Published in final edited form as:

Exp Parasitol. 2011 July ; 128(3): 205–211. doi:10.1016/j.exppara.2011.03.010.

Proinflammatory responses by glycosylphosphatidylinositols (GPIs) of *Plasmodium falciparum* are mainly mediated through the recognition of TLR2/TLR1

Jianzhong Zhu*, Gowdahalli Krishnegowda, Guangfu Li, and D Channe Gowda

Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA 17033, USA

Abstract

The glycosylphosphatidylinositols (GPIs) of *Plasmodium falciparum* have been shown to activate macrophages and produce inflammatory responses. The activation of macrophages by malarial GPIs involves engagement of Toll like receptor 2 (TLR2) resulting in the intracellular signaling and production of cytokines. In the present study, we investigated the requirement of TLR1 and TLR6 for the TLR2 mediated cell signaling and proinflammatory cytokine production by macrophages. The data demonstrate that malarial GPIs, which contain three fatty acid substituents, preferentially engage TLR2-TLR1 dimeric pair than TLR2-TLR6, whereas their derivatives, *sn*-2 lyso GPIs, that contain two fatty acid substituents recognize TLR2-TLR6 with slightly higher selectivity as compared to TLR2-TLR1 heteromeric pair. These results are analogous to the recognition of triacylated bacterial and diacylated mycoplasmal lipoproteins, respectively, by TLR2-TLR1 and TLR2-TLR6 dimers, suggesting that the lipid portions of the microbial GPI ligands play essential role in determining their TLR recognition specificity.

Keywords

Plasmodium falciparum; Glycosylphosphatidylinositol anchors; Toll-like receptors; TLR2-TLR1/TLR6; Recognition specificity; Proinflammatory responses

1. Introduction

Malaria caused by the *Plasmodium* family of protozoan parasites, especially by infection with *P. falciparum* and *P. vivax*, is a major health crisis around the world and is a leading contributor to the death tolls among various infectious diseases. Studies have shown that effective pro-inflammatory responses are essential for controlling parasite growth and for the development of protective immunity (Riley, et al., 2006, Stevenson and Riley, 2004). Studies in both humans and mouse malaria models have demonstrated that the initial efficient production of TNF- α , ILN- γ , IL-12, IL-6 and nitric oxide (NO) directly relates to the effective resolution of infection. However, studies have also shown that excessive production of TNF- α , IFN- γ , IL-12, and NO contributes to severe malaria, including

© 2011 Elsevier Ltd. All rights reserved.

*Corresponding author: Current address: Department of Microbiology, Hillman Cancer Center, University of Pittsburgh, Pittsburgh PA 15232, USA. Ph: 412-623-7729; Fax: 412-623-7715; jzz9@pitt.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cerebral malaria, liver injury, and organ dysfunction (Gowda, 2007, Schofield and Grau, 2005, Torre, et al., 2002). Like in other pathogenic infections, the production of pro-inflammatory responses to malaria infection involves activation of cells by the parasite-specific molecules via host cell receptors, initiating MAPK and NF- κ B signaling pathways, leading to the transcriptional activation for cytokine gene expression. Recent studies have amply demonstrated that the host-pathogen interactions involves the sensing of certain conserved molecules called pathogen-associated molecular patterns (PAMPs)¹ by the Toll like receptors (TLR) family of proteins expressed on the cells of innate immune system such as macrophages and dendritic cells (Akira, 2009). Understanding of the pathogenic molecules of parasites such as glycosylphosphatidyinositol (GPIs) and specific TLR receptors involved in activating the cells of the innate immune system will be valuable for developing strategies for therapeutics and a vaccine for malaria.

GPIs are a special group of glycolipids, consisting of a conserved glycan core structure 6Man α 1-2Man α 1-6Man α 1-4GlcN attached to phosphatidylinositol moiety *via* α (1-6) linkage. GPIs are ubiquitously expressed by eukaryotic cells and their primary function is to anchor proteins onto the cell surface through ethanolamine phosphate substituent at O-6 of the terminal mannose. GPIs from different organisms differ in their acyl/alkyl substituents, and in having additional sugar moieties on the third and/or first mannose, extra ethanolamine phosphate groups on the core glycan structure, and an acyl substituent on C-2 of inositol. Thus, naturally occurring GPIs have broad structural diversity, exhibiting diverse biological activity (Paulick and Bertozzi, 2008). In GPIs of *Plasmodium falciparum*, the core glycan structure is substituted with an additional mannose at O-2 of the terminal mannose of the GPI trisaccharide core, and the inositol residue is substituted predominantly with a palmitoyl moiety and the glycerol residue is substituted mainly with a saturated fatty acid moiety at C-1 and an unsaturated moiety at C-2 (Channe Gowda, 2002). The malarial GPIs are heterogeneous with regard to fatty acid substituents at various positions, the structure of the major molecule is: ethanolamine-phosphate-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6inositol(O-2-pamitoyl)-phosphate-CH₂-CH(O-oleoyl)-CH₂-O-stearoyl. Although, expressed at low levels by animal cells, GPIs are abundantly expressed by protozoan parasites of the genus *Trypanosome* (Butikofer, et al., 2010, Ferguson, 1999), *Leishmania* (Chandra, et al., 2010), *Taxoplasma* (Debierre-Grockiego and Schwarz, 2010) and *Plasmodium* (Gowda, 2007, Schofield and Hackett, 1993). The GPIs purified from these parasites species have been shown to activate cells of the innate immune system such as macrophages and endothelial cells to induce the production of proinflammatory cytokines and the upregulation of cell adhesion molecules; the exacerbated inflammatory responses to these microbial infections are thought to contribute to pathogenesis (Butikofer, et al., 2010, Chandra, et al., 2010, Debierre-Grockiego and Schwarz, 2010, Ferguson, 1999, Gowda, 2007, Schofield and Hackett, 1993).

