Caspase-1 Activation of Interleukin-1β (IL-1β) and IL-18 Is Dispensable for Induction of Experimental Cerebral Malaria[▽][†]

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Malaria infection is initiated by sporozoite invasion of hepatocytes and asexual reproduction of liver stages, processes that are regarded to be "clinically and diagnostically silent." Merozoites, which egress from hepatocytes, infect erythrocytes in periodic cycles and induce disease. How the host innate immune system contributes to disease outcomes and to the induction of effector cells during malaria remains unclear. Likewise, how the initial liver stages may shape responses to blood-stage parasites is unknown. Here, using both sporozoite- and blood-stage-induced infections with the rodent malaria parasite *Plasmodium berghei* ANKA, we show that the MyD88 and Toll-like receptor 2/4 (TLR2/4) pathways play critical roles in the development of experimental cerebral malaria (ECM). Strikingly, an absolute dependence on MyD88 and TLR2/4 was observed when infections were initiated with sporozoites. In addition, we show that caspase-1 activation of interleukin-1β (IL-1β) and IL-18, which is associated with the inflammasome pathway, does not contribute to *P. berghei* ANKA-induced immunopathology. Consistent with these data, prophylactic cover with the IL-1β antagonist anakinra did not reduce the incidence of ECM. Therefore, we propose that protection against ECM due to loss of TLR signaling functions is caused by effector mechanisms other than IL-1β activation.

Malaria, which is caused by infection with *Plasmodium* parasites, is a significant global health problem that is responsible for approximately 250 million infections and 0.9 million deaths annually (51). Malaria infection is initiated following inoculation of sporozoites that invade and differentiate into preerythrocytic forms in the liver. Merozoites egress from hepatocytes and initiate a blood-stage infection. While the sporozoites and liver stages of the parasite are known to induce a "clinically silent" infection, blood stages elicit pathological responses associated with malaria.

Cerebral malaria (CM) is the most severe complication due to infection with the human parasite *Plasmodium falciparum*. Both the adhesion of infected red blood cells (RBCs) in the endothelial microvasculature of the brain and the dysregulation of the host's immune response to the parasite have been implicated in the pathogenesis of CM (34, 50). Infection of susceptible C57BL/6 mice with *Plasmodium berghei* ANKA reproduces the neurological signs associated with human CM; within 6 to 10 days of infection, animals develop ataxia, paraplegia, seizures, and coma and become moribund (12). This experimental model (referred to as experimental cerebral malaria [ECM]) has revealed important roles for proinflammatory cytokines such as gamma interferon (IFN-γ) (3), tumor necrosis factor alpha (TNF-α) (19), and lymphotoxin alpha (LT-α) (15) and for the intravascular arrest of "pathogenic" CD8⁺ T cells to the brain (23, 39) as vital effectors responsible for ECM onset. However, despite the progress made with rodent models, the nature of the innate immune pathways that direct the induction of inflammation and pathological sequelae remains poorly understood.

Innate immune signaling through Toll-like receptors (TLRs), which comprise several molecules using a common adaptor molecule, MyD88 (myeloid differentiation factor 88), invokes inflammation in response to microbial challenge (26). Numerous studies have provided conflicting information regarding the role of TLRs in ECM. Two studies have reported either partial or almost full protection from ECM in *P. berghei* ANKA-infected mice deficient in MyD88 (11, 20); partial protection from ECM was also reported in mice deficient in TLR2 and TLR9 (11, 20). While the ligands for TLR2 and TLR9 in *P. berghei* ANKA remain unknown, these results support *in vitro* studies in *P. falciparum* suggesting a role for glycosylphosphatidylinositol (GPI) and hemozoin as parasite-derived ligands for TLR2 and TLR9, respectively (10, 28). However, the identification of hemozoin as a TLR9 ligand remains controversial (36). In sharp contrast, two studies have reported that ECM development is independent of TLR signaling. Mice deficient in MyD88 or TLR1, -2, -3, -4, -6, -7, or -9 (48) and triple TLR2/4/9-deficient mice (29) have been described to be as susceptible to immune pathology as wild-type (WT) animals. The reasons for these discrepancies remain to be resolved.

The reported partial dependence or nondependence of ECM pathogenesis on *MyD88* suggests that *P. berghei* ANKA infection stimulates inflammation utilizing some other innate immune pathway. The MyD88 adaptor molecule utilized by TLRs is shared with the interleukin-1 (IL-1) and IL-18 signal-

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ing pathways (1, 8). IL-1β and IL-18 are structurally related proinflammatory cytokines (5) that are central to the initiation of the inflammatory cascade during a host's innate response to microbial infection (9, 40). Both cytokines are synthesized as propeptides that are cleaved by active caspase-1 for full cytokine maturation (18, 22). TLR agonists are necessary for pro-IL-1β and IL-18 synthesis, and ATP stimulates caspase-1-dependent cleavage and secretion (33). Cytoplasmic "danger" signals, which are independent of TLR ligation, from microbes, microbe-derived products, and other endogenous stimuli have been shown to activate caspase-1 (from its inactive form pro-caspase-1) within a multiprotein complex termed the inflammasome (25).

In addition to reports of hemozoin being a TLR9 ligand, recent studies have identified its function as a "danger" signal for the Nalp3 inflammasome (13, 21, 42), which is composed of the Nod-like protein Nalp3, the PYRIN-CARD protein ASC, and caspase-1 (37). Additional known activators of the Nalp3 inflammasome include other crystalline structures such as asbestos and silica (14). *In vitro* studies utilizing bone-marrow derived macrophages (BMDM) have shown that pure hemozoin induces the cleavage of caspase-1 into its enzymatically active form, which leads to the production of IL-1β and IL-18 (13, 21, 42). Moreover, *in vivo* studies have highlighted a role for Nalp3, caspase-1, and IL-1 receptor (IL-1R) in a mouse model of hemozoin-induced peritonitis (13). Consequently, the Nalp3 inflammasome has recently been implicated in the development of ECM using Nalp3-deficient mice (13). However, a separate study refuted a role for the Nalp3 inflammasome by showing that mice deficient in caspase-1, ASC, or the IL-1 receptor succumbed to *P. berghei* ANKA infection as WT mice did. Mice deficient in Nalp3 remained susceptible to ECM despite displaying a delayed onset of neurological signs (38). A clarification of the role of caspase-1 dependent activation of IL-1β as well as IL-18 in ECM is therefore warranted.

