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Insights into the immunopathogenesis of malaria using mouse models

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Malaria kills approximately 1–2 million people every year, mostly in sub-Saharan Africa and in Asia. These deaths are at the most severe end of a scale of pathologies affecting approximately 500 million people per year. Much of the pathogenesis of malaria is caused by inappropriate or excessive immune responses mounted by the body to eliminate malaria parasites. In this review, we examine the evidence that immunopathology is responsible for malaria disease in the context of what we have learnt from animal models of malaria. In particular, we look in detail at the processes involved in endothelial cell damage leading to syndromes such as cerebral malaria, as well as generalised systemic manifestations such as anaemia, cachexia and problems with thermoregulation of the body. We also consider malaria in light of the variation of the severity of disease observed among people, and discuss the contribution from animal models to our understanding of this variation. Finally, we discuss some of the implications of immunopathology, and of host and parasite genetic variation, for the design and implementation of anti-malarial vaccines.

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Malaria is a mosquito-borne disease caused by protozoan parasites of the genus *Plasmodium*. Approximately 40% of the world's population is at risk of becoming infected, mainly in Asia and sub-Saharan Africa, and malaria is responsible for over 1 million deaths every year, mostly in children under 5 years old (Refs 1, 2). The immune system plays an important role in the host defence against infection with malaria parasites, and in limiting the extent of infection once it occurs. However, immune responses that have evolved to kill pathogens such as malaria parasites are seldom confined to the destruction of the parasites; excessive immune responses often occur, sometimes in inappropriate locations, resulting in damage to the host, hereafter referred to as immunopathology.

Plasmodium parasites have a heteroxenous life cycle (Fig. 1), whereby infected female mosquitoes inject sporozoites into the mammalian bloodstream upon feeding. Sporozoites migrate to the liver where they multiply in hepatocytes, eventually forming many merozoites, which, after rupture of the infected cell, continue their life cycle in red blood cells (RBCs). It is the continual cycling of malaria parasites within RBCs, and the immune responses directed against this stage of the parasite, which cause most of the pathology observed in malaria infections.

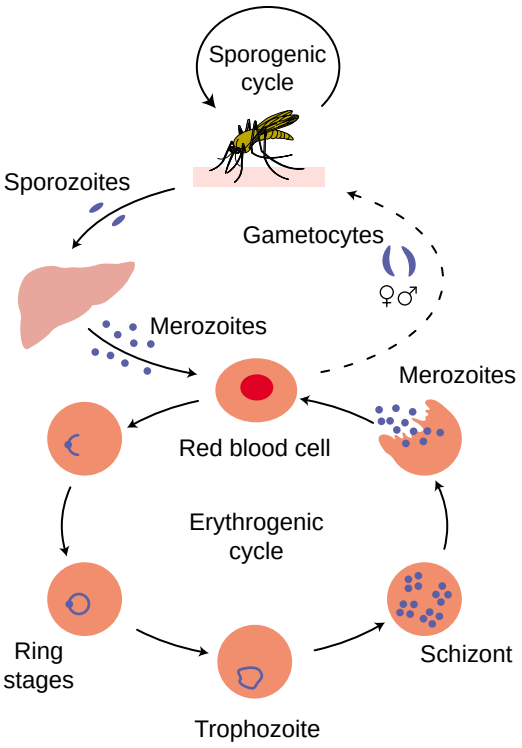

Figure 1. *Plasmodium* life cycle. A bite from an infectious mosquito results in the transfer of sporozoites into the blood stream of the human host, which then travel to the liver. Parasite replication occurs in the liver, leading to the release of merozoites into the bloodstream. The merozoites bind to the surface of the red blood cell (RBC) and enter the RBC via an active invasion process. The parasite then undergoes growth through the ring and trophozoite stages, finally producing schizonts containing multiple merozoites (erythrogenic cycle). Maturation of the schizont leads to the destruction of RBCs and release of merozoites into the bloodstream, which invade further RBCs. Occasionally, parasite maturation will result in the production of gametocytes. These are released into the bloodstream and are subsequently taken up by the mosquito, via a bite, and then undergo the sexual stage of development (sporogenic cycle).

Malarial disease in humans

One of the most characteristic features of malaria in humans is fever, which coincides with the rupture of schizonts every 48 to 72 h, depending on the species of *Plasmodium*. However, there are significant complications of blood-stage infections, which lead to severe morbidity and, in a proportion of cases, mortality. Many factors determine the severity of the pathology, not least the ability of the parasite to sequester on deep intravascular endothelium of the host. Sequestration is mediated via specific parasite molecules expressed on the surface of the infected RBC, which bind to host molecules expressed on the endothelial lining of blood vessels. These sequestered parasitised RBCs (pRBCs) can result in blood vessel blockage and are also implicated in immunopathology as they can be targets of leukocyte accumulation and localised immune responses (Ref. 3).

Sequestration of pRBCs on brain endothelium is associated with cerebral malaria (CM), one of the most severe forms of *Plasmodium falciparum* malaria. Sequestration in CM is speculated to be mediated via *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed on the surface of the pRBC (Ref. 4). Immune complex deposition

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(Ref. 5) together with 'rosetting', where pRBCs surround themselves with uninfected RBCs, can amplify the occlusive effects of sequestering parasites (Ref. 6). A role for PfEMP1 in *P. falciparum* rosetting has been suggested, although host complement receptor 1 (CR1) may also be involved (Ref. 6). Polymorphism in the *CR1* gene in the South Pacific has been found to affect the degree of parasite rosetting observed in patients, and certain alleles correlate with the severity of infection (Ref. 7), possibly via increasing the parasite growth rate (Ref. 8). Clinically, CM is characterised by encephalopathy, with loss of consciousness and muscle tone (Ref. 9). Histological examination of brain sections from fatal cases of CM reveals damage to, and swelling of, the brain vessel walls, with accompanying vascular haemorrhage (Ref. 10), but pathology is not uniform among patients (Ref. 11), indicating several different syndromes are grouped under the definition CM. In addition to pRBCs, platelets and leucocytes can also be seen to adhere to the endothelium of the microvasculature in CM (Refs 12, 13, 14).

The consequences of sequestration of pRBCs to the endothelium of organs such as the lung, liver and bone marrow have not been characterised in great depth. However, organ-specific pathology can in turn lead to organ failure and multiple systemic effects such as metabolic acidosis and respiratory distress, hypoglycaemia, cachexia and anorexia (Refs 15, 16, 17, 18, 19, 20).

In humans, severe malarial anaemia is a major cause of morbidity, contributing to hypoxia, acidosis and cardiac failure (Ref. 21). Other than destruction of RBCs via the maturation of the parasite, little is known about mechanisms of parasite-induced reduction of RBCs. In *P. falciparum* infections, the level of anaemia is often disproportional to parasitaemia, and anaemic persistence or deterioration can occur at low-level parasitaemia (Refs 22, 23). Therefore, direct parasite destruction of RBCs cannot be the only factor contributing to malarial anaemia. Other factors, such as premature RBC destruction and/or inadequate RBC production, must also be involved, as discussed later.

