Trends in Parasitology

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REVIEWS

Review

Blood–Brain Barrier in Cerebral Malaria: Pathogenesis and Therapeutic Intervention

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Cerebral malaria is a life-threatening complication of malaria caused by the parasite *Plasmodium falciparum*. The growing problem of drug resistance and the dearth of new antiparasitic drugs are a serious threat to the antimalaria treatment regimes. Studies on humans and the murine model have implicated the disruption of the blood–brain barrier (BBB) in the lethal course of the disease. Therefore, efforts to alleviate the BBB dysfunction could serve as an adjunct therapy. Here, we review the mechanisms associated with the disruption of the BBB. In addition, we discuss the current, still limited, knowledge on the contribution of different cell types, microparticles, and the kynurenine pathway in the regulation of BBB dysfunction, and how these molecules could be used as potential new therapeutic targets.

Highlights

Cerebral malaria is a life-threatening complication of malaria, particularly in children. The emergence of drug-resistant *Plasmodium* strains is a big challenge to develop new antiparasitic drugs and strategies to combat malaria.

Disruption of BBB integrity results in neurological complications which may ultimately lead to death of the infected individual. Prevention of BBB dysfunction would be an attractive alternative therapeutic strategy to prevent cerebral malaria.

Understanding the underlying mechanisms contributing to the disruption of the BBB, mortality, and also long-lasting neurological sequelae may aid in identifying molecules which could serve as potential new targets for the treatment of cerebral malaria.

Cerebral Malaria – Clinical Significance and the Differences between Murine and Human Studies

Cerebral malaria (CM) is a severe neurological syndrome of human malaria caused by the parasite *Plasmodium falciparum* (*Pf*) affecting mainly children in sub-Saharan Africa and adults in Asia. The complications of CM include clouding of consciousness, cerebral seizures, and coma, and may lead to the death of the infected individual. According to the 2018 WHO *World Malaria Report* for 2017, 435 000 patients died of malaria, with CM accounting for 90% of the deaths. About a quarter of surviving patients suffer from long-term neurological and cognitive deficits such as behavioral abnormality, epilepsy, and impaired motor functions [1,2]. Over the years, **quinine and artemisinin compounds** (see Glossary) have been used for the treatment of severe malaria. The use of these drugs has led to the emergence of resistant strains [3,4]. The problem of drug resistance is ever growing, and novel therapeutic strategies need to be developed, particularly those targeting the host or host–pathogen interaction. Understanding the underlying mechanisms leading to the development of CM would aid in the identification of potential new therapeutic targets. One major limitation of human CM studies is that a detailed analysis of the intracerebral pathogenesis and pathology can be conducted mainly postmortem. Therefore, CM is experimentally studied using a mouse model known as experimental cerebral malaria (ECM). Although *Plasmodium berghei* ANKA (*Pb*A)-induced ECM recapitulates some of the features of human CM, the disease pathology differs considerably. While human CM is characterized by sequestration of **infected red blood cells (iRBCs)** to the cerebral microvasculature, with minute inflammatory changes in the brain, murine ECM shows little or no intracerebral sequestration of iRBCs but a prominent proinflammatory cytokine response in the brain. Moreover, human postmortem reports revealed no intracerebral accumulation of $CD8^+$ T cells, whereas intracerebral accumulation of $CD8^+$ T cells is essential for the development of ECM (reviewed in detail by White *et al.* [5]). These differences need to be considered while translating murine studies to human malaria.

The BBB: The Hotspot in CM

Several studies on patients in Africa and Asia have shown that the disruption of the BBB in CM leads to severe neurological complications including (i) **intracerebral hemorrhage**, (ii) seizures

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resulting from an electrolyte imbalance, and (iii) an increase in intracranial pressure due to widespread edema resulting in axonal damage [6–9]. All of these factors ultimately result in central nervous system (CNS) dysfunction and death. The BBB is a semipermeable membrane which separates the peripheral blood from the brain parenchyma. The BBB comprises a monolayer of endothelial cells (ECs) joined together by tight junctions and the underlying basal lamina. The integrity of the BBB is further supported by pericytes and the astrocyte end-feet (Figure 1). The BBB, together with microglia and neurons, forms the neurovascular unit (Figure 1). Apart from serving as an anatomical barrier, the BBB plays a crucial role in the homeostasis of the CNS by facilitating the transport of nutrients such as glucose and amino acids from the blood to the CNS and by removing metabolic waste products from the CNS into the blood by means of specific transport channels [10]. Up to now, the precise underlying mechanisms leading to the disruption of BBB integrity during CM have remained unclear. Several mutually nonexclusive events have been associated with BBB disturbances in human and murine studies, namely (i) sequestration of iRBCs to the brain ECs [11], (ii) an excessive inflammation resulting from heightened intracerebral proinflammatory cytokine response [12], (iii) disseminated intravascular coagulation in the brain [13], and (iv) dysregulation of vascular ECs [14] (Figure 2, Key Figure). In addition, a growing number of reports implicate two other major factors in the disturbance of the BBB: (a) the host´s pathway for tryptophan metabolism, also known as the kynurenine pathway, and (b) **extracellular vesicles** released by the host and the parasite called **microparticles**.

Mechanisms of Sequestration of iRBCs in CM

Sequestration, that is, accumulation of parasitized erythrocytes to the luminal site of microvessel ECs triggers CM [11]. The *Pf* **erythrocyte membrane protein-1 (*Pf*EMP1)** facilitates the adhesion of the infected erythrocytes on the brain vascular ECs (Figure 2). *Pf*EMP1 is localized to small cup-like protrusions on the plasma membrane of infected erythrocytes called knobs,

