Research Article

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Journal of Pharmaceutical Microbiology

2018
Vol. 4 No. 1:3

Pathogenesis and Experimental Models of Cerebral Malaria: A Review

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Citation: Singh PP, Prakash B (2018) Pathogenesis and Experimental Models of Cerebral Malaria: A Review. J Pharm Microbiol. Vol. 4 No. 1:3

Abstract

Cerebral malaria (CM), a severe complication of the nervous system due to uncontrolled/untreated *Plasmodium falciparum* infection in humans, is characterized by neurological symptoms, hypothermia, and the sequestration of the infected-erythrocytes (IEs) and platelet microparticles in brain. The sequestration of the IEs in the brain capillaries ensues in hemorrhages, hypoxia, hypoglycemia, convulsions, coma, and ultimately, the death of the patient, if left untreated. Our understanding of human CM (HCM) is rather sparse mainly because of the ethical constraints. So far, knowledge about CM has been obtained from autopsy studies of the brain tissue of CM patients. Therefore, there is urgent need for ideal *in vivo* and/or *in vitro* models of CM, which mimic the HCM, as closely as possible. So far, no ideal experimental CM model has been reported. The critical analysis of the data collected from various *in vivo* and *in vitro* models of CM is expected to augment our understaning of several important aspects of HCM pathogenesis. The identification of biomarkers/biosignatures for the prognosis and diagnosis of HCM are very much warranted. The availability of a suitable experimental model(s) of CM will be helpful in understanding the pathogenesis of HCM, and in the discovery and development of novel therapeutic strategies for HCM.

Keywords: Animal models; Biomarkers; Cerebral Malaria; Hypothermia; *Plasmodium falciparum*; Sequestration

Received: September 26, 2017; **Accepted:** April 19, 2018; **Published:** April 26, 2018

Introduction

The malaria parasite *Plasmodium* has a wide range of host specificity in vertebrates; including primates, rodents, aves and reptiles [1]. These *Plasmodium* parasites are transmitted through mosquito vectors, and have constrained host specificity i.e. the *Plasmodium* species which are confined to rodents only infect the rodents [2,3]. *Plasmodium* infection causes malaria, one of the most prevalent infectious diseases in tropics and sub-tropics [4]. For a long time we knew that naturally, only four *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) infect humans, and cause malaria [5]. In the recent past; however, three more species of simian malaria parasites (*P. knowlesi*, *P. brasilianum*, and *P. simium*) have been reported to naturally cause malaria in humans [6].

Nevertheless, *P. falciparum* is the only malaria parasite which is highly virulent and often fatal. Cerebral malaria (CM) is a severe neurological complication of malaria in humans and other hosts. During *P. falciparum* malaria, human CM (HCM) is known to occur which involves the sequestration of IEs in brain capillaries resulting in their blockade [7]. HCM also involves symptoms like hemorrhage, hypoglycemia, hypoxia, convulsions, coma, which eventually lead to the death of the patient if untreated [7]. Several hypotheses have been proposed for the pathogenesis of the HCM, these include (i) mechanical or sequestration hypothesis, (ii) inflammation hypothesis and (iii) hemostasis hypothesis. So far, all the data on HCM has been obtained from the autopsy studies on the brain tissue of CM patients, and, put together, is quite insufficient to draw some meaningful conclusion. Therefore, researchers are still working for an ideal experimental model of CM, which should mimic almost all characteristic features of the HCM. The availability of an experimental model(s) of CM, which closely mimics HCM, will definitely go a long way in augmenting our understanding of the molecular mechanisms of the pathogenesis of HCM, which in turn, is expected to help in the formulation of novel strategies to mitigate and/or to prevent HCM, and also in the discovery and development of novel therapeutic strategies for HCM.

In-vivo/*in-vitro* Models of CM

Several *in-vivo* and *in-vitro* models are available to study the CM pathogenesis; however, all of them are far from satisfactory. Each of the *in-vivo* or *in-vitro* models has their own strengths and weaknesses. The available *in vivo* CM models include non-human primates, rodents and avian. The integration of the data obtained all these models has been quite helpful in the understanding the pathogenesis of HCM.

