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Induction of Proinflammatory Responses in Macrophages by the Glycosylphosphatidylinositols (GPIs) of *Plasmodium falciparum*: CELL SIGNALING RECEPTORS, GPI STRUCTURAL REQUIREMENT, AND REGULATION OF GPI ACTIVITY*

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SUMMARY

The proinflammatory cytokines produced by the innate immune system in response to pathogenic infection protect the host by controlling microbial growth. However, excessive proinflammatory responses could disrupt the host's vital physiological functions, causing severe pathological conditions. In the case of *Plasmodium falciparum*, the protozoan parasite that causes fatal malaria in man, the glycosylphosphatidylinositol (GPI) anchors are thought to be the major factors that contribute to malaria pathogenesis through their ability to induce proinflammatory responses. In this study, we identified the receptors for *P. falciparum* GPI-induced cell signaling that leads to proinflammatory responses, and studied the GPI structure-activity relationship. The data show that GPI-signaling is mediated mainly through recognition by TLR2 and to a lesser extent by TLR4. The activity of *sn*-2 *lyso* GPIs is comparable to that of the intact GPIs, whereas the activity of Man_3-GPIs is about 80% that of the intact GPIs. The GPIs with three (intact GPIs and Man_3-GPIs) and two fatty acids (*sn*-2 *lyso* GPIs) appear to differ considerably in the requirement of the auxiliary receptor, TLR1 or TLR6, for recognition by TLR2. The former are preferentially recognized by TLR2/TLR1, whereas the latter are favored by TLR2/TLR6. However, the signaling pathways initiated by all three GPI types are similar, involving the MyD88-dependent activation of ERK, JNK and p38, and NF-κB signaling pathways. The signaling molecules of these pathways differentially contribute to the production of various cytokines and nitric oxide (Zhu, J., *et al*. (2004) *J. Biol. Chem*., accompanying manuscript). Our data also show that GPIs are degraded by

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the macrophage surface phospholipases, predominantly into inactive species, indicating that the host can regulate GPI activity, at least in part, by this mechanism. These results imply that macrophage surface phospholipases play important roles in the GPI-induced innate immune response and malaria pathogenesis.

Malaria caused by the parasitic protozoa of the genus *Plasmodium* is a major public health problem in Africa, South America, and South Asia (1–4). The disease afflicts about 500 million people and causes ~3 million deaths annually and ranks first among the various infectious diseases, causing global morbidity and mortality. More than 100 *Plasmodium* species exist in nature that can infect various vertebrate animals (1). However, only four species are infectious to man and, of these, *P. falciparum* is responsible for >95% of deaths (1–4).

Plasmodium infection causes a wide range of clinical manifestations, including cerebral malaria, acute respiratory distress, pulmonary edema, renal failure, and severe anemia (1, 2). The acquisition of effective protective immunity to malaria requires repetitive infections over a period of a few years (5, 6). Therefore, during the initial periods of infection, innate immunity plays a crucial role in controlling parasite growth (7–9). Otherwise, the parasite is expected to grow exponentially, leading to the rapid destruction of all the circulatory erythrocytes and death.

Proinflammatory cytokines, such as TNF-α, IFN-γ, IL-12, IL-1 and IL-6, and nitric oxide produced during malaria infection, are critical for controlling parasite growth (2, 8–15). However, excessive production of proinflammatory cytokines could lead to severe pathological conditions (16–19). Therefore, understanding of the mechanism of innate immune responses to *P. falciparum* factors could offer therapeutic targets for malaria. Although the various *P. falciparum* components that are potentially involved in the production of inflammatory responses by the innate immune system remain to be elucidated, the glycosylphosphatidylinositol (GPI)[1] anchor glycolipids of the parasite have been proposed as the prominent parasite components responsible for malaria pathogenesis (20, 21). Accumulated evidence indicates that GPIs of parasitic protozoa contribute prominently to the pathology of parasitic diseases (22). The deleterious effects of the parasite GPIs have been attributed to their ability to induce TNF-α and other proinflammatory cytokines and nitric oxide, which contribute to disease pathology (20–22). It has been shown that the level of TNF-α is markedly elevated in patients with fatal cerebral malaria, and anti-TNF-α antibodies prevent the development of cerebral malaria (23).

GPIs consist of a conserved glycan structure, ethanolamine-phosphate-6Manα1–2Manα1–6Manα1–4GlcN, α (1–6)-linked to the PI (24–27). GPIs are ubiquitous in eukaryotes, where they are primarily involved in anchoring certain cell surface proteins to plasma membranes. Compared to animal cells, GPIs are abundantly expressed in parasites, such as

[1]The abbreviations used are: GPIs, glycosylphosphatidylinositols; TLR, toll like receptor; mTLR, murine TLR; hTLR, human TLR; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor κB; MyD88, myeloid differentiation factor; LPS, lipopolysaccharide; MALP-2, macrophage activating lipoprotein 2-kDa; Pam_3CSK_4, *N*-palmitoyl-*S*-dipalmitoyl-cysteinyl-SKKKK (tripalmytoyl-CSK_4 peptide); PE, phycoerythrin; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; BSA, bovine serum albumin; FBS, fetal bovine serum.

Trypanosome, *Leishmania*, and *Plasmodium*. Therefore, in these parasites, a relatively large pool of GPIs is no longer anchored to proteins and appears to be the direct target of the host innate immune system for inducing proinflammatory responses (22). GPIs from different species differ in the type of acyl/alkyl substituents, the presence of additional sugar moieties on the third and/or first mannose, extra ethanolamine phosphate groups on the carbohydrate moiety, and acyl substituent on C2 of inositol, leading to a broad structural diversity and variation in potency of their biological activity (28, 29).

GPIs of *P. falciparum* have been shown to activate protein tyrosine kinase and protein kinase C, which together regulate the activation of NF-κB/c-*Rel* transcription factor with the downstream expression of proinflammatory responses (30–32). However, the receptors that mediate *P. falciparum* GPI signaling, and how the exogenously induced signal is transmitted into the cells has remained unclear. Research during the past few years has shown that the innate immune responses to various microbial pathogens are mediated by a family of signal-transducing proteins called TLRs (33–35). To date, thirteen TLRs, TLR1 through TLR13, have been identified in mammalian cells, most recognizing specific pathogen-associated molecular patterns (36). For example, TLR4 recognizes enterobacterial LPS, TLR3 double stranded RNA, TLR5 flagellin, and TLR9 CpG-containing motifs of bacterial DNA. TLR2, however, exhibits broad ligand recognition, and the identified ligands include peptidoglycan, lipoteichoic acid, lipoproteins, lipoarabinomannan, zymosan, certain glycolipids, non-enterobacterial LPS, and porins (33–35). Efficient signal-transduction by TLR2 appears to require its heterodimerization with either TLR1 (for triacylated lipoproteins) or TLR6 (for diacylated lipoproteins) (33, 37, 38). The transmission of responses by TLRs involves, in most cases, the recruitment of a shared adaptor protein, MyD88, which interacts with TLRs through Toll-IL-1 receptor (TIR) domains, initiating signaling cascades, engaging various MAPKs and NF-κB (39).

Thus far, there have been no direct studies demonstrating a TLR-mediated immune responses to *P. falciparum* ligands, although MyD88-deficient mice have been reported to be protected from *P. berghei*-induced IL-12-mediated liver injury, suggesting involvement of TLR-mediated immune responses to malarial factors (40). Recently, GPI moieties of the mucin-type glycoproteins of *Trypanosome cruzi* trypamastigotes have been shown to induce proinflammatory responses through TLR2-mediated signaling (41–43). However, *P. falciparum* GPIs are structurally distinct from those of *T. cruzi*; the former contain a diacylated glycerol moiety and fatty acid acylation at C-2 of inositol, whereas the latter have *sn*1-alkyl-*sn*2-acylglycerol and lack inositol-acylation (29, 44, 45). Further, the requirement of TLR1 or TLR6 for heterodimerization with TLR2 for signaling by GPIs is not known. In this study, we show that proinflammatory responses to *P. falciparum* GPIs by macrophages are mediated mainly through TLR2 and to a lesser but significant extent also through TLR4. We also show, for the first time, that the parasite GPIs are degraded by macrophage surface phospholipase A_2 and phospholipase D, and that intact and *sn*-2 *lyso* GPIs are differentially recognized by TLR2/TLR1 and TLR2/TLR6.

EXPERIMENTAL PROCEDURES

Materials

All cell culture reagents, including RPMI 1640, DMEM, FBS, penicillin/streptomycin, and gentamycin were from Invitrogen. In some experiments FBS from Hyclone (Logan, UT) was used. Bee venom phospholipase A_2 (1724 units/mg), jack bean α-mannosidase (20 units/mg), LPS (from *Salmonella minnesota* Re595 strain, catalog number L 9764) were from Sigma. MALP-2 and Pam_3CSK_4 (standard TLR2 ligands) were from EMC Microcollections (Tübingen, Germany). IL-1β was from Pierce (Rockford, IL). Human blood and serum from healthy donors were from the hospital of the Hershey Medical Center. *Limulus* amebocyte lysate assay kit was from Associates of Cape Cod (Falmouth, MA). Mycoplasma Detection Kit (version 2.0) was from American Type Culture Collection (ATCC). Colloidal gold (40–60 nm particle size) was from ImmunoReagent products (Lakeside, AZ). Chemiluminescence substrate kit was from KPL (Gaithersburg, MD). Dual-Luciferase Reporter Assay kit and Passive Lysis Buffer were from Promega. The Golgi Stop (monensin), 2.4 G2 purified antibody against Fc receptor, Cytofix/Cytoperm, and PE-conjugated rat anti-mouse CD11b (clone M1/70) were from PharMingen (San Diego, CA), and FITC-conjugated rat anti-mouse TNF-α (clone MP6-XT22) was from Caltag (Burlingame, CA). Anti-human TLR2 and anti-human TLR4 mouse monoclonal antibodies (both IgG2), clones TL2.1 and HTA125, respectively were from eBioscience, Inc. (San Diego, CA). A mouse monoclonal antibody (IgG2) specific to an ovarian glycoprotein tumor antigen (OVB-3, Ref. 46) was a gift from Dr. Ira Pastan, NCI, NIH, Bethesda. Phospho-specific anti-mouse ERK1/ERK2, p38 and JNK mouse monoclonal antibodies, anti-mouse β-tubulin peptide mouse monoclonal antibody, rabbit polyclonal antibodies against mouse IκBα, ERK1/ERK2, p38, and JNK peptides, and HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgGs were from Cell Signaling Technology, Inc., (Beverly, MA). Mouse monoclonal antibody specific HA (HA.11 from clone 16B12) was from Covance, Richmond, CA. Nitrocellulose membranes were from Bio-Rad. Endotoxin-free reagents, water, and buffers were used for all the experimental procedures.

