REVIEW ARTICLE

Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease

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SUMMARY

Cerebral malaria is a life-threatening complication of malaria infection. The pathogenesis of cerebral malaria is poorly defined and progress in understanding the condition is severely hampered by the inability to study in detail, *ante-mortem*, the parasitological and immunological events within the brain that lead to the onset of clinical symptoms. Experimental murine models have been used to investigate the sequence of events that lead to cerebral malaria, but there is significant debate on the merits of these models and whether their study is relevant to human disease. Here we review the current understanding of the parasitological and immunological events leading to human and experimental cerebral malaria, and explain why we believe that studies with experimental models of CM are crucial to define the pathogenesis of the condition.

Key words: cerebral malaria, murine model, pathogenesis.

INTRODUCTION

Malaria remains a major public health problem in many tropical countries. The World Health Organization (WHO) estimates that 40% of the world's population lives in areas affected by malaria, resulting in approximately 200–300 million clinical cases each year, leading to the deaths of more than 2 million young children every year, mainly in sub-Saharan Africa. The vast majority of cases of severe malaria are caused by infection with the *Plasmodium falciparum* species of the parasite. Clinical presentations of severe malaria vary but include altered consciousness, respiratory distress, severe anaemia (haemoglobin level of <5 g/dl), multi-organ failure and cerebral malaria. The WHO definition of CM is unrousable coma (graded according to either Blantyre or Glasgow coma scale) not attributable to other causes (Teasdale and Jennet, 1974; Molyneux *et al*. 1989). In areas of high malaria transmission, susceptibility to severe malaria varies with age and exposure to the parasite; adults are, in general, resistant to severe malaria whilst infants and very young children are at significantly increased risk of developing severe malarial anaemia. Older children, who have had at least one previous malaria infection, are disproportionately at risk of developing cerebral malaria (CM) (Marsh and Snow, 1999). The epidemiology of severe malaria is highly suggestive of a role for the immune system in both initiation of (in children) and protection from (in adults) cerebral malaria, either indirectly – by selecting for infection by parasites of differing virulence – or directly – by contributing to the pathogenesis of the syndrome.

CEREBRAL MALARIA

The incidence of cerebral malaria is difficult to assess, but hospital admission records indicate that in the region of 1% of all *P. falciparum* infections progress to CM, which is fatal in 10–20% of all cases (300 000–500 000 deaths each year). Moreover, at least 10–20% of individuals who survive and recover from CM display long-term physical or cognitive dysfunction (Carter *et al*. 2005*a*, *b*; Idro *et al*. 2005; Boivin *et al*. 2007). Since the discovery by Marchiafava and Bignami in 1894 of malaria parasites within the brain of humans during infection, attention has focussed on understanding the pathophysiological processes that predispose towards

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CM, with a view to the development of preventative measures or targeted therapies for the condition. Contrasting theories on the roles of parasite sequestration within the brain and the host immune response to the parasite in the pathogenesis of CM were initially proposed (reviewed by Van der Heyde *et al*. 2006), with current understanding suggesting that neuropathology is the result of a combination of both processes, as discussed below.

Cerebral malaria can develop rapidly after initial bouts of fever lasting 2–3 days. Coma is the standard definition of CM, but other symptoms associated with the condition include general malaise, headache, fits, vomiting and diarrhoea. The clinical symptoms associated with early-stage CM are not pathognomonic for the condition and are difficult to differentiate from encephalitis, meningitis and febrile convulsions. This has implications for the rapid and early diagnosis of the condition, which often significantly delays the initiation of treatment. The early symptoms of CM can progress rapidly to increased intracranial pressure, hemiparesis, ataxia and coma if immediate medical treatment is not provided. The diverse set of neurological complications associated with CM indicates that multiple areas of the brain are affected by the condition.

Anti-malarial drug-based therapies are the first-line treatment for patients with cerebral malaria; however, the incidence of neurological deficiencies and mortality remain unacceptably high with fatality rates of around 15% following treatment with Artemisinin compared with 20% for traditionally used quinine-based treatments (Dondorp *et al*. 2005*a*). This is unsurprising as anti-malarial therapy can only be implemented when CM is first suspected or diagnosed at health care centres; CM is at an advanced state in the majority of these individuals and anti-malarial drugs by themselves are often insufficient to reverse and alleviate the symptoms of CM. Therefore, there is an urgent need to develop adjunctive therapies, such as immuno-modulators or neuro-protective agents that may be administered with anti-malarials. At present the lack of understanding of the pathogenesis of CM means that the potentially most efficacious targets for therapeutic intervention remain to be identified.

THE PATHOLOGY AND ASSOCIATED CLINICAL FEATURES OF CM

Post-mortem examinations of brains from individuals that succumbed to CM have helped to uncover the type and distribution of brain pathology that occurs during the condition. Some of the most commonly reported findings include swelling and haemorrhaging in the white matter of the subcortical rim and corpus callosum as well as petechial and ring haemorrhages in both cerebral and cerebellar cortices (reviewed by Haldar *et al*. 2007). In the majority of cases, histopathological examinations reveal cerebral capillaries plugged with parasitized erythrocytes (reviewed by Haldar *et al*. 2007). Margination of monocytes and macrophages within cerebral vessels and the presence of pigmented macrophages sequestered with pRBC are also well described features of CM (Patnaik *et al*. 1994). Due to the lack of detailed comparative histopathological studies of pediatric and adult CM cases it is difficult to conclude whether the pathology of CM varies between children and non-immune adults, but as there are a number of differences in the symptoms of pediatric and adult CM, it is possible there may be some age-related differences in cerebral pathology (Mishra and Wiese, 2009).

