TGF-β1 and IL-4 in the lung [483]. Notably, TGF-β has been implicated in resistance of BALB/c mice to malaria infection. Splenic TGF-β expression decreased in the ECM-susceptible CBA/J strain but not BALB/c mice infected with PbA [47], and inhibition of TGF-β led to elevated levels of TNF and earlier death in BALB/c mice infected with *P. berghei* NK65 and *P. chabaudi* [94]. The mechanisms that regulate TGF-β production in PbA infection are not well-characterized. If, as in the asthma model, CHI3L1 promotes TGF-β expression, *Chi3l1*$^{-/-}$ BALB/c mice may be more susceptible to ECM. Effects in *Chi3l1*$^{-/-}$ C57BL/6 mice would be expected to be small, considering the relative deficiency of TGF-β in this strain during PbA infection [47].

Finally, regardless of whether CHI3L1 plays a role in murine models of malaria, elevation of plasma levels in children with severe and fatal malaria suggests that it may have utility as a prognostic biomarker.

Chapter 5

Combinations of host inflammatory and angiogenic biomarkers predict mortality among Ugandan children with severe malaria

5.1 ABSTRACT

Severe malaria is a leading cause of childhood mortality in Africa. However, at presentation, it is difficult to predict which children with severe malaria are at greatest risk of death. Dysregulated host inflammatory responses and endothelial activation play central roles in severe malaria pathogenesis. We hypothesized that biomarkers of these processes would accurately predict outcome among children with severe malaria. Plasma was obtained from children with uncomplicated malaria (n=53), cerebral malaria (n=44) and severe malarial anemia (n=59) at time of presentation to hospital in Kampala, Uganda. Levels of angiopoietin-2, vWF, vWF propeptide, soluble P-selectin, soluble ICAM-1, soluble endoglin, soluble FMS-like tyrosine kinase-1 (Flt-1), soluble Tie-2, C-reactive protein, procalcitonin, IP-10, soluble triggering receptor expressed on myeloid cells-1 (TREM-1), and Chitinase-3 like-1 (CHI3L1) were determined by ELISA. Receiver operating characteristic (ROC) curve analysis was used to assess predictive accuracy of individual biomarkers. Seven biomarkers (angiopoietin-2, soluble ICAM-1, soluble Flt-1, procalcitonin, IP-10, soluble TREM-1, CHI3L1) discriminated well between children who survived severe malaria infection and those who subsequently died (area under ROC curve>0.7). Combinational approaches were applied in an attempt to improve accuracy. A biomarker score was developed based on dichotomization and summation of the six biomarkers, resulting in 91.3% (95% CI: 72.0-98.9) sensitivity and 90.0% (81.2-95.6) specificity for predicting death. Similar predictive accuracy was achieved with models comprised of 2 or 3

biomarkers. Classification tree analysis generated a 3-marker model with 100% sensitivity and 92.5% specificity (cross-validated misclassification rate: 15.4%, standard error 4.9%). In summary, we identified novel host biomarkers of pediatric severe and fatal malaria (soluble TREM-1 and soluble Flt-1) and generated simple biomarker combinations that accurately predicted death in an African pediatric population. While requiring validation in further studies, these results suggest the utility of combinatorial biomarker strategies as prognostic tests for severe malaria.

5.2 INTRODUCTION

While TREM-1 and CHI3L1 may not contribute to the pathogenesis of malaria, they were both found to be elevated in the plasma of children with severe malaria compared to uncomplicated malaria, and further increased in fatal cases. These data suggest that sTREM-1 and CHI3L1 may have utility as prognostic biomarkers of severe malaria. Studies in malaria and other conditions have demonstrated that combining multiple biomarkers can significantly improve the accuracy of predictive models [14,126,567,568]. Combining markers of different pathophysiological pathways can be particularly useful, as each marker may independently contribute information regarding the nature and severity of disease [569]. Therefore, in addition to sTREM-1 and CHI3L1, we measured other markers of inflammation and endothelial activation in our Ugandan patient population. We hypothesized that combining these plasma markers using different strategies would improve their ability to predict mortality in our sample.

In terms of inflammatory biomarkers, we measured plasma levels of acute-phase response components, C-reactive protein (CRP) and procalcitonin (PCT), which have been shown to increase during malaria infection [570,571]. We also measured IP-10, a chemokine reported to be elevated in fatal CM [504,572]. Given the implication of endothelial activation in CM pathogenesis, we selected an array of endothelial activation biomarkers to assess in our samples. Upon endothelial activation, soluble endothelial cell receptors are released via ectodomain shedding or alternative splicing. We measured the soluble forms of ICAM-1 (sICAM-1) and the TGF-β receptor endoglin (s-endoglin), which have both been shown to be increased in severe

malaria [573,574], and soluble FMS-like tyrosine kinase-1 (sFlt-1), which has been implicated in placental malaria [575]. Endothelial activation also causes exocytosis of WPB [199]. We assayed WBP-associated factors Ang-2, von Willebrand factor (vWF), vWF propeptide, and soluble P-selectin (sP-selectin). Some of these molecules are elevated in CM [200-202] and have been suggested to contribute to pathology: Ang-2 may exacerbate vascular activation in malaria by antagonizing the quiescence-promoting interaction of the endothelial Tie-2 receptor with Ang-1 [205], while vWF may help tether parasitized erythrocytes to endothelial cells via platelets [203]. In addition to CM patients, systemic endothelial activation has been shown to occur in adults with uncomplicated and non-CM severe malaria [574,576]; however, few studies have characterized the extent and significance of this process in pediatric SMA.

First, we determined which markers were elevated in severe disease compared to uncomplicated malaria, and then assessed which markers discriminated between children who survived severe malaria infection and those who subsequently died. Furthermore, we identified combinations of biomarkers from these host pathways that accurately predicted mortality among children with severe malaria.

5.3 METHODS

5.3.1 Biomarker quantification in plasma samples. Study site, study protocol, participants, sample collection, and ELISA analysis are described in Chapter 3 Materials and Methods. The following markers were assayed by ELISA (dilution factor indicated in parentheses): Ang-2 (1:5), CRP (1:40,000), s-endoglin (1:25), IP-10 (1:2), sFlt-1 (1:6), sICAM-1 (1:1000), sP-selectin

(1:50), sTie-2 (1:25; all R&D Systems), PCT (1:5; Ray BioTech), and vWF propeptide (1:400; Sanquin). ELISAs were performed blinded to all associated clinical data, and according to the manufacturers' instructions with the following changes: assays were performed in a volume of 50 µL/well; plasma samples were incubated overnight at 4^{o}C; and ELISAs were developed using Extravidin®-Alkaline Phosphatase (Sigma, 1:1000 dilution, 45 min incubation) followed by addition of p-Nitrophenyl phosphate substrate (Sigma) and optical density readings at 405 nm. s-endoglin and sP-selectin were only measured in samples collected during the first year of the study (n=101). For vWF, plates were coated with anti-human vWF antibody (Dako, 1:600), incubated with samples (1:500 dilution) and serial dilutions of recombinant vWF (American Diagnostica), then incubated with horseradish peroxidase-conjugated anti-human vWF (Dako, 1:8000). Assays were developed with tetramethylbenzidine, stopped with H_2SO_4, and read at 450 nm.

5.3.2 Statistical analysis. GraphPad Prism v4, SPSS v18, and MedCalc software were used for analysis. For clinical and demographic variables, differences between groups were assessed using the Chi-square test (categorical variables) or the Kruskal-Wallis test with Dunn's multiple comparison post-hoc tests (continuous variables). The Mann-Whitney U test was used to compare biomarker levels between groups, and p values were corrected for multiple comparisons using Holm's correction. Receiver operating characteristic curves were generated using the non-parametric method of Delong et. al [577]. Cut-points were determined using the Youden index (J = max[sensitivity + specificity – 1]). For logistic regression, linearity of an independent variable with the log odds of the dependent was assessed by including a Box-Tidwell transformation into the model and ensuring that this term was not significant. Bootstrapping (1000 sample draws)

was used to generate variance estimates for b. Model goodness-of-fit was assessed by the Hosmer-Lemeshow test and calibration slope analysis [578]. Positive and negative predictive values were calculated using the reported case fatality rate of 5.7% for microscopy-confirmed CM and SMA at Mulago Hospital [545]. Classification tree analysis was performed in SPSS with the following settings: minimum 10 cases for parent nodes and 5 for child nodes; customized prior probabilities based on the case fatality rate at Mulago Hospital; customized misclassification costs (as indicated); pruning to reduce overfitting; and cross-validation with 10 sample folds to generate an estimate of the misclassification rate and its standard error. There were no missing values from the dataset.

5.4 RESULTS

5.4.1 Biomarker levels in uncomplicated vs. severe malaria patients. Plasma samples from Ugandan children with malaria infection were obtained at time of presentation to hospital (Table 5.1). Samples were assayed for biomarkers of inflammation and endothelial activation, and results are displayed in Fig. 5.1. As discussed in previous chapters, sTREM-1 and CHI3L1 levels were higher in CM and SMA compared to UM. Other inflammatory markers CRP and PCT were also increased in severe malaria vs. uncomplicated malaria ($p<0.01$), but IP-10 was not ($p>0.05$). Most biomarkers of endothelial activation were significantly elevated in CM and SMA compared to UM, including WPB-associated proteins Ang-2, vWF, and vWF propeptide ($p<0.01$). However, s-endoglin and sP-selectin did not differ between groups ($p>0.05$).

