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Disruption of JNK2 Decreases the Cytokine Response to Plasmodium falciparum Glycosylphosphatidylinositol In Vitro and Confers Protection in a Cerebral Malaria Model

Ziyue Lu,† Lena Serghides,† Samir N. Patel,† Norbert Degousee,‡ Barry B. Rubin,‡ Gowdahalli Krishnegowda,§ D. Channe Gowda,§ Michael Karin,¶ and Kevin C. Kain2*†¶

Host inflammatory responses to Plasmodium falciparum GPI anchors are believed to play an important role in the pathophysiology of severe malaria. However, relatively little is known about the signal transduction pathways involved in pGPI-stimulated inflammatory response and its potential contribution to severe malaria syndromes. In this study, we investigated the role of MAPK activation in pGPI-induced cytokine secretion and examined the role of selected MAPKs in a model of cerebral malaria in vivo. We demonstrate that ERK1/2, JNK, p38, c-Jun, and activating transcription factor-2 became phosphorylated in pGPI-stimulated macrophages. A JNK inhibitor (1,9-pyrazoloanthrone) inhibited pGPI-induced phosphorylation of JNK, c-Jun, and activating transcription factor-2 and significantly decreased pGPI-induced TNF-α secretion. pGPI-stimulated JNK and c-Jun phosphorylation was absent in Jnk2−/− macrophages but unchanged in Jnk1−/− and Jnk3−/− macrophages compared with wild-type macrophages. Jnk2−/− macrophages secreted significantly less TNF-α in response to pGPI than macrophages from Jnk1−/−, Jnk3−/−, and wild-type counterparts. Furthermore, we demonstrate a role for JNK2 in mediating inflammatory responses and severe malaria in vivo. In contrast to wild-type or Jnk1−/− mice, Jnk2−/− mice had lower levels of TNF-α in vivo and exhibited significantly higher survival rates when challenged with Plasmodium berghei ANKA. These results provide direct evidence that pGPI induces TNF-α secretion through activation of MAPK pathways, including JNK2. These results suggest that JNK2 is a potential target for therapeutic interventions in severe malaria.

Plasmodium falciparum malaria is a major cause of global morbidity and mortality, responsible for an estimated 500 million cases and 1.5–2.7 million deaths annually (1). The majority of fatalities are attributable to severe malaria occurring primarily among young children in sub-Saharan Africa. The molecular mechanisms underlying severe malaria and its associated syndromes such as cerebral malaria are not fully understood. However, it is clear that these are complex multisystem disorders involving a number of immunopathogenic processes. These include erythrocyte invasion and destruction, dyserythropoiesis, sequestration of parasitized erythrocytes in the microvasculature, dysregulated cytokine cascades, and metabolic perturbation associated with the release of inflammatory mediators from monocytes/macrophages (mφs) in response to parasite bioactive molecules (2–5).

It is unknown why only a small proportion of infected individuals develop severe or cerebral malaria. A number of studies have reported that marked, sustained, and/or unbalanced proinflammatory cytokine response to infection, including high levels of TNF-α, IL-1, and IL-6, are associated with disease severity and fatal outcomes in both human falciparum malaria and murine models of severe or cerebral malaria (6–11). This has led to the hypothesis that excessive and/or dysregulated secretion of proinflammatory cytokines, in particular TNF-α and related mediators including lymphoxygenase-1 (12), contribute to the development of cerebral malaria. This hypothesis is supported by reports of increased susceptibility to cerebral malaria in patients with specific promoter polymorphisms that lead to increased TNF-α gene expression (13, 14). TNF-α and other mediators such as NO have been reported to up-regulate endothelial cell receptors including ICAM-1 and CD31, which may contribute to sequestration of parasitized erythrocytes in the cerebral vasculature (15, 16). In addition to potential adverse consequences associated with excessive or dysregulated production, inflammatory mediators also play a critical role in host defense against malaria parasites and promote mφ phagocytosis of parasitized erythrocytes (5). Therefore, innate pro- and anti-inflammatory responses to malaria must be tightly regulated to control infection without inducing immunopathology (5, 17–20).