In-vivo models

Rodent malaria models: A rodent CM model has been suggested nearly long ago [8]. However, now there are several rodent CM models available to study various aspects of the biology of HCM. A rodent host and malaria parasite combination, based up on its stringentness, ultimately determines the outcome of the model i.e. whether it mimic the HCM or not [9]. In murine malarias, the chances of the occurrence of CM incidences and their reproducibility are relatively high. In these models, the immune system is highly active in modulating the pathogenesis and the consequent outcome of CM. The HCM and mouse experimental CM models (ECM) have several similarities, which include (i) the pathology of the nervous system and clinical manifestations of the nervous system malfunction, (ii) disturbed cytokine balance induced brain pathology, (iii) brain endothelial cell involvement in disease pathology and as effector cells in the pathophysiology of malaria, and (iv) similarities in the immune activation pathways [10, 11]. However, every model of murine malaria is not suitable to be developed as a model of ECM; and to act as a model for HCM. It requires very stringent host-parasite combination system, a particular strain of the parasite, the size of the inoculum, the diet of host and the environmental conditions [12-14]. The murine CM was characterized by the inflammation, sequestration of the immune cells in the brain microvessels and vascular leakage of the capillaries due to blood-brain barrier (BBB) infiltration. Thus the murine ECM deviated from the HCM, in terms of an absence of the sequestration of IEs in brain microvessels, little or no BBB dysfunction in the case of the HCM; the vascular leakage of the BBB of murine ECM was similar to the pediatric HCM condition. Despite the vast deviations in the disease pathology/characteristics and severity between murine ECM and HCM, still, these murine models of ECM are being used to study the CM pathogenesis, to discover the new interventions, and to develop the strategic therapeutic treatments for the HCM. Usually, it is the combination of the rodent malaria parasite and mouse strain which determines the outcome of a particular ECM model *viz*. i) *P. berghei* ANKA-CBA/ca mice strain represents the neuropathology of the CM characterized by the slight or little IEs sequestration in the microvessels, and cytokines such as TNF-α and IFN-γ are involved in the inflammation and the vascular occlusion of the brain capillaries[15], ii) *P. berghei* ANKA-BALB/c mice strain this combination never been used as model for ECM because BALB/c mice are resistant to develop CM [15], iii) *P.berghei* ANKA-C57BL/6 mice strain also act as a model to study the HCM but the brain vasculature was sequestered with the leucocytes and the cytokines involved in the pathology include TNF-α and IFN-γ [15], iv) *P. berghei* ANKA -DBA/2 mice strain combination represents neuronal pathology of ECM as like as HCM in terms of the recovery from the CM. The neuronal pathology was mild and recoverable [15], v) *P. berghei* ANKA- BALB/c × C57BL/6 mice strain will represent the model of age-dependent neuropathology i. e the development of the ECM is based on the age of the mice chosen for the experiment there are both sequestrations of the IEs and there is infiltration of the leucocytes in the microvasculature of the nervous system, IFN-γ was highly expressive in this system [15], vi) *P. berghei* K173 - C57BL/6 mice strain also stand for the model of ECM to investigate the HCM, but the neurological syndrome is due to the sequestration of the activated leucocytes and the IFN-γ involved in the disease pathology [15], vii) the combination of the *P. yoleii* (17XL) - Swiss mice strain stands for the ECM model to access the HCM pathology, neurological sequel is due to the sequestration of the IEs in the microvasculature of the brain [15], viii) the host-parasite system of the *P. yolei* (17XL) - BALB/c mice strain combination acts as a model of ECM to study the cerebral pathology of the HCM. In this combination system, the IEs were sequestered/cytoadhere to the brain capillary endothelium contributes to pathology [15]. Currently, the *P. berghei* ANKA- C57BL/6 mice model of the ECM considered as a gold standard model to investigate HCM. There are several similarities of characteristics of disease histopathology between *P. berghei* ANKA model ECM and HCM, which are as follows [10,11], i) brain hemorrhage, was observed in both of these two CM conditions as small patches in the brain due to the vascular leakage of the capillaries and venules, ii) cytoadherence/agglutination/sequestration of the IEs due to their interaction with the microvascular endothelium in both humans and mouse but the extent of the sequestration of the IEs in the mice brain microvessels in case of ECM is limited in nature, iii) occlusion and embolization of the brain microvasculature are common in both HCM and ECM, iv) sequestration of leucocytes in the neuro-capillary network of the brain occurs in both cases of the HCM and ECM but the extent of the leucocytes sequestered more in the case of murine ECM, v) necrosis of the damaged microvessels observed in the both cases of the HCM and ECM, vi) major histocompatibility complexes of class I and II highly expressive and overactive in the cerebral pathology such as CM in both mice and humans, vii) overexpression of the TNF receptors takes place in murine ECM and HCM, vii) and excessive stimulation of the cell adhesion molecules (CAMs) such as ICAM-1, VCAM-1, E-selectin and CD36 occurs predominantly in the HCM; in case of murine ECM; ICAM-1 and VCAM-1 are vastly expressed [15-19].

Avian malaria model(s): Avian *Plasmodium* can be easily transferred by the mosquito vectors whenever there are favorable conditions [20]. *Plasmodium* species are pathogenic to domestic poultry found in Africa, Asia, and South America. Clinical symptoms of avian malaria ranging from no clinical manifestations to severe malaria and death. Infections with the *P. gallinaceum*, *P. juxtranucleare*, *and P. durae* are most dangerous for the poultry birds because they produce around 90% mortality. *P. gallinaceum* infects chickens and was first described by Brumpt in 1935, formed a foremost model for the systemic study of human malaria [21-23], and governed as a screening model for the studies of the biology of the parasite, immune responses and chemotherapeutic research from 1890 until 1940 [22-24]. This malaria model has also been used in current researches [24-26].

