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Glycosylphosphatidylinositols in Malaria Pathogenesis and Immunity: Potential for Therapeutic Inhibition and Vaccination

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Abstract Glycosylphosphatidylinositols (GPIs) are found in the outer cell membranes of all eukaryotes. GPIs anchor a diverse range of proteins to the surface of *Plasmodium falciparum*, but may also exist free of protein attachment. In vitro and in vivo studies have established GPIs as likely candidate toxins in malaria, consistent with the prevailing paradigm that attributes induction of inflammatory cytokines, fever and other pathology to parasite toxins released when schizonts rupture. Although evolutionarily conserved, sufficient structural differences appear to exist that impart upon plasmodial GPIs the ability to activate second messengers in mammalian cells and elicit immune responses. In populations exposed to *P. falciparum*, the antibody response to purified GPIs is characterised by a predominance of immunoglobulin (Ig)G over IgM and an increase in the prevalence, level and persistence of responses with increasing age. It remains unclear, however, if these antibodies or other cellular responses to GPIs mediate anti-toxic immunity in humans; anti-toxic immunity may comprise either reduction in the severity of disease or maintenance of the malaria-tolerant state (i.e. persistent asymptomatic parasitaemia). *P. falciparum* GPIs are potentially amenable to specific therapeutic inhibition and vaccination; more needs to be known about their dual roles in malaria pathogenesis and protection for these strategies to succeed.

Abbreviations

GPI	Glycosylphosphatidylinositol
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LT	Lymphotoxin
Man4	Fourth mannose
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
NF	Nuclear factor
NO	Nitric oxide
PKC	Protein kinase C
PTK	Protein tyrosine kinase
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor

1 Introduction

Plasmodium falciparum glycosylphosphatidylinositols (GPIs) became the focus of mainstream media attention in 2002 with the publication in *Nature* of a report demonstrating proof of concept for an anti-toxic vaccine that delayed malaria mortality in a rodent model (Schofield et al. 2002). As the best

characterised of the putative malaria toxins, interest in GPIs has burgeoned since the early 1990s to the point that strategies for creating fully synthetic GPI analogues are advancing rapidly (Lu et al. 2004; Liu and Seeberger 2004; Seeberger et al. 2004). This should soon enable investigators to clarify the role of GPIs in malaria pathogenesis and immunity. Understanding the potential for anti-GPI therapeutics and vaccination in human malaria requires an understanding of the pathophysiology of toxin-mediated events, as well as the nature of human anti-toxic immune responses. The conceptual framework must account for epidemiological phenomena that have been patiently catalogued by malariologists and withstood years of observation. Thus, the present review comprises three main parts: an outline of the toxic basis of malaria disease, with an emphasis on the proposed role of GPIs; a summary of what is thought clinically to represent anti-toxic immunity to malaria; and a discussion of what is presently known about immune responses to plasmodial GPIs.

2 The Toxic Basis of Malaria

Fever and anaemia are common to all forms of human malaria, whereas severe malaria due to *P. falciparum* is additionally characterised by metabolic acidosis, hypoglycaemia, uraemia, pulmonary oedema and/or coma (referred to as cerebral malaria). These features of malaria pathogenesis are thought to result from a number of mechanisms acting in concert: destruction of red blood cells by the parasite, a cell-mediated inflammatory host response, and in the case of *P. falciparum* only, cytoadherence of parasitised erythrocytes to vascular endothelium. Cytoadherence in particular may lead to sequestration of parasites away from the general circulation and localised organ-based immunopathology. This section will focus on the potential roles played by parasite toxin(s) in initiating and/or exacerbating these key events. Readers seeking more information are referred to comprehensive recent reviews (Clark and Cowden 2003; Clark et al. 2004; Maitland and Marsh 2004).

2.1 The Toxic Paradigm in Malaria

A prevailing view of malaria pathogenesis can be stated as follows: that a parasite toxin (or toxins) induces production of host-derived inflammatory cytokines that are directly responsible for the characteristic febrile paroxysms of malaria, and indirectly contribute to other clinical manifestations. The Italian

Nobel laureate Camillo Golgi first established that paroxysms occurred following malarial shizogony in 1886 (Golgi 1886), but it took another 100 years for the physiological basis of this observation to be elucidated. Parallel lines of research converged in the late 1970s and early 1980s, leading to the recognition that endotoxin-mediated bacterial sepsis and malaria shared striking similarities in their pathophysiological features and cytokine profiles (Clark 1978; Clark et al. 1981). Central to this was the discovery and definition of tumour necrosis factor (TNF)-α, followed by recognition that it mediated pathology in rodent malaria models (Clark et al. 1987) and could be induced by malaria parasites and putative malaria toxins in vitro (Kwiatkowski et al. 1989; Scragg et al. 1999). Subsequently, plasma TNF-α levels were shown to correlate with the severity of human malaria (Grau et al. 1989; Kern et al. 1989), and a causal role was suggested by genetic association studies linking polymorphisms in the TNF-α promoter region to disease outcome (McGuire et al. 1994; Wattavidanage et al. 1999; Aidoo et al. 2001).

With time, it has become clear that TNF-α is but one in a milieu of soluble mediators that together influence the pathophysiology of malaria. Monoclonal anti-TNF-α antibodies given to Gambian children with cerebral malaria reduced fever (Kwiatkowski et al. 1993) but had no effect on mortality in a subsequent randomised placebo-controlled trial (van Hensbroek et al. 1996). Similarly disappointing results were observed using polyclonal anti-TNF-α antibodies in Thai adults with severe malaria (Looareesuwan et al. 1999), and the relevance of TNF-α polymorphisms has since been questioned (Bayley et al. 2004). Recent studies have demonstrated that lymphotoxin (LT)-α (formerly TNF-β), rather than TNF-α, appears to be the principle mediator of murine cerebral malaria in the *P. berghei* (ANKA) model (Engwerda et al. 2002; Rae et al. 2004). Interestingly, this is consistent with research conducted 10 years earlier that showed elevated levels of LT-α in human malaria, and induction of interleukin (IL)-6 and hypoglycaemia in mice injected with the cytokine (Clark et al. 1992). Together, these studies highlight that the pivotal role ascribed to TNF-α in malaria pathogenesis may in part have reflected its study in isolation from the complex interplay of other mediators (Dodoo et al. 2002). Evidence from in vitro, animal and human studies (reviewed by Artavanis-Tsakonas et al. 2003; Clark et al. 2004) suggests that at least the following are also involved in determining the outcome of malaria infection: interferon (IFN)-γ, IL-1, 6, 10, 12 (Dodoo et al. 2002), and 18 (Singh et al. 2002), transforming growth factor (TGF)-β (Omer and Riley 1998), chemokines, nitric oxide (NO) (Anstey et al. 1996; Hobbs et al. 2002) and prostaglandins (Perkins et al. 2001). Moreover, in different settings or at different levels, the same cytokine or mediator may be harmful or protective (reviewed by Hunt and Grau 2003). By way of example, TNF-α itself has been ascribed a protective

role in regulating parasite density (Kwiatkowski 1995), which is supported by the demonstration of a parasiticidal effect of TNF-α on *P. falciparum* in vitro (Muniz-Junqueira et al. 2001).

