

Experimental cerebral malaria progresses independently of the Nlrp3 inflammasome

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Cerebral malaria is the most severe complication of *Plasmodium falciparum* infection in humans and the pathogenesis is still unclear. Using the *P. berghei* ANKA infection model of mice, we investigated a potential involvement of Nlrp3 and the inflammasome in the pathogenesis of cerebral malaria. Nlrp3 mRNA expression was upregulated in brain endothelial cells after exposure to *P. berghei* ANKA. Although β -hematin, a synthetic compound of the parasites heme polymer hemozoin, induced the release of IL-1 β in macrophages through Nlrp3, we did not obtain evidence for a role of IL-1 β *in vivo*. Nlrp3 knock-out mice displayed a delayed onset of cerebral malaria; however, mice deficient in caspase-1, the adaptor protein ASC or the IL-1 receptor succumbed as WT mice. These results indicate that the role of Nlrp3 in experimental cerebral malaria is independent of the inflammasome and the IL-1 receptor pathway.

Key words: Brain endothelial cells · Cerebral Malaria · Inflammasome



Supporting Information available online

Introduction

Plasmodium (*P.*) parasites, the causative agents of malaria infect 200–300 million humans annually with 2–3 million deaths *per* year. The most severe form of malaria is cerebral malaria caused by infection with *P. falciparum* parasites [1]. Cerebral malaria is a complication facilitated by excessive inflammatory conditions that contribute to the adhesion of erythrocytes infected with

P. falciparum to endothelial cells in brain capillaries [2]. Infection of rodents with *P. berghei* ANKA serves as a model of cerebral malaria. The underlying pathogenic mechanisms of cerebral malaria are incompletely understood. A Th1 response and recruitment of pathogenic CD8⁺ and CD4⁺ T cells along with the release of pro-inflammatory cytokines such as TNF, lymphotoxin- α and IFN- γ are required for the onset of cerebral malaria [2]. In addition, the activation of the brain microvascular endothelium is of pivotal importance [3, 4].

During the replication of *P.* parasites in erythrocytes, hemoglobin is degraded by the parasite and polymerized to the heme polymer hemozoin [5]. Synthetic hemozoin is called β -hematin.

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Given the limited amounts of natural hemozoin and the contamination of hemozoin by other parasite components make synthetic β -hematin a useful tool to assess the induction of immune responses to this parasite component. Studies revealed that hemozoin can be recognized by TLR9 and contribute to the innate immune sensing of the parasite [6].

The inflammasome is a multi-protein complex activating caspase-1 and releasing bioactive IL-1 β [7]. The Nod-like receptor Nlrp3 mediates inflammasome activation to a variety of microbial and endogenous stimuli [7, 8]. Two signals are required for the cleavage of pro-IL-1 β and the activation of the inflammasome. A first signal provides the transcription of pro-IL-1 β and components of the inflammasome such as Nlrp3 [9]. A second signal, such as ATP, that acts on the P2X7 receptor, or particulate substances lead to the assembly and activation of the Nlrp3 inflammasome and cleavage of caspase-1 [7]. It has been reported that malarial hemozoin can activate the Nlrp3 inflammasome [10, 11]. It was suggested that the Nlrp3 inflammasome was important for the development of experimental cerebral malaria based on studies in Nlrp3-deficient mice. However, the role of the inflammasome in cerebral malaria remained unclear [10, 12]. In this report, we investigated the involvement of Nlrp3, ASC, the P2X7 receptor, caspase-1 and the IL-1 receptor in the pathogenesis of experimental cerebral malaria.