P. gallinaceum infection in chickens represents a satisfactory model for malaria research because of the parasite is in disease pathology and severity such as causing CM [26]. In this study model mortality was proportional to the parasitemia i.e. higher parasitemia correlated to death of infected bird [26]. The body temperature of the bird was positively correlated with parasitemia; increase parasitemia, increase in the body temperature [26]. Hematocrit values were inversely proportional to the parasitemia of the infected bird [26]. The histopathology studies of brain tissue obtained from, the infected bird shown the tissue infiltration of various immune cells and occlusion of the brain microvessels as similar as of the HCM [26].

Non-human primate malaria model(s): A very close genetic and phylogenetic relationship between non-human primate (NHP) hosts and the malarial parasites which infect them, with humans and human malaria parasites makes the NHP malaria models most appropriate and very reproducible models to study various pathogenic, immunological and chemotherapeutic aspects of human malarias. Therefore, it is considered expedient and appropriate to use NHP malaria models as ECM models to mimic human HCM [9]. Though NHPs can be experimentally infected by the malaria parasites despite their natural tendency to get the infection through mosquito bites, the knowledge regarding the disease outcome in these animals remains elusive. Apparently, NHP malaria models seem to be suitable systems for ECM to study HCM; however it is difficult to study the dynamics of CM, especially the onset of the characteristic manifestations. Further, the frequency of occurrence and extent of incidence of CM in NHP malaria models have been observed to be quite diminutive and unpredictable. The acquisition, logistical, maintenance and the related cost considerations involved, coupled with ethical issues regarding their usage for research purposes and the lack of the availability of gene knock-down and knockout models makes NHP malaria models rather unsuitable/un-preferred ECM models [9]. Nevertheless, the NHP host-parasite systems appear suitable for highly effective diagnostic techniques of neuroimaging such as functional MRI etc. to study the disease pathogenesis and to know the degree of severity [27]. There are various host-parasite combinations in NHP malaria models to study the ECM. i) The *P. coatneyi* – *Macaca mullata* (rhesus monkey) shows CM complications such as sequestration of the IEs in the brain microvasculature and expression of cellular adhesion molecules such as ICAM-1, CD36, TSP etc. This model was considered to be an acceptable model to investigate HCM [28-31], ii) *P. fragile* – *Macaca mullata* shows characteristics such as rosetting of IEs and the sequestration of the IEs in the brain microvessels following CAMs are expressed and involved in the pathogenesis like; ICAM-1, CD36; TSP etc. Neurological syndrome characterized by fitting and coma [32-33], iii) *P. falciparum* – *Saimiri sciureus* (squirrel monkey) host – parasite association ECM model characterized by the rosetting of the IE and the sequestration of the IEs occurs in lungs, kidneys and other organs including brain microvessels, but the cell adhesion molecules expression was similar to the other host-parasite systems, expressed CAMs listed as follows ICAM-1, CD36, TSP etc. CM symptoms characterized by fitting and coma [34], iv) *P. knowlesi* – *Macaca mullata* ECM model of NHP malaria model was characterized by the sequestration of the IEs in the neuronal capillary network and neuronal infection characterized by the mild coma in the infected host animal [35-36], v) *P. coatneyi* – *Macaca fuscata* (Japanese macaques) model of NHP malarial model mimics HCM in terms of rosetting, sequestration, and severity of the neurological syndrome [31], vi) *P. knowlesi* - *Paplio anubis* (olive baboon) NHP malaria model has been suggested as an ECM model for HCM, having characteristic sequestration of IEs in brain microvasculature and the neurological symptoms appear during end stage infections [37], vii) *P. falciparum* is known to infect neotropical *Aotus* monkeys (owl monkeys), and causes severe disease complications and death [9].

In vitro models

In vitro models of CM are thought to be very helpful in cell-cell interaction studies *viz*. behavior of IEs, the function(s) of CAMs, the elaboration various of soluble molecules, host cell apoptosis, role of brain parenchymal cells and BBB changes in the CM. Additionally, the *in vitro* models are quite useful in striking a fine balance among the observations of autopsy, animal model and human genetic studies [38-40]. *In vitro* CM models lend additional support to the deep understanding of the different cell-cell interactions, host proteins interactions, and of various variable parasite proteins involved in the cytoadherence. Several *in vitro* models of CM have been reported using different types of cells and cell-lines, which include i) HUVEC (human umbilical vein endothelial cells), ii) HBEC (human brain endothelial cells), iii) HLEC (human lung endothelial cells), iv) HMEC (human mammary endothelial cells), v) stably transfected Chinese hamster ovary (CHO) with CD36 coding genes, vi) stably transfected Chinese hamster ovary (CHO) with ICAM-1 coding genes, vii) monkey brain microvascular endothelial cells (monkey brain MVEC), viii) mouse brain microvascular endothelial cells (mouse brain MVEC), ix) human monocytes, x) human platelets, xi) C32 amelanotic melanoma cells, xii) U937 myelomonocytic cells, xiii) BB19 (immortalized human brain capillary endothelial cell line) and xiv) retinal whole mount method [15].