It is generally thought that the soluble mediators induced by malaria toxins contribute not only to fever, but also to the end-organ, metabolic and haematological consequences of disease. TNF-α and LT-α have been shown to increase expression of parasitised erythrocyte receptors such as intercellular adhesion molecule (ICAM)-1 on endothelial cells (Ockenhouse et al. 1992; Engwerda et al. 2002), thus potentially initiating a vicious cycle that may lead to severe malaria by sequestering more parasitised cells and further increasing local cytokine production. Other mediators, such as NO and prostaglandins, decrease cytokine-induced endothelial ICAM-1 and reduce cytoadherence of parasitised erythrocytes (Xiao et al. 1999; Serirom et al. 2003). Other factors are likely to contribute to protection or pathology in CM, including IFN-γ, migration inhibitory factor (Clark et al. 2003b), carbon monoxide (Clark et al. 2003a) and chemokines. The degree of anaemia in malaria is thought to exceed that which can be explained by destruction of parasitised erythrocytes alone, leading to the proposition that erythropoiesis may be depressed by soluble mediators (reviewed by Menendez et al. 2000). Severe metabolic acidosis with hyper-lactataemia is associated with a very high mortality in malaria (English et al. 1996) and probably results from a combination of tissue hypoxia, direct effects of cytokines, lactate production by parasites, and decreased clearance of lactate by the liver (Marsh and Snow 1997). The pathogenesis of other metabolic derangements such as hypoglycaemia may share similar antecedents (Clark et al. 1997).

2.2 Structure of *P. falciparum* GPIs

GPI molecules are evolutionarily conserved glycolipids present in the outer membranes of eukaryotic cells. Their core structure comprises a single membrane-associated phospholipid head, attached linearly to an inositol ring that is followed by a tetrasaccharide containing one glucosamine and three sequentially numbered mannose residues (Fig. 1). A phosphoethanolamine group attached to the terminal mannose affords stable anchorage for a diverse range of proteins. GPIs vary between eukaryotes in a limited number of ways that nonetheless appear to impart a wide range of functional differences. In mammals and yeast, an additional ethanolamine phosphate is invariably present as a side-chain on the first mannose and occasionally on the second mannose. Protozoa such as *Trypanosoma brucei* may carry an additional carbohydrate modification on the first mannose (Ferguson 1999). In contrast,

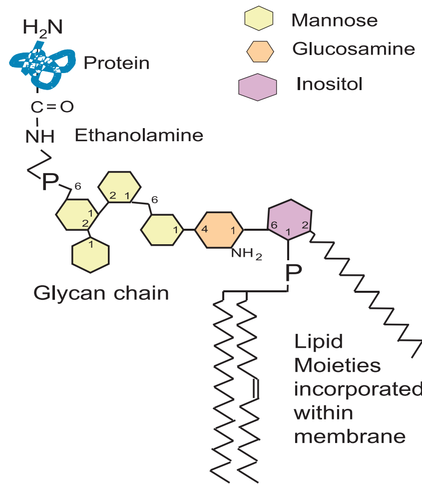

Fig. 1 Schematic representation of *P. falciparum* GPI. The lipid moieties are incorporated into the plasma membrane and joined via glycerol and phosphate (*P*) to an inositol ring. A conserved core glycan chain composed of glucosamine and three mannose residues is attached at the inositol 6 position. The terminal third conserved mannose residue is joined via a P ester to ethanolamine, which facilitates linkage to the C-terminus of a protein via an amide group. *P. falciparum* GPI remains acylated at the inositol 2-position, and a fourth side-chain mannose is typically attached to the ethanolamine-linked mannose

no side-chain modifications of the first two mannose residues have been described in *P. falciparum*. A side-chain fourth mannose (Man_4) is typically attached to the terminal mannose in *P. falciparum* and yeast, whereas this was thought to be uncommon until recently in mammals. It has now been demonstrated that *hSMP3* is the human homologue for yeast *SMP3*, and that it encodes the mannosyltransferase responsible for addition of Man_4 to GPIs (Taron et al. 2004). Although the gene is only weakly expressed in many cultured mammalian cell lines, it is expressed in most human tissues, challenging the previous paradigm that Man_4-GPI formation is relatively unimportant in mammalia.

Mammalian GPI inositol rings may be acylated by palmitate in some cases, although it is common for mammalian inositol to be deacylated following GPI biosynthesis (Chen et al. 1998). In contrast, *P. falciparum* inositol remains acylated, most often with palmitate (90%) but occasionally with myristate (10%) (Naik et al. 2000a). The phosphorylated lipid moiety attached to inositol in *P. falciparum* is invariably diacylglycerol in structure (Naik et al. 2000a), but in mammalian cells is predominantly 1-alkyl, 2-acyl glycerol. Recent

examination by gas chromatography-mass spectrometry of protein-anchored "major" GPIs in *P. falciparum* has demonstrated heterogeneity resulting in at least five different structures, which can be fully ascribed to compositional differences in the fatty acyl substituents (Naik et al. 2000a). Importantly, variation in lipid composition did not appear to influence the likelihood of protein anchoring by the GPIs and it is likely that other biologically active "minor" GPIs with unusual fatty acyl substituents remain to be described (Naik et al. 2000a). Plasmodial GPIs from human and rodent malaria species demonstrate a high level of conservation in structure (Gerold et al. 1997; Naik et al. 2000a; Kimmel et al. 2003), which is also seen in widely dispersed geographical isolates of *P. falciparum* (Berhe et al. 1999). To date, GPIs have not been described in other species of plasmodia that cause malaria in humans.

2.3
Biosynthesis of *P. falciparum* GPIs

The general processes involved in GPI biosynthesis have been comprehensively reviewed (Ferguson 1999; Kinoshita and Inoue 2000; Eisenhaber et al. 2003) and will be only briefly summarised here. GPIs are assembled sequentially in the endoplasmic reticulum by a number of proteins/protein complexes that exhibit a variety of catalytic and regulatory roles. These proteins and their genes are well characterised in mammals, and this has enabled matching of sequence homologues in other eukaryotes in a number of instances through database mining. Homologues have been identified in *P. falciparum* for 8 of the approximately 20 known protein components of human GPI biosynthesis, with a 40%–70% similarity in amino acid sequences (Delorenzi et al. 2002; Shams-Eldin et al. 2002). The existence of other homologues may have been concealed by the level of stringency used for database matching or incomplete annotation of the *P. falciparum* genome at the time; also, non-homologous proteins may perform some of the designated functions (Delorenzi et al. 2002). In general, the *P. falciparum* homologues described are for proteins involved in key catalytic roles, rather than in regulating the rate or stability of reactions. Consistent with the proposed structure of *P. falciparum* GPIs, homologues for the mammalian genes that encoded addition of phosphoethanolamine groups to non-terminal mannoses and deacylation of inositol (which appears not to occur in *P. falciparum*) were not found. It has been suggested that this overall economy may have arisen through an adaptation to rapid growth and from lesser requirements for fine tuning compared to higher eukaryotes (Delorenzi et al. 2002).

The transamidation reaction that covalently anchors proteins to GPI inside the endoplasmic reticulum depends on recognition of a signal sequence and

proteolytic cleavage of a carboxy-terminal pro-peptide (Eisenhaber et al. 2003). This process appears to be intricately mediated in mammalian cells by the protein PIG-T, which possesses both a hook for the key catalytic subunit (PIG-K) and a hole-like beta propeller structure that regulates access of C terminal oligopeptides to the protease site through a tunnel (Eisenhaber et al. 2003). A similar coding region for the *PIG-T* gene was found in *P. falciparum*, but failed stringency tests (Delorenzi et al. 2002). However, it has been noted that sequence similarity BLAST searching may fail to detect *PIG-T* homologues, unless consideration is given to secondary structural preferences or physical property patterns (Eisenhaber et al. 2003). As cells from mammals and parasitic protozoa appear to differ in their preference for particular amino acid coding regions in the signal sequence (Moran and Caras 1994), the transamidation reaction represents a particularly attractive target for species-specific inhibitors.