Cell lines

Raw264.7 and J774A.1 mouse macrophage cell lines, and L929 murine fibroblast cells were from ATCC. HEK-293 human embryonic kidney epithelial cells were originally from Dr. David Schowalter, when he was at the University of Washington. HEK-293, Raw264.7, and J774A.1 cells were cultured in DMEM, 10% FBS, 1% penicillin/streptomycin. L929 cells were cultured as suspension in DMEM, 5% FBS, 1% glutamine, and 1% penicillin/streptomycin in roller flasks at 37 °C. For the preparation of conditioned medium from L929 cells, the cells were cultured in the above medium for 5 days, the supernatant collected, centrifuged at 2,500 rpm for 20 min. The clear solution was used as a source of macrophage colony stimulatory factor.

Mice

The $TLR2^{-/-}$, $TLR4^{-/-}$, and $MyD88^{-/-}$ mice were produced at the Research Institute for Microbial Diseases, Osaka University, Japan. The knockout mice were backcrossed six to eight generations to C57BL/6J mice. The C57BL/6J wild type mice were from The Jackson

Laboratories. TLR2 and TLR4 double knockout (TLR2/4$^{-/-}$) mice were produced by crossing TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. All animals were maintained in a pathogen-free environment.

Parasites

Intraerythrocytic *P. falciparum* (FCR-3 strain) was cultured in RPMI 1640 medium using *O*-type blood and 10% *O*-positive human serum, 50 μg/ml of gentamycin at 3–4% hematocrit (45, 47). Parasite cultures were regularly synchronized with 5% sorbitol (48) and tested for mycoplasma by PCR using ATCC kit (49).

Isolation of GPIs from P. falciparum

Isolation of parasites and purification of GPIs were performed as described previously (45). Briefly, mycoplasma-free parasite cultures with 20–30% parasitemia were harvested at the schizont stage, treated with 0.025% saponin in Trager buffer, and passed through a 26-gauge needle to lyse the erythrocytes. The suspension was centrifuged, and washed several times, the erythrocyte debris were removed by centrifugation on 5% BSA cushion. The parasites were washed three times with PBS, pH 7.4, lyophilized, and stored at –80 °C. The parasites (from 10 ml wet pellet) were extracted 3 times with chloroform, methanol (2:1, v/v) to remove non-glycosylated lipids. GPIs were extracted with chloroform, methanol, water (10:10:3, v/v/v), dried, and partitioned between water and water-saturated 1-butanol. The organic layer was washed 4 times with water and dried. The residue was extracted with 80% aqueous 1-propanol, dried and the GPIs were further purified by HPLC using C_4 Supelcosil LC-304 column (4.6 X 250 cm, Supelco) as described previously (45). In some experiments GPIs were further purified by HPTLC using chloroform, methanol, water (10:10:2.4, v/v/v). The HPLC and HPTLC-purified GPIs were found to be free from endotoxin as tested by the *Limulus* amebocyte lysate assay (50). The purity of the GPIs was confirmed by mass spectrometry and carbohydrate compositional analysis. The GPIs were quantified by determining the amount of GlcN and Man after acid hydrolysis and HPLC as described previously (45, and see also below).

Preparation of Man_3-GPIs

The Man_4-GPIs (10 μg) were treated with jack bean α-mannosidase (40 units/ml) in 100 μl of 100 mM NaOAc, 2 mM Zn^{2+}, pH 5.0, containing 0.1% sodium taurodeoxycholate at 37 °C for 24 h (51). The solutions were heated in a boiling water bath for 5 min, cooled and extracted with water-saturated 1-butanol, washed 4 times with water, and dried. The Man_3-GPIs were purified by HPLC as above and the purity of the samples was ascertained by mass spectrometry and by the carbohydrate compositional analysis (see below).

Preparation of sn-2 lyso GPIs

The GPIs (10 μg) were treated with bee venom phospholipase A_2 (1700 unit/ml) in 100 μl of 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5, at 37 °C for 24 h (44). The solution was heated in a boiling water bath for 5 min, cooled, and was extracted with water-saturated 1-butanol, washed 4 times with water, and dried. The *sn*-2 *lyso* GPIs were purified by HPLC as above

and the purity was determined by mass spectrometry and by the carbohydrate compositional analysis (45).

Mass spectrometry

The solutions of GPIs in chloroform, methanol, water (8:4:3, v/v/v) were with mixed equal volumes of a saturated solution of 6-aza-2-thiothymine in 50% ethanol, deposited on the sample plate, and air-dried. Mass spectra (an average of 50 shots) were acquired in a linear negative ion mode on a DE-PRO MALDI-TOF mass spectrometer (PE-Biosystems, Framingham, MA), equipped with a nitrogen laser (337 nm) at 20-kV accelerating voltage.

Carbohydrate compositional analysis

The GPIs (~1 μg each) were hydrolyzed with 400 μl of either 2.5 M trifluoroacetic acid at 100 °C for 5 h or 3 M HCl at 100 °C for 4 h. The hydolysates were dried in a Speed-Vac, dissolved in water, and analyzed on a CarboPac PA10 high-pH anion-exchange column (2 X 250 mm) using a Dionex BioLC GS50 HPLC system coupled to a ID50 electrochemical detector (52). The elution was performed with 16 mM sodium hydroxide, and the response factors for the monosaccharides were determined using standard sugar solutions.

Coating of GPIs to gold particles

The colloidal gold suspension (1.5 ml) was centrifuged in an Eppendorf centrifuge at 8,000 rpm and washed 3 times with endotoxin-free water. To the particle pellet thus obtained (~8 μl) was suspended in 120 μl of water and mixed with GPIs (5 μg) in 30 μl 80% 1-propanol, and dried in a Speed-Vac. By adding a known amount of [^{3}H]GlcN-labeled GPIs during coating, we found that the GPIs are quantitatively adsorbed by the gold beads. The GPI-coated gold particle were suspended in 13 ml of DMEM, 10% FBS, and used for stimulation of macrophages in 24-well or 96-well microtiter plates.

Preparation of Mouse Bone Marrow Macrophages and Human Peripheral Blood Monocytes, and Stimulation with GPIs

Mouse macrophages were obtained by the differentiation of primary bone marrow cells with 30% of L929 cell conditioned medium as described (53). The macrophages were plated into 96-well plates (2.5 X 10^4 cells/well), and after 24 h, the culture supernatants were removed and incubated with the indicated amounts of GPIs in DMEM medium containing 10% FBS and 1% penicillin/streptomycin. For human monocytes, the whole blood was diluted with 3 volumes of RPMI 1640 medium, and 4 volumes of cell suspension was layered on 1 volume of isolymph, and centrifuged at 1300 rpm at room temperature for 30 min. The buffy layer at the interface was recovered, washed two times with RPMI 1640 medium, and then suspended in RPMI 1640 medium containing 10% FBS (Invitrogen) and 1% penicillin/streptomycin (2.5 X 10^6 cells/ml). The cell suspension (200 μl) was aliquoted into 96-well microtiter plates and incubated overnight at 37 °C under CO_2 atmosphere. The unbound cells were washed off, and the bound cells (~2 X 10^4 cells/well) were stimulated with GPIs. After 48 h, the culture supernatants were collected and TNF-α measured by ELISA. For the analysis of cell signaling molecules, mouse macrophages (5 X 10^5 cells/well) in 24-well plastic plates were maintained overnight in DMEM containing 0.5% FBS and 1% penicillin/

streptomycin, and then stimulated with GPIs. In the case of human monocytes, 2.5 X 10^5 cells in 1640 medium containing 0.5% FBS and 1% penicillin/streptomycin were stimulated with GPIs. In both cases, at the indicated time points, the culture supernatants were removed, and cells washed once with ice-cold PBS, pH 7.4 and lysed with RIPA buffer (52). The cell lysates were used for the analysis of MAPK phosphorylation or IκBα degradation by Western blotting (see below).

Estimation of TNF-α by ELISA

The TNF-α in the culture supernatants of macrophages stimulated with GPIs were determined by Sandwich ELISA with HRP-conjugated streptavidin and TMB 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 with SpectraMax Plus384 plate reader (Molecular Devices). The cytokine concentrations were calculated with reference to the standard curves

Stimulation of bone marrow primary cells, staining of intracellular TNF-α, and FACS analysis

Bone marrow cells were flushed from femurs and tibias, the red blood cells lysed by suspending cells in ammonium chloride hypotonic buffer (0.15 M NH_4Cl, 1 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4). The bone marrow cells were plated in 96-well microtiter plates (1 X 10^6 cells/well) in DMEM containing 10% FBS (Hyclone). The cells were stimulated with 200 nM GPIs at 37 °C for 5 h in the presence of 0.15 μl/well of the Golgi Stop (monensin) to accumulate TNF-α intracellularly. The cells were spun in the plate, and blocked with 1:100 diluted 2.4 G2 antibody against Fc receptors in FACS buffer (1% BSA in PBS plus 0.09% sodium azide), then stained with 1:100 diluted PE-conjugated rat anti-mouse CD11b antibody in FACS buffer. The cells were then fixed and permeabilized with 100 μl/well Cytofix/Cytoperm, and stained with 1:100 diluted FITC-conjugated rat anti-mouse TNF-α. After two washes, the cells were analyzed on a Beckton Dickinson FACScan using CellQuest Pro software for data analysis.

HEK-293 cell transfection and stimulation with GPIs

HEK-293 cells were cultured in DMEM medium, 10% FBS (Hyclone), penicillin/streptomycin, and the confluent monolayer harvested by treatment with trypsin/EDTA. The cells were plated in 96-well microtiter plates (4 X 10^4 cells/well) one day before transfection. The following amounts of DNA per well were transfected by the calcium phosphate precipitation as described (54): 10 ng of *E-selectin*-firefly luciferase, 0.2 ng of β-*actin-Renilla* luciferase, 2.5 ng of h*TLR2*, when alone or 1.25 ng when cotransfected with 1.25 ng of h*TLR1* or 12.5 ng of h*TLR6* (adjusted for equivalent expression of HA-tagged TLR proteins as judged by Western blot analysis using anti-HA antibody), and 2.5 ng of h*CD14*. Total DNA per well was normalized to 50 ng by adding empty vector. Three-hours after transfection, cells were washed, incubated with DMEM, 10% FBS, and 20–24 h later stimulated with 200 nM GPIs or the indicated amounts of control ligands in DMEM containing 10% FBS. After a 5-h incubation, the cells were washed once in PBS and lysed in Passive Lysis Buffer (Promega, Madison, WI). The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's

instructions. The relative light-units (RLU) were quantitated using the formula: (Light output by the *E-selectin* firefly luciferase)/(Light output by the β-*actin-Renilla* luciferase control).