Less than 0.5% of the estimated worldwide clinical cases of malaria infection result in fatality (Refs 1, 2, 24). In the remaining cases, disease varies widely. There are many factors that play a role in this variation, which is nearly always accompanied by differences in measured immune responses. However, analysis of immune responses in humans is confined to the study of immune cells and molecules in peripheral blood. Although these measurements can be statistically modelled alongside the type and degree of severity of malarial syndrome, it is difficult to determine causative mechanisms.

Mouse malaria models offer an opportunity to dissect immunological mechanisms that might be involved in malarial disease since there are similarities in the immune responses and pathological features of malarial infection in humans and mice. Although mouse models cannot reflect all the complexities of the effects of host, parasite and environmental interactions confounding human disease, controlled experimental infections of laboratory mice, in particular genetically manipulated mice with defects in their immune system, allow us to investigate the role of immunopathogenesis in malarial disease. In this review, we discuss some of the mechanisms of immunopathology that have been determined from mouse malaria models, highlighting common pathways involved in the different syndromes of malaria, and the effects of parasite and host variation on the severity of disease.

Mouse models of malaria

Rodent malaria parasites, isolated from *Thamnomys* thicket rats in the African Congo (Refs 25, 26), have provided an invaluable resource for studying the role of inflammatory and immune responses in the pathology of malaria. The characteristics of both infection and disease differ in the four species of *Plasmodium* that infect mice [*P. chabaudi*, *P. yoelii*, *P. berghei* and *P. vinckei* (Ref. 26)], and for each of the species in various strains of mice. The life history of human malaria parasites and pathological symptoms of human malaria can be mimicked with infections of each of these different species of mouse malaria in combination with different strains of mice, allowing for a range of models in which the different syndromes can be studied (Table 1).

Pro-inflammatory immune responses in malaria infection

Pro-inflammatory immune pathways contribute to malaria pathology

Most studies of human malaria infections implicate pro-inflammatory pathways and immune responses in many aspects of malarial

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Table 1. Mouse models of malaria

Species; subspecies; clone	Organs of sequestration	RBC invasion preference	Mouse strain susceptibility to anaemia	Mouse strain susceptibility to CM
berghei ANKA	Brain, lungs and adipose tissue (Ref. 79)	Reticulocytes	C57BL/6: lethal CD-1: lethal C57BL/6J: non-lethal (Refs 159, 160)	C57BL/6: susceptible CBA: susceptible BALB/c: resistant (Ref. 3)
yoelii yoelii 17X	Brain (Ref. 26)	Reticulocytes and mature RBCs	BALB/c: non-lethal (Ref. 160)	Most strains resistant
yoelii yoelii 17XL		Reticulocytes and mature RBCs	BALB/c: lethal C57BL/6: lethal (Ref. 119)	Most strains susceptible
chabaudi chabaudi AS	Liver and brain (Refs 97, 101)	Mature RBCs	A/J: lethal C57BL/6: non-lethal (Ref. 121)	C57BL/6 IL-10 KO: susceptible (Ref. 161)
chabaudi chabaudi AJ	Liver (Ref. 98)	Mature RBCs	BALB/c: non-lethal (Ref. 121)	
chabaudi adami DS		Mature RBCs	C3H: lethal C57BL/6: non-lethal (Ref. 128)	
chabaudi adami DK		Mature RBCs	BALB/c: non-lethal C3H: non-lethal (Ref. 128)	
vinckei vinckei		Mature RBCs	BALB/c: lethal (Ref. 120)	

Abbreviations: IL, interleukin; KO, knockout; RBC, red blood cell.

disease. *Plasmodium* infection induces an acute-phase reaction (Fig. 2) through interleukin 1 (IL-1), IL-6 and tumour necrosis factor α (TNF-α), which can be found in plasma of infected individuals (Refs 27, 28, 29). In addition to acute-phase responses, human immune responses to malaria parasites are also largely pro-inflammatory [T-helper 1 (Th1)], as characterised by elevated serum levels of IL-12 (Refs 28, 30, 31), IL-18 (Refs 31, 32) and interferon γ (IFN-γ) (Refs 27, 29). *P. falciparum* can trigger release of pro-inflammatory cytokines from innate cells such as natural killer (NK) cells (Refs 33, 34, 35) or as a result of recognition of small non-peptidic phosphorylated antigens by γδ T cells (Refs 36, 37). Some components of a Th2 response can also be measured in *P. falciparum* infection, most notably IL-4 (Refs 29, 38) and IgE (Ref. 39).

The cytokine TNF-α has been implicated in the pathogenesis of malaria infection. Elevated serum TNF-α (Ref. 40), in addition to polymorphisms in the promoter for the TNF-α gene (Refs 41, 42), have been associated with susceptibility to CM. Sequestration of pRBCs is central to many syndromes of malarial disease and can be promoted by pro-inflammatory cytokine up-regulation of the ligands that bind pRBCs (reviewed by Ref. 43). In addition to CM, hyperlactaemia (a consequence of acidosis) (Ref. 44), hypoglycaemia (Ref. 40) and fever (Ref. 45) in malaria patients have all been shown to correlate with elevated levels of serum TNF-α. Therapy with a mouse monoclonal antibody that neutralises TNF-α indicates the involvement of this cytokine in malarial fever (Ref. 46).

Immune responses against malaria parasites in mice are similar to those of humans in that a