Although parasite sequestration, haemorrhages and inflammation are found in the majority of CM brains, it is clear that CM is not a homogenous syndrome. For example, 3 different patterns of histopathological changes have been described in African children: in addition to the 'classical' pattern of CM of parasite sequestration, perivascular haemorrhages and immune cell infiltration within brain micro-vessels, parasite sequestration may be observed within the brain in the absence of any other abnormalities and there are cases where individuals with high peripheral parasitaemia develop a syndrome that is clinically defined as CM but where there is no evidence of parasite sequestration within the brain (Clark *et al*. 2003; Taylor *et al*. 2004). The reasons for the variations in pathology of CM are unclear but may be due to genetic variation in hosts or parasites, environmental factors or the host immune response to the parasite.

THE LIMITATIONS OF STUDIES OF HUMAN CEREBRAL MALARIA IN DEFINING THE PATHOGENESIS OF THE CONDITION

Cerebral malaria is likely the result of a complex sequence of inter-related events, most probably beginning either with sequestration of trophozoite-infected red blood cells (pRBC) in the small blood vessels (reviewed by Chakrovorty *et al*. 2008) and/or with the rupture of infected red blood cells and the release of parasite-derived toxins (Bate and Kwiatkowski, 1994; Schofield *et al*. 1996). The relative importance of systemic versus brain-localized events – including pRBC sequestration and rupture, lymphocyte, monocyte, endothelial and glial cell activation and release of inflammatory mediators – their sequence and timing in the pathogenesis of CM are very much unknown. For obvious reasons, histopathological examination of CM brains is limited to post-mortem analysis of fatal cases and it is thus not possible to describe the sequence of events leading to the onset of CM symptoms nor to compare fatal cases with those that resolve in response to treatment. Such investigations and comparisons are

essential to delineate truly pathogenic systemic and intra-cerebral processes from neutral and/or protective responses. Increased utility of non-invasive *in vivo* imaging techniques, such as magnetic resonance imaging (MRI) and spectroscopy (MRS) and computational topography (CT), should hopefully help to address these issues (Kampfl *et al*. 1993; Crawley *et al*. 1996; Patanker *et al*. 2002; Penet *et al*. 2005, 2007), but these studies are severely restricted by ethical constraints and the availability of the expensive specialized equipment in malaria-endemic areas. It is therefore extremely difficult to move beyond purely descriptive and correlative studies in humans: defining the immunological pathways and parasite-driven processes that underlie the pathogenesis of the syndrome, and demonstrating causality, is difficult without direct intervention studies. Moreover, examination of peripheral blood (which is possible in non-fatal as well as fatal cases) may provide limited information on the immunological and parasitological environment in the brain and, again, patients usually present to hospital only once the syndrome is well-established. For example, peripheral blood parasitaemia does not always accurately predict total parasite biomass (Silamut *et al*. 1999) and total parasite biomass is a stronger correlate of severe malarial disease than is peripheral parasitaemia (Dondorp *et al*. 2005*b*). It is clear that other approaches – in combination with human studies – are required to fully understand the pathogenesis of CM.

EXPERIMENTAL MODELS OF CEREBRAL MALARIA

Much of our understanding of mammalian physiology has come from studies of animals and the extent of the conservation of basic immunological and neuropathological processes between laboratory rodents and humans is becoming ever more apparent (Hau and Van Hoosier Jr, 2005). Experimental models have proven invaluable for understanding the pathogenesis of numerous autoimmune and infectious diseases of humans and many vaccines and immune-therapies currently in use were initially developed and tested in experimental models (Hau and Van Hoosier Jr, 2005). It is likely, therefore, that the use of relevant experimental animal models can significantly aid in the study of cerebral malaria. Primate models of CM, including *P. knowlesi* and *P. coatneyi* infections in Rhesus monkeys (Aikawa *et al*. 1992; Ibiwoye *et al*. 1993) and *P. falciparum* infection in squirrel monkeys (Gysin *et al*. 1992), have allowed the investigation of some aspects of CM, but these models are prohibitively expensive and are restricted to low numbers for ethical reasons. Consequently, other experimental models are required. Neuropathological syndromes have been shown to develop in certain strains of inbred mice infected with various strains of *Plasmodium berghei* (Pb) (Rest, 1982; Curfs *et al*. 1993*a*) or the lethal (XL) variant of *P. yoelii* (PyXL) (Yoeli and Hargreaves, 1974); however, there has been – and continues to be – significant disagreement within the malaria research community as to whether the murine models share sufficient similarities with human cerebral malaria to make them relevant or useful. In the remainder of this review we will evaluate the currently available models of ECM and we will attempt to resolve the relevance of experimental models of cerebral malaria to human infection.

Plasmodium yoelii *XL and* Plasmodium berghei *K173*

Although more extensively studied as a model of hyperparasitaemia and failure of parasite control (Couper *et al*. 2007, 2008), PyXL has been shown to sequester within the brain microvasculature and produce a cerebral syndrome comparable with human cerebral malaria (Yoeli and Hargreaves, 1974; Kaul *et al*. 1994); however, the hyper-parasitaemia associated with this infection (rapidly ascending peripheral parasitaemia that can reach 80–100%) is not typical of human CM cases (Silamut *et al*. 1999) and this model is not widely used to study CM. In a few studies, *P. berghei* K173 has been found to induce CM-like signs (Curfs *et al*. 1993*a*; Mitchell *et al*. 2005), but the dose-dependent onset of ECM in this model (inducing cerebral pathology after low dose but not high dose infection) (Mitchell *et al*. 2005) also limits its utility as a model of human CM: indeed *P. berghei* K173 is frequently used as a non-ECM-infection to compare with the most widely used model of ECM, *P. berghei* ANKA infection (Mitchell *et al*. 2005).