TLRs are the evolutionarily conserved signal-transducing transmembrane molecules expressed by the cells of the innate immune system either on the cell surface or in the lumen of endosomes and exhibit distinct specificity in recognizing PAMPs. Interactions of TLRs with PAMPs enable the innate immune system to discriminate various pathogens and produce pathogen-specific immune responses. For example, TLR4 interacts with bacterial lipopolysaccharides, TLR9 with CpG-containing motifs of bacterial DNA, and TLR2 recognizes several ligands, including lipoteichoic acid, lipoproteins, lipoarabinomannan, and GPIs. Upon interactions with microbial components, TLRs transduce signals through their conserved cytoplasmic segments, Toll-IL1 receptor (TIR) domains, thereby activating MAPK and NF- κ B cascades. This leads to the induction of a wide range of immunological

¹Abbreviations used: GPI, glycosylphosphatidylinositol; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; BMDM, bone marrow derived macrophages.

responses, including the production of cytokines, chemokines, cell adhesion molecules, and co-stimulatory molecules (Akira, 2009).

Unlike other TLRs most of which form homodimers for functional activity, TLR2 requires dimerization with either TLR1 or TLR6 for the efficient and specific recognitions of microbial ligands (Takeda, et al., 2002). Studies have shown that functional association with TLR1 or TLR6 enables TLR2 to discriminate microbes expressing triacylated or diacylated lipoproteins. Thus, TLR2-TLR1 can efficiently recognize triacylated lipoproteins, which are expressed by mycobacteria (Drage, et al., 2009, Takeuchi, et al., 2002), whereas TLR2-TLR6 heterodimer exhibits specificity to mycoplasmal diacylated lipoproteins (Takeuchi, et al., 2001). Further, using synthetic lipopeptides that mimic the activity of lipoproteins, it has been demonstrated that TLR2-TLR1 could recognize certain triacylated peptides, conversely, TLR2-TLR6 was shown to recognize some diacylated peptides (Nakao, et al., 2005, Takeda, et al., 2002). Analogous to bacterial and mycoplasmal lipoproteins, the immunostimulatory GPIs of protozoan parasites are found as glycolipids containing either two or three fatty acyl/alkyl substituents (Butikofer, et al., 2010, Chandra, et al., 2010, Debierre-Grockiego and Schwarz 2010, Ferguson, 1999, Gowda, 2007, Naik, et al., 2000, Schofield and Hackett, 1993). Although, the protozoan GPIs have been reported to activate macrophages through TLR2 recognition (de Veer, et al., 2003, Debierre-Grockiego, et al., 2007, Ropert and Gazzinelli, 2004), the specificity with respect to the requirement of TLR1 or TLR6 has not been studied in detail. By expressing TLRs and reporter proteins in HEK-293 cells, we have previously observed that GPIs isolated from malaria parasite, *Plasmodium falciparum* that are triacylated are preferentially recognized by human TLR2-TLR1, whereas *sn-2 lyso* derivatives of malarial GPIs, which contain two fatty acyl moieties, are preferred by the human TLR2-TLR6 dimer (Krishnegowda, et al., 2005). Here, we extended these studies to determine the TLR recognition and signaling specificity of malarial GPIs under physiological conditions using macrophages from gene knockout mice and anti-TLR antibodies. Our results show that, as in the case of microbial lipoproteins, TLR2-TLR1 and TLR2-TLR6 heterodimers discriminate triacylated and diacylated GPIs.

2. Materials and Methods

2.1. Reagents

Standard synthetic TLR2 ligands MALP-2, FSL-1 and Pam₃C_{SK}₄, were purchased from EMC Microcollections (Tübingen, Germany). *E. coli* O₁₁₁:B₄ strain LPS was obtained from Sigma. Dual luciferase reporter assay kit was from Promega. Anti-human TLR1 monoclonal antibody (mouse IgG1, clone GD2.F4) and anti-human TLR6 monoclonal antibody (rat IgG2a, clone hPer6) were from eBioscience, Inc. (San Diego, CA). A mouse monoclonal antibody (IgG2) specific to an ovarian glycoprotein tumor antigen (OVb-3), phospho-specific anti-ERK1/ERK2, p38 and JNK antibodies, anti-I κ B α , ERK1/ERK2, p38, JNK, pan-actin antibodies, and HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgGs, ELISA kits for TNF- α , IL-6 and IL-12 (p40) measurements were same as those described previously (Krishnegowda, et al., 2005, Zhu, et al., 2005). Human blood and plasma from healthy donors were from the Hershey Medical Center. Endotoxin-free reagents, water, and buffers were used for all the experimental procedures.

2.2. Mice

The TLR1^{-/-}, TLR6^{-/-} and TLR2^{-/-} mice (C57BL/6J background), produced at the Research Institute for Microbial Diseases, Osaka University, Japan, were kindly provided by Drs. Shizuo Akira and Satoshi Uematsu, Osaka University, Osaka, Japan. The C57BL/6J wild type (WT) mice were from The Jackson Laboratories. All animals were maintained in a

pathogen-free environment and the animal care was in accordance with the Institutional Guidelines of the Pennsylvania State University College of Medicine.