Western blot analysis of signaling molecules

The cell lysates obtained after stimulation of mouse macrophages and human monocytes with GPIs, as above, were electrophoresed on 10% SDS-polyacrylamide gels in the presence of 2-mercaptoethanol. The protein bands on gels were transferred onto nitrocellulose membranes, blocked with 5% fat-free dry milk, and incubated with 1:500- or 1:1000-diluted anti-peptide rabbit polyclonal or phospho-specific monoclonal antibodies to detect various MAPKs and their phosphorylated forms, respectively. The membranes were also probed with 1:500-diluted anti-IκBα peptide rabbit polyclonal antibody or 1:1000 anti-β-tubulin mouse monoclonal antibody. After washing with Tris-buffered saline, the membranes were incubated with 1:2000-diluted HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG. The bound secondary antibodies were detected with chemiluminescence substrate.

GPI degradation by macrophages

The parasite GPIs (0.1 μg plus 200,000 cpm of [^{3}H]GlcN-labeled GPIs obtained by metabolic labeling) in ethanol, water, 1-proponol (78:20:2, v/v/v) were added to murine macrophages (1 X 10^6 cells) or human monocytes (1 X 10^5 cells) in glass Petri dishes in serum-free medium (Invitrogen) that supports the growth of mammalian cells and incubated under conditions at 37 °C for 48 h. The culture supernatants were collected and extracted with 1-butanol. The organic layer was washed four times with water. The aqueous layer was chromatographed on a Bio-Gel P-4 column (1 X 90 cm) in 0.1 M pyridine, 0.1 M acetic acid, pH 5.0. The radioactivity-containing fractions were pooled and lyophilized. Samples recovered, in each case, from the organic and aqueous layers were analyzed by TLC using chloroform, methanol, water (10:10:2.4, v/v/v).

To determine whether GPIs were degraded by phospholipases on the cell surface or by those released into medium, 200 ng/ml of unlabeled GPIs were incubated with macrophages (2 X 10^6 cells) in 1 ml of serum-free medium at 37 °C for 48 h. The supernatant was collected centrifuged to remove cell debris, and incubated with 400,000 cpm of [^{3}H]GlcN-labeled GPIs at 37 °C for 48 h. The solution was extracted with 1-butanol, and the organic and water layers analyzed as above.

Preparation of glycan and the lipid moieties of GPIs

The GPI portion lacking the lipid moiety was isolated after incubation of the parasite Man_4-GPIs (5 μg plus 400,000 cpm [^{3}H]GlcN-labeled GPIs in 25 μl 78% ethanol, 20% water, 2% 1-propanol) with murine bone marrow-derived macrophages (1 X 10^7 cells) in 5 ml of serum-free medium (see above) at 37 °C for 48 h. The culture supernatant was extracted with 1-butanol, and the samples that remained in the aqueous phase were dried, chromatographed on Bio-Gel P-4 (1 X 90 cm) and further purified by ion-exchange chromatography using AG50w-X12 (H^+) resin.

The PI portion was isolated by treatment of GPIs with HNO_2 followed by extraction with 1-butanol as described previously (45).

Statistical analysis

The data from TLR2/TLR1 and TLR2/TLR6 gain of function assay (see Fig. 5) are presented as mean + SD (n = 3). Raw data between two groups were compared by student's 't' test for paired samples using t test. Data from more than two groups were compared by analysis of variance (ANOVA) using Prism 3 program form GraphPad Software, Inc. (San Diego CA). Probabilities (*p* value) of 0.05 or less were considered statistically significant.

RESULTS

Purification of P. falciparum GPIs

The isolation of GPIs from cultured *P. falciparum* and purification by HPLC using a C4 reversed-phase column and by HPTLC was as previously described (45). Endotoxin-free reagents were used throughout the GPI isolation and purification procedures, and GPI preparations that tested negative for endotoxin activity by *Limulus* amebocyte lysate assay were used for all the studies. The identity and purity of the GPIs were determined by mass spectrometry. As reported previously (45), the spectra showed the presence of multiple GPI species that differ from each other by 28 mass units due to the variation in chain length of acyl fatty acid substitutents. Man_3-GPIs and *sn*-2 lyso GPIs were prepared, purified and characterized by mass spectrometry as reported previously (45).

TNF-α Production By Macrophages Stimulated with P. falciparum GPIs and GPI Structure-Activity Relationship. P. falciparum

GPIs are generally readily soluble in aqueous 80% 1-proponal or water-saturated 1-butanol, but not so easily in 80% ethanol or DMSO. Since 1-propanol and 1-butanol are toxic to cells, they are not suitable solvents for the preparation of GPI stock solutions for activity studies. We found that the *P. falciparum* GPIs are easily soluble in a mixture of ethanol, water, 1-propanol (78:20:2, v/v/v); up to 2 μl of this solvent in 200 μl of culture medium is not toxic to macrophages. However, our initial studies showed that, when macrophages were stimulated with GPI stock solutions prepared in the above solvent, there were considerable variations in the level of TNF-α produced in replicate assays. This was mainly due to the quick evaporation of solvent while dissolving a few microgram quantities of GPIs in a few microliters volume. Evaporation of the stock solution during handling confounded the problem, resulting in inconsistent transfer of aliquots to macrophage culture. We circumvented this problem by coating the GPIs onto colloidal gold particles and stimulating the cells with the coated particles. To determine the validity of this procedure, we initially studied TNF-α production in macrophages stimulated with aliquots of a stock GPI solution in the above organic solvent (prepared using a large batch of GPIs to minimize solvent evaporation) and compared the results with those obtained by treating macrophages with similar amounts of GPIs coated on gold particles. In three different cells types, namely, human peripheral blood monocytes, bone marrow-derived mouse macrophages, and cultured mouse macrophage cell lines (data not shown), the levels of TNF-α produced by stimulation with GPIs dissolved in the above organic solvent were comparable to those produced by

treatment GPI-coated gold particles (Fig. 1). The gold particles alone were unable to activate cells, and the viability of cells was not affected with the amounts of gold particles used in the experiments. We found that GPIs coated onto gold particles offer several advantages over the procedure using GPIs solution in organic solvents: (i) results from replicate and different experimental sets are consistently reproducible, (ii) cell toxicity due to organic solvents is avoided, and (iii) as shown below, the GPIs with fewer sugar residues, such as Man_3-GPIs containing three fatty acid substituents that are insoluble or have very low solubility in 80% ethanol or DMSO can be easily handled to obtain reproducible results.

We have previously reported that the macrophage signaling activity of *P. falciparum* GPIs is markedly affected by the removal of the terminal fourth mannose residue by jack bean α-mannosidase (55). However, in the present study, when assessed by coating onto gold particles, Man_3-GPIs, in three different macrophages types used, showed approximately ~80% of the TNF-α-producing activity of Man_4-GPIs (Fig. 1). Further, we found that the Man_3-GPIs, compared to Man_4-GPIs, is sparingly soluble in 80% aqueous ethanol, the solvent that was used for the preparation of GPI stock solution in the previous study, presumably because of fewer sugar residues in Man_3-GPIs (55). We also found that Man_3-GPIs are soluble in ethanol, water, 1-propanol (78:20:2), and the results obtained by using Man_3-GPIs in this solvent were nearly comparable to those obtained by stimulation of macrophages with Man_3-GPI-coated gold particles (Fig. 1). Therefore, the previously reported inactivity of Man_3-GPIs was most likely due to the failure of transferring Man_3-GPIs from the stock vial to the culture medium because of their low solubility in 80% ethanol. In the case of *sn*-2 *lyso* GPIs, as reported previously (54), the TNF-α-producing activity is comparable to that of the intact Man_4-GPIs (Fig. 1).

TLR2 is the Major Receptor for Macrophage Signaling by P. falciparum GPIs

Previous studies have shown that the GPIs purified from *P. falciparum* can activate macrophages and endothelial cells to produce proinflammatory cytokines and nitric oxide (20, 30–32). Previous studies have also shown that stimulation of cells with the parasite GPIs results in the activation of protein tyrosine kinase and protein kinase C, which together activate NF-κB and the downstream expression of cytokines and nitric oxide (20, 30–32). However, the receptor involved in the recognition of *P. falciparum* GPIs and the signaling pathways that were activated have not been studied. Recent studies have shown that cell signaling by bacterial LPS and *T. cruzi* GPIs is mediated by TLR4 and TLR2, respectively (33–35, 41–43). To determine whether cell signaling by *P. falciparum* GPIs is also mediated through recognition by TLRs and to study the GPI structure-activity relationship with respect to recognition by TLRs, we stimulated bone marrow-derived macrophages from wild-type, $TLR2^{-/-}$ or $TLR4^{-/-}$, and $MyD88^{-/-}$ mice with the parasite Man_4-GPIs, and its derivatives, namely, Man_3-GPIs, and *sn*-2 *lyso* GPIs. In each case, cell activation was assessed by measuring TNF-α secreted into the culture medium. With Man_4-GPIs and Man_3-GPIs, in each case, the level of TNF-α produced by macrophages from $TLR4^{-/-}$ cells was approximately 72% of that produced by the corresponding GPI type in macrophages from wild type animals (Fig. 2*A* and 2*B*). Macrophages from $TLR2^{-/-}$ mice produced ~20% of TNF-α compared with that by macrophages from wild type mice. The macrophages from $MyD88^{-/-}$ mice, however, produced only ~8% of TNF-α compared with that produced by

the cells from wild type animals. These results taken together indicate that *P. falciparum* GPIs are recognized by both TLR2 and TLR4 and these receptors together account for ~92% of the GPI induced activity by macrophages, and that recognition of GPIs by TLR2 is far more efficient than that by TLR4. The results also indicate that parasite GPIs can induce a low level (~8%) of activity either through the TLR4-dependent, MyD88-independent pathway or through recognition by receptors other than TLR2 and TLR4. In the case of *sn*-2 *lyso* GPIs, however, the level of TNF-α produced by TLR4$^{-/-}$, TLR2$^{-/-}$ and MyD88$^{-/-}$ macrophages was ~80%, ~12% and ~8%, respectively, compared with that by wild type macrophages (Fig. 2*C*). These results reveal that the *sn*-2 *lyso* GPIs, containing two fatty acid substituents, also exhibit a low level of TLR4-dependent cell signaling activity.