Plasmodium berghei *ANKA*

The *Plasmodium berghei* ANKA (PbA) model replicates many events seen during human CM and is accepted as the best available experimental model of cerebral malaria. Infection of susceptible strains of mice, including C57BL/6 and CBA, leads to the development of fatal cerebral pathology, with clinical signs including ataxia, fitting, respiratory distress and coma (de Souza and Riley, 2002). The time to onset of clinical signs varies depending on the infection dose, the genetic background of the host and the specific clone of infecting parasites, but is typically between 5 and10 days post-infection (de Souza and Riley, 2002). As in humans, there is a rapid deterioration in the condition of infected animals once clinical signs become apparent, with death often occurring within 4 or 5 h after the onset of neurological signs. Multiple areas of blood-brain barrier disruption with vascular leakage involving the cortex, cerebellum and olfactory bulb are observed in brains of PbA-infected mice displaying signs of ECM

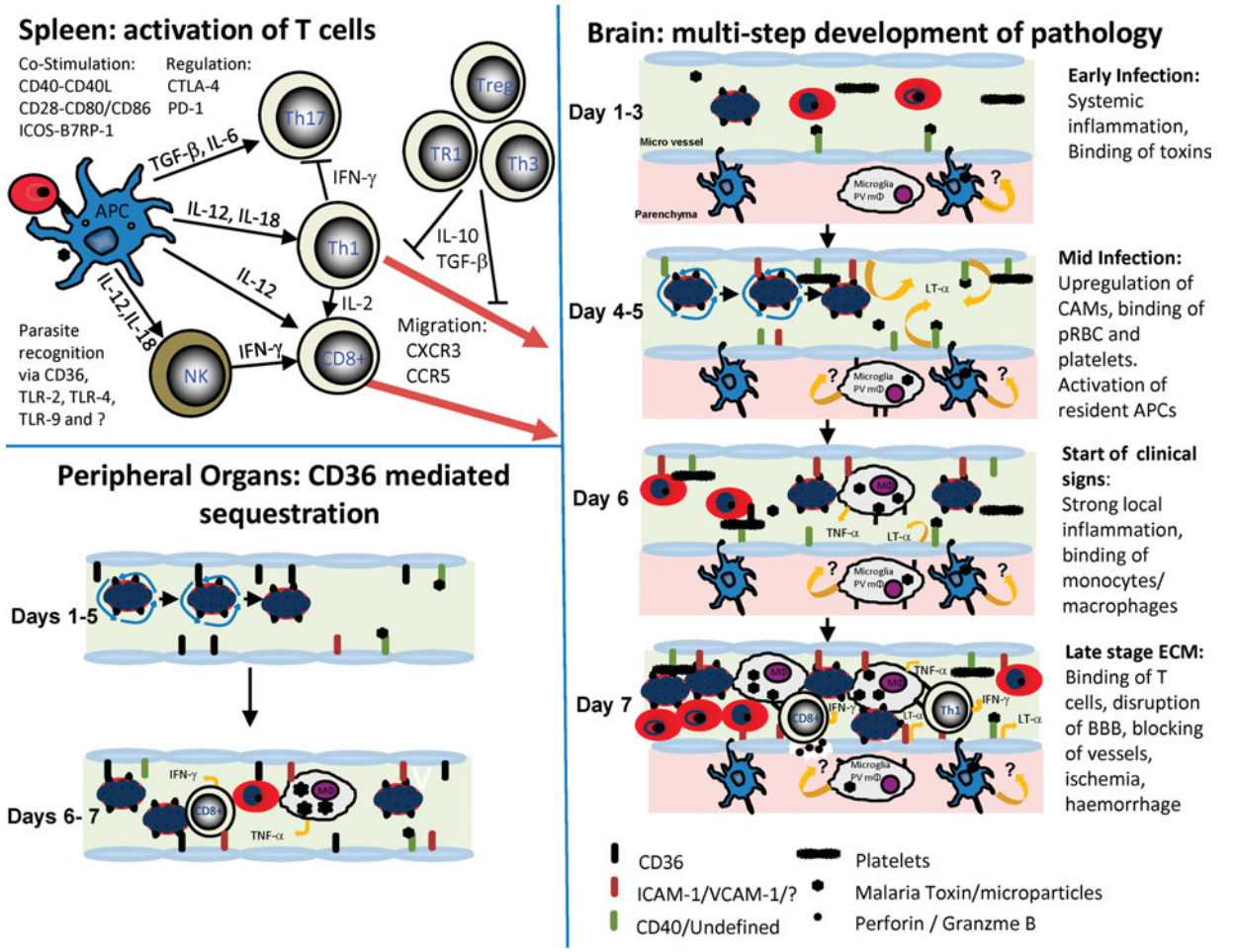

Fig. 1. A hypothetical schema of events leading to the development of experimental cerebral malaria. Rupture of pRBCs releases molecules that activate brain microvascular endothelial cells leading to upregulation of receptors for pRBC. Phagocytosis of parasite moieties in the spleen and liver, priming lymphocytes within the spleen, promotes systemic inflammation which further amplifies endothelial cell activation in the brain and activates brain-resident perivascular macrophages, microglia and astrocytes. pRBCs bind to endothelial receptors; platelets binding to endothelial receptors may provide additional ligands for pRBC adherence. Activated endothelial and glial cells provide chemotactic signals for lymphocytes and myeloid cells. Sequestered pRBCs and leukocytes interfere with cerebral blood flow and, together with cytotoxic molecules, damage the blood-brain barrier leading to oedema and haemorrhage.