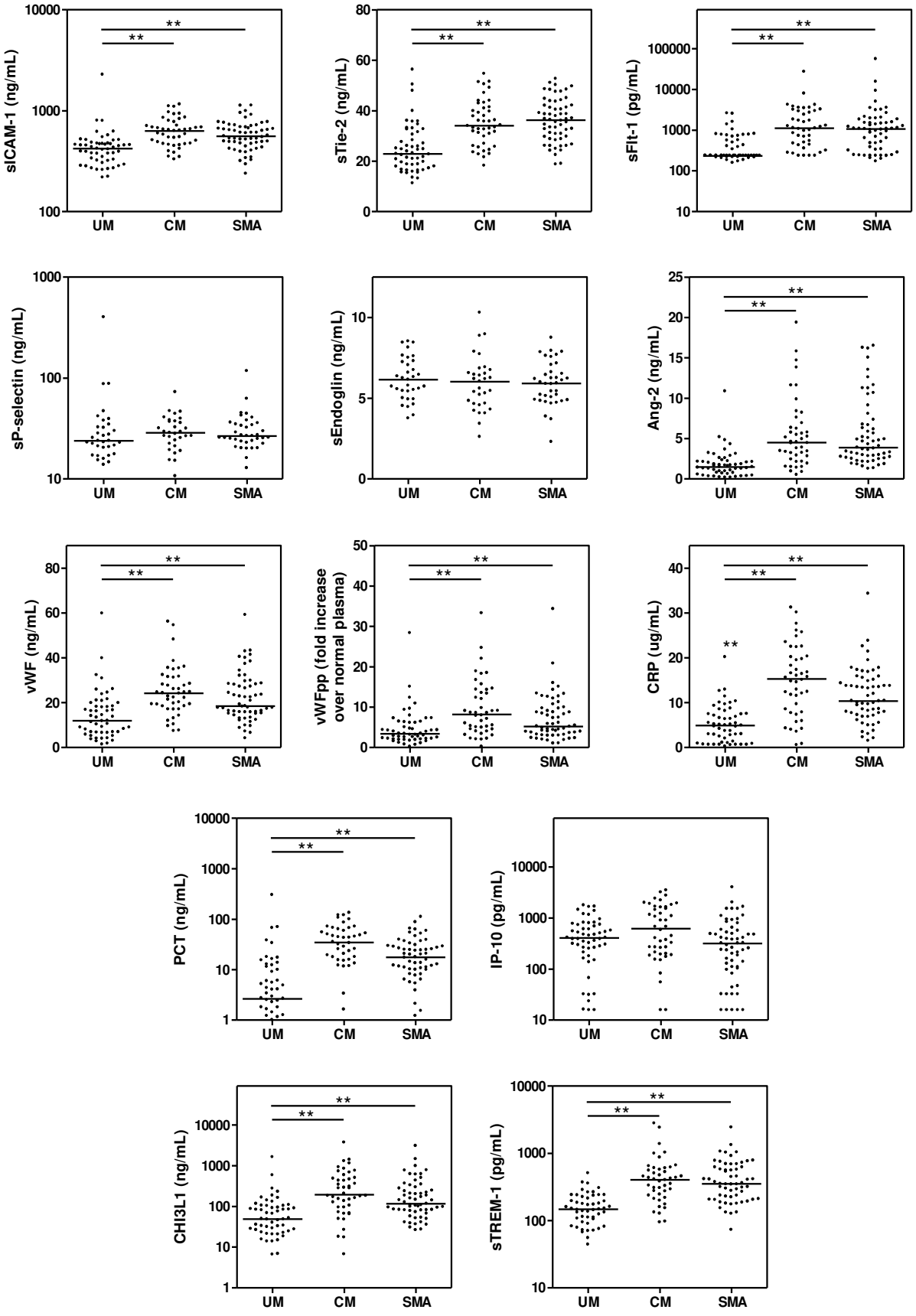

sICAM-1 (ng/mL)
sTie-2 (ng/mL)
sFlt-1 (pg/mL)
sP-selectin (ng/mL)
sEndoglin (ng/mL)
Ang-2 (ng/mL)
vWF (ng/mL)
vWFpp (fold increase over normal plasma)
CRP (ug/mL)
PCT (ng/mL)
IP-10 (pg/mL)
CHI3L1 (ng/mL)
sTREM-1 (pg/mL)
UM
CM
SMA
**

Fig. 5.1: Plasma biomarker levels in Ugandan children with uncomplicated and severe malaria at time of presentation. Biomarkers of inflammation and endothelial activation in the plasma of children presenting with uncomplicated malaria (UM), cerebral malaria (CM), or severe malarial anemia (SMA) were measured by ELISA. Data are presented as dot plots with medians. A Mann Whitney U test was performed for each comparison, and p values were adjusted for multiple comparisons using Holm's correction (n=26). ** $p<0.01$.

5.4.2 Biomarkers as predictors of mortality in children with severe malaria. To evaluate the prognostic utility of these plasma biomarkers, we compared admission levels between children with severe malaria who survived infection and those who subsequently died. After correction for multiple comparisons, admission levels of Ang-2 and CHI3L1 (Fig. 5.2A; $p<0.05$) were significantly increased in CM fatalities compared to survivors, while Ang-2, sICAM-1, IP-10, CHI3L1 ($p<0.01$), sTREM-1 and sFlt-1 ($p<0.05$) were elevated in SMA fatalities compared to survivors (Fig. 5.2B).

The biomarkers that reached significance in the SMA group but not the CM group after correction for multiple comparisons (sICAM-1, IP-10, sTREM-1, sFlt-1) were significant or trending towards significance in the CM group before the correction was applied. This suggests that the apparent differences between syndromes may have been due to low statistical power and therefore we combined all severe malaria patients for further analysis. This strategy also avoids the problem of classifying mixed clinical phenotypes, as occurred in the present study population. Characteristics of survivors and fatalities were similar (Table 5.1), although among fatalities there was a greater proportion of females ($p=0.007$) and increased parasitemia ($p=0.023$). We found that Ang-2, sICAM-1, sFlt-1, IP-10, CHI3L1, and sTREM-1 ($p<0.01$), as well as PCT ($p<0.05$), were elevated in fatal cases of severe malaria compared to survivors (Fig. 5.2C).

Table 5.1: Demographic and clinical characteristics of study participants presenting with uncomplicated and severe malaria.[a]

Characteristic	UM[b] (n=53)	CM[c] (n=44)	SMA (n=59)	Pooled severe malaria	
				Survivors (n=80)	Fatalities (n=23)
Gender (% female)	45.3	52.3	49.2	46.3	65.2§§
Age (years)	4.4 (2.1, 8.1)	3.0 (1.5, 4.3)	1.3 (0.9, 2.0)$^{***\#\#\#}$	1.6 (1.0, 3.1)	1.9 (1.2, 3.3)
Days reported ill prior to presentation	3 (2, 4)	3 (2, 4)	4 (3, 5)$^{***\ \#}$	3 (3, 4)	3 (2, 7)
Parasitemia (parasites/uL)	$3.8x10^4$ ($1.6x10^4$, $1.2x10^5$)	$9.8x10^4$ ($1.5x10^4$, $2.7x10^5$)	$2.6x10^4$ ($7.4x10^3$, $1.2x10^5$)$^{\ \#}$	$3.7x10^4$ ($7.5x10^3$, $1.5x10^5$)	$1.6x10^5$ ($2.2x10^4$, $3.9x10^5$)§
Hemoglobin (g/dL)	10.1 (9.4, 11.3)	6.3 (5. 3, 8.4)***	3.8 (3.2, 4.4)$^{***\#\#\#}$	4.3 (3.4, 5.6)	5.4 (4.2, 8.3)^{§,d}
Platelet count ($x10^9$/L)	166 (107, 219)	73 (47, 128)***	116 (71, 165)*	103 (61, 162)	73 (41, 128)
Fatal cases	0	14	9	0	23

[a]All variables except gender are presented as median (interquartile range). Groups were compared using the Mann Whitney U test or Kruskal-Wallis test with Dunn's post-hoc tests (continuous variables) or Chi-square test (categorical variables). [b]UM, uncomplicated malaria; CM, cerebral malaria; SMA, severe malaria anemia. [c]6 children with concurrent CM and SMA were included in the CM group. 5 children with SMA exhibited decreased consciousness but did not meet criteria for CM. [d]Increased hemoglobin among fatalities was due to the higher CM:SMA ratio in this group vs survivors. * $p<0.05$, *** $p<0.001$ CM or SMA vs. UM. $^{\#}$ $p<0.05$, $^{\#\#\#}$ $p<0.001$ SMA vs. CM. § $p<0.05$, §§ $p<0.01$ fatalities vs. survivors.