Abbreviations used in this paper: mφs, monocytes/macrophages; pGPI, Plasmodium falciparum GPI; ATF-2, activating transcription factor-2; PD98059, 2’-amino-3’-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole; SP600125, 1,9-pyrazoloanthrone; MAPKK, MAPK kinase; WT, wild type.

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GPI anchors from a number of parasites including *Trypanosoma* and *Leishmania* spp. have been implicated in activation of the host innate immune system to produce proinflammatory cytokines in response to infection (21). Similarly, the innate immune system to produce proinflammatory cytokines in response to malaria in vivo. Using MAPK transcription factor-2 (ATF-2). We also demonstrate an important role of MAPK and AP-1 activation in regulating deleterious host inflammatory responses that appear to contribute to adverse clinical outcomes. In this study, we investigated the role of MAPK and AP-1 activation in pGPI-induced TNF-α secretion in mφs and explored the role of individual JNK isoforms or enzymes in the response to malaria in vivo. Using MAPK inhibitors and mφs from Jnk1−/− and Jnk2−/− mice, we demonstrate that pGPI induces TNF-α secretion, at least in part, through activation of the JNK2 pathway, leading to phosphorylation and activation of the transcription factors c-Jun and activating transcription factor-2 (ATF-2). We also demonstrate an important role for JNK2 in mediating severe malaria syndromes in vivo.

**Materials and Methods**

**Reagents and Abs**

DMEM and HBSS were from Invitrogen Life Technologies. FBS was from Sigma-Aldrich and was heat inactivated at 55°C for 30 min before use. Polyvinylidene difluoride membrane was from Millipore. ECL was from PerkinElmer. *Limulus* amebocyte lysate assay kit was from Associates of Cape Cod, 2′-Amino-3′-methoxyflavone (PD08059), 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), and 1,9-pyrazoloanthrone (SP600125) were from Calbiochem. Abs recognizing total and phospho-specific ERK1/2, JNK, p38, c-Jun, ATF-2, and p38 were from Cell Signaling Technology. ERK Ab was from Santa Cruz Biotechnology. HRP-conjugated goat anti-mouse IgGs and goat anti-rabbit IgGs were from Bio-Rad.

**Cells**

RAW264.7 cells, a murine mφ cell line (American Type Culture Collection), were cultured at 37°C in a 5% CO2 humidified incubator and maintained in DMEM supplemented with 10% FBS, 1.5 mM l-glutamine, and 25 ng/ml gentamicin. Peritoneal mφs were harvested in ice-cold HBSS 4 days after the i.p. injection of 2 ml of 3% sterile thioglycollate. The cells were washed twice with cold HBSS and resuspended in DMEM, 10% FBS, and l-glutamine at 1 × 10⁶ cells/ml. Cells were seeded into 24- or 96-well plates and incubated at 37°C with 5% CO2 overnight. The adherent cells were used the following day for experiments.

**Mice**

The JNK1 and JNK2 heterozygous mice were from M. Karin (University of California San Diego, La Jolla, CA) (40). JNK3 heterozygous mice were provided by Dr. R. Davis (University of Massachusetts, Medical School, Worcester, MA). Heterozygous mice were intercrossed to obtain homozygotes, which were identified by genotyping of DNA from tail samples. Jnk1−/−, Jnk2−/−, and Jnk3−/− mice, and their wild-type (WT) littermates from these intercrosses were used for experiments. All animals were maintained under specific pathogen-free conditions in a barrier facility. Mice were 8–12 wk of age at the time of experiments. Animal use was performed in compliance with current University Health Network institutional guidelines. All animal protocols were approved by the Animal Care Committee of the University Health Network.

**Parasite culture**

Parasite cultures were maintained and synchronized as described (41). Parasites from the laboratory clone ItG were used. Parasite cultures were treated with *Mycoplasma* removal agent (ICN Pharmaceuticals) and tested negative for *Mycoplasma* by PCR analysis before use. Culture supernatants were tested by the *Limulus* amebocyte lysate assay (42) for endotoxin. Only *Mycoplasma*- and endotoxin-free parasite culture supernatants were used for experiments. Parasite culture supernatants, which are rich sources of GPI, were collected, aliquoted, and frozen for subsequent use (41).