(Penet *et al*. 2005; Lackner *et al*. 2006; de Souza and Couper, unpublished observations), with loss of specific neuronal populations within the cortex and striatum (Clark *et al*. 2005), accumulation of pRBC within blood vessels (Rest, 1982; Hearn *et al*. 2000) and focal perivascular inflammation (Engwerda *et al*. 2002). ECM is also associated with the significant accumulation of platelets within the brain vasculature (Wassmer *et al*. 2003; von Zur Muhlen *et al*. 2008): platelets have been shown to directly promote endothelial cell damage during infection (Wassmer *et al*. 2006). Cognitive dysfunction during *P. berghei* ANKA infection, as shown by impaired visual memory, is directly correlated with haemorrhage and inflammation, including microglial activation (Desuisseaux *et al*. 2008). Indeed, accumulation of monocytes and macrophages, and activation of brain resident mononuclear cells, including astrocytes and microglial cells is believed to be a key feature of ECM (Grau *et al*. 1987; Medana *et al*. 1997*a*, *b*; Pais and Chatterjee, 2005) (Fig. 1).

As in humans, genetic and environmental factors determine the susceptibility of mice to ECM. For example, the resistance of F1 intercrossed BALB/c (resistant) and C57BL/6 (susceptible) mice to the development of ECM is determined by age, and environmental exposure, with young mice (8–10 weeks) susceptible to ECM and older mice (16–20 weeks) resistant to the development of cerebral signs (Hearn *et al*. 2000). Genetic resistance to ECM and *P. berghei* ANKA infection has been mapped using intercrossed resistant and susceptible strains of mice to loci on chromosomes 1, 9, 11, 17 and 18 (Bagot *et al*. 2002; Nagayasu *et al*. 2002; Ohno and Nishimura, 2004; Campino *et al*. 2005). However, the genes encoded within each of these regions that control resistance to ECM and parasite levels remain to be identified. More recently, micro-array profiling

of susceptible and resistant strains of mice have identified distinct expression profiles in the brain of genes involved in metabolic energy pathways, immune-activation, apoptosis and neuroprotection/neurotoxicity (Delahaye *et al*. 2007; Lovegrove *et al*. 2007; Oakley *et al*. 2008). Differences in the immune response of ECM-susceptible and ECM-resistant strains of mice to infection are discussed in more detail below.

THE ROLE OF PARASITE SEQUESTRATION DURING HUMAN AND EXPERIMENTAL CEREBRAL MALARIA

The sequestration of mature parasites within peripheral tissues via adherence of pRBC to vascular endothelium is a common feature of malaria infections. It is believed that this prevents the clearance of mature-stage parasites by the spleen, allowing the development of sufficient numbers of infectious parasites (gametocytes) to ensure transmission to mosquitoes (Beeson *et al*. 2001; Engwerda *et al*. 2005). Although sequestration is initially beneficial to the parasite, it is widely believed to have significant deleterious consequences for the host. For many years it was assumed that the symptoms of CM were due solely to occlusion of brain microvessels by sequestered pRBC (reviewed by Berendt *et al*. 1994; Van der Heyde *et al*. 2006). In this scenario, parasite adherence to brain endothelial cells, combined with rosetting of uninfected and infected red blood cells, impairs blood flow leading to hypoxia, hypoglycaemia and the buildup of toxic waste products, including lactic acid (Van der Heyde *et al*. 2006), which rapidly leads to irreversible tissue damage. However, the typically quite subtle neurological consequences experienced by CM survivors are not consistent with this simple aetiology and other causes of disrupted neuronal signalling are also likely to play a part (Rae *et al*. 2004; Penet *et al*. 2005; Hunt *et al*. 2006).

Although parasite sequestration is usually seen in CM brains, the association is not absolute and, despite a plethora of associative data, there is very little empirical evidence that parasite sequestration in the brain is either necessary or sufficient to cause CM. Deaths attributable to CM, as defined by WHO guidelines, have been observed in the absence of parasite sequestration within the brain (Clark *et al*. 2003; Taylor *et al*. 2004; Haldar *et al*. 2007). Furthermore, parasite sequestration has been observed in individuals that did not develop severe cerebral malaria (Silamut *et al*. 1999; Seydel *et al*. 2006). This heterogeneous association between cerebral parasite sequestration and clinical outcome raises important questions regarding the precise aetiology of CM (Clark *et al*. 2006). Specifically, we need to consider the possibility that transient interactions between sequestering pRBC and cerebral tissues might be sufficient to trigger downstream immunological and biochemical processes that lead to the development of CM.

The widespread assumption that sustained parasite sequestration in the brain is essential for development of CM has led some researchers to question whether cerebral parasite sequestration occurs during *P. berghei* ANKA infection and thus whether the pathogenesis of ECM is comparable to human CM (Berendt *et al*. 1994; Franke-Fayard *et al*. 2005). Accumulation of PbA pRBCs in cerebral and cerebellar capillaries of mice displaying signs of ECM has been observed at both light and electron microscopic levels (Rest, 1982; Jennings *et al*. 1998; Hearn *et al*. 2000; Beghdadi *et al*. 2008). Detailed investigations on the nature of parasite sequestration during *P. berghei* ANKA infection have, however, yet to be performed and as such it is unknown whether PbA parasites adhere through strong, tight junctions, or via weak easily disrupted interactions. The comparison of parasite sequestration in the brain during ECM and CM is also severely complicated by the method of tissue preparation; mouse brains are routinely perfused prior to histological examination during ECM, but perfusion is seldom performed prior to the examination of brains from individuals with fatal CM. As such, parasite sequestration may be frequently under-estimated (in ECM) or over-estimated (in CM). Nevertheless, most blocked vessels during ECM contain a mixture of parasitized RBC and leukocytes (Hearn *et al*. 2000; Jennings *et al*. 1998). Consequently, parasite accumulation alone may not be sufficient to cause blockage of brain-micro-vessels during *P. berghei* ANKA infection.

