Sterile attenuation of *Plasmodium* parasites at the liver-stage either by irradiation or genetic modification, or at the blood-stage by chemoprophylaxis, has been shown to induce immune responses that can protect against subsequent wild-type infection. However, following certain interventions, parasite attenuation can be incomplete or non-sterile. Instead parasites are rendered developmentally stunted but still capable of establishing an acute infection. In experiments involving *Plasmodium berghei* ANKA, a model of experimental cerebral malaria, it has been observed that several forms of attenuated parasites do not induce cerebral pathology. In this perspective we collect evidence from studies on murine malaria in particular, and attempt to “connect the dots” between early immune responses and protection from severe cerebral disease, highlighting potential parallels to human infection.

**Keywords:** malaria, attenuation, experimental cerebral malaria, early immune response
potential critical factors involved in protection against cerebral malaria.

INCOMPLETE PARASITE ATTENUATION AT THE LIVER-STAGE

Haussig et al. (2011) recently observed that targeting the apicoplast by disruption of a Plasmodium-specific protein that plays a role in liver merozoite formation (PALM) affected liver-stage development and the subsequent onset of blood-stage parasitemia. 30% of the palm(-)-immunized mice became patent following a delay of up to 4 days, of which the majority did not develop cerebral pathology (Haussig et al., 2011).

Another approach described an experimental vaccination regime consisting of sporozoite immunization applied concomitantly with either azithromycin or clindamycin drug cover. This permits full development of the malarial liver-stage, but inhibits the inheritance and biogenesis of the apicoplast, thus preventing the onset of blood-stage infection. While sterile protection was found to depend on IFN-γ producing CD8+ T cells that exclusively targeted the intra-hepatic stages, it was dose-dependent and a reduction in the sporozoite numbers used for immunization led to breakthrough infections. However, all mice that developed blood-stage infection featured a delay in the onset of patency and were apparently protected from ECM (Friesen et al., 2010).

Further it is known that sterile protection conferred by immunization under chloroquine (CQ) cover relies on a critical threshold of intra-hepatic parasites (Nganou-Makamdop et al., 2012). Comparable to Friesen et al. we have observed that a reduction in the sporozoite numbers used for immunization under CQ cover, does not confer sterile protection against a wild type infection, but delays the onset of blood-stages and protects against ECM (Pfeil et al., unpublished).

While the mechanism behind protection against severe cerebral symptoms in the models described above still remains elusive, preliminary data from another experiment suggests a role for an altered host immune response in the modulation of ECM outcome. Incomplete attenuation of PbA parasites achieved via sub-therapeutic administration of a liver acting anti-malarial substance lead to the suppression of intrahepatic development, a delay in prepatency and subsequent abrogation of cerebral pathology. This effect was supported by a robust host immune environment involving a Th1 response and early T-cell activation in both liver and spleen (Lewis et al., unpublished).

A common motif between these observations is development impairment or attenuation during the transition from liver to the intraerythrocytic phase of the malaria parasite. It is conceivable that this altered transition results in a slow trickle of parasites into the bloodstream. How exactly this slow onset of blood-stage parasitemia modulates the immune response in a way that severe disease is prevented, remains unknown.

INCOMPLETE PARASITE ATTENUATION AT THE BLOOD-STAGE

The notion that parasite growth kinetics in the blood can be linked to cerebral malaria, while tenuous, is not entirely novel. In fact, several murine studies have documented early growth defects in the blood that caused an altered disease outcome. One such example was the oral administration of trioxone T-10 thiocetals to C57BL/6 mice 24 h after infection with PbA-infected erythrocytes, which completely abrogated ECM in treated mice (Jacobine et al., 2012). Deletion of certain non-essential blood-stage antigens also achieves the same result. For example P. berghei parasites lacking the endogenous merozoite surface protein 7 (MSP7) remain viable, but are impaired in their multiplication rates in the blood (Tewari et al., 2005). A minor delay in parasite development in vivo, was attributed to enhanced reticulocyte preference but was sufficient to ablate ECM in C57BL/6 mice (Tewari et al., 2005; Spaccapelo et al., 2011). Similar virulence-attenuated phenotypes were also observed in experiments with parasites lacking plasmepsin 4 (Δpm4) or a component of the PTEX, thioredoxin-2 (TRX2) ΔPbTRX-2. Protection from ECM in the case of Δpm4 parasites was associated with a growth defect in the blood (Spaccapelo et al., 2010). While ΔPbTRX2 mutants displayed a marked delay in parasitemia resulting in abrogation of ECM in the majority of mice, variations in virulence were observed between ΔPbTRX2 clones, which the authors hypothesized resulted from differences in the number of times the clones had been passaged (Matthews et al., 2013).