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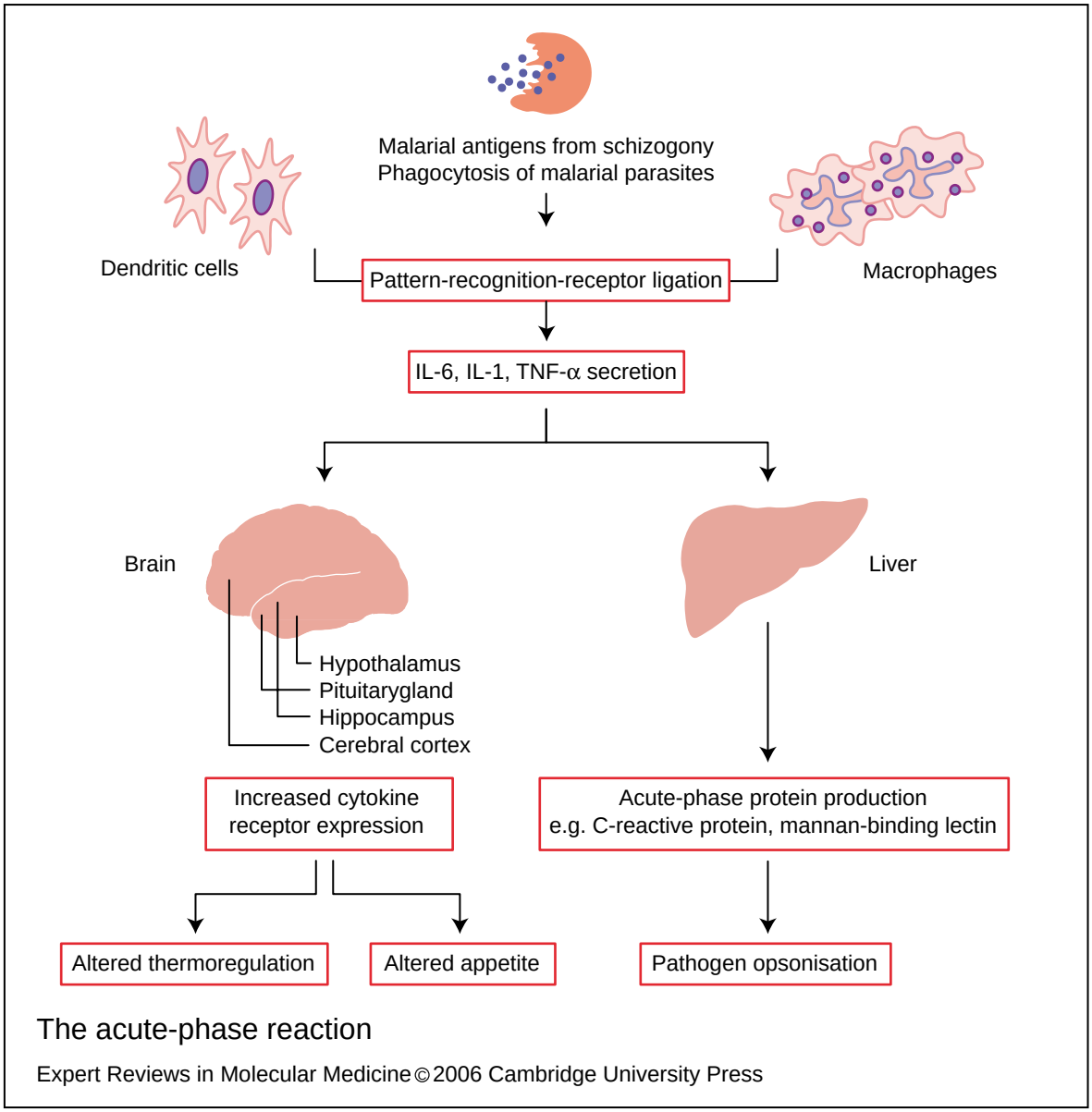

Figure 2. The acute-phase reaction. The acute-phase reaction occurs via the secretion of acute-phase cytokines such as interleukin (IL)-6, IL-1 and tumour necrosis factor α (TNF-α). These cytokines are secreted from antigen-presenting cells such as dendritic cells or macrophages upon recognition of malaria parasites via pattern-recognition receptors. Pro-inflammatory responses upregulate cytokine receptors in different parts of the brain, which mediate the effects of the acute-phase response such as alterations in thermoregulation of the body and loss of appetite. For example, Utsuyama and Hirokawa (Ref. 76) have demonstrated upregulation of the receptors for IL-1 (IL-1R), IL-6 (IL-6) and interferon γ (IFN-γR) in the hypothalmus and pituitary gland, and the receptor for TNF-α (TNF-αR) in the cerebral cortex, hippocampus and pituitary gland of the brains of mice injected with lipopolysaccharide (a mimic for sepsis). The acute-phase-response-associated cytokines also trigger the release of acute-phase proteins such as C-reactive protein from hepatocytes in the liver. Acute-phase proteins are opsonic and might help with clearance of malaria parasites.

pro-inflammatory response can be measured, and this response appears to be associated with disease severity (reviewed in Ref. 47). For example, CM caused by *P. berghei* (ANKA) infection in mice is a consequence of an inflammatory response stemming from inflammatory immune cells (Ref. 3). Studies of the *P. chabaudi* model have shown that type 1 pro-inflammatory responses

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switch to type 2 responses, typified by the appearance of IL-4-secreting Th2 cells after the peak of infection (Ref. 47).

Anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β) can be measured in human *P. falciparum* infection (Refs 28, 30, 31, 48). Regulatory cytokines are generally considered to counteract the effects of pro-inflammatory cytokines such as IFN-γ by down-regulating the effector function of cytokines like TNF-α. Indeed, lower ratios of TNF-α to the anti-inflammatory cytokine IL-10 have repeatedly been found to be associated with protection against severe malaria anaemia in human *P. falciparum* infections (Refs 49, 50, 51), and increased ratios of pro-inflammatory to anti-inflammatory cytokines have been associated with increased risk of fever (Ref. 52). Studies in mouse models demonstrate that the effects of the pro-inflammatory responses induced in malaria infection can be tempered by anti-inflammatory responses, with the induction of cytokines such as IL-10 (Ref. 53) and TGF-β (Refs 53, 54, 55). The induction of anti-inflammatory cytokines has been shown to directly alleviate hypothermia, cachexia and anaemia in mice, with very little effect on circulating parasite levels (Ref. 53), demonstrating the potentially pathogenic nature of a pro-inflammatory immune response. Paradoxically, although these studies show that TGF-β is a player in protection from pathology, there is some evidence in both human (Ref. 56) and mouse (Ref. 57) infections that participation of regulatory T cells in the immune response mounted against malaria parasites results in faster parasite growth rates, possibly via mechanisms involving TGF-β. Thus, anti-inflammatory immune responses, which may alleviate disease severity, can simultaneously result in the presence of more parasites, indicating one immunological mechanism that might play a role in the discordance between parasite number and disease severity.

Induction of pro-inflammatory immune responses by malaria parasites

The first interactions between *Plasmodium* and the host immune system are most likely to occur through dendritic cells (DCs) and other antigen-presenting cells (APCs), which respond to pRBCs by secreting TNF-α, IL-6, IL-12 and IL-18 (Ref. 58). This initially serves to activate effector cells of the innate system such as NK cells (Ref. 34), augmenting any direct NK cell activation by *Plasmodium* (Ref. 59). Cellular recognition of malaria parasites occurs via pattern-recognition receptors (PRRs) expressed on the surface of immune cells, the better studied of which are the Toll-like receptors (TLRs). TLRs recognise molecular patterns on a variety of pathogen molecules such as lipopolysaccharide (LPS) (TLR4), repetitive CpG bacterial DNA (TLR9) and fungal zymosan (TLR2) (reviewed in Ref. 60). The activation of immune cells via TLR ligation occurs via several pathways, with the principal one involving myeloid differentiation factor 88 (MyD88). The MyD88 pathway is implicated in the activation of DCs, macrophages and NK cells in both human and mouse malaria infections (Refs 34, 61, 62, 63). This pathway, alongside the production of IL-18 from macrophages, is essential for activation of human and mouse NK cells by *P. falciparum* (Ref. 34).