Recently, Franke-Fayard *et al*. (2005) reported CD36 (Scavenger type B receptor)-mediated sequestration of luciferase-expressing *P. berghei* ANKA pRBC, visualized by bioluminescent imaging, in lung and adipose tissue but not in the brains of infected mice. This is consistent with the requirement for CD36-mediated PbA pRBC sequestration for initiation of acute lung injury (Lovegrove *et al*. 2008), and with the observation that $CD36^{-/-}$ mice are fully susceptible to ECM. These findings have been interpreted as evidence that pRBC sequestration does not occur in the brain during PbA infection and is not required for initiation of ECM, and thus that ECM has a significantly different aetiology to CM (Franke-Fayard *et al*. 2005). However, whole body imaging and multi-organ comparisons may under-estimate cerebral sequestration since it is likely that the density of sequestered pRBC is much lower in brain than in much more heavily vascularized organs such as lung or spleen, and higher resolution analysis of the brain is required to rule out sequestration; nevertheless, and despite the authors claims, focal parasite sequestration was evident in one of the two examples of day

7 p.i. brains shown by Franke-Fayard *et al*. (2005). Secondly, the lack of a role for CD36 in ECM does not rule out that (as in humans) there are other receptors mediating pRBC sequestration in the brain (as discussed below). Importantly, other studies using the same bioluminescent parasite system have not only shown significant accumulation of *P. berghei* ANKA pRBC in the brains of mice showing signs of ECM, but have also demonstrated that parasite biomass in the brain is directly correlated with risk of ECM (Amante *et al*. 2007; Randall *et al*. 2008*a*; Nie *et al*. 2009).

HOST CELL RECEPTORS MEDIATING CYTO-ADHERENCE DURING CM AND ECM

Although CD36 appears to be the main receptor for *P. falciparum* pRBC sequestration in peripheral organs, CD36-mediated adhesion is not believed to be involved in sequestration in the brain (Newbold *et al*. 1999); CD36 is expressed only at very low levels in healthy brain tissue and it is not upregulated during malaria infection (Turner *et al*. 1994; Newbold *et al*. 1999). Nonetheless, it has recently been postulated that platelets and platelet and endothelial cell derived microparticles – submicron particles generated by vesiculation of cellular membranes (reviewed by Coltel *et al*. 2006) – may bind to brain endothelial cells, providing a source of CD36 that allows indirect CD36-mediated pRBC binding to brain endothelial cells (Wassmer *et al*. 2004; Faille *et al*. 2009); however, this hypothesis remains to be validated *in vivo*.

At present, intercellular adhesion molecule 1 (ICAM-1) is the most studied putative endothelial receptor for the sequestration of *P. falciparum* pRBC within the brain (Newbold *et al*. 1999; Chakravorty and Craig, 2005). The expression of ICAM-1 is significantly upregulated on cerebral vasculature endothelium during malaria infection (Turner *et al*. 1994; Newbold *et al*. 1999), and *P. falciparum* pRBC bind to ICAM-1 *in vitro* under flow conditions (Ockenhouse *et al*. 1991, Udomsangpetch *et al*. 1996; Adams *et al*. 2000). Strains of *P. falciparum* differ in their ability to bind to ICAM-1 and CD36 (Johnson *et al*. 1993; Gardner *et al*. 1996; Udomsangpetch *et al*. 1996) and although there is some evidence that the degree of binding of pRBC to ICAM-1 is associated with risk of development of CM (Newbold *et al*. 1999), this correlation is not absolute (Rogerson *et al*. 1999; Heddini *et al*. 2001). Moreover, there are conflicting data on links between risk of CM and allelic variation in the ICAM-1 gene. Specifically, a non-synonomous single nucleotide polymorphism in ICAM-1 (ICAM-1 $^{\text{kilifi}}$) has been shown to be either associated (Fernandez-Ryes *et al*. 1997), or not associated (Bellamy *et al*. 1998; Fry *et al*. 2008) with the risk of severe malaria. Consequently, it has been proposed that other receptors may facilitate pRBC sequestration within the brain (Ockenhouse *et al*. 1992; Chakravorty and Craig 2005). Upregulated expression of VCAM-1, E-Selectin and ELAM-1 by brain microvascular endothelium is also observed during CM; however, as with ICAM-1, there is significant debate on the role of these receptors (Ockenhouse *et al*. 1992; Silamut *et al*. 1999; Udomsangpetch *et al*. 1996), which may reflect difficulties in comparing *in vitro* studies using plate-bound receptors or (non-cerebral) endothelial cells with what may occur *in vivo* during infection. The failure to identify a single critical receptor mediating pRBC sequestration in the brain during CM may indicate promiscuous or redundant receptor binding by the parasite. This fits the current model where *P. falciparum* cyto-adherence is a multi-step process involving multiple (possibly partially redundant) receptor interactions mediating primary contact, rolling and finally firm adhesion (Udomsangpetch *et al*. 1997; McCormick *et al*. 1997; Yipp *et al*. 2000; Gray *et al*. 2003; Ho *et al*. 1998).

As in human CM, ICAM-1, VCAM-1 and P-selectin are all upregulated on brain vascular endothelium in ECM-susceptible strains of mice during *P. berghei* ANKA infection (reviewed by Schofield and Grau, 2005; Good *et al*. 2005). Moreover, ICAM-1 deficient mice (backcrossed onto the susceptible C57BL/6 background) do not develop ECM, suggesting that ICAM-1 expression is an essential step in the pathway of development of ECM (Favre *et al*. 1999; Li *et al*. 2003). Leukocyte rolling in these mice was unimpaired – indicating that resistance to ECM was not due to decreased leukocyte sequestration in the brain – but pRBC sequestration within the brain was not specifically examined. Similarly, mice with specific defects in endothelial cell expression of P-selectin are also resistant to ECM but, again, pRBC sequestration was not reported (Combes *et al*. 2004). Clearly, more detailed examinations of these mouse strains are required to determine whether ECM resistance is due to reduced pRBC sequestration, attenuation of immune responses (including suboptimal T cell activation) or both.