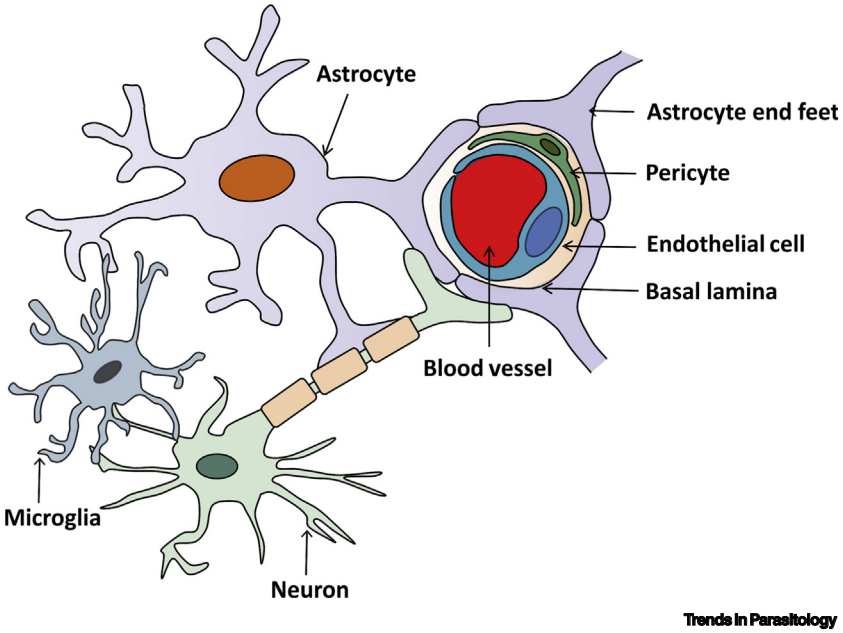

Figure 1. Composition of the Blood–Brain Barrier (BBB). The BBB comprises a monolayer of endothelial cells joined together by tight junctions and the underlying basal lamina. The integrity of the BBB is further supported by pericytes and the astrocyte end-feet. The BBB, together with microglia and neurons, forms the neurovascular unit.

Glossary

Coagulation cascade: coagulation is a process of blood clot formation induced via intrinsic or extrinsic pathways. Clot formation in response to external tissue injury leads to the activation of the extrinsic pathway and involves factor VII. Clot formation in response to abnormality within the vascular system leads to the activation of the intrinsic pathway, which involves factors XII, XI, IX, and VIII. Both pathways activate factors I, II, V, and X, ultimately resulting in fibrin clot formation.
Erythrocyte membrane protein-1 (*Pf*EMP1): a primary *Plasmodium falciparum* antigen and an important virulence factor responsible for cytoadherence of iRBCs. It is encoded by a family of roughly 60 var genes. Transcriptional switching of various var genes results in the generation of different forms of *Pf*EMP1; this helps the parasite to escape detection by the host immune system.
Extracellular vesicles/microparticles: small cell-derived vesicles containing RNA, DNA, protein, lipids, or metabolites; they mediate cell-to-cell communication. Extracellular vesicles can be broadly classified as microparticles and endosomes. While microparticles are heterogeneous particles formed by the outward budding of the plasma membrane, endosomes are homogeneous particles which are end products of the endocytic recycling pathway.
Infected red blood cells (iRBCs): the asexual stage of *Plasmodium*, known as the merozoite, invades red blood cells and replicates inside them.
Intracerebral hemorrhage: bleeding in the brain parenchyma due to the rupture of a blood vessel.
miRNAs (microRNAs): small noncoding RNAs which regulate gene expression by inhibiting translation or degrading their target mRNA.
miR-155: microRNA 155 is known to downregulate cell-adhesion molecules and tight-junction proteins.
MyD88: Toll-like-receptor-associated downstream adaptor molecule.
Quinine and artemisinin compounds: plant-derived antimalarial drugs. Quinine inhibits the parasite's hemoglobin metabolism, resulting in the accumulation of cytotoxic heme. The accumulated heme exerts its antiparasitic activity by lysing the parasite

which mediate the binding of the iRBCs to the ECs [15]. *Pf*EMP1 binds to various receptors on the brain ECs, including the intercellular adhesion molecule-1 (ICAM-1), the vascular cell adhesion molecule-1 (VCAM-1), and the cytokine-activated endothelial protein C receptor (EPCR) [16–18], thereby immobilizing the iRBCs on the vascular endothelium (Figure 3). Patients with CM show an expansion of parasites expressing *Pf*EMP1 that bind to both ICAM-1 and EPCR [17,19,20]. Chan *et al.* have shown that patients with high levels of anti-*Pf*EMP1 antibodies had a significantly reduced risk of developing symptomatic malaria [21]. Platelets induce the adhesion of iRBCs to one another, forming large autoagglutinates [22]. Furthermore, noninfected erythrocytes form

membrane. Artemisinin, by contrast, generates carbon-centered free radicals which alkylate vital parasite proteins, thereby killing them.

Key Figure

Events Associated with Disturbances of the Blood–Brain Barrier (BBB)

