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REVIEW

Dynamic interactions of *Plasmodium* spp. with vascular endothelium

Mark R. Gillrie [a,b] and May Ho[a]

[a]Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada; [b]Department of Medicine, University of Calgary, Calgary, Alberta, Canada

ABSTRACT

Plasmodial species are protozoan parasites that infect erythrocytes. As such, they are in close contact with microvascular endothelium for most of the life cycle in the mammalian host. The host-parasite interactions of this stage of the infection are responsible for the clinical manifestations of the disease that range from a mild febrile illness to severe and frequently fatal syndromes such as cerebral malaria and multi-organ failure. *Plasmodium falciparum*, the causative agent of the most severe form of malaria, is particularly predisposed to modulating endothelial function through either direct adhesion to endothelial receptor molecules, or by releasing potent host and parasite products that can stimulate endothelial activation and/or disrupt barrier function. In this review, we provide a critical analysis of the current clinical and laboratory evidence for endothelial dysfunction during severe *P. falciparum* malaria. Future investigations using state-of-the-art technologies such as mass cytometry and organs-on-chips to further delineate parasite-endothelial cell interactions are also discussed.

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Introduction

Plasmodium species are intracellular protozoan parasites of erythrocytes. The life cycle of malaria parasites is complex, with asexual reproduction occurring in the mammalian host and sexual reproduction in the anopheline mosquito vectors.[1] The parasites are transmitted to humans in the form of sporozoites through the bite of a female anopheline mosquito. After circulating briefly in the bloodstream, sporozoites invade hepatocytes in which they undergo asexual reproduction to form a large intracellular schizont that contains thousands of merozoites when they are mature within 5–15 d of sporozoite inoculation. The enlarged hepatocyte eventually bursts, releasing merozoites into the bloodstream where they rapidly invade erythrocytes via specific surface receptors. Approximately 50% of released merozoites effectively reinvade erythrocytes to initiate the erythrocytic cycle. Inside the erythrocyte, the parasite develops within a membrane-bound parasitophorous vacuole first as a trophozoite and then as a schizont. When the schizont matures, the infected erythrocyte ruptures, liberating merozoites that rapidly invade fresh erythrocytes in the general circulation, thus continuing the erythrocytic life cycle. Some merozoites develop into sexual forms (gametocytes), which are taken into the mosquito mid-gut with a blood meal. These may fuse to form a zygote and then undergo meiosis to form first an ookinete and later an oocyst in the gut wall. The oocysts burst, liberating large numbers of sporozoites that migrate to the salivary glands where they are injected into the human host during the next blood meal to continue the exoerythrocytic life cycle.

Because of the intravascular localization, infected red blood cells (IRBC) are in intimate contact with vascular endothelium throughout the erythrocytic cycle. The host-parasite interactions of this stage of the infection are responsible for the clinical manifestations of the disease that range from a mild febrile illness to severe and frequently fatal syndromes such as cerebral malaria and multi-organ failure. However, despite intense investigations, many questions remain regarding the role of endothelial cells in the pathogenesis of severe malaria. In particular, the part played by endothelial barrier function is controversial. Is barrier function compromised in severe malaria and to what degree? Is barrier dysfunction a direct effect of parasite adhesion or is it secondary to endothelial activation by

CONTACT May Ho, MD mho@ucalgary.ca University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada.

pro-inflammatory mediators? Is there significant endothelial cell death? In this review, we will focus on the endothelial response to *Plasmodium falciparum*, the major cause of mortality and morbidity among the 5 known plasmodial species that infect humans. Endothelial pathology by *P. vivax* that has been associated with severe disease will also be discussed.

Endothelial cell barriers

Endothelial cells that line the vasculature separate the vascular and parenchymal compartments of all organs and thereby regulate gas exchange, metabolism, trafficking of immune cells and dissemination of blood borne infections. The homotypic and heterotypic cell-cell interactions formed on the lateral membrane that maintain paracellular barrier integrity include adherens and tight junctions, with a lesser contribution by gap junctions.[2] Adherens junctions (AJ) are composed of VE-cadherin and nectins while tight junctions (TJ) are composed of junctional adhesion molecules (JAM), claudins, and occludin all of which are anchored to the endothelial cytoskeleton via various plaque proteins such as p120, afadin, catenins, and zonula occludens (ZO) (Fig. 1). Plaque proteins also serve to localize regulators of junction assembly and disassembly, including Src-family kinases (SFK) and vascular endothelial cell–specific phosphotyrosine phosphatase (VE-PTP). In general, endothelial barrier function is maintained by preserving transmembrane AJ and TJ proteins VE-cadherin, claudin-5 and occludin in an unphosphorylated state.[2,3] AJ serve as the primary regulators of endothelial barrier function in most organs, with the notable exception of the blood brain barrier (BBB) where the thin microvascular endothelial cells are bound together to a large extent by TJ, creating a high resistance paracellular barrier. Permeability results when junctional proteins are relocated or their expression downregulated, as occurs in many inflammatory conditions.

In addition to the paracellular barrier maintained by junctional proteins, there has been increasing appreciation of the role of transcytosis that serves to transport albumin across resting endothelium under