PARASITE LIGANDS MEDIATING pRBC SEQUESTRATION DURING CEREBRAL MALARIA

Clonally variant surface antigens that are expressed on the surface of *P. falciparum*-infected erythrocytes are known to facilitate binding of pRBC to endothelial receptors (Newbold *et al*. 1999). The most studied of these is the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) family of proteins. Encoded by *var* genes, PfEMP-1 is a polymorphic, high molecular weight (200–500 kDa) protein comprised of variable numbers and sequences of duffy binding like (DBL) and cysteine-rich interdomain region (CIDR) domains that mediate binding to

various host molecules (reviewed by Scherf, 2008). Infected erythrocytes from most *P. falciparum* isolates bind to CD36 through its interaction with CIDR-1 (Baruch *et al*. 1995, 1997). DBL-1α, with its clusters of glycosaminoglycan (GAG)-binding motifs, is believed to mediate the formation of rosettes (i.e. binding of infected erythrocytes to uninfected erythrocytes) (Chen *et al*. 1998), which have been linked to the pathogenesis of CM (Newbold *et al*. 1999), whereas DBL-1β binds to ICAM-1 (Smith *et al*. 2000; Oleinikov *et al*. 2009) and DBLγ binds to chondroitin suphate A (CSA) (Reeder *et al*. 1999; Buffet *et al*. 1999; Gamain *et al*. 2004), the latter interaction mediating tissue-specific sequestration of pRBC in the placenta. Disease association studies have suggested that differences in CIDRs and DBLs between commonly expressed *var* genes may contribute to variations in parasite virulence (Jensen *et al*. 2004; Normark *et al*. 2007), such that some parasite isolates are more likely than others to sequester in particular tissues and therefore cause differing clinical presentations, but – with the exception of particular PfEMP-1 molecules that favour placental sequestration (Fried and Duffy, 2002; Salanti *et al*. 2004) – direct evidence to support this hypothesis is lacking. Moreover, the potential roles in pRBC sequestration of other parasite-encoded erythrocyte surface antigens such as stevors (subtelomeric variant open reading frame), rifins (repetitive interspersed family of genes), Pfmc-2TM, surfs (surface-associated interspersed genes), reticulocyte-homologue binding proteins, EBA (erythrocyte binding antigen) and RhopH1/clag (high molecular mass rhoptry complex/ cytoadherence linked asexual gene) also need clarification (Scherf, 2008).

There are no known homologues of *var* genes in other malaria species. However, a large multi-gene *Plasmodium* interspersed repeat (*pir*) family has been identified in *P. vivax* (del Portillo *et al*. 2001) and is believed to be involved in antigenic variation. Homologues members of the *pir* family have been discovered in the rodent malaria parasites *P. chabaudi* (*cir*), *P. yoelii* (*yir*) and *P. berghei* (*bir*) (Carlton *et al*. 2002; Janssen *et al*. 2002; Cunningham *et al*. 2005). Whilst clonal antigenic variation has been described in *P. chabaudi* (McLean *et al*. 1986), the role of the *cir* family remains to be determined. Furthermore, whether the *bir* family plays a role in the development of *P. berghei*-induced ECM malaria remains to be elucidated.

THE ROLE OF THE IMMUNE RESPONSE IN THE PATHOGENESIS OF CEREBRAL MALARIA

Human cerebral malaria

The highly characteristic cytokine profiles that are associated with acute severe malaria provide associative evidence for involvement of the host immune response in the aetiology of CM. High plasma TNF, IFN-γ, IL-6 concentrations and elevated ratios of pro-to anti-inflammatory cytokines (including IL-10) are consistently observed in individuals with cerebral malaria when compared with individuals with uncomplicated malaria (reviewed Schofield and Grau, 2005; Good *et al*. 2005) and high concentrations of inflammatory cytokines in the cerebrospinal fluid are associated with a high risk of developing neurological sequelae (John *et al*. 2008). Very recently it has been shown that the binding of pRBCs to brain endothelial cells, causes the activation of the NF-κB pathway, leading to the production of CCL20, CXCL1, CXCL2, IL-6 and IL-8 (Tripathi *et al*. 2009). Despite this, the difficulty of carrying out mechanistic studies in humans means that it is not at all clear whether (and if so, how) these inflammatory responses lead to the onset of CM; however, direct effects, such as upregulation of endothelial ICAM-1 and VCAM-1 expression (Esslinger *et al*. 1994), and indirect effects, such as induction of fever leading to enhanced expression of PfEMP1 on pRBCs (Udomsangpetch *et al*. 2002), either of which might potentiate pRBC sequestration, have been suggested. Inflammatory cytokines may also be responsible for the presence of activated microglial cells (Schluesener *et al*. 1998), the main phagocytic macrophage-like cell population of the brain, and sequestered monocytes (Patnaik *et al*. 1994) in CM. It is possible that activated myeloid cells amplify the local intra-cerebral inflammatory response – by presenting antigen to T cells and/or producing inflammatory cytokines – but definitive exploration of this pathway in human CM is not feasible. The constraints imposed by gaining access to crucial tissues at key time-points in the onset of CM also explains the relative lack of data on the role of T cells in human CM. The only data that are available compare peripheral blood T cell populations in CM and non-CM cases and since it is clear that there is major re-alloacation of T cell subsets between the tissues and peripheral blood during acute malaria infection (Elhassan *et al*. 1994), these data are extremely difficult to interpret. Nevertheless, reductions in numbers of circulating $CD4^+$ T cells (reflecting either sequestration in tissues or activation-induced cell death) (Elhassan *et al*. 1994; Hviid *et al*. 1997) and increased frequencies of $CD4^+$ T cells expressing TCR Vβ21.3 have been correlated with disease severity (Loizon *et al*. 2007). The potential for $CD8^+$ T cells to play a role in the aetiology of human CM has not been systematically evaluated but there is no evidence as yet to implicate this population in the pathogenesis of CM.