An inference drawn from the examples above suggests that chemical or genetic methods of attenuation modify parasite growth in the blood in a way that differs from a natural infection. This form of attenuation could potentially stall parasite development, thereby reducing the burden of viable parasites and the ensuing immunopathogenesis, thus resulting in the abrogation of ECM.

PARASITE ATTENUATION AND CLINICAL OUTCOME IN HUMANS

Although not directly comparable to the examples described above, similar observations have also been reported from human clinical trials. The partially protective effect against clinical and severe disease following immunization of individuals with the leading malaria vaccine candidate RTS,S represents a good example. The fact that a vaccine against pre-erythrocytic stages confers protection against severe malaria was suggested to stem from vaccine-induced immune responses that reduced the number of liver-stage parasites after natural infection. Such partial pre-erythrocytic immunity may result in the “leakage” of small numbers of parasites. This slow onset of blood-stage parasitemia might increase the time frame required to establish innate and adaptive immune responses that inhibit blood-stage growth and consequently limit severe disease (Guinovart et al., 2009). In a similar setting, long-term reduction in the risk of clinical malaria in Tanzanian children was observed following intermittent preventive treatment with the antimalarial sulfadoxine-pyrimethamine (SP). It was proposed that the long half-life and possibly anti-liver-stage acting properties of SP lead to low-dose blood-stage infections that effectively induce prolonged protection from clinical malaria (Schellenberg et al., 2001; Greenwood, 2007; Sutherland et al., 2007). Such clinical studies and many others that test vaccine efficacy or antimalarial drug potency, however, lack a detailed...
understanding of the downstream effects on human cerebral malaria.

**EARLY IMMUNE RESPONSES AND EVENTS THAT MAY AFFECT DOWNSTREAM IMMUNOPATHOGENESIS**

Early immune responses and particularly elements and mechanisms of the innate immune system can influence downstream effector responses and consequently disease outcome (O’Garra and Murphy, 1994; Jankovic et al., 2001; Mitchell et al., 2005).

In vitro observations with *P. falciparum* and also murine studies have shown that infected red blood cells and parasite moieties such as glycosylphosphatidylinositol (GPI) and hemozoin can trigger innate pathways of the immune system, primarily through toll-like receptor signaling (Schofield et al., 1996; Coban et al., 2005). A study in the rodent model, that was published in 2007 identified TLR-2,-9 and MyD88-dependent signaling as mediators of ECM (Coban et al., 2007). However, subsequent studies showed that TLR-deficient mice still succumbed to ECM (Togbe et al., 2007; Lepenies et al., 2008), thus pointing out a controversial role for TLRs in the development of cerebral pathology.

Nevertheless, other components of the innate immune system have been implicated in the induction of ECM (Hansen et al., 2003, 2007; Maglinao et al., 2013; Palomo et al., 2013). For instance, Hansen et al. (2003) showed that susceptibility or resistance to ECM was dependent on CD1d-restricted NKT cells that modulated Th1/Th2 polarization. A subsequent study showed that NK cell depletion negated T cell recruitment to the brains of ECM-affected mice thus substantiating a role for NK cells in the regulation of adaptive immune responses that influence cerebral pathology (Hansen et al., 2007). Additionally, NK cells and γδ T cells, are also known as early sources of IFN-γ that could enhance parasite clearance mechanisms (Seixas and Langhorne, 1999; Artavanis-Tsakonas and Riley, 2002; Ing and Stevenson, 2009; Inoue et al., 2013).

Indeed, there is evidence that very early inflammatory responses are capable of altering downstream immunopathogenesis in a manner that involves CD8⁺ T cells and IFN-γ (De Souza et al., 1997; Mitchell et al., 2005; Lewis et al., unpublished). ECM-susceptible mice, co-infected with *P. berghei* and *P. berghei* K173 are completely protected from ECM and this protection was found to be associated with increased IFN-γ in the blood at 24 h post-infection and an increase in transcriptional abundance of IFN-γ, IL-10 and IL-12 in both the liver and spleen (Mitchell et al., 2005). In this model early production of IFN-γ was attributed predominantly to CD8⁺ T cells that are known for their ability to rapidly produce this cytokine in a non-antigen-specific manner thereby contributing to innate immunity, e.g., in the early phase of bacterial infections (Berg et al., 2002, 2003; Kambayashi et al., 2003).