The GPIs purified from *T. cruzi* mucin-like glycoproteins have previously been shown to activate cells exclusively through TLR2 (41–43). To determine whether the observed TLR4-mediated activity of *P. falciparum* GPIs was due to LPS-contamination, the experiments were repeated with GPIs obtained from three different parasite batches and each purified by HPLC followed by HPTLC (45). These GPI preparations were found to be pure by mass spectral analysis and the mass spectra were similar to those reported previously (45). With all three GPI preparations, the level of TNF-α production by macrophages from TLR4$^{-/-}$ and TLR2$^{-/-}$ macrophages was, respectively, about 72% and 20% that produced by macrophages from wild type mice (not shown). Thus, our study establishes that *P. falciparum* GPIs, which contain three fatty acid substituents, are recognized by both TLR2 and TLR4, although the contribution of the latter is markedly lower compared to that of the former. Further, the results of Man_4-GPIs and *sn*-2 *lyso*-GPIs, with the former exhibiting greater TLR4-mediated activity than the latter, suggest that the fatty acid content is an important factor in recognition of GPIs by TLR2 and TLR4.

To avoid potential confounding effects of *in vitro* growth in macrophages obtained from the differentiation of bone marrow cells with L929 cell conditioned medium, we stimulated primary mouse bone marrow cells directly with *P. falciparum* GPIs. Activation was assessed by FACS analysis after trapping TNF-α intracellularly. Gold particles alone did not induce TNF-α production in the primary cells; 0.1% TNF-α positive cells was background (Fig. 3). In contrast, when stimulated with the Man4-GPIs, about 4%, 3.6% and 0.3% of cells from the wild type B6, TLR4$^{-/-}$, and TLR2$^{-/-}$ mice produced TNF-α. Cells from either TLR2/4$^{-/-}$ or MyD88$^{-/-}$ mice showed only background levels of 0.1% positive cells in this assay (Fig. 3). The lack of responding cells from TLR2/4$^{-/-}$ or MyD88$^{-/-}$ mice in this experiment, despite the detection of low levels of TNF-α in culture supernatant (see Fig. 2), is likely due to the lower sensitivity of the FACS assay, with only a small population of bone marrow cells responding to activation by GPIs. The cells demonstrated predicted responses to control ligands with 6.4% of cells from TLR2$^{-/-}$ mice responding to LPS and no response (background level of 0.1%) with Pam_3CSK_4, a synthetic lipopeptide with exclusive TLR2 activity. Similarly, as predicted, 5% of TLR4$^{-/-}$ cells were responsive to Pam_3CSK_4, and no response (background level of ~0.1% cells) to LPS. These results confirm that *P. falciparum* GPI-induced cell signaling is mediated mainly through TLR2 in primary mouse bone-marrow cells.

To ascertain whether human TLR2 and TLR4 can recognize *P. falciparum* GPIs in a manner similar to those of mouse, we assessed TNF-α production in human peripheral monocytes treated with anti-human TLR2- or TLR4-specific mouse monoclonal antibodies prior to stimulation with the parasite GPIs. Pretreatment with anti-TLR2 monoclonal antibody resulted in an antibody dose-dependent inhibition of GPI-induced TNF-α production by human monocytes; the level of inhibition was as much as 74–86% in cells treated with 5–40 μg/ml of anti-TLR2 antibody compared to cells treated with a non-relevant mouse monoclonal antibody (Fig. 4). On the other hand, pretreatment of monocytes with 5–40 μg/ml of anti-TLR4 antibody caused 33–49% inhibition of TNF-α production (Fig. 4). With both antibodies the inhibition was statistically highly significant ($p = <0.001$). A non-relevant mouse monoclonal antibody (an IgG2 produced against an ovarian tumor glycoprotein antigen, Ref. 46) showed <5–8 % inhibition of TNF-α production and was not statistically significant at any concentration tested ($p > 0.05$). These results demonstrate that the GPI-induced cell signaling in human monocytes is mediated, as in the case of mouse macrophages, mainly through TLR2 and to some extent through TLR4.

The Man_4-GPIs/Man_3-GPIs and sn-2 Lyso-GPIs are Differentially Recognized by TLR2/TLR1 and TLR2/TLR6 Heterodimers

For TLR2 to efficiently recognize its ligand, dimerization with either TLR1 or TLR6 is essential (33). To examine the role of TLR1 or TLR6 in the recognition of *P. falciparum* GPIs by TLR2, we used a gain-of-function assay in HEK-293 cells employing a NF-κB-dependent ELAM-promoter luciferase reporter assay (54). The cells were transiently transfected with human TLR2 alone, TLR2 plus TLR1 or TLR2 plus TLR6. Upon stimulation with intact Man_4-GPIs, cells transfected with TLR2 alone could induce the ELAM reporter response 6-fold higher compared to the respective cells stimulated with gold particles alone (Fig. 5). In contrast, co-transfection of TLR2 with either TLR1 or TLR6 resulted in 56- and 11-fold induction, respectively (Fig. 5). Upon stimulation with Man_4-GPIs, cells transfected with TLR2 plus TLR1 were consistently significantly more efficient in inducing the reporter response than cells transfected with TLR2 plus TLR6. The IL-1 receptor is constitutively expressed in HEK-293 cells and, as expected, IL-1 mediated equivalent activation of the reporter construct in all transfectants (data not shown).

To determine whether TLR2/TLR1 and TLR2/TLR6 heterodimers discriminate between GPIs with different structural features, the tranfectants were stimulated with Man_3-GPIs and *sn*-2 *lyso* GPIs. Similar to intact Man_4-GPIs, Man_3-GPIs induced a significantly higher response with TLR2/TLR1 compared to TLR2/TLR6 (Fig. 5). Strikingly, the *sn*-2 *lyso* GPIs were significantly better recognized by TLR2/TLR6 relative to TLR2/TLR1 (Fig. 5). These results suggest that TLR1 and TLR6 can discriminate GPIs with three or two fatty acid substituents. This GPI structural discrimination by TLR1 and TLR6 resembles the specific recognition of triacylated peptides and diacylated peptides by TLR2/TLR1 and TLR2/TLR6, respectively, in macrophages from TLR6 and TLR 1 knockout mice (37, 38). Interestingly, in our transfection studies using human TLR genes, the discrimination between Man_4-GPIs and Man_4-*sn*-2 *lyso*-GPIs is far more efficient than that between Pan_4CSK_4 and MALP-2, the standard ligands for TLR2/TLR1 and TLR2/TLR6.

Analysis of the Downstream Signaling Pathway Activation in Macrophages Stimulated with P. falciparum GPIs

The TLR/MyD88-mediated cell signaling triggers the activation of various MAPKs and NF-κB, leading to the production of inflammatory mediators (39). To determine the signaling pathways that are specifically activated by *P. falciparum* GPIs, we analyzed the phosphorylation of the downstream MAPKs and the degradation of IκBα in macrophages from wild type and various knockout mice by Western blotting. In macrophages from wild type and TLR4$^{-/-}$ mice, stimulation with parasite GPIs resulted in the efficient activation of ERK1/2, p38, JNK, and NF-κB (Fig. 6*A* and 6*B*). Cells from TLR2$^{-/-}$ mice also showed activation of these signaling molecules, but the efficiency of activation was significantly lower compared with either the wild type or TLR4$^{-/-}$ mice (Fig. 6*C*). Macrophages from MyD88$^{-/-}$ mice, showed a consistently very low level of activation of ERK1/ERK2, p38 and JNK, but no degradation of IκBα (Fig. 6*D*), suggesting that this could be due to either TLR4-mediated, MyD88-independent signaling or the activation by receptors other than TLR2 and TLR4. Together, these results, in addition to identifying the downstream signaling pathways involved in the GPI-mediated inflammatory responses, confirm that the signaling is mainly through TLR2 and to a lower but significant extent also through TLR4.

Further, we analyzed signaling molecules in human monocytes stimulated with *P. falciparum* Man_4-GPIs (Fig. 6*E*). The pattern of signaling pathways that were activated closely resembled those of the signaling pathways activated in macrophages from wild type mice (compare Fig. 6*E* with Fig. 6*A*).

P. falciparum GPIs are Degraded By Macrophage Surface Phospholipases

GPIs have been reported to undergo spontaneous insertion into plasma membranes (56, 57), a property that has been implicated in cell activation (32). However, it was previously reported that, upon incubation with mouse macrophages, about 98% of *P. falciparum* GPIs remained in the medium and only about 2% was associated with cells (55). The fate of GPIs remaining in culture medium after prolonged incubation with macrophages was not previously studied. In this study, we analyzed the GPIs recovered from culture medium after incubation with mouse macrophages or human monocytes for 48 h. Since it is known that animal sera contain various phospholipases, including GPI-specific phospholipase D and phospholipase A_2 (58–61), the cells were cultured in serum-free medium. TLC analysis of the GPIs recovered from macrophage culture medium showed that, in murine macrophages >98% of the GPIs were degraded, and about ~80% of the degradation products lacked the diacylglycerol phosphate moiety and were recovered in the aqueous phase upon extraction with 1-butanol (Table I). This product remained at or near the origin on the TLC plates (Fig. 7), and mass spectral analysis gave a $(M–H)^-$ ion at m/z 1349.5 (calculated mass for the glycan portion with C16:0 on C-2 of inositol is 1350 kDa). Therefore, the product is derived by the action of GPI-specific phospholipase D on the parasite GPIs. The remainder (~20%) of the degradation product had an Rf value similar to that of *sn*-2 *lyso* GPIs, and it was inferred to be the product of phospholipase A_2. In the case of human peripheral monocytes, ~69% of the GPIs were degraded and ~31% remained intact; however, the cell numbers used were 10 times less than murine macrophages. Of the 69% degraded, the majority lacked the diacylglycerol phosphate moiety (present in water phase when extracted with 1-butanol) and

remained at or near the origin on TLC plates and ~10% converted to products with R_f values similar to those of *sn*-2 *lyso* Man_4-GPIs (Table I and Fig. 7). Furthermore, when incubated with culture medium containing 10% fetal bovine serum, the parasite GPIs were degraded into products that were similar to those formed when incubated with macrophages in serum-free medium (not shown). However, when GPIs were incubated with supernatants from mouse macrophages or human monocytes, almost all of the GPIs remained intact. Therefore, the observed GPI degradation is mainly due to phospholipases that are present on the cell surface. This prediction is consistent with the reported presence of phospholipase D and phospholipase A_2 on the cell surface (62, 63). The GPI glycan portion that lacks the lipid moiety and the PI moiety either alone or together were unable to activate macrophages to induce TNF-α, suggesting that GPIs with covalently linked glycan core and lipid moiety are recognized by TLRs. Thus, these results suggest that macrophages can regulate the activity of GPIs, at least in part, by degrading them into inactive products by the action of the cell surface phospholipase D. *In vivo*, the regulation of GPI activity is likely to be much more effective because of the degradation of the GPIs by serum phospholipases.

