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# IMMUNOLOGICAL PROCESSES IN MALARIA PATHOGENESIS

Louis Schofield\* and Georges E. Grau<sup>‡</sup>

**Abstract** | Malaria is possibly the most serious infectious disease of humans, infecting 5–10% of the world's population, with 300–600 million clinical cases and more than 2 million deaths annually. Adaptive immune responses in the host limit the clinical impact of infection and provide partial, but incomplete, protection against pathogen replication; however, these complex immunological reactions can contribute to disease and fatalities. So, appropriate regulation of immune responses to malaria lies at the heart of the host–parasite balance and has consequences for global public health. This Review article addresses the innate and adaptive immune mechanisms elicited during malaria that either cause or prevent disease and fatalities, and it considers the implications for vaccine design.

**Malaria** is transmitted to vertebrate hosts, such as mice, monkeys and humans, by the bite of female *Anopheles* mosquitoes that are infected with protozoan parasites of the genus *Plasmodium*. The inoculated sporozoite stage is transient and causes no pathology. Within a few minutes, it infects liver cells and undergoes a period of intracellular replication, which is also clinically silent. After liver-stage replication is complete, the parasite initiates blood-stage infection, which is the main cause of disease (FIG. 1). There are four *Plasmodium* species that infect humans. *Plasmodium ovale* typically causes a relatively benign infection. *Plasmodium malariae* is also frequently clinically silent, although an immune-complex-associated glomerulonephropathy can develop following chronic infection. Although it is rarely fatal, *Plasmodium vivax* is a common cause of acute febrile illness, especially in Asia, South America and Oceania, and it might contribute to anaemia. However, most cases of severe disease and most deaths are caused by the blood-stage cycle of *Plasmodium falciparum*, which is endemic in most of sub-Saharan Africa and throughout most of the tropics.

Worldwide, most infections with malaria-causing agents are clinically silent, reflecting the ability of adaptive immune mechanisms to prevent disease. In non-immune individuals, however, infections are

more clinically overt, and a minority of these can become severe or life threatening, manifesting a range of discrete and overlapping disease syndromes of complex aetiologies. Those dying of malaria can have single-organ, multiple-organ or systemic involvement (TABLE 1). Overall patterns of disease depend markedly on the age and the previous immunological experience of the host<sup>1</sup>. In areas of high malaria transmission, the burden of disease is borne by infants and young children; life-threatening disease in this setting typically consists of metabolic acidosis (which leads to respiratory distress), cerebral malaria (CM) and severe malarial anaemia (SMA). However, in areas of lower transmission, primary infections might occur in adulthood, in which severe disease more frequently involves additional disturbances, such as renal failure, pulmonary oedema, shock and jaundice. So, transmission dynamics and host age are important determinants of disease, together with host genetics and immunological responses (discussed later).

The diversity of syndromes (TABLE 1) seems to confound the identification of unifying mechanisms of disease. However, the studies reviewed here generally support a scheme in which several important malaria syndromes might arise from the intersection of a few basic processes: the site-specific localization of parasitized red blood cells (PRBCs) among target organs;

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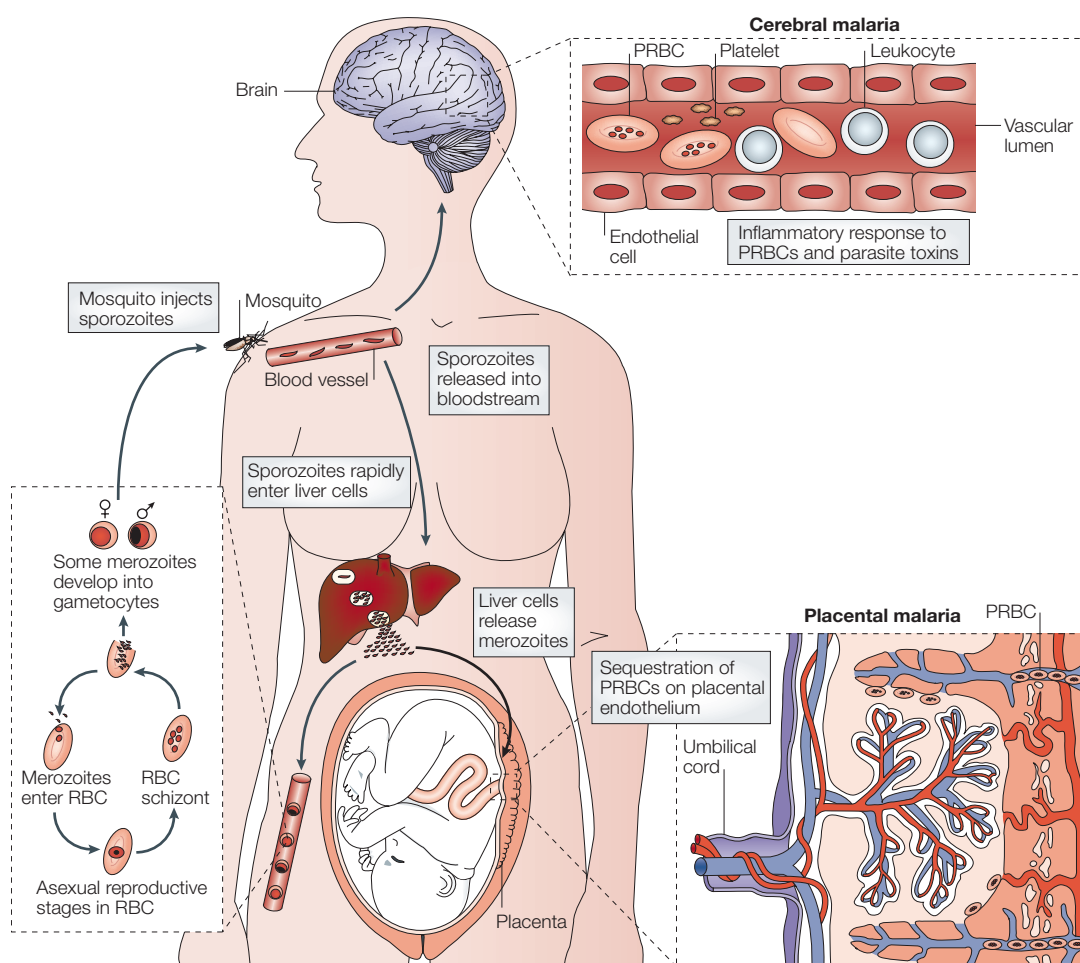
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the local and systemic action of bioactive parasite products, such as toxins, on host tissues; the local and systemic production of pro-inflammatory and counter-regulatory cytokines and chemokines by the innate and adaptive immune systems in response to parasite products; and the activation, recruitment and infiltration of inflammatory cells. According to this view, diverse organ-specific or systemic disease syndromes are end-stage processes of atypical inflammatory cascades that are initiated in target organs by pathogen products and are maintained by infiltrating cells through positive-feedback cycles. In most cases, homeostasis corrects the cascade effect, and responses are adequately downregulated. In severe disease, however, a 'run-away' effect can ensue, with fatal consequences. Appropriate regulation of immune

responses might therefore be a key to healthy outcomes, and understanding these processes might aid in the development of vaccine-based interventions.

#### Initiation of malaria-associated syndromes

**Site-specific localization of PRBCs.** As blood-stage parasites mature through the 48-hour replicative cycle, avoiding passage through the spleen is an essential survival strategy, because this immunological effector organ<sup>2</sup> efficiently filters PRBCs from the bloodstream. Erythrocyte membrane protein 1 (EMPI) is the name given collectively to members of a family of variant cell-surface proteins that are encoded by *P. falciparum* and enable PRBCs to engage multiple receptors — such as intercellular adhesion molecule 1 (ICAM1), vascular cell-adhesion molecule 1 (VCAM1), CD31, CD36,



**Figure 1 | The life cycle of *Plasmodium falciparum*.** Mosquitoes that carry the malaria-causing parasite *Plasmodium falciparum* inject a small number of infectious sporozoites into the bloodstream while feeding. Within a few minutes, they are carried to the liver, where they invade and replicate in liver cells. Then, 10–12 days later, thousands of daughter merozoites are released back into the bloodstream and enter red blood cells (RBCs). The parasites are carried around the circulation within RBCs, but as they grow, they express adherent ligands — such as *P. falciparum* erythrocyte membrane protein 1 — that enable the maturing parasite to bind receptors expressed by endothelial cells that line the blood vessels in the deep vascular beds of organs such as the brain, lungs and placenta. After 48 hours, the parasitized RBCs (PRBCs) rupture and release more daughter merozoites, thereby perpetuating and promoting the blood-stage cycle. The presence of the parasite and the invasion of RBCs might not be sufficient to account for disease; instead, the release of bioactive parasite molecules and an inappropriately regulated host immune response could be the main causes of fatal pathogenesis, which occurs in only a minority of patients. Some merozoites differentiate into gametocytes, which, when taken up by another feeding mosquito, perpetuate the sexual cycle in the insect.