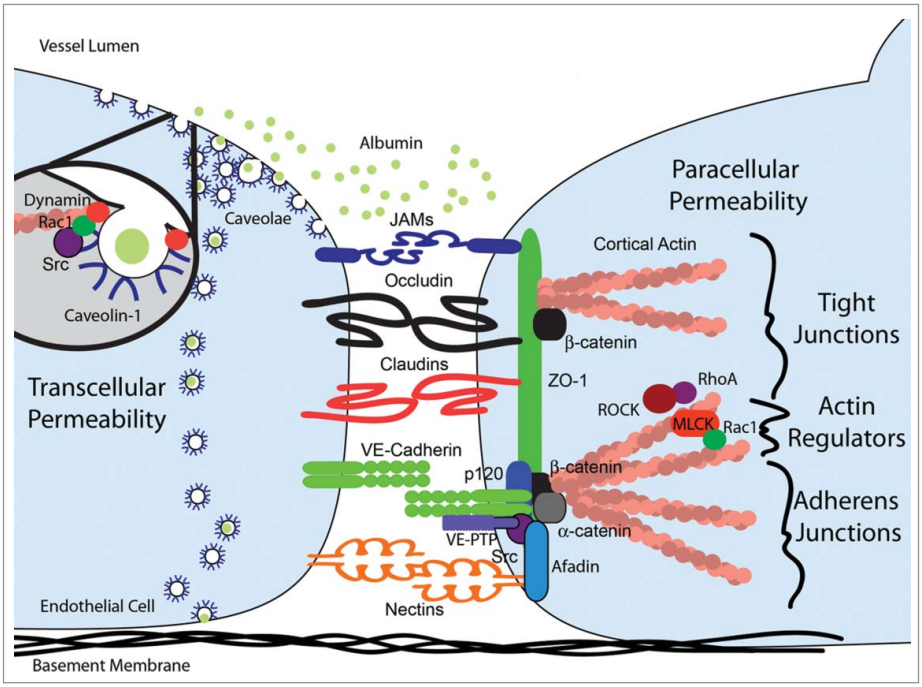

Figure 1. Schematic representation of the transcellular and paracellular endothelial permeability pathways. Transcellular permeability is mediated by the transcytosis of albumin through endothelial cells by membrane invaginations called caveolae. The process is regulated by dynamin which pinches off the neck and thereby internalizes caveolae in response to c-Src and Rac1 signaling. Paracellular permeability is regulated by tight (TJ) and adherens (AJ) junctions. Within each endothelial cell, TJ and AJ transmembrane proteins are anchored to the actin cytoskeleton by a multitude of plaque proteins including α-catenin and p120 plakoglobin for AJ and β-catenin and ZO-1 for both TJ and AJ. Src family kinases critically regulate paracellular permeability by regulating the homotypic or heterotypic association of transmembrane and plaque proteins and the cytoskeleton. The maintenance of cortical actin by myosin light chain kinase (MLCK) is regulated positively by Rac1 signaling and negatively by RhoA and Rho-associated protein kinase (ROCK) signaling.

physiological conditions, but is increased as a result of injury and disease. Transcytosis of albumin, the major oncotic protein of serum, involves the intracellular trafficking of caveolae carrying albumin that has been taken up via multiple receptors.[4] Invagination of caveolae leads to scission from the plasmalemma by the GTPase dynamin 2 and c-src.[5,6] The vesicles move into the endothelial cell and fuse with the basolateral membrane and release albumin into the interstitial space. The process of albumin transcytosis in resting and thrombin-stimulated human lung endothelial cells is regulated by acid sphingomyelinase production of ceramide which results in increases in caveolin-1, the major structural protein of caveolae.[7] Besides SFK, pathologic increases in transcytosis of albumin (transcellular leak) and paracellular leak are co-regulated by dynamin and Rac1.[8] In addition to albumin, transcytosis transports immunoglobulins, transferrin, aminopeptidase P and numerous other molecules. Microvascular endothelial cells can regulate permeability by both transcellular and paracellular routes except for brain microvascular endothelial cells that have few caveolae under basal conditions.

Endothelial barrier function also depends on surrounding cell types which in turn reflect the primary function of a given organ system. For example, in the brain where maintaining strict metabolic homeostasis while preserving a high integrity barrier is paramount, the microvascular endothelial cells are surrounded by pericytes, a basement membrane, and astrocytes with foot processes that are in contact with neurons.[9] The relative importance of stromal cells in maintaining the BBB phenotype is supported by recent evidence that pericyte-deficient mice have increased permeability to water and different solutes through an increase in transcytosis, a process that can be inhibited by the tyrosine kinase inhibitor imatinib.[10] Interestingly, the distribution of junctional proteins in the mutant cells remained unaltered, although irregular stretches of endothelial overlap were commonly seen. An absence of pericytes also affected the normal polarization of the astrocyte end-feet, while the deposition of basement membrane proteins did not change. These results confirm that the fine regulation of endothelial barrier function is organ-specific and involves multiple cell types.

In vascular beds such as the lung, the thick cortical actin layer in microvascular endothelial cells contributes to determining cell shape and stabilizing endothelial AJ, TJ and focal adhesions through protein bridges to the actin cytoskeleton.[11] Under basal conditions, cortical actin is preserved by Rac1 signaling which inhibits myosin light chain kinase (MLCK) and actin stress fiber formation. However, under pathologic conditions, the inflammatory mediators thrombin and TNF-α can activate intracellular SFK, calcium release, protein kinase C and RhoA signaling which together result in Rho-associated protein kinase (ROCK)-mediated phosphorylation of MLCK. MLCK activation generates actinomyosin-mediated contractile units with subsequent changes in cell shape, disassembly of the cortical actin layer and the formation of cytoplasmic actin stress fibers. Concurrently, pathways upstream of ROCK/MLCK also induce tyrosine phosphorylation of claudin-5, occludin, VE-cadherin and plaque proteins. Phosphorylation is driven by SFK and RhoA activation and the inhibition of phosphatases such as VE-PTP. The result is disassembly of junctional protein complexes and internalization of transmembrane receptors that, coupled with stress fiber induced cell retraction, lead to increased paracellular permeability.

In addition to well established endogenous barrier protective signaling pathways such as the ligand/receptor pairs sphingosine-1-phosphate (S1P)/S1P receptor 1(S1P1R), Angiopoietin-1 (Ang-1)/TEK receptor tyrosine kinase2 (Tie2), activated protein C (APC)/endothelial protein C receptor (EPCR) and Slit guidance ligand 2 (Slit2)/Roundabout guidance receptor 4 (Robo4),[12,13] new barrier protective pathways have been described. These include Frizzled7 which localizes to endothelial cell-cell junctions and prevents β-catenin activation and VE-cadherin disruption through Wnt signaling.[11] Adrenomedulin, and the related protein intermedin or adrenomedulin 2, are also known to enhance barrier function through the GPCR calcitonin receptor-like receptor (CRLR).[14] In response to adrenomedulins, CRLR partners with receptor activity-modifying proteins or RAMPS to increase cAMP and Rac1 signaling while decreasing p38MAPK signaling to reduce endothelial actin stress fiber formation, permeability and pro-inflammatory molecule expression. These complex pathways display significant crosstalk where, for example, EPCR signaling regulates S1P1R activation through PAR-1[15] and Tie2 transactivation through PAR-3 cleavage[16] to maintain barrier function. The maintenance of endothelial integrity ultimately depends on the balance of disruptive and protective pathways.