**Isolation and purification of GPIs from *P. falciparum***

Protein-free pGPIs were isolated and purified by HPLC as described (27). Briefly, *Mycoplasma*-free parasite cultures with 20–30% parasitemia were harvested at the schizont stage, treated with 0.025% saponin in Trager buffer, and passed through a 26-gauge needle to lyse the erythrocytes. The suspension was centrifuged and washed several times, and the erythrocyte debris was removed by centrifugation on a 5% BSA cushion. The parasites were washed three times with PBS, lysophylized, and extracted three times with chloroform/methanol (2:1, v/v) to remove nonglycosylated lipids. GPIs were extracted with chloroform/methanol-water (10:10:3, v/v/v), dried, and partitioned between water and water-saturated 1-butanol. The organic layer was washed four times with water and dried. The residue was extracted with 80% aqueous 1-propanol and dried, and the GPIs were further purified by HPLC. All preparations of purified pGPIs were tested for endotoxin before use. The purity of the GPIs was confirmed by mass spectrometry and carbohydrate compositional analysis.

**ELISA assay for TNF-α**

Murine peritoneal mφs from WT and JNK-deficient mice were seeded in 96-well plates (2.5–5 × 10⁴ cells/well) and allowed to adhere overnight. Unattached cells were washed away, and the remaining cells were stimulated with *P. falciparum* culture supernatants (1/5 to 1/500 dilution) or HPLC-purified GPIs (80–160 nM/ml). LPS (100 ng/ml) was used as a positive control to confirm the capacity of the mφ preparations to produce proinflammatory cytokines. For inhibition studies, cells were treated for 1 h with appropriate inhibitors before stimulation with pGPI. After different incubation periods at 37°C, the supernatants were collected, cleared by centrifugation, and assayed for TNF-α (eBioscience).

**ImmunobLOTS**

The mφs were cultured in 24-well plates (0.5–1 × 10⁵ cells/well) overnight in DMEM, 10% FBS and then stimulated with pGPI for various periods. For inhibition studies, the inhibitors were added to the culture medium 1 h before stimulation with pGPI. Cells were harvested and lysed at various
times. Lysates were separated on 12% SDS-PAGE and transferred to poly-vinylidene difluoride membrane. Blots were then probed with different specific antisera directed against phosphorylated or total proteins. After incubation with the appropriate HRP-conjugated secondary Ab, blots were developed using an ECL-based system.

**P. berghei ANKA infection**

*P. berghei ANKA* parasites were maintained by passage through C57BL/6 mice. Infections were initiated by i.p. injection of 1.5 × 10⁶ parasitized erythrocytes per mouse (*Jnk1^−/−*, *Jnk2^−/−*, and their WT littermates). After infection, blood was collected at days 3, 5, and 7 from each infected mouse, and parasitemia was calculated by counting at least 1000 cells on Giemsa-stained smears. Morbidity and mortality were assessed at least twice daily.

**Statistical analysis**

Experiments were performed in duplicate or triplicate and repeated at least three times. Differences between groups were analyzed using Student’s *t* test. Data are represented as mean ± SD, unless otherwise noted. Differences with *p* < 0.05 were considered significant.

**Results**

**pfGPI induces TNF-α secretion in a time- and dose-dependent manner**

Excessive or dysregulated secretion of proinflammatory cytokines such as TNF-α in response to infection, have been implicated in the pathophysiology of severe malaria syndromes (5, 18). We initially examined time- and dose-dependent induction of TNF-α from both RAW264.7 and murine peritoneal mφs (primed and unprimed with IFN-γ) using supernatant-derived and HPLC-purified pfGPI. Consistent with previous studies, both supernatant-derived and HPLC-purified pfGPI induced TNF-α secretion in a time- and a dose-dependent manner in RAW264.7 and murine peritoneal mφs (data not shown) (26).