Mechanisms of CM Pathology

The mechanism(s) CM pathogenesis still remain debatable. However, a few mechanisms have been proposed which include following three hypotheses.

Sequestration hypothesis

In early 1894, in order to explain CM pathogenesis, marchiafava and bignami hypothesized that during *P. falciparum* malaria, IEs sequestered deeply inside the brain microvasculature causing the vascular blockade, hypoxia, hypoglycemia and accumulation of the toxic products such as lactic acid resulting in confusion, convulsions, coma and ultimately death [41,42]. The accumulation of the lactic acid leads to lactic acidosis in the CM; due to hypoperfusion of the particular brain tissue, tissue generates its energy needs through anaerobic respiration. Though this contention appeared to support sequestration hypothesis, the exact mechanism of hyperlactatemia in CM appear to be quite complicated and need not to be linked to anaerobic respiration [43]. Elevated lactate levels seem to play a significant role in CM pathogenesis [44] can be correlated with;

i) decreased oxygen supply, ii) alterations in the redox status, iii) convulsions and iv) may be due to decreased clearance by the liver [45]. *P. falciparum* infection causes structural and functional alterations in both uninfected and infected erythrocytes, hindering their movement in brain capillaries of 3-7 micron diameter; the rigid RBCs of diameter 7.5 microns will occlude the capillaries and resulting in the mechanical obstruction of the brain capillaries [46]. The integration of the parasitic proteins in the RBC membrane results in the increased rigidity of the membrane of IEs [47]. The uninfected erythrocyte rigidity is due to the excessive oxidative stress [46]. In ECM, there appears to be an inverse relationship between capillary occlusion and the concentration of the functional capillaries [48]. The sequestration hypothesis thus appears to be based on the proportionality of levels of parasitemia and the degree of obstruction of the brain microvasculature. However, on the contrary, no such clinical relationship between the higher parasitemia and the mortality has been reported. It should be noted here that to observe sequestration is not a practical possibility in postmortem studies of each and every *P. falciparum* malaria patient [48,49].

Inflammation hypothesis

In 1948, Brian Maegraith proposed the involvement of inflammatory mediators in systemic inflammatory reaction to malaria pathogenesis leading to failure of vital organs and death [50]. The malaria toxins, which mainly contain glycophosphoinositol, are involved in causing disturbance in the balance of the pro-inflammatory/anti-inflammatory mediators. The increase in oxidative damage is due to the excessive production of the superoxide and nitric oxide (NO) molecules [51]. The so generated pro-inflammatory cytokines such as TNF-α involved in the enhancement of the turnover of the endothelial cell adhesion molecules (eCAMs) such as ICAM-1, one of the key molecules involved in CM pathogenesis. The regulating cytokines for the production of the eCAMs are specific to particular adhesion molecule and to a particular tissue [19]. Experimental proofs have been generated by using the knockout models which suggest the association of immune activated inflammatory cascade and the progression of the CM pathogenesis [52]. The presence of high levels of inflammatory mediators in *P. vivax* malaria indicates that though the participation of the inflammatory cytokines required, but not enough for CM pathogenesis. The outcomes from the clinical trials involving the anti-inflammatory therapy including the antibody (anti-TNF-α monoclonal antibody), pentoxifylline (synthesis inhibitor) and corticosteroid (dexamethasone) have shown that anti-inflammatory therapy seem to be unable to provide the protection from the disease pathology. Moreover, there are some irrational observations during clinical trials [53,54]. The high NO production is also thought to play a role in the CM pathogenesis, which in turn supports, the inflammatory hypothesis. The pleiotropic functions of NO are also being considered to be responsible for pathogenesis of CM [55-61]. The heme released during rupture of the IEs reacts with NO and scavenges it, and thus makes it less available for biological functions [62,63]. Free NO is quenched by superoxide ion due to rigorous oxidative stress in the malignant *P. falciparum* infections [64-66].

Hemostasis hypothesis

The presence of small patches of hemorrhages in the brain, retinal hemorrhages, and vascular leaks are signs of the hemostatic dysfunction in CM. *P. falciparum* malaria patient's abnormalities such as prolonged bleeding, prolonged prothrombin, partial prothrombin times and presence of coagulopathy have been observed, and point out the faults in the factors V, VII, and IX required for the coagulation [67,68]. The low concentrations of anticoagulant proteins and C-reactive protein have been observed in *P. falciparum* malaria [69,70]. Platelets are chief effector cells of hemostasis system and associated with pathogenesis of CM. Immune activated platelets release chemokines, cytokines and other immune modulator molecules from their cytoplasmic granules [71]. Thrombocytopenia and its extent have always been linked to the pathogenesis of the CM [72-74]. Certainly, thrombocytopenia enhances the probability of the bleeding. The platelet adhesion to the endothelium of the brain microvasculature contributes to the occurrence of CM, similarly to the sequestration of the IEs. Further, platelet microparticles may also mediate between endothelium and leukocytes [75]. Microparticles of EC origin are found extensively in the patients with *P. falciparum* infection, can be linked with the progression of the pathology [76,77]. It is important to note here that no single hypothesis can explain the mechanisms of the CM pathogenesis; apparently, at best, these proposed hypothetical mechanisms seem to be dependent on each other and are not exclusive. Extensive studies to unravel the underlying mechanisms are very much warranted.

