Host response to cytoadherence in Plasmodium falciparum

Srabasti J. Chakravorty1, Katie R. Hughes and Alister G. Craig2
Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, U.K.

Abstract
Cytoadherence of PRBCs (Plasmodium falciparum-infected red blood cells) to host endothelium has been associated with pathology in severe malaria, but, despite extensive information on the primary processes involved in the adhesive interactions, the mechanisms underlying the disease are poorly understood. Endothelial cells have the ability to mobilize immune and pro-adhesive responses when exposed to both PRBCs and TNF (tumour necrosis factor). In addition, there is also an up-regulation by PRBCs and TNF and a concurrent down-regulation of a range of genes involved in inflammation and cell death, by PRBCs and TNF. We propose that the balance between positive and negative regulation will contribute to endothelial pathology during malarial infection. Apposition of PRBCs has been shown by a number of groups to activate signalling pathways. This is dependent, at least in part, on the cytoadherence characteristics of the invading isolate, such that the avidity of the PRBC for the receptor on host endothelium is proportional to the level of activation of the signalling pathways. An understanding of the post-adhesive processes produced by cytoadherence may help us to understand the variable pathology seen in malaria and to design appropriate therapies to alleviate severe disease.

Adhesion receptors
Sequestration of infected red blood cells in the microvasculature is associated with severe disease in Plasmodium falciparum malaria as a result of cytoadherence of infected red blood cells to the ECs (endothelial cells) lining blood vessels. ECs express a wide array of adhesion receptors, which serve as ligands for adhesion. The list of molecules for which adhesion has been shown includes: (i) thrombospondin [1]; (ii) CD36 [2,3]; (iii) Ig superfamily cell adhesion molecules ICAM-1 (intercellular adhesion molecule 1) [4], VCAM-1 (vascular cell adhesion molecule 1) [5], PECAM-1 (platelet/EC adhesion molecule)/CD31 [6] and NCAM (neural cell adhesion molecule) [7]; (iv) selectins P-selectin [8] and E-selectin [5]; (v) integrin αvβ3 [9]; (vi) gC1qR (globular C1q receptor)/HABP1 (hyaluronan-binding protein 1)/p32 [10]; and (vi) carbohydrates CSA (chondroitin sulfate A) [11] and HA (haemagglutinin) [12]. However, over recent years, research has focused particularly on a small selection that are thought to be important for pathology. In the present article, we discuss some of these adhesion receptors and their ability to modulate host EC function.

CD36
Most patient isolates tested show ability to bind to CD36, which was one of the first PRBC (P. falciparum-infected red blood cell) adhesion receptors to be identified [2,3]. CD36 is an 88 kDa highly glycosylated cell-surface class B scavenger receptor expressed on several ECs. Alternative phosphorylation of an external threonine residue is important in regulating substrate specificity, and dephosphorylation of this residue is also required for optimal adhesion of PRBC [13]. Furthermore, recent studies have demonstrated that platelet-expressed CD36 can mediate binding of PRBCs to endothelium devoid of CD36 using platelets as a bridging interaction [14,15], for instance in the brain.

ICAM-1
Adhesion to ICAM-1 has been linked with CM (cerebral malaria), on the basis of post-mortem studies showing increased local ICAM-1 expression which correlated with increased parasite sequestration in the brain [16]. Furthermore, isolates from patients with CM have a higher preference for adhesion in vitro to ICAM-1 [17], although this has not been a consistent finding (for a review, see [18]). ICAM-1 forms homodimers on the EC surface and is involved in leucocyte recruitment during inflammation. The binding site for PRBCs is on the N-terminal external Ig domain of the protein, but is distinct from the binding sites for rhinovirus and LFA-1 (lymphocyte function-associated antigen 1) [19–21]. Cytokines, including TNF (tumour necrosis factor) and
IL (interleukin)-1, which are up-regulated during malaria infection, can increase ICAM-1 levels on many types of endothelium. PRBCs can also increase ICAM-1 levels directly [22–25], as discussed below.

CSA
Another molecule that has been linked to a specific disease phenotype is the carbohydrate CSA [11,26]. CSA is found on placental syncytiotrophoblasts, and binding of PRBCs to CSA is uniquely linked to placental malaria, which leads to low birth weight and concomitant increase in neonatal mortality (see [27] for a review).