**pfGPI-induced activation of MAPK pathways results in AP-1 activation and proinflammatory cytokine secretion**

pfGPI has recently been shown to activate MAPKs and NF-κB and contribute to the production of proinflammatory mediators, such as TNF-α (26). In addition to NF-κB, the TNF-α promoter contains recognition sites for the transcription factor complex AP-1. However, the role of AP-1 in pfGPI-mediated cytokine response has not previously been reported. We examined the requirement for MAPK pathways and phosphorylation of AP-1 components in pfGPI-induced cytokine response using specific MAPK inhibitors and mφs from mice with targeted gene deletions.

To initiate investigation into the role of MAPKs in pfGPI-induced signal transduction, we examined RAW264.7 and murine peritoneal mφs treated with supernatant-derived and HPLC-purified pfGPI over time. Cell lysates were examined for the activated forms of MAPKs by immunoblot analysis. ERK1/2, JNK, and p38 MAPK became phosphorylated in cells stimulated with pfGPI (Fig. 1A and data not shown) (26). Phosphorylation of ERK1/2, JNK, and p38 peaked at 15 min and was sustained at 60 min after exposure to pfGPI. To further characterize the role of pfGPI-induced MAPK activation, mφs were pretreated with inhibitors of JNK (SP600125) (43), MEK1/2 (PD98059) (44), or p38 MAPK (SB203580) (45), followed by pfGPI stimulation. PD98059, which inhibits MEK1/2 activity and blocks ERK1/2 phosphorylation, inhibited pfGPI-induced ERK1/2 phosphorylation (Fig. 1B and data not shown). Of interest, PD98059 also decreased pfGPI-induced JNK phosphorylation. SB203580, the p38 inhibitor, caused an increase in pfGPI-induced ERK1/2 and JNK phosphorylation. Pretreatment with SP600125 resulted in partial inhibition of JNK phosphorylation and an increase in p38 phosphorylation. Thus, the pattern of phosphorylation observed in the presence of these MAPK inhibitors suggests that cross-talk occurs between ERK1/2 and JNK and between JNK and p38 pathways during pfGPI-induced signaling. However, the observed effects may also be attributable to the lack of specificity of the inhibitors used. SP600125 has been shown to inhibit multiple kinases at concentrations approximating the IC₅₀ for inhibition of JNK activity (46).

We next investigated the effect of pfGPI-induced MAPK signaling on phosphorylation of transcription factors c-Jun and ATF-2, because both have previously been shown to be activated by MAPKs, resulting in the induction of TNF-α mRNA transcription (35). Using RAW264.7 and murine peritoneal mφs, we found that pfGPI stimulation resulted in the phosphorylation of c-Jun and ATF-2 (Fig. 1C and data not shown). SP600125 blocked the pfGPI-stimulated activation of both c-Jun and ATF-2, consistent with previous reports that activation of the JNK pathway may result in phosphorylation of c-Jun and ATF-2 (36, 47). The MEK1/2

**FIGURE 1.** *pfGPI induces the phosphorylation of MAPKs, c-Jun, and ATF-2. Effects of specific inhibitors on pfGPI-induced phosphorylation. A, RAW264.7 mφs were stimulated with pfGPI for the time indicated. Cells were harvested, and lysates were resolved on 12% SDS-PAGE and blotted with phospho-ERK1/2 (top), phospho-JNK (middle), or phospho-p38 Abs (bottom). B, RAW264.7 mφs were treated for 1 h with PP98059 (50 μM, inhibits MEK upstream of ERK1/2), SB203580 (25 μM, inhibits p38), and SP600125 (25 μM, inhibits JNK) before stimulation with pfGPI, and cell lysates were immunoblotted with phospho-ERK1/2 and phospho-c-Jun using specific antibodies. C, RAW264.7 mφs were treated as in B, and cell lysates were immunoblotted with phospho-c-Jun and phospho-ATF-2.
inhibitor PD98059 decreased pGPI-induced c-Jun phosphorylation but to a lesser extent than SP600125. PD98059 had only minimal effects on pGPI-induced ATF-2 phosphorylation. p38 activation has previously been reported to result in phosphorylation of ATF-2 (48), but in this case, the p38 inhibitor SB203580 had no effect on pGPI-induced ATF-2 phosphorylation. However, SB203580 increased pGPI stimulated c-Jun phosphorylation at 30 and 60 min, consistent with its ability to increase pGPI-induced JNK activation.