Clinical evidence of barrier dysfunction in severe malaria

By far the most studied and most controversial endothelial barrier in patients with falciparum malaria is the BBB that is linked to the clinical syndrome called cerebral malaria (CM). The complication is characterized by coma, seizures and focal neurological deficits. This complication carries a mortality rate of 15–20% despite optimal therapy. CM also results in long-term cognitive defects in about 20% of survivors. CM is more common in children than adults. Both pediatric and adult BBB function has been studied clinically using fluorescein angiography of the retina,[13] cerebrospinal fluid (CSF) protein analysis,[17-19] and more recently magnetic resonance imaging (MRI) studies of the brain.[20,21] In children with cerebral malaria, there is clinical evidence of increased intracranial pressure leading to brainstem herniation.[22,23] In a recent antemortem MRI study of 168 children with CM in Malawi, brain swelling emerges as the most reliable predictor of severity.[20] The cause of brain swelling is postulated to be due to cytotoxic edema from impaired perfusion, metabolic injury and cell death. Endothelial cell activation leading to barrier disruption may also occur. Increase in blood volume due to vascular congestion and increased cerebral blood flow are other possibilities. The absence of classical postmortem findings of cerebral edema is attributed to the rapidity with which death occurs in children with CM.

In adults, cerebral edema has not been seen in several imaging studies.[21,24] Mild diffuse brain swelling is found in a variety of anatomical sites. This swelling is partly attributed to venous congestion by the sequestered IRBC mass causing increased cerebral blood volume.[25] Brain herniation is not seen, nor is there evidence of toxigenic or vasogenic edema.[21] This would be in keeping with a lack of detection of albumin or IgG leakage into the CSF of adult patients with CM, in contrast to the significant leakage seen during bacterial meningitis.[19] More importantly, none of the MRI findings are different between patients with cerebral and non-cerebral malaria, and there are no differences between fatal and nonfatal infections. However, more marked changes in BBB have been observed in postmortem samples, including endothelial vacuolar degeneration and junctional protein disruption.[26-28] Whether these findings reflect the disease process or postmortem changes in the BBB is unclear. To date, the only adequately powered randomized trial on modulating BBB function during cerebral malaria was on the use of mannitol. Unfortunately, mannitol infusion failed to show therapeutic benefit but instead was associated with prolongation of coma.[24]

The contribution of leukocytes to endothelial barrier function in CM is also controversial. Histological sections of the adult brain in CM are notable for the almost complete absence of leukocyte infiltrates.[26] In fact, the intriguing question has been why leukocytes are not recruited when the pro-inflammatory milieu for recruitment to occur is present. More recent studies in pediatric patients have revealed the presence of some monocytes and platelets in the cerebral microvascular.[17,27,29] There is also a suggestion that there may be systemic neutrophil activation which may contribute to cerebral pathology through soluble mediators such as myeloperoxidase and elastase that are released.[30]

In contrast to the findings in the brain, features consistent with acute lung injury are frequently found in the pulmonary microvasculature at autopsy of both adult and pediatric patients with severe falciparum malaria.[31-33] The findings include edema, microthrombi and leukocyte infiltration. The microvessels are densely packed with IRBC. Leukocytes, particularly malaria pigment laden monocytes and less so neutrophils, are also frequently found. Clinical studies on lung function in adult and pediatric patients support the presence of capillary dysfunction manifested as impaired gas exchange and reduced peripheral reactive hyperemic index.[34,35] However, despite evidence of acute lung injury, the presence of overt pulmonary edema in severe malaria varies from 5 to 25% in adults and less than 10% in children.[32] In the latter group, respiratory distress is believed to be mainly central nervous system (CNS)-driven as a response to systemic metabolic acidosis.[36]

Pulmonary manifestations are also associated with infection with the parasite *P. vivax*, an infection that has often been considered mild and chronic rather than fulminant. There is evidence of alveolar-capillary membrane permeability[37,38] and systemic endothelial activation[39] despite a lower parasite burden and the relative absence of significant IRBC sequestration.

In addition to the brain and lung as major sites of pathology, acute kidney injury leading to renal failure approaches 40% in adults and 10% in children under 5 years of age.[40,41] Renal pathology has been largely

attributed to acute tubular necrosis secondary to sequestration of IRBC and accumulation of mononuclear cells within both glomerular and peritubular capillaries.[42] Indirect measures of parasite sequestration and monocyte activation levels have been found to correlate with renal failure requiring replacement therapy.[43] Mild to moderate glomerulonephritis has also been observed as seen by glomerular hypercellularity and collapse.[40] Histological assessment of renal tissue from severe malaria patients for ZO-1 distribution reveals marked and diffuse reductions in glomerular ZO-1 staining including within glomerular endothelial cells suggestive of extensive paracellular junction disruption.[44]

Mechanisms of endothelial dysfunction in severe malaria

Endothelial permeability in response to IRBC has been studied using different sources of primary endothelial cells or endothelial cell lines in vitro.[45-49] Permeability is assessed either as a change in transendothelial resistance, or the flux of fluorescein-labeled solutes such as dextran or albumin. While there is general agreement that IRBC do induce permeability, the mechanisms appear to differ depending on the experimental design and cell types used. The unresolved issues include i) whether direct contact between IRBC and endothelium is necessary; ii) what inflammatory mediators are involved; and iii) whether there is associated endothelial apoptosis or cell death. Each of these issues will be discussed in the following sections, with a summary schematic representation in Fig. 3.

