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

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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.

1. Introduction

Malaria caused by the *Plasmodium* family of protozoan parasites, especially by infection with *Plasmodium falciparum* and *Plasmodium 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, IFN-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 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-κB signaling pathways, leading to the transcriptional activation for cytokine gene expression. Recent studies have amply demonstrated that the host–pathogen interactions involve the sensing of certain conserved molecules called pathogen-associated molecular patterns (PAMPs) 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 glycosylphosphatidylinositols (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 6Manα1-2Manα1-6Manα1-4GlcN 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 *P. 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

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

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regard to fatty acid substituents at various positions; the structure of the major molecule is: ethanolamine-phosphate-6Manα1-2Manα1-6Manα1-4GlcNα1-6inositol(O-2-pamitoyl)-phosphate-CH_2-CH(O-oleoyl)-CH_2-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-κB cascades. This leads to the induction of a wide range of immunological 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, *P. 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 Pam_3CSK_4, were purchased from EMC Microcollections (Tübingen, Germany). *E. coli* O111:B_4 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-IκBα, 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/6 J 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 Man_3-GPIs and sn-2 lyso GPIs

The HPLC-purified parasite GPIs were treated separately with jack bean α-mannosidase or bee venom phopholipase A2 as reported previously (Naik et al., 2000; Vijaykumar et al., 2001). The Man_3-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,