Results and discussion

β -Hematin but not *Plasmodium* parasites induce IL-1 β secretion *in vitro*

It has been reported that β -hematin can induce the activation of the inflammasome and release of IL-1 β [10, 11]. When macrophages were incubated with β -hematin, release of IL-1 β was not detected (Fig. 1). The addition of 5 mM ATP as a second signal for inflammasome activation did not trigger IL-1 β release either. Only the co-incubation of β -hematin with LPS triggered the release of IL-1 β in an Nlrp3-dependant manner. LPS but not β -hematin or *P. falciparum* crude extracts triggered IL-6 production, which was further enhanced by the co-incubation of with β -hematin or *P. falciparum* crude extracts (Fig. 1). Our data are in accordance with recently published studies and confirm the release of IL-1 β in response to high concentrations of β -hematin when primed with LPS [10–11]. We next incubated *P. falciparum* 3D7 crude extracts or natural hemozoin with macrophages for 16 h. Neither *P. falciparum* crude extracts nor natural hemozoin triggered detectable release of IL-1 β from macrophages (data not shown). When *P. berghei* ANKA infected erythrocytes were incubated with macrophages at a multiplicity of infection of 100 parasitized erythrocytes *per* macrophage, the release of IL-1 β was not detected either (data not shown). Thus, although high concentrations of the synthetic compound β -hematin induced the release of IL-1 β by macrophages when co-incubated with LPS *in vitro*, this response was not induced by *P. berghei* ANKA parasites

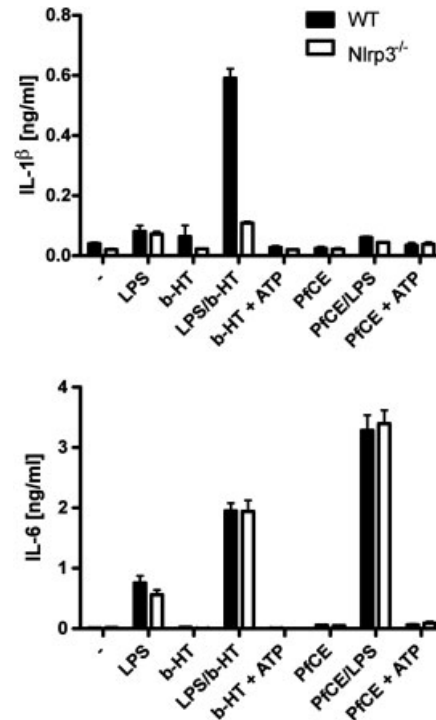


Figure 1. The role of the Nlrp3 inflammasome in response to β -hematin and *Plasmodium* extracts. Macrophages from WT or Nlrp3^{-/-} mice were incubated in the presence of 1 μ g/mL LPS, 250 μ g/mL β -hematin (β -HT) or 1 mg/mL *P. falciparum* 3D7 extracts (PfCE). As indicated, either 1 μ g/mL LPS or 5 mM ATP was added to the β -hematin and *P. falciparum* culture, respectively. LPS was added for the entire incubation period whereas ATP was only added for the last 30 min. After 16 h, culture supernatants were collected and IL-1 β and IL-6 release were assessed by ELISA. Data show mean + SEM and are representative of three independent experiments.

or *P. falciparum* parasite extracts. As the serum concentrations of hemozoin *in vivo* are estimated to be 1.9 μ g/mL in the case of mild anemia and 12.9 μ g/mL in the case of severe anemia [13], the concentrations of β -hematin that elicited an IL-1 β response *in vitro* was of a 20–130 fold excess of the concentrations encountered *in vivo*.

Nlrp3 is involved in the pathogenesis of cerebral malaria

We next tested if Nlrp3 and the inflammasome are involved in the pathogenesis of cerebral malaria *in vivo*. Therefore, we infected WT, Nlrp3^{-/-}, P2X7R^{-/-}, ASC^{-/-}, caspase-1^{-/-} and IL-1R^{-/-} mice with 10⁶ *P. berghei* ANKA parasitized red blood cells (Fig. 2). WT mice developed cerebral malaria and succumbed to infection with a median survival of 6 days. The median survival of Nlrp3^{-/-} mice was prolonged by 2 days and Nlrp3^{-/-} succumbed to *P. berghei* ANKA infection after 8 days. Mice deficient in the P2X7 receptor, ASC, caspase-1 and the IL-1 receptor succumbed to *P. berghei* ANKA infection as WT mice. The caspase-1 survival data are in accordance with Griffith *et al.* who previously reported that cerebral malaria develops independently of caspase-1 [12].