CHAGAS' DISEASE

A disease that is caused by *Trypanosoma cruzi*. In chronic cases, it is associated with autoimmune damage to various organs.

thrombospondin, endothelial-cell selectin (E-selectin), chondroitin sulphate A (CSA) and hyaluronic acid — that are expressed by vascular endothelial cells in deep-organ microvascular beds. Binding to these receptors by cell-surface *P. falciparum* EMP1 sequesters parasites so that they are removed from the circulation and, consequently, do not travel to the spleen. Although this is advantageous for the survival of the parasite, this strategy has the pathological consequence of concentrating parasites in various target organs, and the precise locations depend on the differential expression of the various *P. falciparum* EMP1 members and their diverse endothelial-cell-expressed receptors.

**Production of bioactive parasite products.** As sequestered parasites mature, they produce a variety of bioactive molecules that either upregulate or downregulate pathogenic processes, largely through their effects on the innate immune system (TABLE 2). Immune responses to infectious insults are mainly initiated by the interaction of pathogen-associated molecular patterns (PAMPs) with receptors expressed by host cells. For viruses, bacteria and yeast, PAMPs include modified lipids (such as bacterial lipopolysaccharides), carbohydrates (such as yeast zymosan), proteins (such as flagellin) and nucleic acids (such as unmethylated CpG-motif-containing DNA and double-stranded RNA). Many studies implicate glycosylphosphatidylinositol (GPI) of *P. falciparum* as a malaria PAMP and as a toxin. Purified GPI induces the expression of many genes that are implicated in malaria pathogenesis: for example, genes that encode pro-inflammatory cytokines — such

as tumour-necrosis factor (TNF), interleukin-1 (IL-1) and IL-12 (REFS 3–6) — inducible nitric-oxide synthase<sup>7</sup>, and various adhesion molecules that are expressed at the surface of the vascular endothelium and are recognized by *P. falciparum* EMP1 (REF. 8), which increases endothelial-cell binding by PRBCs<sup>8</sup>. In a sepsis–shock model, GPI alone is sufficient to cause symptoms that are similar to those of acute malaria, such as transient pyrexia, hypoglycaemia and death of recipients as a consequence of TNF-mediated coagulopathy, as seen in the malarial shock-like syndrome<sup>3</sup> (TABLE 1). The GPIs from *Trypanosoma brucei*, *Trypanosoma cruzi* and *Toxoplasma gondii* all have similar properties to the GPI from *P. falciparum*<sup>9–11</sup>, and this might account for some pathogenic features of trypanosomiasis, CHAGAS' DISEASE and toxoplasmosis.

Other potential *P. falciparum* PAMPs include phosphorylated, non-peptidic antigens, to which  $\gamma\delta$  T cells respond with slow kinetics<sup>12</sup>, and haemozoin, the insoluble, crystalline residue of parasite-mediated haemoglobin digestion, which is long-lived and accumulates in phagocytes. Haemozoin has interesting, although seemingly contradictory, bioactivities. It has been reported to induce<sup>13</sup> or inhibit<sup>14</sup> dendritic cell (DC) maturation and to induce either the production of the T helper 1 (T<sub>H</sub>1) cytokines TNF<sup>15</sup> and IL-12 (REF. 13) or the T<sub>H</sub>2 cytokine IL-10. It also inhibits general proliferative responses by human leukocytes<sup>16</sup>. In addition, it has been shown to promote monocyte and macrophage dysfunction, by impairing phagocytosis and the expression of MHC class II molecules, CD11c and ICAM1 (REF. 17). Overall, haemozoin seems to be highly immunosuppressive<sup>18,19</sup>.

Table 1 | Severe and fatal disease syndromes in malaria

Syndrome	Clinical features	Possible sequence or mechanism of disease
Cerebral malaria	Sustained impaired consciousness, coma, long-term neurological sequelae	Cerebral parasite sequestration; bioactive GPI; pro-inflammatory cytokine cascade; endothelial-cell activation; natural killer T-cell activation; T <sub>H</sub> 1/T <sub>H</sub> 2-cell balance; chemokine production; monocyte, macrophage and neutrophil recruitment; platelet and fibrinogen deposition; CD4 <sup>+</sup> , CD8 <sup>+</sup> and $\gamma\delta$ T-cell involvement; IFN- $\gamma$ production; neurological metabolic derangements; possibly hypoxia
Placental malaria	Placental insufficiency, low birth weight, premature delivery, loss of fetus	<i>Plasmodium falciparum</i> EMP1-mediated binding to placental endothelium and syncytiotrophoblast through chondroitin sulphate A and hyaluronic acid; cytokine production; chemokine-mediated recruitment and infiltration of monocytes; intravascular macrophage differentiation
Severe malarial anaemia	Pallor, lethargy, haemoglobin level of 4–6 g per 10 ml	Erythropoietic suppression by toxins and cytokines; increased RBC destruction, owing to parasitization, RBC alterations, complement and immune complex or antigen deposition, erythrophagocytosis, splenic hyperphagism, CD4 <sup>+</sup> T cells, T <sub>H</sub> 1/T <sub>H</sub> 2 cytokine balance (TNF and IFN- $\gamma$ versus IL-10)
Metabolic acidosis	Respiratory distress, deep breathing (Kussmaul breathing), hypovolaemia	Molecular mechanisms unknown. Possibly widespread parasite sequestration; bioactive toxins; increased vascular permeability; reduced tissue perfusion; anaemia; pulmonary airway obstruction; hypoxia; increased host glycolysis; repressed gluconeogenesis. Some overlap with shock-like syndrome
Shock-like syndrome (systemic inflammatory-response-like syndrome)	Shock, haemodynamic changes, impaired organ perfusion, disseminated intravascular coagulation	Bioactive toxins; T <sub>H</sub> 1 cytokines; acute-phase reactants

EMP1, erythrocyte membrane protein 1; GPI, glycosylphosphatidylinositol; IFN- $\gamma$ , interferon- $\gamma$ ; IL-10, interleukin-10; RBC, red blood cell; T<sub>H</sub>, T helper; TNF, tumour-necrosis factor.

## ERYTHROPOIETIC SUPPRESSION

The inhibition of normal production of fresh red blood cells in the bone marrow or spleen. This occurs by various mechanisms, including inhibition of precursor-cell responsiveness to erythropoietin.

Table 2 | Malaria products and their bioactivities

Parasite product	Receptor and cell type	Pathological and cellular effects
<i>Plasmodium falciparum</i> EMP1-family members	ICAM1, VCAM1, CD36, thrombospondin, E-selectin, chondroitin sulphate A, hyaluronic acid and CD31 on endothelial cells and trophoblast cells; CD36 on DCs	Binding directs parasite to the brain, placenta and possibly other target organs; CD36 engagement proposed to suppress DC and macrophage activation
GPI	TLR2, TLR4 and/or possibly C-type lectins on several cell types, including DCs, macrophages, endothelial cells and adipocytes; CD1d and V $\alpha$ 14-V $\beta$ 8 TCR on NKT cells	Induces widespread expression of genes encoding pro-inflammatory proteins (including TNF, IL-1, IL-6, IL-12, iNOS, ICAM1, VCAM1); activates NKT cells; induces T <sub>H</sub> 1- or T <sub>H</sub> 2-cytokine production
Haemozoin	TLR9 on DCs	Contradictory reports: both T <sub>H</sub> 1- and T <sub>H</sub> 2-cell activities; induces and inhibits DCs; suppresses macrophages; induces IL-10 production; broadly immunosuppressive
Unknown ligands	NKC-encoded receptors on NK and NKT cells	Activates NK cells; induces IFN- $\gamma$ production; regulates balance of T <sub>H</sub> 1 and T <sub>H</sub> 2 cytokines produced by NKT cells
Isopentenyl pyrophosphate	$\gamma\delta$ TCRs	Activates $\gamma\delta$ T cells; induces IFN- $\gamma$ production
Protein antigens	Diverse TCRs on CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells	Activates $\alpha\beta$ T cells; induces T <sub>H</sub> 1- or T <sub>H</sub> 2-cytokine production
Unknown sugar(s)	MBL in plasma	Possible binding provides protection; low levels of MBL are associated with disease

DC, dendritic cell; EMP1, erythrocyte membrane protein 1; E-selectin, endothelial-cell selectin; GPI, glycosylphosphatidylinositol; ICAM1, intercellular adhesion molecule 1; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; iNOS, inducible nitric-oxide synthase; MBL, mannose-binding lectin; NK, natural killer; NKC, natural killer complex; NKT, natural killer T; TCR, T-cell receptor; T<sub>H</sub>, T helper; TLR, Toll-like receptor; TNF, tumour-necrosis factor; VCAM1, vascular cell-adhesion molecule 1.