Several TLRs and other receptors recognising different components of *Plasmodium* have been described. Free glycosylphosphatidylinositols (GPIs) anchored on the surface of the parasite can activate mouse and human macrophages, B cells and DCs to initiate the pro-inflammatory response (Ref. 64) via TLR2 and to a lesser extent TLR4 (Ref. 62). Haemozoin pigment, formed as a by-product from the breakdown of haem in pRBCs, activates DCs through TLR9 (Ref. 65), presumably after phagocytosis since TLR9 is expressed intracellularly. In particular, recognition of malaria parasites by plasmacytoid DCs (pDCs) triggers proliferation of, and IFN-γ secretion from, γδ T cells (Ref. 61). More recently, TLR11 expressed on mouse DCs has been shown to be a receptor for profilin of *Toxoplasma gondii*, which has a homologue in *P. falciparum* (Ref. 66). However there is no TLR11 homologue predicted in the human genome, suggesting that if profilin is a TLR ligand playing a role in the induction of immune responses to pRBCs it would have to be via ligation to another PRR. TLRs 6 and 2 can be associated with the CD36 scavenger receptor (Ref. 67) found on DCs and macrophages. Since CD36 is able to bind pRBCs, as evinced by its role in mediating sequestration of pRBCs to the endothelium, this raises the possibility of parasite recognition via heterodimeric TLR–CD36 complexes. It will be interesting to see the direct effects of absence of these TLRs on immunity and pathogenesis of mouse malarias in future studies.

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The mechanics of immunopathogenesis in mouse malarial infection

Fever

Like humans, mice show altered thermoregulation in malaria infection, but develop hypothermia (Refs 53, 68, 69, 70, 71). Neutralisation of TNF-α reduced hypothermia in *P. chabaudi* infections (Ref. 53), and hypothermia was not observed in *P. berghei* (ANKA) infections of genetically engineered mice lacking the p75 TNF receptor 2 (TNFR2) (Ref. 72). However, deficiency in p55 TNF receptor 1 (TNFR1) did not affect the hypothermia induced by either *P. berghei* (ANKA) (Ref. 72) or *P. chabaudi* (Ref. 73) infections. This finding might be related to differences in the cell signalling molecules recruited intracellularly by the two different TNF receptors (reviewed in Ref. 74). Currently there are no reports of a febrile response in mouse malarias.

Although thermoregulation in mouse models of malaria has not been studied in great detail, it is tempting to speculate that there are similarities between mouse models of malaria and sepsis in thermoregulation. Sepsis in mice is associated with upregulation of acute-phase cytokines (Fig. 2) and alterations in body temperature (Ref. 75). Of particular interest, LPS injection, a mimic for sepsis, results in the upregulation of mRNA for both the IL-1 and IL-6 receptors in the hypothalamus, the organ responsible for thermoregulation (Ref. 76). As reviewed by Leon (Ref. 75), it has also been shown that the TNF-α induced in experimental sepsis in mice is responsible for hypothermia, rather than fever.

Cerebral malaria

Much of the work on malarial pathogenesis in mice has concentrated on CM, as this is the most severe clinical syndrome of human *P. falciparum* malaria. CM is most often studied using *P. berghei* (ANKA) because this species of mouse malaria induces symptoms similar to the human disease. However, different cloned lines of *P. berghei* ANKA differ in the induction of CM in mice (Ref. 70). *P. berghei* K173 is also used in the study of CM in mice, although this parasite line does not result in all the neurological signs of CM. The aptness of *P. berghei* and other *Plasmodium* infections of mice as models for CM, and details of mechanisms of pathogenesis, have recently been the subject of two extensive reviews (Refs 3, 77) and are not covered in depth here. However, since the mechanisms and host responses resulting in CM may be relevant to other complications of malaria infections, we discuss here some aspects of the pathogenesis of *P. berghei* CM.

P. berghei pRBCs and host leukocytes sequester via a number of adhesion molecules expressed on the endothelial cells lining the vasculature of the brain (Ref. 78). Sequestration of *P. berghei* ANKA is mediated, at least in part, by CD36 (Ref. 79). The sequestered parasites trigger a series of potentially immunopathological events, many of which are thought to involve macrophages (Fig. 3). Resident macrophages in the brain microvasculature can recognise sequestered parasites via PRRs and, following activation, they secrete chemokines such as CCL5/RANTES (Ref. 80), which trigger migration of additional macrophages and lymphocytes onto the endothelium (Ref. 81). Macrophages in *P. berghei* ANKA infection are activated before any sequestering leukocytes can be observed (Ref. 81), and their elimination before infection protects against CM (Ref. 82). This protection is not observed if macrophages are eliminated after infection begins and the recruiting of lymphocytes into the microvasculature has already commenced (Refs 82, 83). Mice deficient in CCR5 (the receptor for CCL5/RANTES) have a decreased susceptibility to CM (Ref. 80), and susceptible C57BL/6 mice demonstrated prolonged expression of RANTES in the brain compared with resistant BALB/c mice (Ref. 84). Sequestering cells adhere to the endothelium via adhesion molecules, and also become activated via interaction with sequestered parasites. The sequestration of macrophages in particular has been shown to be dependent on CD40 (expressed on endothelial cells, platelets and mast cells) binding to CD40 ligand (CD40L) on macrophages in *P. berghei* infection (Ref. 85). The best correlate for death from CM in this study was with macrophage sequestration, and not pRBC sequestration (Ref. 85).

Activated macrophages also amplify the acute-phase response by secreting IL-1, IL-6 and TNF-α. The sequestration of pRBCs and leukocytes is intensified by this action since, as discussed earlier, the expression of many of the adhesion molecules on the endothelium that mediate binding can be upregulated by TNF-α. Indeed, susceptible mice have been shown to have increased sensitivity to TNF-α-induced up-regulation of adhesion molecules compared with resistant animals (Ref. 86), possibly as a result of different levels of TNF receptor. Indeed the symptoms of CM in *P. berghei* have been shown

