Induction of Proinflammatory Responses in Macrophages by the Glycosylphosphatidylinositol anchors of *Plasmodium falciparum*

THE REQUIREMENT OF EXTRACELLULAR SIGNAL-REGULATED KINASE, p38, c-Jun N-TERMINAL KINASE AND NF-κB PATHWAYS FOR THE EXPRESSION OF PROINFLAMMATORY CYTOKINES AND NITRIC OXIDE*

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The glycosylphosphatidylinositol (GPI) anchors of *Plasmodium falciparum* have been proposed to be the major factors that contribute to malaria pathogenesis by eliciting the production of proinflammatory cytokines and nitric oxide by the host innate immune system. In this study we demonstrate that the parasite GPIs can effectively induce the production of TNF-α at 5–20 nM concentrations in interferon-γ-primed monocytes and macrophages. The potency of the parasite GPIs activity is physiologically relevant to their ability to contribute to severe malaria pathogenesis. More importantly, we investigated the requirement of the extracellular signal-regulated kinase (ERK)-, c-Jun N-terminal kinase (JNK)-, p38-, and NF-κB-signaling pathways that are activated in response to *P. falciparum* GPIs through toll-like receptor-mediated recognition (Krishnegowda, G., Hajjar, A. M., Zhu J. Z., Douglass, E. J., Uematsu, S., Akira, S., Wood, A. S., and Gowda, D. C. (2005) *J. Biol. Chem.* 280, 8606–8616) for the proinflammatory responses by macrophages. The data conclusively show that the production of TNF-α, interleukin (IL)-12, IL-6, and nitric oxide by macrophages stimulated with parasite GPIs is critically dependent on the NF-κB and JNK pathways. NF-κB is essential for IL-6 and IL-12 production but not for TNF-α and nitric oxide, whereas NF-κB/c-Rel appears to be important for all four proinflammatory mediators. JNK1 and JNK2 are functionally redundant for the expression of TNF-α, IL-6, and nitric oxide, whereas JNK2 but not JNK1 is essential for IL-12 production. The ERK signaling pathway is not involved in TNF-α and nitric oxide production, but, interestingly, negatively regulates the expression of IL-6 and IL-12. Furthermore, p38 is critical for the production of IL-6 and IL-12 but is only marginally required for the production of TNF-α and nitric oxide. Thus, our data define the differential requirement of the downstream signaling molecules for the production of key proinflammatory cytokines and nitric oxide by macrophages in response to *P. falciparum* GPI stimuli. The data have important implications for the development of therapeutics for malaria treatment.

Received for publication, December 1, 2004
Published, JBC Papers in Press, December 15, 2004, DOI 10.1074/jbc.M413539200

*The study was supported by NIAID, National Institutes of Health Grant AI41139. 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.

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‡The abbreviations used are: TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; GPI, glycosylphosphatidylinositol; TLR, toll-like receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; Tpl2, tumor progression locus 2 (a member of MAPK kinase family); MAP3Ks or Cot; Erg-1, early growth response 1 (transcription factor); iNOS, inducible nitric-oxide synthase; FBS, fetal bovine serum; PD98059, 2-amino-3-(methoxyflavone; U0126, 1,4-diazino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; SP600125, 1,9-pyrazoloanthrone; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1-(4-pyridyl)-1H-imidazole; SN50, NF-κB inhibitor peptide with sequence AVAVLPAVLLAPQQRKQRKLLMP; PDTC, pyrrolidinedithiocarbamate; MyD88, myeloid differentiation factor; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription.

The process of malaria pathogenesis is very complex and, despite malaria being one of the most extensively studied infectious diseases during the past decades, the precise molecular basis for disease progression remains poorly understood. However, based on our current knowledge, several key processes can be recognized (1). These include the rapid destruction of infected and uninfected erythrocytes, dyserythropoiesis, metabolic acidosis, hypoglycemia, and adherence of infected erythrocytes to the microvascular capillary, vascular obstruction, and production of high levels of proinflammatory mediators in response to parasitic factors (1–5).

Studies from various laboratories have demonstrated that proinflammatory responses, including the production of TNF-α,1 IFNγ, IL-12, and reactive oxygen and nitrogen intermediates by the innate immune system in response to the microbes are critical for killing the pathogenic organisms (5–8). The inflammatory cytokines can also control infection by promoting macrophage phagocytosis and by complement activation (7, 9, 10). In addition, the innate immune response is critical in determining the nature of adaptive immune responses, i.e. the specificity of the adaptive immunity against pathogens depends on the pattern of early cytokine expression (5, 8).

Accumulated evidence also demonstrated that excessive production of proinflammatory mediators leads to systemic and organ-related pathological conditions (5, 11–19). Malaria-infected individuals have been shown to produce high levels of TNF-α and IFNγ and nitric oxide, which are associated with fever and cerebral and other forms of malaria (5, 11–19). In the case of *Plasmodium falciparum* infection, adherence of the parasite-infected erythrocytes to the microvascular capillaries of various organs and in the dermal tissues potentially promotes pathogenesis because of the high parasite burden and severe proinflammatory responses in localized infection, adherence of *Plasmodium falciparum* to the microvascular capillaries of various organs and in the dermal tissues, poten-
areas, leading to endothelial damage and organ dysfunction (15). Furthermore, up-regulation of endothelial cell adhesion molecules in response to TNF-α potentially augments the adherence, spiraling the destruction of the endothelia and causing organ-related pathological conditions (20). Understanding the underlying mechanism that controls the expression of the proinflammatory responses to *P. falciparum* components is likely to provide therapeutic avenues for the prevention of malaria pathogenesis.

Although it is known that excessive production of proinflammatory mediators is a key process that contributes to the *P. falciparum* malaria pathogenesis, very little is known about the nature of various potential ligands of the parasite and cell-signaling mechanisms involved. However, about a decade ago, it was shown that the glycosylphosphatidylinositols (GPIs) of *P. falciparum* could induce the expression of proinflammatory cytokines and nitric oxide in macrophages (21). GPIs administered to animals have been shown to cause transient pyrexia and hypoglycemia, cachexia, and death in galactosamine-sensitized animals, the events that are reminiscent of acute malaria (21). Thus, GPIs have been proposed as the dominant parasite factors responsible for malaria pathogenesis. Later studies have shown that the GPIs of *P. falciparum* can induce the expression of iNOS, up-regulate the expression of intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin in endothelial cells, implicating these processes in malaria pathogenesis (20).