2.4
Specific Roles of *P. falciparum* GPIs in Malaria Pathogenesis

A range of in vitro and in vivo animal studies have demonstrated that purified *P. falciparum* GPIs can induce the pathophysiology ascribed to putative malaria toxins and/or that anti-GPI antibodies can neutralise these effects. Mouse macrophages were first shown to produce TNF-α after incubation with erythrocytes parasitised with either *P. yoelii* or *P. berghei in vitro* in 1988 (Bate et al. 1988). Consistent results were soon demonstrated for human monocytes in the presence of *P. falciparum* in vitro (Kwiatkowski et al. 1989). This was followed by partial characterisation of the likely toxin using physical and chemical extraction procedures (Bate et al. 1992b) coupled with monoclonal and polyclonal antibody neutralisation studies (Bate et al. 1992a; Bate and Kwiatkowski 1994a). By 1993, it was clear that the putative toxin was a GPI and that its injection into thioglycollate-primed rodents could reproduce major features of acute clinical malaria, including pyrexia and hypoglycaemia (Schofield and Hackett 1993); similar findings have subsequently been reported in unprimed mice (Elased et al. 2004). Concurrently, it was shown that a monoclonal antibody to *P. falciparum*-derived GPIs could neutralise the TNF-α-inducing activity of whole-parasite extracts in vitro (Schofield et al. 1993), suggesting that GPIs alone may be responsible for TNF-α induction. Polyclonal antibodies raised in T-cell-deficient mice (Bate et al. 1990) and sera from human patients infected with both *P. falciparum* and *P. vivax* (Bate and Kwiatkowski 1994b) were reported to have similar activity. However, we have been unable to confirm this ourselves, with toxin-neutralisation assays showing no clear-cut correlation between anti-GPI an-

tibody titres in serum and the ability of purified immunoglobulin (Ig)G from the same serum to neutralise TNF-α induction by whole-parasite extracts from a macrophage cell line in vitro (J.B. de Souza et al., unpublished data). These data suggest either that GPI is not the only TNF-inducing component of parasite extracts or that not all anti-GPI antibodies are able to neutralise GPI activity.

By the mid-1990s, *P. falciparum* GPIs had been shown capable of inducing macrophage production of IL-1 (Schofield and Hackett 1993) as well as production of NO by macrophages and vascular endothelial cells in a process enhanced by IFN-γ (Tachado et al. 1996). *P. falciparum* GPIs were then shown to increase endothelial cell expression of ICAM-1, vascular cell adhesion molecule-1 and E-selectin (receptors implicated in cytoadherence of parasitised erythrocytes) in a process enhanced by TNF-α and IL-1 (Schofield et al. 1996). This process was also blocked by monoclonal anti-GPI antibodies. Consistent with the ability to induce hypoglycaemia in mice, *P. falciparum* GPIs were shown to possess insulin-mimetic activity through increasing glucose oxidation in murine adipocytes in vitro (Schofield and Hackett 1993). The culmination of these studies was the recent demonstration that murine antibodies raised through vaccination with a *P. falciparum* GPI glycan analogue completely abolished whole schizont extract-induced TNF-α release from mouse macrophages (Schofield et al. 2002). To the extent that the in vitro model matches the clinical situation in humans, this is the most suggestive evidence to date that GPIs are the predominant pro-inflammatory toxins of *P. falciparum*.

It has not yet been determined precisely how *P. falciparum* GPIs initiate the intracellular signalling that results in expression of cytokines, NO and adhesion molecules. Early studies using specific inhibitors supported a two-step model of cellular activation that ultimately ends with activation of the transcription factor nuclear factor (NF)-κB (Schofield et al. 1996; Tachado et al. 1996, 1997; Vijaykumar et al. 2001); the first signal provided by the glycan moiety [activating protein tyrosine kinase (PTK)] and the second by the diacylglycerol [activating protein kinase C (PKC)]. The issue of whether the two signals require insertion and/or endocytosis of whole GPIs or GPI substructures into the plasma membrane (Vijaykumar et al. 2001) or can be provided entirely extracellularly (Vijaykumar et al. 2001) remains unresolved. More recently it has been suggested that *P. falciparum* GPIs can initiate intracellular signalling—without internalisation—through toll-like receptors (TLRs), although the extent to which signalling via the PTK, PKC and TLR pathways may overlap is still unclear. In vitro studies examining TNF-α production by GPI-stimulated macrophages derived from the bone marrow of TLR knockout mice have demonstrated a major role for TLR2, a lesser role

for TLR4, and a potential minor role for other pathways not dependent on the TLR2/TLR4 shared adapter protein MyD88 (Krishnegowda et al. 2004). Similar results were apparent in experiments using human monocytes that were pre-treated with monoclonal antibodies to TLR2 and/or TLR4. Subsequent intracellular signalling in both the mouse and human cells involved activation of a number of second messengers, including ERK1/ERK2, JNK and p38, and ultimately the NF-κB pathway. Parallel experiments by the same investigators demonstrated that the pattern of TNF-α, IL-6, IL-12 and NO production by GPI-stimulated mouse macrophages depended on differential activation of these signalling molecules and a variable requirement for co-stimulation with IFN-γ (Zhu et al. 2004). This may have relevance in vivo, as it has previously been shown that $CD3^+$ T cells are required for optimal TNF-α production by human monocytes stimulated by *P. falciparum* ex vivo (Scragg et al. 1999), which may also indicate the involvement of IFN-γ (reviewed by Artavanis-Tsakonas et al. 2003).

The exact structural requirements of *P. falciparum* GPIs for activation of intracellular signalling also require further clarification. The issue of whether glycan-induced signalling is dependent on the third (Tachado et al. 1997) or fourth terminal (Vijaykumar et al. 2001) mannose appears to have been resolved in favour of the former, with Man_3 GPIs shown to possess approximately 80% of the TNF-α-inducing activity of Man_4 GPIs (Krishnegowda et al. 2004). Insolubility of Man_3 GPIs in the solvent (80% ethanol) used for transfer from the stock vial to the culture medium is now thought to explain the previous results (Vijaykumar et al. 2001). More in keeping with their previous findings (Vijaykumar et al. 2001), diacylated *sn*-2 *lyso*-GPIs (resulting from partial deacylation of native GPIs by phospholipase A_2) induced levels of TNF-α from mouse macrophages and human monocytes that were similar to those from the intact molecules (Krishnegowda et al. 2004). Interestingly, heterodimerisation of TLR2 (a basic requirement for TLR2-induced signalling) involved TLR1 in the case of triacylated GPIs but TLR6 for the diacylated *sn*-2 *lyso*-GPIs, thus implicating lipid composition as a basis for antigenic discrimination by macrophages—a phenomenon that the authors point out has a precedent in the case of bacterial lipoproteins (Takeda et al. 2002; Akira and Hemmi 2003). Just as intriguing was the finding that GPIs were degraded and inactivated in the presence of mouse macrophages and human monocytes, consistent with cell-surface related activity of phospholipase A_2 and phospholipase D (Krishnegowda et al. 2004). To what extent human monocyte phospholipases may be able to regulate the activity of *P. falciparum* GPIs in vivo, and whether this may further be influenced by phospholipases released into serum during human malaria (Vadas et al. 1993), remains to be determined.