Divergent results might be a consequence of differences in haemozoin preparations, because these preparations are heterogeneous, containing uncharacterized bioactive contaminants, such as non-covalently associated phospholipids, hydroxylated fatty acids, carbohydrates and glycolipids. Removal of non-covalently associated lipids from haemozoin preparations abolishes their bioactivity<sup>20,21</sup>. However, a synthetic version of haemozoin,  $\beta$ -haematin, has pro-inflammatory activity on mouse monocytes and macrophages<sup>13,22</sup>.

At high PRBC to target-cell ratios, PRBCs can inhibit the maturation of DCs and reduce their ability to stimulate T cells<sup>23,24</sup>. DC exhaustion could result from antigen overload, but it has been suggested, although not proven, that these activities result from the binding of *P. falciparum* EMP1 to CD36 and CD51 (REFS 23,24). If this is the case, this important molecule would also participate in the negative regulation of the immune system, in contrast to PAMPs, which only induce activation of the innate immune system.

**Recognition of parasite molecules by innate immune receptors.** The various members of the mammalian Toll-like receptor (TLR) family are important receptors that are responsible for recognition of microbial PAMPs. Mice that have a mutant version of TLR4, which binds lipopolysaccharide, are responsive to GPI from *P. falciparum*<sup>3</sup> and *T. cruzi*, indicating that this glycolipid triggers a different receptor that results in the expression of pro-inflammatory genes. GPI from both pathogens can activate TLR2, and this requires the crucial TLR adaptor protein MyD88 (myeloid

differentiation primary-response gene 88)<sup>25,26</sup>. However, TLR2-deficient mice produced pro-inflammatory cytokines when stimulated with live *T. cruzi*, and they were almost as resistant to infection as wild-type mice<sup>27</sup>. Evidence for the involvement of TLR mechanisms in the immunoregulation and immunopathogenesis of malaria is still limited. MyD88-deficient mice that are infected with *Plasmodium berghei* have less liver injury and produce less IL-12 but not IL-18 than wild-type mice<sup>28</sup>. However, the liver injury in this experimental system does not model the pathophysiological disease processes that occur in humans. Haemozoin activates mouse DCs through TLR9 (REF 13). TLR9 is expressed by monocytes, macrophages, B cells and DCs in mice, but it is restricted to B cells and plasmacytoid DCs in humans<sup>29</sup>. So, TLR9 agonists (and presumably haemozoin) do not activate monocytes or macrophages in humans. TLR9 functions through a strictly MyD88-dependent pathway. However, data indicate that there is no significant difference in peak parasitaemia, ERYTHROPOIETIC SUPPRESSION, interferon- $\gamma$  (IFN- $\gamma$ ) production and CM fatality rates between *P. berghei*-infected, *Myd88*<sup>-/-</sup> mice and their *P. berghei*-infected, *Myd88*<sup>+/-</sup> litter-mates (L.S., unpublished observations). If this observation is confirmed, then MyD88-independent pathways would seem to dominate disease processes in this model. Other lectin-like receptors might also function as pattern-recognition receptors or modulatory receptors, including calcium-dependent C-type lectins such as soluble mannose-binding lectin (MBL), which is present in the plasma. MBL binds sugars that are present at the surface of PRBCs but have yet to be characterized<sup>30</sup>, and a low level of MBL in the



plasma is associated with severe disease in humans<sup>31</sup>. Nonetheless, lectin-like-receptor signalling and bioactivity remain poorly defined. Further studies are clearly required to dissect the role of purified, structurally defined malaria PAMPs in the activation of TLR-signalling pathways, both those that are MyD88 dependent and MyD88 independent, and to elucidate the possible contributions of these pathways to relevant pathophysiological processes.

**'Intermediate' pathways between innate and adaptive immunity.** Many studies show that pro-inflammatory  $T_H1$  cytokines are crucial determinants of malaria disease states. In addition to acute-phase monokines, the production of which is induced rapidly by parasite toxins, IFN- $\gamma$  levels can increase very early during malaria, indicating that non-conventional lymphoid populations that can function with accelerated kinetics account for this production. CD1d-restricted natural killer T (NKT) cells are such an 'intermediate' arm between innate and adaptive immunity, and these cells are particularly important in regulating the downstream differentiation of CD4<sup>+</sup> T cells into  $T_H1$  and  $T_H2$  cells<sup>32</sup>. As well as having toxic bioactivity, GPI is a natural glycolipid ligand for NKT cells<sup>33</sup>, together with closely related mycobacterial phosphatidylinositol mannosides<sup>34</sup>. In mice with CM, NKT cells were shown to be a crucial determinant of cytokine levels, the pro-inflammatory cascade, pathogenesis and fatality<sup>35</sup>. The CD1d-NKT-cell pathway also upregulates and downregulates acute malarial splenomegaly in mice and is an important determinant of B-cell responses<sup>36</sup>. The CD1d-NKT-cell pathway either prevents or promotes fatality, and it determines the differentiation of immune cells, depending on which alleles of the natural killer complex (NKC), which is located on mouse chromosome 6 (REF. 37), are expressed. The loci in the NKC are differentially expressed by natural killer (NK) cells and NKT cells, and they control the production of pro-inflammatory  $T_H1$  cytokines and counter-regulatory  $T_H2$  cytokines by NKT cells<sup>35</sup>. NKC loci also determine the level of malarial anaemia, the isotypes of malaria-specific antibodies and the  $T_H1/T_H2$  profile of conventional T cells that is induced during infection<sup>37</sup>. C57BL/6 mice, which are  $T_H1$ -cell-response prone, are susceptible to CM, whereas BALB/c mice, which have a genetically determined  $T_H2$ -cell bias, are resistant<sup>38</sup>, and these different profiles reflect the substantial contribution of polymorphic NKC loci<sup>37</sup>. Infection with malaria-causing agents imparts NKC-dependent signals to NKT cells that influence their differentiation into cells that secrete  $T_H1$  or  $T_H2$  cytokines, but the specific receptor-ligand interactions that are involved in this process are unknown. So, the NKC is a crucial genetic determinant of malaria pathogenesis in mice, with an important role in controlling NK- and NKT-cell function. Not all NKC loci are involved, however, because injecting certain NK-cell-specific monoclonal antibodies does not affect pathogenesis in mice<sup>39</sup>. Human NK cells also become activated early during malaria<sup>40</sup> and are activated rapidly by parasites

*in vitro*<sup>41</sup>, which requires direct contact of PRBCs with NK cells and results in IFN- $\gamma$  production<sup>42</sup>. However, the relevance of NK cells to human disease remains unclear.