DISCUSSION

Studies from different laboratories during the past decade demonstrated that the GPIs of various protozoan parasites, particularly, those of *P. falciparum*, *Trypanosome* and *Leishmania*, can activate host macrophages, triggering the production of proinflammatory cytokines and nitric oxide. These responses, which are similar to those elicited by bacterial LPS, can contribute to disease pathogenesis and thus, GPIs have been recognized as the dominant factors that contribute to various parasitic diseases. However, the mechanism of cell signaling by GPIs of various parasitic protozoa with regard to the receptors that transduce signals and the signaling pathways that mediate cytokine responses remained sparsely studied. In this respect, to date, the only GPIs that have been studied are those of *T. cruzi* (41–43). Cell signaling in response to these GPIs has been shown to be through the exclusive recognition by TLR2, resulting in the activation of the MyD88-dependent MAPK and NF-κB pathways and downstream expression of cyokines and nitric oxide. However, the GPIs of various organisms differ markedly in their overall structural complexity and exhibit varied potency with regard to their ability to activate macrophages (28, 29). Therefore, one would expect that GPIs from different parasitic organisms are differentially recognized by TLRs, similar to the case of LPS from various gram-negative bacteria (33). While LPS from some bacteria are recognized exclusively by TLR4, those from other bacteria display TLR2 activity (38). Further, although previous studies have shown that *P. falciparum* GPI-induced proinflammatory responses involves the activation of protein kinase C and protein tyrosine kinase in mouse macrophages, leading to NF-κB nuclear translocation with downstream cytokine expression (30–32), the receptors that recognize GPIs and cell-signaling pathways involved have not been studied. In this study, we conclusively show that cell signaling by *P. falciparum* GPIs is mediated mainly through the recognition by TLR2 and to a lesser extent by TLR4 as well.

The evidence that supports the above conclusion include: (i) Direct measurement of the level of TNF-α produced by macrophages from wild type, $TLR2^{-/-}$, $TLR4^{-/-}$ and $MyD88^{-/-}$ macrophages in response to stimulation with highly purified GPIs of *P. falciparum*. As

shown in Fig. 2, upon stimulation with *P. falciparum* GPIs, TLR4$^{-/-}$ macrophages consistently produced approximately 72% of TNF-α compared with that produced by macrophages from the wild type mice. Conversely, the amount of TNF-α elicited by TLR2$^{-/-}$ macrophages was about 20% of that secreted by the wild type macrophages. (ii) When primary bone marrow cells were directly stimulated with parasite GPIs, the percentage of cells responding in TLR4$^{-/-}$ cells was 90% of the wild-type levels whereas the percentage in TLR2$^{-/-}$ cells was 10% of wild type. In contrast to these results, negligible number of the primary bone marrow cells from TLR2/TLR4 double knockout and MyD88$^{-/-}$ mice produced TNF-α in response to the parasite GPIs. (iii) Blocking of TLR2 and TLR4 by incubation of human monocytes with anti-TLR2 and anti-TLR4 antibodies caused, respectively, up to 86% and 49% decrease in the GPI-induced production of TNF-α. Taken together, these results clearly demonstrate that *P. falciparum* GPI-induced cell signaling in macrophages is mediated mainly through TLR2 and to a lesser degree through TLR4. However, it is not clear from the results of this study whether the low level of MyD88-independent activation of macrophages (see Fig. 2) is mediated by TLR4 or by receptors other than TLR2 and TLR4.

The *P. falciparum* GPI-induced signaling in macrophages leads to the MyD88-dependent activation of MAPKs and NF-κB pathways. This is evident from the observed phosphorylation of the downstream MAPKs, namely, ERK1/ERK2, p38, and JNK, and degradation of IκBα (Fig. 6). The efficient activation of various MAPKs and NF-κB pathways in the wild type macrophages, and negligible level of activation of these signaling molecules in MyD88$^{-/-}$ cells further demonstrates that cell signaling by *P. falciparum* GPIs is predominantly through TLRs. Furthermore, activation of the various downstream MAPKs and NF-κB by TLR4$^{-/-}$ and TLR2$^{-/-}$ macrophages and lack of activation of TLR2/TLR4 double knockout macrophages as measured by FACS (see Fig. 3) are consistent with our conclusion that cell signaling by the parasite GPIs is mediated by both TLR2 and TLR4, although the former is much more efficient than the latter.

Our observation that the *P. falciparum* GPIs, in addition to being efficiently recognized by TLR2, are also recognized, albeit to lower degree, by TLR4, contrast the property of *T. cruzi* mucin GPIs (40–42). The latter GPIs have been shown to activate macrophages exclusively through TLR2, and the GPIs are unable to activate TLR2$^{-/-}$ macrophages. Furthermore, as reported in the accompanying paper, while *P. falciparum* GPIs can significantly activate cells at 10–40 nanomolar concentrations, the *T. cruzi* GPIs are at least 10 times more potent in their ability to activate macrophages and the level of inflammatory cytokines and nitric oxide produced (28, 29). This difference in the activity of *P. falciparum* and *T. cruzi* GPIs is likely related to their characteristic structural features. The GPIs of *P. falciparum* have a diacylglycerol moiety, whereas *T. cruzi* GPIs contain a glycerol residue with *sn*-1-alkyl and *sn*-2 acyl substituents. Further, while the GPIs of *P. falciparum* are inositol acylated and lack substituents on the tetramannose core, the GPIs of *T. cruzi* lack inositol acylation and their tetramannose core is substituted with 0–4 galactosyl residues (64).

Despite the above noted structure-activity differences, however, the GPIs of *P. falciparum* and *T. cruzi* GPIs resemble each other closely with regard to the activation of the ERK1/ERK2, p38, JNK, and NF-κB pathways. This is because in both cases the GPI-induced

signal transduces to cells mainly through a MyD88-dependent upstream signaling pathway, which is common to both TLR2 and TLR4 ligands. In the case of TLR4 ligands, a MyD88-independent pathway is also involved in the activation of cells (65). However, since TLR4-mediated activation in response to *P. falciparum* GPIs is relatively low, the contribution by this pathway to the overall level of cell activation by the parasite GPIs appears to be negligible or very low, as indicated by the low level of proinflammatory cytokine production in MyD88$^{-/-}$ macrophages.

The results of this study show that TLR2/TLR1 and TLR2/TLR6 heterodimers can differentially recognize GPIs containing three and two fatty acid substituents, in a manner similar to the discrimination of triacylated bacterial lipoproteins and diacylated mycoplasmal lipoproteins. It has been previously shown that triacylated lipoproteins are recognized by TLR2/TLR1, whereas diacylated lipoproteins are recognized by TLR2/TLR6 (37, 38). Thus, our data show that *P. falciparum* GPIs, containing overall three fatty acid substituents, are preferentially recognized by TLR2/TLR1 and activate mouse macrophages and human monocytes to induce cytokine production. Similarly, the *sn*-2 *lyso* GPIs, prepared from the parasite GPIs by removing the fatty acid at the C-2 position of glycerol with phospholipase A_2, are the preferred ligands for human TLR2/TLR6 compared to human TLR2/TLR1. It is interesting that the GPIs containing three and two fatty acid substituents, present structural patterns similar to the triacylated and diacylated lipoproteins, respectively, for differential recognition by TLR1 and TLR6, even though GPIs and lipoproteins are totally different classes of compounds. The implication of these observations is that the fatty acid disposition in the ligand, regardless of whether it is a GPI or a lipoprotein, is the critical structural pattern that is favored by TLR2/TLR1 and TLR2/TLR6, and this preferential recognition might represent the first level of pathogen discrimination by macrophages. The glycan and protein structures of GPIs and lipoproteins might then represent higher levels of pathogen-specific pattern recognition for ligand-specific innate responses. Furthermore, our results are also consistent with previous observations that an intact conjugate of the lipid and glycan portions is essential for the GPI activity, and the lipid or glycan moiety alone is not active (28, 55, 64).

The results of this study demonstrate that macrophage/monocyte cell surface and/or secreted phospolipases regulate the activity of GPIs. Our results show that the *P. falciparum* GPIs exposed to macrophages are degraded by the cell surface or secreted phospholipases, including phospholipase A_2 and phospholipase D. Upon exposure to macrophages in culture medium with or without serum, the majority of the GPIs were converted to products lacking PI formed by the action of GPI-specific phospholipase D. Since the products of phospholipase D are unable to elicit the production of proinflammatory responses, our data indicate that macrophages regulate the activity of GPIs, at least in part, by degrading them into inactive products. The regulation of GPI activity is expected to be even more efficient in *in vivo* situations because the mammalian sera contain significant amounts of phospholipase A_2 and phospholipase D activity and therefore, GPIs are more efficiently degraded. This could be one of the mechanisms by which the host immune system modulates its activity that is induced in response to GPIs. Thus, our observations have uncovered the mechanism of innate immune response to *P. falciparum* GPIs and the ability of host macrophages to

modulate the GPI activity during parasite-host interactions. These findings have important implications in parasite survival, host resistance and the process of pathogenesis.

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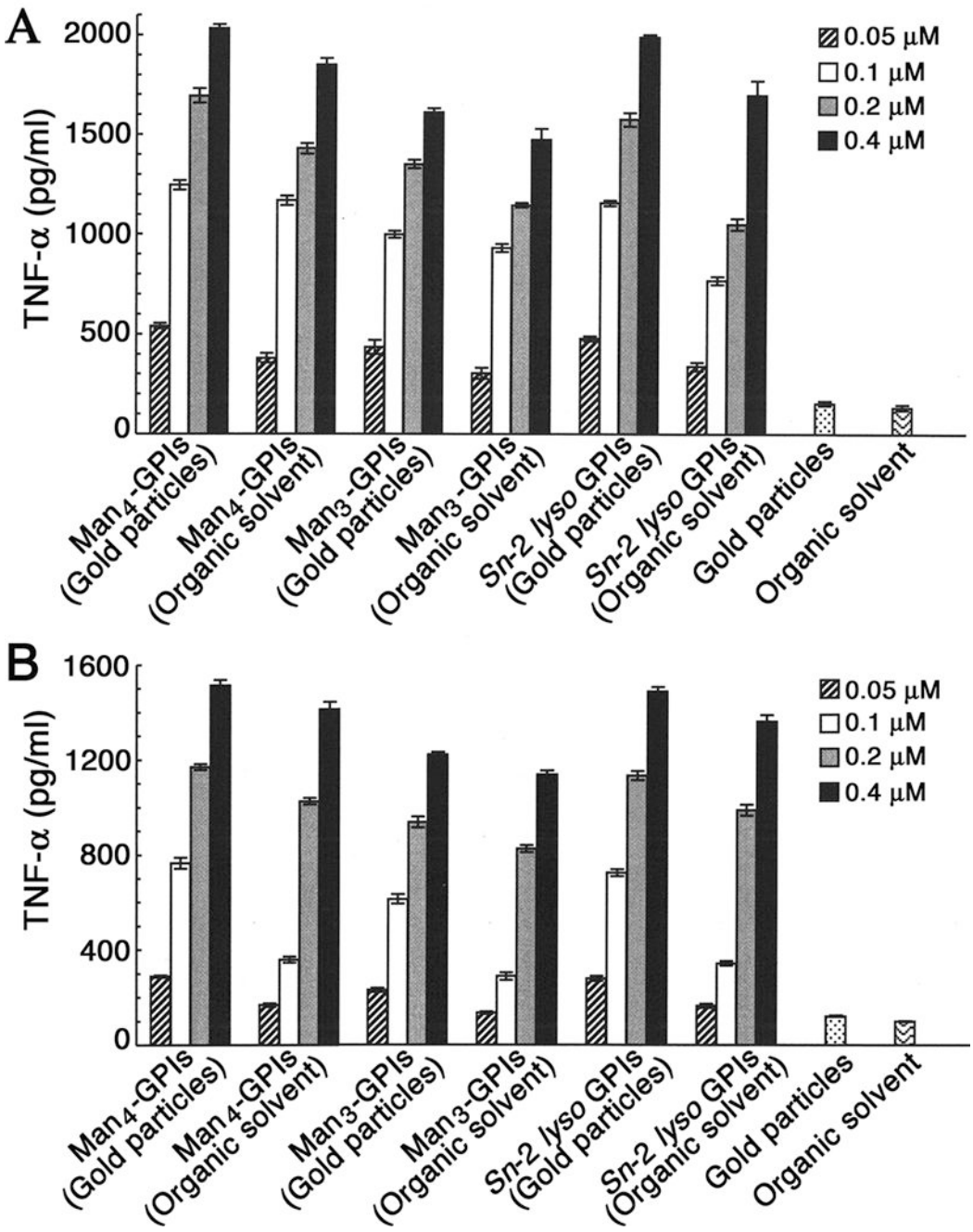