In addition to their putative roles as toxins, GPIs may indirectly contribute to malaria pathogenesis both by anchoring protein determinants of parasite virulence and altering their pathological and immune functions. In *P. falciparum*, as in all eukaryotes, the fundamental physiological role of GPIs is to stably anchor a diverse range of proteins on cellular plasma membranes. A number of important proteins appear to be GPI-anchored in *P. falciparum,* including merozoite surface protein (MSP)-1, MSP-2 and MSP-4 (Chatterjee and Mayor 2001; Gowda 2002). In addition, a large pool of *P. falciparum* GPIs exist free of protein attachment (Gerold et al. 1994), which is also common in other parasitic protozoa (Ropert and Gazzinelli 2000). It is possible, although not yet proven, that it is the free GPIs released at the time of schizont rupture that are predominantly responsible for toxin-mediated effects. It has been reported that the presence of GPI anchors on sporozoite protein vaccines impairs the development of antibody (Martinez et al. 2000; Scheiblhofer et al. 2001) and T-cell responses (Bruna-Romero et al. 2004) with consequent attenuation of vaccine-induced immunity; similar observations have been made in other systems (Wood and Elliott 1998). However, following DNA vaccination, antibody responses to a gametocyte-specific antigen are enhanced by the retention of the GPI anchor signal sequence (Fanning et al. 2003). These data suggest that the presence of glycans alters the solubility and membrane interactions of proteins, and thus the pathway of protein trafficking within an antigen-presenting cell. Additionally, GPI attachment may alter the conformation of anchored proteins in a manner that influences the proteins' function and/or immunogenicity. These observations may have important implications for the development of recombinant protein vaccines, many of which are based on GPI-anchored proteins (reviewed by Gowda 2002).

2.5
Putative Malaria Toxins Other than GPI

In addition to GPI, haemozoin (or "malaria pigment"; an insoluble digestion product of trophozoites comprising detoxified haemoglobin, as well as remnants of host and parasite membranes) has been implicated as a malaria toxin (Arese and Schwarzer 1997). Purified and synthetic haemozoin (β-haematin) were linked to inflammatory cytokine production in vitro in a number of studies (Pichyangkul et al. 1994; Prada et al. 1995; Sherry et al. 1995; Mordmuller et al. 1998) conducted in the period preceding identification of *Mycoplasma* contamination of parasite cultures (Turrini et al. 1997; Rowe et al. 1998). Subsequently, de-proteinated haemozoin enriched from *P. falciparum* culture was shown to induce TNF-α and IL-1β production by human peripheral blood mononuclear cells *ex vivo,* which was able to be blocked by naturally occur-

ring IgM antibodies (Biswas et al. 2001). More recently, it was demonstrated that synthetic haemozoin induced inflammatory cytokines (including IL1-α, IL1-β, IL-6, and IL-12), chemokines and their receptors, and migration of neutrophils and monocytes, following injection into mice (Jaramillo et al. 2004). Unlike the case with GPIs, it appears that *P. falciparum* haemozoin is more likely to activate host cells through a hitherto unique TLR9-mediated, MyD88-dependent pathway that is sensitive to inhibition by chloroquine (Pichyangkul et al. 2004; Coban et al. 2005). In addition to induction of soluble mediators, haemozoin-laden macrophages have been shown to produce large quantities of the free fatty acids 12- and 15-hydroxy-arachidonic acid, which may be pathogenic by causing vascular damage and increased cytoadherence (Schwarzer et al. 2003).

Others have shown that *P. falciparum*-derived haemozoin induced NO synthase-2 expression in cultured human blood monocytes ex vivo, which was more pronounced in cells from children with anaemia (Keller et al. 2004). A similar effect has been shown in mouse macrophages with *P. falciparum*-derived and synthetic haemozoin, although a requirement for co-stimulation with IFN-γ was apparent in that study (Jaramillo et al. 2003). In contrast, β-haematin was shown to suppress TNF-α and NO production by lipopolysaccharide (LPS)-stimulated mouse macrophages (Taramelli et al. 1995); an effect later attributed to β-haematin-induced oxidative stress (Taramelli et al. 2000). Another study showed that high levels of haemozoin ingestion by intervillous blood monocytes were associated with suppression of TNF-α, prostaglandin-E_2 and IL-10 in women with placental parasitaemia (Perkins et al. 2003). The source of mononuclear cells in these different studies, and the nature of the interactions between the cells and haemozoin, may be factors to consider in resolving these apparent contradictions (Basilico et al. 2003; Jaramillo et al. 2005).

Other candidates for "malaria toxins" are anti-malarial IgE-antigen complexes and IgE-anti-IgE complexes, which may also induce TNF-α release from peripheral blood mononuclear cells in vitro (Perlmann et al. 1997). These complexes have been proposed to activate NF-κB transcription factors by cross-linking the macrophage low-affinity Fcε receptor for IgE (Perlmann et al. 1997). Using an in vitro toxin-neutralisation assay, which measures TNF-α in supernatants of cultured macrophages incubated with malaria-immune sera and *P. falciparum* schizont lysates, we have shown that some malaria-immune sera themselves induce TNF-α in the absence of exogenous malaria antigen; this activity is absent from purified IgG (J.B. de Souza et al., unpublished data). Although this might be explained by trace amounts of GPI in the serum (as the sera were taken from residents of highly malaria-endemic areas), it is possible that serum IgE complexes induce TNF-α; this notion is fur-

ther supported by the observation that non-IgG antibodies in other malaria-immune sera synergise with parasite extract to enhance TNF-α production (J.B. de Souza et al., unpublished data). These data are consistent with the likely involvement of an immune complex-mediated TNF-α triggering pathway. Anti-malarial IgE/antigen complexes may exert pathogenic effects by depositing on cerebral microvasculature (Maeno et al. 2000), leading to local induction of pro-inflammatory cytokines, with consequent vascular damage. This would explain the recent finding that West African children with severe malaria had higher anti-*P. falciparum* IgE levels than age-matched children with mild malaria (Calissano et al. 2003).

2.6 Summary

It should be recognised that, at present, the evidence that *P. falciparum* GPIs act as toxins in humans in vivo is circumstantial. Purified or synthetic GPIs have not been injected into humans, and nor have anti-GPI antibodies been shown to inhibit the pathogenesis of *P. falciparum* in vivo; thus, the level of evidence is less than that associating bacterial endotoxin with the clinical manifestations of sepsis. Regardless, in vitro studies and data from animal models of malaria have identified clear avenues of investigation into the role of GPIs in human malaria, as well as other putative toxins such as haemozoin and IgE complexes.

3 The Nature of Anti-toxic Immunity to Malaria

Bewilderment regarding the mechanisms of anti-malarial immunity is reflected by the number of terms used to describe it; among others, natural, clinical, innate, acquired, specific, non-specific, anti-parasitic, anti-disease, anti-toxic, cell-mediated, antibody-mediated, tolerance, premunition and semi-immune. In part, confusion in the use of these terms (sometimes interchangeably) has arisen through a desire to explain observable epidemiological phenomena on the one hand, within the confines of existing immunological paradigms on the other. As the focus of this review is the putative malaria toxin GPI, and immune responses to it, we will generally refer to anti-toxic immune responses, and dispense with the commonly used epithet “anti-disease”, as the latter may be considered the sine qua non of all forms of immunity. Hence, this section concerns differences in the clinical condition that can reasonably be expected to infer the existence of anti-toxic immunity, which may in theory act by inhibition of parasite metabolic pathways leading to reduced or

defective toxin production (of which little is presently known), neutralisation of toxin at or shortly after the time of release, interference with the cellular mechanisms triggered by toxin activation that lead to production of inflammatory mediators, and/or diminished host responsiveness to one or more of the inflammatory processes that follow.