2.3. *P. falciparum* culturing and purification of GPIs

The malaria parasite, *P. falciparum* (3D7 strain), was cultured using O-positive human red blood cells in RPMI 1640 medium containing 10–20% human O-positive plasma and 50 µg/ml gentamycin under 90% nitrogen, 5% oxygen and 5% carbon dioxide atmosphere as described previously (Krishnegowda, et al., 2005). Synchronous cultures (20–30% parasitemia) were harvested at the late trophozoite stage of parasites. The parasites were released from the infected red blood cells by treatment with 0.05% saponin and the released parasites purified by centrifugation on cushions of 5% BSA in PBS, pH 7.4. GPIs from parasites were extracted with chloroform/water/ethanol (10:10:3, v/v/v), dried, partitioned to water-saturated 1-butanol, and purified by high pressure liquid chromatography (HPLC) as previously reported (Krishnegowda, et al., 2005).

2.4. Preparation of Man₃-GPIs and sn-2 lyso GPIs

The HPLC-purified parasite GPIs were treated separately with jack bean α-mannosidase or bee venom phospholipase A2 as reported previously (Naik, et al., 2000, Vijaykumar, et al., 2001). The Man₃-GPIs and sn-2 lyso GPIs formed were purified by HPLC (Krishnegowda, et al., 2005).

2.5. TLR gain of function assay

HEK-293 cells were plated in 96-well microtiter plates (4×10^4 cells/well) were cultured in DMEM containing 10% FBS, 1% L-glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, nonessential amino acids, and 10 units/ml penicillin/streptomycin (complete medium). After 24 h, the cells were transfected with 10 ng of ELAM-luciferase, 0.3 ng of β-actin-*Renilla* luciferase, 2.5 ng of mouse CD14, 2.5 ng of mouse TLR2, 1.25 ng of mouse TLR1 and 1.25 ng of mouse TLR6 using lipofectamine 2000 (Invitrogen). The amount of DNA used was adjusted for equivalent expression of HA-tagged TLR proteins as judged by Western blot analysis using anti-HA antibody. Total DNA per well was normalized to 50 ng by the addition of empty vector. The stimulation of transfected cells with malarial GPIs and measurement of luciferase activity were described in our previous paper (Krishnegowda, et al., 2005).

2.6. Preparation of bone marrow macrophages and stimulation with GPIs

The mouse bone marrow cells were differentiated into macrophages with macrophage-colony stimulating factor by culturing in DMEM (complete medium) containing 30% L929 cell culture conditioned medium at 37 °C for seven days (Krishnegowda, et al., 2005, Zhu, et al., 2005). The macrophages were harvested by scraping, suspended in complete DMEM medium, seeded into microtiter plates, and stimulated with GPIs coated on gold particles (Krishnegowda, et al., 2005, Zhu, et al., 2005). Cells treated with uncoated gold particles were used as controls.

2.7. Preparation of human peripheral blood monocytes and stimulation with GPIs

Peripheral blood mononuclear cells were isolated from the human blood buffy coat by centrifugation on isolymp (CTL Scientific Supply, Deer Park, NY) cushions, washed, suspended in DMEM (complete medium), and seeded into 96-well microtiter plates. After 24 h of incubation at 37 °C, the unbound cells were removed and the bound monocytes were stimulated with GPIs for 48 h. The culture supernatants were collected and TNF-α was measured by ELISA (Krishnegowda, et al., 2005).

2.8. Measurement of TNF- α , IL-6 and IL-12 (p40) and nitrite

The TNF- α , IL-6 and IL-12 (p40) secreted into culture medium by GPI-stimulated cells were determined by sandwich ELISA using DuoSet ELISA development kits (R&D Systems, Inc., Minneapolis, MN) as reported previously (Zhu, et al., 2005). The nitrite levels were assayed by microcolorimetric assay using the Griess reagents (Zhu, et al., 2005).

2.9. Western blot analysis of signaling molecules

Macrophages in microtiter plates were cultured overnight in DMEM (complete medium) but containing 0.5% FBS and stimulated with GPIs in complete medium containing 10% FBS. At various time points, the culture supernatants were removed and cells were washed with ice-cold PBS and lysed with ice-cold RIPA 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 mixed with equal volumes of 2X 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 nitrocellulose membranes, blocked with 5% non-fat milk, and incubated with primary antibody followed by HRP conjugated anti-mouse or anti-rabbit secondary antibodies. The membranes were treated with chemiluminescent substrate (LumiGLO, from KPL Gaithersburg, MD), and exposed to X-ray films.

2.10. Gene expression analysis by RT-PCR

Total RNA from macrophages or monocytes was reverse transcribed using oligo-dT₁₄ primers. Aliquots of cDNA samples were analyzed for TNF- α , IL-6, IL-12 (p40) and iNOS genes, and the β -actin gene by PCR using the following primers: TNF- α , sense cggtgcctatgtctcagcct and anti-sense ttgggcagattgacctcagc; IL-6, sense gatgtctacaaactggatataatc and anti-sense ggtccttagccactcctctgtg; IL-12p40, sense cagaagctaaccatctcctggttg and anti-sense ccggagtaatttggtgcttcacac; iNOS, sense ctgcagcacttgatcaggaaactg and anti-sense gggagtagcctgtgtgcacctggaa; β -actin, sense accctaaggccaacctgaa and anti-sense ccgtcgttgccaatagtga (custom synthesized by Invitrogen). The PCR conditions used were: TNF- α and β -actin, 94 °C 30 s, 50 °C 30 s, and 72 °C 60 s for 30 cycles; IL-6 and iNOS, 94 °C 30 s, 60 °C 45 s, and 72 °C 60 s for 35 cycles; IL-12p40, 94 °C 30 s, 60 °C 30 s, and 72 °C 60 s for 38 cycles.