Previously, it was proposed that parasite GPI-induced proinflammatory responses involve the activation of protein kinase C and protein-tyrosine kinase-signaling pathways that collaboratively activate NF-κB/C-Rel, which translocates to the nucleus, initiating the expression of proinflammatory molecules (22). It has also been postulated that the two signaling pathways are activated by the distinct structural elements of GPIs, namely the conserved glycan and diacylglycerol moieties, respectively (23). Several hypotheses, including the insertion of GPIs into cell membrane microdomains rich in GPI-anchored proteins and membrane perturbation, hydrolysis of GPIs, and internalization of the diacylglycerol moiety or GPI translocation across the cell membrane initiating the activation of protein kinase C-dependent second messengers, have been proposed for cell signaling by GPIs (23). However, in recent years it has become clear that the proinflammatory responses by the innate immune system are mediated through the recognition of specific molecular patterns of microorganisms by the TLR family of proteins (24–26). Recently, cell signaling by GPIs of *Trypanosoma cruzi* to produce proinflammatory mediators has been shown to be through the exclusive recognition of TLR2 (27). In the preceding paper (28) we have demonstrated that *P. falciparum* GPI-induced proinflammatory responses in macrophages proceed through the recognition of mainly TLR2 and to a lesser extent through TLR4, and the cell signaling involves the activation of MAPK-, ERK-, p38-, JNK-, and NF-κB-signaling pathways.

A number of studies have shown that ERK-, p38-, JNK-, and NF-κB-signaling pathways are activated by TLR/MyD88-mediated cell signaling in response to various ligands of pathogenic organisms (29). However, the requirement of various signaling molecules of these pathways for the expression of cytokines appears to be dependent on the nature of ligands and the host cell types. In many cases the reported results are controversial (30). With regard to GPIs, a recent study reported the requirement of ERK, p38, and NF-κB pathways with GPIs of *T. cruzi*; however, the information is very limited (31). In this study we investigated the requirement of the MAPK- and NF-κB-signaling pathways for the *P. falciparum* GPI-induced expression of proinflammatory mediators using macrophages from mice deficient in the activation of ERK1/ERK2, JNK1, and JNK2 and by using specific inhibitors. Our data show that the ERK1/ERK2-, p38-, and JNK1/JNK2-signaling molecules and NF-κB family proteins are differentially involved in the GPI-induced production of TNF-α, IL-6, IL-12, and nitric oxide by macrophages.

**EXPERIMENTAL PROCEDURES**

**Materials**—PDTC, SN50 peptide, PD98059 and U0126, SB203580, and SB202190 were from Calbiochem. Griese reagent was from Sigma. SP600125 was from Toronto Cookson, Inc. (Ellisville, MO). The chemiluminescence substrate kit was from KPL (Gaithersburg, MD). Nitrocellulose membranes were from Bio-Rad. The anti-peptide and phospho-specific antibodies that recognize ERK1/ERK2, p38, JNK, anti-IκBα, and anti-β-tubulin antibodies and horseradish peroxidase-conjugated goat anti-mouse IgGs and goat anti-rabbit IgGs were from Cell Signaling Technology Inc. (Beverly, MA). Anti-Tpl2 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Kits for the estimation of TNF-α, IL-6, and IL-12 (p40) by ELISA (DuoSet ELISA Development System) were from R&D Systems, Inc. (Minneapolis, MN). FBS, DMEM, and Trizol RNA isolation reagent were from Invitrogen. L929 murine fibroblast cells were from American Type Culture Collection. The PCR primers used were custom-synthesized by Invitrogen. Human blood and serum from healthy donors were from the hospital of the Hershey Medical Center. Endotoxin-free reagents, water, and buffers were used for all the experimental procedures.

**P. falciparum Culture and Induction of GPIs**—The parasites culturing and purification of GPIs are described in the preceding paper (28).

**Mice**—NF-κB1/p105 knock-out mice and the corresponding wild type control mice (B6.129PF2/J) were used for cytokine and nitric oxide measurements. The parasites cultured and the corresponding wild type control mice (C57BL6/J) were purchased from The Jackson Laboratories. The animals were housed in a germ-free environment, and the animal care was in accordance with the Institutional Guidelines of the Pennsylvania State University College of Medicine.

**Preparation of L929 Cell Supernatant**—L929 cells were cultured in DMEM, 5% FBS, 1% penicillin/streptomycin, and 1%L-glutamine in roller flasks at 37 °C for 4–5 days. The supernatant were collected, centrifuged at 2500 rpm for 20 min, and used as a source of macrophage colony-stimulating factor.

**Preparation of Bone Marrow-derived Macrophages**—Bone marrow-derived macrophages were obtained as described (32). Briefly, the murine bone marrow was flushed from the femurs of mice with DMEM, 10% FBS, and penicillin/streptomycin. The tissue debris in each case was removed by centrifugation at 300 rpm, and cells were spun down at 1200 rpm. The cell pellets were washed twice with DMEM, 10% FBS, and penicillin/streptomycin and resuspended in DMEM, 5% FBS, 30% L929 cell supernatant, and 1% penicillin/streptomycin and cultured at 37 °C for 2 days. The non-adherent cells were removed, and the adherent cells were cultured by changing the medium every 2 days. Seven days later the macrophages were harvested using phosphate-buffered saline, pH 7.2, containing 10 mM EDTA and 20% FBS and used for stimulation with the parasite GPIs.