3.1 Tolerance of Malaria Parasitaemia Without Symptoms

Despite thousands of publications related to anti-malarial immunity, it is probably just as true to say today as it was 100 years ago that the best correlate of immunity to malaria is the presence of malaria parasites in the blood of individuals without symptoms (Sinton 1938; McGregor et al. 1956). It is generally accepted that the likelihood of observing asymptomatic parasitaemia within a population correlates with the intensity of exposure, and that it is unlikely to be observed in previously malaria-naïve individuals. It has been shown by some that asymptomatic infections can become established and persist at reasonably stable densities over time (Sowunmi 1995; Farnert et al. 1997; Smith et al. 1999; Bruce et al. 2000), but others have challenged this notion in longitudinal studies demonstrating an increased future risk of symptoms (Missinou et al. 2003; Njama-Meya et al. 2004). It is evident though, from our own studies in regions of intense malaria transmission, that a high prevalence of asymptomatic infections can be expected in individuals of all ages in whom signs and symptoms are objectively evaluated on multiple occasions over 24 h (Boutlis et al. 2002, 2003b). The levels of parasitaemia observed, especially in children (Rogier et al. 1996; Boutlis et al. 2002), are often much higher than those recorded to cause disease in previously malaria-naïve subjects (Gatton and Cheng 2002; Molineaux et al. 2002). Given that it has long been assumed that malaria parasites release pyrogenic substances (i.e. toxins) during schizogony (Golgi 1886), and there is no evidence to suggest that field strains can become avirulent, it seems reasonable to suppose that host immune responses maintain the healthy phenotype during asymptomatic infection.

Through detailed epidemiological studies conducted in regions of high malaria endemicity, it has been observed that young children will tolerate high levels of malaria parasitaemia; levels that are much more frequently associated with disease in adults (Smith et al. 1994; Rogier et al. 1996). By mathematical modelling, the risk of symptoms has been shown to correlate with the level of parasitaemia in an age-dependent manner, with a peak at approximately 1 year of age that declines steeply through early childhood to a plateau in adolescence (Smith et al. 1994; Rogier et al. 1996). The exponentially decaying curve that describes the relationship between the level of

parasitaemia associated with fever and increasing age has been referred to as defining a "pyrogenic threshold" (Rogier et al. 1996). These results were generally in keeping with observations such as those made by Miller in Liberia in 1958: "...while adults were more efficient in suppressing parasite levels and suffered less from clinical attacks of malaria, children could tolerate higher parasite burdens without showing clinical evidence of disease" (Miller 1958). In contrast, the application of mathematical modelling techniques in regions of low or unstable malaria transmission has shown a less discrete relationship between the level of parasitaemia and the risk of symptoms, with generally lower pyrogenic thresholds and less dependence on age (Prybylski et al. 1999; Boisier et al. 2002). This suggests that fully effective anti-toxic immunity may only become manifest in areas of quite high and/or stable malaria transmission. Interestingly, studies of experimental infection in previously malaria-naïve subjects have shown that an individual's pyrogenic threshold may increase following an initial infection, as well as being influenced by host genetics and the "strain" of parasite (Gatton and Cheng 2002; Molineaux et al. 2002).

Considered together, these observations are consistent with a model in which repetitive and ongoing exposure to malaria infection results in relatively short-lived anti-toxic immune responses that abrogate inflammatory pathology. It is evident from studies in hyper-endemic regions that the efficacy of these responses is highest in early childhood and is lowest in adulthood. This creates an apparent paradox in relation to the fact that, in endemic areas, malaria severity is highest in young children and the frequency of malaria attacks reduces with age. This can be resolved, however, by conceptualising disease as resulting from uncontrolled expansion of parasite densities that overcome an individual's pyrogenic threshold. The immune responses that act to limit parasite replication (sometimes referred to as "anti-parasitic" responses) appear to increase with age, in contrast to the anti-toxic responses that maintain tolerance of parasitaemia. The ability to limit the severity of disease in the face of unchecked parasite growth may be expected to result from a number of factors, some of which may be anti-toxic in nature (Sect. 3.2), but in addition may involve prevention of other critical events that are not primarily toxin-mediated, such as cytoadherence and anaemia.

3.2 Limitation of Malaria Severity in the Face of Disease

The model proposed in the previous section is broadly consistent with the concept that anti-malarial immune responses can be categorised as anti-parasitic, anti-fever (i.e. parasite tolerance), and anti-severe disease (Snow

and Marsh 1998). The latter may involve mechanisms that reduce cytoadherence (e.g. antibodies to cytoadherence ligands on infected erythrocytes), whilst alterations in the balance of inflammatory and anti-inflammatory cytokines may effect both anti-fever and anti-severe disease immunity (Omer et al. 2000). To better understand the influence of age itself (separated from exposure) on malaria severity, Baird and colleagues have prospectively studied recently arrived Indonesian transmigrants from very low-endemic Java into hyper-endemic Papua Province (Baird 1998). Previously malaria-naïve children appeared to manifest relative resistance to the severity of malaria compared to adults (Baird et al. 1998). The explanation for these findings is not entirely clear, and results may have been biased in part by differences in the use of prophylaxis or in treatment-seeking behaviour in adults and children. Alternatively, it is plausible that children make lower levels of inflammatory cytokines in response to a given "dose" of parasites (Riley 1999).

Of the numerous candidate host responses proposed to limit malaria severity, some may be predominantly anti-toxic in nature, whereas others may act more broadly. For example, a growing body of literature (reviewed by Anstey et al. 1999a) suggests that genetic (Hobbs et al. 2002) and acquired influences regulating NO production act to reduce malaria severity through cross-talk with other soluble mediators, such as TNF-α (Iuvone et al. 1996) and prostaglandins (N.M. Anstey et al., unpublished data), in addition to reducing expression of endothelial cytoadherence receptors (Serirom et al. 2003). It had previously been suggested that individuals in malaria-endemic regions produced NO in an age-dependent manner that was correlated with the age-dependent pyrogenic threshold (Clark et al. 1996; Anstey et al. 1999b). However, a recent detailed longitudinal examination of NO production in malaria-exposed children and adults living in Madang, Papua New Guinea has shown that NO production differs little across age groups from age 2 to 60 years (Boutlis et al. 2004).

Additionally, the anti-inflammatory cytokines IL-10 and TGF-β have repeatedly been shown to reduce the toxic effects of malaria infections in mice and in humans. The pathology of *P. chabaudi chabaudi* infection and the mortality in IL-10-deficient mice are ameliorated by anti-TNF-α and exacerbated by anti-TGF-β antibodies (Omer and Riley 1998; Dodoo et al. 2002; Li et al. 2003), and recent data suggest an important role for regulatory T cells in setting the pro-inflammatory/anti-inflammatory cytokine balance (Omer et al. 2003; Hisaeda et al. 2004). Disentangling the influence of a raft of other cell-mediated and cytokine responses on malaria severity is difficult in isolation, but has recently been reviewed in the context of an overall model of anti-malarial immunity that also considers factors such as nutrition (Artavanis-Tsakonas et al. 2003). The subtlety and importance of cross-species interactions between

malaria and other infections, such as intestinal helminths, that may also influence cell-mediated phenomena, are beginning to be appreciated (Nacher 2002; Boutlis et al. 2003b; Le Hesran et al. 2004; Nacher 2005; Hesran 2005), but further discussion is beyond the scope of the present review.