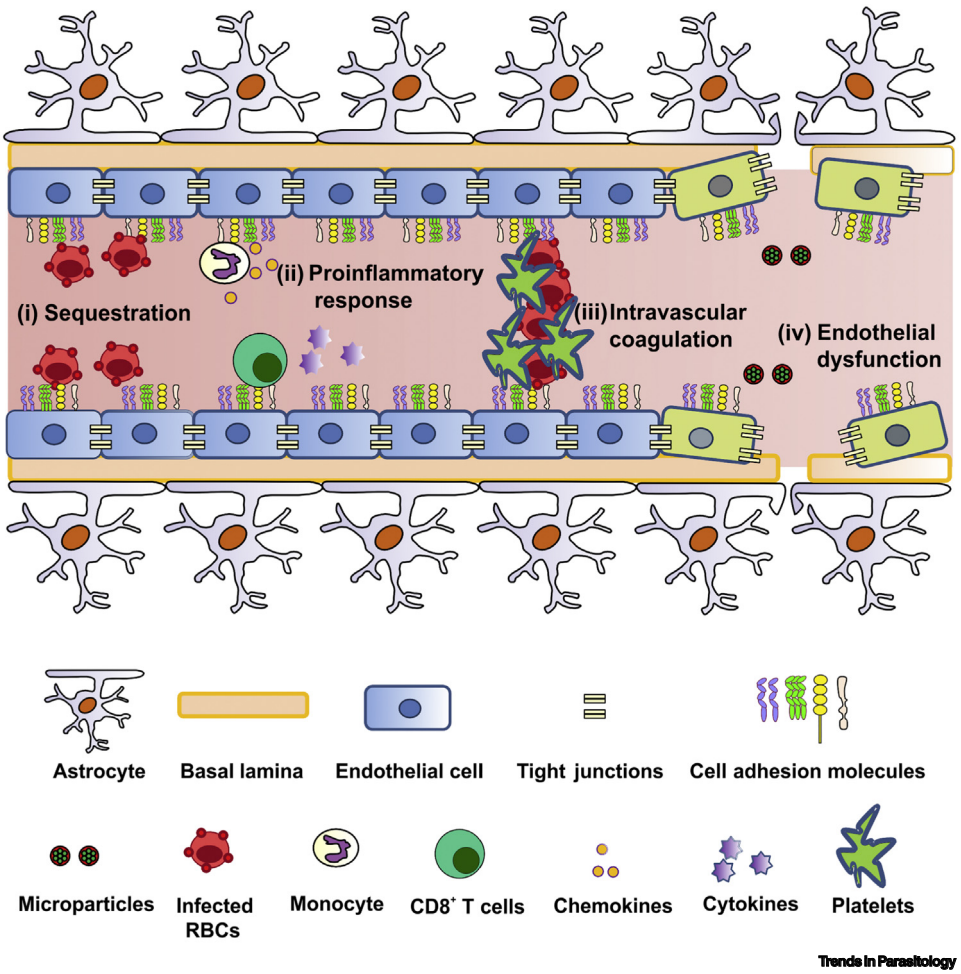

Figure 2. (i) Sequestration of (*Plasmodium falciparum*)-infected red blood cells to the brain endothelial cells. (ii) Excessive inflammation resulting from heightened intracerebral proinflammatory cytokine response. (iii) Disseminated intravascular coagulation in the brain. (iv) Dysregulation of vascular endothelial cells.

rosettes around the iRBCs, further contributing to microvascular obstruction [23]. Since sequestration of iRBCs to the brain endothelium plays a critical role in the development of CM, prevention of sequestration and desequestration of the iRBCs are attractive therapeutic approaches, discussed in detail by Glennon *et al*. [24].

Binding of *Pf*EMP1 to the receptors on the ECs may trigger multiple signaling pathways in ECs, resulting in the reorganization of tight-junction complexes, ultimately leading to the leakiness of the BBB (Figure 3). *In vitro* studies on transendothelial migration of leukocytes, using human and murine ECs, have shown that engagement of ICAM-1 induces the phosphorylation of cytoskeleton-associated proteins, including FAK, paxillin, p130Cas, and cortactin, resulting in remodeling of the endothelial cytoskeleton to facilitate BBB opening (reviewed by Wittchen [25] (Figure 3). Lennartz *et al*. [17] have shown that the DBLβ motif of *Pf*EMP1 is responsible for interaction with ICAM-1; the authors further show that antibodies against the DBLβ motif of *Pf*EMP1, purified from human serum, inhibited ICAM-1-specific adhesion of erythrocytes *in vitro*. On a similar line, use of antibodies against EPCR inhibited *Pf*EMP1 binding to human ECs [18]. Furthermore, studies on human ECs showed that cross-linking of VCAM-1 results in activation of Rac1 signaling, which induces weakening of tight junctions through Rho-dependent induction of stress fibers [25] (Figure 3). Preclinical studies have shown that activated protein C (aPC), an anticoagulant serine protease, binds to EPCR and exerts its anti-inflammatory/antiapoptotic activities, thereby protecting endothelial barrier functions [26,27]. Binding of *Pf*EMP1 to EPCR prevents interaction of aPC with EPCR and thereby has the following two effects. (i) Impairment of the aPC-mediated anticoagulative pathway by fostering the activation of tissue factors VIIIa and Va; activation of these tissue factors leads to thrombin generation, which ultimately results in fibrin deposition. (ii) Activation of Rho A and NF-κB pathways via thrombin-mediated cleavage of PAR1 which, in turn, induces a proinflammatory response leading to the disruption of the BBB [19,27,28] (Figure 3). Administration of aPC may prevent the BBB dysfunction by inhibiting thrombin activity [24]. Sequestration of iRBCs can also be prevented by the use of drugs targeting cytoadherence of iRBCs. Rapamycin prevents the cytoadherence of iRBCs via reduction of ICAM-1 and VCAM-1 expression in ECM [29]. Moreover, ethanolic extracts of the fungus *Trichoderma stromaticum* decrease inflammation and ameliorate ECM by reducing cerebral expression of ICAM-1 and VCAM-1, thereby preserving BBB integrity [30]. Although the role of iRBC sequestration in the pathology of CM is well defined, the role of iRBC sequestration in ECM is controversial [5]. This is one of the major limitations of the murine model of ECM.

A Proinflammatory Response Contributes to Brain Pathology in CM

Another feature associated with CM is the intracerebral inflammation caused by a heightened proinflammatory immune response (Figure 2). Proinflammatory cytokines, such as tumor necrosis factor (TNF), lymphotoxin α (LTα), interferon-γ (IFN-γ), interleukin-1α (IL-1α), and IL-1β, are upregulated during CM and have been implicated in the pathogenesis of the disease [12]. Although the levels of TNF are elevated in the serum during human CM, inhibition of TNF by anti-TNF antibodies does not confirm protection from CM [31].