3. Results and discussion

Previously, we have demonstrated that *P. falciparum* GPIs can activate macrophages and induce the production of proinflammatory cytokines through the engagement of TLR2 (Krishnegowda, et al., 2005). Further, using a gain of function assay in HEK-293 cells lacking endogenous TLRs, we observed that human TLR2-TLR1 and TLR2-TLR6 could differentially recognize the malarial GPIs and their *sn*-2 lyso-derivatives (Krishnegowda, et al., 2005). As a preliminary step toward our efforts to perform a detailed study on the receptor specificity for GPI recognition by macrophages, we determined GPI recognition specificity of mouse TLR using a cell transfection system. HEK-293 cells were transiently transfected with TLR2 alone, TLR2 and TLR1 or TLR2 and TLR6. When stimulated with *P. falciparum* GPIs and their derivatives, the intact GPIs (Man₄-GPs that contain three fatty acid substituents), Man₃-GPIs (the structures that lack the terminal fourth mannose of malarial GPIs), and *sn*-2 lyso GPIs (that lack the fatty acid substituent at the *sn*-2 position of malarial GPIs) were efficiently recognized TLR2-TLR1 and TLR2-TLR6 heterodimers, activating NF- κ B (fig.1). Using a similar gain of function assay, we have previously shown that, in the case of human TLRs, Man₄-GPIs and Man₃-GPIs are recognized mainly by TLR2-TLR1 heterodimer, but scarcely by TLR2-TLR6 dimer (Krishnegowda, et al., 2005). In contrast, the diacylated GPIs (*sn*-2 lyso GPIs) exhibited higher preference to be recognized by TLR2-TLR6 than TLR2-TLR1. In the assay involving cells transfected with

mouse TLRs in this study, preferential recognition of triacylated and diacylated GPIs by, respectively, mouse TLR2-TLR1 and TLR2-TLR2 dimers were not evident (fig. 1). The observed difference in the recognition specificity of malaria GPIs by the mouse versus human TLR heterodimers could be due to the inherent species specific disparity in the structural features of TLRs. Alternatively, it is possible that in the transfection experiment, the mouse and human TLR2, TLR1 and TLR6 were either differentially expressed as compared to their expression pattern under physiological conditions or structurally differently disposed due to different cell environment. Note that in the gain of functional assay, the control ligands, Pam3CSK4 and MALP-2, were also not distinctively recognized by mouse TLR2-TLR1 and TLR2-TLR6 (fig.1), even though, in immune cells, these analogous tri- and di-acylated peptides (Pam3CSK4 and MALP-2) have been previously shown to be distinctively recognized by mouse TLR2-TLR1 and TLR2-TLR6 (Nakao, et al., 2005, Takeda, et al., 2002). Therefore, we further assessed the GPI activity in macrophages obtained by the differentiation of bone marrow cells from WT, TLR1^{-/-}, TLR2^{-/-}, and TLR6^{-/-} mice.

To determine the GPI recognition specificity by endogenous TLRs, we stimulated macrophages from TLR1^{-/-}, TLR2^{-/-}, TLR6^{-/-} and wide type (WT) mice with Man₄-GPIs or *sn-2 lyso* GPIs and measured TNF- α , IL-6, IL-12 and nitric oxide (NO) production. Both triacylated and diacylated GPIs induced the production of cytokines and NO by macrophages in a dose-dependent manner. Compared to wild type macrophages, in TLR1^{-/-} macrophages, the triacylated GPI-induced production of TNF- α and NO was decreased by 50–60% and that of IL-6 and IL-12 was decreased by 70–80% (fig. 2). In the case of TLR6^{-/-} macrophages stimulated with triacylated GPIs, the production of cytokines and NO was decreased only by 10–20% (fig. 2). In contrast to these results, when stimulated with *sn-2 lyso* GPIs, the production of TNF- α , IL-6, IL-12 and NO by TLR1^{-/-} macrophages was decreased only marginally (~10%) when compared with cytokines produced by the WT cells. In TLR6^{-/-} macrophages, the production of TNF- α and NO in response to the diacylated GPIs was decreased by 20–30% and that of IL-6 and IL-12 was reduced by 40–50% (fig. 2). Further, we measured mRNA levels induced by macrophages stimulated with Man₄-GPIs. Consistent with the results of cytokine measurements, mRNA induction of TNF- α , IL-6, IL-12 and inducible nitric oxide synthase (iNOS) were decreased and/or delayed considerably in macrophages from TLR2^{-/-} and TLR1^{-/-} mice but not in cells from TLR6^{-/-} mice (fig. 3A and 3B). Together these results demonstrate that the triacylated GPIs are also mainly recognized by TLR2-TLR1 than by TLR2-TLR6. On the other hand, diacylated GPIs are preferentially recognized by TLR2-TLR6 heterodimeric pairs than TLR2-TLR1, although the extent of discrimination by these TLR dimer pairs is only modest.