A potentially critical issue which also needs to be addressed in malaria research is that almost all the studies mentioned above utilized an artificial method of parasite infection by either intraperitoneal or intravenous injection of asexual blood-stage parasites. This type of inoculation bypasses the obligate sporozoite and liver-stage phases of the life cycle. How and to what extent malaria preerythrocytic stages shape the immune responses to the subsequent blood-stage infection remain largely unknown. Thus, this lack of knowledge warrants a reevaluation of immune responses to blood stages initiated by sporozoite infection. Indeed, very few studies have investigated immune responses to blood-stage parasites that are initiated with sporozoites (4, 16).

In the present study, we reexamined the roles of MyD88 and caspase-1 activation of the IL-1β and IL-18 pathways during ECM and contrasted infections initiated with sporozoites versus parasitized erythrocytes. Finally, we ascertained whether the IL-1β pathway is a potential target for therapeutic and/or prophylactic intervention against severe disease caused by *Plasmodium* infection.

MATERIALS AND METHODS

Animals. Procedures with experimental animals were performed according to the German Animal Protection Law, United Kingdom Home Office, and European regulations. C57BL/6AnNCr mice (6 to 10 weeks old) were obtained from

Charles River Laboratories, Sulzfeld, Germany. Mice deficient in MyD88 (obtained from S. Akira [1]), TLR2/4 (originally obtained as singly deficient mice from S. Akira [47]), caspase-1 (obtained from BASF [30]), IL-1β (obtained from D. Chaplin [43]), or IL-18 (obtained from S. Akira [46]) in the C57BL/6 background were bred in a pathogen-free animal facility at the Max-Planck-Institute for Infection Biology and have been backcrossed to the C57BL/6 background for at least 10 generations.

***P. berghei* ANKA infection.** The complete life cycle of *P. berghei* ANKA was maintained by passage through naïve mice and *Anopheles stephensi* mosquitoes. The *P. berghei* ANKA parasites utilized in this study were clonal parasites which are passaged at regular intervals through the entire life cycle and originate from clone 15cy1, which had been engineered to express the green fluorescent protein (17). Infections were initiated by intravenous (i.v.) inoculation of 10^4 sporozoites, purified from mosquito salivary glands, or similar numbers of infected red blood cells (iRBCs), obtained from infected C57BL/6 mice. Infected mice were observed daily for the manifestation of ECM, which was defined as the sudden onset of ataxia, paralysis, convulsion, or coma (12). The presence of ECM signs served as the ethical endpoint criterion for culling of animals. Spleens and brains were removed for analysis by flow cytometry. Infected mice that did not develop ECM until day 18 after infection were considered resistant and were sacrificed in order to avoid suffering from high parasitemia and severe anemia. The presence of blood-stage parasites was determined by daily examination of Giemsa-stained blood smears by oil immersion microscopy. For all infection experiments, parasitemia was assessed every other day by counting up to 50 microscopy fields.

Quantification of parasite development in the liver. Total RNA was extracted from mouse livers at 44 h after infection using the RNeasy kit (Qiagen) and treated with DNase I. cDNA was synthesized using the RETROscript kit (Ambion). Hepatic parasite development was assessed by quantitative reverse transcription-PCR (RT-PCR) using SYBR green I master mix (Applied Biosystems). Reverse transcripts of *P. berghei* ANKA 18S rRNA were amplified in parallel with murine glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) in a final volume of 25 µl in 96-well plates (Applied Biosystems). Oligonucleotide primers (Eurofins MWG Operon) for mGAPDH were 5′-CGT CCC GTA GAC AAA ATG GT-3′ (forward) and 5′-TTG ATG GCA ACA ATC TCC AC-3′) (reverse), and those for *P. berghei* ANKA 18S rRNA were 5′-AAG CAT TAA ATA AAG CGA ATA CAT CCT TAC-3′ (forward) and 5′-GGA GAT TGG TTT TGA CGT TTA TGT G-3′ (reverse).

Cytokine measurement. Cytokine measurement was performed using the mouse inflammation cytometric bead array kit from BD Biosciences according to the manufacturer's instructions on a LSR II cell analyzer (BD Biosciences). Raw data were analyzed with FlowJo software (Tree Star). Plasma samples for *in vivo* measurement of cytokines were prepared from heparinized blood collected from naïve and infected mice.

Preparation of brain-sequestered lymphocytes. Brain-sequestered lymphocytes were isolated as described previously (6). Perfused brains were digested in collagenase D buffer and lymphocytes isolated on a 30% Percoll (GE Healthcare) gradient. The isolated brain-sequestered lymphocytes were surface stained according to standard protocols. Antibodies for cell surface staining were obtained from eBiosciences (anti-mouse CD4 [GK1.5] and CD8 [53.6-7]). Antibodies for intracellular staining of IFN-γ (XMG1.2) were obtained from eBioscience. Cells were analyzed using an LSR II cell analyzer (BD Biosciences) and FlowJo software (TreeStar).

Statistical analysis. Statistical significance was assessed using the Mann-Whitney test, with a *P* value of <0.05 taken as indicating a significant difference. Survival curves were compared by using the log rank (Mantel-Cox) test. All statistical tests were computed with GraphPad Prism 5 (GraphPad Software).

RESULTS

ECM pathogenesis is dependent on MyD88 and TLR2/4 signaling. We first evaluated whether the MyD88 and TLR2/4 signaling pathways are involved in the development of ECM. For this purpose, we compared the courses of *Plasmodium berghei* ANKA infection in WT, MyD88-deficient, and TLR2/4-deficient C57BL/6 mice and contrasted infections caused by inoculation of 10^4 sporozoites or the same number of infected red blood cells (iRBCs) (Fig. 1).

