Chapter 4
Role of White Blood Cells in Immunopathogenesis of Cerebral Malaria

Ruchika Saroa and Upma Bagai

Abstract Cerebral malaria (CM) is a fatal complication caused by *Plasmodium falciparum* in humans claiming two million lives annually. The pathogenesis of this disease yet remains partially understood. Various studies have been carried out to understand the exact processes of CM which indicate towards the involvement of the immune response in the neurological complications. It has been hypothesized that CM occurs due to the over-vigorous immune response which originally evolved for the protection of the host against malaria. Some studies also examined immune-pathological responses occurring during CM and focused on reactions being carried out primarily in the systemic circulation. But these findings are not able to fully account for the development of neurological complications in malaria. There are multiple mechanisms which are involved in the induction of cerebral complications which contribute to the pathogenesis of CM. In the present study, results from human and mouse model demonstrating the contribution of various cells and cytokines in the development of CM and neurological complications have been summarized.

Keywords Cerebral malaria · Cytokines · Malaria · T cells

4.1 Introduction

Malaria, Tuberculosis and Acquired immunodeficiency syndrome (AIDS) are three most deadly diseases across the world causing millions of deaths every year. *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* are five species of malaria parasites infecting humans. Among these species *P. falciparum* causes malignant malaria and is responsible for almost all malaria deaths worldwide (Kang et al. 2010).

In India, maximum mortality occurs in Orissa, West Bengal, Jharkhand and the central states of Chhattisgarh, Madhya Pradesh, Gujarat, Karnataka and Rajasthan.

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P. falciparum infections alone are responsible for 88% of deaths which mark it as the main target for vaccine trials of the different initiatives and eradication programmes launched against malaria. In endemic areas, newborns are resistant to malaria due to the effect of mother's antibodies transferred through the placenta. When this immunity declines, the risk of contracting the disease increases. Therefore, young children are at high risk of malaria until they have developed their own protective immunity (Das et al. 2012).

Major problem in malaria control today is the increasing resistance of *P. falciparum* to various antimalarial drugs, lack of cost-effective drugs and non-availability of suitable vaccine against deadly parasite. Severe malarial anaemia, metabolic acidosis, placental-associated malaria and cerebral malaria are various complications associated with this disease (Medana et al. 2001a).

4.2 Life Cycle of *P. falciparum*

Anopheles mosquitoes are vectors of *P. falciparum*. Infected mosquito injects sporozoites into human skin during blood meal which travel to the liver for invading hepatocytes. Within 2 days, one merozoite transforms into a trophozoite and then into a schizont (Siciliano and Alano 2015).

After tissue schizogony, merozoites invade red blood cells (RBCs), and erythrocytic schizogony takes place. Inside the host, *P. falciparum*-infected red blood cells escape from immune surveillance and send adhesive proteins to the host's cell membrane. These proteins make the cells adhere to small blood vessels, which pose a threat to the human host since the clustered red blood cells create a blockage in the circulation system especially in the brain. Some schizonts develop into male and female gametes which are ingested by a mosquito when it feeds on infected blood (Fig. 4.1).

Inside the mosquito's midgut, male and female gametocytes fuse and develop into ookinete. The motile ookinetes penetrate the midgut wall and release sporozoites, which migrate to the salivary glands from where they are injected into humans during the next blood meal (Fig. 4.1; Siciliano and Alano 2015).

4.3 Signs and Symptoms of Cerebral Malaria

The major clinical symptoms of malaria include fever, malaise, splenomegaly, anaemia, convulsions, muscle pain and bloody stool due to the cyclic multiplication of the parasite into RBCs. During *P. falciparum* infection, the spectrum of severe pathology is broad and includes metabolic acidosis, cerebral malaria (CM) and severe malaria anaemia (SMA) accompanied by hypoxia, hypoglycaemia, lactic acidosis and multi-organ failure which may result in coma and death (McCall and Sauerwein 2010).