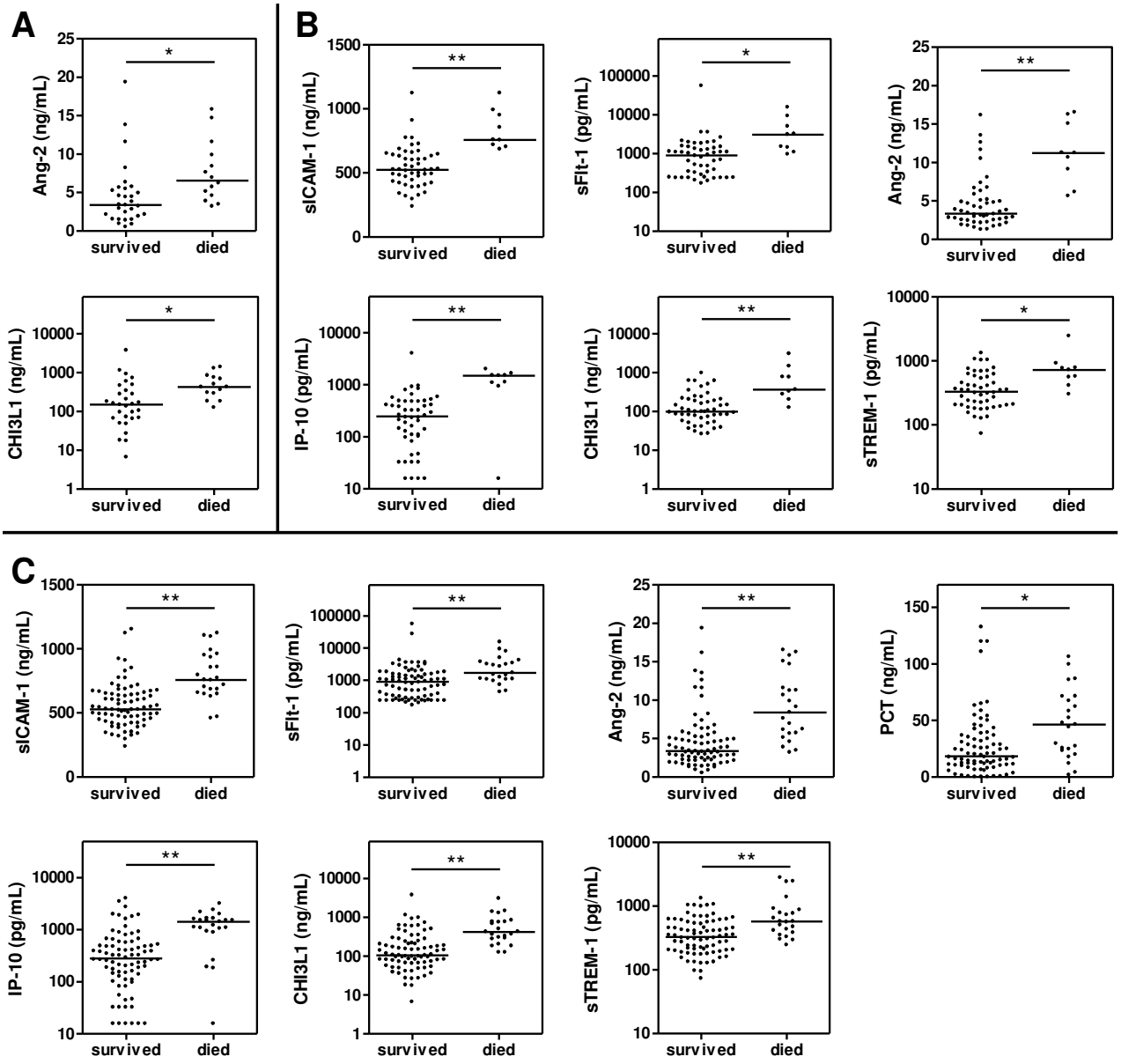


Fig. 5.2: Plasma biomarker levels in children presenting with severe malaria who survived or subsequently died from infection. Presented are biomarkers that were significantly different for (A) CM patients only, (B) SMA patients only, and (C) all severe malaria patients combined. Biomarkers were measured by ELISA. Data are presented as dot plots with medians. A Mann Whitney U test was performed for each comparison, and p values were adjusted for multiple comparisons using Holm's correction (n=13 for each group). * $p<0.05$ and ** $p<0.01$.

To assess how well these biomarkers discriminated between survivors and fatalities, we generated receiver operating characteristic (ROC) curves and determined the area under the curve (AUC) (Fig. 5.3). Ang-2, sICAM-1, IP-10, and CHI3L1 had excellent predictive ability (AUC 0.8-0.9), and sTREM-1, sFlt-1 and PCT had acceptable predictive ability (AUC 0.7-0.8) [579]. The AUC for parasitemia, which is used in clinical practice as a prognostic factor [580], was 0.66.

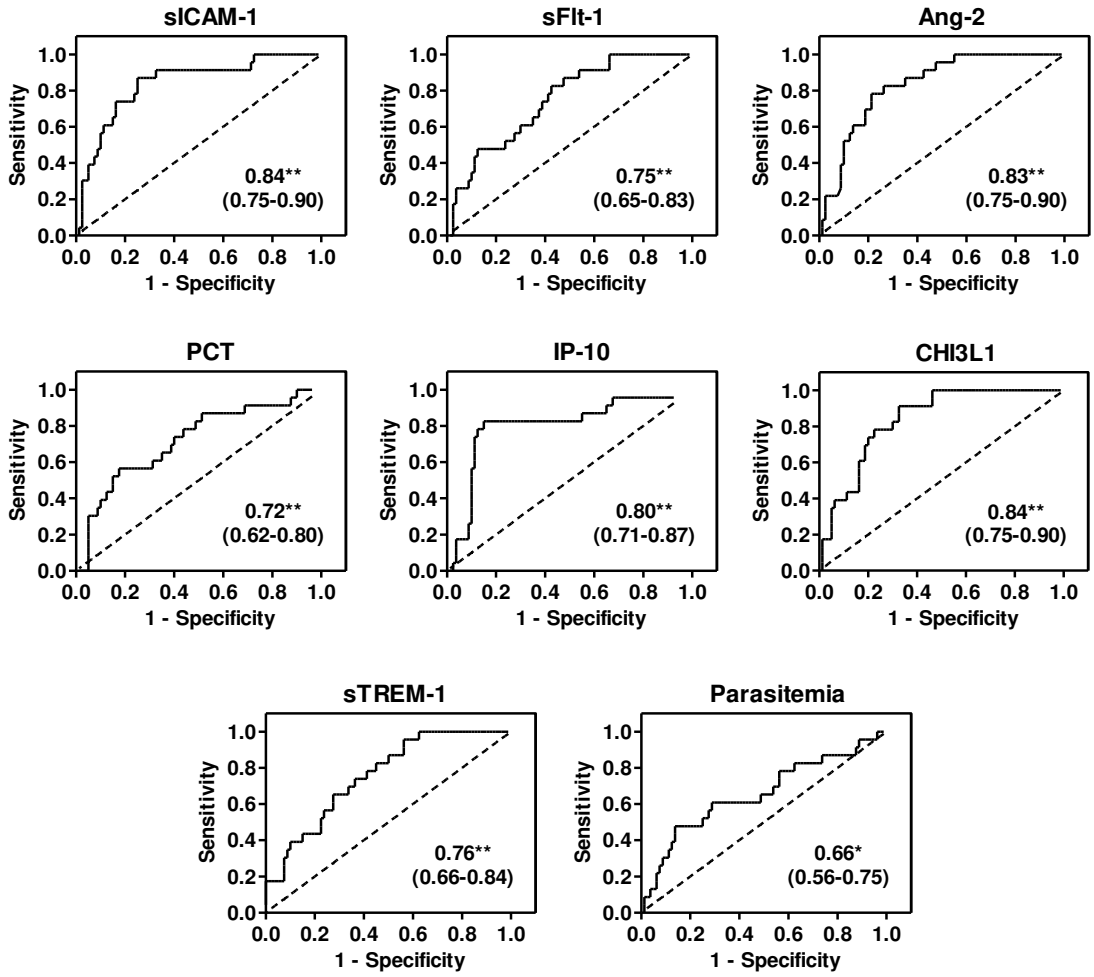


Fig. 5.3: Assessment of biomarker utility in predicting outcome in children with severe malaria. A receiver operating characteristic (ROC) curve was generated for each biomarker. Dashed reference lines represents the ROC curve for a test with no discriminatory ability. Area under the ROC curve is displayed with 95% confidence intervals in parentheses. p values were adjusted for multiple comparisons using Holm's correction (n=7). * $p<0.05$, ** $p<0.01$.

We used the Youden index to obtain a cut-point for each biomarker, and evaluated classification performance of these dichotomized biomarkers (Table 5.2). sTREM-1 achieved the highest sensitivity (95.7%) but had low specificity (43.8%), while IP-10 predicted death with the highest overall accuracy (82.6% sensitivity, 85% specificity).

5.4.3 Predicting mortality using a "biomarker score". We hypothesized that combining biomarkers would improve predictive accuracy. The small number of deaths in the study precluded multivariable logistic regression analysis with more than 2-3 independent variables [581]. Therefore, as performed in other conditions [568], we combined the biomarkers into a score. For each marker, one point was assigned if the measured value was greater than the corresponding cut-point, and zero points were assigned if it was lower. A cumulative "biomarker score" was calculated for each patient by summing the points for all six markers. No two dichotomized biomarkers were highly correlated (Spearman's rho <0.5 for all; Table 5.3), suggesting that each biomarker would contribute some unique information to the score.