Furthermore, Engwerda *et al*. [32] have shown that LTα knockout mice are completely protected from ECM, and the sources of LTα during ECM are the radio-resistant intracerebral parenchymal cells. LTα upregulates the expression of ICAM-1 and thereby fosters the intracerebral recruitment of leukocytes. These data suggest that depletion of LTα in the parenchymal cells could be an option to prevent the neurological complication of ECM. The role of LTα in the context of CM has not been explored yet. Monocytes and macrophages rapidly respond to infection by plasmodia and play an important role in the control of the infection. During CM, the macrophages and monocytes produce a plethora of cytokines, including IL-1α and IL-1β (Figure 4) [12,33]. These cytokines activate the ECs, which release chemokines, including CCL2, CCL4, CXCL4,

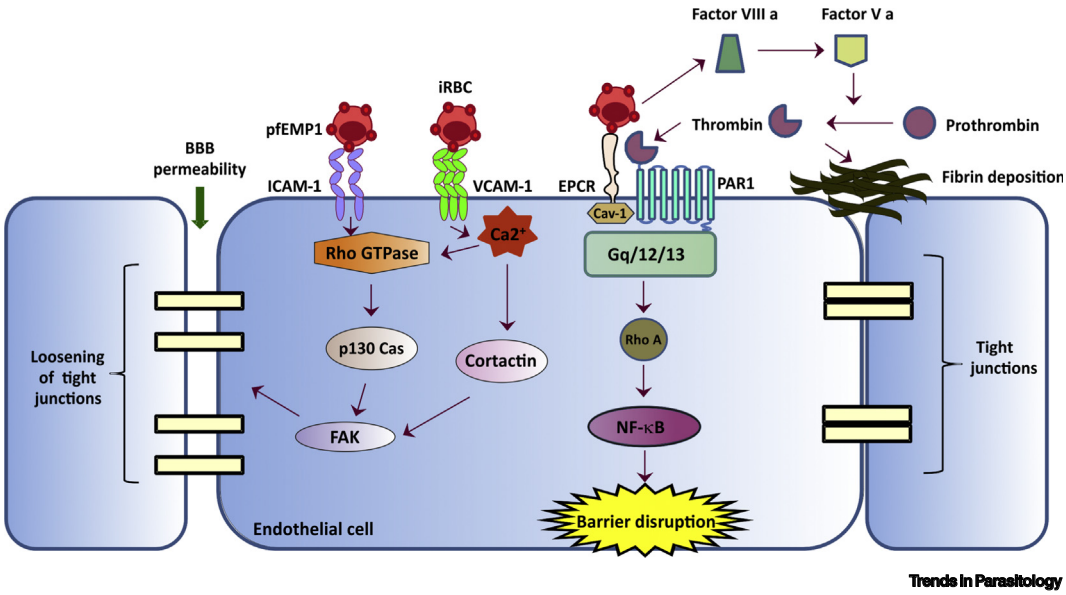

Figure 3. Sequestration of Infected Red Blood Cells (iRBCs) to the Brain Endothelial Cells (ECs). *Pf*EMP1 of the iRBCs binds to various receptors on the brain ECs, including ICAM-1, VCAM-1, and EPCR. This triggers multiple signaling pathways in ECs, resulting in the reorganization of tight-junction complexes and enhancing permeability of the blood–brain barrier (BBB). ICAM-1 induces remodeling of the endothelial cytoskeleton through Rho-dependent phosphorylation of cytoskeleton-associated proteins, including FAK, paxillin, p130 Cas, and cortactin, thereby facilitating BBB opening. Ligation of *Pf*EMP1 to VCAM-1 results in weakening of tight junctions through Rho-dependent induction of stress fibers. Binding of *Pf*EMP1 to endothelial protein C receptor (EPCR) impairs the anticoagulative pathway by fostering the activation of tissue factors VIIIa and Va. Activation of these tissue factors leads to thrombin generation, which ultimately results in fibrin deposition. Binding of *Pf*EMP1 to EPCR also activates Rho A and NF-κB pathways via thrombin-mediated cleavage of PAR1, inducing a proinflammatory response leading to the disruption of the BBB.

CXCL8, and CXCL10, and induce the recruitment of leukocytes to the brain (reviewed by Dunst *et al*. [12]; Figure 4). Studies using knockout mice have shown that deletion of CCL2, CCL4, CXCL4, CXCL8, and CXCL10 reduces the pathology of ECM [12]. For instance, CCL2 increases endothelial permeability by redistribution of tight-junction proteins [34]. Therefore, the abovementioned chemokines could serve as potential therapeutic targets.

Postmortem studies from the brains of patients with CM have shown intracerebral accumulation of leukocytes (reviewed by Renia *et al*. [35]) and platelets [36] along with iRBCs. Concomitantly in ECM, intracerebral accumulation of multiple leukocyte populations, including macrophages, monocytes, neutrophils, NK cells, T cells, and platelets, have been observed [35]. The recruited leukocytes further produce cytokines and chemokines which induce local inflammation and disruption of the BBB (Figure 4) [12]. Neuregulin-1 treatment protected from ECM by reducing the intracerebral levels of the proinflammatory cytokines TNF, IL-6, and IL-1α and the chemokine CXCL10, thus decreasing leukocyte accumulation in brain [37]. It is well documented that $CD8^+$ T cells play an important role in the pathology of ECM [35]; they mediate the disruption of the BBB by fostering leukocyte accumulation, and by inducing apoptosis of ECs through granzyme B and perforin-mediated cytotoxicity (Figure 4) [35,38]. The chemokine receptor CXCR3, expressed on leukocytes, is responsible for their intracerebral trafficking. Therefore, CXCR3 is an ideal candidate for therapeutic targeting. Inhibition of protein tyrosine phosphatase activity using the inhibitor potassium bisperoxo (1,10-phenanthroline) oxovanadate (V) trihydrate [bpV (phen)] prevented ECM, by reducing CXCR3 expression on T cells, thereby, impairing their recruitment to the brain [39]. Moreover, deficiency of IRF1 reduced the expression of CXCR3 on pathogenic $CD8^+$ T cells, suppressed interferon-γ production, and delayed $CD8^+$ T cell proliferation during ECM [40]. The antibiotic doxycycline reduces cerebral infiltration of

