Stimulation of innate immune responses by malarial glycosylphosphatidylinositol via pattern recognition receptors

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SUMMARY

The glycosylphosphatidylinositol (GPI) anchor of *Plasmodium falciparum* is thought to function as a critical toxin that contributes to severe malarial pathogenesis by eliciting the production of proinflammatory responses by the innate immune system of mammalian hosts. Analysis of the fine structure of *P. falciparum* GPI suggests a requirement for the presence of both core glycan and lipid moieties in the recognition and signalling of parasite glycolipids by host immune cells. It has been demonstrated that GPI anchors of various parasitic protozoa can mediate cellular immune responses via members of the Toll-like family of pattern recognition receptors (TLRs). Recent studies indicate that GPI anchors of *P. falciparum* and other protozoa are preferentially recognized by TLR-2, involving the MyD88-dependent activation of specific signalling pathways that mediate the production of proinflammatory cytokines and nitric oxide from host macrophages *in vitro*. However, the contribution of malaria GPI toxin to severe disease syndromes and the role of specific TLRs or other pattern recognition receptors in innate immunity *in vivo* is only just beginning to be characterized. A better understanding of the molecular mechanisms underlying severe malarial pathogenesis may yet lead to substantial new insights with important implications for the development of novel therapeutics for malaria treatment.

Key words: Malaria, GPI, *P. falciparum*, *T. cruzi*, *T. brucei*, *T. gondii*, pathology, Toll receptors, pattern recognition.

INTRODUCTION: MALARIA DISEASE

Malaria is an infectious disease that causes enormous global medical and economic burdens. Of the four species of malaria parasites known to infect humans, the bulk of severe disease and complications is caused by the species *Plasmodium falciparum*. More than 80% of the approximately 2 million estimated cases of malaria-related mortality worldwide occur in Africa, mostly affecting infants and pre-school children aged 6 months to 5 years. Malarial fatalities are associated with a spectrum of discrete and overlapping disease syndromes of complex aetiologies. Humans affected and dying of malaria may variously suffer systemic, single- or multi-organ involvement, including acute respiratory distress, coagulopathy, shock, metabolic acidosis, hypoglycaemia, renal failure, pulmonary oedema and cerebral involvement including seizures and coma (White & Ho, 1992). Basic mechanisms controlling these processes are thought to be the site-specific localization of parasites (by cytoadherence to vascular endothelial markers such as the adhesin ICAM-1 (Berendt *et al*. 1989), and both local and systemic inflammatory responses arising from the action of cytokines produced predominantly by the innate immune system (Stevenson & Riley, 2004). The ability of the intraerythrocytic parasites to cytoadhere to endothelium and sequester in the microvasculature of vital organs (Miller *et al*. 2002) is thought to promote the accumulation of high local concentrations of bioactive parasite products (GPI-anchored antigens and glycolipids, metabolite toxins) that can trigger a cascade of pro-inflammatory immune responses in the brain, lungs, spleen, liver, kidney and placenta. This cytokine-dependent inflammatory cascade is believed to contribute to the spectrum of acute clinical symptoms that manifest during severe malaria bouts. In order to better understand the molecular mechanisms underlying severe malarial pathogenesis, our group has focused its attention on the interaction between *P. falciparum* and the immune system of the vertebrate host in the context of malaria infection.

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MOLECULAR BASIS OF SEVERE MALARIA – CYTOKINE CONTRIBUTIONS

When examining the molecular basis of malarial pathogenesis it is clear that many factors from both the parasite and the host immune system contribute to the development of severe disease and there is an intricate relationship between these factors (reviewed by Chen, Schlichtherle & Wahlgren 2000; Stevenson & Riley, 2004). Both human and murine malaria infections elicit cells of the innate immune

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compartment to produce high levels of pro-inflammatory cytokines such as interferon-gamma (IFN-γ), interleukin-1 (IL-1), IL-6, IL-12 and tumor necrosis factor alpha (TNF-α) (Grau *et al*. 1987, 1989*b*; Lucas *et al*. 1997; Lyke *et al*. 2004). These pro-inflammatory cytokines appear to be essential for mediating acquired immunity to the invasive sporozoite during the early stages of infection (Schofield *et al*. 1987), but their role in mediating host resistance to blood stage infection is less clear.

The over-production of inflammatory cytokines can also be harmful to the host. Serum IFN-γ levels are elevated in patients with acute *P. falciparum* malaria (Ringwald *et al*. 1991), and death resulting from cerebral malaria (CM) was found to be associated with heterozygosity for an IFN-γ receptor-1 polymorphism in Gambian children (Koch *et al*. 2002). In the murine model both IFN-γ and IFN-γ receptor knock-out mice are resistant to *Plasmodium berghei*-mediated CM (Amani *et al*. 2000), and injection of neutralizing monoclonal antibody (mAb) against IFN-γ was shown to protect against CM in C57BL/6 susceptible mice (Grau *et al*. 1989*a*). These data suggest a role for IFN-γ as a key regulator of downstream immune system processes in the pathogenesis of severe malaria. IFN-γ has multiple activities in both the innate and acquired compartments of the immune system. Importantly, IFN-γ induces macrophages to become highly activated and, when exposed to live parasites, to generate high levels of free radical nitrogen intermediates (RNIs) via an inducible nitric oxide synthase (iNOS). RNIs can cause irreversible damage to neuronal tissues and have been implicated in the pathogenesis of CM (Al Yaman *et al*. 1996). In addition, when stimulated with parasite products *in vitro*, IFN-γ-primed macrophages can produce high levels of other pro-inflammatory cytokines associated with severe malaria, including IL-1, IL-6, IL-10, IL-12 and TNF-α (Grau *et al*. 1987, 1989*b*; Lucas *et al*. 1997; Lyke *et al*. 2004). Severe malaria is also associated with polymorphisms in the TNF-α promoter region, which may be linked to increased TNF-α expression (Mcguire *et al*. 1994), and high circulating levels of TNF-α are a prognostic indicator of fatality (Grau *et al*. 1989*b*). In concert, IFN-γ and TNF-α cytokines are capable of synergistically up-regulating intracellular adhesion molecules such as ICAM-1, VCAM-1, CD36 and thrombospondin on various cell types, including vascular endothelium and leukocytes. This augments sequestration of parasitized erythrocytes to endothelium and intensifies the deposition of malaria toxins in the deep vasculature of organs such as the brain. Malaria fatalities are thus strongly associated with an exacerbated systemic or organ-specific inflammatory cascade, but the molecular nature of the parasite toxin(s) that trigger the overproduction of pro-inflammatory cytokines has for a long time remained unclear.

MALARIAL GPIS AS CANDIDATE TOXINS AND PATHOGENICITY FACTORS

Several years ago, *in vitro* and *in vivo* studies suggested that glycosylphosphatidylinositol (GPI)-anchored molecules of *P. falciparum* function as the dominant malarial toxin in the context of infection. This hypothesis was based on the finding that purified GPI-anchored glycoproteins and glycolipids of *P. falciparum* and *P. berghei* are very potent activators of innate immune cells, capable of inducing pro-inflammatory cytokines TNF-α and IL-1 from macrophages (Schofield & Hackett, 1993). This activity has subsequently been confirmed by others (Naik *et al*. 2000; Vijaykumar, Naik & Gowda, 2001) (Table 1). The original study also showed that when administered to mice, GPI alone is sufficient to cause symptoms similar to acute malaria infection such as transient fever and hypoglycemia and death of recipients due to TNF-α-mediated sepsis (Schofield *et al*. 1993). Initial data showed that the endotoxin activity of major *P. falciparum* surface antigens released during schizont rupture (i.e. MSP-1, MSP-2) is restricted to the GPI moiety, since protein denaturation and exhaustive digestion with pronase does not abolish or reduce the potency of these molecules for the induction of cytokines *in vitro* (Schofield & Hackett, 1993). In contrast, enzymatic cleavage with phospholipase A_2 or chemical treatment of the GPI moiety with mild alkali (for selective removal of fatty acid esterified at the glycerol *sn*-1 and *sn*-2 postitions) or nitrous acid (for selective breakage of the glucosamine-*myo*-inositol bond) reduces most of the pro-inflammatory activity (Schofield & Hackett, 1993) – indicating a requirement for intact GPI-associated glycolipid structure. Additional studies found that purified *P. falciparum* GPI glycolipids were also able to induce, either directly or in synergy with IFN-γ, a range of other activities in various host tissues and cell types, including insulin-mimetic signalling in adipocytes (Schofield *et al*. 1994, 1996), the induction of iNOS in macrophages and vascular endothelium (Tachado *et al*. 1996), and the up-regulation of cell adhesion molecules on the surface of host leukocytes and endothelial cells (Schofield *et al*. 1996). The bioactivity of purified GPI glycolipid was comparable to that obtained with live parasites *in vitro*, in the range of 0·1–10 parasite equivalents per macrophage (Schofield & Hackett, 1993; Schofield *et al*. 1996). Importantly, monoclonal or polyclonal antibodies that recognize malarial GPI completely block the induction of TNF-α or iNOS output from macrophages and ICAM-1 expression on endothelial cells by crude extracts of *P. falciparum* (Schofield *et al*. 1994, 1996, 2002). Thus, *Plasmodium* GPIs appear both necessary and sufficient for the induction of pro-inflammatory host responses by malarial parasites *in vitro*.