3.3 Summary

It appears that anti-toxic immune responses to malaria may contribute to the maintenance of malarial tolerance on the one hand, while contributing to resistance to severe disease in those unable to limit exponential expansion of parasitaemia on the other. It should be recognised that the potential for multiple anti-toxic effector mechanisms exists, but that they do not necessarily have the same relationship with age. For example, those that mediate tolerance may decrease with age, whereas those underlying resistance to disease severity may increase, with some degree of overlap in the transition from one to another. While an individual's genetic make-up and parasite strain differences may contribute to the overall level of toxicity manifest in the host–parasite relationship, it is likely that the level of exposure to malaria and other infections, and factors related to age per se, are also involved. It seems logical that the sum of an individual's anti-toxic immune responses will act in concert to protect the individual by either neutralising the parasite toxin(s) and/or diminishing the host's response to toxin-triggered events. Furthermore, it is apparent that not all immune responses acting to reduce the severity of malaria need be thought of as being primarily anti-toxic in nature.

4 Immunity to *P. falciparum* GPIs

Assuming that the preparations used were pure and free from contamination (Naik et al. 2000a), it has been consistently demonstrated in a number of studies done in malaria-endemic regions that GPIs purified from *P. falciparum* are recognised by human antibodies (Naik et al. 2000a; de Souza et al. 2002; Boutlis et al. 2002; Hudson Keenihan et al. 2003; Suguitan et al. 2004). This has raised the possibility that these naturally occurring antibodies could neutralise GPIs and influence the outcome of human malaria. The presence of antibodies implies that GPIs are recognised by immune cells; hence, GPIs may also potentially elicit other cellular immune responses that are not antibody-mediated. It is important to understand how GPIs may initiate these immune responses, and how they are effected, if vaccination against GPIs is to be used for protection against malaria.

4.1 GPI Induction of Tolerance-Like Immunity

Obvious parallels exist between the phenomena of malarial tolerance and bacterial endotoxin tolerance, which has led to the proposition that they are mediated by common molecular pathways (Clark et al. 2004). The phenomenon of endotoxin tolerance was initially defined in the 1960s on the basis that rabbits could be effectively immunised with a low dose of bacterial endotoxin (LPS) against death from the subsequent injection of a potentially lethal dose (reviewed by West and Heagy 2002). Similarly, febrile responses in humans decrease with subsequent injections of endotoxin (van der Poll and van Deventer 1999). The tolerant state in animals was later shown to correlate with a reduction in TNF-α production in response to repeated endotoxin injection that persisted for several weeks (Sanchez-Cantu et al. 1989). Subsequently, it was shown that LPS-stimulated production of TNF-α, IL-1β, IL-6, and IL-10 in whole blood taken from human volunteers 3 h after an intravenous injection of *Escherichia coli* LPS was significantly reduced compared to baseline, but restored at 24 h (van der Poll et al. 1996). Further in vitro experiments using human and animal monocytes/macrophages defined the characteristic physiological changes accompanying repeated exposure to endotoxin: inhibition of TNF-α; augmentation of NO and prostaglandin-E_2; and variably altered IL-6, IL-1 and IL-8 secretion (West and Heagy 2002). Given the timescale of the induction of endotoxin tolerance, the simplest interpretation of these data is that cells become temporarily refractory to endotoxin after stimulation.

Optimal responses to LPS derived from *Enterobacteriaceae* depend on cellular recognition by TLR4 complexed with CD-14 and the membrane-associated molecule MD-2 (Latz et al. 2002). Binding of LPS leads to induction of protein kinases, which in turn activate nuclear factors including NF-κB (Ziegler-Heitbrock et al. 1994) that influence transcriptional activation of numerous inflammatory cytokine genes (Dobrovolskaia and Vogel 2002). Desensitised human monocytes rendered tolerant by pre-treatment with LPS accumulate an excess of functionally inactive NF-κB complexes comprising p50 homodimers (Ziegler-Heitbrock et al. 1994; Kastenbauer and Ziegler-Heitbrock 1999) and have elevated concentrations of the NF-κB-inhibitory protein Iκ-Bα (Wahlstrom t al. 1999; Ferlito et al. 2001); the activation of other kinases in the signal transduction cascade is also inhibited in tolerant cells (West and Heagy 2002). Alternatively, although alteration of intracellular signal transduction pathways appears likely to mediate tolerance, down-regulation of LPS receptors on the surface of immune cells or over-production of anti-inflammatory mediators such as IL-10, TGF-β and NO may also be in-

volved (Zingarelli et al. 1995; Fahmi et al. 1995; Dobrovolskaia and Vogel 2002). Indeed, NO had been proposed as a candidate mediator of malarial tolerance in endemic regions on the basis of studies suggesting a role in the mediation of endotoxin tolerance (Zingarelli et al. 1995) and preliminary observations in human studies (Clark et al. 1996; Anstey et al. 1999b). The subsequent demonstration that NO is dispensable to the development of endotoxin tolerance in NO synthase-2-knockout mice (Zingarelli et al. 2002) and recent contradictory data from human studies (Boutlis et al. 2004) casts doubt on these suggestions.

The recent demonstration that *P. falciparum* may initiate intracellular signalling through TLR2 and TLR4 (Krishnegowda et al. 2004) provides insight into the mechanisms that may underlie tolerance to malaria infection. Like *P. falciparum* GPIs, the GPI-mucin of *T. cruzi* appears to be recognised by both TLR2 and TLR4 (Campos et al. 2001; Ropert and Gazzinelli 2004; Oliveira et al. 2004); indeed, detailed studies have demonstrated that *T. cruzi* GPI-mucin and *E. coli* LPS use functionally similar pathways to induce TNF-α and IL-12 production in murine macrophages, with levels of both cytokines reduced in response to secondary stimuli (Ropert et al. 2001). This mirrors other models of cross-tolerance induced by differential induction of TLR2/TLR4 (Lehner et al. 2001; Beutler et al. 2001), consistent with these receptors sharing common intracellular pathways. The practical relevance of these studies to human malaria may have been hinted at as long as 50 years ago when it was shown that even a first malaria infection could induce cross-tolerance to endotoxin in experimentally infected humans (Heyman and Beeson 1949; Rubenstein et al. 1965). It is unclear, however, whether possible LPS contamination of the parasite preparations used in these studies may have confounded this effect. Considering the accumulated evidence, it would appear the most likely candidate model for malarial tolerance is one that parallels bacterial endotoxin tolerance.