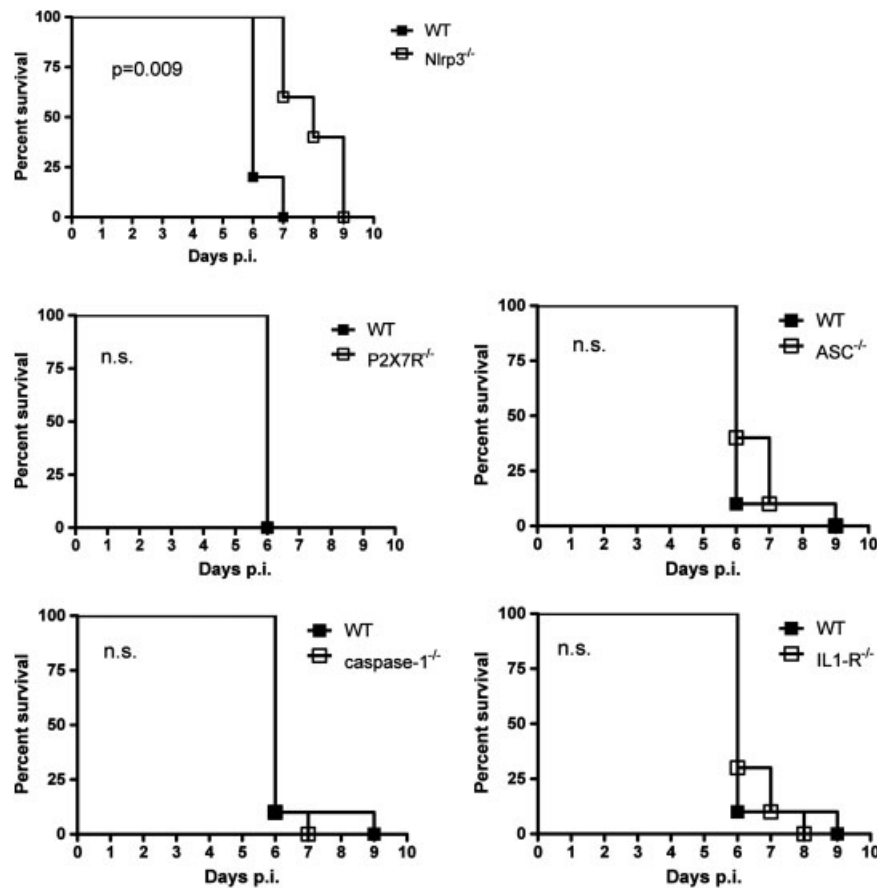


Figure 2. Nlrp3 functions independently of the inflammasome in experimental cerebral malaria. WT, Nlrp3^{-/-}, P2X7R^{-/-}, ASC^{-/-}, Caspase-1^{-/-} and IL-1R^{-/-} mice were intraperitoneally infected with 10⁶ *P. berghei* ANKA infected erythrocytes and monitored for survival. Ten mice per group were used for all groups, except for the P2X7 receptor mice. A representative survival pattern of at least two independent experiments is shown, except for the P2X7R^{-/-} mice, which has only been performed once with five mice per group. Statistical differences were assessed using Gehan-Breslow-Wilcoxon test and are indicated in the figures.

Orengo *et al.* observed the induction of proinflammatory cytokines by *Plasmodium* derived uric acid *in vitro* [14]. We collected serum samples to determine cytokine levels induced by *P. berghei* ANKA infection in WT and Nlrp3^{-/-} mice. Serum levels of IFN- γ , IL-10, TNF and IL-6 were induced in a similar manner in WT and Nlrp3^{-/-} mice. IL-1 β serum levels were not induced within 5 days of infection in WT or Nlrp3^{-/-} mice (Supporting Information Fig. 1A). Similarly, we did not detect differences in the development of blood parasitism in WT and Nlrp3^{-/-} mice (Supporting Information Fig. 1B). We isolated brain tissue mRNA from WT mice after 5 days of infection with *P. berghei* ANKA and assessed the expression of Nlrp3. Nlrp3 mRNA was approximately twofold induced in the brain of WT mice (Supporting Information Fig. 2). The chemokine IP-10, which has been shown to mediate the recruitment of disease inducing leukocytes to the brain [15], as well as markers of endothelial activation such as P-selectin and ICAM-1 were induced in a comparable manner in WT and Nlrp3^{-/-} mice (Supporting Information Fig. 2). Although the delayed onset of cerebral malaria in Nlrp3^{-/-} mice is comparable with data reported by Dostert *et al.* [10], we provide evidence that inflammasome components

are not involved in the pathogenesis of experimental cerebral malaria.