All infections initiated with sporozoites in WT, $MyD88^{-/-}$, and $TLR2/4^{-/-}$ mice became patent at 3 to 4 days after infection (Fig. 1A). The majority of the sporozoite-infected WT
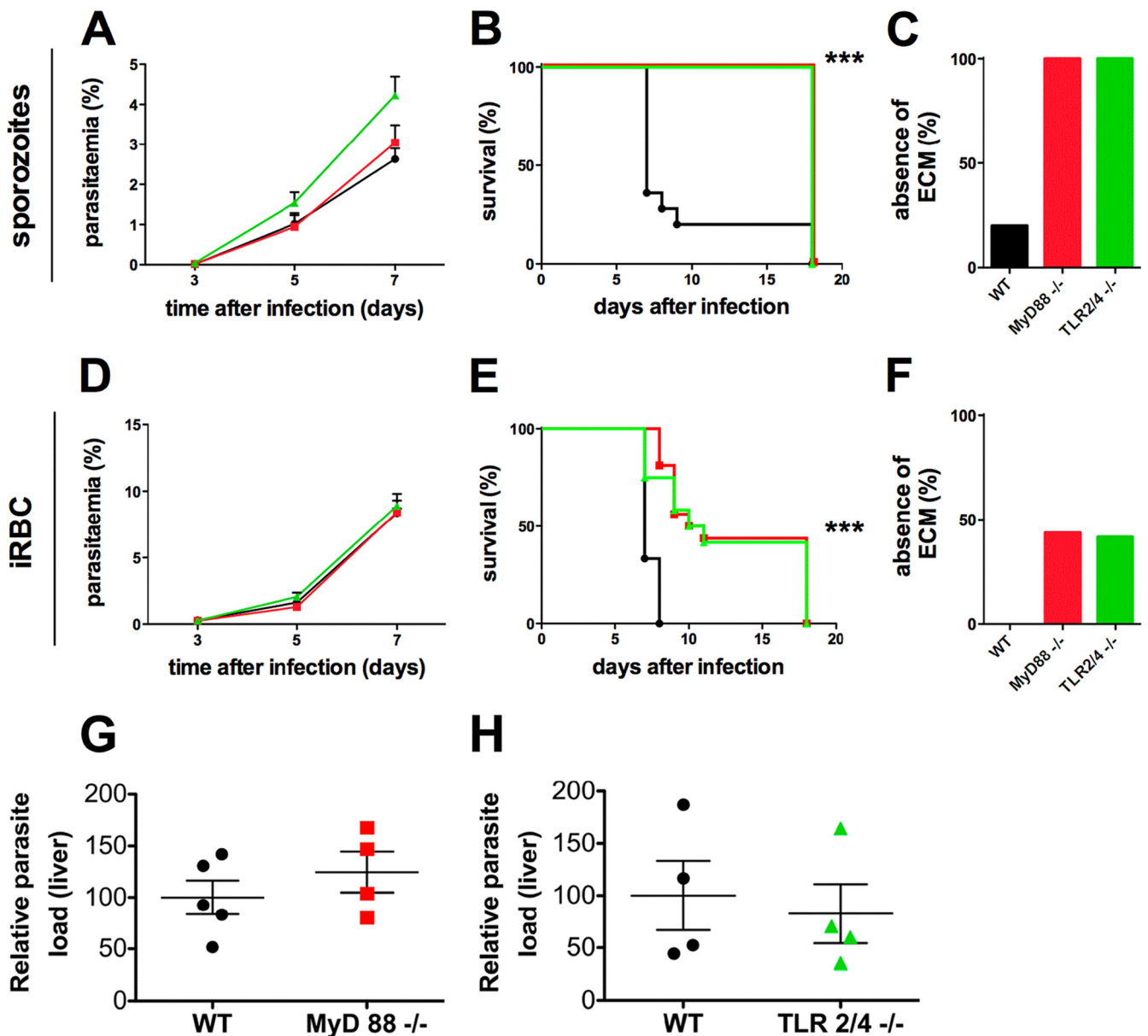
FIG. 1. $MyD88^{-/-}$ and $TLR\,2/4^{-/-}$ mice show an increased resistance to experimental cerebral malaria. WT (black, ●) $MyD88^{-/-}$ (red, ■), and $TLR\,2/4^{-/-}$ (green, ▲) mice were infected with 10^4 $P.\ berghei$ sporozoites (A to C) or iRBCs (D to F) by i.v. injection. (A and D) Average parasitemia during the course of infection. Error bars indicate the standard errors of the means (SEM). Results of a single representative experiment are displayed for one sporozoite-induced and one blood-stage-induced infection ($n = 5$). No significant differences were observed between any groups of mice. (B and E) Kaplan-Meier analysis of the time from infection to development of signs of ECM. Data were cumulative from multiple experiments. For sporozoite-induced infections: WT, $n = 25$; $MyD88^{-/-}$, $n = 18$; $TLR2/4^{-/-}$, $n = 13$. For iRBC-induced infections: WT, $n = 15$; $MyD88^{-/-}$, $n = 16$; $TLR\,2/4^{-/-}$, $n = 12$. Survival rates were analyzed by using the log rank test. ***, P < 0.001. (C and F) Absence of ECM shown as the percentage of infected animals based on cumulative data. (G and H) $MyD88^{-/-}$ (G) and $TLR2/4^{-/-}$ (H) mice were infected with 10^4 $P.\ berghei$ sporozoites. The parasite load in the liver was measured by real-time PCR at 42 h postinfection. No significant differences were observed between groups of mice.

mice ($n = 25$) developed ECM on day 7 or 8 after sporozoite inoculation (Fig. 1B). These results indicate that a blood-stage infection of as short as 4 to 5 days is sufficient to induce ECM. In sharp contrast, none of the sporozoite-infected $MyD88^{-/-}$ ($n = 18$) and $TLR2/4^{-/-}$ ($n = 13$) mice developed ECM. All animals that did not develop ECM were carefully culled on day 18 postinfection to avoid severe anemia and high parasitemia. There was no difference in parasitemia levels during the first

7 days of infection among sporozoite-inoculated WT, $MyD88^{-/-}$, and $TLR2/4^{-/-}$ mice (Fig. 1A). Thus, we conclude that the MyD88-dependent TLR2/4 pathway is critically involved in induction of ECM upon a sporozoite-induced malaria infection.