To examine the role of pGPI-induced MAPK activation in TNF-α production, RAW264.7 and murine peritoneal mds were pretreated with MAPK inhibitors and then stimulated with pGPI for 24 h. Both PD98059 and SP600125 significantly inhibited pGPI-stimulated TNF-α secretion in a dose-dependent manner (Fig. 2, A and B). In contrast, the p38 inhibitor SB203580 had no significant effect on pGPI-induced TNF-α secretion. Addition of the MAPK inhibitors in the absence of pGPI did not induce TNF-α secretion (data not shown). As a positive control for the activity of all inhibitors RAW264.7 mds were pretreated with PD98059, SP600125, or SB203580 and then stimulated with LPS (Fig. 2C). All inhibitors exhibited a dose-dependent inhibition of LPS-induced TNF-α. In summary, these data demonstrate that pGPI stimulates the phosphorylation of ERK1/2, JNK, and p38 MAPK. However, pGPI-induced TNF-α secretion appears to be correlated with JNK activation and c-Jun and ATF-2 phosphorylation.

Role of JNK isoforms

JNK2 mediates pGPI-induced responses and phosphorylation of c-Jun. The preceding experiments demonstrate that pGPI induces the phosphorylation of JNK, c-Jun and ATF-2 and that inhibition of JNK, c-Jun, and ATF-2 phosphorylation was associated with attenuated pGPI-induced TNF-α secretion. These data support a role for JNK signal transduction in AP-1 activation and proinflammatory cytokine secretion in the mφ response to malaria bioreactive molecules.

Members of the JNK family comprise 10 different isoforms created by alternative splicing of three JNK genes Jnk1, Jnk2, and Jnk3. The inhibitor SP600125 inhibits all JNK isoforms; therefore, in experiments using this compound, it is not possible to discern which JNK isoforms mediate pGPI-induced TNF-α secretion (43). We therefore used Jnk1−/−, Jnk2−/−, and Jnk3−/− mice to further define the role of individual JNK isoforms in mediating pGPI-induced cellular responses and signal transduction in vitro and in vivo using the P. berghei ANKA murine model of cerebral malaria.

Peritoneal mds from Jnk1−/−, Jnk2−/−, and Jnk3−/− and corresponding WT mice were initially used to determine whether individual JNK isoforms regulate pGPI-induced TNF-α secretion by mds. After exposure to pGPI, the secretion of TNF-α in WT, Jnk1−/− (Fig. 3A), and Jnk3−/− mds (data not shown) was not significantly different. In contrast, pGPI-induced TNF-α secretion from Jnk2−/− mds was significantly lower than from WT mds at 24 h (Fig. 4A). These data provide direct evidence that JNK2 participates in the regulation of TNF-α secretion by mds.