To determine whether endogenously expressed human TLR1 and TLR6 also discriminate tri- and di-acylated GPIs, we tested human peripheral monocytes using anti-human TLR1 and anti-human TLR6 monoclonal antibodies. Upon stimulation with triacylated GPIs, monocytes pretreated with anti-human TLR1 antibody produced significantly lower levels of TNF- α compared to untreated monocytes or cells pretreated with a non-relevant monoclonal antibody (fig. 4). These data are consistent with the results of cytokines produced by mouse macrophages that triacylated malarial GPIs are preferentially recognized by TLR2-TLR1 than by TLR2-TLR6. In contrast to the above results, monocytes pretreated with anti-human TLR6 monoclonal antibody showed only a marginal decrease in the production of TNF- α in response to stimulation by triacylated malarial GPIs.

The ligation of microbial ligands to TLRs leads to intracellular signaling and the activation of ERK, p38 and JNK, and NF- κ B pathways, resulting in the downstream production of inflammatory mediators (Krishnegowda, et al., 2005, Zhu, et al., 2005). We examined these

cell signaling pathways in TLR1^{-/-}, TLR2^{-/-}, and TLR6^{-/-} macrophages stimulated with malarial GPIs. As expected, the GPIs could stimulate macrophages, leading to the phosphorylation of ERK, p38 and JNK, and degradation of IκBα. Compared to WT macrophages, the phosphorylation of MAPKs and the IκBα degradation were markedly decreased in TLR2^{-/-} macrophages (fig. 5). In TLR1^{-/-} macrophages, the phosphorylation of all three MAPK and degradation of IκBα noticeably decreased or delayed. However, in TLR6^{-/-} cells, little or no changes in the activation of various signaling molecules were apparent as compared to those in WT cells (fig. 5). Therefore, while the preferential recognition of triacylated GPIs by TLR2-TLR1 was clearly seen, discriminative recognition by TLR2-TLR6 was not evident.

Studies have identified several components of malaria parasites as the activators of the innate immune system to produce pro-inflammatory responses. These include: (i) GPIs (Krishnegowda, et al., 2005, Zhu, et al., 2005) that interact mainly with TLR2 and to a lesser extent with TLR4; (ii) heme and microparticles released from the *P. falciparum*-infected erythrocytes that recognize TLR4 (Couper, et al., 2010, Figueiredo, et al., 2007); (iii) hemozoin alone, hemozoin-DNA or protein-DNA complex that exhibit specificity to TLR9 (Coban, et al., 2007, Parroche, et al., 2007, Pichyangkul, et al., 2004, Wu, et al., 2010). Among these, protein-DNA complex is the major component that dominantly activates DCs (Wu, et al., 2010). Recently, it has also been reported that the mice deficient in TLR2, TLR4 or TLR9 individually or those lack the expression of all three TLRs were not protected against cerebral malaria caused by *P. berghei* ANKA infection (Lepenies, et al., 2008). In contrast to these studies, in another study, mice lacking either TLR2 or TLR9 have been reported to protected against cerebral malaria (Coban, et al., 2009). Further, mice deficient in MyD88 were protected from IL-12-dependent liver injury (Adachi, et al., 2001) in infection with lethal *P. berghei* NK65 infection. Yet in another study, using *P. chabaudi chabaudi* AS, it has been shown that MyD88 and to some extent TLR9 mediate malaria symptoms, but were not required for the development of immunity required for parasite growth control (Franklin, et al., 2007). Thus, the contributions of the TLRs that recognize different components of malaria parasite toward malaria pathogenesis/development of protective immunity remain unclear. Further detailed studies are needed to gain deeper insight into the roles of TLRs in malaria pathogenesis or protective immunity to malaria.

4. Conclusion

The ability of TLRs to discriminate various pathogenic organisms by specifically recognizing PAMPs has been amply demonstrated by a number of studies during the past decade. Thus, it is known that TLR2 collaborates with either TLR1 or TLR6 to discriminate pathogens that express triacylated or diacylated lipoproteins on the surface. While TLR2-TLR1 specifically recognizes triacylated lipoproteins, TLR2-TLR6 exhibits specificity to diacylated lipoproteins. In contrast to lipoproteins, very little is known about the requirement of TLR1 or TLR6 by TLR2 to discriminate GPIs and mycobacterial lipoglycans, lipoarabinomannans. Although TLR2-TLR1 has been shown to recognize the lipoarabinomannans of mycobacteria (Tapping and Tobias, 2003, Wieland, et al., 2004), it is not known whether or not TLR2-TLR6 recognizes these glycolipids.

Our previous study using TLR mediated reporter assay in HEK-293 cells suggested that *P. falciparum* GPIs that are known contain three acylated moieties preferentially activate TLR2-TLR1 dimer, whereas the derivatives of the parasite GPIs that lacked the acyl moiety at the *sn*-2 position preferentially activate TLR2-TLR6 dimer (Krishnegowda, et al., 2005). The results of the present study, obtained using macrophages from the gene knockout mice as well as human monocytes and anti-human TLR1 and anti-human TLR6 antibodies are consistent with the previous results. Thus, the results of this study and those of the previous

study together conclusively demonstrate that in both human and mouse macrophages, *P. falciparum* GPIs are preferentially recognized by TLR2/TLR1, pointing out the physiological relevance of our observations. Our results also support the notion that the lipid moieties of microbial components represent crucial recognition markers for TLRs.

