Short Report: Disruption of Nod-like Receptors Alters Inflammatory Response to Infection but Does Not Confer Protection in Experimental Cerebral Malaria

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Abstract. Research relating to host inflammatory processes during malaria infection has focused on Toll-like receptors, membrane-bound receptors implicated in innate sensing, and phagocytosis of parasitized erythrocytes by host cells. This is the first study to examine the role of Nod proteins, members of the Nod-like receptor (NLR) family of cytoplasmic proteins involved in pathogen recognition, in a murine model of cerebral malaria (Plasmodium berghei ANKA, PbA). Here, we find that nod1nod2−/− mice infected with PbA show no difference in survival or parasitemia compared with wild-type infected animals. However, cytokine levels, notably those associated with NLR activation including interleukin (IL)-1β, KC, and MCP-1, and proteins linked to malaria pathogenesis, such as interferon-γ (IFN-γ), were decreased in the nod1nod2−/− animals. We therefore demonstrate for the first time that Nod proteins are activated in response to parasites, and they play a role in regulating host inflammatory responses during malaria infection.

Severe malaria can present as a variety of clinical syndromes, including both severe malarial anemia and cerebral malaria (CM), to which the majority of deaths are attributable.† Plasmodium berghei ANKA infection represents an experimental model of CM, with associated neurologic symptoms.‡,§ Mice susceptible to this pathogen (e.g., C57BL/6 strain) succumb to infection within 6–10 days as a result of excess, dysregulated inflammation mediated by pro-inflammatory cytokines, such as tumor necrosis factor (TNF)/lymphotoxin-α and interferon-γ (IFN-γ).∥,‡ It is the first study to demonstrate NLR signaling during a parasitic infection, specifically implicating the Nod proteins in malaria disease processes but not outcome.

Pattern recognition receptors play a critical role in the inflammatory response and the activation of the host innate immune response. 15–19 Although pro-inflammatory cytokines are essential for parasite clearance, the overproduction of cytokines through the stimulation of immune cells by parasite products can contribute to CM and fatal outcomes. 10–14 Current research is focused on discovering the host receptors responsible for mediating this immunopathology, with particular attention to pattern recognition receptors.

Pattern recognition receptors play a critical role in the activation of signaling pathways leading to the activation of transcription factors such as NF-κB and ultimately the production of pro-inflammatory cytokines and chemokines. 22,24 The roles of TLRs and NLRs in generating/amplifying this inflammatory response during malaria infection, however, have yet to be fully defined. TLR2, 4, and 9 have been shown to recognize various Plasmodium-associated molecules, including the malaria toxin glycoprophosphorylidyinositol (GPI) and malarial DNA complexed to the parasite waste product hemozoin, leading to the secretion of pro-inflammatory cytokines by host cells. 25–27 However, at present their contribution to the inflammatory response remains controversial. Coban and colleagues demonstrated that the survival of susceptible mice infected with P. berghei ANKA was increased in animals lacking tlr2 or tlr9, but not tlr4; these results have since been contested. 29–31 Furthermore, in human studies polymorphisms in the tlr4 and tlr9, but not tlr2 genes, have so far been associated with disease severity. 32,33 Whereas a functional variant of MAL, a signaling molecule in the TLR2/4 pathway, has been linked to protection from severe malaria, 33 thus, although it is clear that TLRs do play a role during malaria infection, the nature and extent of their function remain to be elucidated.