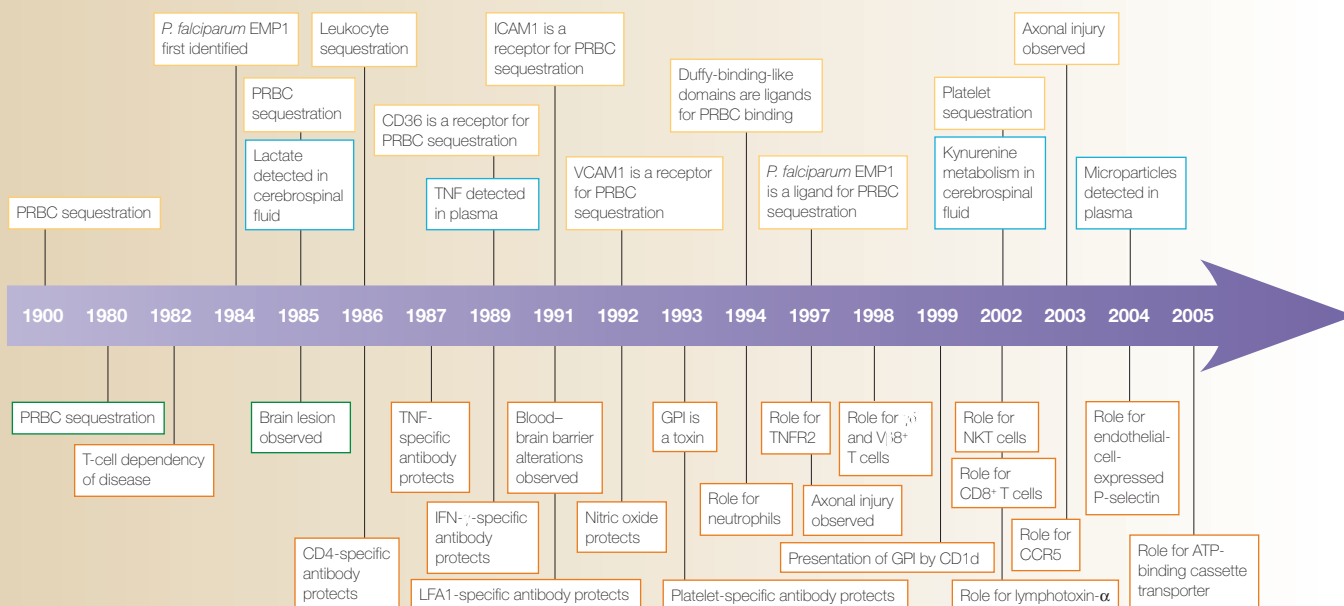
### Progression to cerebral malaria

The histopathology of CM is associated with the accumulation of mature PRBCs in cerebral microvessels, through sequestration. This feature was first described in 1894 (REF. 43) and has since been confirmed by numerous studies<sup>44</sup>. This has led to the dominant theory of CM pathogenesis: that, because PRBCs are sequestered in brain capillaries and post-capillary venules, they induce flow perturbations that eventually lead to obstruction and hypoxia of the surrounding brain parenchyma<sup>45,46</sup> and to haemorrhages. Nevertheless, as early as 1944, there were doubts about any causal relationship between CM and PRBC sequestration<sup>44</sup>. Indeed, there is, for the most part, no proof that PRBC sequestration is sufficient to cause CM<sup>47</sup> and even less that it is a cause of death; this issue has been debated previously<sup>48,49</sup>. Mature-stage parasites are absent from the peripheral blood of patients infected with *P. falciparum*. Clearly, sequestration of PRBCs in deep microvascular beds occurs routinely in all of these patients, although only 1% of these individuals develop CM. So, PRBC sequestration might not be sufficient to cause CM, but it might be necessary. Subsequently, it has become apparent in humans with CM, as well as in mouse models of CM, that host cells, such as leukocytes or platelets, might also be sequestered in brain microvessels, in addition to PRBCs<sup>50,51</sup>. These host cells might be involved in the pathogenesis of CM, either through local effects in brain microvessels or through distant effects mediated by the production of potentially deleterious mediators, such as pro-inflammatory cytokines, which can be detected in the circulation. These leukocytes, however, show little evidence of endothelial extravasation and therefore cannot be described as classic inflammatory cells. The TIMELINE shows how the study of mouse and simian models, *in vitro* assays and human infections has contributed over time to the elucidation of the complex cascade that controls CM pathogenesis.

**Accumulation of intravascular infiltrates.** The intravascular accumulation of monocytes in the brain has long been recognized in mice<sup>52-54</sup> and humans<sup>51,55-60</sup> with CM. Sequestered monocytes and macrophages are more abundant in paediatric patients with CM than in those with SMA or non-malarial encephalopathy<sup>44</sup>. This feature has largely been ignored as a possible contributor to the pathogenic process in CM in humans.

In CM, some cells of the monocyte-macrophage lineage within the microvasculature show characteristics that are normally associated with tissue macrophages (including an increased size and the presence of phagocytosed material, vacuoles, a ruffled plasma membrane and pseudopods). For this reason, we refer to these cells as intravascular macrophages. Therefore, CM is a rare situation in which monocytes

## Timeline | Contribution of animal models and human-based assays to our understanding of cerebral malaria



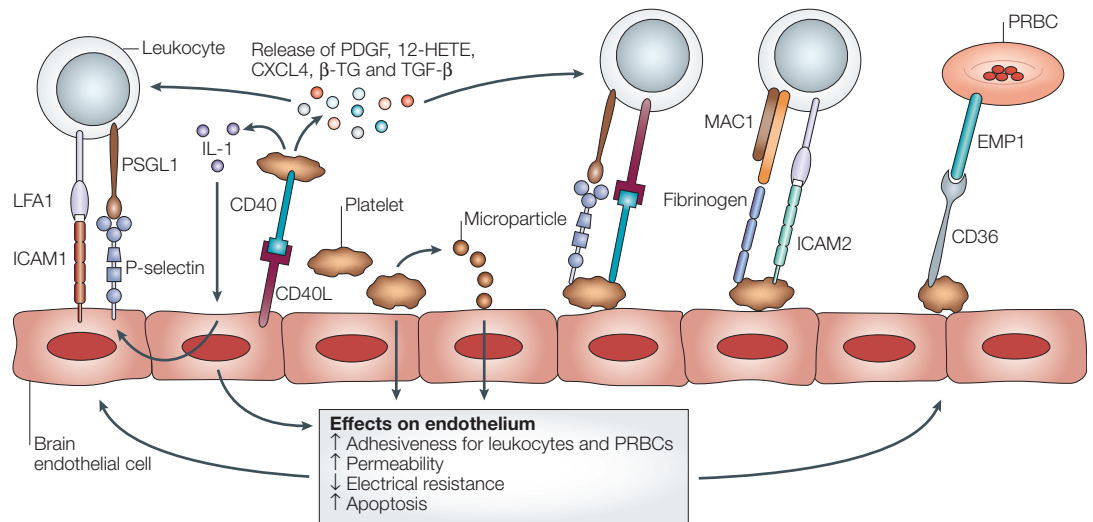
Research on cerebral malaria has been carried out in mouse *in vivo* infection models (orange), simian *in vivo* infection models (green), *in vitro* models of human infection (yellow) and *in vitro* assays involving human tissues (blue). CCR5, CC-chemokine receptor 5; EMP1, erythrocyte membrane protein 1 of *P. falciparum*; E-selectin, endothelial-cell selectin; GPI, glycosylphosphatidylinositol; ICAM1, intercellular adhesion molecule 1; IFN- $\gamma$ , interferon- $\gamma$ ; LFA1, lymphocyte function-associated antigen 1; NKT, natural killer T; *P. falciparum*, *Plasmodium falciparum*; PRBC, parasitized red blood cell; P-selectin, platelet selectin; TNF, tumour-necrosis factor; TNFR2, TNF receptor 2; VCAM1, vascular cell-adhesion molecule 1.

differentiate into macrophages in the intravascular compartment and not in the tissues. Several modifications of monocyte–macrophage phenotype and functions are known to occur in severe malaria. For example, coagulation factors secreted by monocytes, in addition to their role in blood-clot formation, might contribute to the sequestration of cells in brain microvessels of patients with CM, at least in children. Tissue factor can itself function as an adhesion molecule, and it has coagulation-independent roles in cell adhesion and migration. Furthermore, upregulation of tissue-factor expression has a central role in driving a thrombosis–inflammation circuit. Coagulant mediators (such as factor VIIa and factor Xa) and the end-product fibrin are also pro-inflammatory, eliciting the expression of TNF and other cytokines, as well as chemokines and adhesion molecules, and this has recently been reviewed in REFS 50,51.

The accumulation of leukocytes in the brains of patients with CM is evidence of a considerable chemokine cascade, which has been shown in experimental studies, including DNA-microarray analyses of mouse disease<sup>61,62</sup>. The expression of monocyte-secreted cytokines and chemokines, such as TNF, CXC-chemokine ligand 10 (CXCL10; also known as IP10), CC-chemokine ligand 2 (CCL2; also known as MCP1) and CCL5 (also known as RANTES), varies with mouse genotype and correlates with resistance versus susceptibility to disease<sup>62</sup>. Neutrophils also contribute to the brain lesions in mice and are an important source of cytokines

(including the p40 subunit of IL-12, IL-18, IFN- $\gamma$  and TNF) and chemokines (including CCL3 (also known as MIP1 $\alpha$ ), CXCL9 (also known as MIG) and CXCL10) that participate in pathogenesis. Indeed, depletion of neutrophils early in malaria prevents the development of CM in mice, downregulates the expression of T<sub>H</sub>1 cytokines in the brain, and markedly decreases the sequestration of monocytes and the incidence of microhaemorrhages in the brain<sup>63</sup>. Although the contribution of neutrophils to CM in humans is unknown, the neutrophil-specific activation marker and recruitment agent lipocalin was found at a higher concentration in plasma from patients with severe malaria<sup>64</sup>, and transcription of the gene encoding lipocalin is upregulated in the brain during cerebral disease in mice<sup>61</sup>.