Cytoadherence

Over a century ago, Marchiafava and Bignami made the seminal observation in postmortem examination that IRBC are sequestered in the capillaries and postcapillary venules of the brain, resulting in the reduction of the vascular lumen to create a mechanical obstruction to blood flow (Fig. 2).[50] Detailed histopathological studies of human postmortem tissues performed in the past 3 decades show that sequestration is in fact widespread in infected patients, and the degree and organ specificity of sequestration correlated with clinical manifestations.[50-53] More recently, obstruction of the microcirculation that is reversed after anti-malarial therapy has also been documented in vivo in acutely infected patients.[25]

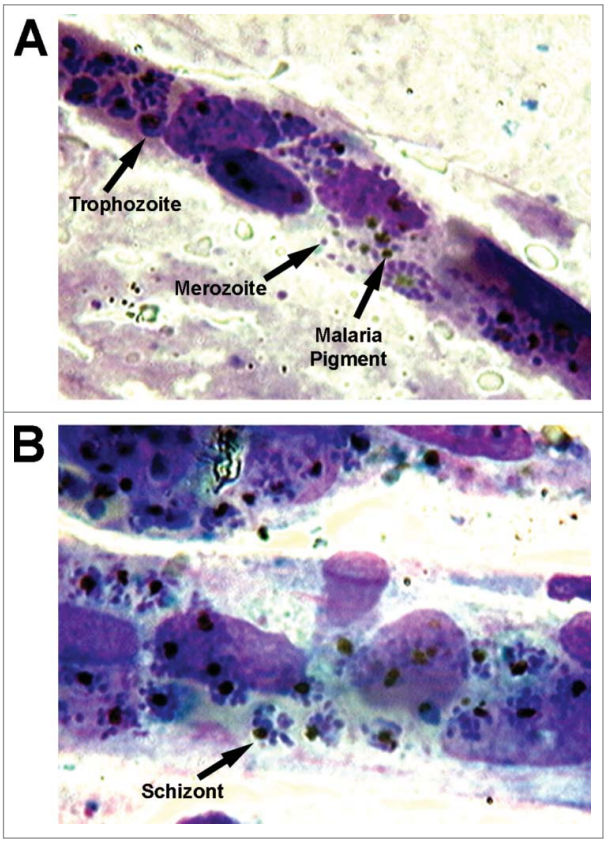

Figure 2. Sequestration of *P. falciparum*-infected erythrocytes in human brain microvessels. Post-mortem brain smears taken from an adult Vietnamese patient who died from cerebral malaria showing intense packing of IRBC in the microvasculature. The brain smears were made by placing brain tissue obtained within 6 hours of death between 2 glass microscope slides, pressing them together, and then sliding them apart to obtain thin smears.[51] This method preserves long fragments of capillaries and venules. Slides were fixed in absolute methanol and stained by the reverse Field's method. The blood vessels were examined at 1000x magnification under oil immersion using an Olympus BH2 microscope. The arrows in (A) indicate an IRBC with a mature trophozoite, and a free merozoite and malaria pigment after schizont rupture. The arrow in (B) indicates an intact schizont with a cluster of merozoites within an IRBC. Note the absence of any inflammatory infiltrate. The micrographs, courtesy of Dr. K. Silamut, Mahidol Oxford Research Unit, Bangkok, Thailand, were originally published in *Blood*. Ho M. EPCR: Holy Grail of Malaria Cytoadhesion? 2014;123:157–159 © the American Society of Hematology.

Sequestration results from the adhesion, or cytoadherence, of IRBC to vascular endothelial cells, and the process is mediated by the variant parasite ligand *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed on the surface of IRBC, and endothelial receptors, of which a number have been implicated.[54,55] Cytoadherence occurs to any significant extent only with *P. falciparum* that infects human

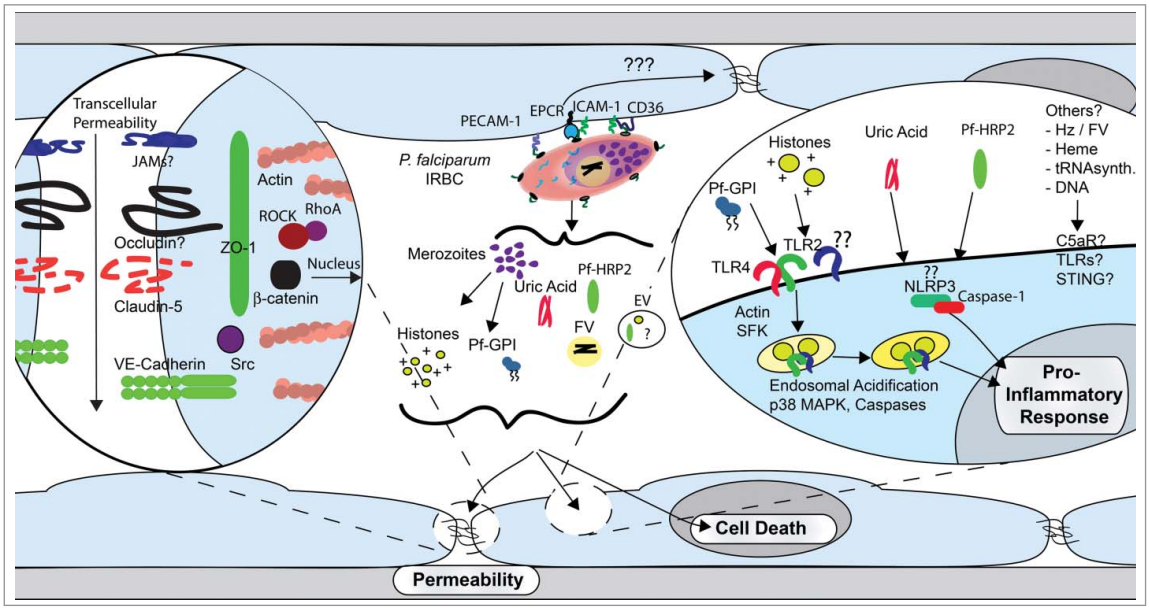

Figure 3. Proposed model of the induction of endothelial proinflammatory response and barrier dysfunction by *P. falciparum*. In the second half of the erythrocytic life cycle, *P. falciparum*-infected erythrocytes (IRBC) either adhere to endothelial receptors or are in close contact with microvascular endothelium. Adhesion through receptors such as CD36, ICAM-1 or $\alpha_5\beta_1$ integrin may lead to intracellular signaling events with resultant actin cytoskeletal changes and disruption of junctional protein expression. In addition, numerous host and parasite products are released in the proximity of endothelial cells at IRBC rupture, where they may induce a pro-inflammatory response or exert a disruptive effect on endothelial barrier function. Transcytosis of albumin and other host molecules may further add to vascular leakage.