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Table 1. Support for pro-inflammatory bioactivity of protozoal GPIs

GPI preparation	Activity	Reference
GPI purified from pronase-digested *P. falciparum* MSP-1 and MSP-2 and *T. brucei* VSG	Induction of TNF-α and IL-1 production in mouse peritoneal macrophages	Schofield & Hackett (1993) Tachado & Schofield (1994)
Octylsepharose and TLC-purified GPIs from *P. falciparum*	Induction of iNOS and ICAM expression in mouse peritoneal macrophages and human vascular endothelium cells	Tachado *et al*. (1996) Schofield *et al*. (1996)
Octylsepharose and TLC-purified *P. falciparum* and *T. brucei* GPIs	Activation of PTK, PKC and NF-κB signalling pathways and TNF-α, IL-1 and iNOS production in murine RAW264 macrophages	Tachado *et al*. (1997)
Octylsepharose-purified *T. cruzi* trypomastigote mucin GPIs	TNF-α and iNOS output in IFN-γ-primed peritoneal macrophages from C3H/HeJ mice	Camago *et al*. (1997*a*,*b*)
Highly purified GPIs and GIPLs of *T. cruzi* trypomastigote forms	TNF-α, IL-12 and iNOS production in peritoneal macrophages from LPS-hyposensitive C3H/HeJ mice	Almeida *et al*. (2000)
HPLC and TLC-purified *P. falciparum* GPIs	TNF-α output in murine J774A.1 macrophages	Vijaykumar *et al*. (2001) Naik *et al*. (2000)
Purified *T. brucei* VSG GPI anchors	TNF-α and IL-1 production in IFN-γ-primed peritoneal macrophages from C3H/HeJ mice and bovine peripheral blood monocytes	Magez *et al*. (1998) Sileghem *et al*. (2001)
HPTLC-purified and chemically synthesized GPIs of *Toxoplasma gondii*	TNF-α production in murine RAW264 macrophages	Debierre-Grockiego *et al*. (2003)
Octylsepharose-purified *T. cruzi* mucin GPI anchors	Activation of TLR-2, MAPK and NF-κB signalling pathways required for IL-12, TNF-α and iNOS production in mouse macrophages and gene induction in transfected CHO cells	Ropert *et al*. (2001) Campos *et al*. (2001)
HPLC and HPTLC-purified *P. falciparum* GPIs	Activation of TLR-2/MyD88, MAPK, JNK and NF-κB signalling pathways required for the expression of TNF-α, IL-6, IL-12 and iNOS in murine bone marrow-derived macrophages and human peripheral blood monocytes	Krishnegowda *et al*. (2005) Zhu *et al* .(2005)

These findings were then extended to GPIs of other parasitic protozoa (Table 1). For example, the GPI anchor of *Trypanosoma brucei* VSG and highly purified GPIs of *Trypanosoma cruzi* trypomastigote origin are potent macrophage activators (Tachado & Schofield, 1994; Magez *et al*. 1998; Almeida & Gazzinelli, 2001), and this may account for many features of pathogenesis of trypanosomiasis and Chagas disease. More recently, it was shown that highly purified GPIs of *Toxoplasma gondii* tachyzoites, the infective form of the parasite which causes severe encephalitis, are bioactive factors that participate in the production of inflammatory cytokines from macrophages during toxoplasmal pathogenesis (Debierre-Grockiego *et al*. 2003). In contrast, GIPLs and lipophosphoglycans derived from infective promastigote forms of *Leishmania* spp. suppress several functions of the host immune system – thereby allowing this parasite to evade the innate immunological control of infection (Camargo *et al*. 1997*b*; Tachado *et al*. 1997). Taken together, these data support the view that GPIs of the parasitic protozoa are both immunostimulatory and immunoregulatory components in the context of protozoal infections and the important differences in bioactivity that exist depend upon GPI fine structure.

GPI ACTS AS A CRITICAL TOXIN IN THE CONTEXT OF SEVERE DISEASE SYNDROMES

The data reviewed so far indicate that malaria GPI is sufficient to act as a toxin but do not establish that it plays this role in the context of authentic disease processes. We therefore sought to test this proposition in a credible pre-clinical model of disease. *P. berghei* ANKA murine malaria has salient features in common with several aspects of the human severe

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and cerebral malaria syndromes and is thus an accepted model for certain important aspects of the human disease (De Souza & Riley, 2002). It manifests a cytokine-dependent encephalopathy associated with up-regulation of adhesins on the cerebral microvascular endothelium and attendant neurological complications (Grau *et al*. 1987, 1989*a*; 1991; Jennings *et al*. 1997). The more generalized syndromes of pulmonary oedema, lactic acidosis, coagulopathies, shock and renal failure are also observed associated with fatalities (Chang *et al*. 2001), indicating that the *P. berghei* ANKA infection models many aspects of clinically severe malaria beyond narrow definitions of cerebral symptomology. Unlike most, but not all, human cerebral malaria cases, however, there is a macrophage infiltrate and compromised blood-brain barrier in the terminal or agonal stages of the murine syndrome. Nonetheless, in the proximal or developmental stages the murine disease is accepted to reflect more accurately the cytokine-dependent inflammatory cascade leading to cerebral and systemic involvement in humans. As recently reviewed (De Souza &Riley, 2002; Miller *et al*. 2002), *P. berghei* ANKA thus appears the best available small animal model of clinically severe malaria, suitable for investigations into proximal events controlling the systemic inflammatory cascade, and particularly the regulation of cytokine-dependent events and pathology.

Chemical synthesis of the non-toxic glycan moiety of mature *Plasmodium* GPI has recently provided a means to test the hypothesis that malaria GPI is causally involved in rodent malaria pathogenesis (Schofield *et al*. 2002). To prepare an immunogen, the synthetic GPI glycan was conjugated to the carrier protein keyhole limpet haemocyanin (KLH-glycan). Antibodies from mice immunized with KLH-glycan reacted only with malaria-infected rather than uninfected red blood cells, as demonstrated by immunofluorescence and Western blot analyses (Schofield *et al*. 2002). These results suggested that GPI of parasite origin was sufficiently different from host endogenous GPI molecules (which differ by virtue of amino-sugar or phosphoethanolamine modifications to the core glycan) to be recognized as foreign (non-self). These antibodies specifically neutralized the production of TNF-α from macrophages induced by *P. falciparum* extracts, confirming their anti-toxic properties *in vitro*. When challenged with *P. berghei* ANKA, naïve and sham-immunized mice all died within 5–8 days post infection (p.i.) with severe cerebral syndrome, whereas 75% of those mice immunized with the KLH-glycan were protected (Fig. 1a). There were no significant differences in parasite counts evident at this early stage of infection, but ultimately all mice succumbed at ~15 days p.i. to severe haemolytic anaemia (Fig. 1b) (Schofield *et al*. 2002) – indicating that parasite growth was unaffected by the antibodies. In studying the effectiveness of KLH-glycan vaccination, the immunized mice were found to be protected against occlusion of brain vasculature (Fig. 1c), pulmonary oedema (Fig. 1d) and blood acidosis (Fig. 1e) (Schofield *et al*. 2002). These findings not only demonstrated the efficacy of this prototype anti-toxic vaccine, but also showed that *Plasmodium* GPI is central to the pathogenesis of systemic disease endpoints and the development of a lethal cerebral syndrome in this model. Thus, GPI of *P. falciparum* satisfies the criteria for a predicted malaria toxin and may thereby contribute to life-threatening disease in humans, albeit a requirement in human malarial pathogenesis and fatality remains to be proven (Boutlis *et al*. 2002; Suguitan *et al*. 2004).