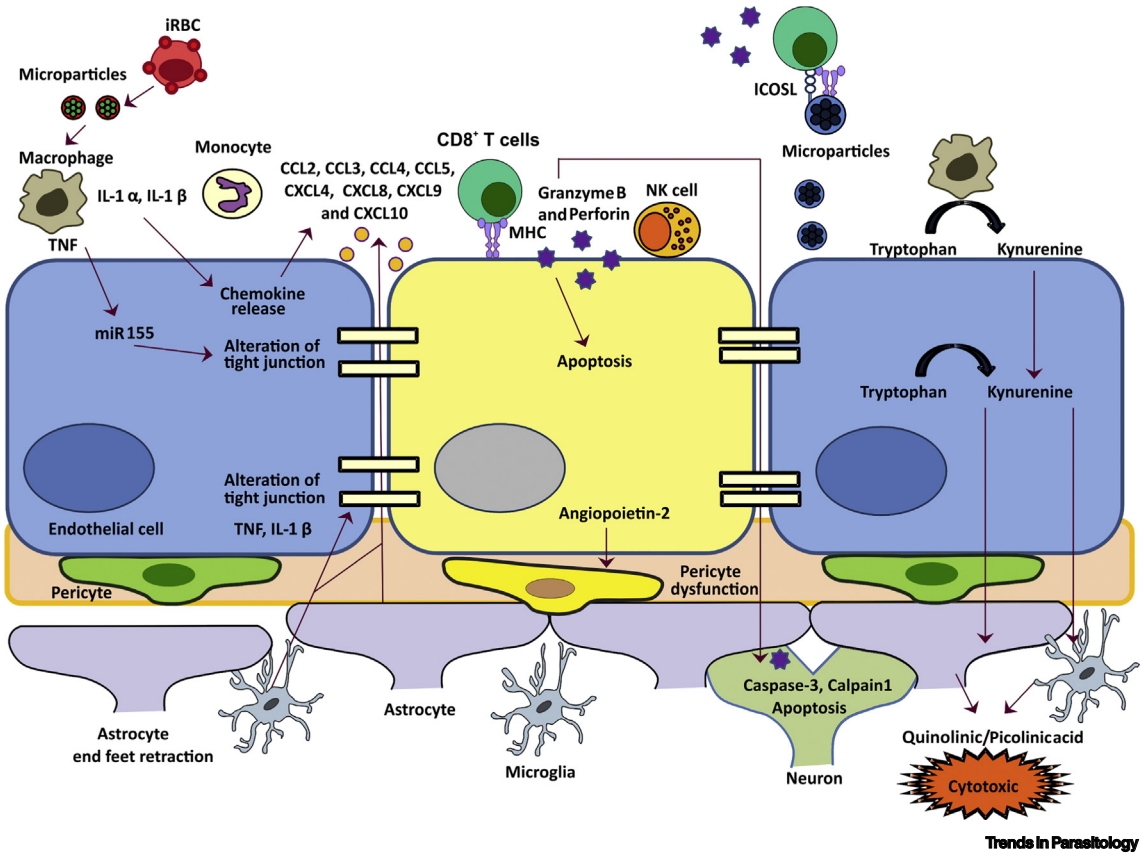

Figure 4. Molecular Mechanisms Contributing to Blood–Brain Barrier (BBB) Dysfunction. Infected red blood cell (iRBC)-derived microparticles induce tumor necrosis factor (TNF) production by the macrophages. TNF upregulates miR-155 expression by the endothelial cells (ECs) which, in turn, leads to the disturbance of BBB integrity by altering the tight junctions. Macrophages and monocytes produce IL-1α and IL-1β, which further activate the ECs to release chemokines, including CCL2, CCL4, CXCL4, CXCL8, and CXCL10. These cytokines foster the accumulation of leukocytes, including NK cells and $CD8^+$ T cells. The infiltrating leukocytes induce apoptosis of ECs through granzyme B and perforin-mediated cytotoxicity. The EC-derived microparticles prime the T cells by the expression of antigen-presenting molecules, including MHC and ICOSL. The $CD8^+$ T cells induce neuronal cell death directly by their cytotoxic function and by activating neuronal caspase-3 and calpain1. In addition to endothelial cells, astrocytes and microglia also contribute to the infiltration of leukocytes by chemokine production. Astrocytes retract their processes from the ECs, leading to reduced wrapping of the blood vessels. Angiopoietin-2, produced by the ECs, also causes reduced wrapping of the blood vessels by inducing pericyte dysfunction. Microglia disrupt the BBB by producing TNF and IL-1β which alter the expression of tight-junction proteins. Kynurenic acid, produced by the macrophages and endothelial cells during tryptophan metabolism, is further converted into cytotoxic quinolinic and picolinic acids by the astrocytes and microglia. These molecules induce the disruption of the BBB.

T cells and expression of matrix metalloproteinase 2 (MMP2), IFN-γ, and granzyme B, the factors associated with ECM pathology [41]. In an interesting study, Gordon *et al*. [42] showed that ECM could be reversed by treating mice with the glutamine analog 6-diazo-5-oxo-L-norleucine (DON). Although DON treatment did not prevent the proliferation of intracerebral accumulation of $CD8^+$ T cells, it inhibited the effector function of $CD8^+$ T cells by blocking their degranulation. In addition to $CD8^+$ T cells, DON treatment also induced about 81 metabolic changes in the brain, resulting in reduced brain swelling and restoration of BBB integrity. This is the only study showing the reversal of late-stage ECM by therapeutic intervention.

Molecular Mechanisms of Intravascular Coagulation in CM

Dysregulation of coagulation is frequently found in patients suffering from CM (Figure 2). Aberrant and widespread activation of the coagulation system, also called disseminated intravascular coagulation, leads to severe complications such as cerebral thrombosis, hemorrhage, intracerebral