4.2
Anti-GPI Antibodies in Human Malaria

The recent purification of GPI in sufficient quantities for analysis has enabled the study of anti-GPI responses in human malaria. The strong theme to emerge from all studies conducted in malaria-endemic regions to date is that both the population prevalence and level of antibody responses increases with age (Naik et al. 2000a; de Souza et al. 2002; Boutlis et al. 2002; Hudson Keenihan et al. 2003; Suguitan et al. 2004). That this was true in a population exposed to perennially intense malaria transmission from birth (Boutlis et al. 2002), as well as Javanese transmigrants experiencing their first malaria

attacks in hyper-endemic Indonesian Papua (Hudson Keenihan et al. 2003), would suggest that immunological changes related to ageing are at least as important as those relating to cumulative exposure in determining antibody production. Antibodies to *P. falciparum* GPIs are infrequent in children less than 2 years of age (Naik et al. 2000a), possibly due to the inability of the immune system of small children to respond to carbohydrate antigens, but the age-relationship of anti-GPI antibody production thereafter closely mirrors that of a number of other blood-stage malaria peptide antigens (Al Yaman et al. 1994, 1995a,. 1995b; Johnson et al. 2000). Field studies have shown that the anti-GPI antibody response is boostable by recent infection (de Souza et al. 2002; Hudson Keenihan et al. 2003), predominantly IgG rather than IgM (Naik et al. 2000a; Boutlis et al. 2002), and highly skewed towards the IgG3 subclass, especially in children (Boutlis et al. 2003a). Anti-GPI antibody responses decay rapidly after elimination of parasites by chemotherapy, suggesting that ongoing antigenic stimulation is required to maintain antibody production; the very rapid decline in treated children compared to treated adults (Boutlis et al. 2002) probably reflects the preponderance of IgG3 in children's plasma (Boutlis et al. 2003a), as IgG3 has a shorter half-life in serum than other IgG subclasses.

The relationship between anti-GPI antibodies and clinical malaria has been evaluated in a number of studies. In a study of Kenyan children and their mothers, seropositivity for anti-GPI antibodies was associated with lower body temperature and higher haemoglobin levels after adjusting for age and parasitaemia (Naik et al. 2000a). However, it was unclear from the data presented whether the association with lower body temperatures directly translated to a lower risk of acute febrile illness. In a prospective study in Gambian children aged 3–8 years, antibody levels at the beginning of the transmission season were not significantly predictive of the subsequent risk of mild malaria after controlling for age (de Souza et al. 2002); nor was there any significant difference in anti-GPI response between hospital-admitted Gambian children with severe (mainly cerebral) malaria or mild malaria in an independent case-control study (de Souza et al. 2002). Interestingly, several children with severe malaria had exceedingly high titres of anti-GPI antibodies in this study, which may have been due to rapid boosting of anti-GPI titres by the current infection. A study of Javanese transmigrants appeared to show that children aged 6–12 years (but not adults) with a positive anti-GPI response following infection had reduced subsequent risk of symptomatic disease (Hudson Keenihan et al. 2003). However, the analysis may have been influenced by the use of multiple measurements from the same child and the lack of adjustment for age; a similar finding was initially noted in the Gambian children, but disappeared after adjusting for the confounding effect of age (de

Souza et al. 2002). A cross-sectional study from Cameroon of anti-GPI antibody responses in pregnant women at delivery showed no relationship with acute or chronic placental pathology, TNF-α levels or pregnancy outcomes (Suguitan et al. 2004). Again, boosting of anti-GPI antibody concentration by the current malaria infection may have obscured important differences in GPI levels earlier in pregnancy.

In summary, investigators have consistently shown that the prevalence, persistence and level of anti-GPI antibodies increase with age in malaria-exposed populations. To date, however, there is little evidence to show that the antibody responses measured correlate with protection against clinical malaria or disease severity. However, it should be borne in mind that none of these studies was primarily designed to evaluate the potential clinical roles of anti-GPI antibodies, and that future hypothesis-driven studies will likely provide more robust results. Crucially, more prospective studies are required in which measures of anti-GPI antibodies prior to infection can be tested for association with outcome of subsequent infection; notwithstanding the major logistical problems associated with prospective studies of severe malaria. The use of fully synthetic GPIs (Lu et al. 2004; Liu and Seeberger 2004; Seeberger et al. 2004) will help dispel any doubt that the responses measured are specific, and combining clinical studies with functional assays (such as toxin neutralisation) will provide the clearest outcomes.

4.3 Antigen Recognition and Processing of GPIs

Although several life cycle stages of *Plasmodium* express GPIs, initiation of immune responses likely occurs during asexual schizogony, when the highest concentrations of free GPI are released (Fig. 2). Early studies, conducted in immunocompetent and T-cell-deficient mice, suggested that non-protein antigens derived from the boiled supernatants of in vitro *P. falciparum* cultures induced a predominantly IgM response (Bate et al. 1990; Playfair et al. 1991). Serum from the mice could be used to block toxin-induced TNF-α production in vitro and in vivo (Playfair et al. 1991); this response appeared rapidly after immunisation and did not appear to be enhanced by boosting (Bate et al. 1992a) or common adjuvants (Playfair et al. 1990). This is typical of T-cell-independent antibody responses (Baumgarth 2000), but is at odds with the typical antibody responses to *P. falciparum* GPIs described in humans, which are IgG dominated (Naik et al. 2000a; Boutlis et al. 2002), characterised by skewing toward IgG3 (Boutlis et al. 2003a), and rapidly boosted by reinfection. This contrasts with the IgG2- and IgG1-dominated responses that have been described toward carbohydrate antigens such as bacterial polysaccha-

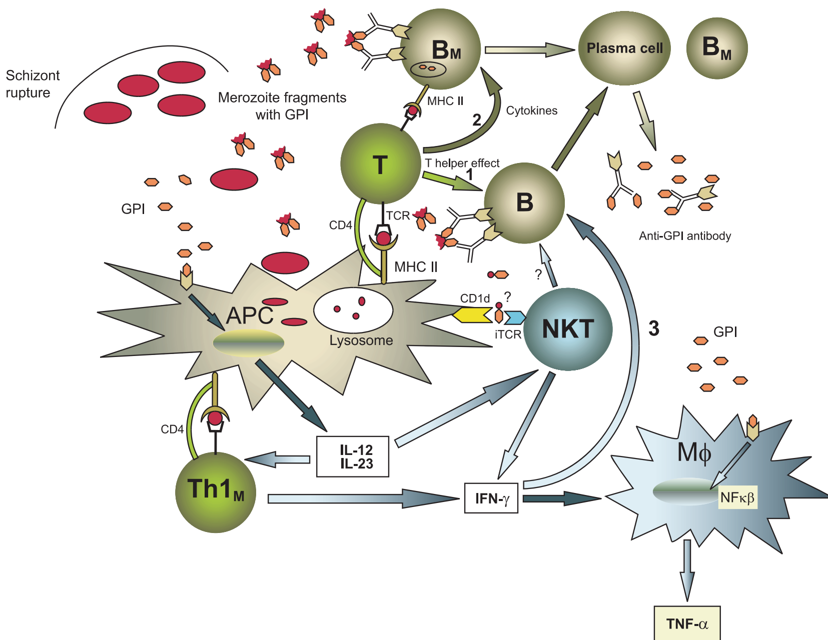

Fig. 2 Proposed cellular interactions in the generation of anti-GPI antibodies. Schizogony is accompanied by the release of merozoites with GPI-linked surface proteins and by free GPI. GPIs provide activation signals for antigen presenting cells (*APC*) and macrophages (*M*ϕ). Parasite material is internalised by APCs and after processing through the endosomal/lysosomal pathway, peptides are presented in association with MHC class II to T cells. Meanwhile B cells may recognise free GPI or GPI bound to merozoites and become activated through cross-linking of the B cell receptor and by helper cytokines released by T cells (*arrows 1* and *2*). B cell activation leads either to plasma cell differentiation and large scale production of anti-GPI antibody or to formation of GPI-specific memory B cells (B_M). Alternatively, GPI-specific B cells may internalise GPI-containing parasite material and present processed MHC class II-linked peptides to T cells. GPIs may also trigger APCs to produce IL-12 and IL-23 which regulate the function of other classes of T cell. IL-23-activated memory T cells ($Th1_M$) produce IFN-γ which in turn acts on B cells [(*arrow 3*), contributing to affinity maturation and Ig class switching) or macrophages (inducing anti-parasitic effector activity)]. The role of CD1d in the presentation of GPI-linked peptide to NKT cells remains controversial; however, it is likely that NKT cells respond following activation by IL-12/23 released by GPI-activated APC

rides (reviewed by Ferrante et al. 1990) and may indicate that GPI-anchored proteins act as natural adjuvants in eliciting immunological memory to *P. falciparum* GPIs. The apparent absence of IgG2 and IgG4 and the high ratio of IgG3 to IgG1 subclasses that we have observed (Boutlis et al. 2003a) are re-

markably similar to the pattern of responses previously described in Solomon Islanders (Rzepczyk et al. 1997) and Gambians (Taylor et al. 1998) to MSP-2, which is a GPI-linked protein.