**Stimulation of Macrophages with P. falciparum GPIs**—Freshly harvested macrophages were seeded in 96-well microtiter plates (2.5 × 10^4 cells/well) and cultured overnight in DMEM, 10% FBS, and penicillin/streptomycin. The cells were treated with varying amounts of GPIs coated onto gold particles as described in the preceding paper (28). Cells treated with uncoated gold particles were used as controls. For inhibition studies the inhibitors were added to the culture medium 1 h before the addition of GPIs. In some experiments cells were primed with IFNγ (100 units/ml) before stimulation with GPIs. The culture supernatant were collected at the indicated time points and stored at −70 °C until used for cytokine and nitric oxide measurements.

For the analysis of signaling proteins, macrophages in 24-well microtiter plates (0.5 × 10^5 cells/well) were cultured overnight in DMEM, 0.5% FBS and then stimulated with 200 ng GPIs. For inhibition studies cells were treated for 1 h with appropriate inhibitors before stimulation with 200 ng GPIs. At various time points, the cells were washed with ice-cold phosphate-buffered saline and then lysed with ice-cold radio-immune precipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.2, containing 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100). The lysates were centrifuged at 4 °C, and the supernatant was used for Western blotting.
RT-PCR analysis of gene transcription, macrophages in 24-well plates (1 x 10^5 cell/well) were cultured overnight in DMEM, 0.5% FBS, then stimulated with 200 nM GPIs in DMEM, 10% FBS; at various time points culture supernatants were removed, and cells were washed with ice-cold phosphate-buffered saline, pH 7.2. Cells were dissolved in Trizol, and total RNA was isolated and used for cDNA preparation.

**Cytokine and Nitrite Measurement**—The TNF-α, IL-6, and IL-12 (p40) in the culture supernatants of macrophages stimulated with GPIs were determined by sandwich ELISA with horseradish peroxidase-conjugated streptavidin and 3,3′,5,5′-tetramethylbenzidine color reagent using the Duoset ELISA development kit (R&D Systems). After stopping the color development with 1 M sulfuric acid, the absorbance at 450 nm was measured using SpectraMax Plus384 plate reader (Molecular Devices). The cytokine concentrations were calculated with reference to the standard curves.

The nitrite in the macrophage culture supernatants was assayed by microcolorimetric assay using the Griess reagent (33). The absorbance at 540 nm was determined, and the nitrite concentrations were estimated with reference to a graph generated using a standard solution of sodium nitrite.

**Cell Viability Assay**—The viability of cells treated with various inhibitors was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as described previously (34). With all inhibitors, cells treated for 4 h were 100% viable. During 48 h of incubation, B203580, SB202190, SP600125, and SN50 did not show noticeable cytotoxicity, whereas PDT, PDB0509, and U0126 showed 5–10% cell toxicity.

**Western Blotting**—The macrophage lysates obtained as outlined above were made to 1 x with respect to the SDS-PAGE sample buffer containing 2-mercaptoethanol, boiled for 5 min, and electrophoresed on 10% SDS-polyacrylamide gels. The protein bands in the gels were transferred onto polyvinylidene difluoride or nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat dry milk in phosphate-buffered saline, pH 7.4, containing 0.1% Tween 20 at room temperature for 1 h and then incubated with either anti-peptide or phospho-specific antibodies in the above buffer. After washing the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, treated with chemiluminescent substrate system (LumiGLO from KPL), and exposed to x-ray films.

**RT-PCR and Real-time PCR**—Total RNA samples from wild type and gene knock-out mice were reverse-transcribed using the oligo-dT 14 primers. Aliquots of cDNA samples were analyzed for TNF-α, IL-6, IL-12, and iNOS genes by real-time PCR. The primers used were: Tpl2 forward 5′-GCT CTC GTT GCC AAT AGT GA-3′, reverse 5′-CAT GCA GAC AGG CAC CCC AC-3′; IL-12, forward 5′-AAG TGC ATC ATC GTT CAT ACA-3′, reverse 5′-GTG CTG AAG GAG CTG CTG ACG-3′; Egr-1, forward 5′-CAA G-3′, reverse 5′-TGA CTT TCT CCT CTT CAA G-3′, reverse 5′-GGT CTT CTT CCT GGT ATG AGA TAG CA-3′; IL-6, forward 5′-AAG TCG GAG GCT TAA TTA CAC ATG T-3′, reverse 5′-AAG AAC ATG TTC AGC GTT CTT CAT ACA-3′; IL-12 (p40), forward 5′-ACA AGA CCT TCC TGA AGT GGT CAC C-3′, reverse 5′-GCT CTT GAT GTT GAA CTT CCA GTC C-3′; iNOS, forward 5′-AAT GTA TGG AAG AGG AAC A-3′, reverse 5′-GGT GGT ACT ACT GTT AGC GTC ATC TTG TA-3′; B-actin, reverse 5′-ACC AGA CTC CAT GCC ATC AC-3′, reverse 5′-TCC ACC ACC CTT CTG CTG TA-3′. The conditions used for PCR were 40 cycles of 94 °C denaturation for 30 s, 50 °C annealing for 30 s, and 72 °C extension for 60 s.

Primers for real-time PCR analysis of TNF-α, IL-6, IL-12 (p40), iNOS, and β-actin transcripts were designed with Primer Express® Software version 2.0 from Applied Biosystems and custom-synthesized by Invitrogen: TNF-α, forward 5′-TGT CTA CTA CCA GCT TCT CTT CAA G-3′, reverse 5′-TGA TCT TCT CTT GGT ATG AGA TAG CA-3′; IL-6, forward 5′-AAG TCG GAG GCT TAA TTA CAC ATG T-3′, reverse 5′-AAG AAC ATG TTC AGC GTT CTT CAT ACA-3′; IL-12 (p40), forward 5′-ACA AGA CCT TCC TGA AGT GGT CAC C-3′, reverse 5′-GCT CTT GAT GTT GAA CTT CCA GTC C-3′; iNOS, forward 5′-AAT GTA TGG AAG AGG AAC A-3′, reverse 5′-GGT GGT ACT ACT GTT AGC GTC ATC TTG TA-3′; β-actin, forward 5′-ACC AGA CTC CAT GCC ATC AC-3′, reverse 5′-TCC ACC ACC CTT CTG CTG TA-3′. The real-time PCR was performed in an ABI Prism 7700 sequence detection system (PerkinElmer Life Sciences) using QuantiTect™ SYBR® Green PCR kit from Qiagen. Amplification was performed in a total volume of 25 μl containing 2x QuantiTech SYBR Green PCR Master Mix, 0.6 μM each primer, 100 ng of cDNA. The PCR conditions used were 45 cycles of 95 °C for 15 s, 52 °C for 30 s, and 72 °C for 30 s. The increase in cytokine mRNA in GPI-stimulated macrophages compared with unstimulated cells was calculated from threshold cycle number (Ct) according ABI Prism 7700 sequence detection system Version 1.6 software (PerkinElmer Life Sciences).