Fig. 1. Measurement of TNF-α produced by human monocytes and mouse macrophages stimulated with *P. falciparum* GPIs and their derivatives

Human monocytes (*panel A*) and mouse bone marrow-derived macrophages (*panel B*) were plated into 96-well microtiter plates (in each case ~2.5 X 10^4 cells/well). After overnight culturing, the cells were stimulated with the indicated amounts of Man_4-GPIs, Man_3-GPIs, or *sn*-2 *lyso* GPIs (either coated on gold particles or solubilized in ethanol, water, 1-propanol (78:20:2, v/v/v). After 48 h, the culture supernatants were collected, and assayed for TNF-α by ELISA. The experiments were performed three times, each in duplicate, and shown are the results of representative experiments. Error bars indicate the range of duplicate wells.

The TNF-α production in mouse macrophage cell lines (J774A.1 and RAW264.7 cells) stimulated with the above GPIs was also studied. In both cell type, the results (not shown) were similar to those obtained for mouse bone marrow derived macrophages.

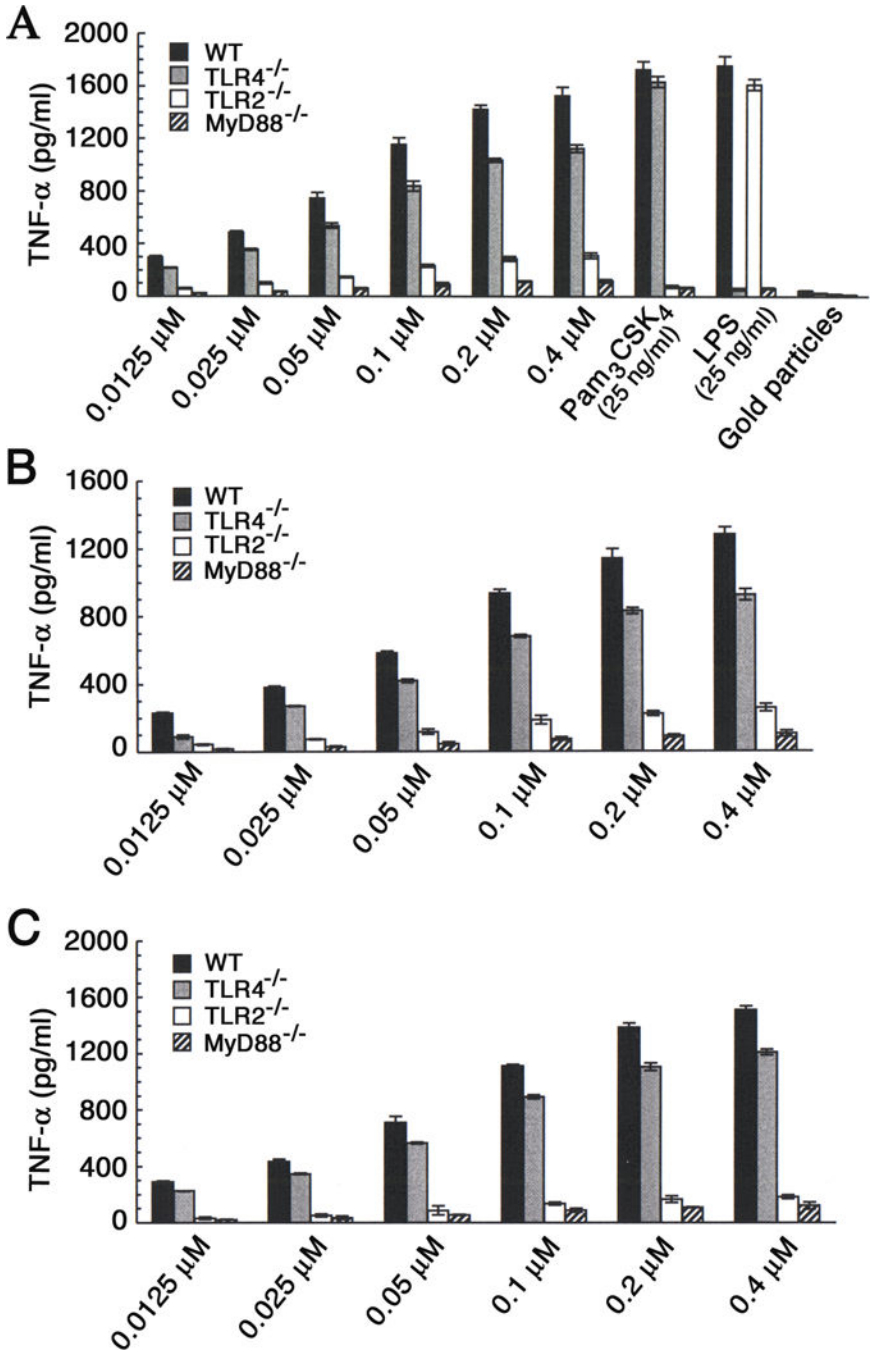

Fig. 2. Assessment of TNF-α produced by macrophages from the wild type, TLR4$^{-/-}$, TLR2$^{-/-}$ and MyD88$^{-/-}$ mice treated with *P. falciparum* GPIs and their derivatives

Bone marrow-derived macrophages from the wild type, TLR4$^{-/-}$, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice were plated into 96-well microtiter plates (2.5 X 10^4 cells/well). The cells were cultured overnight, and then stimulated with the indicated amounts of Man_4-GPIs, Man_3-GPI, and *sn*-2 *lyso*-GPIs (all coated on gold particles). After 48 h, the cultured supernatants were collected, and TNF-α measured by ELISA. The experiments were performed three times, each in duplicate and shown are the results of representative experiments. Error bars indicate the range of duplicate wells. *Panel A*, Stimulation with intact parasite GPIs (Man_4-

GPIs); *panel B*, Stimulation with Man_3-GPIs obtained by treatment of Man_4-GPIs with jack bean α-mannosidase; *panel C*, Stimulation with *sn*-2 *lyso*-GPIs obtained by treatment of Man_4-GPIs with phospholipase A_2.

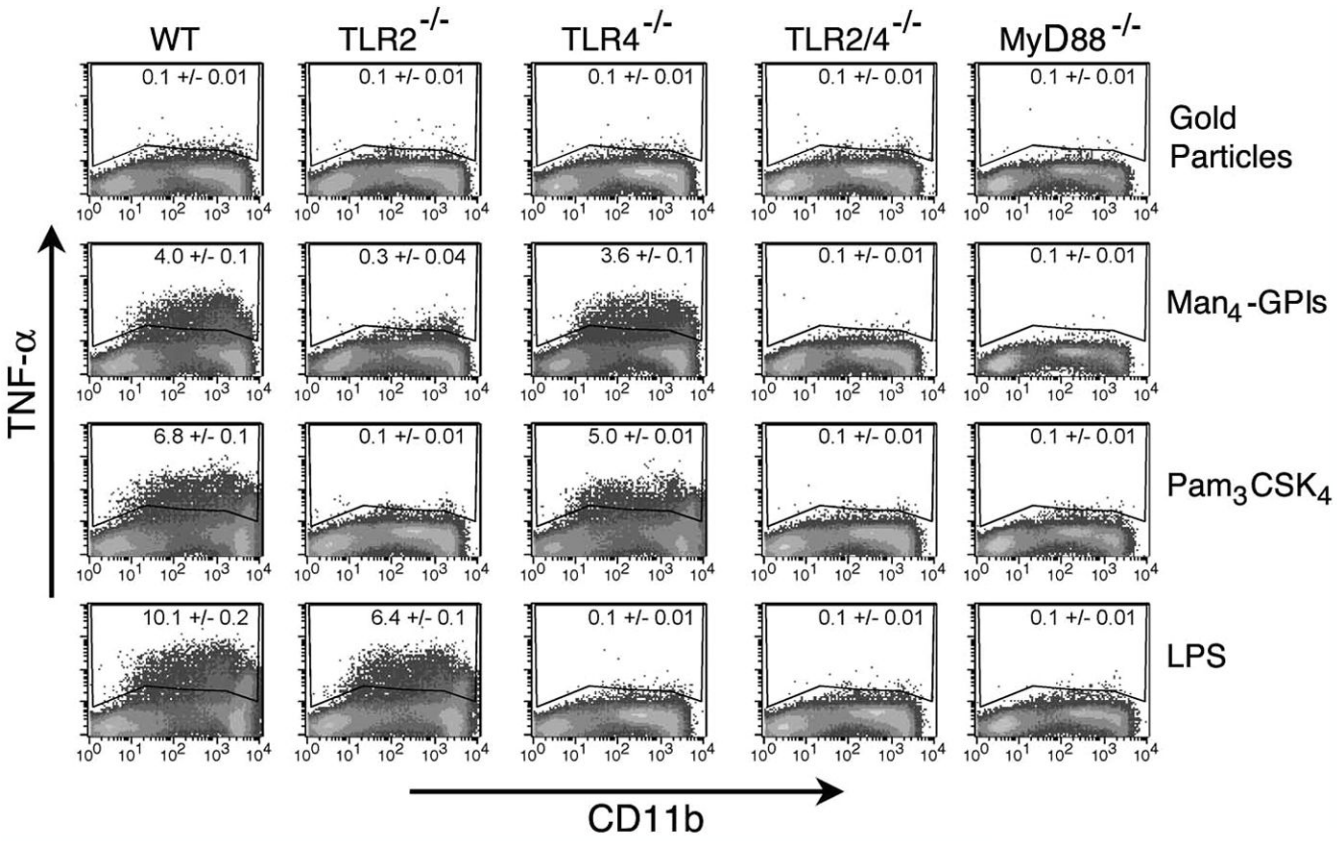

Fig. 3. Analysis of TNF-α production in primary bone marrow cells from wild type, TLR4$^{-/-}$, TLR2$^{-/-}$, TLR2/4$^{-/-}$, and MyD88$^{-/-}$ mice stimulated with *P. falciparum* GPIs