Evidence for a pathogenic role of platelets, both in CM in mice and in *in vitro* models of CM in humans, has been summarized elsewhere<sup>65</sup>. As illustrated in FIG. 2, there are several possible ways through which platelets could affect endothelial-cell function and viability, and promote leukocyte adhesion. First, platelets, together with other cell types, can modulate the expression of adhesion molecules, such as ICAM1, and the production of cytokines, such as IL-6, by endothelial cells<sup>66</sup>, through the release of IL-1. Second, platelet-derived microparticles modulate endothelial-cell metabolism, by regulating the production of cyclooxygenase-2 and prostaglandins<sup>67</sup>, and increase the adhesiveness of the endothelial-cell–leukocyte–platelet interaction,



**Figure 2 | Mechanisms of platelet-endothelial-cell interactions in cerebral malaria.** Platelets, after activation by tumour-necrosis factor, can markedly alter the functions of brain endothelial cells, either directly, by binding to the endothelium, or indirectly, by releasing molecules from their secretory granules. For example, platelets release interleukin-1 (IL-1), which increases the expression of adhesion molecules (such as intercellular adhesion molecule 1, ICAM1) and the production of cytokines (such as IL-6) by endothelial cells. Platelet-derived microparticles also alter endothelial-cell metabolism by regulating the production of cyclooxygenase-2 and prostaglandins, which might affect endothelium permeability, electrical resistance and apoptosis. One of the earliest events in inflammation or tissue injury is the adhesion of activated platelets to the endothelium (possibly through CD40 ligand (CD40L)-CD40 interactions). Importantly, in cerebral malaria, this provides additional receptors at the endothelial-cell surface for the adhesion of leukocytes, which bind platelet surface molecules such as platelet selectin (P-selectin) and CD40L, through P-selectin glycoprotein ligand 1 (PSGL1) and CD40, respectively. Furthermore, platelet-derived microparticles can increase the adhesiveness of endothelial cells to leukocytes and the adhesiveness of the platelets themselves to the endothelium. Finally, activated platelets can indirectly alter endothelial cells through the complex effects of their granule-derived mediators on leukocytes, including platelet-derived growth factor (PDGF), 12-hydroxyeicosatetraenoic acid (12-HETE), CXC-chemokine ligand 4 (CXCL4; also known as PF4),  $\beta$ -thromboglobulin ( $\beta$ -TG) and transforming growth factor- $\beta$  (TGF- $\beta$ ). The exact mechanisms that are involved have yet to be defined. EMP1, erythrocyte membrane protein 1 of *Plasmodium falciparum*; LFA1, lymphocyte function-associated antigen 1; MAC1, macrophage receptor 1; PRBC, parasitized red blood cell.

by directly upregulating the expression of adhesion molecules and integrins such as ICAM1 and macrophage receptor 1 (MAC1; CD11b-CD18), both by the endothelium and by adherent monocytes and other leukocytes<sup>68</sup>. Consistent with this, microparticles have been shown to be crucial for the development of CM in mice<sup>69</sup>, and the level of microparticles is markedly increased in the plasma of children with CM<sup>70</sup>. Third, the adhesion of activated platelets to the endothelium (which is an early event in inflammation and possibly occurs through CD40-ligand-CD40 interactions) can indirectly mediate leukocyte-endothelial-cell adhesion, by providing additional receptors, such as fibrinogen and ICAM2 (REF. 71), which are ligands for leukocyte MAC1 and lymphocyte function-associated antigen 1 (LFA1; CD11a-CD18), respectively. In addition, platelet selectin (P-selectin) is released from secretory granules of activated platelets and can be expressed at their surface, and leukocytes that express P-selectin glycoprotein ligand 1 can then adhere to this surface P-selectin<sup>72</sup>. Last, platelets can also modulate the sequestration of normal RBCs and of PRBCs, by surface expression of CD36, which binds *P. falciparum* EMP1 at the PRBC surface, and they can directly modulate cytokine production by circulating leukocytes and the endothelium, as has been shown in mouse models of CM<sup>73</sup>.

A key unresolved question is how much of the accumulation of parasites in cerebral vessels is associated with adhesion mediated by classic receptor-ligand interactions and how much is associated with non-specific deposition of activated platelets, deposition of fibrin and the presence of other markers of the host inflammatory response. Evidence from P-selectin- or ICAM1-deficient mice indicates that most platelet binding is likely to be ligand mediated (not non-specific), because platelet binding is undetectable by intravital microscopy in these mutant mice<sup>74</sup>. Nonetheless, the relative importance of the numerous effects of platelets in the pathogenesis of CM remains to be further elucidated<sup>50</sup>.

**Role of T cells.** In mice, CM has been known to be a T-cell-dependent disease for two decades. NUDE MICE and mice that are deficient in the  $\alpha\beta$ -TCR are resistant to disease. In addition, *in vivo* depletion using specific monoclonal antibodies showed that CD4<sup>+</sup> T cells are required for pathogenesis<sup>75</sup>. However, MHC-class-II-deficient mice, which lack conventional CD4<sup>+</sup> T cells, still develop CM<sup>39</sup>, probably by retaining CD1d-restricted, CD4<sup>+</sup> NKT cells that have a crucial role in disease<sup>35</sup>. Subsequently,  $\gamma\delta$  T cells have been shown to participate in CM, because although mice that are deficient in the  $\gamma\delta$ -TCR are susceptible to CM, depletion

**NUDE MICE**  
Mice with a mutation that causes both hairlessness and defective formation of the thymus, which results in a lack of mature T cells.



of  $\gamma\delta$  T cells from wild-type mice (using specific antibodies) confers resistance to CM.  $\gamma\delta$ -TCR-deficient animals therefore seem to develop a functional redundancy that is absent in conditions of normal immunological ontogeny<sup>76</sup>. In humans, there are few data that address T-cell involvement in CM.

IFN- $\gamma$  seems to be the most important T-cell-secreted cytokine. *In vivo* neutralization of IFN- $\gamma$  in mice infected with *P. berghei* strain ANKA prevented TNF overproduction and CM development<sup>77</sup>. The central role of IFN- $\gamma$  in the pathogenesis of CM was confirmed by experiments in mice that were deficient in IFN- $\gamma$  or the IFN- $\gamma$  receptor, which are resistant to the development of experimentally induced CM<sup>78</sup>. The cell-surface antigen CTLA4 (cytotoxic T-lymphocyte antigen 4; CD152) negatively regulates T cells and therefore might downregulate T-cell responses and prevent immunopathology. As expected, blockade of CTLA4 exacerbated CM in mice<sup>79</sup>, which highlights the contribution of T cells to disease.

CD8<sup>+</sup> T cells might also be effectors in pathogenesis<sup>39,80,81</sup>. In mice, the number of cytotoxic CD8<sup>+</sup> T cells infiltrating the brain during CM is increased, and these cells contribute to permeability changes of the mouse blood–brain barrier through perforin-dependent mechanisms<sup>82,83</sup>. CM is also associated with an increase in the number of peripheral CD8<sup>+</sup> T cells that have TCRs using the V $\beta$ 8.1 or V $\beta$ 8.2 segments<sup>84</sup>, and disease was reduced when mice were treated with antibodies that specifically neutralize these T cells. Because of the hypothesis that CD8<sup>+</sup> T cells contribute to the pathology of CM<sup>39</sup>, the role of CC-chemokine receptor 2 (CCR2), which is expressed by CD8<sup>+</sup> T cells, was evaluated<sup>85</sup>. Mice that were deficient in CCR2 remained susceptible to CM, indicating that this receptor is not directly involved in cerebral pathology. However, the number of CD8<sup>+</sup>CCR5<sup>+</sup> T cells in the brain increased after infection with *P. berghei* strain ANKA, and CCR5-deficient mice were partially protected against the neurological syndrome<sup>85</sup>. CD8<sup>+</sup> T cells sequestered in the brain were proposed to be responsible for the neurological syndrome and for death, but there has been no report that directly shows the presence of these cells, using histopathology, at the site of neurovascular lesions. Direct contact has been shown *in vitro* between brain endothelial cells and activated T cells, and this contact might also be crucial in cerebral pathogenesis<sup>86</sup>. CD8<sup>+</sup> T cells also have a role in circulatory shock and in respiratory distress in mice that are infected with *P. berghei*<sup>87</sup>.