Nlrp3 does not contribute to T-cell activation

IFN- γ and Th1 cells are essential for cerebral malaria pathogenesis [2]. We assessed the activation of T cells in response to infection with *P. berghei* ANKA. Mice were infected with 10⁶ *P. berghei* ANKA parasitized erythrocytes. The activation of T cells was assessed by the expression of CD69 and CD62L. Nlrp3^{-/-} T cells showed a similar upregulation of CD69 and downregulation of CD62L when compared with WT T cells (Fig. 3A). We next incubated splenocytes and stained for IFN- γ , CD4 and TCR β . We did not detect a difference in the frequency of IFN- γ producing CD4⁺ T cells between WT and Nlrp3^{-/-} mice (Fig. 3B). To assess the ability of WT and Nlrp3^{-/-} T cells to induce cerebral malaria *in vivo*, adoptive transfer experiments were performed. A mixture of 10⁶ CD4⁺ and 10⁶ CD8⁺ cells were transferred into TCR β ^{-/-} recipient mice. Subsequently, mice were infected with 10⁶ *P. berghei* ANKA parasitized erythrocytes

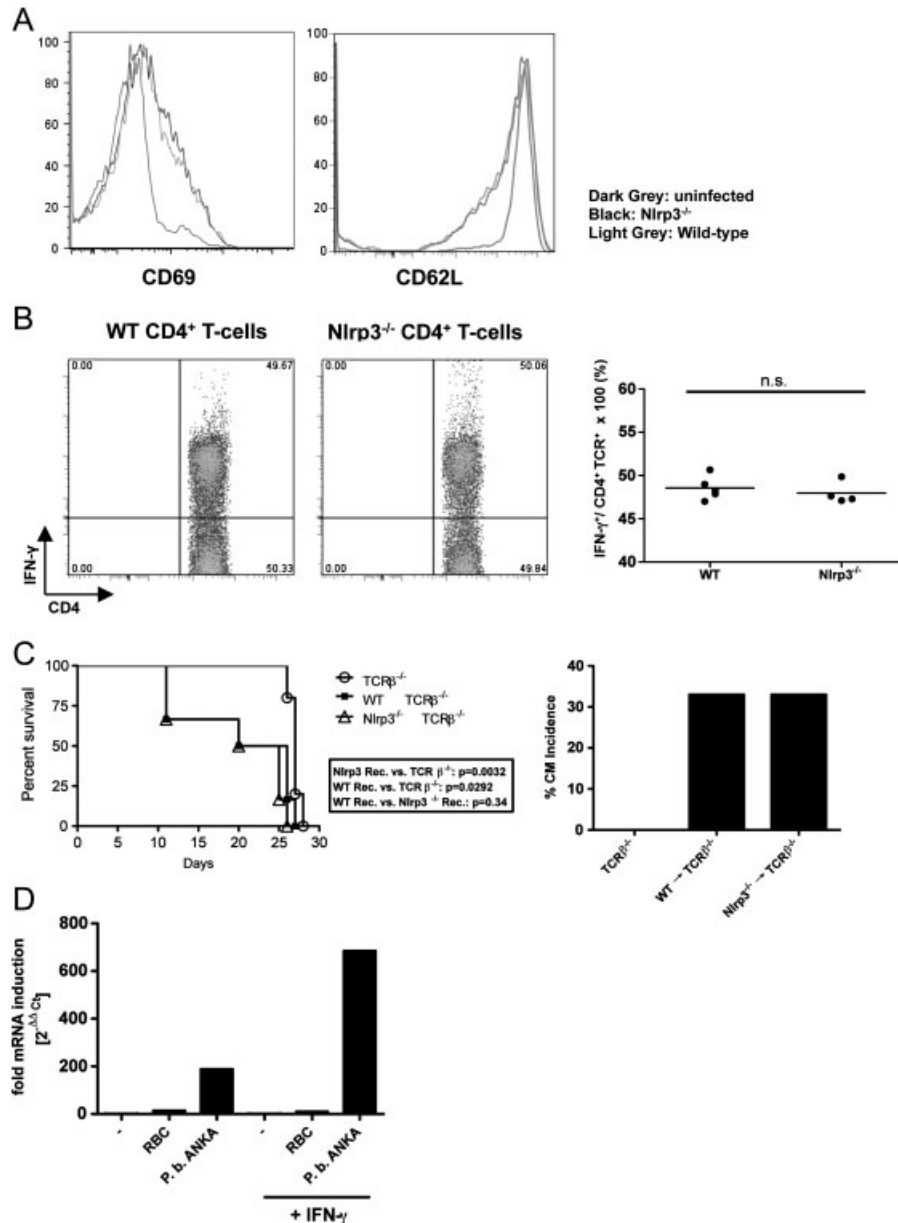


Figure 3. Nlrp3 does not function in T cells but is induced in brain endothelial cells. Splenocytes were isolated from WT and Nlrp3^{-/-} mice after 5 days of infection with *P. berghei* ANKA and stained for the expression of CD69, CD62L (A), and the expression of IFN- γ (B). Data are representative of three independent experiments. Data for individual mice are plotted as a dot plot. Statistical differences were assessed using the unpaired Student's t-test. (C) Purified CD4⁺ and CD8⁺ T cells from WT and Nlrp3^{-/-} mice were adoptively transferred into TCR β ^{-/-} recipients. Briefly, 24 h after transfer, recipients were infected with 1 \times 10⁶ *P. berghei* ANKA infected red blood cells and monitored for survival (left panel) and incidence of cerebral malaria (right panel) at day 10. Statistical differences were assessed using Gehan-Breslow-Wilcoxon test and are as indicated. Data are representative of one experiment. (D) Mouse brain microvascular endothelial cells were incubated with uninfected erythrocytes or *P. berghei* ANKA parasitized erythrocytes for 16 h. As indicated, 100 ng/mL r murine IFN- γ was added. RNA was collected, and Nlrp3 expression was assessed using quantitative PCR. Data are expressed as fold mRNA induction over untreated control samples (2^{ΔΔCt}). Data are representative of two independent experiments.