Newly recognized receptors
The recent identification of two further receptors for adhesion of PRBCs illustrates the diversity of receptors for *P. falciparum* adhesion. The first of these is NCAM, another member of the Ig superfamily. NCAM is known to be expressed on endothelium in two sites where sequestration is commonly observed in severe disease, skin and brain, tempting speculation that it may be an important receptor in severe disease [7]. The second recently identified receptor for PRBC adhesion is gC1qR/HABP1/p32. This membrane protein is expressed on resting ECs and resting platelets, as well as other cell types. gC1qR/HABP1/p32 is also involved in platelet-mediated clumping of PRBCs (as is CD36), so may prove to be an important receptor for malaria pathogenesis [10].

The *P. falciparum* adhesion ligand: PfEMP-1 (*P. falciparum* erythrocyte membrane protein 1)
Adhesion to host receptors has, in most cases, been shown to be mediated by PfEMP-1. This protein is displayed on the surface of the PRBC and is localized to structures known as knobs. Both knobs and PfEMP-1 are visible on the surface of PRBCs from approx. 16–18 h after invasion at the same time as sequestration occurs [28]. The presence of knobs is essential for optimal display of PfEMP-1 for cytoadherence [29,30].

var genes and antigenic variation
PfEMP-1 is encoded by *var* genes; out of a family of 60 *var* genes in a parasite, only one is expressed at a time, the others being silenced at a transcriptional level (for a review, see [31]). *P. falciparum* undergoes antigenic variation, which is a mechanism to evade host adaptive immune responses. This involves switching expression to an alternative *var*, resulting in expression of an antigenically distinct PfEMP-1 protein. Switching occurs at a rate of up to 2% per generation [32], although this rate differs between variants. In addition, recombination between different *var* genes results in a potentially vast repertoire of PfEMP-1 proteins within a population.

PfEMP-1 structure
PfEMP-1 comprises a small cytoplasmic domain, a transmembrane region and a large extracellular domain. This extracellular part consists of several DBL (Duffy-binding like) and CIDR (cysteine-rich interdomain region) domains (see [33] for a review). The number and types of these DBL domains vary; however, the N-terminal domain is almost always a DBLα type combined with a CIDR domain. The latter is known to be responsible for binding to CD36 [34]. Several adhesive properties have been mapped to individual DBLs, such as the DBLβ type that, in some cases, mediates binding to ICAM-1 [35], and DBLα, which can mediate rosetting, a process of binding to uninfected red blood cells via CR1 (complement receptor 1) [36]. Adhesion in the placenta to CSA is thought to be mediated by a conserved set of PfEMP-1 genes encoded by var2csa [37]. These genes, unusually conserved between different isolates, uniquely appear to be specifically up-regulated (or selected for) in pregnant women, and are rarely seen expressed elsewhere. Other associations of specific *var* genes with disease are less precise, although there is increasing evidence to support the role of some subclasses of *var* genes in severe malaria [38,39].

The variation in different PfEMP-1 proteins determines the ability of parasite isolates to adhere differentially to the wide array of host receptors as described above. The diversity generated by antigenic variation and the preponderance of mixed infections in areas of medium to high transmission may explain why, in clinical isolates, parasites expressing different *var* genes are found sequestered in different tissues [40].

At specific vascular sites, unique arrays of adhesion receptors are expressed, resulting in preferential sequestration of parasites expressing different PfEMP-1 variants in different locations, potentially contributing to differential pathology.

Methods to study cytoadherence
A variety of technologies have been used to study adhesion of PRBCs to ECs *in vitro*. These studies have been performed under static conditions using ECs grown to confluence on coverslips or culture plates. Alternatively, ECs have been grown on microslides or parallel plate chambers and assays carried out under laminar flow conditions to achieve a defined physiologically relevant wall shear stress [14]. A skin-graft technique has been used where human skin tissue has been grafted on to mice to allow an interesting way to assess cytoadherence *in vivo* [41]. Adhesion of PRBCs under flow conditions is a complex process. Under these conditions, binding mimics mechanisms described for leucocyte recruitment. Briefly PRBCs are tethered from flow, followed by a rolling interaction along the endothelial surface before firm adhesion occurs. Microfluidics systems have been developed recently that mimic adhesion under both physiologically relevant shear stress and also in response to changes in dimensions of capillaries, typically seen in capillary beds. In this model, the rolling interaction was abolished while PRBCs traversed a narrow constriction; however, it
was quickly restarted when the channel opened out again. This could be due to the increased shear stress in these constrictions, or physical constraints reducing the interaction between receptor and ligand [42].