On the basis of the previous finding, we next investigated pGPI-induced phosphorylation of MAPKs and c-Jun in WT, Jnk1−/−, and Jnk2−/− mds (Figs. 3B and 4B). The induction of phosphorylation of ERK1/2, JNK, p38, and c-Jun by pGPI was similar in WT and Jnk1−/− mds (Fig. 3B). In contrast and in agreement with our observations with TNF-α secretion, induction of JNK and c-Jun phosphorylation by pGPI was almost completely abrogated in Jnk2−/− mds in comparison with WT mds (Fig. 4B), indicating a key role for JNK2 in mediating pGPI-induced mφ responses in vitro. Of additional interest and in support of our earlier findings of cross-talk between pGPI-induced MAPK pathways, pGPI-stimulated Jnk2−/− mds, but not Jnk1−/− mds, exhibited decreased phosphorylation of ERK1/2 in comparison with WT mds. This finding indicates that JNK2 likely mediates the observed cross-talk between JNK and ERK1/2 pathways in pGPI-stimulated mds.
**Jnk2<sup>−/−</sup> mice are protected from severe and fatal malaria infection.** To confirm and extend our results to the in vivo setting, we examined the role of individual JNK isoforms in mediating severe malaria in the *P. berghei* ANKA murine model of experimental cerebral malaria. As with severe *P. falciparum* infection in humans, mice susceptible to the *P. berghei* ANKA malaria (e.g., C57BL/6 mice) develop symptoms of severe malaria including a cytokine-associated encephalopathy, acidosis, coagulopathy, shock, and pulmonary edema, culminating in death (23). Further, mice immunized with synthetic pfGPI were protected from severe complications and fatality induced by *P. berghei* ANKA infection, suggesting functional similarity exists between pfGPI and *P. berghei* GPI (23). To define the role of JNK1 and JNK2 in the response to malaria infection in vivo, Jnk1<sup>−/−</sup> and Jnk2<sup>−/−</sup> mice and their WT littermates were inoculated with *P. berghei* ANKA. We examined parasitemia by microscopy, serum TNF-α levels by ELISA (also measures LT-α), morbidity, and survival (Figs. 3C and 4C). Parasitemia did not differ significantly between Jnk1<sup>−/−</sup>, Jnk2<sup>−/−</sup>, and WT mice (Figs. 3D and 4D). WT and Jnk1<sup>−/−</sup> mice began to demonstrate signs of cerebral involvement including paralysis, loss of coordination, and seizures by day 6; by day 11, 90% of WT and Jnk1<sup>−/−</sup> mice had succumbed to infection (Fig. 3C). There was no significant difference in survival between WT and Jnk1<sup>−/−</sup> mice after infection with *P. berghei* ANKA. In contrast, Jnk2<sup>−/−</sup> mice had a significantly improved survival rate compared with WT mice (41% vs 13%; log rank test: *p* = 0.014; Fig. 4C) and had significantly lower serum TNF-α levels compared with WT and Jnk1<sup>−/−</sup> mice (Fig. 5). Taken together with the in vitro studies above, these data provide direct evidence that JNK2, but not JNK1, contributes to malaria-induced TNF-α secretion and cerebral malaria in vivo.
Discussion

pfGPI is believed to contribute to the pathogenesis of cerebral malaria by inducing the release of inflammatory cytokines and mediators, such as TNF-α and NO, from mφs and by up-regulating cerebral endothelial adhesion molecules which may result in increased sequestration of parasitized erythrocytes in the brain (2). However, relatively little is known about the pfGPI-induced signaling cascades that mediate or contribute to these responses. In the present study with the mφ cell line RAW264.7, specific MAPK inhibitors, primary mφs from Jnk1−/−, Jnk2−/−, and Jnk3−/− mice, and a murine model of cerebral malaria, we demonstrate that pfGPI activates MAPKs, phosphorylates the transcription factors c-Jun and ATF-2, and induces cytokine secretion. Furthermore, we show an important and biologically relevant role for JNK2 in both regulating TNF-α secretion in response to pfGPI in vitro and mediating fatal cerebral malaria in vivo.

