Disruption of CD36 Impairs Cytokine Response to Plasmodium falciparum Glycosylphosphatidylinositol and Confers Susceptibility to Severe and Fatal Malaria In Vivo

Samir N. Patel, Ziyue Lu, Kodjo Ayi, Lena Serghides, D. Channe Gowda and Kevin C. Kain

J Immunol 2007; 178:3954-3961; doi: 10.4049/jimmunol.178.6.3954
http://www.jimmunol.org/content/178/6/3954

References
This article cites 72 articles, 44 of which you can access for free at:
http://www.jimmunol.org/content/178/6/3954.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2007 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Disruption of CD36 Impairs Cytokine Response to Plasmodium falciparum Glycosylphosphatidylinositol and Confers Susceptibility to Severe and Fatal Malaria In Vivo

Samir N. Patel,†‡ Ziyue Lu,* Kodjo Ayi,* Lena Serghides,* D. Channe Gowda,‡ and Kevin C. Kain2*†§

CD36 is a scavenger receptor that has been implicated in malaria pathogenesis as well as innate defense against blood-stage infection. Inflammatory responses to Plasmodium falciparum GPI (pGPI) anchors are believed to play an important role in innate immune response to malaria. We investigated the role of CD36 in pGPI-induced MAPK activation and proinflammatory cytokine secretion. Furthermore, we explored the role of this receptor in an experimental model of acute malaria in vivo. We demonstrate that ERK1/2, JNK, p38, and c-Jun became phosphorylated in pGPI-stimulated macrophages. In contrast, pGPI-induced phosphorylation of JNK, ERK1/2, and c-Jun was reduced in Cd36−/− macrophages and Cd36−/− macrophages secreted significantly less TNF-α in response to pGPI than their wild-type counterparts. In addition, we demonstrate a role for CD36 in innate immune response to malaria in vivo. Compared with wild-type mice, Cd36−/− mice experienced more severe and fatal malaria when challenged with Plasmodium chabaudi chabaudi AS. Cd36−/− mice displayed a combined defect in cytokine induction and parasite clearance with a dysregulated cytokine response to infection, earlier peak parasitemias, higher parasite densities, and higher mortality rates than wild-type mice. These results provide direct evidence that pGPI induces TNF-α secretion in a CD36-dependent manner and support a role for CD36 in modulating host cytokine response and innate control of acute blood-stage malaria infection in vivo. The Journal of Immunology, 2007, 178: 3954–3961.

Malaria caused by Plasmodium falciparum remains a leading cause of global morbidity and mortality accounting for an estimated 500 million infections and over 1 million deaths annually (1). Young children and other non-immune individuals are at the greatest risk of developing severe and fatal malaria (2). Various rodent malaria models have been used to examine components of human malaria infections including severe and cerebral malaria (3–7). In both human infections and experimental malaria models, survival appears to be critically linked to the ability of the host to control blood-stage parasite replication within the first 7–14 days of infection (8). Because malaria-specific Ab and cellular responses are largely absent during the acute stage of infection, innate immune mechanisms appear to be essential in controlling early parasite replication and decreasing the risk of progression to severe and fatal disease (8, 9). Although much work has focused on acquired immune responses to malaria, less is known about the effectors of this protective early innate immune response.

The molecular basis of innate immunity to acute malaria infection has been elucidated in mouse models, and in particular Plasmodium chabaudi chabaudi AS (PCCAS)3 infection in susceptible (A/J) and resistant (C57BL/6) mice. Susceptible A/J mice quickly develop high parasitemia and succumb to infection within 7–14 days. In contrast, resistant C57BL/6 mice are able to control blood-stage parasite replication and survive the infection. Previous studies have established a role for the early and balanced induction of proinflammatory cytokines including IL-12, IFN-γ, and TNF-α, in resistance to acute PCCAS infection (8, 10–16). However, the innate effector mechanisms that actually mediate control of blood-stage parasitemia during acute infection are not fully understood. Ab-mediated mechanisms do not appear to play a major role in initial PCCAS infection because control of acute parasitemia occurs before the production of significant levels of parasite-specific IgG and because B cell-deficient mice are able to control acute PCCAS infection as well as intact mice (17, 18). Similarly, studies indicate that the complement system plays only a minor role in controlling initial PCCAS infection. Mice defective in both classical and alternative complement pathways exhibit only slightly higher parasitemia levels and show only minor delays in the resolution of the acute phase of parasitemia compared with controls (19).

Although opsonic-based systems appear to make only minor contributions, several lines of evidence support a role for opsonin-independent mechanisms, including macrophage phagocytosis, in

---

* McLaughlin-Rotman Centre, University Health Network-Toronto General Hospital, McLaughlin Centre for Molecular Medicine, University of Toronto, Toronto, Ontario, Canada; † Institute of Medical Sciences, University of Toronto, Toronto, Ontario, Canada; Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA 17033; and § Tropical Disease Unit, Division of Infectious Diseases, Department of Medicine, University Health Network-Toronto General Hospital, Toronto, Ontario, Canada

Received for publication September 25, 2006. Accepted for publication January 1, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a Canadian Institutes of Health Research Team Grant in Malaria (to K.C.K.), Operating Grant MT-13721 (to K.C.K.), Genome Canada Ontario through the Ontario Genomics Institute (to K.C.K.), Canadian Genetics Disease Network (to K.C.K.), Physicians Services Inc. (to K.C.K.), and a Canadian Institutes of Health Research Canada Research Chair (to K.C.K.).