We next compared disease progression in infections initiated with iRBCs that bypasses the initial liver stage of infection (Fig. 1D to F). All of the WT mice developed ECM on day 7

or 8 after infection. In contrast, 38% (6/16) of the infected *MyD88*$^{-/-}$ mice had a significant delay in the onset of neuro-logical signs (day 9 to 11 after infection; $P < 0.0001$ by log rank [Mantel-Cox] test), and 44% (7/16) did not develop ECM. Similarly, 33% (4/12) of the infected *TLR2/4*$^{-/-}$ mice had a significant delay in the onset of neurological signs (day 9 to 11 after infection; $P = 0.0002$), and 42% (5/12) did not develop ECM. The survival curves for infected *MyD88*$^{-/-}$ and *TLR2/4*$^{-/-}$ mice did not differ significantly ($P = 0.8$). We did not observe any difference in parasitemia levels during the first 7 days of infection between WT, *MyD88*$^{-/-}$, and *TLR2/4*$^{-/-}$ mice (Fig. 1D). A follow-up of the remaining mutant mice, which stayed free of ECM signs, revealed an increasing vari-ability of parasitemia levels after day 7. All animals developed severe anemia and parasitemia above 20% by day 18 postin-fection (data not shown).

One possible explanation of why we obtained disparate ECM levels when infection was initiated by sporozoite versus blood stages is that liver-stage development is different in WT versus *MyD88*$^{-/-}$ or *TLR2/4*$^{-/-}$ mice. To examine this possi-bility, we infected WT, *MyD88*$^{-/-}$, or *TLR2/4*$^{-/-}$ mice with 10^4 sporozoites. The livers of the animals were excised at 42 h postinfection and processed for quantitative real-time PCR to measure parasite development in the liver. As shown in Fig. 1G and H, we did not observe any difference in the development of the parasite in the liver.

Taken together, our results concur with reports indicating a partial dependency of ECM on MyD88 and TLR2/4 signaling following an iRBC-induced infection (11, 20). Strikingly, an absolute dependency is observed when infection is initiated with sporozoites, leading to a complete protection against ECM in the *MyD88*$^{-/-}$ and *TLR2/4*$^{-/-}$ mice. This difference cannot be attributed to differential development of parasites in the liver when infection is initiated with sporozoites.

Correlation of disease outcome with plasma cytokine levels. ECM is characterized by excessive production of proinflam-matory cytokines (41). We measured the systemic levels of various cytokines in the plasma of sporozoite-infected animals (Fig. 2). Infected WT mice had significantly elevated levels of plasma IFN-γ and monocyte chemotactic/chemoattractant protein 1 (MCP-1), which peaked on day 5 after sporozoite infection. Expression of TNF-α was significantly elevated on day 5 and even further increased on day 7. The anti-inflam-matory cytokine IL-10 only showed a significant increase on day 7.

In infected *MyD88*$^{-/-}$ mice, however, significantly lower IFN-γ and MCP-1 levels ($P = 0.036$ and $P < 0.0001$, respec-tively, by the Mann-Whitney test), which corresponded to sig-nificantly higher IL-10 levels ($P = 0.0072$), were observed on day 5 postinfection. In contrast, infected *TLR2/4*$^{-/-}$ mice pre-sented significantly elevated plasma concentrations of IFN-γ, TNF-α, and IL-10 ($P = 0.0046$, $P = 0.042$, and $P = 0.0097$, respectively) that exceeded those of infected WT mice on day 5 after infection. While beyond the scope of the current study, it will be interesting to analyze the underlying molecular mech-anisms that can explain these differential effects.

Inoculation of iRBCs induced plasma cytokine kinetics (see Fig. S1 in the supplemental material) that were comparable to those after infection initiated with sporozoites. However, while day 5 MCP-1 levels were significantly lower in *MyD88*$^{-/-}$ mice
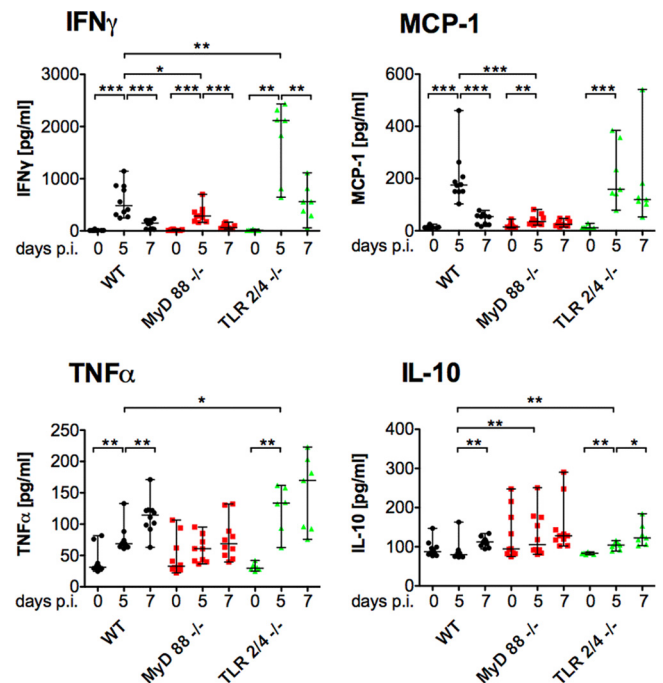

FIG. 2. Plasma concentrations of cytokines correlate with the course of *P. berghei* infection. Plasma samples were collected before and on day 5 and day 7 after i.v. infection with 10^4 sporozoites, and the concentrations of IFN-γ, TNF-α, IL-10, and MCP-1 were measured. Data points represent individual animals; median and ranges are dis-played for each group at the individual time points. Changes over the course of infection within a group (day 0 versus day 5 and day 5 versus day 7) as well as differences between WT and deficient mice on day 5 and day 7 after infection were assessed using the Mann-Whitney test. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

than in WT mice, no difference in the IFN-γ and IL-10 levels in the two groups was observed. The disparate IFN-γ and IL-10 levels in infections initiated by different stages of the *Plasmodium* parasite in WT and *MyD88*$^{-/-}$ mice may possibly explain the partial susceptibility of mutant mice to ECM when infected with blood stages. Overall, our data demonstrate that the innate host defense system contributes critically to ECM.