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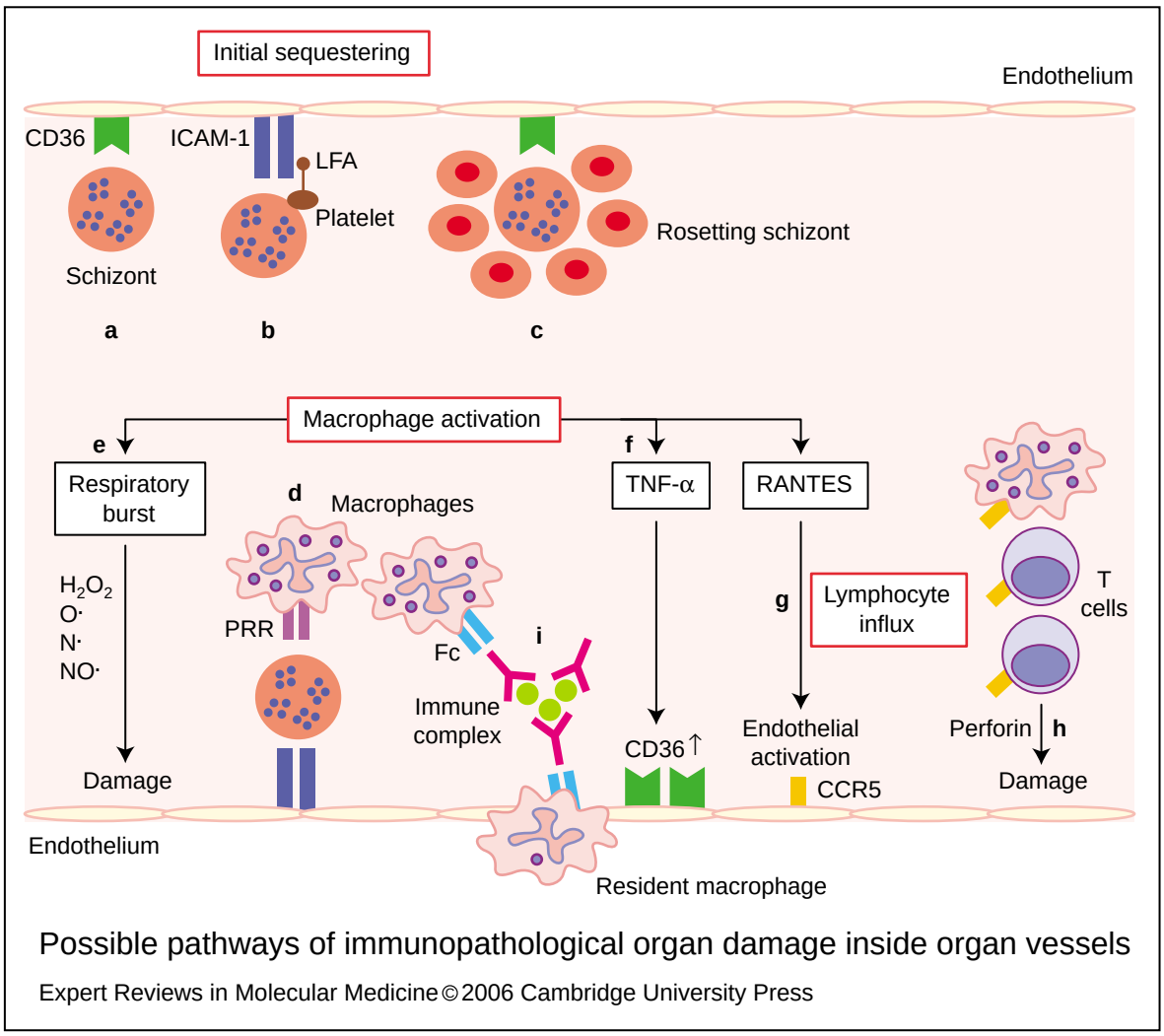

Possible pathways of immunopathological organ damage inside organ vessels

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Figure 3. Possible pathways of immunopathological organ damage inside organ vessels. Macrophages can mediate damage to the endothelial barrier via several different mechanisms. (a) Parasites may sequester via a variety of adhesion molecules and receptors such as CD36. (b) The parasitised red blood cells (pRBCs) may also stick to the endothelium via attached platelets, and (c) might be in a rosetting format. (d) Macrophages recognise pRBCs via pattern-recognition receptors (PRRs) on their surface. Activation of macrophages results in amplification of the acute-phase reaction or respiratory burst. The respiratory burst (e) can cause lesions in the endothelium. In the case of cerebral malaria this can result in haemorrhaging. (f) Tumour necrosis factor α (TNF-α) secreted by activated macrophages up-regulates adhesion molecule expression on endothelial cells, amplifying sequestration; in addition, secretion of RANTES attracts additional macrophages and other lymphocytes from the blood stream (g), and can also activate the endothelial cells to become pro-inflammatory. Incoming $CD8^+$ T cells cause damage via a perforin-dependent pathway (h), although the mechanism by which they become activated is unknown. Immune complexes are deposited on the endothelium (i), which will activate macrophages via Fc-receptors, again amplifying all of the responses described above. Abbreviations: CCR5, chemokine (CC motif) receptor 5 (receptor for RANTES); ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte-function associated antigen 1 (integrin β2; ligand for ICAM-1); RANTES, 'chemokine regulated on activation, normal T cell expressed and secreted'.

to be dependent on signalling of lymphotoxin α (LT-α), a molecule related to TNF-α, through TNFR2 (Ref. 87). Activated macrophages undergo respiratory burst, releasing superoxide anion (O_2-) and nitric oxide (NO), which could harm the endothelial barrier. However, studies in mice largely agree that NO is not the principal mediator of CM (Refs 88, 89, 90) (Fig. 3).

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Platelets can sequester on brain endothelium (Ref. 91) and contribute to CM (Ref. 92) possibly by acting as a vehicle for further sequestration of pRBCs. The contribution of rosetting to pathology of mouse malaria is little studied, with only one recent report describing rosetting in *P. chabaudi* parasites (Ref. 93).

Lymphocytes migrating to the brain ($CD4^+$, $CD8^+$ and γδ T cells), in particular $CD8^+$ T cells (Refs 83, 94, 95), have crucial roles in the development of *P. berghei* (ANKA) CM. The mechanism of $CD8^+$ T-cell-mediated endothelial damage is not known. These cells normally recognise intracellularly derived peptides presented on major histocompatibility complex (MHC) class I molecules, which are not thought to be expressed on pRBCs. Although the specificity of the $CD8^+$ T cells that migrate to the brain in CM is unclear, these cells are likely to cause damage via the perforin pathway (Ref. 94).

Immune complexes of IgG and IgE are found on the microvasculature in both human (Ref. 5) and mouse (Ref. 96) infections. In the former, complex deposition has been positively correlated with CM. Concerning the latter, the observation that infections of B-cell-deficient mice with *P. berghei* ANKA show reduced incidence of the symptoms of CM (Ref. 95) is consistent with the hypothesis that immune complexes may play a role in the pathogenesis of CM. This observation could be explained by the absence of activation of macrophages via Fc-receptor crosslinking, which may contribute to CM via amplification of the acute-phase response.

Other manifestations of malaria that might be related to host (inflammatory) responses

Sequestration of parasites on endothelium is unlikely to be restricted to the brain. Mechanisms of immunopathogenesis in organs other than the brain, and in systemic severe disease, are considerably less well characterised. However, the studies available suggest pro-inflammatory mechanisms occur that are similar to those proposed for CM.

Mouse blood-stage malaria parasites can sequester on endothelium in several organs such as the liver (Refs 97, 98), kidneys (Ref. 99) and lungs (Refs 79, 100) (Table 1), as well as accumulating in the spleen (Ref. 101). Like sequestration of *P. berghei* in the brain, much of the parasite sequestration in the liver and lungs appears to be mediated by CD36 (Refs 79, 101). Although *P. berghei* has not been described as sequestering in the liver, damage to the liver in *P. berghei* infection does occur (Ref. 102) and appears to be mediated by IL-12 generated via the MyD88 pathway (Refs 63, 102).