erythrocytes, which explains why there is no appropriate animal model to study this phenomenon. The majority of PfEMP1 variants bind to CD36, an 88-k_D class B scavenger protein expressed on a number of cell types, including macrophages and endothelial cells.[54] Adhesion of IRBC to CD36 has been shown to recruit CD36 and actin to the site of attachment in human dermal microvascular endothelial cells through the activation of p130 CAS and Src family kinases.[56] Although there is no clear evidence of stress fiber formation, the actin cytoskeletal rearrangement may lead to a change in cell shape and hence affect the integrity of adherens junctions. Moreover, adhesion of IRBC to CD36 recruits $\alpha_5\beta_1$ integrin to the same site, which strengthens the adhesive force.[57] $\alpha_5\beta_1$ has been shown to induce breakdown of endothelial barriers through binding to cANGPTL4 which can directly activate Rho-GTPase Rac1/PAK signaling to cause downstream disruption of VE-cadherin and claudin 5.[58] Paradoxically, $\alpha_5\beta_1$ also appears to have a critical role in barrier maintenance through localization of the integrin to cell-cell contacts and adhesion to extracellular matrix such as fibronectin.[59] These divergent results suggest that the subcellular location of integrin engagement (junctional/basolateral versus apical) may differentially regulate endothelial permeability. Whether engagement of $\alpha_5\beta_1$ by PfEMP1 affects endothelial permeability has not been explored.

IRBC adhesion to ICAM-1 can also lead to similar cytoskeletal changes. Induction of ICAM-1 clustering by antibody-coated beads on human umbilical vein endothelial cells (HUVEC) that do not express CD36 results in Src- and Pyk2-dependent phosphorylation of VE-cadherin that is implicated in the promotion of neutrophil transmigration via the paracellular pathway.[60] Binding of ICAM-1 on human lung endothelial cells by neutrophils also leads to increased transcellular permeability through activation of caveolin-dependent albumin transport.[61] Interestingly, IRBC adhesion to ICAM-1 on a brain endothelial cell line (hCMEC/D3) has been linked to the formation of endothelial cup-like structures and trogocytosis of membrane fragments of IRBC, and the processes are associated with disruption of barrier integrity.[62] As endothelial docking structures or transmigrating cups are well described during leukocyte transmigration,[63] their formation around adherent IRBC that do not transmigrate is somewhat surprising. Moreover, transmigrating cups around neutrophils has been shown to be a mechanism for the maintenance rather than

disruption of barrier function.[64] These discrepancies will need to be resolved in future studies.

IRBC adhesion may also affect permeability indirectly by inducing tissue factor production on endothelial cells and subsequent activation of the coagulation cascade,[65] as evidenced by elevated levels of circulating thrombin-antithrombin III complex,[66,67] the presence of activated thrombin[68] and fibrin deposition[69] on brain microvascular endothelium in postmortem brain tissues. Thrombin is known to induce permeability through PAR-1 which heterodimerizes with PAR-3 leading to $G_{\alpha 13}$-dependent calcium signaling, actin stress fiber formation and transient increases in endothelial permeability.[70] Moreover, a subset of parasites from patients with CM has been shown to bind to EPCR, and may compete with APC for the binding to this receptor.[55,71,72] As APC exerts a protective effect on thrombin induced barrier dysfunction, IRBC adhesion may exaggerate the barrier dysfunction elicited by thrombin.[71,73,74]

It should be emphasized that the in vitro evidence for the disruption of barrier function as a direct effect of cytoadherence is not robust, as each process has been demonstrated by only a few IRBC, or by using surrogates, such as antibody or recombinant PfEMP1-coated beads. It is conceivable that the number of interactions could be much higher in vivo, in view of the fact that microvessels are densely packed with IRBC (Fig. 2) compared with the relatively sparse IRBC adhesion to endothelial monolayers in vitro. It has also been demonstrated that IRBC adhesion at a given time may be mediated by multiple, some as yet unidentified, endothelial receptors,[75] so that the downstream effect is likely the summation of the activation of multiple pathways.

Host inflammatory mediators

A growing number of host-derived pro-inflammatory cytokines, chemokines, metabolic and lipid mediators known to increase endothelial permeability has been shown to be elevated in severe malaria.[76,77] These inflammatory mediators are identified mostly by measuring circulating levels using various immunoassays, or more recently by transcriptomics. Very few of the mediators have been studied functionally in relation to the infection, and as a result, the elevated levels do not distinguish between whether a particular inflammatory mediator actually contributes to disease pathology or merely serves as a biomarker of severity.

The most studied pro-inflammatory cytokine in relation to severe falciparum malaria is tumor necrosis factor-α (TNF-α) following reports of elevated levels of this cytokine in African children that correlated with severity and mortality.[78] Cytokine levels have also been measured in *P. vivax* malaria. TNF-α levels during paroxysms of fever in vivax malaria are either much higher than in severe and fatal falciparum malaria,[79] or levels are similar in the 2 infections.[39] Among its protean effects, TNF-α could induce adhesion molecule expression and endothelial barrier dysfunction. However, although animal studies with the murine parasite *P. berghei* suggested the efficacy of anti-TNF-α therapy,[80] results of anti-TNF-α in human clinical trials in both adult and pediatric patient populations were disappointing.[81,82] The lack of efficacy of the monotherapy, as in the case of sepsis,[83] was likely due to the fact that by the time patients present for treatment, other inflammatory or inhibitory pathways would have been activated. In at least the adult patients, the anti-inflammatory cytokine IL-10 was also found to be markedly elevated,[84] which suggests that perhaps the ratio and time course of TNF-α to IL-10 production may be more predictive of outcome.