PLASMODIUM FALCIPARUM GPI DISTRIBUTION, METABOLISM AND STRUCTURE

GPI molecules represent a ubiquitous class of glycolipids that function as anchors for transmembrane proteins in eukaryotic cells. The parasitic protozoa are unusual in that they express very high levels of GPI-anchored proteins, GPI-anchored glycoconjugates and a range of unconjugated 'free' GPIs and GPI-like structures (GIPLs). Compared with mammalian cells, which typically express in the order of 10^5 GPI copies per cell, various parasitic protozoa express over 10^7 GPI copies per cell and most of these GPI-anchored molecules are displayed at the cell surface (Ferguson *et al*. 1994). In *P. falciparum*, which shows uniquely low levels of *N*- and *O*-linked glycosylation, GPI anchors constitute over 95% of the post-translational carbohydrate modification of parasite proteins (Davidson & Gowda, 2001; Gowda, Gupta & Davidson, 1997). Recent genome-wide sequence analyses predict that approximately 0·5% of the *P. falciparum* proteome may be post-translationally glycosylated with a GPI anchor, which is typical for a lower eukaryote (Eisenhaber, Bork & Eissnhaber, 2001). From microarray transcription data about 2/3 of these proteins are thought to be expressed in asexual blood stages (P. Gilson, personal communication). Among the GPI-anchored proteins in *Plasmodium*, the most important are the circumsporozoite protein (CS-2) which coats the insect stage sporozoites, and the merozoite surface proteins (e.g. MSP-1, MSP-2, MSP-4, MSP-5, MSP-10) expressed at the extracellular blood stage of the parasite. All of these proteins are of particular interest because of their central role in host/parasite interactions and in the response to host immunity, and several are under consideration as vaccine candidates. In addition to GPI-anchored proteins, *Plasmodium* species also contain an abundant pool of structurally identical GPI molecules which are not conjugated to proteins or other structures but exist free in the plasma membrane (free GPIs). In fact, quantification of the relative

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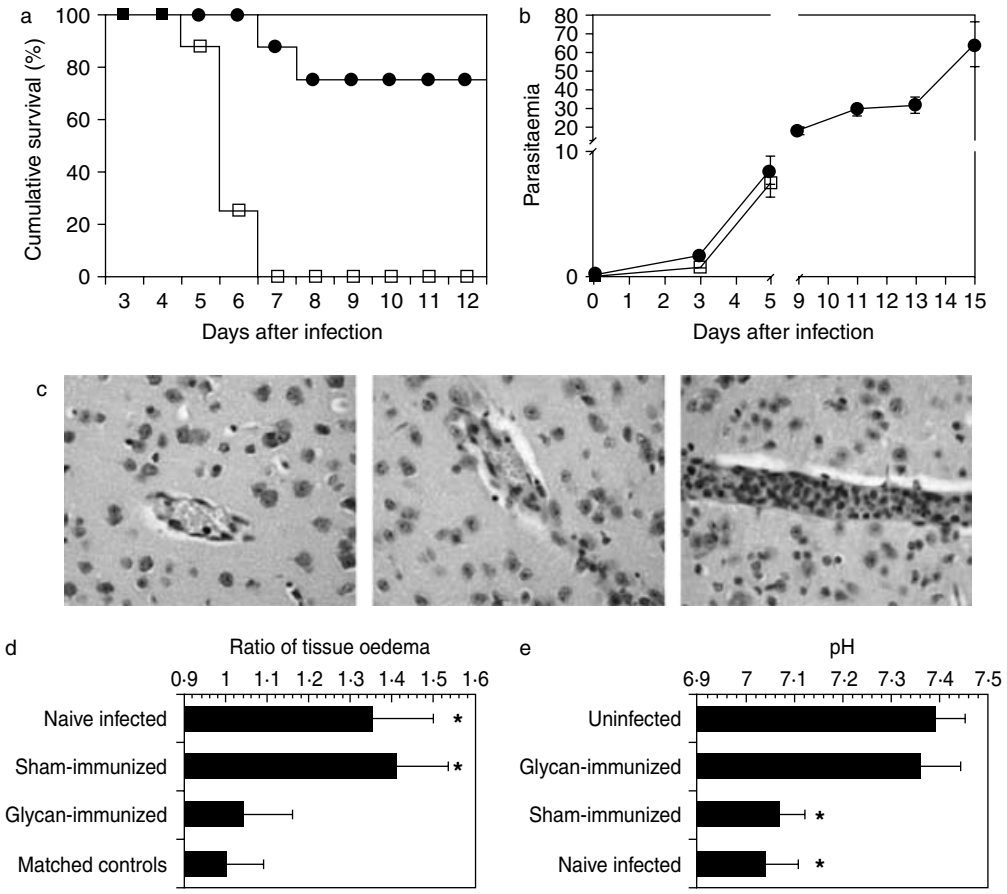

Fig. 1. Immunization against synthetic malaria GPI glycan substantially protects against murine cerebral malaria, pulmonary oedema and peripheral blood acidosis. (a), (b) Kaplan-Meier survival plots and parasitemia levels of KLH-glycan-immunized (filled circles) and sham-immunized (open squares) C57BL/6 mice challenged with *P. berghei* ANKA. (c) Haeomotoxylin and eosin-stained sections of brain tissue showing blood vessels from KLH-glycan immunized (left and centre panels) and sham-immunized (right panel) mice killed 6 days p.i. Pulmonary oedema and blood acidosis indices given as a proportion of the lung wet:dry weight ratio (d) or serum pH (e) form KLH-glycan-immunized and, sham-immunized and naïve infected mice at day 6 p.i. compared to age/sex-matched uninfected mice. Taken from Schofield *et al*. (1993). Permission pending.

amount of GPI molecules purified from late schizonts shows a 4-fold molar excess of free versus serine-linked GPIs (resulting from exhaustive pronase digestion of GPI-anchored proteins) by gas chromatography mass spectrometry (GC/MS), suggesting that free GPIs are the predominant GPI-anchored molecules present at the time of schizont rupture (K. Evans, personal communication). These free GPIs appear to be metabolic end-products and functionally important components in their own right.

Despite the diversity of GPI-anchored molecules found in parasitic protozoa, fungi and mammalian cells, all eukaryotic GPI membrane anchors have a common core structure, consisting of a conserved trimannosylglucosaminyl glycan (Man_3-GlN: Manα1,2-Manα1,6-Manα1,4-GlcN; Fig. 2, bold letters) linked to the inositol residue of a phosphatidylinositol (Man_3-GlN-PI). The glycan core of GPI anchors carries an ethanolamine phosphate (EtN-P) group on the third mannose (Etn-P-Man_3-GlN-PI), making it a substrate for GPI transamidase. This enzyme cleaves the peptide bond at the GPI-anchor attachment site near the carboxy-terminal end and creates an amide linkage between the ethanolamine of GPI and the newly generated carboxyl group of the cleaved protein precursor. GPI molecules are assembled in the endoplasmic reticulum (ER) by the sequential addition of *N*-acetylglucosamine, core mannose and EtN-P residues to PI through the coordinated action of a series of glycsyl- and ethanolaminephosphate-transferases. Critical evaluation of sequence similarity data for malaria homologues of proteins essential for GPI synthesis indicates that the machinery for GPI assembly has been reduced to minimal requirements in *Plasmodium* (Delorenzi *et al*. 2002). Although the essential features of this pathway are conserved in all eukaryotes, considerable differences occur in the extent and nature of side chain additions to the glycan backbone as well as the nature of the lipid anchor (for excellent reviews on GPI structure and biosynthesis in parasitic protozoa see (McConville & Ferguson, 1993; McConville & Menon, 2000). As a result, the core glycan may be