2 Address correspondence and reprint requests to Dr. Kevin C. Kain, University Health Network-Toronto General Hospital, EN 13-214, 200 Elizabeth Street, Toronto, Ontario, Canada M5G 2C4. E-mail address: kevin.kain@uhn.on.ca

3 Abbreviations used in this paper: PCCAS, Plasmodium chabaudi chabaudi AS; pGPI, Plasmodium falciparum GPI; PEs, parasitized erythrocyte; Mø, monocyte/macrophage.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00
mediating protective innate immune responses to PCCAS malaria. In nonopsonic phagocytosis, macrophages recognize and ingest pathogens via pattern-recognition receptors such as scavenger receptors (20–23). Studies by Stevenson et al. (24) demonstrated that macrophage activation in susceptible A/J mice reduced acute blood-stage parasitemia and improved survival, whereas silica treatment to deplete macrophages in resistant C57BL/6 mice resulted in increased parasitemia and higher mortality. More recent work has implicated macrophage scavenger receptors in mediating nonopsonic phagocytosis and innate control of acute blood-stage PCCAS infection. Studies by Su et al. (25) demonstrated that blockade of macrophage scavenger receptors, including class B receptors, with polyinosinic acid (poly (I)), significantly inhibited nonopsonic phagocytosis of parasitized erythrocytes in vitro and treatment of mice with poly (I) resulted in significantly higher levels of ascending parasitemia in vivo compared with control mice (25).

One member of the class B family of scavenger receptors is CD36. Interest for a role for CD36 in malaria was first sparked by the observation that it was a receptor primarily expressed on dermal microvascular endothelium that supported adhesion of most natural isolates of *P. falciparum* malaria. Its initial identification as a sequestration receptor for parasitized erythrocytes (PEs) led to the assumption that it contributed to the pathophysiology of severe malaria and resulted in the development of a number of antiadherence therapies designed to disrupt CD36-PEs interactions (26–28). Conversely, CD36 is also a pattern-recognition receptor that has been shown to participate in macrophage-mediated phagocytosis of apoptotic cells and, more recently, the uptake of PEs by human and murine monocytes/macrophages (Mφ) (29–31). Consequently there remains some uncertainty as to whether CD36 contributes primarily to innate host defense or rather the opposite, malaria pathophysiology (32).

Recent studies have begun to examine the role of host innate sensing receptors such as TLRs in the recognition of parasite GPI and subsequent induced signaling events (33–39). In vitro studies using murine Mφ indicate that *P. falciparum* GPI (pGPI) is recognized primarily by TLR2 and induces activation of three major MAPK families, JNK, ERK1/2, and p38, resulting in the induction of proinflammatory gene expression through, at least in part, the JNK and NF-κB pathway (34, 35, 40). Recently, CD36 has been shown to cooperate with TLR2 in innate sensing and induction of inflammatory cytokines in response to intact bacteria and bacterial ligands (41, 42). However, to date, no studies have reported on the role of CD36 in the secretion of malaria-associated inflammatory cytokines.

The objective of this study was to examine the role of CD36 in: 1) pGPI-induced MAPK activation and cytokine response and 2) innate immunity to malaria in vivo using the PCCAS murine model (43). We hypothesized that CD36 would play a dual role in the innate response to malaria by contributing to the induction of cytokines in response to GPI and through phagocytic control of acute blood-stage infection. In this study, we show that pGPI-stimulated CD36+/− Mφ displayed impaired MAPK phosphorylation and TNF-α response compared with wild-type Mφ. In addition, we demonstrate that CD36+/− mice developed dysregulated cytokine responses to infection, higher parasite burdens, and more severe and fatal malaria than control mice.

Materials and Methods

**Media and reagents**

Endotoxin-free RPMI 1640, heat-inactivated group AB human serum, and gentamicin were purchased from Invitrogen Life Technologies. The Diff-Quik Staining kit was purchased from Fisher Scientific. Mouse Fc fragment (FcγRIIa) was purchased from Jackson Immunochemicals. ERK (PD98059), p38 (SB203580), and Syk kinase (piceatannol) inhibitors were purchased from Calbiochem. Anti-CD36 clone 63 was obtained from Cascade BioScience, anti-TLR2 clone TLR2.1 and ERK were obtained from Santa Cruz Biotechnology, anti-TLR4 clone MTS510 was obtained from eBioscience, β-actin Ab, goat anti-mouse IgG (γ-chain-specific, FITC labeled), and goat anti-mouse IgA (chain-specific, FITC labeled) were purchased from Sigma-Aldrich. Phosphospecific ERK1/2, JNK, p38 e-Jun, and p38 Abs were purchased from Cell Signaling Technology. Goat anti-mouse and goat anti-rabbit IgG (H+L)-HRP conjugate Abs were purchased from Bio-Rad. Mouse IFN-γ, TNF-α, and TGF-β ELISA kits were purchased from R&D Systems. All other reagents were confirmed by mass spectrometry and carbohydrate compositional analysis.