**Cytoadherence to multiple receptors**

Cytoadherence under flow conditions is thought to involve multiple adhesion receptors acting co-operatively, shown with CD36, ICAM-1 and P-selectin [43–45]. A model has been widely proposed where PRBCs are captured from flow by one receptor, roll along ECs before firm adhesion, possibly being mediated by a different receptor. This may separate out roles for different receptor families, where a selectin (e.g. P-selectin) or Ig superfamily receptor (e.g. ICAM-1) may be required to capture PRBCs from flow followed by firm adhesion to a different class of receptor, e.g. CD36. Firm adhesion may also require or be enhanced by interactions with more than one receptor concurrently (e.g. ICAM-1 and CD36).

**Direct activation of ECs**

Several studies on post-mortem tissue have demonstrated modification of the endothelium in malaria associated with cytoadherence. Widespread up-regulation of adhesion receptors ICAM-1 and E-selectin in cerebral vessels has supported the view of endothelial activation in fatal malaria [16], as well as a subsequent study that demonstrated a positive correlation between disease severity and induction of TNF, ICAM-1, VCAM-1, E-selectin and P-selectin in muscle biopsies, indicating widespread systemic EC activation [46].

Studies on post-mortem brain tissue have also revealed evidence of disruption of the BBB (blood–brain barrier). Haemorrhages are often seen, but they are small and not usually associated with necrosis. Thus it is evident that the processes regulating BBB integrity are compromised, leading to accumulation of some plasma proteins and fluids into the perivascular space and brain parenchyma and contributing to cerebral oedema which can lead to fatal consequences [47,48]. However, it is also clear that the degree of vessel leakage is relatively small and that a major contributor to brain swelling is the sequestered parasite mass. There is accumulating in vitro evidence to suggest that the endothelium is activated during a malaria infection and this can lead to changes in endothelial junctions, leading to increased endothelial permeability.

The last 5 years have seen considerable interest in investigating how the sequestered PRBCs can modulate EC phenotype and function directly. A number of groups are concentrating their efforts on investigating the EC changes that follow interaction with PRBCs and on elucidating the mechanisms underlying these changes, using in vitro coculture systems. It has also become apparent that the changes induced by PRBCs, while in some cases being detrimental to the endothelial monolayer by increasing their permeability, can, at the same time, protect ECs from generalized cell death. Although malaria infection has been closely associated with widespread EC activation, this phase encompasses a broad range of changes seen in ECs, including signal transduction, phenotype and functional changes. A number of in vitro studies (including those of our group) have demonstrated up-regulation of adhesion receptor expression, namely ICAM-1 [23,25]; however, in vitro, this response is dictated by the culture conditions, presence of TNF and also the source of the EC. For example, in contrast with Viebig et al. [23], we did not observe any direct induction of ICAM-1 by PRBCs as there was a critical requirement for basal levels of TNF [25]. This was also seen in a separate study demonstrating direct activation of brain ECs but not HUVECs (human umbilical vein endothelial cells) by PRBCs [24]. Although different subsets of ECs may respond differently, the unifying observation in all these studies is the ability of PRBCs to induce changes to ECs.

**Intracellular signalling**

EC surface-adhesion receptors not only are mediators of cell–cell adhesion, but also can mediate intracellular signalling events in EC. The adhesion receptors which have received the most interest in this respect are ICAM-1 and CD36, which are also two of the main receptors for PRBC sequestration. Antibody cross-linking of the CD36 receptor can activate platelets and monocytes. Furthermore, in platelets and ECs, immunoprecipitation with anti-CD36 antibodies co-precipitates the Src-related protein tyrosine kinases [49]. Recent studies have demonstrated that cross-linking of CD36 or interaction between PfEMP-1 and CD36 can mediate ERK1/2 (extracellular-signal-regulated kinase 1/2) phosphorylation in ECs. In addition, it was shown that ERK1/2 activation was important in supporting firm adhesion of PRBCs via an ecto-alkaline phosphatase that dephosphorylates CD36 and hence increases the binding affinity of CD36 [13,41]. Indeed, a recent randomized clinical study demonstrated the inhibition of PRBC sequestration in patients with uncomplicated malaria by the administration of the alkaline phosphatase inhibitor, levamisole [50].