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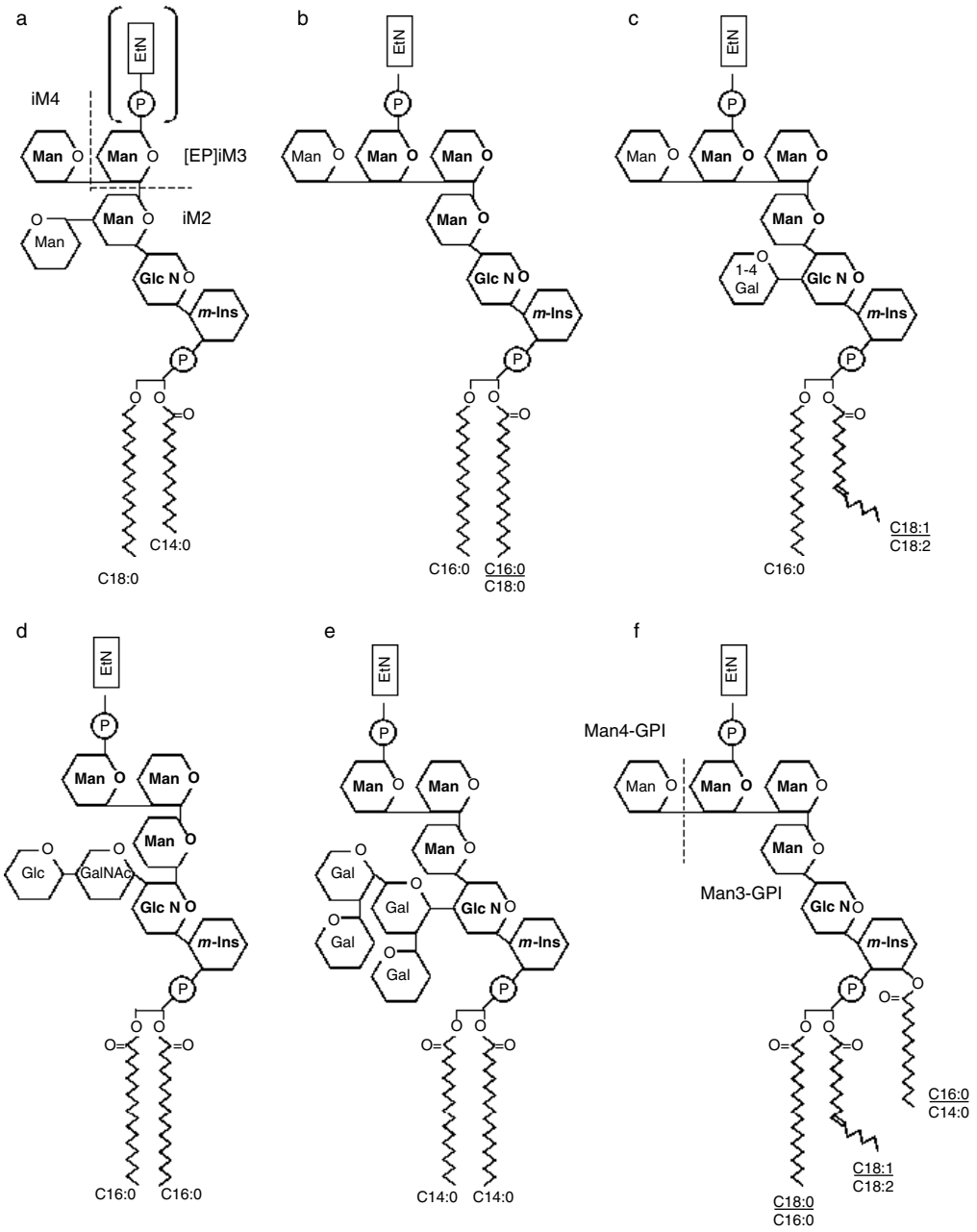

Fig. 2. Schematic structure of parasitic GPI-anchors: (a) *Leishmania mexicana* GIPLs (free iM2, iM3, [EP]iM3 and iM4 GPI forms); *Trypanosoma cruzi* epimastigote (b) and trypomastigote (c) mucin-derived GPI anchors; (d) *Toxoplasma gondii*-derived GPI anchor (e) *Trypanosoma brucei* VSG-derived GPI anchor (f) immature (Man_3-GPI) and mature MSP-1-derived (Man_4-GPI) GPI anchors of *Plasmodium falciparum*. Abbreviations: EtN, ethanolamine; P, phosphate; Man, mannose; GlcN, unacetylated glucosamine; Gal, Galactose; GalNAc, acetylated galactosamine, Glu, glucose; *m*-Ins, *myo*-Inositol; O, ether and ester bonded Oxygen molecules; Cn:n, number of saturated/ unsaturated carbon molecules in the lipid chain.

decorated with accessory carbohydrate (galactose, *N*-acetylglucosamine, sialic acid) side branches or EtN-P groups in a species- and tissue-specific manner (Fig. 2a-f). Other variations in the GPI structure are found in the lipid backbone (glycerol versus ceramide) and the number (mono-, di-), linkage (acyl versus alkyl), as well as the length and degree of saturation of the lipid chains (i. e. C14:0; C16:0, C18:0, C18:1, C18:2) in various GPIs (Fig. 2a–f).

The structure of *P. falciparum* GPIs was first characterized by Schwarz and colleagues (Gerold, Diekmann-Schuppert & Schwartz, 1994), and is one of the simplest described to date (Fig. 2f). Analysis of radiolabeled GPIs suggested that the parasite synthesizes two mature GPI species, which were shown to correspond to a trimannosyl GPI (Fig 2f, Man_3-GPI) and a tetramannosyl GPI (Fig 2f, Man_4-GPI). The Man_3-GPI consists of the evolutionarily conserved core glycan linked to a diacylglycerol (EtN-P-Man_3-GlN-PI-DAG), whereas the Man_4-GPI carries a terminal fourth mannose residue in α1,2 linkage to the third mannose residue

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(EtN-P-Man_4-GlN-PI-DAG). A report that the terminal fourth mannose residue carries an additional charged substituent (Gowda *et al*. 1997) remains unconfirmed. Detailed structural analysis by GC-MS has essentially confirmed the monosaccharide composition of the glycan moiety, but found considerable structural heterogeneity of native *P. falciparum* Man_4-GPI due to variation in fatty acid substituents of the lipid moiety (Naik *et al*. 2000). GC-MS analysis of HPLC-purified parasite GPIs treated with hydrofluoric acid or phospholipase A2 (for selective removal of fatty acids at the sn-1 and/or sn-2 position of the PI moiety, respectively) detected the presence of at least five distinct GPI diacylglycerol structures in the total free Man_4-GPI pool (Naik *et al*. 2000). These contained predominantly saturated stearic (C18 : 0) acid and unsaturated oleic (C18 : 1) acid esterified at the sn-1 and sn-2 positions, respectively, and had mainly palmitic (C16 : 0) acid on C2 of the inositol ring of the core glycan (Naik *et al*. 2000). The differences in fatty acid composition compared with earlier reports may reflect the preferential incorporation of various fatty acids present in different growth media into early GPI precursors (Mitamura *et al*. 2000; Krishnegowda & Gowda, 2003). Importantly, parasite proteins were found to be anchored almost exclusively by the Man_4-GPIs (Naik *et al*. 2000; Gerold *et al*. 1994), indicating a high degree of selectivity towards the presence of the fourth, terminal mannose residue in the GPI anchor moiety. By contrast, all five Man_4-GPI structures were found to be used for protein anchoring (Naik *et al*. 2000), irrespective of their lipid composition. Overall, *Plasmodium* GPIs therefore appear to represent an evolutionarily conserved glycolipid structure with a high level of variation in the lipid moiety (the precursors to GPI synthesis) and a structural requirement for the mature Man_4-GPI species (the substrate of GPI transamidase) with regard to protein anchoring.

STRUCTURE: ACTIVITY

The studies described in the previous sections demonstrated that *Plasmodium*-derived GPIs display potent pro-inflammatory activities that contribute to parasite-associated pathogenesis. Understanding the molecular details of the GPI structure-activity relationship and cell signalling mechanisms involved in these processes may therefore offer valuable insights that could lead to the development of novel therapeutics for malaria. Initial work on murine macrophages and human endothelial cells demonstrated that GPIs of *P. falciparum* and *T. brucei* origin can impart at least two discrete signals through structurally distinct elements. The core GPI glycan moiety was found to act as an agonist for the activation of protein tyrosine kinase (PTK), whereas the GPI diacylglycerol moiety was shown to function as a second messenger substrate required for protein kinase C (PKC) activation (Tachado *et al*. 1997). Of the seven or so PKC members detectable in macrophages, activation by GPI diacylglycerols was restricted to the ε isoform. Activation was associated with a translocation from the cytoplasm to detergent-insoluble membranes. Transient tyrosine phosphorylation of *src*-family non-receptor tyrosine kinases (e.g. hck, lyn), which peaks within minutes after the addition of low concentrations (10 nM – 1 μM) of GPI to cells, is the earliest measurable event in GPI-mediated signal transduction (Tachado *et al*. 1997). Although *lyso*-GPI and deacylated inositolglycan generated by specific enzymatic degradations (i.e. PLA_2, PLD) were shown to be sufficient to activate PTKs, they were found not to promote the full range of signalling events required for the activation of macrophages. In addition, inhibition of signal transduction by specific PKC and NF-κB antagonists could block the production of cytokines in response to *P. falciparum*-derived GPIs (Tachado *et al*. 1997). Taken together, these data suggested that GPI-mediated PTK and PKC signals synergise to activate transcription factors from the NF-κB family for the induction of *de novo* expression of pro-inflammatory loci (e.g. TNF-α, IL-1, IL-6, iNOS, ICAM-1).