Another mediator that has been studied extensively in malaria is nitric oxide (NO). NO could derive from different nitric oxide synthase (NOS) isoenzymes in neuronal tissue (nNOS), or vascular endothelium (eNOS), or from an inducible form found in phagocytic cells (iNOS). The isoenzyme eNOS is most concerned with regulation of microvascular hyperpermeability. eNOS mediates increased endothelial permeability in response to VEGF through tyrosine phosphorylation of VE-cadherin and Rho GTPase-dependent actin stress fiber formation.[85]

Nitric oxide has been postulated to have a protective role in severe falciparum malaria. Similar to its anti-inflammatory role on leukocyte recruitment,[86] it inhibits adhesion of IRBC on HDMEC in vitro.[87] In patients with both *P. falciparum* and *P. vivax* malaria, an inverse relationship between the severity of the infection and nitric oxide production/nitric oxide synthase expression has been demonstrated by several studies.[35,88] The decreased NO production and its precursor L-arginine were attributed to a deficiency of arginine and increased arginase activity. These

derangements are compounded by the increased cell-free hemoglobin circulating in malaria patients secondary to hemolysis, leading to increased NO scavenging and plasma L-arginine catabolism, and an overall reduction in NO bioavailability as seen in sickle cell disease.[89] Increased arginase activity has also been demonstrated in monocytes of the M2 phenotype in children with falciparum malaria.[90] However, in all the above studies concerning the role of NO in severe malaria, microvascular permeability was not directly measured. The readouts were reactive hyperemia-peripheral arterial tonometry, which is more a measure of vascular tone, and gaseous exchange that could be affected by perfusion. Clinical trials of L-arginine in patients with severe falciparum malaria have led to some improvement in vascular tone,[35] but larger randomized trials will be needed to assess effects on disease severity or mortality. A clinical trial in pediatric malaria showed that inhaled nitric oxide did not affect biomarkers of severe disease.[91]

More recently, the role of NO in severe malaria has also implicated the angiogenic factor Ang-2 which is stored in Weibel-Palade bodies (WPB) in endothelial cells and secreted in conjunction with von Willebrand Factor.[92] The decreased vascular NO bioavailability found in severe malaria is proposed to increase exocytosis of WPB with a resultant increase in Ang-2 release. Independently of NO, elevations in Ang-2 in severe malaria are strongly predictive of disease severity, particularly when measured as a ratio with its barrier protective homolog Ang-1.[93] Ang-2 is known to disrupt AJ and can lead to lung injury in mice by mediating the dissociation of Ang-1 from its receptor Tie2 leading to VE-cadherin disruption.[94,95] While murine models of cerebral malaria suggest the Ang-1/-2 axis plays a role in severe disease,[96] analysis of altered Ang-1, Ang-2 and Tie2 protein levels in human brain specimens from adults patients with CM did not discriminate CM from non-CM cases.[97] At present, Ang-2/Ang-1 ratios remain an excellent marker for severe disease, but whether this pathway contributes critically to human cerebral malaria or acute lung injury as seen in sepsis remains unclear.[98]

Host-derived products released at the time of red blood cell rupture such as free heme and its parent compound, hemoglobin, have also been implicated in pediatric[99] and adult[100] severe malaria as well as in endothelial dysfunction in malaria.[101] Heme is known to disrupt endothelial barriers directly through induction of free radical generation and mitochondrial reactive oxygen species signaling in endothelial cells.[102] Heme can also indirectly induce endothelial permeability through induction of histone- and DNA-rich neutrophil extracellular traps[103] or by complement activation which induces the formation of interendothelial gaps in HUVEC.[104] In addition, the barrier disruptive effects of heme could be mediated by NLRP3,[105] TLR4[106] or other MyD88 signaling pathways.[107] Heme induced inflammatory effects can be inhibited by hemoglobin- and heme-binding proteins such as haptoglobin[108] and hemopexin,[109] or detoxification of hemoglobin breakdown products by nitric oxide,[110] heme oxygenase-1 or carbon monoxide which converts toxic methemoglobin to carboxyhemoglobin.[111]

Parasite products

In addition to host pro-inflammatory molecules, parasite products may be released as soluble factors during the intra-erythrocytic growth phase, or released as insoluble factors when the infected red cell and mature parasite ruptures, releasing merozoites, food vacuoles and hemozoin. *Plasmodium falciparum* histidine rich protein 2 (PfHRP2) is an example of a parasite product that is transported from the parasitophorous vacuole to the cytoplasm and is released throughout the life cycle and at the time of red cell rupture.[112] PfHRP2 has been shown to activate the NLRP3 inflammasome activation in the brain endothelial cell line hCMEC/D3, leading to the production of IL-1β- and MyD88-dependent induction of vascular permeability.[113]

More potent parasite products known to mediate endothelial leakage are released on IRBC rupture. In *P. falciparum* infection, merozoites are released every 48 hours and contain glycophosphatidyl inositols (GPI) that anchor parasite proteins to the plasma membrane. GPI are true pathogen associated molecular patterns that are unique to protozoan parasites, and are recognized by TLR2/TLR1 and to a lesser extent TLR4 on human and mouse macrophages.[114] Malarial GPI have been shown to stimulate NO production and upregulate the expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on HUVEC, leading to increased leukocyte and parasite adhesion.[115] The effect of GPI can be inhibited by the Src-family kinase antagonist herbimycin A. Merozoite proteins, independently of TLR2 stimulation, can also increase endothelial permeability in a Src family

kinase-dependent manner by inducing the redistribution of ZO-1, VE-cadherin and claudin-5 away from cell-cell junctions in HDMEC.[49]

As part of hemoglobin degradation, *P. falciparum* generates a relatively insoluble crystal of heme monomers called hemozoin or malaria pigment in parasitophorous food vacuoles. Similar to merozoites, food vacuoles are released every 48 hours at the time of IRBC rupture. Purified food vacuoles have been found to induce endothelial cell death and barrier dysfunction in HDMEC.[49] Food vacuoles may also activate complement and coagulation pathways,[116] both of which could increase endothelial permeability through the production of C5a[117] and thrombin, respectively. Although hemozoin itself does not induce endothelial barrier dysfunction,[47,49] it has been shown to activate NF-κB signaling pathways as shown by increased IL-8 and MCP-1 (CCL2) production in HUVEC.[118]