Table 5.2: Classification performance of biomarkers for predicting mortality among children with severe malaria.[a]

Biomarker	Cut-point[b]	Sensitivity (%)	Specificity (%)	PLR[c]	NLR	PPV (%)[d]	NPV (%)
Ang-2	>5.6 ng/mL	78.3 (56.3-92.5)	78.8 (68.2-87.1)	3.7 (2.9-4.7)	0.3 (0.1-0.7)	18.2 (5.8-38.7)	98.4 (92.4-99.9)
sICAM-1	>645.3 ng/mL	87.0 (66.4-97.2)	75.0 (64.1-84.0)	3.5 (2.8-4.3)	0.2 (0.06-0.5)	17.4 (5.9-35.9)	99.0 (93.2-100)
sFlt-1	>1066.3 pg/mL	82.6 (61.2-95.0)	57.5 (45.9-68.5)	1.9 (1.5-2.5)	0.3 (0.1-0.8)	10.5 (3.4-23.1)	98.2 (90.4-100)
PCT	>43.1 ng/mL	56.5 (34.5-76.8)	82.5 (72.4-90.1)	3.2 (2.2-4.7)	0.5 (0.3-1.0)	16.3 (3.8-39.5)	96.9 (90.5-99.5)
IP-10	>831.2 pg/mL	82.6 (61.2-95.0)	85.0 (75.3-92.0)	5.5 (4.5-6.8)	0.2 (0.07-0.6)	25.0 (8.3-49.8)	98.8 (93.4-100)
CHI3L1	>177.5 ng/mL	91.3 (72.0-98.9)	67.5 (56.1-77.6)	2.8 (2.3-3.4)	0.1 (0.03-0.5)	14.5 (5.0-30.3)	99.2 (93.1-100)
sTREM-1	>289.9 pg/mL	95.7 (78.1-99.9)	43.8 (32.7-55.3)	1.7 (1.3-2.2)	0.1 (0.01-0.7)	9.3 (3.3-19.6)	99.4 (90.5-100)

[a]All parameters are presented with 95% CIs in parentheses. [b]Cut-points were determined using the Youden Index (J = max[sensitivity + specificity – 1]). [c]PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value. [d]PPVs and NPVs were based on estimates that 5.7% of CM and SMA patients at Mulago hospital die of the malaria infection [545].

Table 5.3: Correlations between biomarkers predicting fatality among children with severe malaria.[a]

	Ang-2	sICAM-1	sFlt-1	PCT	IP-10	CHI3L1	sTREM-1
Ang-2	---	0.31	0.41	0.18	0.42	0.37	0.27
sICAM-1		---	0.26	0.16	0.35	0.35	0.21
sFlt-1			---	0.27	0.47	0.27	0.47
PCT				---	0.24	0.43	0.25
IP-10					---	0.29	0.26
CHI3L1						---	0.30
sTREM-1							---

[a]Spearman's rho is displayed for each pair-wise comparison. None of the correlations was significant after correction for multiple comparisons using Holm's correction.

Biomarker score was highly positively correlated with risk of death (Fig. 5.4A; Spearman's rho=0.94, p=0.001). Scores were elevated among fatalities compared to survivors (Fig. 5.4B; median (interquartile range): 5 (4-6) and 1 (0-2.5), respectively). In a univariate logistic regression model, the biomarker score was a significant predictor of death with an odds ratio of 5.2 (95% CI 3.5-19.1) (Table 5.4, Model 1). After adjustment for parasitemia and age, which have been associated with malaria mortality, the score remained significant with an adjusted odds ratio of 5.1 (95% CI 3.6-56.0) (Table 5.4, Model 2).

Table 5.4: Association of biomarker score with outcome among children with severe malaria: logistic regression.[a]

								Hosmer-Lemeshow test		
	Variable	b (95% CI)	SE	Wald	df	p value	OR (95% CI)	Chi square	df	p value
Model 1[b]	Biomarker score	1.7 (1.3-3.0)	1.7	18.8	1	0.001	5.3 (3.5-19.1)	3.3	6	0.77
Model 2[c]	Biomarker score[d]	1.6 (1.3-4.0)	1.9	18.5	1	0.001	5.1 (3.6-56.0)	4.8	8	0.78
	Log parasitemia[e]	0.27 ((-0.59)-1.4)	0.58	0.30	1	0.46	1.3 (0.55-4.1)			
	Age	-0.005 ((-0.63)-1.1)	0.49	0.001	1	0.99	1.0 (0.53-3.1)			

[a]The reference category was "survival." [b]Pseudo-R^2 (Cox & Snell) 0.475 and calibration slope 1.0. [c]Pseudo-R^2 (Cox & Snell) 0.477 and calibration slope 1.0. [d]Biomarker score and log parasitemia had a significant but low correlation (Spearman's rho 0.276, $p<0.01$). [e]Parasitemia was log-transformed in order to achieve linearity with the log-odds of the dependent variable. SE, standard error; OR, odds ratio.

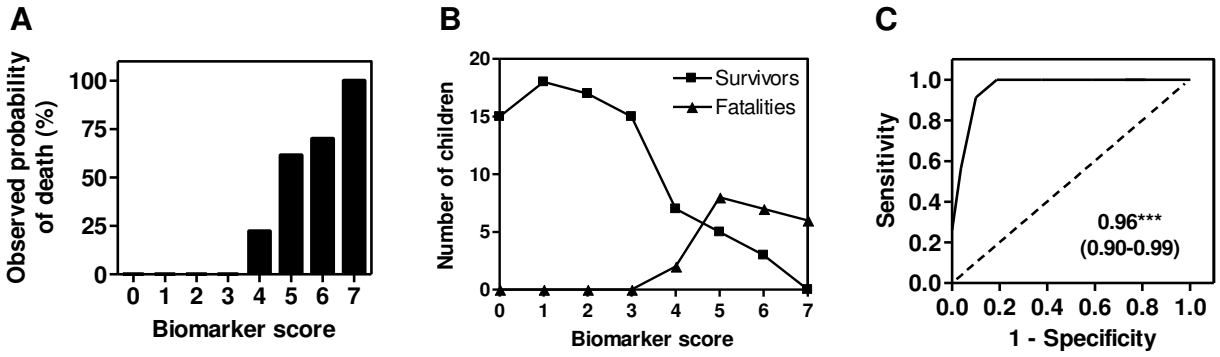


Fig. 5.4: The biomarker score is significantly associated with risk of fatality among children with severe malaria. The biomarker score for each patient was calculated as detailed in the text. (A) Biomarker scores were plotted against observed probability of death. The two variables were significantly related (Spearman's rho=0.94, p=0.001). (B) Biomarker score distributions were plotted for severe malaria survivors and fatalities. (C) A receiver operating characteristic (ROC) curve was generated for the biomarker score. The dashed reference line represents the ROC curve for a test with no discriminatory ability. Area under the ROC curve is displayed on each graph with 95% confidence intervals in parentheses. *** p<0.001.

ROC curve analysis and cut-point determination were performed as above for the biomarker score. The AUC was 0.96 (0.90-0.99) (Fig. 5.4C), and we found that a score $\geq$ 5 was 91.3% sensitive and 90.0% specific for predicting death in our sample (Table 5.5, row 1). While the positive predictive value was low (35.6%) given a fatality rate of 5.7%, the negative predictive value (NPV) was 99.4%, indicating that a child with a score $\leq$ 4 will likely respond well to standard treatment protocols.

A score involving fewer biomarkers might be expected to improve practicality and facilitate potential translation to a clinical application. Using the same scoring scheme, 2-marker combinations performed poorly (data not shown). However, some 3-marker combinations yielded sensitivity >90% and specificity >80% (Table 5.5). Notably, the Ang-2/IP-10/CHI3L1 combination (cut-point $\geq$ 2) had moderate specificity (81.2%), but 100% sensitivity and an NPV of 100%.

To assess whether an accurate 2-marker combination could be achieved using alternative cut-points, we evaluated combinations of the two biomarkers with the greatest AUCs (sICAM-1 and CHI3L1) using all possible cut-points with >90% sensitivity. Combining sICAM-1 (cut-point 605.4 ng/mL) and CHI3L1 (cut-point 116.3 ng/mL) yielded 91.3% sensitivity and 85.0% specificity (Table 5.5).

5.4.4 Predicting mortality using classification tree analysis. To explore another combinatorial strategy, we used classification tree analysis, which selects and organizes independent variables into a decision tree that optimally predicts the dependent measure. Initially, a model based on IP-10 and sTREM-1 was generated with 43.5% sensitivity and 100% specificity for predicting mortality (data not shown). Since high sensitivity would be a crucial feature of a prognostic test for severe malaria, we repeated the analysis assigning the cost of misclassifying a death as a survivor as 10 times greater than the cost of misclassifying a survivor as a death. A model based on IP-10, Ang-2, and sICAM-1 was generated (Fig. 5.5), with 100% sensitivity and 92.5% specificity for predicting outcome (cross-validated misclassification rate 15.4%, standard error 4.9%). In summary, combining dichotomized biomarkers using a scoring system or a classification tree predicted severe malaria mortality in our patient population with high accuracy.