One of the important questions raised by these studies relates to the fine structure requirements with respect to the bioactivity of *P. falciparum* GPIs on host cells. Using structurally defined *Plasmodium* GPI precursors which differ in the degree of glycosylation together with specific enzymatic and chemical treatments for the selective removal of diacylglycerol or PI-diacylglycerol structures, it was possible to map the minimum structural requirement for PTK activation to the evolutionarily conserved Man_3-GlcN-PI moiety (Tachado *et al*. 1997). Consistent with this result, the biological activity of total parasite extract, including TNF-α output, can be inhibited by high concentrations of GPI partial structures such as α-methyl mannoside, glucosamine, and phosphatidylinositol. Vijaykumar *et al*. essentially confirmed and extended these findings by studying the inhibition profile of *P. falciparum* GPI bioactivity (Vijaykumar *et al*. 2001). In this study, authors used chemically defined GPI fragments generated by enzymatic treatment of HPLC-purified Man_4-GPI with α-mannosidase (for selective removal of the terminal fourth mannose residue on the core glycan) or chemical treatment with hydrofluoric acid or nitrous acid (for selective cleavage of phosphate or amino bonds of the core glycan, respectively). Consistent with results by Tachado *et al*. (1997), none of the GPI partial structures alone were able to induce TNF-α production by macrophages (Vijaykumar *et al*. 2001). Prior incubation of macrophages with inactive Man_4-containing carbohydrate fragments (but not the PI or DAG

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fragments) released by HF or nitrous acid was found to effectively inhibit the activity of intact Man_4-GPI (Vijaykumar *et al*. 2001). In contrast to intact Man_4-GPI, the authors reported that *P. falciparum* Man_3-GPI failed to activate macrophages, nor did Man_3-containing carbohydrate fragments inhibit the ability of Man_4-GPI to induce TNF-α production (Vijaykumar *et al*. 2001). Surprisingly, the same group subsequently published a paper reporting 80% of TNF-α output from macrophages in response to Man_3-GPI compared to Man_4-GPI (Krishnegowda *et al*. 2005). The data are therefore inconsistent and at odds with the different inhibition profile of soluble Man_4-containing and Man_3-containing carbohydrate fragments on *P. falciparum* GPI activity (Vijaykumar *et al*. 2001). Although discrepancies with regard to the bioactivity of intact Man_3-GPI remain to be resolved, current observations indicate that the fourth, terminal α-mannose residue linked to the core glycan moiety may be critical for the activity of *P. falciparum* GPIs.

The precise structural requirement of the lipid moiety for the bioactivity of *P. falciparum* GPIs still remains to be determined. Structure-activity data from different protozoan parasites indicates that ceramide or saturated lipid chains in the monoalkyl or 1-alkyl-2-acyl-glycerol GPIs moieties of *Leishmania* spp (Fig. 2a) or *T. cruzi* epimastigote forms (eGPI, Fig. 2b) induce relatively low or no activity in macrophages (Camargo *et al*. 1997*b*), compared to the bioactive *T. gondii* (Fig. 2d), *T. brucei* (Fig. 2e) and *P. falciparum* (Fig. 2f) diacylglycerol-containing GPI structures. Moreover, the bioactive GPIs of *T. cruzi* trypomastigote forms (tGPI, Fig. 2c) and *P. falciparum* GPI anchors (Fig. 2f) contain mainly unsaturated fatty acid chains at the sn-2 position in the 1-alkyl-2-acyl-glycerol and 1,2-diacylglycerol moieties, respectively (reviewed by (Almeida & Gazzinelli, 2001; Ropert *et al*. 2002). Whether the presence of the unsaturated fatty acid chain at the sn-2 position of *P. falciparum* GPI is actually required for its bioactivity is however unclear. Tachado *et al*. (1997) found that the potency of phospholipase A_2-generated sn-2 lyso GPIs of *P. falciparum* is drastically reduced, whereas Vijaykumar *et al*. (2001) and Krishnegowda *et al*. (2005) reported that these molecules retained nearly all activity. The latter findings are at odds with the observation that chemical removal of fatty acids at the sn-2 position is sufficient for a drastic ($\sim$100fold) reduction in the ability of highly purified *T. brucei* and *T. cruzi* trypomastigote GPIs to activate macrophages (Camargo *et al*. 1997*b*; Tachado *et al*. 1997, Almeida *et al*. 2000). The reason for this discrepancy is not clear, but is likely to be due to the difficulty in obtaining adequate amounts of pure, structurally-defined native GPIs. The preparation of native malarial GPI is a labour-intensive process that requires the large-scale growth of *P. falciparum* in human red blood cell cultures. All growth media used must be endotoxin-free and cultures need to be rigorously tested for mycoplasma contamination before being processed, to eliminate the likelihood of potential contamination by bacterial lipopolysaccharides and mycoplasma-derived lipopeptides. The preparation of malarial GPI itself involves a series of optimized glycolipid extraction and fractionation steps using organic solvents and at least one round of RP-HPLC followed by analytical HP-TLC and GC-MS analyses. The yield of pure native GPI resulting from this procedure is typically low – in the range of 10 μg per 1 litre of culture – and this has proven to be probably one of the most limiting factors in the laboratory setting. The chemical synthesis of partial *Plasmodium* GPI structures (Schofield *et al*. 2002) as well as entire protozoan GPI-anchors (Debierre-Grockiego *et al*. 2003) may soon help to resolve some of the questions regarding the detailed structural requirements for malarial GPI bioactivity.

SIGNALLING PATHWAYS TRIGGERED BY *PLASMODIUM* GPI – THE TWO-SIGNAL MODEL

The molecular mechanisms of protozoan glycolipid-host cell interaction are poorly understood. In particular, how activation signals mediated by protozoan GPIs are transmitted across the plasma membrane to the interior of host immune cells remains unclear. Tachado *et al*. (1997) proposed a working hypothesis to explain how bioactive GPIs of *T. brucei* and *P. falciparum* can activate host macrophages. The initial interaction is predicted to involve the binding of parasite-derived glycolipids to a putative glycan-specific transmembrane receptor present in the surface of host macrophages. This agrees with the observation that stimulation of macrophages with parasite GPI glycan fragments alone is sufficient to cause rapid transient activation of *src*-family non-receptor PTKs in host macrophages. Although PTK activation does not provide the full range of signalling events leading to downstream inflammatory gene expression, capture by a glycan-specific receptor is predicted to catalyze the transfer of GPI from the extracellular space to the host membrane. Within the plasma membrane, GPI anchored molecules are known to diffuse laterally and partition into sphingolipid- and cholesterol-rich liquid-ordered microdomains which function both as signalling platforms and endosomal entry and sorting sites. Membrane-permeant diacylglycerol, formed via the hydrolysis of parasite-derived GPI molecules by PLD present in the serum or on the surface of mammalian macrophages (Krishnegowda *et al*. 2005), could cross cellular membranes to activate an intracellular PKC. Both PTK and PKC signalling pathways were shown to collaborate to activate transcription factors of the NF-κB/rel family and thereby promote expression of pro-inflammatory cytokines. Although certain

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aspects of this model remain unclear, it has provided a useful hypothetical framework that agrees with the observed structural requirement for both carbohydrate and fatty acid moieties of intact GPI-anchors of *P. falciparum*, *T. brucei*, *T. cruzi* and *T. gondii* origin for their full pro-inflammatory activity on macrophages (Almeida *et al.* 2000; Sileghem *et al.* 2001; Debierre-Grockiego *et al.* 2003). The major unresolved issues concerning this two-signal model for GPI-specific signal transduction are the identities of the glycan-specific transmembrane receptor and the molecular mechanism by which GPI-derived lipids contribute to the signalling pathways triggered by parasite-derived GPIs.