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

Protein-free pGPIs were isolated and purified by HPLC as described (35, 40). Briefly, mycoplasma-free parasite cultures with 20–30% parasitemia were harvested at the schizont stage, treated with 0.025% saponin in Trager buffer (56 mM NaCl (3.3 g/L), 59 mM KCl (4.4 g/L), 0.9 mM NaH2PO4·H2O (0.14 g/L), 9 mM K2HPO4 (1.57 g/L), 10.7 mM NaHCO3 (0.9 g/L), and 13.8 mM glucose (2.5 g/L)) (pH 7.0) 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, lypophilized, and extracted three times with chloroform/methanol (2:1, v/v) to remove nonglycosylated lipids. GPs 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 GPs were further purified by HPLC. All preparations of purified pGPIs were tested for the presence of endotoxin using *Limulus* amebocyte lysate test assay before use. By this assay, the positive detection limit for LPS was 0.01 ng/ml. The purity of the GPs was confirmed by mass spectrometry and carbohydrate compositional analysis.

**Mice, parasites, and experimental infections**

CD36−/− C57BL/6 and CD36+/− C57BL/6 mice were bred in the animal facility at the University of Toronto. All mice were bred and kept under pathogen-free conditions with a 12-h light cycle. Animal protocols were approved by the Animal Care Committee of the University of Toronto and all experiments involving animals were performed in compliance with current university institutional guidelines. Male mice of 8–12 wk of age were used in all experiments. PCCAS parasites were provided by Dr. M. Stevenson (McGill University, Montreal, Quebec, Canada). Blood-stage PCCAS was maintained in our laboratory as described (44). Infections in experimental animals were initiated by i.p. injection of 104 PCCAS. Course of infection was monitored daily for 18 days by determining parasitemia on blood smears stained with Diff-Quik. Morbidity was assessed at least twice daily. For in vitro assays, *P. falciparum* (ITG clone) was cultured as previously described (29, 31) and used as a control.

**Flow cytometry**

To determine Mφ surface levels of CD36, TLR-2, and TLR-4, thiglycolate-elicited peritoneal Mφ were isolated from CD36−/− and CD36+/− mice, and were stained with either monoclonal anti-CD36 Ab (1/100), monoclonal anti-TLR-2 Ab (1/100), or monoclonal anti-TLR-4 Ab (1/100) for 30 min, and then with a secondary anti-mouse IgA (1/16) or anti-mouse IgG-FITC conjugate (1/20) overnight. Controls were stained with the secondary Ab alone. Mφ were then fixed with 0.5% paraformaldehyde/PBS and analyzed using the EPICS ELITE flow cytometer and software (Beckman Coulter).

**Immunoblots**

To determine the activation of MAPKs, both CD36−/− and CD36+/− peritoneal Mφ were seeded onto 12-well plates (1 × 106 cells/well) overnight in RPMI 1640 with 10% FBS and then stimulated with HPLC-purified pGPI (80–160 nM/ml) for various time points (0, 15, 30, and 60 min). Cells were harvested, lysed, and lysates were frozen at −80°C. Immunoblot analysis was conducted as described (40). Briefly, lysates were separated on 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Blots were then probed with specific Abs recognizing total and phosphospecific-ERK1/2 (p42/44 MAPK, Th18/20/Tyr185), phospho-stress-activated protein kinase/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182) and phospho-c-Jun (Ser73). Total ERK1/2, p38, and β-actin were used as loading controls.
Phagocytosis assay

Phagocytosis assays were performed as previously described using thioglycolate-elicited Mφ from Cd36+/+ and Cd36−/− mice (31). Briefly, Mφ were purified from peritoneal exudates cells by adherence to glass coverslips. Cells were allowed to adhere for 30 min followed by stringent washing to remove nonadherent cells. Over 99% of adherent cells were macrophages, as assessed by microscopic examination of Giemsa-stained coverslips. Mφ were plated at a density of 1.5 × 10^4 cells on autoclaved glass coverslips in 24-well polystyrene culture plates and cultured for 5 days in RPMI 1640 with 10% FBS and gentamicin (RPMI-10-G) at 37°C and 5% CO₂ before use. To block FcRs, plated Mφ were pretreated with mouse Fc fragments (20 μg/ml) for 20 min at 4°C. Mature-stage PCCAS PEIs (trophozoites and schizonts) were obtained from an infected C57BL/6 mice with ~30% parasitemia at ~6 P.M. by cardiac puncture (45). Mature-stage P. falciparum ITG PEIs were used in phagocytosis assays as positive controls. Mφ were exposed to mature-stage parasites at a ratio of 50 PEIs to 1 Mφ in 500 μl of RPMI-10-G, and incubated for 2 h at 37°C, 5% CO₂ with gentle rocking. Coverslips were then washed with ice-cold water for 45 s to lyse nonphagocytosed PEIs, fixed, and stained with Diff-Quik. Phagocytosis was quantified by light microscopy, counting 500 Mφ per coverslip. Criteria for phagocytosis required the PEIs to be contained completely within the Mφ cell outline. Phagocytic index was calculated as: phagocytic index % = (number of Mφ with at least one ingested PEIS (PCCAS or P. falciparum) over total number of Mφ counted) × 100. Where indicated, MAPK activation was blocked with specific inhibitors to examine the role of Cd36-induced MAPK activation (29) in the phagocytosis of PCCAS PEIs. Mφ were pretreated either with a selective MEK-1 inhibitor (PD98059; 50 μM), p38 inhibitor (SB203580; 30 μM), or a Syk kinase-specific inhibitor (piceatannol; 30 μM) for 1 h at 37°C. Mφ were then washed with RPMI 1640 and phagocytosis assays were conducted as described above.