Table 5.5: Classification performance of biomarker combinations for predicting mortality among children with severe malaria.[a]

Combination	Cut-point[b]	Sensitivity (%)	Specificity (%)	PLR[c]	NLR	PPV (%)[d]	NPV (%)
Biomarker score (7 markers)	≥5	91.3 (72.0-98.9)	90.0 (81.2-95.6)	9.1 (7.9-10.6)	0.01 (0.02-0.4)	35.6 (13.3-63.7)	99.4 (94.7-100)
Ang-2, IP-10, CHI3L1	≥2	100 (85.2-100)	81.2 (71.0-89.1)	5.3 (4.8-5.9)	0	24.4 (9.4-46.0)	100 (95.4-100)
Ang-2, PCT, sICAM-1	≥2	91.3 (72.0-98.9)	88.8 (79.7-94.7)	8.1 (7.0-9.4)	0.1 (0.02-0.4)	32.9 (12.1-60.3)	99.4 (94.7-100)
Ang-2, IP-10, PCT	≥2	91.3 (72.0-98.9)	86.3 (76.7-92.9)	6.6 (5.7-7.7)	0.1 (0.02-0.4)	28.6 (10.2-54.4)	99.4 (94.6-100)
sICAM-1, IP-10, CHI3L1	≥2	91.3 (72.0-98.9)	83.8 (73.8-91.1)	5.6 (4.8-6.6)	0.1 (0.03-0.4)	25.4 (9.2-48.8)	99.4 (94.4-100)
PCT, IP-10, sTREM-1	≥2	91.3 (72.0-98.9)	81.3 (71.0-89.1)	4.9 (4.1-5.7)	0.1 (0.03-0.4)	22.7 (8.1-44.8)	99.4 (94.2-100)
sICAM-1, PCT, CHI3L1	≥2	91.3 (72.0-98.9)	80.0 (69.6-88.1)	4.6 (3.9-5.4)	0.1 (0.03-0.4)	21.6 (7.6-43.0)	99.3 (94.1-100)
sICAM-1, CHI3L1[e]	2	91.3 (72.0-98.9)	85.0 (75.3-92.0)	6.1 (5.2-7.1)	0.1 (0.02-0.4)	26.9 (9.5-51.8)	99.4 (94.5-100)

[a]All parameters are presented with 95% CIs in parentheses. [b]Cut-points were determined using the Youden Index (J = max[sensitivity + specificity – 1]). [c]PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value. [d]PPVs and NPVs were based on estimates that 5.7% of CM and SMA patients at Mulago hospital die of the malaria infection [545]. [e]Cut-points were 605.4 ng/mL for sICAM-1 (sensitivity 91.3%, specificity 67.5%) and 116.3 ng/mL for CHI3L1 (sensitivity 100%, specificity 53.8%).

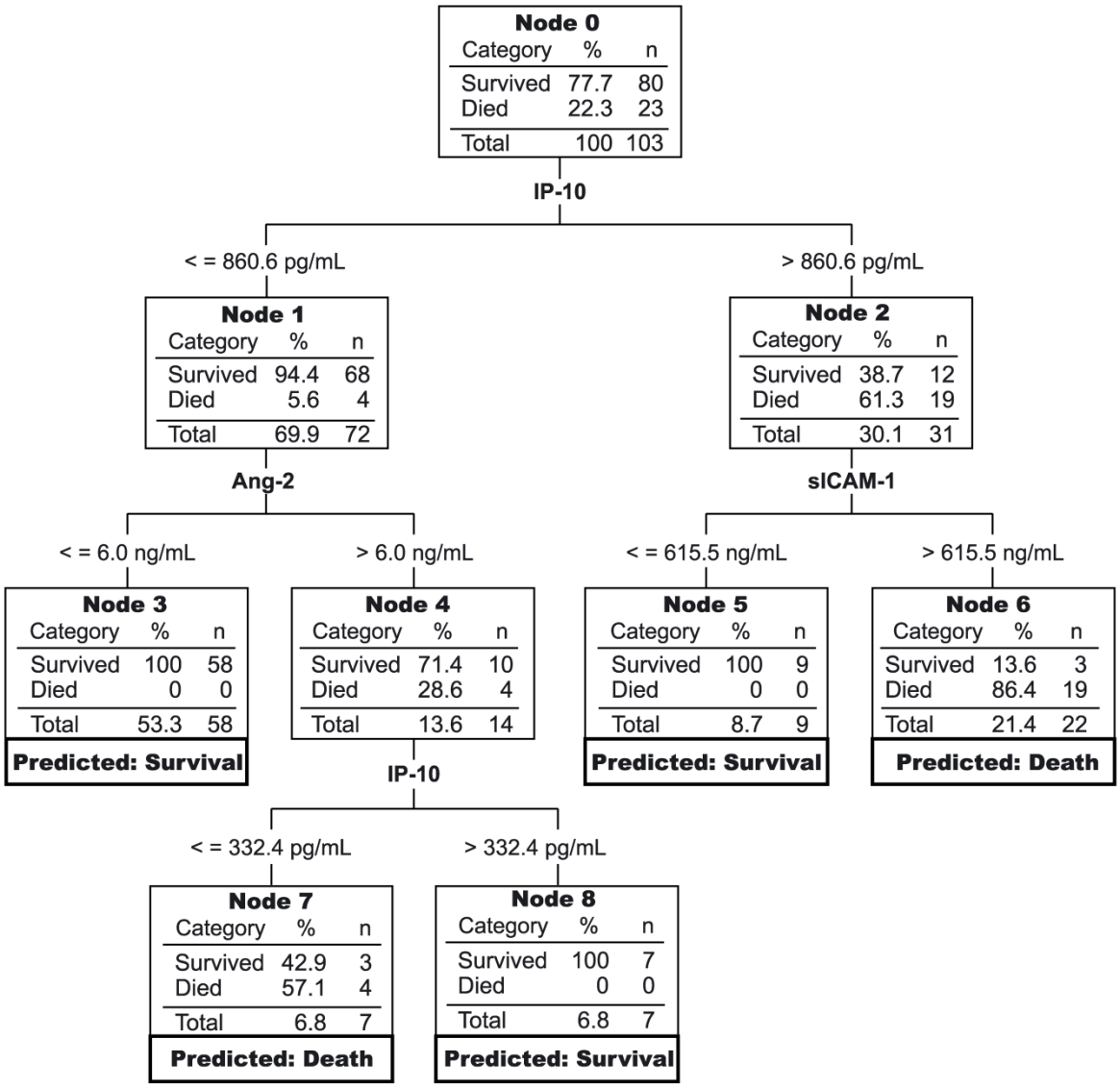


Fig. 5.5: Classification tree analysis to predict outcome of severe malaria infection with host biomarkers. All six biomarkers that discriminated survivors from fatalities were entered into the classification tree analysis. Prior probabilities of survival and death were specified (94.3% and 5.7%, respectively). The cost of misclassifying a true death was designated as 10 times the cost of misclassifying a true survivor. The cut-points selected by the analysis are indicated between parent and child nodes. Below each terminal node (i.e. no further branching), the predicted categorization of all patients in that node is indicated. This model yielded 100% sensitivity and 92.5% specificity for predicting mortality (cross-validated misclassification rate 15.4% with standard error 4.9%).

5.5 DISCUSSION

Combinations of prognostic biomarkers, particularly if drawn from distinct pathobiological pathways, have been found to improve predictive accuracy over individual biomarkers [568,569]. In this study, we demonstrated that simple schemes combining as few as 2-3 host biomarkers of inflammation and endothelial activation predicted mortality with high accuracy among a group of Ugandan children with severe malaria. These findings provide support for the development of prognostic tests for severe malaria based on host biomarker combinations. Moreover, we identified sFlt-1 as a novel biomarker of severe and fatal malaria in children and further characterized WPB exocytosis in malaria infection.

sFlt-1 is generated by alternative splicing of VEGF receptor-1 mRNA and antagonizes the pro-inflammatory and pro-angiogenic effects of VEGF. Our observation of increased sFlt-1 in severe malaria parallels findings in sepsis patients [582]. Data from murine models of sepsis suggest that sFlt-1 may have a protective role in this disease, as sFlt-1 administration reduced VEGF-mediated vascular permeability and mortality [583]. VEGF expression in the brain was increased in European travellers who died of CM compared to controls with non-neurological causes of death [584], and plasma VEGF levels positively correlated with neurological complications in African children with CM [585]. Thus, similarly to sepsis, elevated sFlt-1 in severe malaria may represent a host response to counter the pathological effects of excess VEGF. However, the role of VEGF in malaria infection is controversial: VEGF can also have neuroprotective effects, and some reports have demonstrated decreasing plasma VEGF with

increasing malaria severity [200,504]. Further studies are required to delineate the roles of sFlt-1/VEGF in severe malaria.

As previously described [200-202], we observed increased plasma levels of WPB components Ang-2, vWF, and vWF propeptide in CM vs. UM. We also demonstrated for the first time that these factors are specifically elevated among children with SMA, suggesting that extensive WPB exocytosis occurs not only in CM but also in SMA. Few studies have directly addressed endothelial activation in SMA [508]. WPB exocytosis can be induced by factors generated during malaria infection (e.g., cytokines, histamine, reactive oxygen species) that have been shown to be more elevated in SMA compared to UM [234,238]. It is biologically plausible that increased circulating levels of WPB contents could directly contribute to the pathogenesis of SMA. Ang-2 sensitization of endothelial cells to TNF [204] may amplify secretion of endothelial cytokines, such as IL-6, that can promote anemia [586]. Interestingly, Ang-2 can impair maintenance of long-term hematopoietic stem cells (LT-HSCs) in bone marrow by inhibiting the Tie-2/Ang-1 interaction [587]. While the role of LT-HSCs in SMA requires clarification, it is interesting to speculate that dysregulated Ang-2 levels may contribute to anemia via LT-HSC depletion. Unfortunately we are unable to comment on the Ang-1 levels or Ang-2/Ang-1 ratios in these children due to poor detectability of Ang-1 in citrated plasma.