Toll-like receptor signalling pathways activated by parasite glycolipids

Recent efforts to identify the parasite glycolipid-specific receptor responsible for GPI-mediated transmembrane signalling have focused predominantly on certain members of the evolutionary conserved Toll-like receptor family. TLRs have emerged as key receptors responsible for recognizing conserved pathogen-associated molecular patterns (PAMPs) on microbes including modified lipids (e.g. bacterial lipopolysaccharides and lipoproteins), carbohydrates (e.g. yeast zymosan), proteins (e.g. flagellin), and nucleic acids (e.g. unmethylated CpG DNA and double-stranded RNA) (reviewed by Takeda, Kaisho & Akira, 2003; Akira & Takeda, 2004). The ten members of the human TLR family of receptors (TLRs 1-10) can form homo- or heterodimers with other TLR members or specific accessory proteins like CD14 to form a high-affinity binding site for multiple diverse ligands. This dimerisation triggers association with a family of adaptor molecules including MyD88, Mal/TIRAP and TRIF (O'Neill, 2003; McGettrick & O'Neill, 2004) that mediate downstream activation of IL-1R-associated kinases (IRAKs) and mitogen-activated protein kinases (MAPKs) that lead to the activation of NF-κB-mediated transcription of inflammatory cytokine genes.

The proposed involvement of different members of TLRs in cell signalling by parasite glycolipids was based on the observation that the pattern of macrophage activation by *T. cruzi* mucin-derived GPI-anchors is analogous to that of bacterial lipopolysacharide (LPS). Initial studies showed that, trypomastigote mucin GPIs are capable of triggering different MAP kinases (e.g. ERK1-2, MKK4 and p38) and NF-κB with kinetic and inhibition profiles similar to those triggered by LPS (Ropert *et al.* 2001). It was further demonstrated that an efficient response to bacterial lipopolysaccharide *in vivo* requires the formation of non-covalent CD14/LPS receptor interactions with the TLR-4/MD2 transmembrane receptor complex (Wright *et al.* 1990; Poltorak *et al.* 1998; Nagai *et al.* 2002). However, macrophages from LPS-hypo-responsive C3H/HeJ mice with mutations in the TLR-4 receptor were still responsive to *T. cruzi*-derived GPI anchors or GPI-mucins (Ropert *et al.* 2001) – suggesting that these glycolipids trigger another member of the TLR family. Subsequent *in vitro* studies in mammalian cell lines transfected with different TLR expression plasmids and NF-κB-specific reporter constructs suggested that *T. cruzi* parasites GPI activate TLR-2 (Campos *et al.* 2001). Interestingly, GPI anchors derived from trypomastigote and epimastigote stages had variable activities in activating NF-κB-dependent gene expression in cell lines expressing CD14 and TLR-2 (Campos *et al.* 2001) – depending on the presence of extra galactose residues in the glycan core and unsaturated fatty acids in the sn-2 position of the alkylacylglycerolipid moiety for maximum activity. This agrees with the different potency of tGPIs and eGPIs in mouse macrophages and suggested that *T. cruzi* GPI-activation of TLR-2 is essential for the production of proinflammatory cytokines *in vitro*.

However, *in vivo* studies in TLR and MyD88 knockout mice in the susceptible C57BL/6 background have provided a rather more complex picture of the role of TLR-mediated signalling during acute *T. cruzi* infection. For example, when exposed to live *T. cruzi* trypomastigotes *in vitro*, macrophages from MyD88-deficient mice responded to neither purified trypomastitote GPIs nor live *T. cruzi* parasites (Campos *et al.* 2004). By contrast, macrophages from TLR-2 null mice, despite being less responsive to trypomastigote GPIs, were found to still produce pro-inflammatory cytokines when stimulated with live parasites (Campos *et al.* 2004). Moreover, whereas both MyD88 and IFN-γ knockout mice showed signs of enhanced susceptibility to *T. cruzi*–mediated lethality, TLR-2-deficient mice were almost as resistant to infection as wild-types (Campos *et al.* 2004). Taken together, the results indicate that the deficiency of a single TLR, such as TLR2, may not have a major impact during acute *T. cruzi* infection *in vivo*. This implies the existence of multiple TLR-dependent signalling pathways being activated during acute infection with live *T. cruzi* trypomastigotes, involving both mucin GPI anchors and probably other parasite-derived bioactive molecules. Consistent with similar findings in *Toxoplasma gondii* (Chen *et al.* 2002; Scanga *et al.* 2002) and *Leishmania major* (Hawn *et al.* 2002; De Veer *et al.* 2003; Muraille *et al.* 2003) infections, these data for the first time implied a major role for a TLR/MyD88-dependent signalling pathway in the early production of pro-inflammatory cytokines and host resistance to different parasites.

Current evidence for the involvement of TLR/MyD88-dependent mechanisms in the immunoregulation and immunopathogenesis of *Plasmodium*

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malaria is still sparse. A study in the BALB/c mouse model of *P. berghei* liver-stage infection demonstrated that cytokine-mediated liver injury depends on the TLR/MyD88 signalling pathway. This study found that both IL-12 and MyD88-deficient BALB/c mice infected with *P. berghei* parasites were unaffected by liver damage with reduced production of IL-12 but not IL-18 (Adachi *et al.* 2001). *P. berghei* infection therefore appears to activate a MyD88-dependent pathway to specifically induce IL-12, which in turn results in cytotoxic T cell lysis of hepatocytes and liver injury in experimental animals. However, none of the examined Toll-receptor-deficient strains (TLR-1, TLR-2, TLR-4, TLR-6 or TLR2/4 double-knockout mice) were found to affect IL-12 production or protect from liver damage (Adachi *et al.* 2001). The particular TLR involved in this process remains to be elucidated. It should be noted that cytokine-induced liver injury in this experimental system does not appear to model any known pathophysiological disease processes occurring in human malaria infections.

In contrast to malarial liver stages, which are clinically silent and short lived, severe malarial pathogenesis is largely mediated by the *Plasmodium* asexual blood stages. Therefore, it is of great interest to investigate the role of the TLR/MyD88 pathways during *P. berghei* ANKA-mediated malaria in the C57BL/6 strain susceptible to cerebral syndromes. A recent study reported that both human and murine plasmacytoid dendritic cells (pDCs) are activated by *Plasmodium* schizonts via a TLR-9/MyD88-dependent mechanism (Pichyangkul *et al.* 2004) to produce IFN-α. Coban *et al.* (2005) went on to show that haemozoin, a crystalline by-product derived from the digestion of erythrocytic haemoglobin is responsible for the activation of TLR-9 by malarial extracts. Members of the TLR-7 and TLR-9 subfamily are known to sense viral and bacterial CpG-DNA at the endosomal subcellular compartment via a strictly MyD88-dependent signalling pathway (Latz *et al.* 2004; Lund *et al.* 2004). Pro-inflammatory cytokine production, including TNF-α and IL-12p40 from Flt ligand-derived dendritic cells treated with malarial extracts is totally dependent on TLR-9 and MyD88 and independent of TRIF, another TLR adaptor involved in MyD88 independent TLR signalling (Coban *et al.* 2005). Production of IL-6 in sera of mice injected with purified haemozoin was dependent on MyD88 and TLR-9 (Coban *et al.* 2005) – suggesting the *in vivo* response to haemozoin is also MyD88 dependent (Coban *et al.* 2005). It should be noted however that haemozoin preparations vary in purity and may also be associated with bioactive contaminants.