Similarly, it has been widely established that cross-linking ICAM-1 or binding to ICAM-1 can lead to intracellular signalling in ECs, especially in the context of leucocyte adhesion in inflammation. Activation of MAPKs (mitogen-activated protein kinases) following ligation of surface ICAM-1 can lead to a number of phenotypic and functional changes, including tyrosine phosphorylation of cytoskeletal proteins, such as fac, paxillin and p130, resulting in cytoskeletal rearrangement mediated by Rho activation [51], modulation of junctional proteins, migration of neutrophils towards junctions for transendothelial migration, and up-regulation of VCAM-1, mediated by ERK1 and AP-1 (activator protein 1) [52]. There is now evidence to suggest that the intracellular domain of ICAM-1 is crucial for mediating intracellular signalling following ICAM-1 ligation [53]. ICAM-1 signalling is complex and encompasses activities such as MAPK and transcription factor activation, cytokine production, protein expression, oxidative stress via...
activation of xanthine oxidase and subsequent ROS (reactive oxygen species) production [54], and modulation of cell–cell junctions [55]. Ligation of ICAM-1 was also shown to increase ICAM-1 expression and affect leucocyte adhesion [56]. We have shown that the interaction between PRBCs and ECs can lead to activation of ERK1/2, JNK (c-Jun N-terminal kinase) and p38 MAPKs in ECs, which was dependent on the avidity of the interaction between PIEMP-1 and ICAM-1 [57], and, in separate studies, we have also demonstrated up-regulation of ICAM-1 on HUVECs following interaction with PRBCs in the presence of basal levels of TNF (Figure 1) [25]. Tripathi et al. [24] described nuclear translocation of the transcription factor NF-κB (nuclear factor κB) as part of the signalling cascade that results in up-regulation of ICAM-1, following interaction with PRBCs [24], which fits well with the occurrence of at least one NF-κB-binding site in the promoter element of the ICAM-1 gene. Interestingly, they also observed blockade of ICAM-1 induction, but not constitutive levels of ICAM-1, using inhibitors of ROS.

**Indirect activation of ECs**

Although evidence supports a direct interaction between PRBCs and ECs and other host cells in the modulation of the endothelium, it is also apparent that *P. falciparum*-derived proteins can interact with ECs or macrophages to initiate signalling cascades that can modulate these cells without cytoadherence. For example, this is supported by the observation that the parasite-derived factor PfGPI (*P. falciparum* glycosylphosphatidylinositol), which is released during schizont rupture, can induce release of TNF from macrophages. This TNF release can be inhibited using a JNK inhibitor, 1,9-pyrazoloanthrone [58]. Parasite proteins were also shown to induce disruption of endothelial barrier function in a Src-family kinase-dependent manner, but surprisingly not the intact infected red blood cells [59]. Yet another mechanism for modulation of the endothelium was identified whereby endothelial permeability was increased following interaction with PBMCs which were previously exposed to parasite proteins [60].

**Modulation of endothelial permeability**

Modulation of endothelial permeability in cerebral and pulmonary microvasculature following PRBC sequestration could be implicated in the development of CM and pulmonary oedema respectively, two of the major symptoms of severe malaria. Loss of EC TJPs (tight-junctional proteins) such as ZO-1 (zonula occludens 1), occludin and vinculin, which are critical for maintaining the integrity of the vascular endothelium, was observed by immunohistochemical analysis, most notably in vessels containing sequestered parasites in post-mortem tissue from Vietnamese adults and Malawian children with CM [61–63]. More recently a study in Thailand, demonstrated a decrease in the expression of the TJPs occludin, vinculin and ZO-1 when PRBCs isolated from patients with CM were co-cultured with ECs [64].

In vitro studies have also demonstrated an increase in the permeability of lung and brain ECs mediated by contact with PRBCs. BBB resistance was significantly reduced by up to 70% following contact with intact PRBCs and also on exposure to PRBC-derived soluble factors from the culture supernatant which could be precipitated with 40% ammonium sulfate [65], suggesting that it has at least a protein component. Another study using lung ECs also demonstrated increased endothelial permeability following exposure to PRBCs [66]. Decreases in EC resistance have also been observed in platelet-mediated cytoadherence [67].

**Modulation of endothelial apoptosis**

A number of in vitro studies have suggested that EC apoptosis is induced by PRBCs in brain microvasculature in CM. Although 40% of CM patients show caspase 3 activity, a marker of apoptosis, there was no evidence of specific irreversible damage in CM brain [68]. Thus EC apoptosis is not a widespread feature of CM and hence cannot be attributed a critical role in the breakdown of the BBB. Similarly, development of pulmonary oedema resulting from increased permeability of the lung capillaries is also a common feature of severe malaria [69], but is not thought to be linked to extensive host cell death. Vessel leakage in vivo during malaria infection has been observed directly in the retina [70], which resolves on successful anti-malarial treatment.