ECM pathogenesis is independent of caspase-1, IL-1β, and IL-18. Other receptors that are known to signal through MyD88 are the IL-1 and IL-18 receptors (1). Synthesized as propeptides, mature IL-1β and IL-18 are products of activated caspase-1, which in turn is tightly regulated by the Nalp3 in-flammasome, another innate immune pathway involved in in-flammatory responses (32).

To determine a role for caspase-1 activation of IL-1β and IL-18 during ECM, we compared the courses of *P. berghei* ANKA infection in WT, *caspase-1*$^{-/-}$, *IL-1β*$^{-/-}$, and *IL-18*$^{-/-}$ mice inoculated with sporozoites (Fig. 3A to C). Infected en-zyme/cytokine-deficient mice developed ECM as WT mice did (Fig. 3B). Furthermore, we did not observe any difference in parasitemia levels during the first 7 days of infection in the different groups (Fig. 3A). Similar results were obtained when infections were initiated with iRBCs (Fig. 3D to F). Collec-tively, these results indicate that caspase-1 activation of IL-1β and IL-18 is dispensable for the development of effector re-sponses associated with ECM.

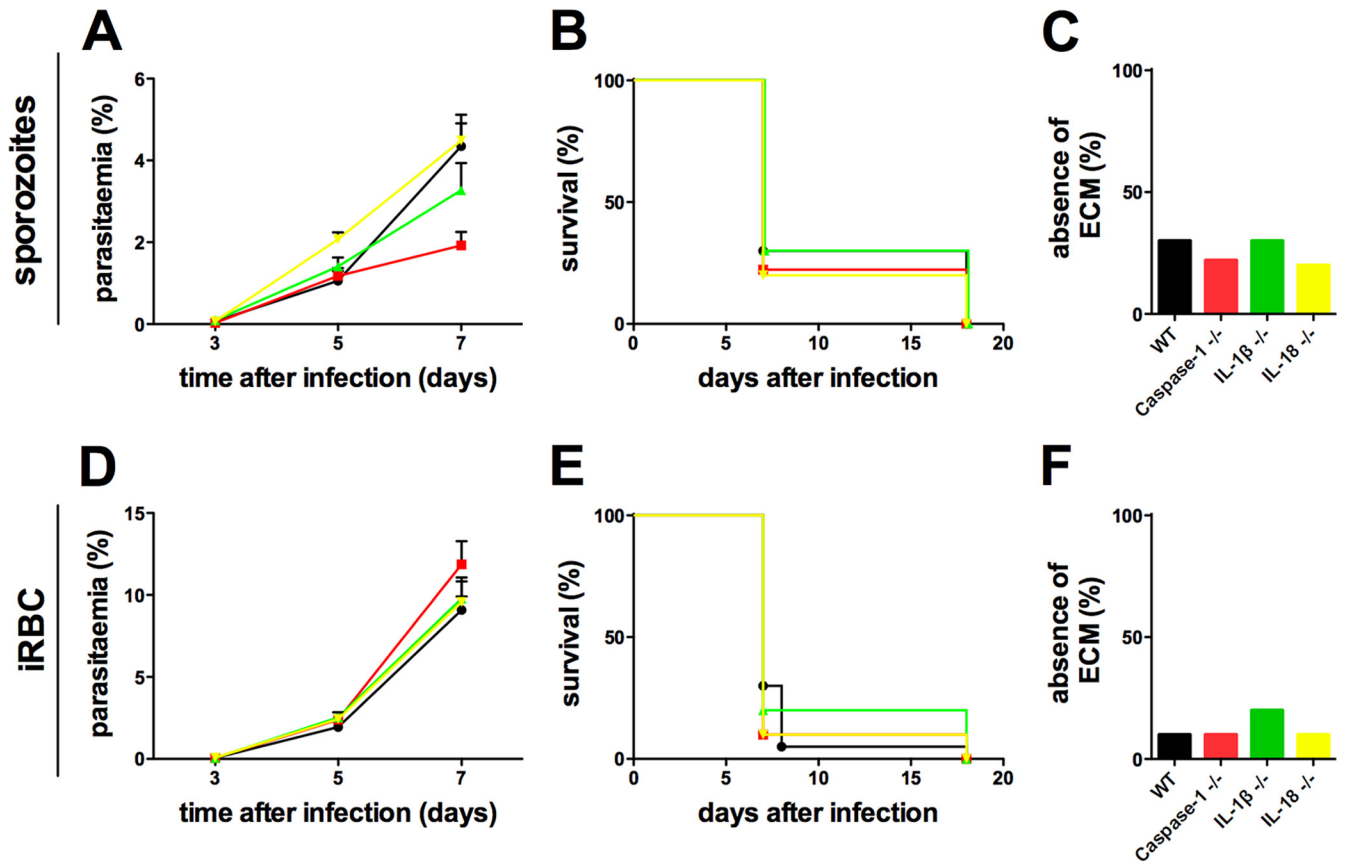FIG. 3. *P. berghei* ANKA infections are unaltered in *caspase-1*$^{-/-}$, *IL-1β*$^{-/-}$, and *IL-18*$^{-/-}$ mice. WT (black, ●), *caspase-1*$^{-/-}$ (red, ■), *IL-1β*$^{-/-}$ (green, ▲), and *IL-18*$^{-/-}$ (yellow, ▼) mice were i.v. injected with 10^4 sporozoites (A to C) or iRBCs (D to F). (A and D) Average parasitemia during the course of infection. Error bars indicate the SEM. Results of 1 representative experiment from 2 experiments are displayed ($n = 4$ or 5 each). No significant differences were observed between any groups of mice. (C and E) Kaplan-Meier analysis of the time from infection to development of signs of ECM. Data were cumulative from two experiments. For sporozoite-induced infections: WT, $n = 10$; *caspase 1*$^{-/-}$, $n = 9$; *IL-1β*$^{-/-}$, $n = 10$; *IL-18*$^{-/-}$, $n = 10$. For iRBC-induced infections: WT, $n = 20$; *caspase 1*$^{-/-}$, $n = 10$; *IL-1β*$^{-/-}$, $n = 10$; *IL-18*$^{-/-}$, $n = 10$. Survival rates were analyzed by using the log rank test. No significant differences were observed between any groups of mice ($P > 0.05$). (C and F) Absence of ECM shown as the percentage of infected animals based on cumulative data.