Regardless of whether these biomarkers mediate or simply reflect pathology, combinations of biomarkers accurately predicted mortality among children with severe malaria in our sample. Notably, some biomarker combinations showed excellent sensitivity, ensuring that the majority of children at high risk of death would be identified. While an effective adjunctive therapy for

severe malaria remains elusive, prognostication could allow triage of patients for closer monitoring or intensive care resources, as available. Such a test may also assist in risk stratification and patient selection for clinical trials of adjunctive therapies, which are ongoing [109,588].

Previous studies have developed clinical scores to prognosticate outcome in pediatric severe malaria [14,126,500]. These scores incorporate clinical features such as prostration, coma, and respiratory distress. The simplicity and negligible cost of these tests are important features. However, a prognostic assay would ideally predict mortality with both high sensitivity and specificity based on a single criterion to avoid the uncertainty associated with non-extreme scores. The biomarker combinatorial strategies presented here appear to possess this attribute, although further studies are required to confirm our findings. Another advantage of a biomarker-based prognostic test over clinical assessment is its objective quality, which is unaffected by between-clinician variability. Furthermore, advances in point-of-care platforms [509,510] may enable development of affordable tests that integrate malaria diagnostics with prognostic biomarkers.

There were some discrepancies between our data and previous studies of biomarkers in pediatric severe malaria. s-endoglin was found to be increased in Gabonese children with severe malaria compared to UM [573], but we did not replicate these results. We observed similar levels of IP-10 in CM and SMA fatalities, in contrast to a report that serum IP-10 was specifically elevated in Ghanaian children who died from CM [572]. However, these studies may not be comparable since blood was obtained post-mortem in the Ghanaian study rather than at admission. It is also

possible that the above discrepancies are due to regional differences in parasite strains, host genetics, and/or common co-infections.

The limitations to our study include a small sample size and the use of non-consecutive samples, which may have introduced a selection bias. Co-infections with pathogens other than HIV were not assessed, and thus it is unclear how they may have affected biomarker levels. Biomarker combinations that accurately predicted mortality require external validation in larger prospective studies with adjustment for potential demographic and clinical confounders. Head-to-head prospective comparisons with known prognostic factors (e.g. lactate) or scores is required to determine the ultimate clinical utility of this biomarker strategy. Validation is also needed in children with non-CM/non-SMA severe malaria syndromes, such as respiratory distress. Further studies should be performed at multiple sites in sub-Saharan Africa, as it is possible that optimal cut-points may vary between populations due to differences in ethnicity, co-infection rates, or overall health. For instance, in a multi-centre study evaluating prognostic factors in severe malaria, the optimal cut-point for base excess varied between sites [502]. Moreover, inter-site differences in treatment guidelines or access to supportive care (e.g. blood transfusions) could affect mortality patterns and thus the generalizability of a prognostic test. Nevertheless, this study identified novel biomarkers in African children, who are at the greatest risk of malaria mortality, and specifically in SMA, for which few informative biomarkers have been described. We provide proof-of-concept that combining as few as 3 biomarkers using simple schemes may be able to accurately predict outcome in severe malaria infection.

Chapter 6

General Discussion and Future Directions

For almost a century, *Plasmodium* was believed to cause severe disease primarily through widespread parasitization of host erythrocytes, accumulation of these infected cells in vital organs, and direct damage to host tissues. It has since been realized that the host is not entirely passive during malaria infection, but rather may contribute to pathology by responding to and interacting with the parasite, as in other severe infections such as sepsis and severe influenza. Presumably, hosts respond as they do with the intent of self-preservation. Humans and *Plasmodium* species have co-evolved over millennia and have largely learned to co-exist. However, the innate immune pathways that can control infectious burden – such as endothelial ICAM-1 upregulation to recruit phagocytes to sites of pathogen localization, or production of pro-inflammatory mediators to activate the defenses of immune and parenchymal cells – can also mediate damage. This may be due to overexuberant immune activation, in the wrong place or at the wrong time; a particular parasite strain that exploits these host responses for its own survival; synergy with an independent insult (e.g. co-infection, malnutrition); or some combination of these factors.

The host response best characterized for its dual nature in malaria infection is inflammation. Inflammation is considered a target for therapeutic modulation in severe malaria, although this strategy has had limited success. New approaches – derived from an improved understanding of the stimuli, receptors, and pathways that contribute to the inflammatory response – may hold promise [267,342]. In this work, we sought to better characterize malaria-induced inflammation with respect to its relationship to phagocytosis, as well as novel candidates

that may play a role. We also exploited the excessive activation of inflammation and endothelial activation in severe malaria to generate combinatorial biomarker tools for predicting outcome.

6.1 Links between inflammatory and phagocytic pathways in malaria infection

6.1.1 General discussion

Studies in murine models have implicated TLR-mediated inflammation in the pathogenesis of severe malaria. In our study, we uncovered a potentially beneficial role for TLR activation by parasite components: enhanced non-opsonic PE internalization by macrophages. The relative importance of CD36-mediated PE uptake during malaria infection in non-immune hosts is not known. However, TLR activation may also promote clearance of PEs opsonized with autologous IgG: we found that FSL-1 stimulation increased phagocytosis of RBCs opsonized with low – but not high – levels of IgG (data not shown). Thus, TLR signalling may serve to boost pathogen clearance prior to development of a robust acquired immune response. Consistent with our findings, SNPs that enhance TLR signalling have been associated with protection from clinical and placental malaria in human populations [321,322] – although TLR-mediated inflammatory responses were likely a major contributor. Contrary to what our data would predict, TLR/MyD88-deficient mice did not exhibit increased parasitemia during *P. chabaudi* infection. Our *in vitro* findings, using purified, bead-conjugated *Pf*GPIs, may not reflect interactions *in vivo*. It is also possible that other pathways have compensated for TLRs in these genetically altered animals, or that other processes affected by TLR deletion obscure any reduction in parasite clearance.

While inflammation and phagocytosis were integrated to some degree in our model system, we did not observe pro-inflammatory cytokine production upon PE internalization by macrophages. This is unusual: while some pathogens have evolved mechanisms to dampen inflammation upon phagocytosis [380,383], at least some response is generally detected. While the reason for unproductive phagosomal TLR signalling is unknown, this finding may have implications for the development of adaptive immunity to malaria. Blander *et. al.* demonstrated that antigen presentation on MHCII can occur in a TLR-dependent and phagosome-autonomous manner: phagocytosis of particles lacking TLR agonists (e.g. apoptotic cells) did not lead to antigen presentation, even if DCs were stimulated with exogenous LPS or simultaneously engulfed TLR-stimulating particles [589]. If our observation hold true for DCs (the more efficient antigen-presenting cell), ineffective phagosomal TLR signalling during PE uptake may weaken T cell priming for PfEMP-1 or other blood-stage antigens.

Our findings emphasize that the characteristics of purified PAMPs may not always be penetrant in more complex models, as in the case of hemozoin and Caspase-1 inflammasome activation [352]. Although purified *Pf*GPIs stimulate TLR2-mediated inflammation *in vitro*, endogenous *Pf*GPIs do not seem to induce cytokine production during PE phagocytosis, and *Tlr2*$^{-/-}$ mice do not show a phenotype in murine malaria models. The latter finding may relate to compensation by other TLRs or differences between *P. falciparum* and *P. berghei* GPIs. However, it was shown in that while TLR2 mediates pro-inflammatory responses to *T. cruzi* GPIs *in vitro*, *Tlr2*$^{-/-}$ mice exhibited increased inflammation during *in vivo* infection [590]. As discussed, the context of PAMP presentation to PRRs is critical, and the myriad pathways activated during infection may lead to masking or unexpected synergies.

The apparent segregation of PE internalization and cytokine production lessens concerns that therapeutic enhancement of innate PE clearance will promote immunopathology. Our group has previously demonstrated that peroxisome proliferator-activated receptor (PPAR)-gamma agonists specifically upregulate CD36 expression and CD36-dependent PE uptake *in vitro* [591]. Moreover, treatment of acute mild malaria patients led to more rapid parasite clearance without exacerbating inflammation [588]. However, these PPAR agonists have independent anti-inflammatory effects [591], and thus the present study confirms that CD36-mediated PE internalization *per se* does not induce cytokines. It should be noted that therapeutic manipulation of CD36 in severe malaria is controversial. Due to its role in mediating PE cytoadhesion to endothelial cells directly [121] or via platelets [216], as well as PE-induced inhibition of DC maturation [592], agents have been developed to disrupt the PE-CD36 interaction [593]. Human genetic studies have been variable, reporting association of CD36 nonsense mutations with susceptibility to or protection from severe malaria, or no relationship at all [594-597]. While differences in study design or population may have contributed to these conflicting results, it is plausible that CD36 plays multiple roles in malaria infection due to its ability to bind PEs and various endogenous ligands, and may exert different effects on disease progression in a context-dependent manner.

6.1.2 Future directions

A number of questions merit further investigation. For example, why is phagosomal TLR signalling unproductive during PE internalization? To determine whether TLR signalling is initiated at all, proximal events could be examined, such as recruitment of TLRs and adaptor

molecules to the phagosome (if technically feasible) and IRAK-1 phosphorylation. As a complementary approach, the *P. falciparum* genome could be scanned for homologs of TLRs or TLR adaptors. This strategy successfully identified a TIR domain homolog in *Paracoccus denitrificans* bacteria that interacts with mammalian MyD88 and may be a competitive inhibitor for TLR signalling [598]. Recently, a herpesvirus NLR homolog was found to block NLR signalling to promote its own persistence [599]. Malaria may make use of a similar strategy to limit inflammation.