While haemozoin is a TLR 9 ligand little else was known about other TLR activating components present in malarial extracts. A recent publication from Krishnegowda *et al.* (2005) has shown that purified *P. falciparum* GPI coupled to gold particles efficiently activates *in vitro*-derived bone marrow macrophages to produce pro-inflammatory cytokines in a MyD88 dependent manner. TNF-α production was shown to be 80% and 20% of that produced in macrophages from wild type mice when macrophages were derived from TLR4 and TLR2-null mice respectively. No significant activation of TNF-α was detected in macrophages isolated from either mice deficient in both TLR2/TLR4 or MyD88-null mice. This suggests that malarial GPI signalling in isolated bone marrow derived macrophages is primarily dependent on TLR2 and absolutely requires MyD88. Additional studies in HEK-293 cells transfected with different TLR expression plasmids and dual luciferase reporter constructs suggest that TLR2/TLR1 and TLR2/TLR5 heterodimers can differentially recognize GPIs containing three and two fatty acid substituents (Krishnegowda *et al.* 2005), in a manner similar to the discrimination of triacylated bacterial lipoproteins and diacylated mycoplasma lipoproteins (Takeda, Takeuchi & Akira, 2002). It would have been of interest to know the effects of other TLR-activating protozoal GPIs coupled to gold particles, as well as non TLR-activating glycolipids as a more relevant control than just gold particles alone. Importantly, whether TLR activation by *Plasmodium* GPI is a significant factor contributing to the pathogenesis of severe malaria syndromes *in vivo*, as implied by these studies, remains to be addressed.

It appears that GPI is important in the pathogenic cascade initiated by malarial infection as vaccination with the glycan moiety dramatically protects mice from cerebral symptoms (Schofield *et al.* 2002). Whether this pathogenic activity is mediated by TLR activated pathways or other mechanisms, is a crucial issue that remains to be established. To help address whether TLRs play a role in malarial pathogenesis we infected mice deficient in MyD88, which are unresponsive to all known TLR-2 and TLR-9 stimuli (McGettrick & O'Neill, 2004) including all the assays performed with malarial GPI (Krishnegowda *et al.* 2005) and haemozoin (Coban *et al.* 2005). Preliminary data indicate that while these mice fail to induce IL-12p40 in response to infection there is no significant difference in peak parasitaemia or death rates in MyD88$^{-/-}$ null mice compared to MyD88$^{+/-}$ heterozygous littermates (unpublished observation). This is in stark contrast to the protective effect observed in both IFN-γ and IFN-γ receptor knockout mice (Amani *et al.* 2000) and in mice immunized with the glycan portion of malarial GPI (Schofield *et al.* 2002). These data suggest that MyD88-independent pathways are the primary effectors involved in the initiation and amplification of the acute cerebral syndrome that is the pathogenic mode of action in this model of cerebral malaria.

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A similar MyD88-independent mechanism appears to be responsible for the induction of IL-18 after *P. berghei*-infection of the resistant BALB/c strain (Adachi *et al*. 2001). Indeed, there are at least four additional MyD88-like adaptor moleules that may associate with TLRs to induced systemic inflammation (e.g. TIRAP/Mal, TRIF/TICAM-1 and TRAM/TICAM-2) (Horng *et al*. 2002; Yamamoto *et al*. 2002, 2003*a*, *b*). Together, they are capable of providing specificity for MyD88-independent signalling by TLRs 1-4 and 6, but whether *Plasmodium* schizonts or purified glycolipids activate any of these TLRs is currently unknown. The activation of NKT-cells is important in the pathogenesis of rodent malaria (Hansen *et al*. 2003) and this offers another TLR-independent mechanism for the production of IFN-γ and initiation of the cascade that ultimately can lead to cerebral syndrome in the *P. berghei* model of malaria. Further studies will be required to dissect the potential role of *P. falciparum* GPI in the activation of multiple MyD88-dependent and -independent pathways in *P. berghei*-infected C57BL/6 mice and to elucidate the possible contribution of TLRs to severe malarial pathogenesis.

RECOGNITION OF PARASITE GPIS BY NON-TLR PATTERN RECOGNITION RECEPTORS

In addition to Toll-like receptors, cells of the immune system are equipped with many other glycan-specific surface proteins that function as pattern recognition and uptake receptors on macrophages and DCs. Many of these are members of the Ca^{2+}-dependent C-type lectin family and recognize their ligands through the structurally related Ca^{2+}-dependent carbohydrate-recognition domains (CRDs) (Cambi & Figdor, 2003). Pathogen recognition by lectin-like receptors (LLRs), such as the soluble serum mannose-binding protein (MBP) (Gadjeva, Takahashi & Tniel, 2004), the macrophage cell-surface mannose receptor (MMR) (Ezekowitz *et al*. 1990) or the dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) receptor (Cambi *et al*. 2003), is mediated by binding of terminal mannose residues characteristic of viral, bacterial, fungal and parasite cell surfaces. Ligand binding most likely depends on subtle differences in the arrangements of carbohydrate residues and their branching – creating unique sets of carbohydrate recognition profiles.

Current observations support the idea of carbohydrate-specific interactions in the recognition of protozoan glycolipids by macrophages. It has been suggested, for example, that the potent cytokine producing activity of *T. cruzi* mucin GPIs purified from trypomastigote forms may be due to the precise number and/or structure of carbohydrate residues, since mild treatment with periodate largely destroys this activity (Camargo *et al*. 1997*a*, *b*). Compared to the low potency of *T. cruzi* mucin GPIs purified from epimastigote forms the high potency of trypomastigote GPIs is probably due to up to four extra α-galactosyl residues linked to the first mannose residue of the glycan core (Almeida & Gazzinelli, 2001). In agreement with this hypothesis, the presence of the galactose side-chain additions to the core glycan of highly purified GPI anchors of *T. brucei* origin were also shown to be essential for optimal TNF-α output from macrophages (Magez *et al*. 1998). It is possible that this carbohydrate pattern is recognized by TLR2, as evidenced by the potent induction of NFκB-dependent gene expression in transfected cell lines by trypomastigote compared to epimastigote GPIs (Campos *et al*. 2001). Recent work by Vijaykumar *et al*. (2001) suggested that cell signalling by *P. falciparum* GPI molecules, which lack the galactosyl side chain modifications of *T. cruzi* and *T. brucei* GPIs, may represent a novel mechanism involving the initial recognition of the distal fourth α-mannosyl residue. This proposal is based on the carbohydrate inhibition profile of *P. falciparum* Man_4-GPI activity on macrophages *in vitro*, as discussed above.

A good example of a recognition molecule able to differentiate between defined mannose-terminating patterns is the hepatocyte-derived soluble mannan-binding protein. MBP is an abundant C-type lectin found in various mammalian sera including human sera, and plays an important role in innate immunity. It is able to specifically recognize terminal mannose-, *N*-acetyoglucosamine and fucose-rich carbohydrate patterns on the surfaces of pathogenic microorganisms and initiates complement deposition on relevant (non-self) surfaces (reviewed by Gadjeva *et al*. 2004). In addition to carbohydrate patterns present on bacteria, viruses and fungi, it has been observed that MBP is also able to recognize major surface glycoconjugates and glycoproteins of *Leishmania* and *P. falciparum* parasites. For example, using thin layer chromatogram-overlay assays it was shown that MBP binds to the mannose-terminating di-, tri- and tetrasaccharide fragments derived from purified LPG and GIPLs that are highly expressed on promastigote and amastigote stages of most *Leishmania* species (Green *et al*. 1994). As demonstrated by immunofluorescence microcopy of live *L. major* and *L. mexicana* promastigotes (Green *et al*. 1994), FACS analyses show that human MBL may also function as an opsonin for schizont-infected erythrocytes (Klabunde *et al*. 2002). Attempts to identify specific parasite-derived surface ligands found that MBP binds to at least two glycoproteins that incorporated ^{3}H-glucosamine and were immunogenic in humans (Klabunde *et al*. 2002; Garred *et al*. 2003). In patients with homozygous mutations in the MBL gene complicated malaria was associated with significantly higher parasite burdens compared to MBL-competent counterparts (Garred *et al*. 2003), but whether MBP deficiency is a significant

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risk factor for severe malaria is still a matter of debate (Bellamy *et al*. 1998).

Whether tissue macrophages or dendritic cells express a specific lectin-like receptor, such as cell-surface mannose receptor or DC-SIGN capable of recognizing the terminal mannose residue on the core glycan of *Plasmodium* GPIs remains to be determined. Evidence is emerging that LLRs not only play a role as phagocytic antigen uptake receptors, but may allow signalling upon fusion of late endosomes/lysosomes via TLRs expressed on endosomal membranes (i.e. TLR-3, TLR-7, TLR-9). Whereas TLR signalling through MyD88, IRAK and MAPK resulting in the activation of NF-κB is quite well understood, signalling through lectin-like receptors via alternative pathways is still relatively obscure. Nevertheless, in view of the different dose-response and TLR/MyD88 signalling profiles of *T. cruzi* trypomastigote and *P. falciparum* GPIs, it appears that cellular immune responses to GPIs of various parasitic protozoa may involve multiple lipid- and carbohydrate-specific pattern recognition and signal transduction mechanisms.