Primary bone marrow cells from the wild type, TLR4$^{-/-}$, TLR2$^{-/-}$, TLR2/4$^{-/-}$, MyD88$^{-/-}$, mice were plated into 96-well microtiter plates (1 X 10^6 cells/well) in DMEM medium, 10% FBS, and then stimulated with 200 nM of Man_4GPIs (coated on gold particles) in the presence of monensin. Similar number of cells, treated with 10 ng/ml LPS (TLR4 ligand), and 1 μg/ml Pam_3CSK_4 (TLR2 ligand) both in the presence of monensin, were used as controls. The cells were blocked with anti-Fc receptor antibody, stained with PE-conjugated rat anti-CD11b antibody, fixed, permeabilized, and then stained with FITC-conjugated rat anti-mouse TNF-α. The cells were stained, and analyzed by FACS. The experiments were repeated four times, and data from a representative set is shown.

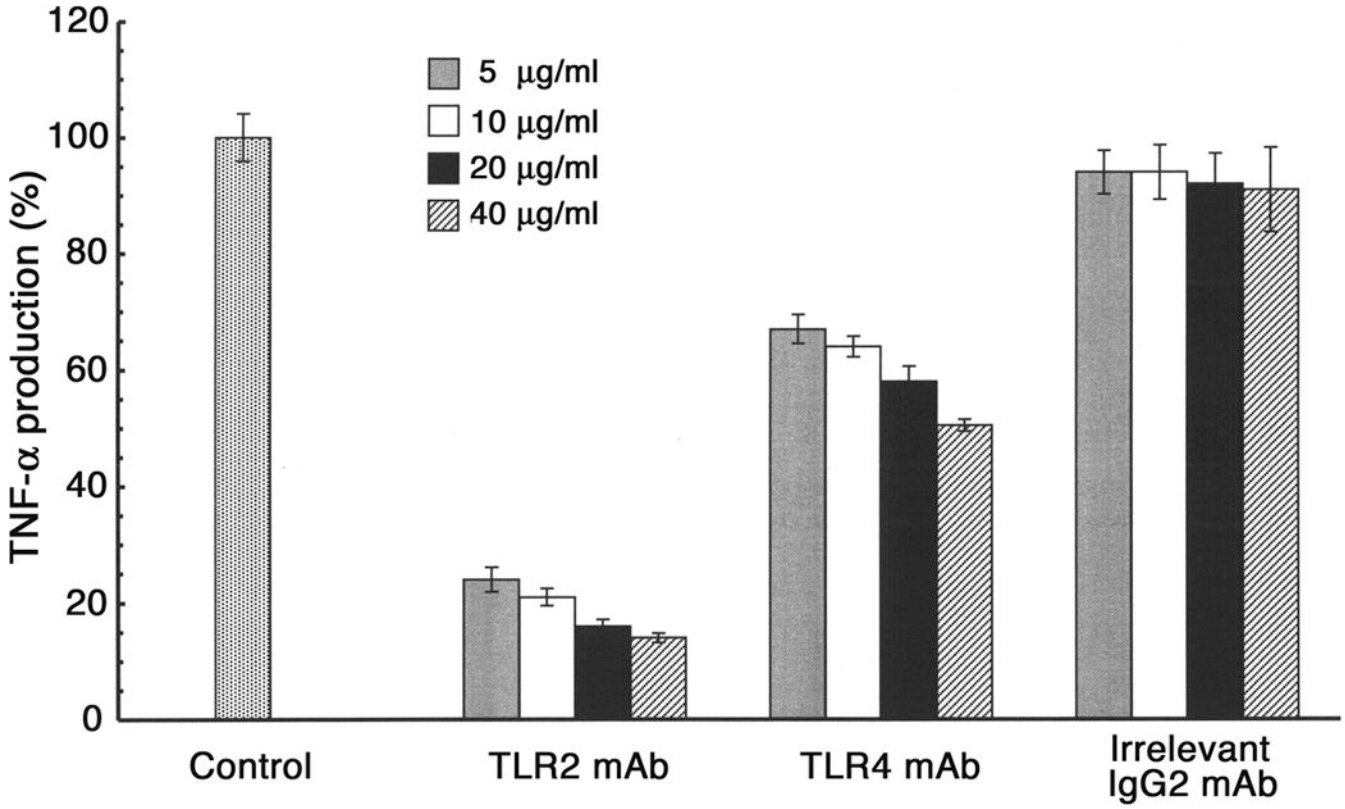

Fig. 4. Inhibition of *P. falciparum* GPI-induced TNF-α production in human monocytes by anti-TLR2 and anti-TLR4 monoclonal antibodies

Human monocytes, plated in 96-well microtiter plates (2.5 X 10^4 cells/well), were incubated with the indicated amounts of anti-TLR2 IgG2 or anti-TLR4 IgG2 monoclonal antibodies in 1640 RPMI medium containing 10% FBS and 1% penicillin/streptomycin. Cells similarly treated with a mouse IgG2 monoclonal antibody against a human tumor-specific glycoprotein (Ref. 46) was used as controls. After 1 h at 37 °C, the cultures were stimulated with 100 nM Man_4-GPIs for 24 h. The culture supernatants were recovered, and the level of TNF-α assayed by ELISA. The experiments were performed two times, each in duplicate, and the average values are plotted. At all concentrations tested, inhibition by anti-TLR2 and anti-TLR4 antibodies was highly significant (p <0.001), whereas inhibition by control antibody was not significant (p >0.05) by ANOVA.

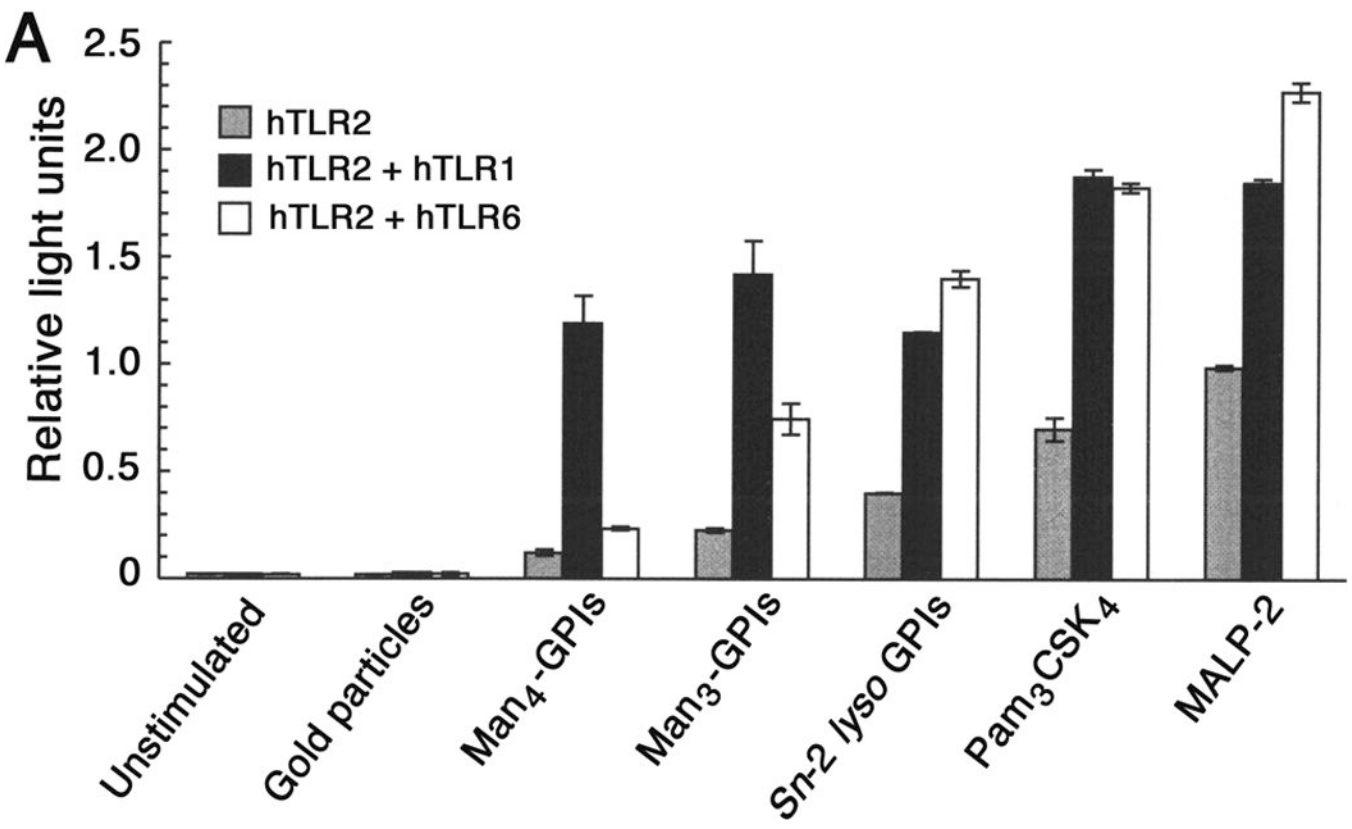

Fig. 5. TLR2/TLR1 and TLR2/TLR6 are differentially recognized by GPIs with three and two fatty acids

HEK cells were plated in 96-well microtiter plates (4 X 10^4 cells) and transfected with appropriate human TLRs, CD14 genes plus E-selectin-firefly luciferase, β-actin-*Renilla* luciferase genes as outlined in "Experimental Procedure." The transfected cells were stimulated with 400 nM GPIs. Transfected cells were also stimulated with Pam3CSK4 and MALP-2 as controls. After 5 h, the cells were washed, lysed and luciferase activity measured. Experiments were repeated three times, each in triplicates, and in each case similar results were obtained. Data from a representative experiment is shown. Error bars indicate the standard deviation of triplicate wells. The results were analyzed by Student's t test.