Consistent with the above results, the plasma cytokine profiles of IFN-γ, MCP-1, TNF-α, and IL-10 in sporozoite-inoculated *caspase-1*$^{-/-}$, *IL-1β*$^{-/-}$, and *IL-18*$^{-/-}$ mice were indistinguishable from those in infected WT mice (Fig. 4). Similarly, using iRBCs for infection, we did not observe any significant differences in the cytokine profiles among the different groups (see Fig. S2 in the supplemental material).

To examine the induction of IFN-γ responses, we additionally performed short-term stimulation of day 7 splenocytes from WT and enzyme/cytokine-deficient mice in the presence of phorbol myristate acetate (PMA)/ionomycin. In good support of our findings from the plasma cytokine profiling, intracellular staining analysis of CD4$^+$ and CD8$^+$ cells from infected WT and enzyme/cytokine-deficient mice showed comparable frequencies of cells producing IFN-γ (see Fig. S3 in the supplemental material).

The IFN-γ-controlled trafficking of pathogenic CD8 T cells in the brain is a canonical feature of ECM (6, 7, 23). Therefore, we isolated brain-infiltrating lymphocytes from WT and enzyme/cytokine-deficient mice at the time the animals were committed to neurological signs (Fig. 5). As predicted from the

clinical outcome, WT and enzyme/cytokine-deficient mice had similar proportions of brain-infiltrating CD8$^+$ T cells. This infiltration was independent of the induction of infection by either sporozoites (Fig. 5A and B [left panel]) or iRBCs (Fig. 5B [right panel]). Therefore, we conclude that caspase-1 activation of IL-1β and IL-18 plays no pathological role in ECM *in vivo*.

Interference with IL-1β signaling does not offer an experimental therapeutic approach. Our findings of a dispensable role for IL-1β signaling in ECM further substantiate previous work showing that IL-1R-deficient mice succumb to ECM indistinguishably from WT mice (38). To obtain further independent evidence and to examine a potential drug target to prevent severe forms of malaria, we tested whether treatment with the IL-1β antagonist anakinra (24) modulates the clinical outcome of a malaria infection (Fig. 6). High doses of anakinra (250 mg/kg) were administered to mice for 7 consecutive days following infection with *P. berghei* ANKA sporozoites. In agreement with our data from the knockout mice, treated and untreated mice showed similar courses of infection (Fig. 6A). Importantly, animals in both groups displayed signs of ECM at
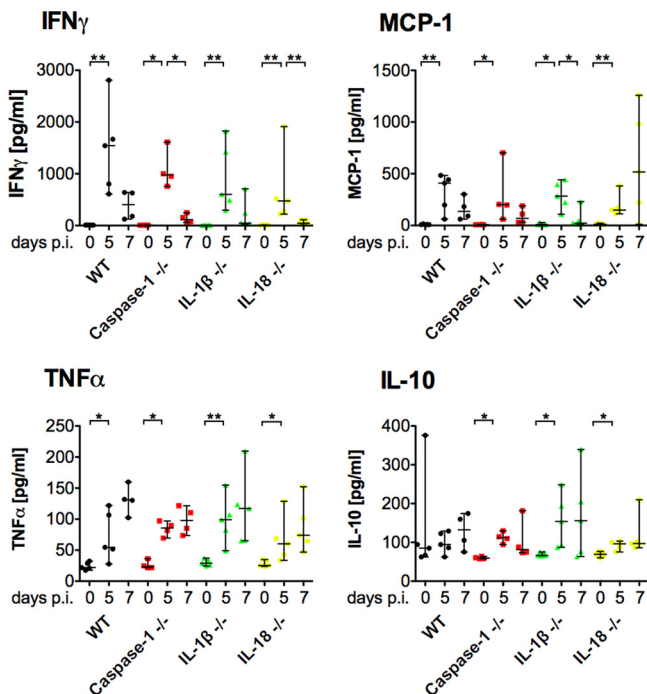
FIG. 4. Effect of deficiency in caspase-1, IL-1β, or IL-18 on the kinetics of cytokines over the course of *P. berghei* infections. Plasma samples were collected before and 5 and 7 days after i.v. infection with 10^4 *P. berghei* sporozoites, and levels of IFN-γ, TNF-α, IL-10, and MCP-1 were measured. Data points represent individual animals; medians and ranges are displayed for each group at the individual time points. Changes over the course of infection within a group (day 0 versus day 5 and day 5 versus day 7) as well as differences between WT and deficient mice on day 5 and day 7 after infection were assessed using the Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$.

7 days after infection to similar degrees (Fig. 6B). As a consequence, no protection against ECM was detected in anakinra-treated animals (Fig. 6C). These findings independently reject the hypothesis that the IL-1β signaling pathway plays an important role in sporozoite-induced ECM.