Cytokine ELISAs

To determine pfGPI-induced TNF-α secretion in vitro, peritoneal Cd36+/+ and Cd36−/− Mφ, prepared as above, were seeded in 24-well polystyrene plates (200,000 cells/well) and allowed to adhere for 1 h. Unattached cells were washed away and the remaining cells were exposed to HPLC-purified pfGPI (80–160 nM/ml) as described (35, 40), or no additions for 24 h. LPS (100 ng/ml) was used as a positive control to confirm the capacity of the Mφ preparations to produce proinflammatory cytokines. Following incubation at 37°C, the supernatants were collected, cleared by centrifugation, and assayed for TNF-α. To determine circulating serum cytokine levels, Cd36−/− and wild-type mice were injected with 1 × 10^8 PCCAS-PEIs i.p. On days 1, 3, 5, 7, and 10, blood was collected from the mice by cardiac puncture. Blood was allowed to clot, cleared by centrifugation, and serum was stored at −80°C and later assayed for IFN-γ, TNF-α, and TGF-β using commercially available standard ELISA kits.

Statistical analysis

All experiments were performed in duplicate or triplicate and repeated at least three times. Data are presented as the mean ± SD, unless otherwise noted. Differences between groups were analyzed using the Student t test, Mann-Whitney rank-sum test, or the log-rank test.

Results

pfGPI-induced MAPK activation and TNF-α production is Cd36 dependent

pfGPI has recently been shown to activate MAPKs and contribute to the production of proinflammatory mediators such as TNF-α, in a TLR2-dependent manner (34, 35, 40). Cd36 has been shown to cooperate with TLR2 in mediating host inflammatory responses to intact Staphylococcus aureus and some, but not all, TLR2 ligands (41, 42). To determine whether Cd36 is involved in pfGPI-induced signaling and cytokine induction, we investigated the pfGPI-induced activation of three major MAPK pathways—ERK1/2, JNK, and p38—and the induction of TNF-α in Cd36+/+ and Cd36−/− Mφs. Consistent with previous studies, HPLC-purified pfGPI induced TNF-α secretion in a time- and a dose-dependent manner in Cd36+/+ Mφ (Fig. 1A and data not shown) (34, 35). In contrast, Cd36−/− Mφ secreted significantly less TNF-α (Fig. 1A). To ensure these differential responses were not attributable to differences in expression of TLR2 or TLR4 between Cd36+/+ and Cd36−/− Mφ, we examined receptor levels by flow cytometry. Both Cd36+/+ and Cd36−/− Mφ had equivalent levels of TLR2 and TLR4 on their surface (Fig. 1, B–D) and respond equally well to LPS (data not shown).

We next investigated the requirement for Cd36 in pfGPI-induced MAPK activation. Peritoneal Mφ from Cd36+/+ and Cd36−/− mice were treated with HPLC-purified pfGPI and cell lysates were examined for the activated forms of MAPKs by immunoblot analysis. ERK1/2, JNK, p38 MAPK, and the transcription factor c-Jun, became phosphorylated in Cd36+/+ Mφs stimulated with pfGPI (Fig. 2). We have previously shown a role for JNK in the phosphorylation of c-Jun, a component of the transcription factor complex AP-1, in response to pfGPI (40). Following phosphorylation and activation of its components, AP-1 can stimulate transcriptional activity and the induction of inflammatory mediators including TNF-α (46, 47). In Cd36+/+ Mφ, phosphorylation of ERK1/2, JNK, p38, and c-Jun peaked at 30 min and was sustained at 60 min after exposure to pfGPI. In contrast, Cd36−/− Mφ displayed reduced phosphorylation of ERK1/2 and JNK and had delayed and reduced phosphorylation of c-Jun (Fig. 2).

Statistical analysis

All experiments were performed in duplicate or triplicate and repeated at least three times. Data are presented as the mean ± SD, unless otherwise noted. Differences between groups were analyzed using the Student t test, Mann-Whitney rank-sum test, or the log-rank test.