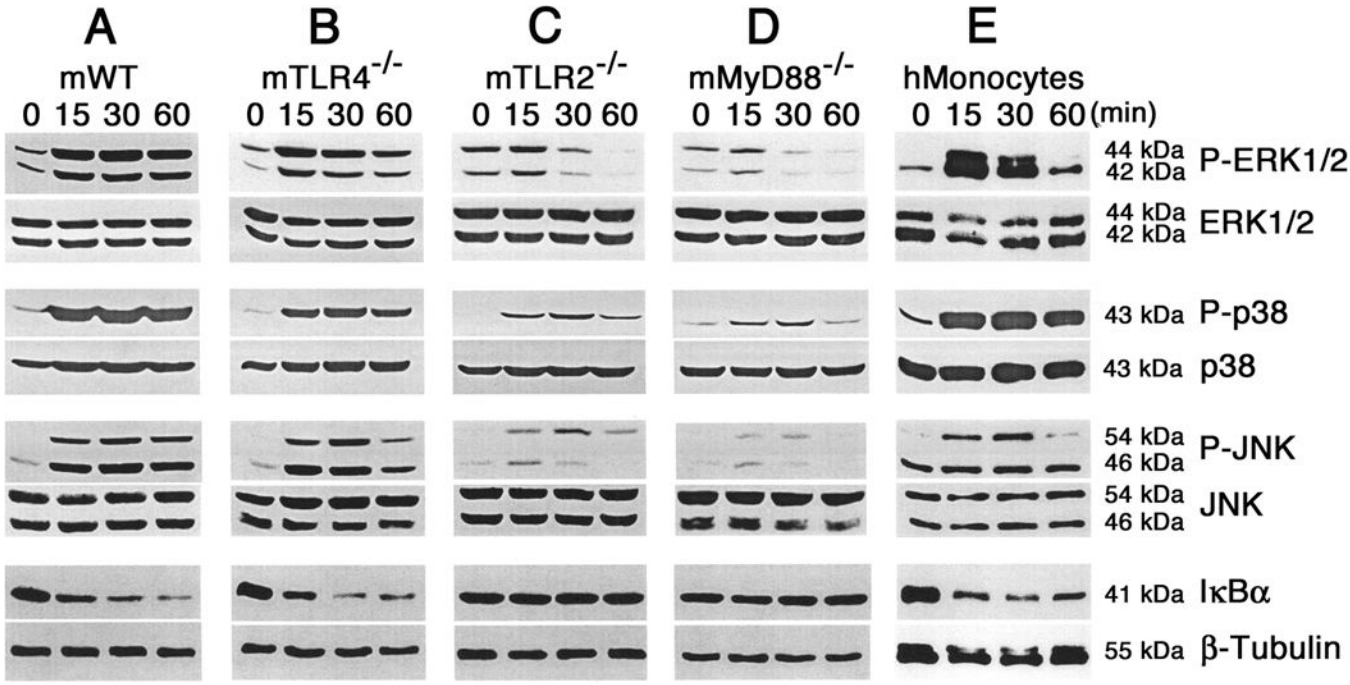

Fig. 6. Analysis of downstream MAPKs phosphorylation and IκBα degradation in murine bone marrow macrophages and human monocytes treated with *P. falciparum* GPIs

Bone marrow-derived macrophages (5 X 10^5 cells/well) from wild type, $TLR2^{-/-}$,$TLR4^{-/-}$, and $MyD88^{-/-}$ mice and human monocytes (2.5 X 10^5 cells/well) were plated in 24-well microtiter plates. The cells were cultured overnight as described under "Experimental Procedures", and then stimulated with 200 nM Man_4-GPIs (coated on gold particles). At the indicated time points, the cultured supernatants were removed, and cells were washed and lysed. The cell lysates were electrophoresed on 10% SDS-polyacrylamide gels under reducing conditions. The protein bands on gels were transferred onto nitrocellulose membranes, blocked with 5% fat-free dry milk, and probed with antipeptide and phospho-specific antibodies against ERK1/ERK2, p38, and JNK. The membranes were also probed with antibodies against IkBα and β-tubulin. The bound antibodies were detected with HRP-conjugated goat anti-mouse IgGs and chemiluminescence substrate. P, phospho-specific antibodies.

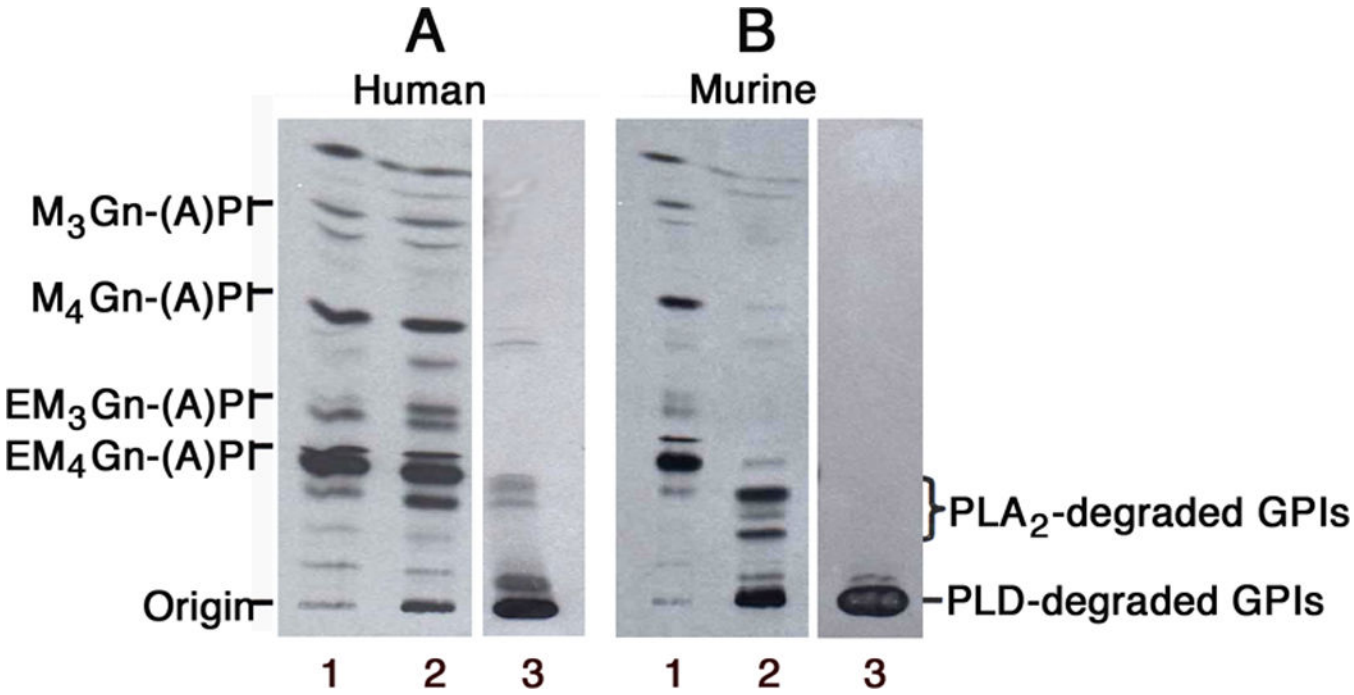

Fig. 7. *P. falciparum* GPIs exposed to human monocytes or murine macrophages are degraded by cell surface phospholipases

P. falciparum GPIs (0.1 μg + 200,000 cpm of [^{3}H]GlcN-labeled GPIs) were incubated with human monocytes or murine macrophages in serum-free medium. After 48 h, the culture supernatants were extracted with 1-butanol, and the organic layers were washed with water, dried. The aqueous layers were chromatographed on Bio-Gel P-4, and the radioactivity-containing fractions were pooled and dried. The residues from both the organic and aqueous layers were analyzed by HPTLC using $CHCl_3$, MeOH, H_2O (10:10:2.4, v/v/v), and GPI bands were visualized by fluorography. *Panel A*, GPIs incubated with human monocytes; *panel B*, GPIs incubated with murine bone-marrow derived macrophages. In both cases: *Lane 1*, untreated *P. falciparum* GPIs and GPI biosynthetic intermediates; *lane 2*, GPI recovered in organic layer*; lane 3*, GPIs recovered in aqueous layers. The positions corresponding to the mobility of *sn*-2 *lyso* GPIs (the product of phospholipase A_2 on Man_4- and Man_3-GPIs) and GPI glycan core lacking the PI moiety (the product of phospholipase D) are indicated in the right margin. The identities of the GPIs are indicated in the left margin. *EtP-M_4Gn-(A)PI*, inositol acylated EtN-*P*-Man_4-GlcN-PI; *EtP-M_3Gn-(A)PI*, inositol acylated EtN-*P*-Man_3-GlcN-PI; *M_4Gn-(A)PI*, inositol acylated Man_4-GlcN-PI; *EtP-M_3Gn-(A)PI*, inositol acylated Man_3-GlcN-PI. The experiments were also performed with J774A.1 and RAW264.7 murine macrophage cell lines, and the results (not shown) were similar to those of murine bone marrow-derived macrophages.

Table I

Yield of GPIs and their degradation products recovered from the culture supernatants of human monocytes and mouse macrophages incubated with P. falciparum GPIs[a]

Human monocytes and mouse bone marrow-derived macrophages were incubated with the *P. falciparum* GPIs (0.1 μg plus 200,000 of [^{3}H]GlcN-labeled GPIs) in serum-free medium. After 48 h, the culture supernatants were collected and the radioactive GPIs and their degradation products were recovered as described in "Experimental Procedures", and quantified by measuring radioactivity of aliquots using a liquid scintillation counter. The yields of products in the organic and aqueous layers are expressed as percentage of total ^{3}H-activity recovered.

Cells	Yield of GPIs and degradation products (% total)	
	1-Butanol layer	Aqueous layer
Human monocytes	51[b]	49
Mouse macrophages	49[c]	53

[a] About 5–10% of the GPIs were associated with the cells; of which ~60% was in 1-butanol layer and ~40% was in aqueous layer. Because of low yield these were not studied further.

[b] Approximately 20% of the ^{3}H activity in the 1-butanol layer corresponded to the phospholipase D-degradation products and a similar amount represented the phospholipase A_2-degaradation products. The remainder of the ^{3}H activity in the 1-butanol layer represented undegraded GPIs. However, note that the cell number was about 10-times less than that of murine macrophages.

[c] About 45% of the ^{3}H activity in 1-butanol layer corresponded to the phospholipase A_2-degaradation products and the remaining ~55% corresponded to the phospholipase D-degradation products. Only negligible amounts of undegraded GPIs were present.