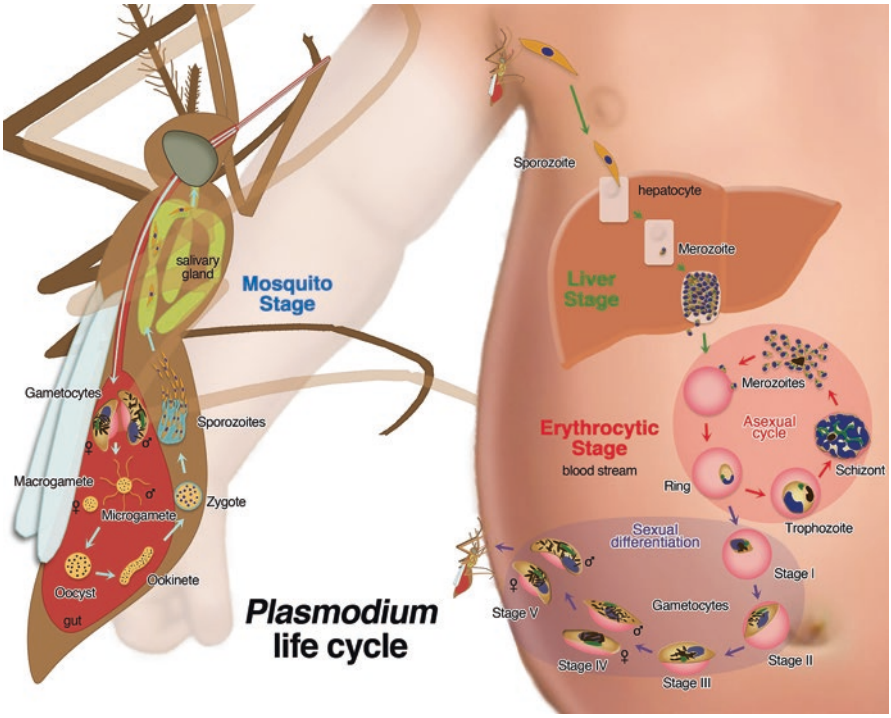

Fig. 4.1 Pictorial representation of the life cycle and different development stages of *P. falciparum* inside humans as well as mosquitos. (Source: Le Roch Laboratory, University of California, Riverside)

Fig. 4.2 African child suffering from cerebral malaria. (Source: Larry Johnson and Mike Urban: Insecticide-resistant mosquitoes challenging Gates malaria efforts)

The clinical hallmark of cerebral malaria is impaired consciousness with coma being the most severe manifestation. Patients may develop coma following progressive weakness, brain swelling, intracranial hypertension and retinal changes (Figs. 4.2 and 4.3; Idro et al. 2010).

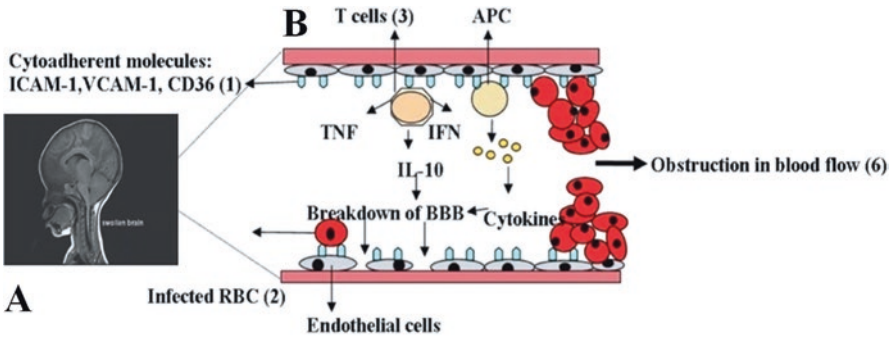

Fig. 4.3 (**a**) Magnetic resonance imaging (MRI) of child who died due to cerebral malaria (Left) having swelling in the brain (Source: Dr. Terrie Taylor from Michigan State University holds a child in the Queen Elizabeth Hospital in Blantyre, Malawi. Photo by Jim Peck, MSU.). (**b**) A hypothetical explanation of infected red blood cells (IRBCs) and leukocytes adherence to the endothelial cells via *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) mediated parasite adhesion by Intercellular adhesion molecule (ICAM-1) leading to attachment of *Plasmodium*-infected red blood cells to endothelial cells of the brain (2), adhered lymphocytes and APCs (3) release inflammatory cytokines (TNF-α, IL-10) (4) which can lead to breakdown of blood-brain barrier (BBB) permeability (5) allowing leakage of plasma proteins into the perivascular space and obstruction in blood flow (6) aggravated by platelets due to release of fibrinogen resulting in neurological syndrome