As with TLRs, activation of NLRs has been shown to contribute to pathogenesis in several infectious diseases. 34 More specifically, both Nod1 and Nod2 recognize bacterial molecules produced during the synthesis and/or degradation of peptidoglycan, 20,21,35,36 and as such, mice lacking these proteins show increased susceptibility to and impaired clearance of bacteria. 37,38 However, little is known about the involvement of NLRs during malaria infection. Recent studies have shown that Nod proteins are upregulated when peripheral blood mononuclear cells (PBMCs) are exposed to malaria sporozoites, 39 but the role of NLRs during blood-stage infection, and their contribution to severe malaria pathogenesis, have not yet been examined. This was the focus of our study, and using the P. berghei ANKA murine model of CM, we found that Nod1 and Nod2 have no direct effect on survival (Figure 1A) or parasitemia (Figure 1B) of infected C57BL/6 mice. However, levels of cytokines/chemokines associated with NLR activation (IL-1β, KC, and MCP-1) and, interestingly, malaria pathogenesis (IFN-γ) were influenced by the absence of Nod proteins (Figure 2). As such, this is the first study to demonstrate NLR signaling during a parasitic infection, specifically implicating Nod proteins in malaria disease processes but not outcome.

The production of IL-1β, KC, and MCP-1, known to be induced by Nod1 and Nod2, was observed during malaria infection in wild-type mice. The nod1nod2−/− animals, however, had reduced levels of IL-1β (baseline differences observed at D0 are not significantly different, Student’s t test, P = 0.12), KC, and MCP-1 over the course of infection (Figures 2A and 2C), indicating that Nod proteins are activated by malaria infection, through currently unknown pathways or in response to yet unidentified parasite-derived agonists. The reduced IL-1β levels observed in nod1nod2−/− mice (Figure 2A) during infection are of particular interest in light of recent
Influence of Nod proteins on susceptibility to cerebral malaria. The 8–12-week-old C57BL/6 mice (+/+ and nod1nod2−/− (+/−, backcrossed 9 times to the C57BL/6 background) mice were infected with 1 × 10⁸ Plasmodium berghei ANKA parasitized erythrocytes by intraperitoneal injection. Parasitemia was measured every 2 days by thin blood smear. (A) Survival data are combined data from three experiments (N = 21 mice per group; Log rank test: P = 0.62). (B) Parasitemia (N = 10 mice per group; mean ± SEM; Student’s t test on sum values of parasitemia over time for each animal: P = 0.94). Data shown are representative of three independent experiments with a minimum of 5 animals per group.

Publications implicating IL1-β and IL1-β signaling in the pathogenesis of human cerebral malaria⁴⁶,⁴⁷ and severe malarial anemia.⁴² Therefore, Nod proteins may be involved in malaria pathogenesis through their strong induction of IL1-β.

The decreased levels of IFN-γ recorded in the nod1nod2−/− animals could be attributed to IL1-β because this cytokine was implicated in the production of IFN-γ.⁵³ However, the lower levels of both plasma IL1-β and IFN-γ had no effect on plasma TNF or IL-6 levels, which remained similar in both wild-type and nod1nod2−/− animals over the course of infection.

Numerous studies have demonstrated that IFN-γ plays a critical mechanistic role in the pathogenesis of CM. High levels of this protein lead to excessive host inflammation and development of severe disease.¹²-¹⁴ Treatment with anti-IFN-γ antibody protects animals from CM,⁴¹ IFN-γ, and IFNγR−/− mice show resistance to CM, and recent microarray analysis implicates IFN response mechanisms in susceptibility to CM.⁴⁶ The IFN-γ not only impacts the host inflammatory response, but is also required for the control of blood stage parasite replication during P. berghei ANKA infection.⁴⁵ Previously, treatment with anti-IFN-γ antibody was shown to have no effect on parasitemia.⁷ However, later work conducted by Amani and colleagues using IFNγR−/− animals rather than anti-IFN-γ antibody treatment demonstrated that parasitemia is affected by IFN-γ levels, but only at high parasite burdens.⁴⁶

In concordance with our findings, other members of the NLR signaling pathway have been shown to regulate IFN-γ production during bacterial infection.⁴⁷ However, despite the decreased IFN-γ levels observed in our study, nod1nod2−/− animals did not possess a survival advantage over their wild-type counterparts, nor was their parasitemia affected. The low levels of IFN-γ detected in the nod1nod2−/− animals may therefore have been sufficient for initiating host inflammatory responses, ultimately leading to CM, without affecting parasitemia. Furthermore, the lack in early IFN-γ production in the nod1nod2−/− animals, which was shown to be protective against progression to CM in non-cerebral P. berghei models,⁴⁸ may have contributed to CM pathology. Indeed, observing the brains of all animals collected upon euthanasia, no differences in pathology were observed between those of wild-type versus nod1nod2−/− mice (data not shown).