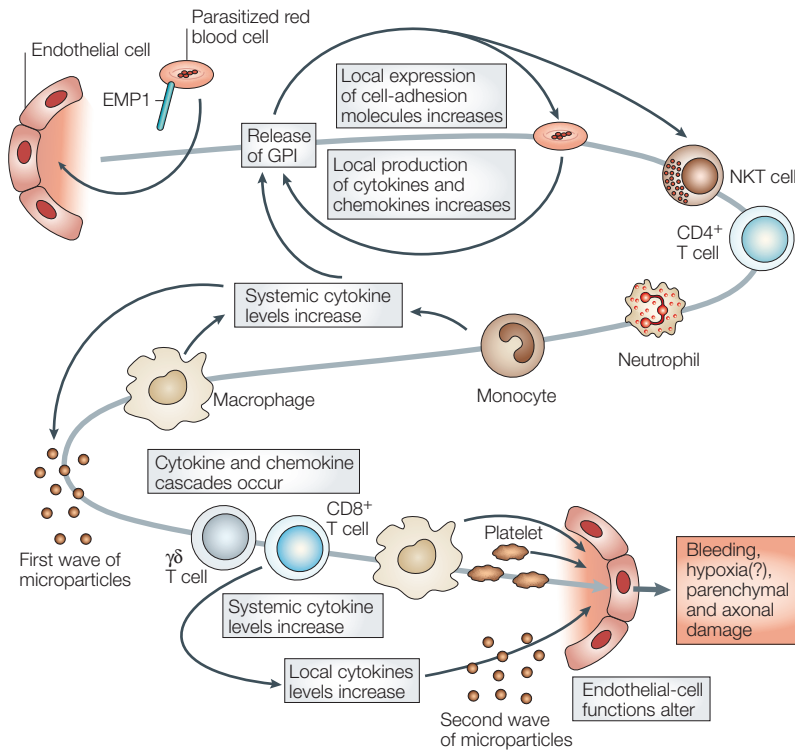
**Microvascular obstruction: what is the sequence of events?** In conclusion, histopathological studies have uncovered the presence of PRBCs, platelets and leukocytes, each of which might contribute to disease, in the cerebral vascular lumen in humans and mice with CM (FIG. 3). What cannot be deduced from such observations is the order in which the cells and platelets are sequestered in a vessel and the nature of the ensuing interactions. There are three possibilities: namely, that PRBCs bind brain endothelial

cells first, followed by leukocytes or platelets; that leukocytes bind first; or that platelets make the initial interaction. This might vary in different areas of the brain and over time, thereby leading to a mosaic of arrested cells. So, conclusions drawn from histology, even in post-mortem analyses of multiple sites, might be influenced by sampling variables. In addition, the transient nature of the binding events, the temporal pattern of expression of adhesion molecules, and competition among PRBCs and leukocytes for binding sites is likely to determine the sequence of adhesive events. For example, P-selectin and E-selectin are expressed transiently, which probably influences the binding of circulating cells. Endothelial-cell, leukocyte and platelet adhesion molecules are likely to participate in cell–cell interactions in this dynamic environment. After cells have accumulated, they produce chemotactic, inflammatory and toxic mediators that further contribute to the pathogenesis, and this can lead to positive-feedback cycles (FIG. 3).

Judgement must remain reserved, however, on the contribution of each factor in different age groups, in different transmission settings and across the spectrum of disease. What is cause and what is effect in this complex picture? The difficulty of ascribing function applies particularly to post-mortem histological analyses of human cerebral disease states, because processes that are observed to be associated with disease might be corrective attempts that have been made by the host to control infection or to downregulate pathogenesis, or might be pathogenically neutral. A recent histological study points to the possibility of a disease spectrum in paediatric patients with CM<sup>60</sup>. There are three defined disease categories (CM1, CM2 and CM3), and they occurred in 15%, 56% and 29% of clinically defined cases, respectively. Patients with CM1 have only PRBC sequestration, whereas patients with CM2 have PRBC sequestration plus other intra- and perivascular pathology, including immune-cell infiltrates. Patients with CM3 fulfilled the complete World Health Organization clinical criteria for CM, including unrousable coma associated with infection, but they died of non-malarial causes. So, what seems to be a defined syndrome appears heterogeneous with respect to the underlying pathogenesis<sup>88</sup>. Key histological studies have been undertaken in South-East Asian adults. These implicate a role mainly for PRBC sequestration in CM, and it seems probable that there are substantial differences between CM in adults and in children (studied in Africa), with intravascular leukocyte accumulation being more pronounced in children. Fortunately, animal models allow hypotheses to be tested by experimental intervention, and CM in rodents seems to mirror the human disease with reasonable accuracy, including presenting a disease spectrum that depends on host genetics. Because disease progression in animal models evidently requires several sequential steps, multiple points of intervention might also be possible in the treatment of humans with CM.

**Immunology of placental malaria**

In areas with high rates of transmission of malaria, women have considerable immunity to malaria by the time they reach child-bearing age. However, during their first or second pregnancy, they are susceptible to placental malaria, and this becomes less common with subsequent pregnancies. CSA and hyaluronic acid



**Figure 3 | Schematic representation of events that are likely to lead to severe malarial disease, particularly in the brain.** First, parasitized red blood cells (PRBCs) adhere to receptors expressed by brain microvascular endothelial cells, such as intercellular adhesion molecule 1 (ICAM1), through surface expression of *Plasmodium falciparum* erythrocyte membrane protein 1 (EMP1). When merozoites are released from PRBCs ~4 hours later, parasite glycosylphosphatidylinositol (GPI), which is either released into the blood or present in parasite membranes, functions as a pathogen-associated molecular pattern and toxin, thereby inducing an inflammatory response. A local acute-phase response then occurs, which involves activation of the endothelium and local production of cytokines and chemokines, and this results in upregulation of expression of cell-adhesion molecules by endothelial cells. Within the next ~24 hours, this cycle is perpetuated and exacerbated, owing to increasing parasite numbers and further binding of PRBCs to endothelial cells that have upregulated expression of cell-adhesion molecules. GPI can also function as a ligand for CD1d-restricted natural killer T (NKT) cells, leading to their activation. Activated NKT cells can regulate the differentiation of CD4<sup>+</sup> T cells into T helper 1 (T<sub>H</sub>1) or T<sub>H</sub>2 cells, depending on which natural-killer-complex loci are expressed, so activation and involvement of CD4<sup>+</sup> T cells occurs. In addition, chemokines recruit monocytes and activate neutrophils (although neutrophils are not known to infiltrate brain microvessels in humans or mice with cerebral malaria). Recruited monocytes can then differentiate into macrophages and become arrested in brain microvessels. Macrophages can also be activated by GPI, a process that is amplified by interferon- $\gamma$ . Local activated macrophages produce more chemokines, which are released systemically, thereby amplifying infiltration of cells, sequestration of PRBCs and release of microparticles (which are probably of endothelial-cell origin). After several more cycles,  $\gamma\delta$  T cells and CD8<sup>+</sup> T cells might become involved, releasing more chemokines and cytokines both systemically and locally and possibly inducing perforin-mediated lesions in the endothelium. Together with locally arrested macrophages, platelets are sequestered and participate in altering endothelial-cell functions. More microparticles of platelet, endothelial-cell and monocyte origin are released, which leads to the dissemination of pro-inflammatory and pro-coagulant effects. Finally, damage to the endothelium, with possible perivascular haemorrhage, axonal injury, and neurotransmitter and metabolic changes, can ensue. The overall disease spectrum in humans might depend on whether all of these processes occur or only some of them.

are receptors that are expressed by endothelial cells, preferentially in the placenta. PRBCs that display *P. falciparum* EMP1 proteins that bind these receptors can be sequestered, allowing parasite maturation on the placental endothelium<sup>89</sup> and thereby validating the link between an adhesive phenotype and organ-specific disease<sup>90</sup>. As is the case for CM in mice<sup>52,54,91</sup> and humans<sup>51,55,60</sup>, severe placental malaria (which results in low birth weight) is associated with substantial macrophage infiltration into the placenta and high chemokine expression<sup>92,93</sup>, indicating that adhesive phenotype and chemokine-driven cellular infiltration are key determinants of organ-specific disease syndromes. Interestingly, women with placental malaria remain afebrile, indicating that the condition is mainly a local pathology, with some aspects of acquired clinical immunity remaining unaffected by placental parasitization.