Factors Influencing the Outcome of CM

A large number of factors can be surmised which influence, directly and/or indirectly, the onset, severity, and outcome in ECM models and HCM. These factors may influence CM both to detriment (aggravate the disease) or benefit (resolve the disease) of the host. These factors may helpful for the parasite in its efforts to evade the mounting immunological onslaught of the host.

Cell adhesion molecules (CAMs)

Cell adhesion molecules are major factors which influence the sequestration of IEs, adhesion of the immune cells and platelets to the endothelium of the brain capillaries. The elevated CAM levels are thought to be augment the communication between the attracted immune cells and endothelium, and are also considered to be instrumental in plugging the leakage in cerebral capillaries [19,78]. ICAM-1, CD36 are prominent among the CAMs act as ligands for the *Pf*EMP-1 protein on the surface of *P. falciparum* IEs; mediates the sequestration and adhesion of the IEs to the endothelium of the brain capillaries. Increased IEs sequestration ensues in vascular occlusion, reduced flow of blood, hypoxia, and some other characteristic features (acidosis, hypoxia, and ischemia) of HCM [19,78,79]. Sequestration thus causes damage to the host endothelium, and leads to the apoptosis of the EC of brain microvasculature which may results in vascular leakage, ultimately the dysfunction of the BBB [19]. The elevated CAM level-induced amplification of sequestration may augment elaboration of inflammatory cytokines, and chemokines from

the EC, which, in turn, may trigger uncontrolled inflammatory cascade causing additional damage to the BBB [80]. Significantly increased levels of ICAM-1 expression, and TNF-α and IL-β levels especially in the cerebellum, have been observed in the brain tissues of children who died of CM [81]. Further, the role of ICAM-1 has been documented in the development of the CM. ICAM-1 deficient mice display resistance to the CM, showed diminished levels of serum TNF-α, lack of immune cell sequestration in brain microvasculature, and no or reduced damage to the BBB when compared to their wild-type littermates [82]. These observations suggest that the CAMs are detrimental in CM.

Cytokines

Cytokines are low molecular weight glycoprotein molecules, regulators of the host response to infection, inflammation and immune response and several other processes. Whereas the induction and involvement of pro-inflammatory cytokines ensues in pathological conditions which are detrimental to host, anti-inflammatory cytokines involved in the reduction of inflammation and augmentation of healing process. Thus, during infection and inflammatory conditions, anti-inflammatory cytokines play protective role(s), pro-inflammatory cytokines aggravate both infection and disease progression. Nevertheless, in *P. falciparum* malaria patients, both pro- and anti-inflammatory cytokines play their specific role(s) both in the onset and progression of CM, and also influence the end result of CM [83].

TNF-α

Tumor necrosis factor- α (TNF-α, catechin) is an important pro-inflammatory Th_1 cytokine which is involved in several biological functions and is extensively involved inflammation. There are several lines of evidence which support the role of TNF-α in the pathogenesis of the CM [83]. Elevated levels of TNF-α was recorded in several cases of CM, and administration of the anti-TNF-α antibody has been reported to mitigate neuronal symptoms and pathology [83]. Elevated levels of soluble forms of $TNFR_1$ and $TNFR_2$ are found in the plasma samples of both adult and paediatric patients [84], and also in the case of murine CM [85]. $TNFR_2$ has proven the potential to cause CM pathology because the $TNFR_2$ gene knockout and not the $TNFR_1$ knockout mice were protected from the CM pathology [85]. The elevated expression of the $TNFR_2$ has been reported in the brain endothelium of the Malawian children with fatal malaria [86].

IFN-γ

The interferons (IFNs) were discovered because of their property to interference with the viral replication [87]. IFNs were categorised into two groups i) type I and ii) type II based on their receptor pharmacology, specificity and sequence homology. The type I interferons includes IFN-α, IFN-β, IFN-ω and IFN-τ. IFN-γ is the only type II interferon and it phenotypically dissimilar to the type I interferons [88]. IFN-γ acts in autocrine or paracrine manner, locally [89]. IFN-γ has immunomodulatory functions, and its production is regulated by the cytokines IL-12 and IL-18 [90]. In murine ECM, IFN-γ has been observed to be related with mortality and disease pathology, and antibodies against the IFN-γ are known to protect the mice from mortality and the CM development. Mice knockout for both IFN-γ and IFN-γR have been found defiant to develop CM [79,91]. Acute malaria patients from both South East Asia [92] and African regions [93] have been reported to have high IFN-γ plasma levels. The Gambians, heterozygous for the IFN receptor polymorphism, show low probability of developing CM and its related mortality [94]. Thus observations from both human and murine malaria studies strongly suggest the role of IFN-γ in the CM pathogenesis.