Our experiments indicate that Nod proteins are not responsible for the onset or course of CM, but that they are activated by infection. Therefore, our results are consistent with current thinking that inflammation during malaria occurs through mainly TLR signaling.¹⁰,²⁶-²⁸,³³ Parasite-infected erythrocytes are recognized by the host immune system through the interaction between TLRs on the surface of host cells and parasite proteins. These interactions mediate inflammatory responses to infection and may augment phagocytosis, and contribute to clearance of parasites by host cells. Other models of pathogen-phagocyte interaction have demonstrated that NLRs can recognize their cognate ligands after pathogen internalization. This process could explain how host inflammation was altered during P. berghei ANKA infection in the nod1nod2−/− animals.

Activation of innate immune responses during bacterial infection requires both TLRs and NLRs,⁴⁶ and this may also be true during malaria infection. Several studies have linked Nod responses with TLR stimulation. Treatment of epithelial cells with TNF and IFN-γ upregulates the expression of nod2, amplifying their inflammatory response to TLR ligands, such as LPS.⁵⁰ Similarly, another study has shown that treating murine macrophages with LPS, IL-6, or TNF increases Nod2 protein and nod2 mRNA expression.⁵¹ Finally, when using malaria sporozoites to stimulate PBMCs, examination of the transcriptional profile of these cells indicated elevated levels of nod2, tlr2, and myd88.³⁹ Therefore, TLRs and NLRs may both contribute to CM, although to a different extent; teasing out their respective roles will prove vital in understanding how the dysregulated host inflammatory response to malaria parasites is generated. Future work should therefore consider disease pathology and cytokine levels in target organs, such as the brain, lung, spleen, and liver, which have proved informative in dissecting differences in susceptibility to CM,³⁵ and may yield more information as to the effect of Nod proteins at the inflammatory sites during malaria infection.

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Figure 2. Plasma cytokine levels during *Plasmodium berghei* ANKA infection. The 8–12-week old C57BL/6 mice (+/+) and *nod1nod2*−/− (−/−) mice were infected with $1 \times 10^6$ parasitized erythrocytes. Plasma was collected on days 1, 5, and 6, and cytokines measured using the CBA mouse inflammation kit (BD Pharmingen) and ELISA IL1-β and KC kits (R&D Systems). Parametric data were analyzed using Student's $t$ test, whereas the Mann–Whitney test was used for non-parametric data. Data are representative of two independent experiments (with $N \geq 5$ mice per group). (A) Plasma levels of interferon-γ (IFN-γ) (left, data are represented as mean ± SEM, $P = 0.03$, Mann–Whitney test on sum values of cytokine level over time for each animal, $N = 10$ per group), and IL1-β (right, data are represented as mean ± SEM, $P = 0.001$, Students $t$ test on sum values of cytokine level over time for each animal, $N = 10$ per group) over the course of infection. (B) Plasma levels of tumor necrosis factor (TNF) (left, data are represented as mean ± SEM, $P = 0.08$, Students $t$ test on sum values of cytokine level over time for each animal, $N = 10$ per group), and IL-6 (right, data are represented as mean ± SEM, $P = 0.79$, Students $t$ test on sum values of cytokine level over time for each animal, $N = 10$ per group) over the course of infection. (C) Plasma levels of MCP-1 (left, data are represented as mean ± SEM, $P = 0.01$, Mann–Whitney test on sum values of cytokine level over time for each animal, $N = 10$ per group), and KC (right, data are represented as mean ± SEM, $P = 0.002$, Mann–Whitney test on sum values of cytokine level over time for each animal, $N = 10$ per group) over the course of infection.
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