Additionally, we show in this study that TLR1 contributes prominently to GPI mediated intracellular cell signaling and downstream pro-inflammatory gene expression, suggesting TLR1 biological relevance with regard to pathology of malaria. Analysis of TLR gene polymorphisms in malaria patients showed that TLR2, TLR4 and TLR9 play important roles in human malaria (Khor, et al., 2007, Mockenhaupt, et al., 2006, Mockenhaupt, et al., 2006, Parroche, et al., 2007, Wu, et al., 2010). Since TLR1 heterodimerizes with TLR2 and contributes to TLR2 mediated cell signaling and proinflammatory cytokine production, TLR1 probably have a role in malaria pathogenesis. A more recent study showed that TLR1 variant with S248N mutation influences placental malaria (Hamann, et al., 2010). Further, it has been recently found that the TLR1 mutation, A7202G, is associated with higher TLR1 induced NF- κ B activation, leading to elevated TLR1-mediated cytokine production, increased susceptibility to organ dysfunction, and death in sepsis caused by gram-positive bacteria (Wurfel, et al., 2008). Thus, these results suggest that further investigation on the TLR1 gene polymorphism in malaria populations may provide new insight into malaria pathogenesis.

Acknowledgments

The study was supported by the grant AI41139 from the National Institute of Infectious Diseases and Allergy, NIH, and funding from the Pennsylvania Department of Health. We thank Drs. Shizuo Akira and Satoshi Uematsu, Research Institute for Microbial Diseases, Osaka University, Japan, for providing TLR gene knockout mice.

References

- Adachi K, Tsutsui H, Kashiwamura S, Seki E, Nakano H, Takeuchi O, Takeda K, Okumura K, Van Kaer L, Okumura H, Akira S, Nakanishi K. Plasmodium berghei infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. *Journal of Immunology*. 2001; 167:5928–5934.
- Akira S. Innate immunity to pathogens: diversity in receptors for microbial recognition. *Immunological Reviews*. 2009; 227:5–8. [PubMed: 19120470]
- Butikofer P, Greganova E, Liu YC, Edwards IJ, Lehane MJ, Acosta-Serrano A. Lipid remodelling of glycosylphosphatidylinositol (GPI) glycoconjugates in procyclic-form trypanosomes: biosynthesis and processing of GPIs revisited. *Biochemical Journal*. 2010; 428:409–418. [PubMed: 20345369]
- Chandra S, Ruhela D, Deb A, Vishwakarma RA. Glycobiology of the Leishmania parasite and emerging targets for antileishmanial drug discovery. *Expert Opinion on Therapeutic Targets*. 2010; 14:739–757. [PubMed: 20536412]
- Channe Gowda D. Structure and activity of glycosylphosphatidylinositol anchors of Plasmodium falciparum. *Microbes and Infection*. 2002; 4:983–990. [PubMed: 12106792]
- Coban C, Ishii KJ, Akira S. Immune interventions of human diseases through toll-like receptors. *Advances in Experimental Medicine and Biology*. 2009; 655:63–80. [PubMed: 20047036]
- Coban C, Ishii KJ, Uematsu S, Arisue N, Sato S, Yamamoto M, Kawai T, Takeuchi O, Hiseada H, Horii T, Akira S. Pathological role of Toll-like receptor signaling in cerebral malaria. *Intestinal Immunology*. 2007; 19:67–79.
- Couper KN, Barnes T, Hafalla JC, Combes V, Ryffel B, Secher T, Grau GE, Riley EM, de Souza JB. Parasite-derived plasma microparticles contribute significantly to malaria infection-induced inflammation through potent macrophage stimulation. *PLoS Pathogens*. 2010; 6:e1000744. [PubMed: 20126448]
- de Veer MJ, Curtis JM, Baldwin TM, DiDonato JA, Sexton A, McConville MJ, Handman E, Schofield L. MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and