Further characterization of the signalling pathway mediating TLR-enhanced phagocytosis could also be pursued to identify points of divergence from pro-inflammatory TLR signals. If TLRs are indeed valid therapeutic targets in CM or other severe infections, it would be ideal to spare their pro-phagocytic effects. Systematic blockade of TLR downstream signalling elements using chemical inhibitors or siRNA could be assessed for effects on phagocytosis and inflammation using EBABs or other model systems.

One TLR signalling molecule likely to be identified by this strategy is PI3K. PI3K is critical for phagocytosis of most particles, including EBABs and PEs (data not shown). The role of PI3K in TLR-mediated inflammation has been controversial, possibly due to off-target effects of chemical PI3K inhibitors. However, mice genetically deficient in PI3K subunits generally show increased inflammation in various models (reviewed in [600]), indicating that PI3K normally suppresses TLR-mediated inflammatory responses. It appears that PI3K manipulation can have opposing effects on inflammation and phagocytosis *in vivo*. In a murine sepsis model, PI3K inhibition was linked to enhanced inflammation, depressed neutrophil phagocytosis, and

increased susceptibility to disease [601]. Conversely, mice with a myeloid-specific deletion of PI3K regulator phosphatase and tensin homologue deleted on chromosome Ten (PTEN) showed increased PI3K activity, reduced inflammation, enhanced phagocytic clearance, and prolonged survival during infection with *Streptococcus pneumoniae* (which, similarly to malaria, activates TLR2 and TLR9) [602]. The roles of PI3K and PTEN in the inflammatory and phagocytic responses to malaria *in vitro* and *in vivo* could be investigated using genetic and pharmacological strategies (e.g. PI3K and PTEN inhibitors, targeted knockdown of myeloid PTEN *in vivo*). Certainly, PI3K activation is by no means specific to TLRs, and thus potential side effects of its modulation must be evaluated.

The ability of TLRs to enhance phagocytosis of IgG-opsonized RBCs, as aforementioned, raises the possibility that TLR stimulation may enhance clearance of uRBCs opsonized by IgG/complement during infection, thereby potentially contributing to SMA. We have begun preliminary studies assessing the effects of exogenous TLR agonists on macrophage internalization of uRBCs isolated from *P. falciparum* culture or *P. chabaudi* infection. However, we have been unable to observe uRBC phagocytosis even in the absence of TLR stimulation. This suggests that the uRBCs have not been sufficiently modified by malaria exposure to be recognized by macrophages, which may be a requirement for TLR-mediated enhancement of phagocytosis. Incubation of uRBCs with serum from semi-immune individuals may enhance uptake, due to binding of antibodies to parasite proteins deposited on the uRBC surface [232]. To assess TLR-enhanced erythrophagocytosis *in vivo*, biotin-labelled RBCs could be injected in wild-type and TLR-null *P. chabaudi* mice and monitored for more rapid turnover in TLR-sufficient animals. While this approach does not formally distinguish between clearance by

phagocytes or other means (e.g. hemolysis), erythrophagocytosis in spleen and bone marrow can be evaluated. If TLR stimulation does promote uRBC clearance, this may provide some rationale for TLR-inhibitory adjunctive therapy in SMA. Effects of TLR activation on dyserythropoiesis in malarial anemia models also warrant examination.

6.2 Investigating novel candidates in the inflammatory response to malaria

6.2.1 General discussion

TREM-1 signalling has been causally implicated in rodent models of inflammatory pathologies, including multiple sepsis models, and this is consistently linked to elevated monocyte TREM-1 and plasma sTREM-1 in the corresponding human condition. In this work we substantially add to the characterization of TREM-1 in malaria infection by demonstrating increased monocyte TREM-1 levels and plasma sTREM-1 in (severe) malaria infection in human and murine systems. However, contrary to our hypothesis, TREM-1 blockade did not reduce cytokine responses or immunopathology in the murine malaria models we examined.

These negative findings reinforce that, despite similarities in clinical presentation and pathobiological pathways, sepsis and severe malaria are not wholly comparable. The strategy of drawing from the sepsis literature to guide malaria research has been instructive in some cases (e.g., C5a). Excessive TH1 inflammatory responses and endothelial dysfunction have been found to contribute to both sepsis and severe malaria, and considering the finite number of innate immune pathways available to respond to danger signals, there is likely to be overlap. Indeed, most PRRs and inflammatory mediators have been implicated in a range of infectious and non-infectious inflammatory models. However, the diversity of the innate immune system and its

ability to discriminate between threats is becoming increasingly apparent as novel PRRs are discovered and synergies between PRR signalling pathways identified [269,297]. As a result of the different structural elements and life cycles of malaria and bacterial species, the complement of activated PRRs and the context of PAMP recognition will differ, and thus so may the dysregulated inflammatory responses contributing to pathology. (Diversity amongst bacterial species causing sepsis may similarly underlie the heterogeneity of this condition.) Ultimately, these differences may account for the low prevalence of typical sepsis complications (e.g. hypotension, renal failure, disseminated intravascular coagulation) in severe malaria, at least in pediatric populations [34]. While analogizing malaria and sepsis has yielded some useful information, this is not a given, and unbiased approaches may be more informative (discussed below).

Selection of the other novel candidate, CHI3L1, was based on its ability to modulate inflammation and other host pathways relevant to severe malaria. We found that CHI3L1 was induced in both human and murine malaria, which to our knowledge is the first characterization of CHI3L1 in protozoal infection. While CHI3L1 deficiency may increase susceptibility to ECM, it did not appear to affect malaria-induced inflammation as we hypothesized. The promotion of TH2 adaptive immune responses by CHI3L1 in asthma models [483] may not be directly relevant to the TH1 inflammatory response in primary malaria infection. However, CHI3L1 could exert similar effects in these two contexts (e.g. TGF-β induction). Moreover, the role of CHI3L1 in inflammatory responses as a result of its pro-survival activity, as well as the pro-survival activity itself, seemed worthwhile to investigate in PbA-induced ECM, considering the implication of endothelial and neuronal apoptosis in this model. The slightly earlier death of

Chi3l1$^{-/-}$ mice may have been a result of earlier apoptosis of these cells in the brain, though this requires experimental validation.

6.2.2 Future directions

Future studies will address the hypothesis that TREM-1 contributes to the severity of malarial-bacterial co-infection, as we found that TREM-1 activation heightened the pro-inflammatory response to *P. falciparum* by decreasing IL-10 production. Moreover, upregulation of monocyte TREM-1 expression by malaria ([445], Fig. 3.4) may facilitate TREM-1 activation by co-infecting bacteria. First, to extend our *in vitro* findings, ECM-resistant BALB/c mice will be infected with PbA and injected with an agonistic TREM-1 antibody. We predict that TREM-1 activation will suppress IL-10 production, which contributes to protection in this model [47,174], and thereby increase susceptibility to ECM. Cytokine levels and TNF/IL-10 ratios will be compared between uninfected and infected mice with or without antibody treatment, to determine whether pro-inflammatory responses are synergistically enhanced by PbA infection and TREM-1 activation. Monocyte TREM-1 upregulation by PbA infection will be evaluated as a potential basis for any synergy observed.

This question could also be addressed in the *P. chabaudi*-endotoxemia model described by Franklin *et. al.* [344]. Mice resistant to either *P. chabaudi* or low-dose LPS alone show excessive inflammation and reduced survival upon combination of these insults, due to upregulation of TLRs and other immune-related genes by *P. chabaudi* via TLR9 and IFNγ. If TREM-1 is also found to be upregulated by *P. chabaudi*, its contribution to LPS hyperresponsiveness could be assessed using *Trem1*$^{-/-}$ mice (although careful controls will be required since LPS can also

upregulate TREM-1). Cytokine levels would be analyzed as described above. If these studies indicate a role for TREM-1 in malaria-bacterial co-infection, monocyte TREM-1 expression could be compared in patients with malaria, bacterial infection, or co-infection, and correlated to plasma levels of pro- and anti-inflammatory cytokines.

Regarding CHI3L1, a potential role in protection from severe malaria will be investigated using PbA infection of *Chi3l1*$^{-/-}$ BALB/c mice, as it is easier to observe increased susceptibility to ECM in a normally resistant strain. We will also determine whether provision of exogenous recombinant CHI3L1 to susceptible mice improves outcome. Lee *et. al.* generated a mouse strain with a lung-specific transgene encoding human CHI3L1, which can replace the function of murine CHI3L1 [483] and is thought to travel systemically (C. Lee, personal communication). Transgene expression is under the control of a doxycycline-inducible promoter, which may complicate PbA challenge due to the anti-malarial properties of doxycycline. The use of doxycycline-resistant *P. yoelii* strains may enable use of these transgenic mice to address the role of CHI3L1 in malaria infection.

If CHI3L1 is found to mediate protection against severe malaria in murine models, several lines of investigation may be pursued with respect to mechanism. These include the capacity of CHI3L1 to induce TGF-β [483] during malaria infection, thereby dampening pathological inflammation; the pro-survival effects of CHI3L1, which may protect neurons or endothelial cells from apoptosis; and CHI3L1 regulation of MMPs [476], which have been proposed to contribute to ECM pathogenesis [603]. Finally, *Chi3l1* promoter haplotypes linked to altered protein expression could be assessed for their association with malaria severity in a case-control

study: variants mediating increased CHI3L1 expression would be expected to be overrepresented in uncomplicated versus severe malaria patients. If these combined investigations reveal a role for CHI3L1 in protection from severe disease, therapeutic strategies based on enhancement of CHI3L1 levels or activity could be considered.