Other parasite derived products such as DNA,[119,120] histones,[121] tRNA synthetase,[120] uric acid[122] and heat shock proteins (HSP)[123] have been shown to activate the host immune system. In the case of histones, they can disrupt endothelia into renal arteries l barrier function. *P. falciparum* histones H3 and H4, which share >95% homology with human histones, were shown to increase endothelial permeability in primary human lung cells based on their strongly cationic charge.[121] Histone-induced disruption of VE-cadherin, claudin-5 and ZO-1 expression was partially Src family kinase-dependent, and may also involve caspase-independent cell death. In vivo, the direct injection of bovine histones, which share high homology to *P. falciparum* histones, into murine renal arteries induced TLR2/4-dependent endothelial cell death and edema culminating in acute kidney injury.[124] Other soluble parasite proteins, such as the DNA-binding proteins high mobility group box (HMGB) 1 and 2, may induce permeability through activation of RAGE as does its human counterpart.[125] Plasmodia also produce HSP, and PfHSP70 is thought to have adjuvant and pro-inflammatory activities through TLR2/4 and TLR4/MyD88 activation respectively.[123]

The importance of parasite products in inducing endothelial permeability is underscored in a recent study which demonstrated that as yet unidentified product(s) released on IRBC rupture activated β-catenin signaling and subsequent disruption of claudin-5 expression in primary human brain endothelial cells.[126] Inhibition of β-catenin–induced T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor activity in the nucleus prevented the disruption of endothelial junctions. Nuclear translocation of β-catenin and TCF/LEF is known to mediate crosstalk between AJ and TJ following VE-cadherin disruption.[127] Nuclear β-catenin stabilizes TCF/LEF transcription factor binding to the claudin-5 promoter and suppresses claudin-5 production. These studies are consistent with previous results showing late stage (schizont) parasite extracts and merozoite proteins decreased endothelial claudin-5 production in conjunction with disruption in VE-cadherin localization to cell-cell junctions.[49] Interestingly, the blockade of the angiotensin II type 1 receptor (AT1) or stimulation of the type 2 receptor (AT2) abrogated IRBC–induced activation of β-catenin and prevented the disruption of brain endothelial monolayers.

Most recently, IRBC-derived extracellular vesicles (EV) have been shown to transfer host microRNA451a (miR451a) that silences cav-1 and atf2 mRNA.[128] Endothelial permeability in bone marrow endothelial cells was augmented by the transfer of EV containing RNA-induced silencing complex (RISC) proteins, including the Argonaut protein Ago2. The findings are consistent with the reported occurrence of trogocytosis,[62] and the contact-dependent but cytoadherence-independent activation of endothelial cells by intact IRBC. Parasite-derived proteins are also contained within EV,[129,130] so that there may be intracellular delivery of parasite products to endothelial cells. Depending on the route of delivery, parasite products may elicit different signaling pathways with potentially different outcomes. A difference in the outcome of intracellular vs. extracellular activation has been shown for LPS that activates via TLR4 on the cell surface, but induces non-canonical inflammasome activation involving caspase 4/11 when presented intracellularly.[131] These findings suggest that *P. falciparum* products may target multiple pathways in the maintenance of endothelial integrity.

Cell death

Closely linked to permeability is whether endothelial activation is associated with cell death. Endothelial cell apoptosis has been implicated as a mechanism of increased endothelial permeability,[46,132-134] and different clinical isolates of *P. falciparum* appeared to induce apoptosis to varying degrees in primary

endothelial cells derived from human lung and brain.[133] The induction of endothelial apoptosis was sensitive to the environmental pH and required direct contact between the parasite and the endothelial cell, although adhesion to specific receptors was not essential. Moreover, the extent of induced apoptosis in the 2 endothelial cell types was inhibited by pan-caspase or caspase-8 inhibitors and varied with the isolate. Analysis of parasite gene transcripts suggest that the activation of different parasite pathways, such as plasmodium apoptosis–linked pathogenicity factors, could play a part in the observed susceptibility to cell death. The increase in endothelial permeability and apoptosis induced by IRBC has also been linked to Rho A activation and was partially inhibited by the ROCK inhibitor fasudil but the parasite agonists, host receptors and downstream effectors, such as actin stress fiber formation, were not determined.[134]

In other studies, cell death is either not observed[47,49,135] or could not be attributed to the activation of caspases. Treatment of primary dermal and lung endothelial cells with *P. falciparum* histones has been shown to induce cell death which was insensitive to a pan-caspase inhibitor.[121] In addition, PfHRP2 has been shown to induce redistribution of the tight junction protein claudin-5 leading to compromised integrity of the brain endothelial cell line hCMEC/D3.[113] Although the parasite protein activated caspase 1, apoptosis and pyroptosis were excluded by the absence of DNA fragmentation and the lack of an inhibitory effect of a pan-caspase inhibitor at 6 hours when a change in permeability was first detectable. Nicking of DNA was however evident at 24 hours.

The extent of endothelial cytotoxicity in vivo also remains to be determined. Based on histopathological[53] and transmission electron microscopy[26] studies of postmortem tissues, generalized endothelial cell death is not a prominent feature of acute falciparum malaria, consistent with the largely reversible nature of neurologic dysfunction in those that survive the infection. However, in a small autopsy series of pediatric and adult with CM, cleaved caspase-3, the terminal effector of apoptosis pathways, was seen in >70% of brain endothelial cells examined.[136] The presence of ring hemorrhages around cerebral microvessels at autopsy,[53] or as part of the retinopathy observed in patients[137] would also be compatible with the occurrence of endothelial cell death. Indeed, the high levels of circulating nucleosomes of both parasite and human origins detected in the plasma of patients with severe falciparum malaria, compared with the much lower levels detected in septic patients,[138] would suggest that cell death in this infection may be more extensive than previously appreciated. The host cell types that are affected and cell death pathways involved remain to be defined.

Future directions

Imaging studies

Current understanding of the pathophysiology of severe malaria is largely based on autopsy studies. New imaging techniques are now available to perform some of the studies in the clinical setting. Novel ultrasound techniques that allow high resolution imaging of microvessels (10μm) could be performed non-invasively to quantify microvascular perfusion within the CNS in severe malaria patients in conjunction with MRI studies to better correlate the relationship between brain swelling and sequestration.[139] In fatal cases, ante-mortem ultrasound imaging would also be useful to correlate microvascular perfusion deficits with abnormalities found at autopsy.