- Toll-like receptor 2 signaling. *European Journal of Immunology*. 2003; 33:2822–2831. [PubMed: 14515266]
- Debierre-Grockiego F, Campos MA, Azzouz N, Schmidt J, Bieker U, Resende MG, Mansur DS, Weingart R, Schmidt RR, Golenbock DT, Gazzinelli RT, Schwarz RT. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from *Toxoplasma gondii*. *Journal of Immunology*. 2007; 179:1129–1137.
- Debierre-Grockiego F, Schwarz RT. Immunological reactions in response to apicomplexan glycosylphosphatidylinositols. *Glycobiology*. 2010; 20:801–811. [PubMed: 20378610]
- Drage MG, Pecora ND, Hise AG, Febbraio M, Silverstein RL, Golenbock DT, Boom WH, Harding CV. TLR2 and its co-receptors determine responses of macrophages and dendritic cells to lipoproteins of *Mycobacterium tuberculosis*. *Cellular Immunology*. 2009; 258:29–37. [PubMed: 19362712]
- Ferguson MA. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *Journal of Cell Science*. 1999; 112(Pt 17):2799–2809. [PubMed: 10444375]
- Figueiredo RT, Fernandez PL, Mourao-Sa DS, Porto BN, Dutra FF, Alves LS, Oliveira MF, Oliveira PL, Graca-Souza AV, Bozza MT. Characterization of heme as activator of Toll-like receptor 4. *Journal of Biological Chemistry*. 2007; 282:20221–20229. [PubMed: 17502383]
- Franklin BS, Rodrigues SO, Antonelli LR, Oliveira RV, Goncalves AM, Sales-Junior PA, Valente EP, Alvarez-Leite JI, Ropert C, Golenbock DT, Gazzinelli RT. MyD88-dependent activation of dendritic cells and CD4(+) T lymphocytes mediates symptoms, but is not required for the immunological control of parasites during rodent malaria. *Microbes and Infection*. 2007; 9:881–890. [PubMed: 17537666]
- Gowda DC. TLR-mediated cell signaling by malaria GPIs. *Trends in Parasitology*. 2007; 23:596–604. [PubMed: 17980663]
- Hamann L, Bedu-Addo G, Eggelte TA, Schumann RR, Mockenhaupt FP. The toll-like receptor 1 variant S248N influences placental malaria. *Infection, Genetics and Evolution*. 2010; 10:785–789.
- Khor CC, Chapman SJ, Vannberg FO, Dunne A, Murphy C, Ling EY, Frodsham AJ, Walley AJ, Kyrieleis O, Khan A, Aucan C, Segal S, Moore CE, Knox K, Campbell SJ, Lienhardt C, Scott A, Aaby P, Sow OY, Grignani RT, Sillah J, Sirugo G, Peshu N, Williams TN, Maitland K, Davies RJ, Kwiatkowski DP, Day NP, Yala D, Crook DW, Marsh K, Berkley JA, O'Neill LA, Hill AV. A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. *Nature Genetics*. 2007; 39:523–528. [PubMed: 17322885]
- Krishnegowda G, Hajjar AM, Zhu J, Douglass EJ, Uematsu S, Akira S, Woods AS, Gowda DC. Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *Journal of Biological Chemistry*. 2005; 280:8606–8616. [PubMed: 15623512]
- Lepeniev B, Cramer JP, Burchard GD, Wagner H, Kirschning CJ, Jacobs T. Induction of experimental cerebral malaria is independent of TLR2/4/9. *Medical Microbiology and Immunology*. 2008; 197:39–44. [PubMed: 17668237]
- Mockenhaupt FP, Cramer JP, Hamann L, Stegemann MS, Eckert J, Oh NR, Otchwemah RN, Dietz E, Ehrhardt S, Schroder NW, Bienzle U, Schumann RR. Toll-like receptor (TLR) polymorphisms in African children: Common TLR- 4 variants predispose to severe malaria. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:177–182. [PubMed: 16371473]
- Mockenhaupt FP, Hamann L, von Gaertner C, Bedu-Addo G, von Kleinsorgen C, Schumann RR, Bienzle U. Common polymorphisms of toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy. *Journal of Infectious Diseases*. 2006; 194:184–188. [PubMed: 16779724]
- Naik RS, Branch OH, Woods AS, Vijaykumar M, Perkins DJ, Nahlen BL, Lal AA, Cotter RJ, Costello CE, Ockenhouse CF, Davidson EA, Gowda DC. Glycosylphosphatidylinositol anchors of *Plasmodium falciparum*: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. *Journal of Experimental Medicine*. 2000; 192:1563–1576. [PubMed: 11104799]

- Nakao Y, Funami K, Kikkawa S, Taniguchi M, Nishiguchi M, Fukumori Y, Seya T, Matsumoto M. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. *Journal of Immunology*. 2005; 174:1566–1573.
- Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, Visintin A, Halmen KA, Lamphier M, Olivier M, Bartholomeu DC, Gazzinelli RT, Golenbock DT. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:1919–1924. [PubMed: 17261807]
- Paulick MG, Bertozzi CR. The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. *Biochemistry*. 2008; 47:6991–7000. [PubMed: 18557633]
- Pichyangkul S, Yongvanitchit K, Kum-arb U, Hemmi H, Akira S, Krieg AM, Heppner DG, Stewart VA, Hasegawa H, Looareesuwan S, Shanks GD, Miller RS. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. *Journal of Immunology*. 2004; 172:4926–4933.
- Riley EM, Wahl S, Perkins DJ, Schofield L. Regulating immunity to malaria. *Parasite Immunology*. 2006; 28:35–49. [PubMed: 16438675]
- Roport C, Gazzinelli RT. Regulatory role of Toll-like receptor 2 during infection with *Trypanosoma cruzi*. *Journal of Endotoxin Research*. 2004; 10:425–430. [PubMed: 15588426]
- Schofield L, Grau GE. Immunological processes in malaria pathogenesis. *Nature Reviews Immunology*. 2005; 5:722–735.
- Schofield L, Hackett F. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. *Journal of Experimental Medicine*. 1993; 177:145–153. [PubMed: 8418196]
- Stevenson MM, Riley EM. Innate immunity to malaria. *Nature Reviews Immunology*. 2004; 4:169–180.
- Takeda K, Takeuchi O, Akira S. Recognition of lipopeptides by Toll-like receptors. *Journal of Endotoxin Research*. 2002; 8:459–463. [PubMed: 12697090]
- Takeuchi O, Kawai T, Muhlratt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *International Immunology*. 2001; 13:933–940. [PubMed: 11431423]
- Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *Journal of Immunology*. 2002; 169:10–14.
- Tapping RI, Tobias PS. Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. *Journal of Endotoxin Research*. 2003; 9:264–268. [PubMed: 12935358]
- Torre D, Speranza F, Martegani R. Role of proinflammatory and anti-inflammatory cytokines in the immune response to *Plasmodium falciparum* malaria. *Lancet Infectious Diseases*. 2002; 2:719–720. [PubMed: 12467687]
- Vijaykumar M, Naik RS, Gowda DC. *Plasmodium falciparum* glycosylphosphatidylinositol-induced TNF-alpha secretion by macrophages is mediated without membrane insertion or endocytosis. *Journal of Biological Chemistry*. 2001; 276:6909–6912. [PubMed: 11152670]
- Wieland CW, Knapp S, Florquin S, de Vos AF, Takeda K, Akira S, Golenbock DT, Verbon A, van der Poll T. Non-mannose-capped lipoarabinomannan induces lung inflammation via toll-like receptor 2. *American Journal of Respiratory and Critical Care Medicine*. 2004; 170:1367–1374. [PubMed: 15447943]
- Wu X, Gowda NM, Kumar S, Gowda DC. Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses. *Journal of Immunology*. 2010; 184:4338–4348.
- Wurfel MM, Gordon AC, Holden TD, Radella F, Strout J, Kajikawa O, Ruzinski JT, Rona G, Black RA, Stratton S, Jarvik GP, Hajjar AM, Nickerson DA, Rieder M, Sevransky J, Maloney JP, Moss M, Martin G, Shanholtz C, Garcia JG, Gao L, Brower R, Barnes KC, Walley KR, Russell JA, Martin TR. Toll-like receptor 1 polymorphisms affect innate immune responses and outcomes in sepsis. *American Journal of Respiratory and Critical Care Medicine*. 2008; 178:710–720. [PubMed: 18635889]