DISCUSSION

Innate immune pathways triggered by pathogen-associated molecular patterns or "danger" signals are pivotal for the first-line defense against various microbial infections, but their roles during blood-stage malaria are either poorly understood or controversial. Moreover, the potential of preerythrocytic stages, which precede a *Plasmodium* blood-stage infection, to modulate innate immune responses and disease outcomes has typically been neglected. Our present study addressed the pathological roles of central players of innate defense mechanisms in malaria infections initiated with the preerythrocytic parasite stages, i.e., sporozoites, in side-by-side comparisons to infections induced by transfer of infected blood. Inoculation of WT mice with iRBCs bypasses the 2 to 3 days of liver-stage development (Fig. 1 and 3) but does not translate into an altered onset of ECM. Additionally, all mice displayed the effector response characterized by induction of proinflammatory cytokines at 5 days after infection (Fig. 2 and 4; see Fig. S2

in the supplemental material) regardless of the route of infection.

In line with this result, it has been demonstrated previously that *MyD88*-deficient C57BL/6 mice have a higher hepatic parasite load than WT animals (49). In contrast to the published data, we did not observe any difference in the development of liver-stage parasites between WT and *MyD88*$^{-/-}$ (or *TLR2/4*$^{-/-}$) mice. Consequently, we did not observe any difference in prepatency between WT animals and the different mice with deficiencies in innate defense mechanisms that we utilized in this study (Fig. 1A and 3A). It is conceivable that immune responses that direct the development of ECM may already be primed during the "clinically silent" liver stage. Therefore, it is possible that the pathological role of MyD88-dependent signaling in natural infections may have been underestimated.

Our infection experiments with *MyD88*$^{-/-}$ and *TLR2/4*$^{-/-}$ mice substantiate a role for TLR signaling in severe rodent model malaria. Protection against ECM was partial after inoculation of iRBCs, as reported previously (11, 20). Notably, protection in knockout mice was complete when infections were initiated by sporozoite inoculations. An unexpected finding of our study is that despite comparable clinical protection in *MyD88*$^{-/-}$ and *TLR2/4*$^{-/-}$ mice, the proinflammatory cytokine profiles were very distinct and hence may not qualify as signatures of pathogenesis. While inactivation of the downstream adaptor molecule MyD88 correlated with reduced IFN-γ and MCP-1 plasma levels, there was a corresponding significant increase in IL-10 levels. Similarly, a significant increase in IL-10 was associated with increased IFN-γ levels in *TLR2/4*$^{-/-}$ mice. These observations are consistent with the reported protective role for IL-10 in ECM (27). Slightly different results were obtained with infections initiated with blood stages in *MyD88*$^{-/-}$ mice, as significantly reduced levels of only MCP-1 were observed at day 5 postinfection. We conclude that MyD88 and TLR2/4 may trigger inflammatory responses that ultimately lead to ECM when infections are associated with blood stages. However, the associated cytokine response is largely dependent on MyD88 alone. In the absence of *TLR2/4*, high levels of proinflammatory cytokines can be compensated for by a yet-unknown mechanism that is beyond the scope of this study. Irrespective of the underlying mechanisms, the observed dichotomy of protection against ECM in cytokine^{high} as well as cytokine^{low} infection models underscores the complexity of this acute neuroinflammatory complication, as documented for human cerebral malaria (2, 50).

The recent description of a role for the Nalp3 inflammasome in response to hemozoin as a malarial "danger signal" (13, 42) prompted us to test whether caspase-1-dependent activation of IL-1β and IL-18 is a central pathway which triggers ECM pathology. In this study, we found caspase-1, IL-1β, and IL-18 to be dispensable for the induction of ECM. Knockout mice succumbed to cerebral signs indistinguishably from WT mice. In support of earlier findings that IL-18 is dispensable for ECM caused by *P. berghei* ANKA blood stages (44), we now extend this conclusion to sporozoite-induced *Plasmodium* infections. We also demonstrated that IL-18 is not a key trigger for high IFN-γ production. Our results also support very recent findings that IL-1β receptor-deficient mice infected with 10^6 iRBCs are susceptible to ECM (38). Based on our findings, the validity of this study can likely be extended to sporozoite-
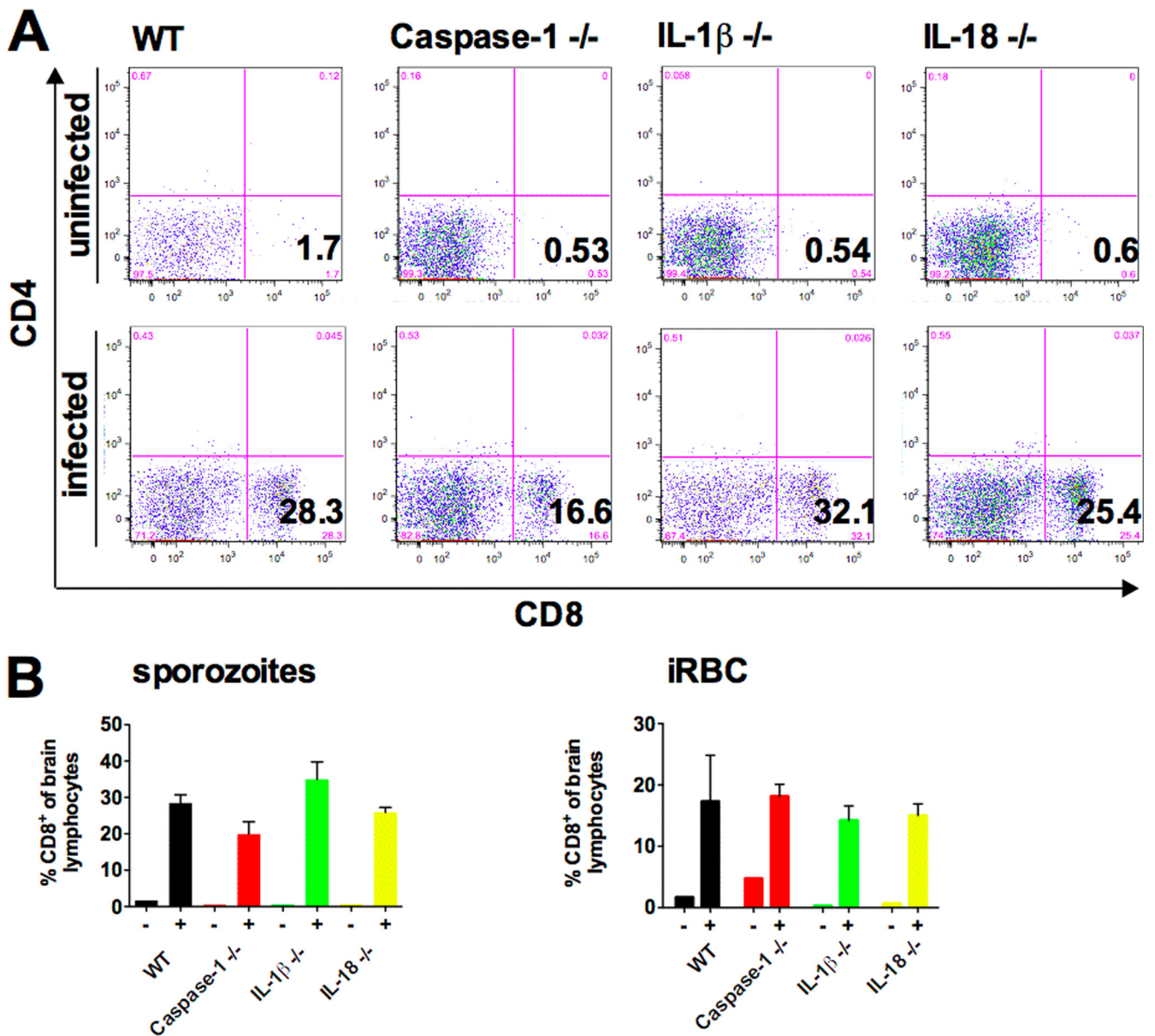
FIG. 5. CD8^{+} T cells sequester to the brains of *caspase-1*^{−/−}, *IL-1*β[−/−], and *IL-18*^{−/−} mice suffering from ECM. (A) Representative dot plots of leukocytes from brains of naïve mice and of mice suffering from ECM at 7 days after *P. berghei* sporozoite injection. (B) Percentages of CD8^{+} cells among total brain-derived leukocytes from mice that developed ECM (+) and naïve age-matched control mice (−).