hypoxia, and reduced blood flow (Figure 3) [43]. Moxon *et al*. [13] suggest that intravascular coagulation in CM may be caused by reduced expression of iRBC binding receptor EPCR and anticoagulant/thrombin receptor thrombomodulin (TM). The **coagulation cascade** is triggered by iRBCs, which activate both the extrinsic and intrinsic pathways of blood coagulation by inducing the expression of tissue factor (TF) by the ECs [44,45]. TF initiates the coagulation cascade which results in the conversion of fibrinogen to fibrin, and, finally, thrombosis (Figure 3) [44]. Studies using purified systems *in vitro* show that, in addition to the induction of TF, *Pf* releases a procoagulation factor called histidine-rich protein II (HRPII). HRPII fosters coagulation by binding to antithrombins, including dermatan sulfate, heparan sulfate, and heparin, inhibiting their activity [46]. Blocking of HRPII could aid in targeting the coagulation process, thereby preventing cerebral thrombosis. Furthermore, *Pf* infection also fosters the coagulation process by reducing the levels of anticoagulative factors, including aPC and antithrombin [45], and by increasing the levels of plasminogen activator inhibitor-1, an inhibitor of fibrinolysis [47]. aPC downregulates the coagulation cascade by proteolytically inactivating coagulation factors Va and VIIIa in CM [48]. Therefore, as suggested by Glennon *et al.* [24], administration of aPC would be an attractive therapeutic target. Interestingly, host metabolism also influences the coagulation process during CM. Using 2-deoxy glucose (2DG), a modified form of glucose, Wang *et al*. [49] have shown that inhibition of glycolysis prevented the formation of thrombin and thrombotic complications during ECM. Plasma exchange transfusion could also be a treatment option as it normalizes coagulation by a reduction of coagulation factors in CM [50].

Factors Governing Endothelial Dysfunction in CM

During CM, dysfunction of the ECs may occur due to adhesion of iRBCs to ECs, the local proinflammatory cytokine response, and increased coagulation of the blood in the microvasculature leading to cerebrovascular constriction (Figure 2). Synthetic oleanane triterpenoids have been shown to reduce plasma levels of the cytokines IL-10, TNF, and IFN-γ, thereby enhancing the integrity of the BBB during ECM [51]. Recently, Barker *et al.* [52] have implicated **micro RNA** in the activation of ECs (Figure 4). Using knockout mice, the authors have shown that deficiency of **miR-155** preserved the BBB integrity by reducing the activation of ECs, even in the presence of proinflammatory cytokines. Furthermore, using an *ex vivo* endothelial microvessel model, the authors have shown that pretreatment with anti-miR-155 reduced the vascular leakage induced by serum from malaria patients, thereby identifying miR-155 as a potential target to prevent endothelial dysfunction during CM [52]. Endothelial dysfunction in malaria is also associated with reduced levels of nitric oxide (NO) and its precursor, L-arginine [53]. NO is essential for the proper functioning of ECs [54]. NO protects the ECs by (i) reducing the expression of cytokine-inducible adhesion molecules like ICAM-1, VCAM-1, and E-selectin on ECs [53,55], which, in turn, impairs the proinflammatory cytokine response by limiting the intracerebral accumulation of leukocytes, and (ii) reducing the adherence of iRBCs to the microvascular endothelium [56], the main trigger for CM-associated pathology. Endothelial dysfunction can also be prevented by supplementing L-arginine, the substrate for the synthesis of NO. L-arginine supplementation resulted in the dilation of constricted arterioles and increased regional cerebral blood flow during ECM [57]. Furthermore, increased NO levels reduce the intracerebral leukocyte accumulation by downregulating cell-adhesion molecules [53,55]. In addition to its procoagulative function, HRPII also induces endothelial dysfunction in human ECs via activation of the inflammasome [58]. This study further highlights HRPII as a potential drug target. The sphingolipid sphingosine-1-phosphate (S1P) is known to foster BBB integrity by inducing cytoskeleton rearrangement and upregulating tight junctions on the ECs [59]. Oggungwan *et al*. [60] have shown that *in vitro* treatment of ECs with FTY720, a synthetic sphingosine-1-phosphate analog, restores endothelial cell permeability induced by serum from malaria patients, suggesting potential clinical applications.

Altered Astrocyte Activation and Distribution in CM

Astrocytes form the most abundant glial cell population in the CNS, and they contribute to the formation of the BBB (Figures 1 and 2). In addition to directly constructing the BBB by building the glia limitans with their end-feet, astrocytes also induce BBB properties in ECs. A recent study by Barrera *et al*. [61] on pediatric CM autopsies reported increased activation of ICAM-1 and glial fibrillary acidic protein, the prototypic cytoskeleton protein of astrocytes, by the astrocytes. Hayashi *et al*. [62] have shown that contact with astrocytes increased the expression of tight junctions by the ECs. We and others have shown that astrocytes are activated during *Pb*A infection, further pointing towards a role in ECM [35,38]. Medana *et al*. [63] identified that astrocytes retract their processes during ECM and are unevenly distributed, leading to reduced wrapping of the blood vessels. The loss of ensheathed vessels might contribute to the disruption of the BBB (Figure 4). Combes *et al*. [64] proposed that astrocytes could be the potential convergence point of the two major proposed mechanisms of CM pathogenesis, that is, the cytokine response and the resulting cerebral hypoxia. During ECM, astrocytes are a source of CXCL9 (Miu *et al*. [65]), the chemoattractant which recruits the pathogenic T cells to the brain. Furthermore, Shrivastava *et al*. [66] have demonstrated that astrocytes uptake vesicles derived from iRBCs and upregulate the chemokine CXCL10. Upon *in vitro* stimulation with iRBC, astrocytes upregulate the production of leukocyte-recruiting chemokines, including CCL2, CCL5, CXCL9, and CXCL10 (Figure 4) [67]. In the context of ECM, chemokine release by astrocytes could exacerbate the disease by recruitment of pathogenic $CD8^+$ T cells and by impaired ensheathment of blood vessels. Thus, strategies reducing astrocyte pathology appear as an attractive therapeutic option, although our knowledge of astrocytes in ECM and, in particular, CM, is very limited.