Nephritic damage is a complication of malaria in humans infected primarily with *Plasmodium malariae* (Refs 15, 103). Although mild nephropathies occur in *P. falciparum* and *P. vivax* infection, they appear to be related to renal failure resulting from hypovolaemia, and are reversible by rehydration. Very little is known of the mechanisms involved in nephritic damage in *P. malariae* infections, although cytokines and immune-related responses are suggested by several observations in human infections (reviewed in Ref. 103) and in two studies of *P. berghei* infections in mice (Refs 104, 105). In *P. berghei* infections, the glomeruli of the kidneys were seen to be infiltrated with macrophages producing cytokines involved in the acute-phase reaction. Furthermore, the levels of these cytokines were positively correlated with proteinuria (a measure of kidney damage). Expression of the anti-inflammatory cytokine IL-10 was also observed in both of these studies, although an inverse correlation with protection from proteinuria was found only in the latter study (Ref. 105). Similar to immune complex deposition in the vasculature of the brain in CM, immune complexes containing parasite antigens are deposited in the glomeruli of the kidney in mice infected with *P. berghei* (Ref. 96). Desposition of immune complexes in the kidneys is involved in the pathology of several other inflammatory conditions, most notably systemic lupus erythromatosis, although in this case the immune complexes are against self-antigen rather than exogenous antigen. In one model of immune nephritis, initiation of disease by immune complexes was dependent on Fc-mediated respiratory burst on bone-marrow-derived cells (Ref. 106). To our knowledge, no studies have utilised animals deficient in Fc-receptors to assess whether a similar mechanism might contribute to kidney-specific pathology in malaria infection.

Severe malarial anaemia

Several mouse models have been used to study malarial anaemia (Table 1), and a variety of factors have been shown to be important: haemolysis coupled with dyserythropoiesis; bone marrow

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and splenic haematopoietic cellularity; and altered levels of a range of cytokines, chemokines and hormones. Maximum anaemia occurs after the peak of parasitaemia in mouse models studied (Refs 107, 108), which suggests that premature RBC destruction is not the major contributory factor (Table 2). As with *P. falciparum* infection, reticulocytosis is increased in non-lethal mouse infections, which eventually leads to recovery from anaemia by boosting the number of circulating RBCs (Refs 107, 109). However, anaemia is more severe in lethal mouse infections, resulting in death before the onset of reticulocytosis (Refs 108, 110).

Erythropoietin (EPO) is central to recovery from anaemia in both human and mouse infections, and an elevated level of serum EPO is a physiological indicator of an increased rate of erythropoiesis. It was therefore postulated that an inadequate EPO response in malaria causes anaemia (Refs 111, 112, 113). However, during *P. chabaudi* infection the upregulation of EPO levels seems to be adequate to replace the loss of RBCs and instead it appears that there is a suboptimal response of erythropoietic progenitors to EPO stimulation (Ref. 110), the mechanisms of which remain unclear.

Accelerated clearance of non-parasitised RBCs (npRBCs) in *P. falciparum* infections has also been suggested as a causal factor (Refs 23, 114). More recently, this has been shown in a semi-immune mouse model and offers an explanation as to why anaemia can be disproportional to parasitaemia (Ref. 115). In addition, the sequestration and accumulation of pRBCs in bone marrow (Ref. 116) and spleen could play a role in altering erythropoiesis in infection, either by stimulating local inflammatory and cytokine responses and thereby blocking erythropoiesis directly or by reducing the number of supportive stromal niches. It has also been proposed that infected RBCs adhere in the spleen via proteins encoded by the multigene family *pir*. This would lead to the release of merozoites in a reticulocyte-rich environment and thus enhance infection of the population of RBCs required to compensate for anaemia (Refs 117, 118), at least in infections with malaria parasites that invade reticulocytes (*P. vivax* and *P. yoelii*).

In mouse malarias, a decrease in bone marrow cellularity of up to 75% is observed (Ref. 119), reflecting a decrease in erythroid progenitor populations, with greater reduction as disease severity increases (Refs 120, 121). TNF-α (Ref. 122) and IFN-γ (Ref. 123) were initially suggested to be responsible, but antibody neutralisation of either or both had no effect on the differentiation of multipotent progenitors to erythroid precursors in vitro (Ref. 123). TNF-α is more likely to have an effect on anaemia in partnership with IL-10 (Refs 50, 51), as IL-$10^{-/-}$ *P. chabaudi*-infected mice displayed increased anaemia (Ref. 124), an effect that was reversed in the presence of anti-TNF-α (Ref. 53). Additionally IL-12 appears to stimulate erythropoiesis and is found in increased amounts in non-lethal, compared with lethal, mouse infections (Refs 125, 126). Another cytokine, macrophage-migration inhibition factor, has been identified as an in vitro haematopoietic

Table 2. Mechanisms leading to anaemia in malaria

Mechanism	Details	Refs
Destruction of pRBCs	Haemolysis: parasite maturation, haemoglobin metabolism by the parasite Immune recognition: parasite antigen present on RBC surface	162
Destruction of npRBCs	Reticuloendothelial hyperplasia: increased removal of RBCs from circulation Immune recognition: uptake of circulating parasite antigen by npRBCs	163, 164
Erythropoietic suppression	Decreased RBC production resulting from pro-inflammatory cytokine production, reduced response to EPO, other factors?	162
Dyserythropoiesis	Abnormal Fe^{2+} incorporation, inefficient maturation	162

Abbreviations: EPO, erythropoietin; npRBC, non-parasitised red blood cell; pRBC, parasitised red blood cell.

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suppressor, with elevated levels identified during mouse infection (Ref. 127).

Simultaneous with decreased bone marrow cellularity, foci of extramedullary haematopoiesis emerge in the spleen, accompanied by splenomegaly and a 60-fold increase in erythroid progenitor populations (Refs 107, 119, 128). In contrast to the bone marrow, an increase of splenic progenitors is inversely proportional to disease severity, indicating that an insufficient increase in splenic erythropoiesis is a crucial determinant of the fatal outcome of malaria infection (Ref. 121). In this case, the differences between lethal and non-lethal infections, such as the ability of erythroid precursors to home to the spleen or the erythroid-supportive capability of stromal cells, might help elucidate the situation. However, the precise factors involved remain unknown.

Despite the upregulation of splenic erythropoiesis, total production of RBCs is still not sufficient to prevent anaemia. This could be due to the production of abnormal RBCs, (dyserythropoiesis), which is displayed in both humans (Ref. 129) and mouse (Ref. 130) malarial anaemia. Initially, TNF-α was a candidate for this because injection of infected mice with TNF-α resulted in dyserythopoiesis in vivo (Refs 122, 130). However, infected TNFR1 knockout mice display no differences in anaemia, compared with wild-type mice (Ref. 73), and antibody neutralisation of TNF-α had no effect on erythropoiesis (Ref. 123). These studies demonstrate that much work is required to exploit mouse models for the study of malaria anaemia.