Further evidence that the immune response plays a role in the pathogenesis of severe malaria comes from a series of studies designed to identify genetic polymorphisms that influence the risk of developing

CM (Verra *et al*. 2009). Although there is a bias within the literature towards publication of positive associations the vast majority of reported associations involve genes that either affect parasite development within the red blood cell (e.g. haemoglobinopathies) or that moderate the strength and character of the immune response, for example TNF gene promoter variants (Knight *et al*. 1999; Cabantous *et al*. 2006; Clark *et al*. 2009) and interferon regulatory factor-1 gene variants (Koch *et al*. 2002; Mangano *et al*. 2008, 2009). The details of these associations vary from one population to another, likely reflecting differences in genetic background, but a clear message is beginning to emerge that is consistent with traits that lead to higher than average inflammatory responses being linked to increased risk of CM (Verra *et al*. 2009).

The idea that excessive pro-inflammatory immune responses pre-dispose to CM is also consistent with the clear age-related susceptibility to the development of CM in malaria endemic areas: very young children who have yet to acquire malaria-specific cellular immune responses are relatively resistant to CM (presenting instead with severe malarial anaemia) whereas older children – in whom previous malaria infections will have primed Th-1-like adaptive immune responses – are at increased risk of CM. Epidemiological studies suggest that repeatedly exposed individuals eventually develop protective immunity (such that adults in endemic areas rarely develop CM), which may be characterized by the ability to control parasite replication (keeping parasite densities below the critical threshold for induction of inflammation or impairment of cerebral blood flow), to prevent pRBC sequestration or to regulate the inflammatory process (Artavanis-Tsakonas *et al*. 2003; Walther *et al*. 2009).

Experimental cerebral malaria

The vast majority of the immunological features of human CM are recapitulated during *P. berghei* ANKA infection. For example, the susceptibility of various inbred mouse strains to ECM has been directly correlated with the strength of the pro-inflammatory immune response to the parasite and to the response of microglial and cerebral endothethelial cells (e.g. upregulation of MHC Class I and Class II molecules, ICAM-1 and VCAM-1) to these inflammatory mediators (Lou *et al*. 1998, 2001; Monso-Hinard *et al*. 1997; Randall *et al*. 2008*a*). Moreover, experimental manipulation of PbA-infected mice has allowed causal relationships to be established between specific immune responses and the development of ECM; in the main these relationships are entirely consistent with the associative observations from human studies. For example, administration of LPS, neutralization of IL-10 or heme oxygenase 1 or inhibition of CTLA-4 signalling during PbA infection all lead to the development of ECM in normally resistant mice (Kossodo *et al*. 1997; Neill and Hunt, 1995; Pamplona *et al*. 2007; J. Hafalla manuscript in preparation), whereas neutralization or ablation of IFN-γ, TNF and LT-α signalling or depletion of macrophages (by administration of clodronate liposomes) prevents the development of ECM in susceptible mouse strains (Grau *et al*. 1987, 1989; Curfs *et al*. 1993*b*; Rudin *et al*. 1997; Randall *et al*. 2008*b*; Engwerda *et al*. 2002; Amani *et al*. 2000; Togbe *et al*. 2008). Taken together, the wealth of experimental data indicates that the balance of Th-1 to T regulatory responses is critical in determining the outcome of PbA infection (Kossodo *et al*. 1997; Amante *et al*. 2007; Nie *et al*. 2007), whereas manipulation of Th-2 responses (for example by ablation of IL-4R signalling) does not substantially affect the outcome of infection (Saeftel *et al*. 2004). As in humans, circulating cytokines seem to activate cerebral endothelium, leading to increased expression of adhesion receptors, as well as upregulating chemokine production and chemokine receptor expression on leukocytes (Lou *et al*. 1998; Schofield and Grau, 2005; Good *et al*. 2005; Weiser *et al*. 2007) (Fig. 1).

It is well established that $CD8^+$ T cells play an essential role in the development of ECM: this has recently been reviewed in detail elsewhere (Renia *et al*. 2006). In summary, however, $CD8^+$ T cells accumulate in the brains of susceptible but not resistant mice, in a CXCR3-, IP-10- (CXCL9), MIG- (CXCL10) and platelet factor-4-dependent manner (Hansen *et al*. 2007; Miu *et al*. 2008; Van Den Steen *et al*. 2008; Campanella *et al*. 2008; Srivastava *et al*. 2008; Nie *et al*. 2009), immediately before the onset of neurological signs, and are believed to directly cause disruption of the blood-brain barrier and endothelial cell damage via perforin production (Nitcheu *et al*. 2003; Potter *et al*. 2006) (Fig. 1): depletion of $CD8^+$ T cells either early (from start of infection) or late (from day 4 or 5 post-infection) in infection completely inhibits the development of ECM (Yanez *et al*. 1996; Belnoue *et al*. 2002; Hermsen *et al*. 1997). $CD8^+$ T cells migrate to the brain in a largely antigen-specific manner, following cross-presentation of malaria antigens by classical $CD8^+$ dendritic cells (deWalick *et al*. 2007; Lundie *et al*. 2008; Miyakoda *et al*. 2008). The recent demonstration that NK cell-derived IFN-γ is required for upregulation of CXCR3 on $CD8^+$ T cells and for their subsequent migration to and accumulation within the brain (Hansen *et al*. 2007) is consistent with the observation that IFN-γR signalling regulates sequestration of $CD8^+$ T cells within the brain in susceptible mice (Belnoue *et al*. 2008), and reveals important interactions between innate and adaptive immune responses in the pathogenesis of ECM, opening up potential new avenues of research into the role of innate immune responses, and of

genetic variation in innate response genes, in the pathogenesis of human CM.