MAPKs play an important role in inflammatory gene regulation and have been implicated in signaling cascades initiated by parasite GPIs (26, 29, 32). Consistent with a recent report (26), we demonstrate that pfGPI activated three major MAPK pathways, ERK1/2, JNK, and p38 (Fig. 1A). The availability of partially selective inhibitors allowed us to assess the relative role of each of these MAPKs in the activation of the transcription factors c-Jun and ATF-2 and the induction of TNF-α biosynthesis (Figs. 1 and 2). Although all three MAPK pathways were activated, inhibiting ERK1/2 or JNK signal transduction reduced pfGPI-stimulated TNF-α secretion in a dose-dependent manner, whereas inhibition of p38 had no significant effect on TNF-α secretion. Previous studies concluded that ERK1/2 is less likely to be involved in mediating inflammatory responses and suggested a role for JNK-dependent activation of NF-κB/c-Rel in mediating proinflammatory responses to GPI (26, 37, 49).
In addition to NF-κB, the TNF-α promoter contains recognition sites for other transcription factor complexes including AP-1. After phosphorylation and activation, AP-1 can result in increased transcriptional activity and the induction of inflammatory mediators (34, 35). However, the role of AP-1 in pGPI-mediated cytokine response has not previously been reported. Our data support a role for JNK signal transduction in the activation of the AP-1 complex and the secretion of proinflammatory cytokines in response to malaria bioreactive molecules (Figs. 1C and 2). We demonstrate that pGPI induces JNK-dependent phosphorylation of the transcription factors c-Jun and ATF-2 and that inhibition of JNK, and consequently c-Jun, attenuates pGPI-stimulated TNF-α secretion. Upon phosphorylation, c-Jun can form homo- or heterodimers with JunD or Fos to form the AP-1 complex. Similarly, upon activation, ATF-2 can homodimerize to bind to cAMP response element motifs or can heterodimerize with c-Jun to bind to AP-1 motifs (35). cAMP response element and AP-1 motifs are found within the promoter of the TNF-α gene and other inflammatory genes (37, 50), and could serve as targets for pGPI-induced expression of inflammatory mediators. c-Jun and ATF-2 are both activated by JNK suggesting that both proteins are targets of the same signaling pathway. Our data suggest that pGPI induces c-Jun and ATF-2 activation via JNK, although ERK1/2 may also have some influence on c-Jun activation either directly or by influencing the activity of JNK. Whether c-Jun dimerizes with ATF-2 to form the AP-1 complex is unclear; thus, ATF-2 may contribute to the induction of TNF-α by pGPI, or it may be involved in other pGPI-stimulated events.

The JNK subgroup consists of JNK1, JNK2, and JNK3, each with multiple isoforms that have been postulated to have arisen for specific biological functions (37). A number of studies have reported that JNKs may have both overlapping and distinct biological functions depending on the cell type, the stage of differentiation, and the activating stimuli (51–55). To investigate whether different JNK isoforms might mediate distinct biological responses to pGPI, we investigated the response of Jnk1+/−, Jnk2+/− or Jnk3+/− mds to pGPI in vitro and the response of Jnk1−/− and Jnk2−/− mice to P. berghei ANKA malaria infection in vivo (Figs. 3 and 4). pGPI-induced phosphorylation of MAPKs and c-Jun and TNF-α secretion from Jnk1−/− and Jnk3−/− mds were largely unchanged compared with WT counterparts, whereas the inductions of phosphorylation of JNK, c-Jun, and TNF-α were significantly reduced in Jnk2−/− mds. Consistent with these in vitro data, Jnk2+/− mice experimentally infected with P. berghei ANKA malaria had significantly lower serum TNF-α levels (Fig. 5) and significantly higher survival rates than either WT or Jnk1−/− mice. JNK phosphorylation in Jnk2−/− mice was abrogated, but TNF-α secretion was only decreased by ~20% in vitro and ~50% in vivo, indicating that JNK2 plays only a partial role in the regulation of TNF-α secretion. These observations are consistent with the hypothesis that Jnk2 gene disruption may contribute to improved in vivo survival to malaria by mechanisms that are independent of its effect on TNF-α secretion, perhaps including effects related to impaired ERK1/2 phosphorylation. Taken together, our data demonstrate that JNK1 and JNK2 differentially respond to pGPI and that JNK2 contributes to malaria-induced TNF-α secretion and the outcome of infection in experimental murine cerebral malaria. Our findings are in agreement with a number of studies reporting differential functions of JNK1 and JNK2 (54, 56–59) but differ somewhat from a previous in vitro study using bone marrow-derived macrophages reporting that JNK1 and JNK2 were functionally redundant for the secretion of pGPI-induced TNF-α (26). The discrepancies between our data and the previous study may be attributable to technical differences including the use of IFN-γ priming; however, it is important that our in vivo data confirm our in vitro data that JNK1 and JNK2 are not functionally redundant.