IL-6

IL-6 is an anti-inflammatory cytokine involved in host immune responses, acute inflammatory reactions and hematopoiesis [95]. Significant increase in the serum IL-6 levels has been observed *P. berghei* ANKA infected mice, with or without CM involvement. Severe *P. falciparum* malaria patients also show elevated IL-6 levels [96]. IL-6 has been reported to be involved in the stimulation of the polyclonal B cells, in severe malaria patients [97]. Contrastingly, there are evidences which suggest that IL-6 has nothing to do with the pathogenesis of murine CM [98] There is no deviation of the expression of IL-6 mRNA levels in both CM vulnerable and CM defiant infections of *P. berghei* ANKA [99]. The administration of IL-6 neutralizing antibodies during murine CM did not protect the mice from the disease symptoms and associated mortality, which demonstrates that IL-6 is not invovled in CM [98].

IL-10

IL-10 is known to downregulate the production of TNF-α and IL-1 [100]. In malaria, IL-10 has been shown to have some protective roles. The exogeneous administration of IL-10 has been shown to protect against the development of CM in susceptible *P. berghei* ANKA infected mice; however, paradoxically, in mice resistant to *P. berghei* ANKA infection, administration of neutralising antibody lead to development of CM pathology [101]. Human malaria studies indicate the host protective activity of the IL-10, as shown by the low levels of IL-10 in CM patients as compared to that in uncomplicated malaria [102]. Further, IL-10 seems to protect against CM pathology by restraining the production of TNF-α and GM-CSF, both of which considered responsible for the CM complications [83,103]. Additionally, IL-10 exerts protection against CM by blocking the production of the several chemokines [104], which attract monocytes and $CD8^+$ T cells responsible for the blockade of cerebral microvasculature during CM [79, 105-107]. IL-10 thus appears to be a host protective cytokine during the CM pathogenesis.

IL-1

IL-1 is a pro-inflammatory cytokine, a principal mediator in the acute inflammatory response, and potential mediator of tissue malfunction and devastation. IL-1 is responsible for mediating neuroinflammation and neurodegeneration [108]. IL-1 plays a highly critical role in the production of the Th17 cells from the T cell population [109]. The role of IL-1 in the CM pathogenesis is quite complicated. The administration of the low doses of IL-1 offered protection against lethal CM [110]. Treatment with recombinant IL-1 receptor antagonist inhibited the progression of infection, and averted the development of the CM complications [97].

IL-4

IL-4 is involved in I_g isotype switching, expression of major histo-compatability complex class II by B cells and the differentiation of of T cell subsets [111]. Th_2 cell activation seems important for the clearance of the late stage malaria parasites, and IL-4 activation thus appears to protect against malaria, but even IL-4 scarce mice are able to clear the primary infections of *P. chaubadi* [112]. Addition of recombinant human IL-4 to the *in vitro* culture of the *P. falciparum* inhibited macrophage killing of parasite, thus helped the parasite to escape the immune system [113]. There is decreased expression of IL-4 mRNA in the brains of the mice with CM, which indicates the protective role of IL-4 during CM pathology [99]. Nevertheless, uncertainty continues regarding the role of the IL-4 in the pathogenesis, and regulation of the outcome of the CM.

Vasomodulatory agents

The pathophysiological changes of the CM are associated with the brain microvasculature, thus the recent research regarding the CM primarily focused on the endogenous Vasomodulatory agents such as endothelin-1 and NO.

Endothelin-1

Endothelin-1 (ET-1) is a potent vasoconstrictor secreted by the endothelial cells of the vasculature. The increased ET-1 levels have been observed in the brains of *P. berghei* ANKA-infected mice [114], which are associated with cerebral hypoperfusion and immunopathology of CM [114, 115]. The vasculopathy of CM is mediated by the ET-1, through its action on the endothelin receptor type A (ETA) [116]. The ETA antagonism decreased the haemorrhage and vascular occlusion during CM, and enhanced the survival rate of mice given artemisinin supportive therapy [116]. The IE sequestration-induced endothelium over stress causes noticebale raise in ET-1 levels, and up-regulates the CAM expression [114]. The CAM up-regulation triggers the cerebral sequestration of the IEs, monocytes and platelets and other immune cells, which results in the vascular occlusion, cerebral hypoperfusion, hypoxia, ultimately leads to ischemia and death [117]. Thus it can be concluded that the hyper-expression of the ET-1 is detrimental in CM.