Microglia Are Key Mediators of Neuroinflammation in CM

Microglia are yolk-sac-derived brain parenchymal cells sharing many features with bone-marrow-derived macrophages [68]. They are a key cell type contributing to neuroinflammation. During hemorrhage, the microglia migrate to the site of infection and may phagocytose iRBCs [69]. da Fonseca *et al*. [70] showed that microglia produce the proinflammatory cytokines TNF and IL-1β which impair BBB integrity by altering the expression of tight-junction proteins such as zona occludens-1, claudin-5, occludin, and P-glycoprotein 1 (Figure 4). Since these cytokines are highly upregulated in the brains of patients with CM [71], it is likely that microglia regulate BBB integrity during CM. Microglia are also the major producers of intracerebral NO [70], which could preserve the BBB by preventing the activation of ECs, as discussed above [53,55]. Several studies, including ours, have shown that microglia are activated early during ECM [35,38]. During ECM, microglia retract their ramified processes and migrate towards the venous side of the circulation [63]. *In vitro* studies suggest that microglia may contribute to the control of *Plasmodium* by phagocytosis of iRBCs [66]. Moreover, upon stimulation, microglia can produce a wide variety of chemokines, including CCL2, CCL3, CCL4, CCL5, CXCL8, and CXCL10 (Figure 4) [72]. These chemokines recruit leukocytes to the BBB. The recruited leukocytes, additionally produce chemokines thereby exacerbating the local inflammation and potentiating the disruption of the BBB. Therefore, inhibition or blocking of chemokines and chemokine receptors would be a potential therapeutic approach [12].

Pericyte Dysfunction Impairs Barrier Integrity in CM

Pericytes are perivascular cells which form a sheath around the ECs (Figure 1). They regulate the blood flow by moderating the dilation and constriction of the blood vessels. Another important function of pericytes is removal of the metabolic toxic wastes, thereby maintaining the homeostasis of the CNS. Disruption of pericytes has been reported in CM [61]. Pericytes stabilize the integrity of the BBB in two ways: (i) they induce the expression of tight junctions on the ECs [73], and (ii) they regulate the expression of plasmalemmal vesicle-associated protein and angiopoietins,

which induce vascular permeability [74]. Angiopoietin-1 stabilizes the BBB while angiopoietin-2 weakens the pericyte–endothelial cell interaction, resulting in BBB dysruption. During CM, the levels of angiopoietin-1 are reduced while levels of angiopoietin-2 are elevated, which might be an indication for pericyte dysfunction (Figure 4) [75–78]. Therefore, inhibition of angiopoietin-2 could be an option to prevent pericyte dysfunction. In the context of malaria, the role of pericytes is not well known. It has been reported that TGF-β induces the expression of proinflammatory cytokines by the pericytes, which may contribute to the disruption of the BBB [79]. By contrast, pericytes downregulate the expression of chemokines and adhesion molecules; therefore, they might prevent the recruitment of the leukocytes and thereby protect BBB integrity [79]. Further studies are needed to better characterize the role of pericytes in CM.

Neuronal Injury Contributes to the Complications of CM

Neurons are vitally important brain cells which process and transmit information to other cells. They directly regulate the BBB permeability through innervations of ECs (reviewed by Mizee and de Vries [80]). Additionally, neurons regulate CNS blood flow and the transport of oxygen and glucose to different brain regions. The neurological complications of CM may be attributed to neuronal dysfunction and cell death. Postmortem analysis of children with CM showed widespread axonal and myelin damage in the cerebrum, cerebellum, and brain stem [81]. Similar to CM, axonal injury and demyelination have been reported in ECM [82]. During ECM, granzyme-B produced by pathogenic $CD8^+$ T cells kills neurons directly by its cytotoxic function and by activating neuronal caspase-3 and calpain1 (Figure 4) [83]. Therefore, targeting granzyme-B would be an option to prevent neuronal cell death. Treatment of mice with the drug minocycline, an anti-inflammatory tetracycline, prevented neurodegeneration and disruption of the BBB by reducing levels of inflammatory cytokines and chemokines [84]. Administration of ethanolic extracts of the plant *Azadirachta indica* reduced neuroinflammation and brain edema, and protected neurons from apoptosis during ECM [85]. Simhadri *et al*. [86] have suggested that dysregulation of the LIMK-1/cofilin-1 pathway could lead to alteration of neuronal morphology and may be the reason for cognitive defects in patients surviving CM, thereby identifying the LIMK-1/cofilin-1 pathway as a potential target.

Microparticles and the Kynurenine Pathway as Regulators of the BBB

In addition to the cellular components of the BBB, other factors, such as the host-derived microvesicles known as microparticles, and the host metabolic pathways such as the kynurenine pathway, play a crucial role in the regulation of BBB integrity. Mantel *et al*. [87] have shown that human iRBC-derived microparticles alter endothelial gene expression, thereby causing disruption of the BBB. Microparticles have also been found in patients suffering from CM, and the number of microparticles positively correlates with the severity of the disease [88]. Microparticles are small membrane-derived vesicles with a diameter of less than 1 μM. They contain cellular substances, such as proteins and nucleic acids, and they function as biological messengers between cells. They are released from activated or dying cells as a result of membrane remodeling [89]. Under resting conditions, phosphatidylserine (PS), a component of the cell membrane, is present on the inner leaflet. Upon activation, or during cell death, the PS moves to the outer membrane, buds off, and thereby forms microparticles with antigens on their surface [89]. Microparticles can arise from a variety of cell types, including platelets, erythrocytes, macrophages, monocytes, and ECs. Platelet-derived microparticles (PDMPs) play a key role in the pathogenesis of CM. They stimulate the iRBCs and enhance their binding to the ECs [90]. In addition, the antigens derived from PDMP can alter the endothelial phenotype, thereby fostering the sequestration of iRBCs on the brain endothelium. Studies on patients with CM have identified iRBCs as a major source of microparticles [91,92]. Erythrocyte-derived microparticles induce inflammation by activating macrophages through the TLR4/**MyD88** axis, resulting in upregulation of CD40 and production

of TNF (Figure 4) [92]. Blockage of TLR4 during ECM limits inflammation by impairing macrophage activation [92]. Nantakomol *et al*. [91] have shown that both parasite and heme molecules induce the production of microparticles from human erythrocytes, which can be inhibited by the administration of *N*-acetylcysteine. Interestingly, EC-derived microparticles prime the T cells by the expression of antigen-presenting molecules, including β2 microglobin, MHC II, CD40, and inducible costimulator ligand (ICOSL) (Figure 4) [93]. Noteworthy, the ATP-binding cassette

Table 1. Pathogenic Mechanisms Underlying Human and Murine Cerebral Malaria with Possible Therapeutic Interventions