**RESULTS**

**Production of Proinflammatory Cytokines and Nitric Oxide in IFN-γ-primed Macrophages Stimulated with P. falciparum GPIs**

Previous studies have reported that 0.1–1 μM P. falciparum GPIs are required for the efficient production of TNF-α in macrophage cell lines, and an appreciable level of nitric oxide was evident only at >1 μM GPIs (22, 23, 35, 36). In the case of *T. cruzi* trypomastigote GPIs, however, subnanomolar concentrations are sufficient to induce high levels of proinflammatory cytokines and nitric oxide production in IFN-γ-primed peritoneal macrophages, and priming was essential for the expression of IL-12, IL-6, and nitric oxide (31, 37). Because in previous studies with *P. falciparum* GPIs the activity was studied in macrophage cell lines without IFN-γ priming (22, 23, 35, 36), the reported comparative assessment of the potency of GPIs of these two protozoan parasites measured under different conditions does not accurately reflect the relative potency of malarial GPIs. Therefore, in this study we tested *P. falciparum* GPIs for the production of proinflammatory cytokines and nitric oxide in mouse bone marrow-derived and peritoneal macrophages before and after priming with IFN-γ (Fig. 1 and not shown). Additionally, to determine the pathophysiological relevance of *P. falciparum* GPIs in malaria, we tested the activity of the GPIs in IFN-γ-primed and -unprimed human peripheral monocytes. The *P. falciparum* GPIs induced efficient production of TNF-α at 5–20 nM in IFN-γ-primed and -unprimed murine macrophages and human monocytes (Fig. 1, A and E). The *P. falciparum* GPIs exhibited an order of magnitude higher activity in macrophages than that previously reported under the conditions used for measuring the activity of *T. cruzi* mucin GPIs. Therefore, the potency of the *P. falciparum* GPIs is about 10-fold but not 100-fold lower than that of *T. cruzi* GPIs as reported previously (37). Furthermore, as in the case of *T. cruzi* GPI (31, 37), the *P. falciparum* GPIs were unable to induce IL-6, IL-12, and nitric oxide expression in unprimed macrophages but could induce the production of these proinflammatory mediators in IFN-γ-primed macrophages (Fig. 1, B–D). Although 5–20 nM GPIs were sufficient to produce high levels of TNF-α and nitric oxide in macrophages, 20–320 nM GPIs were required for the efficient production of IL-12 and IL-6 (Fig. 1, B and C).

**The Requirement of NF-κB- and MAPK-signaling Pathways for the *P. falciparum* GPI-induced Production of Proinflammatory Cytokines and Nitric Oxide by Macrophages**

In the preceding paper (28) we have shown that stimulation of macrophages with *P. falciparum* GPIs results in the activation of ERK-, p38-, JNK-, and NF-κB-signaling pathways through the recognition mainly by TLR2 and to some degree by TLR4 (28). In this study we investigated the requirement of these downstream signaling pathways for the production of proinflammatory cytokines and nitric oxide using specific inhibitors of signaling molecules and macrophages from gene knock-out mice. The results of these studies are described below.

**The Requirement of NF-κB Activation for the *P. falciparum* GPI-induced Proinflammatory Responses**—The activation and nuclear translocation of NF-κB is crucial for the production of proinflammatory molecules in response to various microbial ligands (38–41). NF-κB comprises a family of proteins, which differentially regulate the expression of proinflammatory mediators in response to activation by various microbial ligands. For example, NF-κB1/p105 is not essential for the LPS-induced expression of TNF-α and nitric oxide (41, 42), but p50 derived
from NF-κB1/p105 appears to be crucial for the κBζ-dependent expression of a subset of genes in cells activated through TLR-signaling pathways (43). To determine the role of NF-κB1/p105 in the GPI-induced proinflammatory responses, we used macrophages from NF-κB1/p105−/− mice (41, 42). The levels of TNF-α and nitric oxide produced by NF-κB1/p105−/− macrophages were comparable with those by the wild type macrophages (Fig. 2). In both cells types similar levels of TNF-α and nitric oxide were also produced in response to different concentrations of GPI stimulation (not shown). Consistent with these results, the real time RT-PCR analysis showed that the mRNA levels of TNF-α and iNOS in GPI-treated NF-κB1/p105−/− macrophages were comparable with those of the GPI-treated wild type macrophages (Fig. 3). These data indicate that the p50-containing NF-κB complex has little or no role in the production of TNF-α and nitric oxide but is crucial for the production of TNF-α and nitric oxide by macrophages stimulated with GPIs. In contrast to PDTC, SN50 only marginally inhibited the expression of GPI-induced production of TNF-α and nitric oxide in macrophages (Fig. 4B). These results indicate that the p50-containing NF-κB complex has little or no role in the production of TNF-α and nitric oxide, and the data are consistent with the results from NF-κB1/p105−/− macrophages (see Fig. 2). In the case of IL-6 and IL-12, 4.5 μM SN50 inhibited their production by ~40 and 50%, respectively (Fig. 4B). These results are consistent with the requirement of NF-κB1/p50 for κBζ-dependent production of IL-12 and IL-6 (43). Unexpectedly, however, at higher concentrations of SN50, the inhibitory effect on IL-6 and IL-12 expression was nearly abolished, and the reason for this effect is not clear.