The upstream activators of pGPI-induced JNK phosphorylation are currently unknown. JNK protein kinases are activated by dual phosphorylation on threonine and tyrosine, by the MAPK kinases MAPKK4 (also known as SEK-1) and MAPK7 (47). MAPKK4 is primarily activated by stress factors, whereas MAPK7 is primarily activated by cytokines TNF-α and IL-1. MAPK4, but not MAPK7, can activate p38. The observed activation of p38 and the failure of the MEK/ERK inhibitor PD98059 to inhibit p38 activation suggest that pGPI-induced JNK activation occurs via MAPK4 and not via secondary activation of MAPK7 by proinflammatory cytokines. Upstream activators of MAPK4 have yet to be fully elucidated but may include the mixed lineage kinase group of MAPKK kinases, the Rho family of GTPases, protein kinase C, protein tyrosine kinase, and Src family kinases (47).

MAPKs may participate in a number of cellular processes. For example, ERK1/2 signaling can induce cell survival, whereas p38 and JNK have been implicated in apoptosis pathways (47, 60). However, the biological consequence of activating any specific MAPK pathway is dependent on the cellular context and the state and duration of activation of other signaling pathways. Consistent with this observation, modulation of and cross-talk between MAPK pathways has previously been reported (61–63). For example, the JNK-dependent apoptosis pathway may be blocked by prior activation of ERK-associated survival pathways and vice versa (47, 63). Our data with pGPI-induced signaling add additional evidence for regulation by and cross-talk between MAPK pathways. Although we found no evidence that p38 inhibition decreased pGPI-induced secretion of TNF-α, p38 may be involved in other pGPI-associated downstream events and may influence ERK and JNK activation. For example, inhibition of p38 resulted in increased pGPI-stimulated ERK1/2 and JNK phosphorylation, associated with a consequent increase in phosphorylation of downstream target c-Jun. Similar cross-talk regulation has previously
been described between p38 and ERK during osteoclast differentiation, where p38 inhibitors resulted in increased phosphorylation of ERK and vice versa (61). We also provide evidence for possible cross-talk between MEK/ERK and JNK pathways occurring during pGPI-induced signaling (Figs. 1B and 4B). PD98059, a MEK1-specific inhibitor, not only inhibited ERK phosphorylation but also inhibited pGPI-induced JNK phosphorylation. Conversely, SP600125, a specific JNK inhibitor, inhibited pGPI-induced ERK phosphorylation. Although these effects may be attributable, at least in part, to potential nonspecific effects of these inhibitors, the observed changes in phosphorylation of ERK in Jnk2−/− mels support the biological relevance of these interactions.

In summary, severe and cerebral malaria are complex multisystem diseases with immunopathogenic processes involving dysregulated cytokine cascades and associated metabolic perturbation (2–5, 23). Fatality rates are not likely to decline significantly until we have a detailed understanding of the underlying processes and innate host responses that contribute to fatal outcomes and how these might be modulated to improve survival. High or dysregulated levels of inflammatory cytokines such as TNF-α appear to be central to the pathophysiology of severe malaria. Not only lead to cytokine-associated tissue damage but also enhance the expression of cytoadherence receptors, such as ICAM-1 and VCAM-1, which may lead to enhanced sequestration and contribute to complications such as cerebral malaria. Several lines of evidence indicate that pGPI is at least partially responsible for the induction of inflammatory cytokines and increased expression of cytoadherence receptors (2, 15, 23) and lend support to the hypothesis that strategies targeting pGPI-activated processes may be of clinical use in the therapy of severe or cerebral malaria. We provide evidence that pGPI induces the activation of MAPKs leading to JNK-dependent activation of c-Jun and ATF-2, and the release of TNF-α. Although additional studies are needed to fully elucidate the biological consequences of pGPI-induced MAPK activation, it will be of interest to evaluate whether MAPK signaling inhibitors are of value in the treatment of severe malaria in vivo. In this context, several JNK-specific inhibitors are currently under evaluation for potential therapeutic application in a variety of disease states, including Alzheimer’s disease and type 2 diabetes (37). Given our data supporting a role for JNK in pGPI-induced signaling, such JNK-specific inhibitors, may be of therapeutic use for severe malaria syndromes.

Disclosures
The authors have no financial conflict of interest.

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