Nitric oxide

The activities of nitric oxide (NO) include immunomodulatory, vasomodulatory, signal transduction and cell growth regulatory functions [118]. The role of NO in CM is matter of conjecture and incongruity, as both up- and down-regulation in NO levels cause damaging effect of CM. Increased NO levels result in higher intracranial pressure, interference in the neuronal signal transmission and weight loss [119]. On the other hand, decreased NO levels results in endothelial damage and dysfunction. Decrease in NO levels during severe malaria includes hypoarginemia and NO quenching [120,122]. Decreased NO levels lead to CM, haemorrhages, and vessel collapse [16,80,114,120]. Curiously, in both HCM and murine CM, arginine treatment restored the NO levels, reorganised endothelial function, and thus normalized BBB functions [120-124]. In *P. gallinaceum*-infected chickens, treatment with amino guanidine, an NO synthesis inhibitor, increased resistance to infection due to reduced inflammation, anaemia and thrombocytopenia [125].

Prostaglandins

Prostaglandins (PG) are generated by the action of cyclic oxygenases (COX) on the arachidonic acid (AA), and are involved in macrophage stimulation, vascular integration, erythropoiesis, and fever, and pro-inflammatory responses to the infection [126]. The children having malaria-induced severe malaria have concealed bicyclo PGE_2 plasma levels and repressed leukocyte COX-2 gene [126]. Suppressed bicyclo PGE_2 levels lead to severe malaria and CM [126]. Aspirin treatment of ICR mice during ECM aggravated the mortality rate due to inhibition of the PG synthesis [127]. These observations indicate a protective role of PG in CM.

Leukotrienes

Leukotrienes (LT) have an important role in innate and adaptive immune system responses, and are implicated in several inflammatory and pathological conditions, and infectious diseases [128,129]. Cysteine Leukotrienes are responsible for the vascular permeability and edema and the expression of the adhesion molecules and NO production [128]. In *P. berghei* ANKA-induced murine ECM, elevated serum LTB4 levels have been observed, and aspirin treatment drives the AA metabolism towards the 5-LOX enzyme [130], leading to rapid rise in parasitemia and aggravated mortality during CM [127]. LTB4 is a potent inducer of Th_1 cytokines such as IFN-γ [131] overproduction of this Th1 cytokine-associated with the severity of CM pathogenesis. LT may thus have a deleterious role(s) in the CM.

Glycophosphatidylinositol

Glycophosphatidylinositol (GPI), a ubiquitous molecule, is present in outer cell membrane of all eukaryotes. Studies related to *In vivo* and *in-vitro* studies suggest a potential role(s) of *Plasmodium* GPIs in pathogenesis and severity of the disease. Interaction of *P. berghei* and *P. yoelli* IEs with macrophages, *in vitro*, ensued in the elaboration of TNF-α [132], and similar results were obtained with human monocytes with the *P. falciparum* IEs [133]. Purified GPIs, following injection in thioglycolate-primed mice, caused acute malaria manifestations such as fever and hypoglycemia [134], similar results were observed in unprimed mice [135]. Neutralisation of the *Plasmodium* GPIs with monoclonal antibodies nullified the TNF-α production induced by the whole parasite extracts *in vitro* [136]. *P. falciparum* GPIs cause the elevation of the endothelial cell expression of the ICAM-1, VCAM-1 and E-selectin via TNF-α and IL-1[137]. GPIs of *Plasmodium* appear to cause pathogenesis of CM.

Hemozoin

Hemozoin (Hz), the malaria pigment, is a metabolic waste product produced due to digestive catabolism of the haemoglobin in IEs by parasite [138]. The Hz taken-up by phagocytic cells via phagocytosis of the IEs or free Hz released after schizont rupture [139]. Hz thus released alters the cellular metabolism through generation of free radical lipid peroxides from the AA

[140]. Phagocytic up-take of the Hz by circulating neutrophils and macrophages has been linked to the severity of the malaria [141,142]. The Hz formation has also been linked with severe malarial anemia [143], immunosuppression [139], and cytokine dysregulation [144]. Cytokine dysregulation leads to the alterations in the balance of the pro/anti-inflammatory cytokines, which results in aggravating CM during *P. falciparum* malaria.

Alterations in signalling and biological pathways

In severe malaria, parasite-induced disturbances in the host homoeostasis system lead to the alterations in the signalling and biological pathways, which in turn, badly influence the pathophysiology of the CM.

Kynurenine pathway of tryptophan metabolism

Currently, there appears to be sufficient information which supports the view that during HCM and Murine CM, the kynurenine pathway of tryptophan metabolism is stimulated. The Indoleamine oxygenase (IDO), rate-limiting enzyme of the pathway, has been reported to be up-regulated in the brains of mice suffering from CM [145]. Excessive stimulation of the enzyme IDO alters the ratio of the neuroprotective metabolite kynurenic acid (KA) and neuro-excitotoxic metabolite quinolinic acid (QA), and accumulation of QA is known to occur during CM, which leads to neuronal damage [145]. The heightened QA levels have been observed in the cerebrospinal fluid of adult human CM [146], and paediatric CM patients [147]. Murine CM studies, therefore, suggest that inhibition of the Kynurenine pathway extended the survival of the treatment mice three-times longer, as compared to the untreated ones [148].