The Requirement of the ERK-signaling Pathway for the P. falciparum GPI-induced Proinflammatory Responses—Recently, it has been shown that NF-κB1/p105−/− mice are deficient in the activation of the ERK-signaling pathway but not those of p38 and JNK (46, 47). Therefore, we used bone marrow-derived macrophages from NF-κB1/p105−/− mice to study the requirement of ERK activation for the expression of proinflammatory cytokines, TNF-α, IL-6, and IL-12, and nitric oxide in response to P. falciparum GPIs. Macrophages from the NF-κB1/p105−/− mice were, respectively, 5–10- and 2–4-fold lower than that in GPI-treated wild type macrophages at various time points after stimulation (Fig. 3). These data indicate that the observed lower level of IL-12 and IL-6 expression (see Fig. 2) in NF-κB1/p105−/− macrophages compared with wild type macrophages is due to the marked low levels of mRNA transcripts in the former. The data also suggest that p50 (the matured form of NF-κB1/p105) has little or no role in the expression of TNF-α and nitric oxide but is crucial for the expression of IL-6 and IL-12, presumably by the κBζ-mediated gene expression as reported recently for LPS (42, 43).
nB1/p105−/− and wild type mice were stimulated with P. falciparum GPIs, and cell lysates were analyzed for the activation of ERK, p38, JNK, and NF-κB pathways by Western blotting. The macrophages from NF-nB1/p105−/− mice responded normally to the parasite GPI-induced stimulation with regard to p38, JNK activation, and IκBα degradation (indicative of NF-κB activation) but were deficient in the activation of ERK1/ERK2 even after prolonged (2 h) stimulation with the parasite GPIs (Fig. 5A and data not shown). NF-nB1/p105 is known to be involved in the stabilization of Tpl2, a MAP3K (MAPK kinase kinase) upstream of ERK1/ERK2 (46, 47). Consistent with this finding, the level of Tpl2 was also markedly low in macrophages from NF-nB1/p105−/− mice even though the Tpl2 mRNA level was normal (Fig. 5B and C). Furthermore, macrophages from NF-nB1/p105−/− mice were defective in the transcription of Egr-1, a transcription factor that is phosphorylated by ERK (Fig. 5C and see Ref. 46).

It has been previously shown that macrophages from NF-nB1/p105−/− mice when stimulated with LPS showed very low levels of ERK2 activation but not ERK1 (46). In contrast, in this study NF-nB1/p105−/− macrophages stimulated with P. falciparum GPIs exhibited a very low level of ERK1 activation but not ERK2 (Fig. 5A). Nevertheless, because NF-nB1/p105−/− macrophages are defective in ERK1/ERK2 activation, these cells served as a useful model for studying the requirement of ERK-signaling pathway for the expression of P. falciparum GPI-induced proinflammatory cytokine and nitric oxide production.

As described above (see Figs. 2 and 3), the production of TNF-α and nitric oxide as well as the mRNA levels of TNF-α and iNOS in NF-nB1/p105−/− macrophages was normal despite the cells being defective in the activation of the ERK-signaling pathway. These data show that the ERK-signaling pathway is not involved in the expression of TNF-α and nitric oxide by macrophages stimulated with GPIs. In contrast, the GPI-induced NF-nB1/p105−/− macrophages produced significantly lower levels of IL-12 and IL-6 compared with wild type macrophages at both protein and mRNA levels (see Figs. 2 and 3). These data suggest at a first glance that ERK1/ERK2 activation is critically required for the production of IL-12 and IL-6 in macrophages. However, when the activation of ERK1/ERK2 in wild type macrophages was inhibited with specific inhibitors, the production of IL-12 and IL-6 was markedly enhanced (see Fig. 6A). Therefore, as shown recently (43), the observed strikingly low level expression of IL-12 and IL-6 in NF-nB1/p105−/− macrophages is likely due to the functional impairment of IκBζ rather than the lack of ERK1/ERK2 activation in NF-nB1/p105−/− macrophages.

The requirement of the ERK-signaling pathway for the parasite GPI-induced proinflammatory responses was also assessed using the specific inhibitors of ERK1/ERK2 activation, namely PD98059 and UO126. Pretreatment of macrophages with inhibitors followed by stimulation with P. falciparum GPIs led to a 20–30% inhibition of TNF-α and nitric oxide production compared with control macrophages (Fig. 6A). However, the normal levels of TNF-α and nitric oxide production by NF-nB1/p105−/− macrophages deficient in ERK1/ERK2 activation in response to parasite GPIs argue that the observed marginal inhibition of these inflammatory mediators is likely due to some level of either nonspecific inhibition or cytotoxicity. In contrast to these results, treatment of macrophages with PD98059 and UO126 led to markedly elevated levels of GPI-
induced expression of IL-6 and IL-12 compared with cells stimulated with GPIs in the absence of inhibitors (Fig. 6A). With both inhibitors the effect was much more pronounced for the production of IL-12 than that of IL-6. These results agree with the reported negative regulation of IL-12 by T. cruzi GPIs and LPS in macrophages (31, 48). Western blot analysis of the cell lysates showed that, as expected, both PD98059 and UO126 substantially inhibited the GPI-induced activation of ERK1/2 lysates showed that, as expected, both PD98059 and UO126 LPS in macrophages (31, 48). Western blot analysis of the cell lysates from macrophages stimulated with GIs in the presence of inhibitors. Cells primed and stimulated but not pretreated with inhibitors were used as controls. After 48 h of stimulation, the cell culture supernatant was collected and assayed for TNF-α, IL-6, and IL-12 by ELISA and for nitric oxide with the Griess reagent. The experiments were performed three times, the average values were plotted, and the error range is indicated.