The basis of this pathophysiology is unclear, and currently there is considerable disparity in the evidence for PRBC-mediated apoptosis in vascular ECs. There is some evidence to suggest direct induction of apoptosis by PRBCs, mediated through the caspase pathway involving activation of caspase 3, whereas other evidence has suggested that EC apoptosis is an indirect result of oxidative stress due to PRBC-induced generation of ROS [66]. Indeed, studies using
Figure 2 | Diversity of direct and indirect effects on the vascular endothelium in response to PRBC cytoadhesion that can mediate endothelial dysfunction and endothelial survival

superoxide dismutase or other antioxidant supplements have demonstrated protection of ECs following interaction with PRBCs [66,71]. Other studies have demonstrated induction of EC apoptosis indirectly in the absence of physical interaction with PRBCs. For instance, apoptosis was induced in ECs when co-cultured with neutrophils in the presence of sera from *P. falciparum*-infected patients, which was shown to be mediated by neutrophil secretory products [72]. Activated platelets, which are often co-localized with sequestered PRBCs in the CM brain, release TGF-β (transforming growth factor β) which can induce apoptosis in TNF-activated ECs [73]. This is in contrast with other work showing that protozoan parasites, including *Plasmodium*, can suppress apoptosis (for reviews, see [74,75]) and the lack of histological evidence for significant apoptosis in severe malaria. Our own studies have supported modulation of caspase 3 activity in ECs by PRBCs [25], and the activation of NF-κB seen by others [24] could also result in suppression of apoptosis during cytoadherence.

There is therefore an increasing belief that the inflammatory response induced in the host following malarial infection is a critical factor in mediating malaria pathology [80]. It seems likely that systemic activation plays an important role in pathology, as shown by a recent study using a synthetic transcriptional inhibitor of TNF which resulted in inhibition of ICAM-1 up-regulation and also inhibition of PRBC cytoadherence to brain EC [81]. However, localized interactions between PRBCs and ECs may also contribute to disease, particularly during the early stages of infection.

**Modulation of endothelial inflammatory response**

Endothelial activation following interaction with PRBCs can lead to the induction of a range of cytokines and inflammatory mediators. Indeed, there is a huge body of evidence describing up-regulated serum levels of pro-inflammatory cytokines such as TNF [76], IFNγ (interferon γ), IL-1β, IL-8 [77] and IL-6 in studies of severe malaria. Furthermore, TNF, IL-1β and TGF-β have been demonstrated in post-mortem brain tissue in human CM [61]. A recent study, on post-mortem tissue from paediatric CM patients with no pathological diagnosis except malaria, demonstrated expression of HO-1 (haem oxygenase-1), an inflammatory mediator, in brain, liver and lung tissue, suggesting widespread systemic inflammation [78]. An additional result of EC activation in malaria is endothelial vesiculation and the generation of microparticles. These can interact with other host cells such as leucocytes and platelets, to induce cytokine release, thus maintaining and propagating an inflammatory environment [79].

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**Modulation of endothelial survival**

Our recent microarray analysis of transcriptional changes in ECs following exposure to PRBCs in the presence of basal levels of TNF have demonstrated modulation of several genes that can mediate cell survival. This includes overexpression of *UCP3* (uncoupling protein 3) mRNA, which detoxifies ROS to prevent oxidative stress on ECs [82] and superoxide dismutase, which is a scavenger of ROS, and underexpression of caspase 3 mRNA, which is a direct mediator of apoptosis. The protection of the endothelium...
may be a mechanism to effectively protect the parasite and promote survival of the transmissible phenotypes.

Thus it is feasible that, while PRBCs initiate a pro-inflammatory and pro-adhesive response in ECs, there is a concomitant suppression of genes that are involved in mediating apoptosis and overexpression of genes that have a role in protection and survival of the endothelium. It is conceivable that the balance between endothelial activation and endothelial protection is a critical factor in dictating the severity of malaria pathology.

Conclusion

Severe malaria has often been considered as a simple syndrome, but it is increasingly seen as a collection of disease states, not only between adults and children, but also within paediatric disease. This complex clinical picture may well reflect equally complex molecular mechanisms based on a balance between the protective and detrimental host responses (Figure 2). This in turn may be contributed to by a range of parasite properties, including the ability of PRBCs to adhere to host endothelium. As research turns towards discovery of new therapies aimed at alleviating severe disease, it is important that we understand how the parasite is modulating the host response to infection.

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