Pathogenic events	Possible intervention	CM/ECM	Refs
Sequestration of iRBCs	(i) Desequestration		
	(a) Antibodies against DBLβ motif of *Pf*EMP1	CM	[17]
	(b) Antibodies against EPCR	CM	[18]
	(ii) Reduction of ICAM-1 and VCAM-1 expression		
	(a) Rapamycin treatment	ECM	[29]
	(b) Administration of ethanolic extracts of the fungus *Trichoderma stromaticum*	ECM	[30]
Proinflammatory response	(i) Neutralization of cytokines and chemokines		
	Neuregulin-1 treatment	ECM	[37]
	(ii) Prevention of intracerebral accumulation of $CD8^+$ T cells		
	(a) Inhibition of LTα	ECM	[32]
	(b) PTP inhibition by bpV(phen)	ECM	[39]
	(c) IRF1 inhibition	ECM	[40]
	(d) Doxycycline treatment	ECM	[41]
	(e) Administration of 6-diazo-5-oxo- L-norleucine	ECM	[42]
Intravascular coagulation	Prevention of intravascular coagulation		
	(a) Blocking histidine-rich protein II	CM	[47,58]
	(b) Administration of aPC	CM	[24]
	(c) Administration of 2-deoxy glucose	ECM	[49]
	(d) Plasma exchange transfusion	CM	[50]
Endothelial dysfunction	(i) Prevention of activation of ECs		
	(a) Administration of synthetic oleanane triterpenoids	ECM	[51]
	(b) anti-miR155 treatment	ECM	[52]
	(c) Supplementing L-arginine	ECM	[57]
	(ii) Restoration of tight junction		
	Administration of FTY720	CM	[60]
Astrocyte activation	Neutralization of cytokines and chemokines	ECM	[12]
Microglia activation	Blocking chemokine and chemokine receptors	ECM	[12]
Pericyte dysfunction	Inhibition of angiopoietin-2	CM	[75–78]
Neuronal damage	(i) Prevention of neuronal death		
	Targeting granzyme-B	ECM	[83]
	(ii) Prevention of neurodegeneration		
	Administration of minocycline	ECM	[84]
	(iii) Reduction of neuroinflammation		
	Administration of extracts of the plant *Azadirachta indica*	ECM	[85]
Microparticles	(i) Inhibition of production of microparticles		
	(a) Administration of *N*-acetylcysteine	CM	[91]
	(b) Inhibition of ATP-binding cassette transporter A1	ECM	[94]
	(ii) Prevention of activation of macrophages by microparticles		
	Blockage of TLR4	ECM	[92]
Kynurenine pathway	Inhibition of quinolinic and picolinic acids	CM	[96–98]

transporter A1 (ABCA1) is a key molecule modulating the formation of microparticles. ABCA1 regulates the movement of PS from the inner to the outer leaflet. Deletion of ABCA1 protected mice from ECM [94]. The number of microparticles was drastically reduced in the plasma of ABCA1-deficient mice, resulting in impaired activation of ECs and reduced accumulation of leukocytes, thereby identifying ABCA1 as a potential therapeutic target in CM. El-Assaad *et al.* [95] have shown that transfer of microparticles from an infected mouse into a healthy recipient mouse induced CM-like brain and lung pathology, further supporting the pathogenic role of microparticles in the development of CM.

In addition to the discussed factors, metabolic pathways play a key role in the pathogenesis of ECM, and presumably CM. In fact, the abnormal accumulation of metabolites may create a toxic microenvironment fostering damage to the BBB. One such pathway implicated in the BBB dysfunction is the kynurenine pathway involved in the metabolism of the essential amino acid tryptophan. Studies on malaria patients have revealed an increase in levels of kynurenine and kynurenine metabolites – quinolinic acid, kynurenic acid, and picolinic acid – in the cerebrospinal fluid [96–98]. The levels of quinolinic and picolinic acids correlated with the severity of the disease [97]. Quinolinic acid is an excitotoxin exerting its toxic effects on astrocytes, microglia, and neurons (Figure 4). In addition, quinolinic acid can disrupt the integrity and cohesion of the BBB [99]. Picolinic acid is a proinflammatory mediator which induces chemokine production by the macrophages [100]. The chemokines, in turn, could recruit leukocytes, further fostering disruption of the BBB. Using inhibitors of quinolinic and picolinic acids could be an option to maintain the integrity of the BBB during malaria.

Concluding Remarks

The efficacy of the treatment to control CM is threatened by the unresolved problem of drug resistance. Therefore, the development of new adjunct therapeutic strategies manipulating parasite–host interaction provides an attractive additive treatment in combination with antimalarial drugs for the treatment of CM. Since disturbance of BBB integrity leads to the lethal course of CM, prevention of BBB dysfunction may potentially be a life-saving intervention. However, most experimental studies do not prove that the specific therapeutic intervention could prevent lethal progression after onset of ECM. Therefore, interventional studies verifying the therapeutic potential of the identified molecules and mechanisms (summarized in Table 1) are critically important. Understanding the underlying mechanisms leading to the BBB dysfunction may aid in identifying potential new drug targets. Prospectively, further validation of the discussed targets, using murine inhibition studies and innovative human BBB models, is warranted to qualify them as potential therapeutic targets against BBB dysfunction (see Outstanding Questions).

Outstanding Questions

Since the BBB integrity is not uniform throughout the brain, does the cerebrovascular and BBB dysfunction differ only in areas which are more permeable?

Is the integrity of the BBB restored in patients after antimalarial treatment?

Does the BBB dysfunction continue in patients exhibiting neurological sequelae?

Which molecular mechanisms contribute to the neurological sequelae, and can these molecules serve as therapeutic targets to prevent long-lasting disability?

Author Contributions

Both authors have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Acknowledgments

The authors thank Rituparna Bhattacharjee, Kunjan Harit, and Sissy Just (University of Magdeburg, Germany) for critically reading the manuscript and providing their valuable suggestions. Collaborative research of G.N. and D.S. was supported by the Deutsche Forschungsgemeinschaft (SFB 854, TP30 to G.N. and TP 5 to D.S.).

Disclaimer Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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