The Requirement of the JNK Pathway for the P. falciparum GPI-induced Proinflammatory Responses—Previous studies have shown that activation of the JNK-signaling pathway is crucial for LPS-induced TNF-α (51–53). JNK is comprised of 10 isoforms formed by the alternative splicing of mRNA from three genes, JNK1, JNK2, and JNK3 (53). Of these, only JNK1 and JNK2 are expressed in monocytes and macrophages, and JNK3 is selectively expressed in nervous tissues and to a lesser extent in heart and testis (54). We studied the role of JNK1 (MAPK8) and JNK2 (MAPK9) in bone marrow-derived macrophages from the JNK1 and JNK2 knock-out mice. Upon stimulation with P. falciparum GIs, the levels of TNF-α, nitric oxide, IL-6, and IL-12 produced by the JNK1 and JNK2 null mice were comparable with those of the GPI-treated wild type macrophages (Fig. 8A). Because the JNK pathway is thought to be crucial for inflammatory responses (51–53), these results suggest that JNK1 is either not required for the parasite GPI-induced inflammatory responses, or JNK2 compensates for the function of JNK1. In the case of JNK2−/− macrophages, stimulation with the parasite GIs could induce TNF-α, IL-6, and nitric oxide at levels compared with those produced by the GPI-treated wild type macrophages (Fig. 8A). These results together with those of JNK1−/− macrophages indicate that JNK1 and JNK2 are either not involved in the production of TNF-α, IL-6, and nitric oxide by macrophages stimulated with GIs or these signaling molecules are functionally redundant. However, in GPI-treated JNK2−/− macrophages, the level of IL-12 was substantially (∼80%) decreased, suggesting that JNK2 is critical for IL-12 production, and JNK1 does not compensate for JNK2 for IL-12 expression. Consistent with these results, real-time RT-PCR analysis showed that the levels of TNF-α, IL-6, and iNOS transcripts in JNK2−/− macrophages stimulated with GIs were comparable with those in wild type macrophage control (Fig. 8B). However, the level of IL-12 mRNA was markedly lower in GPI-induced JNK2−/− macrophages compared with wild type macrophages (Fig. 8B).

The requirement of JNK for the P. falciparum GPI-induced inflammatory responses in mouse bone marrow-derived macrophages was also studied by using the inhibitor of JNK enzyme activity, SP600125 (55). SP600125 has been shown to specifically inhibit JNK enzymatic activity at <10 μM concentrations (55). SP600125 inhibited the GPI-induced production of TNF-α, IL-6, IL-12, and nitric oxide in macrophages in a dose-dependent manner (Fig. 9A). The inhibitory effect of SP600125 on IL-12 production was much more pronounced than that on the production of TNF-α, IL-6, and nitric oxide. These results taken together with the results of JNK1−/− and JNK2−/− macrophages indicate that JNK1 and JNK2 compensate for each other for TNF-α, IL-6, and nitric oxide production but not for type mice with the parasite GPIs in the presence of SB203580 showed only a marginal decrease in the production of TNF-α and nitric oxide compared with that in GPI-treated control macrophages (Fig. 7A). However, under the same conditions, the production of IL-6 was reduced by 90% and that of IL-12 was completely abrogated (Fig. 7A). The production of IL-6 and IL-12 was markedly inhibited even at 1.25–5 μM concentrations of SB203580. Similar results were obtained with another related p38-specific inhibitor, SB202190 (data not shown). Consistent with the known property of these inhibitors, which is that they interfere with the p38 kinase activity but not p38 phosphorylation, Western blot analysis of the lysates from macrophages stimulated with GIs in the presence 10 μM SB203580 showed normal p38 phosphorylation (Fig. 7B). Together the above results demonstrate that the p38-signaling pathway is differentially required for the GPI-induced proinflammatory cytokine and nitric oxide production.

The Requirement of the JNK Pathway for the P. falciparum GPI-induced Proinflammatory Responses—Previous studies have shown that activation of the JNK-signaling pathway is crucial for LPS-induced TNF-α (51–53). JNK is comprised of 10 isoforms formed by the alternative splicing of mRNA from three genes, JNK1, JNK2, and JNK3 (53). Of these, only JNK1 and JNK2 are expressed in monocytes and macrophages, and JNK3 is selectively expressed in nervous tissues and to a lesser extent in heart and testis (54). We studied the role of JNK1 (MAPK8) and JNK2 (MAPK9) in bone marrow-derived macrophages from the JNK1 and JNK2 knock-out mice. Upon stimulation with P. falciparum GIs, the levels of TNF-α, nitric oxide, IL-6, and IL-12 produced by the JNK1 and JNK2 null mice were comparable with those of the GPI-treated wild type macrophages (Fig. 8A). Because the JNK pathway is thought to be crucial for inflammatory responses (51–53), these results suggest that JNK1 is either not required for the parasite GPI-induced inflammatory responses, or JNK2 compensates for the function of JNK1. In the case of JNK2−/− macrophages, stimulation with the parasite GIs could induce TNF-α, IL-6, and nitric oxide at levels compared with those produced by the GPI-treated wild type macrophages (Fig. 8A). These results together with those of JNK1−/− macrophages indicate that JNK1 and JNK2 are either not involved in the production of TNF-α, IL-6, and nitric oxide by macrophages stimulated with GIs or these signaling molecules are functionally redundant. However, in GPI-treated JNK2−/− macrophages, the level of IL-12 was substantially (∼80%) decreased, suggesting that JNK2 is critical for IL-12 production, and JNK1 does not compensate for JNK2 for IL-12 expression. Consistent with these results, real-time RT-PCR analysis showed that the levels of TNF-α, IL-6, and iNOS transcripts in JNK2−/− macrophages stimulated with GIs were comparable with those in wild type macrophage control (Fig. 8B). However, the level of IL-12 mRNA was markedly lower in GPI-induced JNK2−/− macrophages compared with wild type macrophages (Fig. 8B).
the expression of IL-12. As expected, the SP600125-treated macrophages showed normal or somewhat decreased (at 10 μM SP600125) levels of JNK phosphorylation upon stimulation with GPIs (Fig. 9B). This could be due to the inhibition of an upstream kinase and/or autophosphorylation (55).