This is perhaps contradictory to the received wisdom that ECM is Th1 in nature and responsibility for pathology lies with IFN-γ, CD8⁺ T cells (de Souza and Riley, 2002) and the Th1-biased C57BL/6 mouse (Locksley et al., 1987). The answer partly lies with the opposing roles of IFN-γ or TNF-α depending on the time of their production during infection, i.e., early expression correlates with protection from ECM while later expression promotes ECM (Grau et al., 1989; de Souza and Riley, 2002; Mitchell et al., 2005). One could speculate that an early inflammatory peak disrupts the delicate balance required for ECM immunopathogenesis. A possible explanation is that parasite elimination mechanisms are enhanced, thus preventing the critical antigen threshold required for the onset of immunopathogenesis (Howland et al., 2013).

Alternatively, early inflammatory responses could also induce early production of anti-inflammatory cytokines such as IL-10, a critical regulator in ECM immunopathogenesis (Kossodo et al., 1997; Couper et al., 2008; Niikura et al., 2010). Our preliminary data also indicates that an early acute systemic inflammation may provoke the production of IL-10 (Lewis et al., unpublished). IL-10 may then alleviate CD8⁺ T cell activation, proliferation and down-regulate the expression of adhesion molecules on the vascular endothelium (Renia et al., 2006). Thus the timing and localization of the production of pro- and anti-inflammatory cytokines is crucial to the development of cerebral immunopathogenesis.

Although we are limited in our understanding of the impact of early immune responses on the development of cerebral malaria in humans, studies from mouse models have suggested that an ability to control the initial parasitemia permits the development of adaptive immune responses that support an early inflammatory response and enhance parasite clearance (Meding and Langhorne, 1991; Mohan et al., 1997; van der Heyde et al., 1997; Su and Stevenson, 2000). An early inflammatory response could in turn dampen the immunopathology that otherwise prevails during a natural infection.

Since ECM is likely caused by a series of immunopathogenic mechanisms that are interrelated but not necessarily sequential or reliant upon each other, the disruption of one mechanism in a given model may not necessarily translate into the same outcome in another.

Nevertheless, we propose the following mechanisms by which growth impairment might play a role in the abrogation of ECM.

**A GROWTH DEFECT MAY AFFECT SEQUESTRATION IN PERIPHERAL ORGANS**

Shortly after the onset of blood-stage infection, parasitized erythrocytes adhere to the peripheral tissues (Beeson et al., 2001), inducing the activation of monocytes, neutrophils, and DCs (Renia et al., 2006). The adherence of parasitized erythrocytes to the vascular endothelium has been shown to induce chemokine secretion and provoke an “activated” state in the brain endothelium. Leukocytes and parasitized erythrocytes bound to the endothelium interfere with the circulation and produce cytotoxic molecules. This damages the blood-brain-barrier and causes hemorrhages and oedema (de Souza et al., 2010). A blood-stage growth “defect” or “modification” may alter the kinetics of the replicating parasite, thereby altering the localization, severity or timing of parasite sequestration. In turn, this may modify the induced innate immune response.

**GROWTH KINETICS COULD ALTER ANTIGEN PRESENTATION**

Given the shared antigen repertoire between liver and blood-stage parasites (Belhoue et al., 2008; Tarun et al., 2008), it is
Although the role of regulatory T cells in malaria is controversial, it is conceivable that they play a role in the protection we observe in some models. ECM can be ablated in normal PbA infection by the expansion of regulatory T cells (Haque et al., 2010). One possibility is that regulatory T cells temper the pro-inflammatory response (Riley et al., 2006), which is a key factor in the development of cerebral malaria. In fact, concomitant infection of mice with Schistosoma japonicum and P. berghei reduces ECM mortality by promoting a Th2 response that is supported by proliferating Tregs (Wang et al., 2013). Interestingly, protection from the severe symptoms of malaria in P. falciparum is also associated with the expansion of CD4+CD45RO+FOXP3+ regulatory T cells (Walther et al., 2009).

CONCLUSION
The examples elaborated above substantiate a crucial role of early immune responses in influencing the immunopathogenesis of ECM. While a clear distinction cannot be drawn between the responses toward late liver-stages and those toward the early blood-stages, they both seem to exert an effect on the onset of parasitemia. Attenuated infections could serve as tools to improve our understanding of the mechanisms by which early immune responses regulate downstream adaptive immunity and consequently cerebral pathology. Elucidating these mechanisms could help refine future intervention strategies.

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