It is clear that effector $CD4^+$ T cells also contribute to the development ECM, potentially by providing help to $CD8^+$ T cells (Good *et al*. 2005); thus, it has been shown that depletion of $CD4^+$ T cells during the early (but not later) stages of PbA infection prevents the development of ECM (Belnoue *et al*. 2002). Nevertheless, in separate studies, depletion of $CD4^+$ T cells during the later stages of infection also prevented the development of ECM (Hermsen *et al*. 1997; Belnoue *et al*. 2008), implying that although far fewer $CD4^+$ than $CD8^+$ T cells accumulate in ECM brains (Belnoue *et al*. 2002), CD4+ T cells may also be involved in the effector phase of ECM. On the other hand, adoptive transfer of PbA-specific $CD4^+$ T cells reduces parasite burdens and prevents ECM in semi-susceptible mice (Finley *et al*. 1983). Whether the protective and pathogenic functions of $CD4^+$ T cells are mediated by distinct subpopulations of Th cells, or is a consequence of the cellular location and/or the number of cells – all of which may potentially vary within different strains of inbred mice – requires further investigation.

The above section clearly describes the associated role of the pro-inflammatory immune response in the pathogenesis of CM and ECM. Leukocyte accumulation within the brain is a significant feature of CM and ECM, but, intriguingly, transmigration of leukocytes into the brain parenchyma does not appear to occur in either condition, indicating that the immunopathogenesis of CM is different from other cerebral pathologies, including Experimental Autoimmune Encephalitis and Multiple Sclerosis. Significantly more is understood regarding the immunological basis of ECM compared with human CM, where the relatively few studies performed are by necessity purely correlative. Consequently, it is impossible at present to definitively state whether the pathogenesis of CM is more or less immune-mediated than ECM, and whether cells, such as $CD8^+$ T cells, play comparable roles in the development of pathology in the two conditions. The ECM model provides valuable clues to processes that can lead to the development of pathology during malaria infection (Fig. 1), and should help to direct focused research to define the immunopathogenesis of CM.

IF ECM IS SUCH A GOOD MODEL FOR HUMAN CM WHY DO PREVENTIVE INTERVENTIONS IDENTIFIED IN ECM FAIL TO REDUCE THE MORBIDITY AND MORTALITY OF HUMAN CM?

The most important reason for developing a good model of CM is to identify and test novel therapies for prevention, attenuation or reversal of cerebral pathology. It is therefore disappointing that interventions, such as anti-TNF therapy (Grau *et al*. 1987) and dexamethasone (Neill and Hunt, 1995) that prevent the development of ECM have proven ineffective in humans (Hoffman *et al*. 1988; van Hensbroek *et al*. 1996). However, with hindsight, it is perhaps not surprising that treatments that prevent ECM when given prior to the development of neurological signs may not be able to reverse established CM pathologies, which is when they must be effective in clinical practice. Indeed, ablation of cytokine signalling, depletion of leukocyte populations and administration of blocking antibodies are all able to prevent, but not reverse, ECM (reviewed by Schofield and Grau 2005; Good *et al*. 2005). This does not necessarily mean, however, that findings from experimental models of CM are not relevant to treatment of CM in humans. Indeed, data showing that low bioavailability of NO contributes to the development of ECM in mice are analogous to results obtained in humans during *P. falciparum* infection (Gramglia *et al*. 2006; Yeo *et al*. 2007, 2008) and reversal of low NO bioavailability by administration of L-arginine or exogenous NO is protective in mice and humans (Gramglia *et al*. 2006; Yeo *et al*. 2007). Combined, these data have led to the current consideration of L-arginine therapy for phase II clinical trials in humans with CM. Similarly, the observation that erythropoietin protects susceptible mice from ECM (Kaiser *et al*. 2006) prompted comparison of erythropoietin levels in the plasma of uncomplicated and severe malaria patients CM (Casals-Pascual *et al*. 2008), leading to erythropoietin being considered as a potential adjunct therapy for CM (Casals-Pascual *et al*. 2009).

CONCLUSIONS

New adjunct therapies to improve the outcomes of cerebral malaria are urgently needed. Studies in humans are severely limited by lack of access to tissues, the impossibility of carrying out time-course studies and our inability to infer causality from associative clinical and epidemiological studies. Whilst not perfect, the neurological syndrome that develops in mice infected with *P. berghei* ANKA recapitulates most of the physiological, parasitological and immunological features of human CM. The ECM model has allowed the molecular and cellular basis of CM to be experimentally investigated and explained and has provided clues that have led to clinical trials of several potential new therapies. In the future, the application of increasingly sophisticated experimental techniques, including live imaging of parasite-host interactions (Wilson *et al*. 2009; Schaeffer *et al*. 2009; Ortolano *et al*. 2009), will allow us to develop an even greater understanding of the sequence of events leading to ECM. We will, for example, be able to determine whether cerebral inflammation precedes or follows pRBC sequestration,

and whether brain–resident or brain-homing leukocytes are more important for the development of cerebral pathology, which will inform future decisions about appropriate immune-modulatory therapy. The now routine use of ophthalmoscopic examination of retinal pathology as a diagnostic tool for CM (White *et al*. 2009; Beare *et al*. 2006), which was first described in the experimental *P. berghei* ANKA model (Chang-Ling *et al*. 1992), demonstrates the importance of translational science in the understanding of cerebral malaria.

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