DISCUSSION

It is becoming increasingly clear that GPIs of various parasitic protozoa, including Plasmodia, Trypanosoma, and Leishmania, play a prominent role in the immunopathology of the parasitic infection by their ability to induce potent proinflammatory responses (56). Thus, identification of the signaling molecules involved in the expression of various proinflammatory mediators may offer therapeutic targets for severe malaria. In the preceding manuscript (28) we showed that stimulation of monocytes and macrophages with P. falciparum GPIs leads to the activation of ERK, p38, JNK, and NF-κB downstream signaling pathways through the TLR/MyD88-mediated signaling mechanism. The results are consistent with the notion that the TLR/MyD88-dependent signaling of macrophages by various pathogen-associated molecular patterns, such as LPS, lipopeptides, and T. cruzi GPIs involves the activation of downstream ERK-, p38-, JNK-, and NF-κB-signaling pathways. Although, the activation of these signaling pathways in response to inflammatory stimuli is a common event, their involvement in the expression of various proinflammatory mediators is not clear and in many instances is controversial (30). In this study using macrophages from gene knock-out animals and specific inhibitors, we define the requirement of downstream signaling pathways for P. falciparum GPI-induced expression of proinflammatory cytokines and nitric oxide. Our data indicate that ERK-, p38-, JNK-, and NF-κB-signaling molecules are differentially involved in the expression proinflammatory mediators in macrophages stimulated with GPIs.

The results of this study show that the activation and translocation of NF-κB complex is obligatory for the GPI-induced expression of proinflammatory mediators, TNF-α, IL-6, IL-12, and nitric oxide (see Fig. 4A). These findings are in agreement with the known information that NF-κB is a key transcriptional factor that regulates the gene expression of a number of cytokines, chemokines, and growth factors by macrophages in response to stimulation with microbial agents. However, NF-κB comprises a family of proteins, and much needs to be learned about the involvement of specific NF-κB family members in the TLR/MyD88-mediated expression of proinflammatory mediators in response to microbial ligands, including GPIs. Our data show that the GPI-induced expression of IL-6 and IL-12 but not that of TNF-α and nitric oxide is specifically regulated by p50 derived from NF-κB1/p105. This is evident from 1) the production of significantly lower levels of IL-6 and IL-12 and normal levels of TNF-α and nitric oxide by GPI-stimulated macrophages pretreated with SN50, an inhibitor of translocation of p50-containing NF-κB complexes (see Fig. 4B), and 2) the expression of normal levels of TNF-α and nitric oxide and markedly low levels of IL-6 and IL-12 by NF-κB1/p105−/− macrophages stimulated with GPIs (see Fig. 2). These results are also consistent with the results of a previous study that reported that the GPI-induced expression of TNF-α and nitric oxide is regulated by NF-κB/c-Rel transcription factor (23). We observed that the expression of IL-6 and IL-12 was markedly reduced at both protein and transcription levels in NF-κB1/p105−/− macrophages (see Figs. 2 and 3). These data are consistent with the recent finding that IskB, a new member of the IskB family proteins that is inducible in macrophages upon stimulation with TLR ligands, regulates the expression of a subset of genes, including IL-6 and IL-12, by recruitment to the DNA-binding sites of the gene promoters (43, 57). The function of IskB appears to require its association with the p50-derived form NF-κB1/p105. Thus, our data demonstrate for the first time that NF-κB1/p105 specifically regulates the GPI-induced expression of IL-6 and IL-12 but not TNF-α and nitric oxide by macrophages.

The results presented in this paper show that the activation of ERK1/ERK2 is differentially involved in the production of proinflammatory mediators by macrophages stimulated with GPIs. When macrophages were stimulated with GPIs, Western blotting using antibodies against Tpl2 and Egr-1 genes were amplified by RT-PCR and analyzed on 1% agarose gel. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was analyzed as an endogenous control.
**Cytokine Induction by P. falciparum GPIs**

**Fig. 6.** The effect of ERK inhibitors on the production of proinflammatory cytokines and nitric oxide in macrophages stimulated with *P. falciparum* GPIs. Panel A, bone marrow-derived macrophages from C57BL/6/J mice were plated into 96-well microtiter plates (2.5 \times 10^5 cells/well). After overnight culturing the cells were treated with the indicated concentrations of PD98059 or UO126 for 1 h and then primed with IFNγ and stimulated with the 40 nM *P. falciparum* GPIs in the continued presence of the inhibitors. Cells not treated with inhibitors but primed with IFNγ and stimulated with the 40 nM GPIs were used as controls. After 48 h of stimulation, the culture supernatants were assayed for TNF-α, IL-6, and IL-12 and for nitric oxide. The experiments were performed three times, each in duplicate, average values were plotted, and error range was indicated. Panel B, bone marrow-derived macrophages in 24-well microtiter plates (5 \times 10^5 cells/well) were treated with 20 and 40 μM PD98059 or 10 and 20 μM UO126 for 1 h and then stimulated with 200 nM GPIs for 25 min. The cell lysates were analyzed by Western blotting using anti-peptide and phospho-specific antibodies against ERK1/ERK2.

*P. falciparum* GPIs. Previously, several laboratories have studied the role of the ERK-signaling pathway for the production of TNF-α and other inflammatory mediators in response to various ligands, including LPS (51, 58, 59). It is generally believed that ERK1/ERK2 are activated by Raf isoforms in response to mitogenic or proliferative stimuli and that they are unlikely to be involved in the proinflammatory responses (59). Tpl2/Cot has also been reported to activate ERK1/ERK2 in response to proinflammatory stimulation, and one study showed that Tpl2−/− macrophages were unable to produce TNF-α in response to LPS (58). However, the requirement of Tpl2-dependent activation of ERK1/ERK2 for the expression of TNF-α and other proinflammatory mediators has not been further studied. In this study we assessed the role of ERK1/ERK2 activation in GPI-stimulated proinflammatory responses by macrophages using the inhibitors PD98059 and UO126 that are widely used by various laboratories. As previously reported for LPS and *T. cruzi* GPIs (31), our results with the inhibitors showed a 20–30% decrease in TNF-α and nitric oxide expression in response to *P. falciparum* GPIs. However, in our studies using NF-κB/p105−/− macrophages that are deficient in ERK activation, the cells were able to produce normal levels of TNF-α and nitric oxide compared with wild type macrophages. These results clearly demonstrate that ERK signaling is not involved in the GPI-induced TNF-α and nitric oxide. These results taken together argue that the observed marginal decrease in the TNF-α and nitric oxide production in GPI-treated macrophages in the presence of PD98059 and UO126 is not due to the inhibition of ERK1/ERK2 activation but likely is due to either a partial cytotoxic effect of the inhibitors or a nonspecific inhibition of other signaling molecules.