and monitored for survival (Fig. 3C). TCR β ^{-/-} mice were resistant to the onset of cerebral malaria and succumbed after 4 wk of infection due to anemia. Approximately, 33% of TCR β ^{-/-} mice receiving either WT or Nlrp3^{-/-} T cells developed cerebral malaria (Fig. 3C). Thus, WT and Nlrp3^{-/-} T cells were equally activated in response to *P. berghei* ANKA and induced onset of cerebral malaria after transfer into TCR β ^{-/-} recipient mice.

Nlrp3 expression is induced in brain microvascular endothelial cells

As cerebral malaria is characterized by a break-down of the blood-brain barrier [3], we incubated mouse brain microvascular endothelial cells with *P. berghei* ANKA parasitized erythrocytes and assessed the expression of Nlrp3. Parasitized

erythrocytes but not uninfected erythrocytes induced an the induction of Nlrp3 mRNA (Fig. 3D). When IFN- γ was incubated with *P. berghei* ANKA infected erythrocytes, the upregulation of Nlrp3 mRNA in brain microvascular endothelial cells was enhanced to an approximately 650-fold induction over uninfected controls (Fig. 3D). Thus, Nlrp3 expression is induced in the brain and brain endothelial cells after infection with *P. berghei* ANKA.

Concluding remarks

Although parasite components such as β -hematin induced the release of IL-1 β *in vitro*, we were unable to detect the secretion of IL-1 β *in vivo*. Consistently, mice deficient in components of the inflammasome succumbed to infection with *P. berghei* ANKA with similar kinetics as WT mice. The delayed onset of cerebral malaria in Nlrp3^{-/-} mice and the induction of Nlrp3 in brain tissue and brain microvascular endothelial cells reveals a potential function of Nlrp3 in the pathogenesis of experimental cerebral malaria independently of the inflammasome.