Host and parasite variation: contributions to immunopathology

Variation in malarial disease

The causes of the variability in human malarial disease are numerous: both humans and malaria parasites are genetically variable and both could account for some of the differences in disease severity (Ref. 131). In addition, age and environmental factors such as differences in dynamics and intensity of transmission will also influence disease outcome (Refs 132, 133). There have been attempts to disentangle the roles of the possible determinants in malaria disease in humans by statistical approaches (Ref. 131), but the relative contributions of host and parasite genetics, and of parasite-mediated damage and immunopathology, have been almost impossible to establish from human studies.

Host genetics

In humans, host factors associated with disease severity include genetic polymorphism in the gene encoding the RBC protein haemoglobin (reviewed in Ref. 134) or in genes encoding components of the immune response (Table 3). In mice, most studies on the role of anti-parasite immune responses in pathology have utilised infections with single species and/or clones in different strains of mice known to differ in their susceptibility to infection and pathology. For example, the *P. berghei* model for CM is highly dependent on host genetics since only CBA and C57BL/6 mice (or a background containing C57BL/6 genes) develop symptoms of CM (reviewed in Ref. 3).

P. chabaudi (AS) is commonly used in immunological studies where investigators have made use of AJ, C3H/He or BALB/c mice (susceptible) and compared the responses and infection phenotype to C57BL/6 or CBA mice (resistant). Genetic crosses between susceptible and resistant mouse strains have identified several *P. chabaudi* resistance (*CHAR*) loci. *CHAR2* (Refs 135, 136) and *CHAR4* (Ref. 135) are involved in the control of parasite replication, *CHAR1* and *CHAR2* partly determine susceptibility to death in *P. chabaudi adami* infection (Ref. 136), and *CHAR3* seems to contribute to clearance of blood-stage parasites (Ref. 137). There are several candidate genes within the *CHAR* loci that could contribute to these experimental observations. *CHAR4* contains genes that encode transcription factors involved in pro-inflammatory signalling pathways, such as Wnt and NF-κB. Pro-inflammatory responses have been shown to play a role in parasite replication rate in *P. chabaudi* (discussed below). The gene encoding vascular cell adhesion molecule 1 (VCAM1) is also contained in the *CHAR4* locus and could contribute to sequestration of pRBCs, thus affecting the measured peripheral parasitaemia. *CHAR2* contains the gene encoding the NK-activating cytokine IL-15 as well as several genes encoding erythrocyte structural proteins (Ref. 135) that might affect the efficiency of invasion and growth rate of *P. chabaudi*. *CHAR3* incorporates the mouse *H2* locus, which includes the gene encoding TNF-α.

Comparison of the immune responses mounted by resistant and susceptible mice in response to *P. chabaudi* have revealed that pro-inflammatory responses play a role in controlling parasitaemia and that the initial cytokine response is crucial. Whereas resistant mice mount an early type 1

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Table 3. Polymorphic immune response genes associated with malarial disease

Immune component	Function in malaria	Evidence for polymorphisms
Tumour necrosis factor α (promoter and receptor)	Pro-inflammatory cytokine; endogenous pyrogen	Refs 41, 42, 165, 166
Inducible nitric oxide synthase	Generates nitric oxide from L-arginine	Refs 167, 168
Interleukin 1β	Component of the acute-phase response; involved in fever induction	Refs 169, 170
Interleukin 12 p40 (Interleukin 12) (Interleukin 23)	IL-12: induction of pro-inflammatory responses IL-23: not yet studied in malaria, but induces $CD4^+$ T cells to produce IL-17, IL-6 and TNF-α	Ref. 171
Interleukin 4	Th2 cytokine promoting plasma cell formation and antibody secretion	Ref. 172
Interleukin 13	Anti-inflammatory Th2 cytokine promoting resistance in several other protozoan infections, but function in malaria unknown; promotes B-cell switching to IgE	Ref. 173
Human leukocyte antigens	Present peptides to the immune system	Ref. 174

Abbreviations: IgE, immunoglobulin E; IL, interleukin; Th2, T-helper 2; TNF-α, tumour necrosis factor α.

response, characterised by IL-12 and IFN-γ, followed by a type 2 response after approximately 3–4 weeks, susceptible mice appear to induce type 2 cytokines, such as IL-5, from the beginning of an infection (Ref. 47). Susceptible animals can be 'rescued' by recombinant IL-12 (rIL-12) (Ref. 138), but removal of IFN-γ and TNF-α by monoclonal antibody treatment removes the beneficial effects of rIL-12 treatment, resulting in mortality (Ref. 138). Thus, a defect in IL-12 production may underlie the failure of A/J animals to mount a type 1 response to *P. chabaudi* and control parasitaemia. Indeed macrophages from susceptible A/J mice are less able to phagocytose pRBCs and produce lower levels of TNF-α and IL-12 in response to pRBCs when compared with macrophages from resistant mice (Refs 139, 140). The protective IFN-γ and TNF-α in rIL-12-treated mice have been shown to come from both $CD4^+$T cells (Ref. 133) and NK cells (Ref. 141).

Anti-inflammatory cytokines can also determine susceptibility of different mouse strains to *P. chabaudi*. Susceptible BALB/c mice have an early transient increase in TGF-β, which correlates with decreased IFN-γ production (Ref. 142). Removal of TGF-β in BALB/c mice by antibody treatment converted the susceptibility phenotype to a resistant one, and the opposite was true of resistant C57BL/6 mice given rTGF-β (Ref. 142). As expected, in both cases resistance correlated with elevated pro-inflammatory immune responses. However, too much of an inflammatory response can result in pathology. Resistant C57BL/6 animals deficient in IL-10 become susceptible to *P. chabaudi* infection, and approximately 50% of these animals die from TNF-α-induced pathology (Ref. 53). Thus, the timing and amount of regulatory cytokine produced can be crucial in mounting a pro-inflammatory response of an appropriate magnitude.

Altogether, these data suggest that inadequate pro-inflammatory responses may result in an uncontrolled parasite multiplication rate and death, whereas uncontrolled pro-inflammatory responses can result in immunopathology. It will be important to determine whether similar mechanisms operate in human infections.

Parasite genetics

Parasite factors also contribute to variation in disease severity in malaria. Genetic variation in the ability of pRBCs to adhere to host endothelium

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(Ref. 143) or to bind to uninfected cells (Ref. 93) could possibly affect parasite virulence. In addition, interaction of different strains of parasites with the host innate and acquired immune system can affect the outcome of the immune response, and mixed infections with more than one species (Refs 74, 114, 144, 145, 146) or clone (Ref. 147) can affect the severity of disease. Thus, parasite genetics may in fact play a role in variation of malarial disease.