4.4 Diagnosis of the Disease

Various techniques are available for the diagnosis of malaria: Giemsa-stained blood smears, microhaematocrit centrifugation, fluorescent dyes, polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA) and ParaSight F test (dipstick test). Correct and well-timed treatments are important in malaria which require quick and valid diagnosis. Therefore, dipstick tests are the most important diagnostic tools which are rapid to perform and easy to read by even untrained persons. The most superior and sensitive method is histidine-rich protein II (HRP-2)-based rapid diagnostic test RDT. Some serological assays are also available for malaria detection including indirect immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA) (Batwala et al. 2010).

4.5 Mechanism of Cerebral Malaria

The key feature of this multifaceted, deadly manifestation is the cytoadherence of *Plasmodium falciparum* parasitized erythrocytes (PE) to endothelial cells (EC) and their sequestration in the cerebral microvasculature of the infected host. It has been reported that schizont-infected red blood cells block the brain capillaries causing obstruction in blood flow. Infected erythrocytes also adhere to normal erythrocytes

(rosetting) and to other infected RBCs (autoagglutination) (Fig. 4.3). Vessels in the brain clogged with infected and normal RBCs result in insufficient supply of oxygen and nutrients to the brain leading to coma and death (Renia et al. 2006). Intravascular leukocyte sequestration has also been observed within brain venules from patients who died of CM (White et al. 2010).

4.6 Murine Model of Cerebral Malaria

P. yoelii and *P. berghei* ANKA (PbA) are the main sources of cerebral syndromes in rodent host which have various similarities to the human conditions. *P. berghei* ANKA infected murine erythrocytes sequester in the microvasculature of various organs via CD36 adhesion molecule, but sequestration is more in the lungs than in the brain (Engwerda et al. 2005).

A comparison within the types of immune responses generated in Balb/C mice and C57BL/6 mice infected with PbA led to the conclusion that Balb/C strain of mouse is resistant to the neurological conditions. So, the most widely used model for cerebral malaria is PbA infection in CBA or C57BL/6 mice (Franke-Fayard et al. 2005).

In human cerebral malaria, it is mainly infected RBCs that attach to the cerebral microvascular endothelium, whereas in rodent CM, it is chiefly leukocytes which exhibit adherence (Berendt et al. 1994).

4.7 Role of Adhesion Molecules in Cerebral Malaria

Sequestration of iRBCs into the brain microvasculature and other tissues is mediated by various receptors which are present on endothelial cells of venules and capillaries inside the host. These receptors include adhesion molecules such as CD36, intercellular adhesion molecule (ICAM)-1 and CD31, thrombospondin and chondroitin sulphate A (CSA) (Berendt et al. 1994).

Immunohistochemical staining of *P. berghei* ANKA infected host showed a strong ICAM-1 expression on vessels of the brain containing iRBCs (Silamut et al. 1999). There are many molecules which act as endothelial receptors for lymphocyte adhesion during inflammation and immune surveillance. The ligands for attachment of infected RBCs are produced by the *Plasmodium,* and the best is PfEMP-1 (*P. falciparum* erythrocyte membrane protein) (Favre et al. 1999). The parasite exports and inserts PfEMP-1 into the erythrocyte membrane at adhesive foci or knobs. Variants of PfEMP-1 expressed by different parasite stains have different binding affinities for the various endothelial receptors and influence the cell sequestration, e.g. CSA-binding parasites are implicated in placental malaria, whereas, ICAM-1 binding is important in CM (Favre et al. 1999).