Results

pfGPI-induced MAPK activation and TNF-α production is Cd36 dependent

pfGPI has recently been shown to activate MAPKs and contribute to the production of proinflammatory mediators such as TNF-α, in a TLR2-dependent manner (34, 35, 40). Cd36 has been shown to cooperate with TLR2 in mediating host inflammatory responses to intact Staphylococcus aureus and some, but not all, TLR2 ligands (41, 42). To determine whether Cd36 is involved in pfGPI-induced signaling and cytokine induction, we investigated the pfGPI-induced activation of three major MAPK pathways—ERK1/2, JNK, and p38—and the induction of TNF-α in Cd36+/+ and Cd36−/− Mφs. Consistent with previous studies, HPLC-purified pfGPI induced TNF-α secretion in a time-and a dose-dependent manner in Cd36+/+ Mφ (Fig. 1A and data not shown) (34, 35). In contrast, Cd36−/− Mφ secreted significantly less TNF-α (Fig. 1A). To ensure these differential responses were not attributable to differences in expression of TLR2 or TLR4 between Cd36+/+ and Cd36−/− Mφ, we examined receptor levels by flow cytometry. Both Cd36+/+ and Cd36−/− Mφ had equivalent levels of TLR2 and TLR4 on their surface (Fig. 1, B–D) and respond equally well to LPS (data not shown).

We next investigated the requirement for Cd36 in pfGPI-induced MAPK activation. Peritoneal Mφ from Cd36+/+ and Cd36−/− mice were treated with HPLC-purified pfGPI and cell lysates were examined for the activated forms of MAPKs by immunoblot analysis. ERK1/2, JNK, p38 MAPK, and the transcription factor c-Jun, became phosphorylated in Cd36+/+ Mφs stimulated with pfGPI (Fig. 2). We have previously shown a role for JNK in the phosphorylation of c-Jun, a component of the transcription factor complex AP-1, in response to pfGPI (40). Following phosphorylation and activation of its components, AP-1 can stimulate transcriptional activity and the induction of inflammatory mediators including TNF-α (46, 47). In Cd36+/+ Mφ, phosphorylation of ERK1/2, JNK, p38, and c-Jun peaked at 30 min and was sustained at 60 min after exposure to pfGPI. In contrast, Cd36−/− Mφ displayed reduced phosphorylation of ERK1/2 and JNK and had delayed and reduced phosphorylation of c-Jun (Fig. 2).
summary, these data demonstrate that pfGPI induces the phosphorylation of JNK, ERK1/2, and c-Jun and the production of TNF-α in a CD36-dependent manner.

**Mφ uptake of PCCAS parasitized erythrocytes is CD36 dependent**

Previous studies examining macrophage uptake of *falciparum* PEs (29–32) and the above observations suggest that CD36 may have dual roles in host response to malaria, i.e., CD36 may participate in both cytokine response to infection and Mφ clearance of PEs. To extend these observations, we examined CD36-malaria interactions in the PCCAS murine model of malaria. Previous studies have shown CD36 participates in the phagocytosis of nonopsonized *P. falciparum*-PEs by human and murine Mφ (29–31). Because PCCAS-PEs have been reported to bind CD36 (43), we initially investigated whether CD36 could mediate the uptake of PCCAS-PEs by murine Mφ in vitro. As shown in Fig. 3, A and B, PCCAS-PEs and *P. falciparum*-PEs were avidly phagocytosed by Cd36+/+ Mφ even in presence of FcR blockade and in the absence of complement or prior opsonization of PEs. Although significant uptake was observed over the 2-h assay for both types of PEs, uptake of *P. falciparum*-PEs by murine Mφ was ~3-fold higher than PCCAS-PEs. Phagocytosis by Cd36–/– Mφ was ~80% lower for PCCAS-PEs and ~90% lower for *P. falciparum* PEs compared with their wild-type counterparts, supporting a role for CD36 in mediating Mφ uptake of both *P. falciparum* and PCCAS-PEs.

CD36-mediated uptake of *P. falciparum*-PEs by human Mφ has previously been shown to activate MAPK cascades including ERK1/2 and p38, and blockade of these signal transduction pathways inhibits phagocytosis of *P. falciparum*-PEs by human Mφ (29). Based on these observations, we investigated the role of CD36-induced MAPK activation in the uptake of PCCAS-PEs by murine Mφ. Similar to that previously reported for human CD36, Ab-induced cross linking of murine CD36 and uptake of PCCAS-PEs induced the phosphorylation of ERK1/2 and p38 MAPK as determined by Western blot (data not shown). Pretreatment of

**FIGURE 2.** Cd36+/+ Mφ have impaired phosphorylation of MAPK pathways in response to pfGPI stimulation. Cd36+/+ and Cd36–/– Mφ were analyzed either with or without stimulation with HPLC-purified pfGPI (80 nM) for 0–60 min as indicated. Cell lysates were resolved by 12% SDS-PAGE and immunoblotted with phospho-ERK1/2, phospho-JNK, phospho-p38, and phospho-c-Jun Abs, respectively. Unphosphorylated total ERK, p38, and β-actin were used as loading controls. Data for each time for ERK1/2, JNK, p38, and c-Jun phosphorylation were derived from the same experiment, run on the same gel, and subjected to the same exposure time. Data are representative of results from three independent experiments.