In this work, we attempted to define novel mediators of innate host responses to malaria using a candidate approach. This approach could be continued by regularly consulting the literature for newly discovered PRRs with functions relevant to malaria, or genes/proteins that have been shown to be differentially expressed in mild and severe malaria. Disadvantages of this strategy include investment of time and resources into candidates that may or may not produce useful data, as well as the constraint of selecting only molecules that have already been linked to innate immunity or malaria.

Unbiased approaches, while more time-consuming and resource-intensive, can circumvent the above issues, and are currently being undertaken to identify host resistance and susceptibility determinants in the context of malaria. A large genome-wide association study was recently performed to discover loci linked to severe malaria, although analysis has been complicated by the complex genetic structures in African populations [604]. In mice, an N-ethyl-N-nitrosourea mutagenesis screen is underway, and a forward genetics strategy has yielded many new insights in the PbA and *P. chabaudi* models [605].

High-throughput genome-wide RNA interference (RNAi) screens have been used to identify host factors involved in bacterial and viral infection of target cells *in vitro* [606,607]. In the malaria

field, targeted RNAi screens have identified genes in hepatocyte cell lines that contribute to PbA sporozoite infection, and these were further validated in murine models [608,609]. Similarly, RNAi screens could be developed for probing host factors mediating inflammation, phagocytosis, or other responses during *in vitro* exposure of host cells to blood-stage malaria. Human macrophage and endothelial cell lines could be used to assess phagocytosis and apoptosis, respectively, although these lines have poor inflammatory responses to *P. falciparum*. Macrophage-like Drosophila S2 cells may be a viable option, as they are easily transfected with RNAi libraries and have been used to identify new roles for homologous mammalian receptors (notably, CD36 [381]). S2 cell responses to malaria would first require characterization, and a substitute readout for pro-inflammatory cytokine production would be required (e.g. anti-microbial peptide secretion). While tractable, the above systems do carry a risk of irrelevance to primary human cells. Notably, technology for primary mammalian cell transfection is improving, and high-throughout siRNA transfection protocols have been reported for monocyte-derived human DCs [610]. An *in vitro* screen for host molecules mediating responses to blood-stage malaria may reveal involvement of unexpected factors, that would then require validation in murine and human systems as potential targets for intervention.

6.3 Combinations of host biomarkers accurately predict outcome in Ugandan children with severe malaria

6.3.1 General discussion

We evaluated CHI3L1, sTREM-1 and other biomarkers of inflammation and endothelial activation for their capacity to predict outcome among children with severe malaria. Predictive tools are currently used for triage and admission to intensive care units in developed countries. In

a typical hospital in Africa, which is drastically more overcrowded, underfunded, and poorly systematized, such tools are of even greater importance to ensure appropriate and timely allocation of scarce supportive/intensive care resources to those at highest risk of death [611,612]. A rapid and simple prognostic test for severe malaria would be useful in this context. While previous reports have associated clinical features, laboratory results, or plasma proteins with risk of mortality, test performance characteristics – if evaluated at all – were not ideal. We found that simple combinations of as few as 3 biomarkers showed excellent sensitivity and specificity for predicting death among Ugandan children with severe malaria. There were some important limitations to our study, most notably the non-consecutive nature of the samples. However, these data warrant validation studies with larger numbers and more rigorous design.

6.3.2 Future directions

As discussed in detail in Chapter 5, the biomarker combinations we identified require external validation in large prospective studies with consecutively collected samples, adjustment for relevant confounders, and formal comparison with known prognostic factors. The small sample size in this study precluded division of the data into training and test sets during analysis, and thus it is possible that our models are over-fitted. This may translate into reduced performance characteristics in validation studies. To protect against this (or simply to improve current combinations), biomarkers of other host processes implicated in severe malaria could also be measured, since combining markers from multiple pathological pathways improves accuracy of prediction [568,569]. Markers of platelet activation may be valuable, such as Platelet Factor 4 or beta-thromboglobulin, which were previously found to be increased in uncomplicated malaria compared to healthy controls [613]. Moreover, the soluble form of Fas was recently shown to be

elevated in CM fatalities versus survivors in India [504]. Inclusion of these biomarkers into the validation study may lead to novel combinations with greater accuracy. While hemostatic and apoptotic biomarkers may be most relevant to CM, it is possible that they will also be elevated in SMA, similarly to what we observed for endothelial activation markers. A large study will provide the luxury of meaningful sub-group analysis. Similar patterns amongst severe malaria syndromes would facilitate potential translation of these findings into a prognostic test for use in the field.

As with discovery of novel molecular players in the host response to malaria infection, unbiased approaches may be more effective than hypothesis-driven identification of optimal biomarker combinations. In conditions such as cancer or transplant rejection, prognostic models are being developed based on genomic, transcriptomic, and/or proteomic profiling of blood, urine, or diseased tissue (reviewed in [614-616]). These systems-based strategies are appealing due to the increased probability of generating a highly accurate combination of markers. However, they are also expensive, and proteomic profiling in particular presents both technical and analytical challenges. This strategy may be a consideration if future studies fail to validate the biomarker combinations assessed in this work.

The utility of host biomarkers may extend beyond prognosis of severe malaria. Clinicians in non-malaria endemic countries lack familiarity with malaria and have difficulty recognizing signs of severe malaria in returned travellers and immigrant populations. Moreover, a recent retrospective study in Uganda suggested that a large proportion of children admitted to hospital with a diagnosis of severe malaria likely had uncomplicated disease and could have been sent home

with oral anti-malarial treatments [617]. Thus, identifying biomarker combinations that discriminate between mild and severe malaria may be of use in the selective admission of severe malaria patients to hospital. Moreover, if a novel adjunctive therapy is found to be effective against severe malaria, biomarkers that predict positive response to therapy at presentation or during treatment could be identified and used in clinical decision-making and monitoring. Lastly, biomarker combinations that accurately predicted outcome in severe malaria could be tested for utility in sepsis, which is also characterized by dysregulated inflammation and endothelial activation and requires prompt triage for effective management.

6.4 Final considerations

In recent years, there has been renewed interest in launching malaria control programs with the ultimate goal of eliminating or even eradicating malaria. Distribution of insecticide-treated bed nets, indoor residual spraying of insecticides, and improved case management are believed to have contributed to substantial declines in prevalence of infection, malaria-related hospital admissions, and mortality in children under 5 in many African countries [1,618]. Nevertheless, the threat of parasite resistance to artemisinins looms large, and regions with poor infrastructure or political instability continue to experience high malaria burdens [2]. Effective treatments for severe malaria are still needed. This is particularly true for CM, as falling transmission rates due to control programs could potentially increase the proportion of CM cases among those who progress to severe malaria [619,620].

Previous attempts at immunomodulatory therapies for severe malaria have proved disappointing. There are many potential pitfalls to such strategies. By the time a CM patient presents to

hospital, is the cytokine storm too intense and self-perpetuating to be interrupted? Can anything be effective in SMA patients without rapid provision of new erythrocytes via transfusion? Are the same inflammatory mediators contributing to severe malaria pathogenesis in every case, or is there heterogeneity, thus complicating target selection? Have immunomodulatory therapies failed because they inhibit host responses too severely, and/or suppress beneficial inflammatory responses as well as pathological ones? Have they failed because they are unable to cross the blood-brain barrier, where local cytokine production is mediating direct neuronal damage?

The answers to these questions can only be determined through careful research that defines the timing, nature, and overlap between protective and detrimental host responses to malaria. Lessons can be learned from other fields, including the need for understanding the biological nuances of potential therapeutic targets and using highly specific agents. For example, a clinical trial of a TNF inhibitor is believed to have caused harm in multiple sclerosis patients because in addition to blocking TNFR1, which contributes to nerve demyelination, it counteracted the remyelination-promoting activity of TNFR2 [621]. Any efforts to inhibit pro-inflammatory mediators in malaria should adequately address any additional effects of the target molecule.

The growing literature on physiological mechanisms that govern resolution of inflammation may also be instructive. Early pro-inflammatory mediators in malaria (e.g. C5a) may be difficult to modulate once severe disease has developed. Administration or upregulation of immunoregulatory molecules avoids this concern, and may be more effective than inhibiting a single cytokine. Proof-of-principle for such a strategy in severe malaria has come from studies of HO-1/CO and PPAR-gamma agonists. A variety of other naturally occurring anti-inflammatory

mediators have been identified (reviewed in [622]). For instance, the resolvins are endogenous lipids derived from eicosanoids and docosanoids that both suppress inflammation and promote macrophage phagocytosis, consistent with a role in restoring inflamed tissue to homeostasis [623]. ResolvinD was shown to reduce C5a-mediated pathology, induce endothelial NO production, and improve survival in microbial sepsis models [624]. These qualities, as well as the existence of synthetic versions already in clinical trials, make resolvins attractive candidates for further investigation in malaria models.

There is conflict in the malaria research community regarding the relevance and value of murine models, particularly PbA-induced ECM. As in most fields, these models are imperfect, but replicate different aspects of human disease. While results must be interpreted with caution, they may lend key insights into the sequence and localization of pathological events that could never be ethically appraised in humans. A multi-pronged approach drawing upon all available research strategies – basic, clinical, epidemiological, sociological, organizational – is required to develop effective therapies, systems and policies that will reduce the morbidity and mortality burden of *Plasmodium* infection worldwide.

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