PfEMP-1 is the major receptor for parasite-host interactions and is expressed on the surface of the parasitized RBCs. This is encoded by a multigene family of up to 60 var genes. Antigenic variations allow the parasite to evade the host's immune system promoting PfEMP-1 binding to a range of ligands including ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), P-selectin, CD36, thrombospondin (TSP) and CSA. ICAM-1 on endothelial cells is markedly increased in response to various inflammatory mediators including pro-inflammatory cytokines such as TNF-α (tumour necrosis factor) and IL-1β (interleukin) (Chakravorty and Craig 2005).

Interaction of lymphocytes with ICAM-1 is mediated via receptors such as LFA-1 and Mac-1 which have a major role in the recruitment of various cells and their migration to tissues (Gardner et al. 2002). There seems to be a synergistic interaction between ICAM-1 and CD36 to bind parasitized RBCs because the blocking antibodies against either of the molecules reduce the binding (McCormick et al. 1997).

Histoarchitectural studies of infected rodent brain having CM showed sequestration of leukocytes into the cerebral parenchyma and PE accumulation intravascularly. The main brain-sequestered leukocytes are macrophages, T cells, few dendritic cells and natural killer (NK) cells (Schmutzhard et al. 2011).

4.8 T Helper and T Cytotoxic Cells

T cells consist of various sets of cells ($CD4^+$ and $CD8^+$ T cells) which fight against infection by secreting various cytokines and chemokines. $CD4^+$ T cells have been found to be crucial in malaria immunity. They not only act as helper cells for secreting antibodies but also act as effector cells in killing parasites. It is executed by secreting inflammatory cytokines and activating other cells like macrophages. They are also known as the major source of interferon-γ (IFN-γ) and TNF-α (Muxel et al. 2011) which are required for protection against the disease. Both induce nitric oxide synthase expression in the spleen to control parasite load, generate good antiparasitic response and mediate protection (Perez-Mazliah and Langhorne 2014).

In our laboratory, during lethal NK-65 infection in rodent host, an increase was observed in splenic $CD4^+$ and $CD8^+$ T-cell population during initial days of infection which declined with increase in parasite load. Whereas, mice immunized with parasite constituents exhibited a strong Th1/Th2 immune response and expansion of $CD4^+$ and $CD8^+$ T cells significantly leading to the clearance of the parasite from the host upon challenge (Kumar et al. 2015).

During cerebral complications in Anka-infected rodent host, a significant increase was observed in $CD4^+$ T-cell population leading to the secretion of IFN-γ and TNF-α in the brain which are responsible for the breaching of blood-brain barrier (Belnoue et al. 2002). $CD8^+$ T cells are cytotoxic cells which recognize pathogen via MHC-I molecule and destroy infected cells. During malaria, $CD8^+$ T cells are responsible for providing cell-mediated immunity against asexual stages of

parasite in host (Belnoue et al. 2002). An elevated number of $CD8^+$ T cells were observed during self-clearing *P. chabaudi* infection, whereas animals depleted of these cells exhibited a significant delay in clearance of the parasite (Helmby et al. 2000).

Cytotoxic cells play a major role in the development of neurological complications. Flow cytometric analysis of leukocytes of the brain in mice having CM showed a selective increase in sequestered $CD8^+\alpha\beta$ T cells. This theory was confirmed by the transfer of splenic $CD8^+$ T cells from mice with CM into RAG-2-deficient mice. It was reported that these transferred cells migrate to the brain and induce neurological symptoms (Pais and Chatterjee 2005). A significant proportion of the $CD8^+$ T cells involved in CM development express the Vβ8.1,2 T-cell receptor (Claser et al. 2011).

In our laboratory, higher $CD8^+$ T-cell count was observed in *P. berghei* ANKA infected rodent host's brain with increase in infection as compared to the spleen. It points to the recruitment of cytotoxic $CD8^+$ T cells to the brain microvasculature with increase in parasitaemia (unpublished data).