**FIGURE 3.** Cd36 participates in the phagocytosis of nonopsonized PCCAS-parasitized erythrocytes in vitro. Cd36+/+ and Cd36–/– Mφ were plated onto glass coverslips. FcR-blocked Mφ were incubated with either noninfected RBC (nRBCs), *P. falciparum*-PEs (A) (as a positive control) or PCCAS-PEs (B and C) for 2 h. Where indicated, Mφ were pretreated with piceatannol (C) (pic, inhibitor of syk kinase involved in FcR-mediated uptake), PD98059 (PD, inhibits MEK1 upstream of ERK1/2) or SB203580 (SB, inhibitor of p38). Phagocytosis assay was conducted as described in Materials and Methods. Data are represented as means (±SD) from four independent experiments. *, *p < 0.01 (Mann-Whitney U test), Cd36+/+ vs Cd36–/– Mφ; #, *p < 0.01, Cd36+/+ vs Cd36–/– Mφ treated with SB203580; **, *p < 0.01, Cd36+/+ vs Cd36–/– Mφ treated with PD98059.
Disruption of CD36 confers susceptibility to fatal PCCAS malaria in vivo. Eight- to 12-wk-old male Cd36+/+ (n = 18) and Cd36−/− (n = 18) mice were inoculated with 1.0 × 10⁶ PCCAS-PEs i.p. and were monitored twice daily over the course of infection for 18 days. Cd36−/− mice had significantly lower survival rates than Cd36+/+ mice (37 vs 80%; log-rank test: χ² = 6.92, p < 0.01).

Cd36−/− murine Mφ with the MEK-1-selective inhibitor PD98059 to inhibit ERK1/2 phosphorylation, or the p38 MAPK-selective inhibitor SB203580 decreased the phagocytic uptake of PCCAS to levels observed in Cd36−/− Mφ (Fig. 3C). In contrast, blockade of Syk kinase, a molecule involved in FcR-mediated phagocytosis, with piceatannol, had no significant effect on the uptake of PCCAS-PEs (Fig. 3C). Collectively, these in vitro findings suggest that Mφ uptake of PCCAS-PEs is dependent upon the presence of murine CD36 and the signaling cascades induced by its activation.

Disruption of CD36 confers a dual defect in vivo: dysregulated cytokine response to infection and defective innate clearance of acute blood-stage parasitemia

To confirm and extend our results to the in vivo setting, we examined PCCAS infection in Cd36−/− mice and their wild-type counterparts in vivo. As with severe P. falciparum malaria in humans, mice susceptible to malaria (e.g., PCCAS malaria in A/J mice) are unable to control blood-stage parasite replication and consequently develop high parasite burdens culminating in a fatal outcome; whereas resistant mice (e.g., C57BL/6) control acute parasitemia and survive (8, 10–16). Based on the hypothesis that CD36 plays a central and nonredundant role in the innate immune response to malaria in vivo, we predicted that normally resistant C57BL/6 mice rendered CD36 deficient, would become susceptible, develop higher parasite burdens, more severe and fatal malaria than wild-type control mice.

We inoculated Cd36−/− mice and their wild-type counterparts with PCCAS malaria and monitored the course of infection for 18 days by examining parasitemia by microscopy, serum cytokines including IFN-γ and TNF-α levels (also measures lymphoxygen-α) by ELISA, morbidity, and survival (Figs. 4 and 5). Cd36−/− mice developed early peak parasitemia and higher parasite densities compared with Cd36+/+ mice (Table I). The parasitemia in Cd36−/− mice was higher on days 5 and 7 with peak parasitemia on day 7 (52% parasitemia), whereas infection in Cd36+/+ mice peaked on day 8 (42.5% parasitemia). There was a significant

FIGURE 4. Disruption of CD36 confers susceptibility to fatal PCCAS malaria in vivo. Eight- to 12-wk-old male Cd36+/+ (n = 18) and Cd36−/− (n = 18) mice were inoculated with 1.0 × 10⁶ PCCAS-PEs i.p. and were monitored twice daily over the course of infection for 18 days. Cd36−/− mice had significantly lower survival rates than Cd36+/+ mice (37 vs 80%; log-rank test: χ² = 6.92, p < 0.01).

FIGURE 5. Disruption of CD36 confers dysregulated cytokine response to PCCAS malaria in vivo. Eight- to 12-wk-old male Cd36+/+ (n = 5) and Cd36−/− mice (n = 5) were infected with 1.0 × 10⁶ PCCAS-PEs i.p. Serum cytokine levels were assessed on days 1, 3, 5, 7, and 10. A. Serum IFN-γ and TNF-α levels (B) were analyzed by ELISA and the data are shown as box plots with the medians and ranges. C, TNF-α: TGF-β ratio from days 1, 3, 5, 7, and 10 of infection. Mann-Whitney U test: #, p < 0.05, Cd36−/− vs Cd36+/+; ##, p < 0.01, Cd36−/− vs Cd36+/+. **, p < 0.001, Cd36−/− vs Cd36+/+. **, p < 0.0001, Cd36−/− vs Cd36+/+.
difference in survival between \( C d 3 6 ^ { − / − } \) and wild-type mice after infection with PCCAS malaria (37 vs 80%; log-rank test, \( p = 0.0085 \); Fig. 4).