**Immunology of severe malarial anaemia**

SMA is the most serious and most common pernicious complication of malaria, and it might be the leading cause of deaths from malaria worldwide. Compared with the florid, acute signs of CM, SMA can be chronic or silent and therefore is less well studied. However, that peaks of SMA incidence coincide with peaks of CM incidence during high transmission seasons in endemic areas indicates that SMA might sometimes be an acute syndrome, notwithstanding that, for some cases, this might be the point at which a chronic process flares up. Many cross-sectional studies report a lack of correlation between parasite densities and severity of anaemia, although longitudinal studies indicate that duration of infection might be a better predictor of risk of disease<sup>94</sup>. There is a misconception, however, that SMA arises simply from the destruction of infected RBCs, and over-reliance on inappropriate models might contribute to this view (BOX 1). Many animals with acute infections develop severe haemolytic anaemia, through invasion and rupture of RBCs as a consequence of excessive parasite burdens of a magnitude that is rare in humans with malaria. In humans, SMA is typically associated with parasite burdens that are considerably lower than those required for the marked, direct destruction of RBCs<sup>95</sup>, with ~12 uninfected RBCs lost for every PRBC<sup>95,96</sup>. Because of the limitations of hyperparasitaemic experimental infections, there has been increased interest in *Aotus* spp. monkeys either immunized with experimental vaccines consisting of antigens from blood-stage parasites or rendered semi-immune by previous exposure to infection with *P. falciparum*, as these animals develop marked SMA despite having very low parasite burdens<sup>97,98</sup>. Before the advent of antibiotics, the high fever induced by malaria was used to treat neurosyphilis; the low parasite burdens, and the kinetics and magnitude of SMA, in semi-immune simian models mirror the anaemia that developed in the numerous untreated humans who had malaria that was induced for the management of neurosyphilis<sup>95,99,100</sup>. Therefore, because direct destruction of RBCs by parasitization seems to be a relatively

Box 1 | **Experimental models of malaria**

The study of malaria is aided by the availability of a wide range of experimental models. Various species of the *Plasmodium* genus — such as *Plasmodium chabaudi*, *Plasmodium berghei*, *Plasmodium yoelii* and *Plasmodium vinckei* — naturally infect rodents. Adapted to infect laboratory rats and inbred mouse strains, they have proven enormously useful in the development of drugs and vaccines, and in basic studies of pathogenesis, immunology, and the genetics of susceptibility or resistance to infection and disease. Other models use parasites that cause malaria in simians — such as *Plasmodium knowlesi* and *Plasmodium cynomolgi* — or parasites of humans that are adapted to simian hosts (such as *Aotus* spp. monkeys). Although no two host–parasite combinations are identical in all features of the relationship, some models are better than others at recapitulating the main features of pathology or the immune responses that occur in malaria in humans. There is great diversity of responses and disease outcomes in human populations, so experimental models that use inbred hosts might reflect only a section of the natural spectrum of disease in humans: varying both host and parasite genetics uncovers a diversified disease spectrum in these models.

**Malaria in mice**

Early during infection, *P. berghei* strain ANKA is a good model of cerebral malaria (CM), and processes identified using this model have been subsequently validated in humans. Inbred mouse strains differ markedly in their susceptibility, showing the importance of host genetic variation in immunopathogenesis<sup>133</sup>. Similarly, different strains of *P. berghei* (K173 versus ANKA) differ in some aspects of pathogenesis, indicating the influence of parasite genetic variation in induced pathology.

Infection with *P. yoelii* strain 17XL (a lethal strain) induces CM that is associated with the sequestration of parasitized red blood cells, and it has been used together with *P. yoelii* strain 17XNL (a non-lethal strain) to study experimental vaccine-induced immune responses.

*P. chabaudi chabaudi* strain AS causes a non-lethal infection in resistant mouse strains and a lethal infection in susceptible mouse strains. Lethality, however, results from haemolysis that is secondary to hyperparasitaemia, which might not be relevant to the human disease processes. This *Plasmodium* strain has been used to study experimental vaccines and immunological processes that control hyperparasitaemia. Infections with *P. chabaudi adami* are self-resolving, non-pathogenic and non-lethal.

*P. vinckei vinckei* causes an aggressive, overwhelming hyperparasitaemia.

minor contributory mechanism<sup>101</sup>, SMA is thought to arise mainly from two processes: increased destruction of non-parasitized RBCs, and decreased production of RBCs (also known as erythropoietic suppression).

**Destruction of uninfected RBCs.** In SMA in humans, accelerated RBC turnover is proposed to result from changes to the surface or structure of uninfected RBCs that target them for destruction<sup>102</sup>. The following have been detected at relatively high frequencies in individuals with SMA<sup>101</sup>: oxidation, phosphatidylserine externalization and reduced deformability of RBCs; complement binding by RBCs; complement regulatory deficiencies; and autoantibodies, immune complexes and IgG specific for non-specifically adsorbed parasite antigen at the surface of RBCs. These mechanisms are proposed to target RBCs for destruction by intravascular haemolysis or for clearance mediated by the RETICULOENDOTHELIAL SYSTEM (RES). However, human SMA is not associated with the typical signs of intravascular haemolysis, such as secretion of haemoglobin in the urine. By contrast, homeostatic RES clearance of RBCs is mainly mediated by macrophages in the splenic red pulp, and recruitment to the spleen or

activation of these macrophages might accelerate the process<sup>103,104</sup>. SMA is associated with high serum levels of neopterin, a marker of macrophage activation, the expression of which is induced by IFN- $\gamma$  in particular<sup>103</sup>. Phagocytosis of uninfected RBCs has been documented during human infections<sup>105,106</sup>, and upregulation of RES activity is implicated in malarial thrombocytopaenia<sup>107</sup>. Anaemia often persists after clearance of parasitaemia, and haemoglobin normalization shows delayed kinetics compared with conditions that have similar blood loss, such as trauma<sup>96</sup>. It is possible that clearance by the RES that involves high RBC turnover extends beyond the period of parasitaemia. In untreated malaria used to control neurosyphilis, the rapid onset of severe anaemia was temporally linked to the appearance of a distended spleen, as was subsequently observed in children with malarial anaemia<sup>99</sup>. These observations are consistent with hyperactivation of the RES and with the magnitude of RBC destruction during SMA. This has led to the proposal that SMA has an inflammatory aetiology<sup>103,104</sup>. Counter-regulatory T<sub>H</sub>2 cytokines such as IL-4 (REF 103) or IL-10 (REFS 108,109) might therefore protect against disease. So, SMA might be regulated by diverse factors that control hypersplenism and splenic macrophage recruitment and activation: for example, host CD4<sup>+</sup> T cells, cytokines and chemokines (such as CCL2, CCL3 and CCL4 (also known as MIP1 $\beta$ )) and parasite products (such as haemozoin and GPI). SMA might therefore be precipitated by the rise of parasitaemia above a crucial threshold, as has been observed for experimental infections of simians<sup>97,98</sup> and humans<sup>95,99,100</sup>.

Nevertheless, it might be the immune response of the host to this parasite biomass that is the main cause of anaemia, rather than simply the direct destruction of RBCs by parasitization. In broad terms of immunoregulation, SMA might therefore have similarities to other malaria syndromes. Adoptive transfer of parasite-specific, CD4<sup>+</sup> T<sub>H</sub>1-cell lines promotes anaemia after infection with *P. berghei*<sup>110</sup>, showing that antigen-specific T cells can contribute to the severity of disease. A plausible mechanism lies in the ability of T cells to upregulate macrophage-mediated RBC clearance. Because SMA is marked in *Aotus* spp. monkeys that have been immunized with *P. falciparum* antigens<sup>97,98</sup>, blood-stage vaccines might not necessarily protect against this condition, although they might suppress parasitaemia. Whether T-cell priming by these experimental vaccines promotes the development of SMA is not clear. Such preclinical models might prove useful for further assessing the efficacy of vaccines in the prevention or promotion of this disease state.

**Decreased production of RBCs.** Normal homeostasis of RBC numbers is maintained by balancing destruction of old RBCs by the RES with production of new RBCs through erythropoiesis. Under the influence of factors such as erythropoietin, haematopoietic stem cells in the bone marrow or spleen multiply and differentiate to produce the youngest fully functional RBCs, which

## RETICULOENDOTHELIAL SYSTEM

The general phagocytic system of the host. It is responsible for removal and destruction of foreign material and senescent or dead host cells, such as red blood cells.



are known as reticulocytes and are the earliest cell in this pathway to be released into circulation. The number of reticulocytes in the blood directly reflects recent erythropoietic activity. Reticulocyte levels are often decreased during acute infection with *P. falciparum*, indicating that another mechanism might contribute to SMA — erythropoietic suppression<sup>111</sup>. Animal models seem to accurately recapitulate this phenomenon. Infection with *Plasmodium chabaudi* or *P. berghei* results in decreased proliferation, differentiation and maturation of erythroid precursors<sup>112,113</sup>, and reticulocyte levels are decreased early in infection, with DNA-microarray analysis of splenocytes and bone-marrow cells uncovering reduced transcription of at least 25 erythroid-specific loci<sup>61</sup>. It has been widely proposed that the TNF and IFN- $\gamma$  cytokine cascade that is associated with the immediate early acute phase of infection mediates erythropoietic suppression in mice and humans, by decreasing the responsiveness of erythroid precursors to erythropoietin<sup>114,115</sup>, but this remains unproven. Anaemia in response to acute infection with *P. berghei* strain ANKA is controlled, in part, by genes encoded by the polymorphic NKC loci that are expressed by NK and NKT cells, which control cytokine levels and immune-cell differentiation<sup>37</sup>. Crude *P. berghei* strain ANKA and *P. chabaudi* parasite lysates also induce erythropoietic suppression *in vivo*<sup>116</sup>, reflecting the bioactivity of parasite products (possibly haemozoin or GPI), which either directly affect erythroid precursors or indirectly affect them by influencing macrophages (to secrete TNF<sup>3</sup>) or NKT cells<sup>33</sup>.