Angiopoietin-Tie 2 signalling pathway

The CM pathology, mainly involves the structural and functional alterations in cerebral microvasculature, both of which can be maintained by factors such as NO, ET-1 etc. The angiopoietin-Tie 2 signalling pathway is one of the regulators of the microvasculature of the brain. Basically angiopoietin-1 (Ang-1) interacts with EC tie 2, and controls the stimulation of the endothelium and maintains the survival of the ECs [18]. Paradoxically, angiopoietin-2 (Ang-2) functions just the other way round of Ang-1[18]. Ang-1/Ang-2 ratio change towards Ang-2 causes sensitization of the endothelium, which results in increased generation of the CAMs, and thus, further aggravates the CM complications [149,150].

Rho-kinase signalling pathway

The BBB integrity is maintained by tight junctions and adherent junctions, which are associated with small GTPase protein rho. Stimulated rho A/rho kinase pathway results in the endothelial dysfunction [151]. Fasudil, a rho-kinase inhibitor, reduced the EC apoptosis, NF-κB activation [152], and prolonged the survival, and can thwart the development of the CM in murine ECM [153].

Toll-like receptor signalling pathway

Toll-like receptors (TLRs) play major role(s) in the generation of primary innate immune responses against the pathogens. TLRs stimulation also activates the production of IFNs, and secretion of Th1 and Th2 cytokines [154]. Most of the TLRs intracellular signalling is MyD88 adaptor molecule dependent [155]. Pro-inflammatory responses to the malaria parasites mediated through the TLR4, TLR9 and adaptor molecule MyD88, after interaction between antigen presenting cell-dendritic cell and *P. berghei*/*P. chabaudi* IEs [156]. TLR2 and TLR9 receptors are involved in the pathogenesis of CM, they aggravate CM related mortality [155].

Heme/Heme oxygenase-1 signalling pathway

Heme oxygenase (HO-1) degrades free heme, which ensues in the formation of bile pigment, biliverdin, free iron, and carbon monoxide (CO) [157]. The heightened HO-1 levels during oxidative stress, exposure to UV rays, heavy metal toxicity and inflammatory pathological conditions, contribute to defence against deleterious effects of these factors. Excessive free heme due to severe hemolysis of IEs and uninfected RBCs during malaria, leads to inflammation and contributes cerebral pathology [158-160]. Recent ECM studies *P. berghei* ANKA show that upregulation of HO-1 enzyme levels and its activity protected the host from the developing ECM. Crossing out of HO-1 and inhibition of its activity augmented cerebral pathology by 83% and 78% respectively [159]. Taken together, these observations, in no uncertain terms, suggest the protective role of enzyme HO-1 during CM.

Biomarkers of CM

So far, there are no explicit biomarkers for the diagnosis of the HCM or ECM, but some recent studies have recognised some serological factors to have the potential to function as biomarkers. It is believed that identification and application of potential practical biomarkers/biosignatures will go a long way in the diagnosis and prognosis and for the therapeutic assessment of a drug or vaccine for CM. In order to identify the biomarkers for the particular disease, it is necessary to have a deep knowledge of disease pathogenesis and of pathophysiological changes during the progression of the disease. However, the pathogenesis of the CM still remains to be properly understood and expounded. Some recent studies in Ghana [161] and India [162] have exposed the remarkable relationship between chemokine interferon-inducible protein-10 (CXCL10) and CM severity, which proposes the potential of the CXCL10 to be used as a practical biomarker for the assessment or quantification of the severity of the CM. Further, there are several serological factors whose concentrations vary, which can potentially be used to discriminate CM for non-CM conditions. These serological factors include elevated plasma/serum levels of the CXCL10, sFas, sTNF-R2 [161, 162], IL-8, IL-1ra [163], and diminished levels of RANTES [164] and vascular endothelial growth factor (VEGF) in CM patients. Till date, only the endothelial regulators Ang-1 and Ang-2 [150,165], and chemokine CXCL10 [166] have been evaluated for their potential to diagnose CM. Erythropoietin

circulating levels also have been thought of as a potential prognostic biomarker for CM; higher levels are associated with 70% less chances of developing neurological sequelae, whereas low levels indicate risk of neurological involvement [167-169]. Increased CXCL10 levels in CSF and peripheral serum samples of CM patients who have died, compared to that in the survivors, suggests its potential to be a prognostic biomarker for CM. Thus all these factors can potentially be developed as diagnostic biomarkers/ biosignatures for CM.

Acknowledgment

We are grateful to Prof. Raghu Ramarao Akkinepalli, Director, National Institute of Pharmaceutical Education and Research (NIPER), for his help and encouragement. Mr. Bhanu Prakash is grateful to the NIPER for the award of a Senior Research Fellowship. This is NIPER communication No. 499.

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