Cloned lines of *Plasmodium* with differing virulence in the mouse can be used to dissect and identify important components involved in severity of malaria. The cloned lines 17XL and 17XNL of *P. yoelii* differ in their ability to induce mortality (or not), and have been used to investigate immunological processes leading to lethal infection (described below). Clones of *P. chabaudi* (Ref. 25) differ in virulence in C57BL/6 mice (Ref. 148) and offer an opportunity to study the regulation of non-lethal immunopathology. This might be more akin to the situation in the field, where most people survive malaria infection but the amount of pathology varies from person to person.

The mechanisms by which different parasite isolates could cause differences in disease severity are unknown. However it is tempting to speculate that there might be variation in the nature or amount of the parasite-derived molecules that trigger host innate and acquired immune responses through PRRs, resulting in differences in the regulation of inflammatory responses and host tissue damage. Indeed, parasites isolated from children with acute malaria vary in their ability to induce TNF-α from mononuclear cells (Ref. 149), although possible contamination of these isolates with *Mycoplasma* was not eliminated in this study and might confound this observation (Ref. 150). The magnitude or kinetics of inflammatory cytokine responses could influence up-regulation of adhesion molecules on the endothelium, and thus parasite sequestration, which may be enhanced by a higher rosetting ability in more virulent parasites, at least in *P. chabaudi* infection (Ref. 93).

The observation that parasite variation results in differences in innate immune responses is not confined to *P. falciparum*. Omer and Riley (Ref. 54) were able to show that different species of mouse malaria induce different amounts of TGF-β from mouse splenocytes, and different clones of *P. chabaudi* can induce quantitatively different responses in $CD11c^+$ DCs (T.J. Lamb, C. Voisine and J. Langhorne, unpublished). Beyond malaria, clones of *Toxoplasma gondii* that differ in virulence have been shown to induce differing amounts of IL-12 from mouse macrophages in a TLR-dependent fashion (Ref. 151), and several strains of *Mycobacterium tuberculosis* induced qualitatively different responses from human monocytes, possibly due to differences in lipid composition (Ref. 152).

Studies into the regulation of immune responses during infection with different clones of mouse malaria parasites have thus far yielded similar results to studies using different mouse strains. Although IFN-γ can be measured in lethal *P. yoelii* infection, it is of a lower magnitude than in non-lethal infection (Refs 55, 153). Early production of regulatory cytokines such as TGF-β (Ref. 55) or IL-10 (Ref. 154) has also been linked with fatality and increased parasite growth rate in the *P. yoelii* model. Depletion of regulatory cytokines in lethal *P. yoelii* infections prolongs survival of the host (Ref. 55), again agreeing with work carried out on *P. chabaudi* using different mouse strains (Ref. 142). Interestingly, the cells activated to produce TGF-β in *P. yoelii* lethal and non-lethal parasites were shown to differ in infections of C57BL/6 mice (Ref. 55). The data from parasite and mouse strains agree somewhat that the induction of a pro-inflammatory response early on in infection is crucial for protection against disease, but that too little inflammation can lead to uncontrolled parasite growth and death.

Clinical implications/applications

There are many efforts to develop anti-malarial vaccines, priming the immune system for defence against incoming parasites and/or the effects of damaging immune responses elicited during infection ('anti-toxin' vaccines). We have reviewed some of the evidence that some symptoms of malaria are due to immunopathology. Since the aim of a vaccine is to stimulate an immune response against incoming parasites, it is important that alterations in the responses mounted by vaccinated individuals should not magnify any responses that might be harmful in excessive amounts. IL-12, for example, is effective when elicited in an appropriate amount: too much or too little in C57BL/6 mice results in an adverse outcome (Ref. 138). Likewise, immunological responses that provide some protection against immunopathology in malaria infections, such as the induction of TGF-β from T regulatory cells (Refs 53, 54, 55), also serve to increase parasite growth rate.

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The premise that vaccines could make malaria infection worse is not just hypothetical. Jennings et al. (Ref. 155) carried out a mouse vaccination trial priming with dead whole blood-stages in *P. berghei* ANKA infections of C57BL/6 (susceptible) and A/J (resistant) animals. This vaccine increased the incidence of lethal CM in resistant AJ animals but was protective in susceptible mice, also demonstrating that host variation needs to be considered in designing a malaria vaccine.

Parasite variation could pose problems on several counts. Both clonal and somatic mechanisms of antigenic variation in malaria parasites imply that, for any vaccine to target parasites successfully, it will have to be effective against all the existing variants in populations of malaria parasites. Furthermore, a vaccine will have to consider thresholds of vaccination parameters, such as the critical number of people in the target population that need to be vaccinated, to reduce or eliminate opportunities for the parasite to evolve around the vaccine. Selection pressures such as vaccines could result in selection for more virulent parasites (Refs 156, 157). Therefore, it is essential that virulence management strategies are implemented (Ref. 158).

Studies such as these suggest caution in developing whole attenuated organisms as potential vaccines. Without a detailed knowledge of the variation in host responses and induction of parasite virulence, intervention could be detrimental rather than protective.

Research in progress and outstanding research questions

Several important questions are currently the focus of research into the immunopathogenesis of malaria. First, linear correlations between circulating parasite number and disease severity are almost never found: which immunological parameters mediate the discordance between parasitaemia and disease severity? Second, the role of immunopathogenesis in CM needs to be better understood. How do lymphocytes such as $CD8^+$ T cells mediate the symptoms of CM in mice? What is the specificity of these cells and how do they become activated? Third, although it is clear that haemolysis, caused by parasite maturation, is not the main cause of anaemia, the contribution of immunopathogenesis to anaemia is uncertain. Cytokine balance, immune destruction of npRBCs and the erythroid response to EPO are all likely to be involved; however, the mechanisms remain undefined and should be the focus of future research using appropriate mouse models. A fourth key concern is how multiple infection with malaria parasites might modify virulence of infection. How do genetically variable malaria parasites interact with the immune system of the host to cause differences in the severity of infection?

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Further reading, resources and contacts

Websites

The website of the World Health Organisation and the Centers for Disease Control and Prevention collate all the data from malaria-affected areas and so offer a good resource on up-to-date epidemiology, as well as the latest medical information on what is being done to combat malaria:

http://www.who.int/topics/malaria/en/
http://www.cdc.gov/malaria/

The National Institute for Medical Research website details current research into the malaria parasite:

http://www.nimr.mrc.ac.uk/parasitol/

Animated lifecycle of a malaria parasite in *Expert Reviews in Molecular Medicine*:

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Features associated with this article

Figures

Figure 1. *Plasmodium* life cycle.
Figure 2. The acute-phase reaction.
Figure 3. Possible pathways of immunopathological organ damage inside organ vessels.

Tables

Table 1. Mouse models of malaria.
Table 2. Mechanisms leading to anaemia in malaria.
Table 3. Polymorphic immune response genes associated with malarial disease.

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