Basic questions of whether changes in endothelial permeability in severe malaria are caused by trans- vs. paracellular permeability and in what organs also remain to be addressed. Significant increases in transcellular permeability can occur in conjunction with paracellular permeability as shown in murine models of endotoxin-induced acute respiratory distress syndrome,[61] or transcellular permeability can occur independently as seen during dengue virus infection[140] or HMGB1 stimulation of mouse lung microvascular endothelial cells in vitro.[141] Detailed analysis of clinical tissue samples for the activation of key signaling pathways associated with dissociation of junctions and transcytosis may provide additional insight into potential therapeutic targets. In this regard, mass cytometry has become an extremely powerful tool for the detection of over 100 molcules per cell using metal-labeled antibodies that could allow for the simultaneous characterization of multiple cell types, inflammatory mediators, junctional proteins, and activation of signaling molecules.[142] Moreover, localizing the presence and relative abundance of parasite products such as histones, GPI, and PfHRP2 at sites of apparent barrier dysfunction might also provide insight into which agonists could be targeted for

blockade. Mass cytometry based techniques could be supplemented by CLARITY protocols which entail embedding fixed tissues, primarily brain, into a synthetic hydrogel matrix followed by electrophoresis to remove lipids.[143] The resulting optically transparent tissue allows high resolution 3D imaging of large (cm) sections of tissue and could provide a more comprehensive overview of endothelial junctional protein distribution in severe malaria. Beyond the brain and lung, applying these methods to the analysis of renal tissue from patients with severe malaria could provide additional insight into acute kidney injury in both children[144] and adults[43] which is associated with renal endothelial cell hypertrophy and cytoplasmic vacuolation as well as monocyte infiltration.[42]

Organs-on-chips

The lack of an appropriate animal model has hampered the study of cytoadherence in severe falciparum malaria, including its effect on barrier function. Recent advances in 3D co-culture techniques[145] that can recapitulate the complex interactions of human cells in physiological matrices such as collagen I or fibronectin constitute a promising approach for determining the relative contribution of specific cells to organ function in states of health and disease.[146] Coculture models of the human blood brain barrier including pericytes and astrocytes along with brain endothelial cells under continuous flow (1dyne/cm^2) have confirmed a long suspected role for both stromal populations in regulating BBB function.[147] Cocultures in this model showed a 2-fold increase in barrier integrity as measured by 3kDa dextran flux and 2- to 5-fold increase in responsiveness to TNF-α as measured by IL-6, IL-8 and G-CSF production compared with endothelial cells alone. Neurons have also been integrated into microfluidic chips containing endothelial cells, astrocytes and pericytes, which results in a 5-fold decrease in 10kDa dextran flux in comparison to endothelial cells alone.[148] Moreover, these coculture systems allow for the examination of neuronal calcium signaling.[149] Similarly, 3D cultures of lung epithelial cells, fibroblasts and endothelial cells to recapitulate the features of the small airways including barrier function and ciliary movement on epithelial cells have been used to assess the effects of drugs on leukocyte recruitment in inflammatory lung diseases and their mechanism of action.[150] Microfluidic models have also allowed for analysis of cellular interactions within microvascular networks (10–100μm) using either de novo generated vascular networks in 3D matrices[151] or in patterned devices devoid of matrices.[152]

The microfluidic models would be ideal for studying endothelial-IRBC interactions in capillaries (5–15μm) and post-capillary venules (30–70μm) where host-pathogen interactions occur in vivo. The hemodynamics of IRBC-endothelial interaction in microfluidic microvessels are likely to vary significantly from the more traditional 2D cultures, including changes in shear stress through high variability in hematocrit, viscosity and parasitemia within microvessels which can lead to vessel obstruction, changes in mechanoreceptor signaling and in turn endothelial function.[137] Although not in direct contact with IRBC, stromal cells such as pericytes, astrocytes, neurons, fibroblasts are likely to be exposed to inflammatory mediators that in turn would modulate endothelial function. There is suggestive clinical evidence for abnormalities in some of these cell types in falciparum malaria, as shown by pericyte and axonal degeneration along with microglial and astrocyte end foot retraction,[26,27] but the full extent of the involvement of these cells in endothelial barrier function remains to be systematically examined.

Metabolomics

A relatively unexplored area of study is the effect of metabolites on endothelial barrier function. Imbalances in common metabolites have been shown to affect immune functions.[153] In *P. falciparum* malaria, an increase in permeability of an immortalized brain endothelial cell line (hCMEC/D3) was shown to occur in an acidic environment attributed to parasite metabolic products.[45] As metabolic acidosis secondary to lactate production by both host and parasites is one of the main indicators of a poor outcome of severe malaria[36] lactic acid too could contribute to barrier dysfunction. Moreover, late stage *P. falciparum* in culture releases high levels of amino acids such as histidine and glutamate.[154] Histidine can be converted by histidine-L-decarboxylase to histamine which is elevated in severe malaria[155] and is a well described barrier disrupting agent acting through the G-protein coupled receptor H1 leading to subsequent RhoA/ROCK-dependent actin contraction.[156] Extracellular glutamate can also increase BBB permeability in vitro[148] and in vivo through the GPCR mGluR pathways.[157] In a mouse model of CM with the murine parasite *P. berghei*, CNS levels of glutamate are found to be increased while glutamine is decreased at the onset of

neurologic dysfunction.[158] Blockade of glutamine breakdown to glutamate using chemical inhibitors administered at the time of neurologic deterioration increased glutamine levels, reversed BBB permeability and brain swelling and rescued mice from CM. The source of excess glutamate in vivo remains unclear although both host cells and parasites have been implicated.

In future studies, inhibition of parasite metabolic pathways by genetic manipulation using CRISPR-Cas9 technology may shed further light on the relative importance of specific parasite derived metabolites.[159] As well, confirming the timing, location and severity of metabolic dysfunction in human CM by analyzing tissue and fluid samples (e.g. blood and CSF) with metabolomics techniques including mass spectrometry and nuclear magnetic resonance analysis will be important. Ultimately, advances in metabolomics analysis in vivo and in vitro may provide insight into metabolic pathways with ideal characteristics for intervention, such as the ability to be rapidly turned off or switched on, as has been attempted with L-arginine administration to boost NO production.

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ORCID

Mark R. Gillrie http://orcid.org/0000-0002-5836-5833

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