Recruitment of $CD4^+$ T helper 1 (Th1) in infected host leads to generation of protective immune response and high levels of IFN-γ and pro-inflammatory cytokines like TNF and LT-α which are the major causes of cerebral complications (Hansen et al. 2003). Stimulated cytotoxic cells expand in the brain during the disease. Perforin-dependent cytotoxic mechanism and brain macrophages may be involved in the induction of neurological complications due to cerebral malaria (Hunt and Grau 2003).

4.9 Monocytes/Macrophages and Dendritic Cells

Macrophages recognize microbial product or pathogen via toll-like receptors leading to acute activation of these cells associated with the release of pro-inflammatory cytokines and chemokines (Chua et al. 2013).

Monocytes, dendritic cells and macrophages perform important roles during malaria infection in humans as well as in rodents. Monocytes are formed from haematopoietic lineage in the bone marrow and are released into the bloodstream upon maturation. Their further differentiation depends upon the downstream signals which trigger them to change into dendritic cells or tissue macrophages (Claser et al. 2011).

During *Plasmodium* infection, they are capable of reducing the parasite load in the blood via antibody-dependent cell inhibition (ADCI) which requires the acquisition of antibodies (IgG1 and IgG3 subtypes) against merozoite surface protein-1 (MSP-1) and glutamate-rich protein (GLURP). Antibody-opsonized merozoites further release the soluble mediators for the inhibition of parasite growth and multiplication (Pratt-Riccio et al. 2011).

It has been reported that macrophages are involved in complement-mediated phagocytosis which is necessary for providing protection against asexual stages of the parasite via complement receptor 1 (CR1 or CD35) (Silver et al. 2010).

Patients suffering from cerebral complications reported activation of platelet endothelial cell adhesion molecule -1 (PECAM-1). Activated platelets secrete various chemotactic molecules due to which the recruitment as well as retention of monocytes/macrophages is enhanced leading to complete occlusion of brain capillaries. These monocyte/macrophage sequestration together with attached parasitized RBCs ultimately lead to mechanical blockage of brain microvasculature (Dorovini-Zis et al. 2011).

Post-mortem histological analysis of infected brain expressed high amounts of stress proteins secreted by brain macrophages as well as showed demyelination and neuronal damage (Medana et al. 2001b). Changes in morphology of monocytes and macrophages indicating activation of retinal microglial cells during *Plasmodium falciparum* infection have also been reported (Hunt and Grau 2003).

Dendritic cells (DCs) are a variant type of antigen-presenting cells (APCs) which plays an important role in the initiation and maintenance of CMI (cell-mediated immune response). Based on the expression of their surface markers and response against pathogens, they can be divided into two types: plasmacytoid DC (pDC) and conventional DC (cDC) (Perry et al. 2004). It has been shown that macrophages are capable of initiating cDC population which can activate $CD4^+$ T cells. It has been shown that both $CD8^+$ and $CD8^+$ splenic cDC can activate parasite-specific $CD4^+$ T-cell responses during *P. chabaudi* infection in mice, whereas depletion of cDCs leads to protection of mice against cerebral malaria (Perry et al. 2004).

4.10 T Regulatory Cells (Tregs)

T suppressor cells are the subtypes of $CD4^+$ T cells which are known for their major involvement in generation of the immune response during various infections. Parasite can manipulate T regulatory cells by changing the T-cell immune response to an extent that could lower the parasite burden (Sakaguchi et al. 2006).

The well-defined markers for Tregs are $CD4^+CD25^{+high}$ and represent 10% of total peripheral $CD4^+$ T cells. They exhibit a high expression of Foxp3 which is necessary for differentiation and functioning of Tregs (Stevenson et al. 2011). They are mainly reported to suppress cellular immune responses through direct contact with immune cells via production of various cytokines like TGF-ß and IL-10 (Bacchetta et al. 2007).

During infection, Tregs reduce host immune responses through cell-to-cell contact, inhibitory cytokines or cytokine deprivation and prevent the effective generation of an immune response. Tregs are also known to downregulate Th2 responses such as IL- 5-dependent eosinophil activation which is required to kill parasites. During generation of immune response in host, the interplay and balance among Th1, Th2 and Tregs is crucial to fight against parasitic infection (Maizels and Yazdanbakhsh 2003). It has been observed that children with severe malaria were

unable to control the inflammation during *P. falciparum* infection suggesting that this component may be rapidly overwhelmed by virulent infections (Torres et al. 2014).