Materials and methods

Mice, cells and parasites

Mice have been described previously [7]. C57Bl6/J and TCR β ^{-/-} mice were purchased from Jackson Laboratories. For survival experiments, mice were sacrificed at the onset of neurological symptoms typical of cerebral malaria (ataxia, seizures, convulsions). The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals. Macrophages were isolated as described previously [16]. Mouse brain microvascular endothelial cells were obtained from cell applications. For adoptive transfer experiments, CD4⁺ and CD8⁺ T-cells were labeled with microbeads (Miltenyi Biotec) and sorted through a magnetic column. *P. berghei* ANKA clone c115cy1 was obtained from MR4 (Manassas, VA, USA). Prior to infection experiments, a frozen parasite stock was passed through a C57Bl6/J mouse and parasites were harvested after 6 days of infection. Mice were infected with 10⁶ infected red blood cells in 500 μ L PBS intraperitoneally.

Preparation of β -hematin and *P. falciparum* 3D7 crude extracts

Mycoplasma-free *Pf* parasites (3D7) were maintained as described previously [17]. After synchronization, mature and HZ-rich mostly trophozoite and schizont stage parasites (~4–5%) were purified by 63% Percoll density centrifugation, washed and resuspended in

incomplete medium, freeze-thawed three to four times, and stored at -80°C until use. Each milliliter of HZ-rich *Pf* crude extract preparation contained approximately 1×10^9 infected erythrocytes. β -Hematin was purified from hemin chloride. In total 45 mg of hemin chloride was dissolved in 4.5 mL of 1 N NaOH and neutralized with 1 N HCl. Then, acetic acid was added until the pH reached 4.8, at a constant temperature of 60°C with magnetic stirring. The mixture was allowed to precipitate at room temperature overnight. The precipitate was subjected to washes with 2% SDS-buffered with 0.1 M sodium bicarbonate (pH 9.1) and subsequent extensive washes with 2% SDS, and then six to eight washes with distilled water.

Flow cytometry

Surface staining was performed with FITC-conjugated anti-CD4 (clone GK1.5 from eBioscience) and PE-conjugated anti-TCR β (clone H57-957 from eBiosciences). Intracellular staining for IFN- γ was performed using Allophycocyanin-conjugated anti-IFN- γ Ab (clone XMG1.2 from eBioscience). CD69 and CD62L were stained with a FITC-conjugated anti-CD69 Ab (clone H1.2F3 from eBioscience) and an allophycocyanin-conjugated CD62L Ab (clone MEL-14 from eBiosciences).

RNA isolation and quantitative PCR

Total RNA was isolated using a total RNA isolation kit (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit (BioRad) and SYBRgreen master mix (Applied Biosystems) was used for QPCR. β -Actin was used for normalization. The following primer sequences were used: Nlrp3 F: ATGGTATGCCAGGAGGACAG; Nlrp3 R: ATGCTCCTTGACCAGTTGGA; IP-10 F: GCTGCAACTGCATCCATATC; IP-10 R: TTTTGGCTAAACGCTTTCATT; P-selectin F: CCGGAAAGACTGGATTGTTTC; P-selectin R: TCCTCATTGCAGGAGAAGTG; ICAM-1 F: GAGAGTGACCCAACTGGAA; ICAM-1 R: AGGGTGAGGTCCTTGCCCTAC; β -actin F: AGAGGGAAATCGTGCGTGAC; β -actin R: CAATAGTGATGACCTGGCCGT.

ELISA

Serum was collected from mice prior to infection with *P. berghei* ANKA and 5 days after infection. ELISA was performed as described previously [16].

Statistical analysis

Prism software (GraphPad) was used to determine statistical significances. Data were analyzed by unpaired *t*-test; Survival data were analyzed by Gehan–Breslow–Wilcoxon test. A *p*-value smaller than 0.05 was considered significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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