SIGNALLING BY GPI-ANCHORED PROTEINS AND GLYOLIPIDS THROUGH LIPID-DEPENDENT MICRODOMAINS

A striking property of mammalian GPI-anchored proteins as well as glycolipids is that their ligation on the cell surface by natural ligands or suitable antibodies regulates numerous signal transduction and activation responses (reviewed by (Horejsi *et al*. 1998; Kasahara & Sanai, 2000). Such signalling capacity is surprising, considering that these molecules have no transmembrane or intracellular moieties and thus no direct contact with the cell interior. The signalling capacity of both endogenously expressed and exogenously added GPI-anchored proteins appears in many cases to involve their association with cytoplasmic signalling molecules in detergent-resistant membrane domains (DRMs). DRMs are stabilized against disruption by cold detergents through liquid-ordered packing of component cholesterol, (glycol)sphingolipids and glycerolipids. An important factor in this lipid-dependent organisation appears to be the length and fluidity (saturation) of the fatty acid residues attached with the GPI-anchored molecules (Schroeder *et al*. 1998; Benting *et al*. 1999). Low buoyant density DRMs, often called lipid rafts, can be isolated by density gradient ultracentrifugation. Such membrane fractions are characteristically enriched in GPI-anchored proteins, dually acylated cytoplasmic signalling or adaptor proteins (e.g. Src-family kinases, trimeric G proteins, Ras, LAT) and different raft-organizing proteins (e.g. caveolins, stomatins, flotillins) (Brown & London, 2000). *In vivo*, liquid-ordered membrane domains are thought to be quite small and/or extremely dynamic and consequently below the level of resolution of the light microscope (Jacobson & Dietrich, 1999). Thus, they are optimally investigated with biophysical techniques that can detect short-range interactions. Recent studies employing chemical crosslinking and fluorescence resonance energy transfer (FRET) microscopy techniques estimated that a fraction (20–40%) of GPI-anchored proteins form extremely tight clusters of nanometer size (~5–50 nm), each containing a few (4–15) different molecules on the surface of living cells (Friedrichson & Kurzchalia, 1998; Varma & Mayor, 1998; Sharma *et al*. 2004). Following cross-linking with antibodies or natural ligands these rafts can coalesce into visible and stable functional domains. Lipid-dependent membrane organization has been proposed to be responsible for the endocytosis and sorting of raft-associated proteins, and for providing platforms for the dynamic assembly of GPI-anchored proteins, transmembrane proteins and cytoplasmic signalling molecules in a variety of biological contexts (e.g. T cell receptor, B cell receptor and FcεRI signalling) (Simons & Toomre, 2000; Anderson & Jacobson, 2002; Mayor & Riezman, 2004).

Of particular interest in the present context of *Plasmodium* GPI signalling is accumulating evidence indicating that the innate recognition of different pathogens may involve the interaction of CD14 with various transmembrane receptors within supramolecular activation clusters (i.e. TLR-2, TLR-4, TLR-6, Mac-1, CXCR4, GDF5 and others) (Henneke *et al*. 2001; Pfeiffer *et al*. 2001; Triantafilou *et al*. 2001 *a*, *b*). By analogy to the immunological synapse, it has been proposed that the entire bacterial recognition system may be based around the formation of a signalling complex of receptors at the site of CD14-LPS ligation within membrane microdomains (Triantafilou & Triantafilou, 2002). Consistent with this theory, FRET analyses have shown that ligation of CD14 by LPS and ceramide provokes ligand-specific clustering and translocation of TLR4 into lipid rafts (Pfeiffer *et al*. 2001; Triantafilou *et al*. 2002). A recent study demonstrates that the atypical PKC isoform, PKC-ζ, is critical in the regulation of LPS-induced TLR4 lipid raft mobilization within macrophages (Cuschieri, Umanskiy & Solomkin, 2004). The cellular mechanisms involved in this process remain incompletely understood, but recruitment of PKCζ to plasma membrane lipid raft microdomains during insulin stimulation of adipocytes was shown to be mediated by TC10, a member of the Rho family of small GTP-binding proteins that is constitutively localized to plasma membrane lipid raft microdomains (Kanzaki *et al*. 2004).

It is possible that similar processes are required for signalling by biologically active parasite GPIs. We recently generated fluorescently-labelled semi-synthetic *P. falciparum* GPIs to study the

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specio-temporal distribution of exogenously added parasite glycolipids in the host cell membrane by confocal microscopy. When added to live mammalian tissue culture cells *in vitro*, these molecules were initially distributed homogeneously, but after several hours concentrated at cell-to-cell contacts and acquired a punctuate distribution on the cell surface (unpublished observation). These preliminary data demonstrate that a proportion of *Plasmodium* glycolipids incorporate into host plasma membrane, but whether the observed cell surface puncta represent sites involved in uptake or signalling by *Plasmodium* glycolipids is currently unknown. FRET analyses using fluorescently-tagged semi- or fully synthetic *Plasmodium* GPI molecules may allow us to further investigate the possible association of *P. falciparum* GPI with CD14, TLRs and other signalling molecules within plasma membrane microdomains in live cells.

CONCLUSIONS AND PERSPECTIVES

While our understanding of malarial GPI bioactivity has improved significantly over the past decade, several key issues remain to be resolved. For example, while the concept that the GPI anchors of parasitic protozoa constitute immunostimulatory and regulatory agents in the context of protozoan infections has been widely recognized, it is still difficult to pinpoint the precise molecular features of parasite GPIs that underlie the differences in their bioactivities. This is especially true in the case of malarial GPI toxin, with independent studies coming to different conclusions with regard to the structural requirement for the presence of the terminal fourth mannose residue on the core glycan and/or the unsaturated fatty acid chain at the sn-2 position of *P. falciparum* GPI for full activation of pro-inflammatory responses of macrophages. One of the main factors contributing to inconsistencies in the present data, and at the same time limiting the pace of our research, is the difficulty in obtaining adequate amounts of pure, structurally defined *P. falciparum* GPIs. The chemical synthesis of milligram quantities of partial GPI structures as well as the entire *Plasmodium* GPI-anchor will make this key reagent more accessible and facilitate the creation of novel probes that will help to resolve some of the questions regarding fine structural requirements for malarial GPI bioactivity, in the near future.

A significant development regarding the potential mechanism of GPI bioactivity has been the demonstration that GPI anchors of various parasitic protozoa can mediate cellular immune responses via the Toll-like receptor family. The original observation that *T. cruzi* trypomastigote GPIs can specifically activate TLR2/MyD88-dependent signalling pathways to produce inflammatory responses in host macrophages has recently been confirmed and extended using *P. falciparum* GPI. The use of macrophages from mice deficient in individual or multiple TLRs (e.g. TLR-2, TLR-4, TLR-2 & 4, TLR-9 etc.), essential adaptor molecules (i.e. MyD88), and downstream signalling molecules (e.g. ERK1/2, JNK-1 or JNK-2) has allowed investigators to dissect the relative contributions of specific TLR(s) and downstream signalling pathways activated by *P. falciparum* GPI and other malarial ligands. Recent studies indicate that malarial GPIs and haemozoins are preferentially recognized by TLR2/TLR1 and TLR9, respectively. Both rely on the MyD88-dependent activation of specific signalling pathways that mediate the production of proinflammatory cytokines and nitric oxide. However, the contribution of bioactive malaria products to severe malarial pathology and the role of TLRs or other pattern recognition receptors in innate immunity is only just beginning to be characterized. Importantly, the role of TLR/MyD88 pathways in mediating pro-inflammatory cytokine production and clearance of blood-stage parasites during the acute phase of malaria infections in mice infected with various *Plasmodium* species, as well as the role of GPI as a critical toxin in the context of human malaria infections remains to be firmly established. Given the complexity of the host/pathogen interface, signalling of malarial GPIs via TLR/MyD88-dependent pathways most likely represents only one example of how multiple pattern recognition receptors may function in regulating innate and adaptive immune responses in the context of protozoal infections. Further analysis of the interaction of *P. falciparum* parasites and the host immune system may shed new insights into the molecular mechanisms underlying severe malarial pathogenesis with potentially important implications for the development of novel therapeutics for malaria treatment.

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