Tregs have been reported to inhibit the pathogenic Th cells which are responsible for control of cerebral syndrome in resistant BALB/c mouse infected with ANKA strain. Whereas, in susceptible mouse strain, Tregs were found to be depleted which resulted in an increase in survival of mice with significant reduction in parasitaemia (Lee et al. 2011).

A comparative study on PbA-infected middle-aged CM-resistant and young CM-susceptible mice reported that this cell population has a regulatory involvement in the control of fatal pathogenesis and it worked in an IL-10-dependent manner (Shan et al. 2013).

$CD4^+$ T regulatory cells were found to be increased in *P. berghei* NK-65-infected rodent host, whereas, immunization of mice with parasite constituents showed a decrease in $CD4^+$ T regulatory cells leading to the generation of strong Th2 immune response. It resulted in the clearance of infection from the host upon challenge (Kumar et al. 2015).

4.11 Natural Killer (NK) Cells

During malaria, NK cells generate innate immune response with help of DCs and secrete various cytokines. They are found to be amplified during infection and are capable of lysing parasitized erythrocytes *in vitro*. In the bloodstream, NK cells produce IFN-γ in response to infected RBCs, leading to macrophage activation, and provide innate immunity to malaria. Pro-inflammatory chemokine IL-8 production by NK cells is also induced during malaria infection which leads to the recruitment and activation of other cells (Ariyasinghe et al. 2006).

NK cells are the strong inhibitors of liver-stage parasite and reported to regulate IgG antibody responses, which are significant for unrestricted malaria parasite control. These cells regulate not only cerebral malaria but also other complications such as pulmonary oedema and severe anaemia (Mitchell et al. 2005). In *Plasmodium berghei* ANKA infected mouse, CD1d-restricted NK cells induce early IFN-γ production and promote cerebral syndrome. IFN-γ production by NK cells has an effect on maintaining immune homeostasis in infected mice. It also influences parasite-specific antibody responses and the TH1/TH2 balance during infection (Brown et al. 1990).

4.12 Blood-Brain Barrier (BBB)

Free flow of molecules and ions in and out of brain parenchyma is regulated by the blood-brain barrier. The BBB along with blood-CSF (cerebrospinal fluid) barrier ensures a constant composition of the extracellular fluid in the brain. This is

essential for normal neuronal function, and it also regulates the passage of immune cells into the central nervous system (Tsukita and Furuse 2000). Excessive production of various cytokines and chemokines like TNF-α, IFN-γ and interleukins can be toxic to the brain and may lead to the production of irreversible symptoms of coma during cerebral malaria. Significantly higher levels of TNF-α and IL-1β in the brain of children having cerebral malaria have been reported (Kwiatkowski et al. 1990).

Increase in levels of adhesion molecules during CM cause changes in endothelial cell junctional permeability. It leads to leakage of plasma proteins and fluids into the perivascular space and brain parenchyma causing cerebral oedema. This evidence supported the fact that functional BBB breakdown occurs in CM inducing systemic or local cytokine release from the blood through endothelial cells into the perivascular space and brain parenchyma (Adams et al. 2002).

4.13 Concluding Remarks

Cytokines secreted by white blood cells can be a major source of defence/inflammation to host against malaria. Elevated immune response generated during *Plasmodium* infection is responsible for creating several complications to host especially CM. It has been observed that immune cells providing protection to host are also responsible for secreting the adhesion molecules on the surface of the endothelial cells of capillaries and venules in the brain. Due to the activation of adhesion molecules in CNS, the infected red blood cells and leukocytes start adhering to the brain microvasculature and obstruct blood flow, creating cerebral complications.

This knowledge might lead to better understanding of the unclear mechanism of sequestration of cells in the brain during generation of strong immune response in malaria-infected host. It can be useful to device therapeutic approach to control the devastating pathogenesis during infection.

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