Zhu J, Krishnegowda G, Gowda DC. Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: the requirement of extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase and NF-kappaB pathways for the expression of proinflammatory cytokines and nitric oxide. *Journal of Biological Chemistry*. 2005; 280:8617–8627. [PubMed: 15611045]

Fig. 1.

Recognition of *P. falciparum* GPIs by TLR2-TLR1 and TLR2-TLR6 dimers. HEK293 cells were plated in 96-well plates (4×10^4 cells/well) and transfected with the indicated mouse TLRs plus, in each case, with mouse CD14, E-selectin firefly luciferase, and β -actin-*Renilla* luciferase reporter genes. The transfected cells were stimulated with 400 nM GPIs or GPI derivatives. The transfected cells were also stimulated in parallel with standard TLR2-TLR1 ligand, Pam₃CSK₄ (100 ng/ml), or with TLR2-TLR6 ligand, MALP-2 (10 nM), as controls. After 5 h, the cells were lysed, and luciferase activity was measured.

Fig. 2.

GPIs containing two or three acyl substituents are differentially recognized by endogenous TLR2-TLR6 and TLR2-TLR1. Bone marrow-derived macrophages from the wild type, TLR1^{-/-}, TLR6^{-/-} and TLR2^{-/-} mice were plated into 96-well plates (2.5×10^4 cells/well). The cells were cultured overnight in DMEM (complete medium), primed with IFN-γ (100 U/ml), and stimulated with *P. falciparum* GPIs (40, 80 and 160 nM) or *sn-2 lyso* GPI (40, 80 and 160 nM). The cells were also stimulated with TLR2-TLR1 ligand, Pam₃CSK₄ (10 ng/ml), or TLR2-TLR6 ligand, FSL-1 (10 nM), as controls. After 48 h, the culture supernatants were collected, and assayed for TNF-α, IL-6 and IL-12 by ELISA. Nitric oxide was assayed by colorimetric estimation of nitrite using the Griess reagents. The results are expressed as average values $\pm$ SD of duplicate wells, and a representative of three similar experiments is shown.

Fig. 3.

The *P. falciparum*-GPI induced expression of cytokines and iNOS mRNA in TLR deficient mouse macrophages. (A) Bone marrow cell-derived macrophages from TLR1^{-/-}, TLR6^{-/-}, TLR2^{-/-} and WT mice were plated in 24-well microtiter plates ($\sim 1 \times 10^6$ cells/well). After overnight culturing, the cells were stimulated with 200 nM *P. falciparum* GPIs. At the indicated time points, the cells were harvested, RNA isolated, and analyzed for mRNAs of TNF- α , IL-6 and IL-12, and iNOS by RT-PCR. The β -actin mRNA was analyzed as an endogenous control. The experiments were performed two times, and the representative data are shown. (B) Relative intensities of the PCR products of GPI-induced mRNA in mouse macrophages as measured by densitometric analysis.

Fig. 4.

The inhibition of *P. falciparum* GPI-induced TNF- α in human monocytes treated with anti-human TLR1 antibody. Human blood mononuclear cells were plated into 96-well plates ($\sim 1 \times 10^6$ cells/well). After overnight culturing, the cells were washed and the bound monocytes treated with the indicated concentrations of anti-TLR1, TLR6 or anti-OVB-3 control monoclonal antibody. After 1 h incubation at 37 °C, the cells were stimulated with 100 nM GPIs for 24 h. The culture supernatants were collected and TNF- α measured by ELISA. The level of TNF- α secreted by cells treated with GPIs (control) was considered as 100%. The relative levels of TNF- α produced by cells treated with antibodies prior to GPI stimulation in comparison to the control cells were calculated and plotted. The statistical analysis of the results performed by Student's t test and P values less than 0.05 were considered statistically significant. *, $P < 0.05$; NS, not significant.

Fig. 5.

Analysis of *P. falciparum* GPI-induced intracellular cell signaling in TLR deficient macrophages. Bone marrow cell-derived macrophages from TLR1^{-/-}, TLR6^{-/-}, TLR2^{-/-} and WT mice were plated in 24-well microtiter plates ($\sim 5 \times 10^5$ cells/well). After overnight culturing, the cells were stimulated with 100 nM GPIs for 0, 15, 30 and 60 min respectively. The cells were lysed and cell lysates electrophoresed on 10% SDS-polyacrylamide gels, and the protein bands in the gels were transferred onto nitrocellular membranes and probed for the activation of MAPK using anti-ERK, p38, and JNK phospho-specific antibodies and that of NF- κ B by using anti-I κ B α antibodies. The levels of non-phosphorylated MAPKs and actin controls were analyzed by using MAPK peptide-specific antibodies and anti-pan-actin antibody, respectively.