In addition to phagocytic control of blood-stage parasite replication, a balanced cytokine response is essential for a successful outcome of acute malarial infections (8). Initial inflammatory cytokine responses, in particular IFN-\( \gamma \) and TNF-\( \alpha \), are required to facilitate parasite clearance but must be controlled, in part by immunoregulatory cytokines such as TGF-\( \beta \) and IL-10, to limit host-mediated immunopathology (15, 16, 48, 49). Previous studies have reported that the ratio of proinflammatory cytokines to anti-inflammatory cytokines is an important prognostic indicator of severe malaria with high ratios significantly associated with fatal outcomes (50–52).

Following challenge with PCCAS, \( C d 3 6 ^ { − / − } \) mice displayed an early defect in the production of IFN-\( \gamma \) (Fig. 5A) and TNF-\( \alpha \) (Fig. 5B) in response to infection compared with wild-type mice. This early defect in inflammatory cytokines in response to infection in \( C d 3 6 ^ { − / − } \) mice was followed by a significant elevation in TNF-\( \alpha \) production later in infection compared with wild-type mice. Because a balanced cytokine response is critical in resolving acute infections, we also analyzed the ratio of proinflammatory to anti-inflammatory cytokines (TNF-\( \alpha \):TGF-\( \beta \)) during the course of infection with PCCAS. CD36-sufficient C57BL/6 mice are resistant to PCCAS and display an increase in TNF-\( \alpha \):TGF-\( \beta \) ratio peaking at day 5, followed by marked decline during the resolution of infection (Fig. 5C). In contrast, \( C d 3 6 ^ { − / − } \) mice had a significantly lower TNF-\( \alpha \):TGF-\( \beta \) ratio early in the infection followed by significantly higher and sustained ratio of these cytokines in the later part of the infection (Fig. 5C). These data suggest that an impaired IFN-\( \gamma \) response early in the course infection coupled with higher TNF-\( \alpha \) and TNF-\( \alpha \):TGF-\( \beta \) ratios in later part of the infection contribute to immunopathology and the observed higher fatality rates in \( C d 3 6 ^ { − / − } \) mice. Taken together, these data provide direct evidence that CD36 contributes to control of acute bloodstream parasite replication, malaria-induced cytokine response, and severe malaria in vivo.

**Discussion**

Survival in acute \( P. f a l c i p a r u m \) infection of humans and in murine models of malaria appears to depend upon the ability of the host to control acute blood-stage parasite replication and prevent immunopathologic responses to infection before the onset of specific acquired immunity (8). Innate immune responses are essential in limiting peak parasite density and in preventing progression to severe disease. However, relatively little is known regarding the effector mechanisms of these protective responses (8). In this study, we identify a role for CD36 in mediating both cytokine response and innate control of acute blood-stage infection in vivo. \( p G P I \)-induced phosphorylation of ERK1/2, and JNK, and the transcription factor c-Jun, was reduced in \( C d 3 6 ^ { − / − } \) Mφ. CD36-deficient Mφ secreted significantly less TNF-\( \alpha \) in response to \( p G P I \) and displayed a marked phagocytic defect for parasitized erythrocytes compared with wild-type Mφ. In vivo, \( C d 3 6 ^ { − / − } \) mice displayed a combined defect in cytokine induction and innate control of parasite replication. \( C d 3 6 ^ { − / − } \) mice exhibited an impaired early proinflammatory response to infection, experienced earlier peak parasitemias, higher parasite densities, and higher fatality rates than their wild-type counterparts.

Pattern-recognition receptors such as TLRs play a central role in innate immune response by sensing microbial pathogens and/or their molecular components and inducing host inflammatory responses to infection (53, 54). Similarly, other classes of pattern recognition receptors on macrophages, including scavenger receptors such as CD36, have been implicated in the innate immune response via their capacity to phagocytose microbial pathogens in the absence of specific immune responses (29–31, 41, 42). We show for the first time that CD36 also contributes to the induction of innate inflammatory responses to malaria infection. Recent studies have demonstrated that \( p G P I \) interacts primarily with TLR2 on the surface of macrophages resulting in the induction of proinflammatory cytokines through the activation of selected MAPKs (ERK1/2, p38, and JNK) pathways and the transcription factor complexes, NF-\( \kappa B \) and AP-1 (35, 40). Although both \( C d 3 6 ^ { − / − } \) and \( C d 3 6 ^ { − / − } \) Mφ have comparable surface levels of TLR2 and TLR4 (Fig. 1, B–D), \( C d 3 6 ^ { − / − } \) Mφ stimulated with \( p G P I \) displayed reduced phosphorylation of ERK1/2 and JNK and c-Jun (Fig. 2), indicating that CD36 is necessary for robust activation of MAPKs and subsequent TNF-\( \alpha \) secretion induced by \( p G P I \) (Fig. 1A). These data provide the first evidence implicating CD36 as a facilitator or coreceptor with TLR2 in the induction of TNF-\( \alpha \) by protozoan ligands. How CD36 might interact with TLRs is unknown. Similar to the role of CD14 in LPS presentation to TLR4, CD36 may function to concentrate or cluster \( p G P I \) on the cell surface for delivery to TLR2 or, alternatively, CD36 may internalize \( p G P I \) and deliver it to TLRs in phagosomes or endosomes (55).