In this study the inhibition of ERK1/ERK2 activation by PD98059 and UO126 led to the marked increase in GPI-induced production of IL-6 and IL-12 (see Fig. 6A). Similar results have been observed in macrophages stimulated with *T. cruzi* GPIs and LPS in the presence of PD98059 (31, 48). Although the reason for the negative regulatory effect of the ERK activation inhibitors on IL-6 and IL-12 expression by macrophages remains to be determined, our preliminary studies showed enhanced transcription of IL-6 and IL-12 expression by macrophages in the presence of ERK activation inhibitors. Therefore, we speculate that the observed enhanced IL-6 and IL-12 expression is due to increased level of p50 and/or efficient binding of p50/IκBα to the gene promoters. In contrast to the results from the inhibitors, as mentioned above, the NF-κB/p105−/− macrophages showed marked reduction in the levels of IL-6 and IL-12 produced in response to *P. falciparum* GPIs compared with control macrophages. These results at first glance suggest that ERK1/ERK2 activation is critically required for the production of IL-6 and IL-12. However, based on the results of a recent study, the observed reduction in the expression of these cytokines is most...
likely due to the functional impairment of IκBζ rather than the lack of normal ERK1/ERK2 activation (43). Thus, our data from the inhibitor and NF-κB1/p105−/− macrophage studies together argue that the ERK-signaling pathway is not critical for the production of certain proinflammatory mediators, such as TNF-α and nitric oxide by GPI-stimulated macrophages, but negatively regulates the induction of other proinflammatory mediators, IL-6 and IL-12.

The data presented here demonstrate that the p38-signaling pathway is differentially required for the P. falciparum GPI-induced expression of TNF-α, IL-6, IL-12, and nitric oxide. Inhibition of p38 activity by SB203585 or SB202190 led to the almost complete abrogation of GPI-induced IL-6 and IL-12 expression by macrophages, whereas the production of TNF-α and nitric oxide was only marginally reduced, indicating that the p38-signaling pathway is crucial for IL-6 and IL-12 and is either marginally required or not essential for TNF-α and nitric oxide production (Fig. 7A and data not shown). Our conclusion that p38 is not involved in nitric oxide production agrees with the results of previous studies, which reported that nitric oxide production by macrophages stimulated with T. cruzi GPIs or LPS is not affected by the inhibition of p38 activity (31, 60, 61). However, with respect to the regulation of TNF-α and IL-12 by the p38 pathway, our results are in sharp contrast to those observed in the case of T. cruzi GPIs (31). In macrophages stimulated with T. cruzi GPIs or LPS, the inhibition of p38 activity by 10 μM SB203580 affected TNF-α production by 60%, whereas the expression of IL-12 was only slightly (~25%) reduced (31). In contrast to these results, however, Lu et al. (62) showed that macrophages deficient in p38 activity (by disruption of MKK3, a specific MAPK upstream of p38) expressed drastically reduced levels of IL-12 in response to LPS, whereas TNF-α was normal. Similar results were observed using SB203580 or SB202190 (30, 62). Furthermore, Allen et al. (63) found that the production of IL-1 and IL-6 in p38−/− mouse embryonic fibroblasts was markedly compromised. Thus, these results agree with our conclusion that p38 is crucial for GPI-induced expression by macrophages of IL-6 and IL-12 but not of TNF-α and nitric oxide.

The requirement of the JNK-signaling pathway for the GPI-induced expression of proinflammatory mediators was not studied before. In this study we show that the JNK pathway is critical for P. falciparum GPI-induced proinflammatory responses. As shown in Fig. 9A, pretreatment of macrophages with SP600125 resulted in a complete blockade of IL-12 and a marked decrease in the level of TNF-α and nitric oxide production; interestingly, the production of IL-6 was only moderately inhibited. These results are in general agreement with the notion that the JNK pathway is important for the production of proinflammatory cytokines in response to LPS and double-stranded RNA (51–53, 64). Our results also demonstrate that of the two JNK isoforms, JNK1 and JNK2, present in macrophages are for the most part functionally redundant. This conclusion is evident from the observed normal level of expression of TNF-α, IL-6, and nitric oxide in macrophages from JNK1−/− or JNK2−/− mice (see Fig. 8A). However, for the expression of IL-12, JNK2 but not JNK1 is critical, and this requirement is at the transcriptional level (see Fig. 8B). These
observations agree with the previous findings that the expression of IFN-α, IFN-β, IL-6, and IL-12 was substantially decreased in JNK2−/− fibroblasts in response to double-stranded RNA and that JNK1 does not compensate for JNK2 (64). Although JNK1 is unable to compensate for JNK2 in fibroblasts, as shown here for P. falciparum GPI-induced macrophages, JNK1 efficiently compensated for JNK2 for the expression of IL-6. This difference is likely due to the different cell types and/or the ligands used.

In conclusion, the results of this study show that of the downstream signaling pathways that are activated in macrophages stimulated with P. falciparum GPIs, JNK and NF-κB pathways are crucial for the production of key proinflammatory mediators, TNF-α, IL-6, IL-12, and nitric oxide. The ERK- and p38-signaling pathways and JNK1/JNK2 and NF-κB signaling mechanism are likely to be of therapeutic benefit for severe malaria.

Acknowledgment—We thank Dr. Adeline M. Hajjar, University of Washington, for critical reading of the manuscript.

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Induction of Proinflammatory Responses in Macrophages by the Glycosylphosphatidylinositolsg of *Plasmodium falciparum*: THE REQUIREMENT OF EXTRACELLULAR SIGNAL-REGULATED KINASE, p38, c-Jun N-TERMINAL KINASE AND NF-κB PATHWAYS FOR THE EXPRESSION OF PROINFLAMMATORY CYTOKINES AND NITRIC OXIDE

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*J. Biol. Chem.* 2005, 280:8617-8627.
doi: 10.1074/jbc.M413539200 originally published online December 15, 2004

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