After loss of RBCs in conditions such as trauma, anaemia is normally compensated for by physiological erythropoiesis. However, SMA could be exacerbated if erythropoietic suppression were to prevent adequate reticulocyte compensation during continuing clearance of RBCs. Further investigation of the multiple host and parasite factors that might influence these two contributory mechanisms of SMA *in vivo* is required. Specifically, the impact of vaccination on SMA-related end-points should be examined in more detail in experimental models.

### Clinical immunity to malaria

Epidemiological studies show that, after the initial period in which children are susceptible to severe malaria, protective immunity that is acquired to malaria develops in three sequential phases: first, immunity to life-threatening disease; second, immunity to symptomatic infection; and only then, third, partial immunity to parasitization. In 1899, Robert Koch observed that immunity to disease precedes the ability to control parasite densities, as others subsequently observed<sup>117–119</sup>, proposing that it reflects the primary acquisition of antitoxic immunity. The data from the treatment of neurosyphilis with malaria shows evidence for antitoxic immunity<sup>120</sup>. At the population level, immunity to severe malaria seems to be acquired after only one or two infections<sup>121</sup>, although many children with severe disease have a previous history of multiple mild bouts of malaria.

Several studies report associations between levels of antibodies that are specific for various parasite antigens and reduced risk of infection<sup>122</sup>, but this is not clearly established for disease states such as CM or SMA. So, there is no single correlate of clinical immunity, and those described do not account for the overall variation in susceptibility in a population<sup>123</sup>. However, antibodies specific for the parasite glycolipid GPI have been found to be negatively associated with the risk of developing SMA<sup>5</sup> or CM<sup>124</sup> and with acute febrile episodes<sup>125</sup>, although a cross-sectional study found no association with tolerance for parasitaemia<sup>126</sup>, which might reflect the developmentally compromised acquisition of carbohydrate-specific antibodies in infants. Although clinical immunity might result from adaptive immune responses to GPI, other explanations include the acquisition of physiological non-responsiveness to malaria toxins (which is analogous to tachyphylaxis, the process of downregulation of lipopolysaccharide-responsive signalling pathways following exposure to the agonist). However, this mechanism is unlikely to operate over long time-scales, whereas clinical immunity seems to be relatively robust. A further possibility is that disease susceptibility or resistance is regulated to a considerable extent by the T<sub>H</sub>1/T<sub>H</sub>2-cytokine profile of the NK- and NKT-cell arm of the immune system (which is intermediate between the innate and adaptive immune systems)<sup>32,33,35–37</sup> or of conventional CD4<sup>+</sup> T cells and that clinical immunity is associated with a switch away from the default, T<sub>H</sub>1-cell-biased responses to T<sub>H</sub>2-cell-biased responses, which prevents severe disease but controls parasite densities only after an appropriately diverse antibody repertoire is generated. Establishing whether clinical immunity results from adaptive immune responses to bioactive parasite products, physiological desensitization to malaria toxins, regulation of the balance of T<sub>H</sub>1 and T<sub>H</sub>2 cytokines, or a combination of mechanisms is an important issue for future research because such considerations should inform vaccine development.

### Implications for vaccines

Vaccines against malaria should aim to reduce morbidity and mortality. Traditional approaches seek to achieve this objective by reducing parasite burdens. In support of this, a recent clinical trial shows that reducing the infective inoculum by administration of a sporozoite-specific vaccine reduces the rates of disease<sup>127</sup>. Nonetheless, reducing the replication of blood-stage parasites, although likely to confer protection, will not necessarily reduce morbidity or mortality, because host immune responses, which can be non-linear with respect to parasite densities, are important determinants of these events. Despite the clinical objective of vaccination, there has been no systematic attempt to assess the impact of experimental vaccines in preclinical models that have appropriate disease end-points. Preclinical models that have been used so far include infection of naive mice with *P. chabaudi* and infection of naive *Aotus* spp. monkeys with *P. falciparum*, and these infections result in high rates of parasite

replication but not in clinically relevant syndromes. As mentioned earlier, SMA is marked in *Aotus* spp. monkeys that have been immunized with *P. falciparum* antigens<sup>97,98</sup>, so blood-stage vaccines might not necessarily protect against this condition, although they might suppress parasitaemia. Whether experimental vaccines can induce immune responses that promote SMA is not clear.

The use of vaccines against the disease aims to reduce morbidity and mortality directly, by immunizing individuals with parasite products that contribute to host pathology<sup>128</sup>. For example, vaccines that are designed to prevent malaria during pregnancy target the domains of *P. falciparum* EMP1 that bind placental receptors<sup>129</sup> (that is, CSA and hyaluronic acid). Other organ-specific disease processes could be targeted if a restricted set of *P. falciparum* EMP1 molecules were found to be important<sup>129</sup>, the inherent diversity of these targets being a potential barrier to these strategies. Chemical synthesis of the glycan group of *Plasmodium* spp. GPI, which is highly conserved and non-toxic, has recently provided a means to test the hypothesis that this molecule is causally involved in the pathogenesis of *P. berghei* infection<sup>130</sup>. Vaccination with the GPI glycan protects against blood acidosis, pulmonary oedema, vascular occlusion by macrophages, and cerebral fatalities in a rodent model of severe malaria induced by *P. berghei* infection<sup>130</sup>. These findings show the efficacy of a prototype antitoxic vaccine, and they prove that GPI is an essential parasite product in the pathogenesis of systemic disease and in the lethal cerebral syndrome in this model.

It has been argued that antitoxic vaccines might exacerbate disease by inhibiting the acute-phase responses that limit parasite replication<sup>131</sup>. Indeed, it has been proposed that the function of malaria toxins is to elicit host responses that limit infection densities by killing parasites<sup>131</sup>. However, 'suicide' explanations of biological function in unicellular organisms are not easily reconciled with Darwinian logic, it being difficult to envisage why parasites would produce a molecule to cause their own demise. Furthermore, strong acute-phase immune responses are seen in association with high parasite densities in sick children, and marked inflammatory cascades do not necessarily reduce parasite burdens in humans or in experimental models. Clearly, this is an important area for further investigation. Nonetheless, these speculations help to highlight

that the impact of the current blood-stage vaccines on malaria pathogenesis remains to be determined, even in preclinical models.

## Conclusions

Because they are blood-borne, malaria-causing parasites have access to multiple organs, including the bone marrow, spleen, brain, lungs and placenta. The binding of *P. falciparum* EMP1 to diverse endothelial-cell-expressed receptors concentrates parasites in certain sites. Parasite toxins, such as GPI and haemozoin, induce acute-phase immune responses, with local activation of monocytes and the vascular endothelium. GPI also contributes to early IFN- $\gamma$  and counter-regulatory IL-4 production, by functioning as a ligand for the CD1d-restricted NKT-cell arm of the immune system. The overall balance of T<sub>H</sub>1 and T<sub>H</sub>2 cytokines at this stage of the cascade is determined to a considerable extent by the polymorphic loci in the NKC. Chemokine cascades recruit intravascular macrophages and inflammatory cells to diverse target organs, with subsequent deposition of platelets and fibrin. Various lymphoid-cell lineages might further contribute to disease, through the production of pro-inflammatory cytokines, such as IFN- $\gamma$ . Counter-regulatory T<sub>H</sub>2-cell responses might downregulate disease and promote parasite clearance through antibody formation. Much severe pathology in malaria therefore has an immunological basis, although this remains poorly investigated for certain key processes such as metabolic acidosis, which is the strongest prognostic indicator of malarial fatality<sup>88</sup> and one of the least understood aspects of pathogenesis. The importance of immune processes in malaria pathogenesis in humans is further exemplified by clear associations of genetic polymorphisms in immune loci — such as those encoding MBL, CD36, CD40 ligand, TNF, IFN- $\gamma$ , IL-4 and the p40 subunit of IL-12 — with altered risk of disease<sup>132</sup>. Credible model systems allowing hypothesis testing by experimentation show that multiple convergent factors are required, and few components are sufficient, for disease progression, indicating that there are multiple potential points of intervention in humans. Because T cells contribute to these processes, vaccination strategies should avoid exacerbating disease, by the induction of appropriate counter-regulatory mechanisms or by the neutralization of pathogenic parasite products.

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#### Competing interests statement

The authors declare no competing financial interests.

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