Our data are in agreement with recent evidence linking the phagocytic uptake of microbial pathogens with innate sensing and the induction of inflammatory responses driven by TLRs. CD36 has been shown to function as a coreceptor or facilitator of TLR2 in the induction of inflammatory cytokines in response to intact bacteria and bacterial ligands (lipoteichoic acid; macrophage-activating lipopeptide 2) (41, 42). \( C d 3 6 ^ { − / − } \) macrophages exposed to lipoteichoic acid, macrophage-activating lipopeptide 2, or intact \( S. a u r e u s \) had significantly impaired inflammatory responses and \( C d 3 6 ^ { − / − } \) mice were unable to control \( S. a u r e u s \) infection in vivo (41, 42).

In vivo, the successful control of acute malaria infection requires a carefully regulated cytokine response. Both the type and sequence of cytokine responses are essential in facilitating parasite clearance and also in minimizing malaria-associated immunopathology. Initial TLR-driven inflammatory cytokines (including IFN-\( \gamma \) and TNF-\( \alpha \)) secreted early in response to infection, play important roles in mediating resistance to acute blood-stage malaria (8, 10, 56–59). The inability to initially produce sufficient proinflammatory cytokines in response to acute blood-stage infection results in adverse outcomes, however, equally fatal is the overproduction of these mediators. To modulate potential immunopathologic responses initial inflammatory responses must be countered by the subsequent secretion of immunoregulatory cytokines such as TGF-\( \beta \) (8, 49). Here, we show that \( C d 3 6 ^ { − / − } \) mice

---

**Table I.** \( C d 3 6 ^ { − / − } \) mice challenged with \( P. c h a u b a d u i c h a u b a d u i \) AS develop higher parasite burdens than wild-type counterparts

<table>
<thead>
<tr>
<th>Day(s) Postinfection</th>
<th>Geometric Means (%) ± SE</th>
<th>Wild type</th>
<th>Knockout</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>8.3 ± 1.53</td>
<td>18.6 ± 1.48</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>39.7 ± 2.01</td>
<td>51.7 ± 2.56</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>29.5 ± 2.44</td>
<td>27.8 ± 2.39</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>11.6 ± 2.27</td>
<td>18.0 ± 3.90</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>3.2 ± 0.52</td>
<td>5.0 ± 0</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* Geometric means of parasitemia (±SE), \( n = 5 \) mice per group. Mann-Whitney test was used to obtain \( p \) values.

**ND, Not determined due to infection-associated mortality observed in the \( C d 3 6 ^ { − / − } \) group of mice after day 7.
display an impaired proinflammatory response early in infection followed by significantly elevated TNF-α and decreased TGF-β levels later in infection (Fig. 5). Depressed IFN-γ response in early infection and decreased TGF-β response and elevated TNF-α response later in infection, have been causally associated with fatal outcomes in the PCCAS mouse model (8, 10, 14, 16, 49, 60, 61). In addition, these types of dysregulated cytokine responses have been epidemiologically associated with severe and fatal malaria in humans (50).

In addition to a coordinated cytokine response, innate control of parasite replication in the acute stage of infection is essential for the survival of mice infected with PCCAS. Consistent with the role of CD36 in the clearance of nonopsonized P. falciparum-PEs (29–31, 62), we demonstrate that CD36 is also involved in the nonopsonic uptake PCCAS-PEs by Mβ. Cd36−/− Mø displayed impaired phagocytosis of PCCAS-PEs (Fig. 3B) and blockade of CD36-induced MAPK activation in Cd36−/− Mø reduced the uptake of PCCAS-PEs to the levels observed with Cd36+/+ Mø (Fig. 3C). We extended these observations to examine the role of CD36 in parasite clearance in vivo and demonstrate that disruption of CD36 resulted in earlier peak parasitemias, higher parasite burdens and higher mortality in mice infected with PCCAS malaria (Table I, Figs. 4 and 5). These data provide the first direct evidence that CD36 contributes to survival by mediating parasite clearance and regulating cytokine responses to malaria infection in vivo.

The interaction between CD36 and malaria is complex. The identification of CD36 as a major sequestration receptor initially led to the assumption that it contributes to the pathogenesis of severe malaria (26, 63–65). However, the present study supports a role for CD36 in host defense and adds to the growing evidence that challenges the role of CD36 in the pathophysiology of cerebral malaria (66–72).

In summary, we demonstrate a dual function for CD36 in mediating phagocytosis and cytokine responses to malaria and provide direct evidence for a role this scavenger receptor in innate host defense to PCCAS malaria in vivo. Collectively, our data add to the growing body of evidence linking the phagocytosis of microbial pathogens to innate sensing and cytokine response mediated via cooperation between pattern recognition receptors such as scavenger receptors and TLRs.

Disclosures
The authors have no financial conflict of interest.

References