induced infections. A potential translational aspect of the disruption of IL-1β signaling was ultimately assessed by protective treatment of *P. berghei* ANKA-infected mice with anakinra. We could rule out any beneficial effect on animal survival or reduced parasite growth, although the doses that we applied were several times higher than what proved to be efficient in a mouse model of acute gout (45). The combined results from the infection of knockout mice and pharmacological intervention in infected WT animals suggest that interference with IL-1β signaling is not a promising approach for adjunct therapy against neurological complications of malaria.

It is important to note that our study was not designed to test the role of hemozoin in activation of the Nalp3 inflammasome. However, our *in vivo* studies demonstrate that this pathway is not crucially involved in the immunopathology of *Plasmodium* infections. A reason for this apparent discrepancy may lie in the smaller amounts and accessibility of hemozoin following natural *Plasmodium* infections than after experimental inoculation of pure hemozoin. Another possible explanation is that hemozoin triggers a pleiotropic response that, in addition to activating of IL-1β and IL-18, induces other, yet-unidentified ECM-inducing innate immune pathways.

Most importantly, our findings also reflect inconclusive data from field studies in Africa and Asia, which have so far failed to convincingly correlate serum/plasma levels of IL-1β (31) or IL-18 (35) with the severity of disease.

In conclusion, using comparative experimental *Plasmodium* infections, we found differential immunopathological patterns
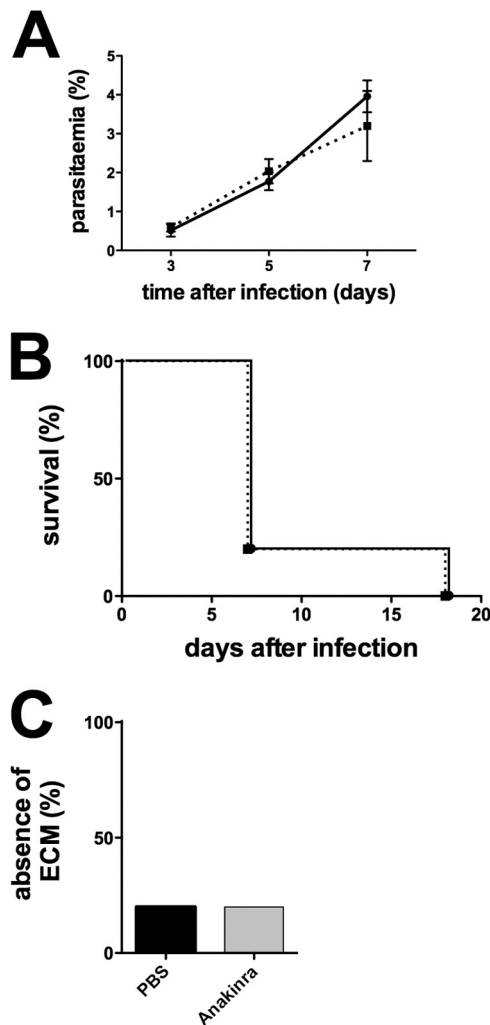
FIG. 6. Treatment with anakinra does not improve the clinical course of *P. berghei* ANKA infection. WT C57BL/6 mice (*n* = 5) were infected with 10⁴ *P. berghei* ANKA sporozoites i.v. and treated for 7 days with 250 mg/kg anakinra i.p. once daily (■). Controls were infected with the same number of parasites and received injections of PBS (●). (A) Average parasitemia during the course of infection. Error bars indicate the SEM. (B) Kaplan-Meier analysis of the time from infection to development of signs of ECM. (C) Absence of ECM shown as the percentage of infected animals.

associated with MyD88 and TLR2/4 and no evidence for an *in vivo* role of proinflammatory cytokines that are under the control of the Nalp3 inflammasome in experimental cerebral malaria.

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