Given the absence of peptide epitopes for conventional T cells, antibody responses to free GPIs are likely to be T-cell-independent during a primary malaria infection. During a secondary response, however, activated GPI-specific B-cells may internalise and process GPI-linked proteins, possibly in the form of membrane vesicles (Hoessli et al. 2003), and subsequently present peptides derived from these GPI-linked proteins to $CD4^{+}$ T cells in the context of major histocompatibility complex class II (MHC-II). This would allow provision of cognate T-cell help to GPI-specific B-cells, via a classical hapten-carrier interaction, leading to immunoglobulin class switching, somatic mutation and affinity maturation of the BCR and generation of long-lived plasma cells and memory B-cells. As cognate interactions are most likely to take place between GPI-specific B cells and T cells recognising peptide epitopes that are physically linked to GPI, it is probable that T-cell help for GPI-specific B cells is provided by T cells specific for GPI-linked proteins and thus that anti-GPI antibodies may share qualitative features with antibodies to, for example, merozoite surface proteins. It is possible, for example, that the predominantly IgG3 subclass response to GPIs is mediated by T cells specific for MSP-2, which is known to induce IgG3 (Rzepczyk et al. 1997; Taylor et al. 1998). In support of this hypothesis, in the absence of covalently linked proteins, GPIs from the protozoal parasite *T. cruzi* can induce a switch from IgM to IgG production in murine B cells only in the presence of the T-cell-derived cytokines IL-4 and IL-5 (Bento et al. 1996).

Little is known of the primary immune response to *P. falciparum* GPIs, although it is likely to involve interactions with pattern recognition molecules (such as TLRs) expressed by dendritic cells and/or macrophages in the spleen (Stevenson and Riley 2004). Various types of lipid and glycolipid molecules can also be presented to NK T cells by CD1 molecules, which are MHC-like molecules encoded by genes outside the MHC (Sieling et al. 1995). CD1 molecules are expressed on the surface of antigen-presenting cells, e.g. macrophages, dendritic cells and B-cells (Kronenberg et al. 2001). Human CD1b has been shown to bind and present the mycobacterial lipoglycans, lipoarabinomannan and PI mannosides, which have a similar basic lipid anchor to *P. falciparum* GPIs (Sieling et al. 1995). In the presence of lipoarabinomannan and $CD1^{+}$ antigen-presenting cells, CD1b-restricted T cells taken from the skin of a patient with leprosy have been shown to induce IgG1 and IgG3 subclass antibody production by B cells at the expense of IgG4 and IgE (Fujieda et al. 1998). Mouse CD1d (which is highly homologous to human CD1d) (Porcelli and Modlin 1999) binds GPI with high affinity via the PI

moiety (Joyce et al. 1998) and has been shown to strongly stimulate natural killer NK T cells in vitro after binding a variety of purified phospholipids (including PI) (Gumperz et al. 2000).

Whether or not CD1d molecules recognise malarial GPIs is controversial. It has been reported that CD1d-restricted presentation of GPI-anchored *P. falciparum* (sporozoite) surface proteins leads to NK T-cell stimulation and that presentation of *P. berghei* circumsporozoite antigen (thought to be GPI anchored) by CD1d elicits NK T-cell help in regulating IgG production (Schofield et al. 1999). However, two independent laboratories, in attempting to replicate these results using CD1d and MHC-II-deficient mice, have demonstrated that the IgG response to the circumsporozoite protein is solely MHC-II-dependent (Molano et al. 2000; Romero et al. 2001), in line with previous evidence (Romero et al. 1988). Genetic differences between the mice used in the three studies were thought not to explain the discrepant results (Romero et al. 2001). Similarly conflicting results have emerged regarding the role for CD1d-restricted NK T-cell-mediated regulation of antibody responses to *T. cruzi* GPIs (Procopio et al. 2002; Duthie et al. 2002), which share structural similarities with those of *P. falciparum* (Naik et al. 2000a). It may be that differences in the process of antigen presentation (exogenous versus endogenous) (Hansen et al. 2003a) or kinetics of the antibody responses (Hansen et al. 2003b) can explain these apparent contradictions. Most recently it has been demonstrated that CD1d-restricted NK T cells appear to influence the balance of IFN-γ/IL-4 cytokine production, pathogenesis and fatality in the murine *P. berghei* (ANKA) model (Hansen et al. 2003a, 2003b), as well as regulating B-cell-mediated antibody responses, which to MSP-1 at least were shown to arise from both CD1d-dependent and MHC-II-dependent pathways (Hansen et al. 2003b).

5
Potential for Therapeutic Inhibition of *P. falciparum* GPI Biosynthesis

GPIs and their anchored proteins appear to mediate critical events in malaria pathogenesis and immunity (Sects. 2 and 4), and their production appears crucial for the survival of *P. falciparum* (Naik et al. 2000b). Thus, therapeutic targeting of *P. falciparum* GPI biosynthesis has the potential to arrest disease by affecting parasite growth, development and virulence. Although the core structure of GPIs is conserved across eukaryotes, available evidence suggests that sufficient differences exist in the specificity and sequence of GPI synthesis reactions to enable species-specific pharmacological manipulation (reviewed by de Macedo et al. 2003). While an ideal inhibitor of *P.*

falciparum GPI synthesis would have little or no effect on mammalian GPI synthesis, absolute specificity may not be essential, given that normal levels of GPI synthesis are in some instances dispensable for mammalian cell survival (Ferguson 1999).

Proof in the concept of selective enzyme inhibition has been demonstrated for a fungal metabolite that inhibited mammalian, but not protozoal, GPIs (Sutterlin et al. 1997) and has been validated at different stages of GPI assembly in *T. brucei* and *Leishmania mexicana* (Smith et al. 1997, 1999, 2001, 2004; Ferguson 2000). These studies have provided the methodological basis to compare GPI synthesis between *P. falciparum* and man. Consequently, a synthetic glucosamine-*N*-acetyl-PI analogue has been shown to irreversibly inhibit the *P. falciparum* de-*N*-acetylase, and an isomer of glucosamine-*N*-PI appears to competitively inhibit either the inositol-transferase or first mannosyltransferase, with little apparent effect on the human orthologues (Smith et al. 2002). Others have shown that early steps in *P. falciparum* GPI assembly can be aborted by using glucosamine (Naik et al. 2003) or modified sugar residues like mannosamine (2-amino-2-deoxy-*d*-mannose), which inhibits parasite growth (Naik et al. 2000b). However, results have been inconsistent between studies and the precise mechanisms of activity remain unclear (Naik et al. 2000b; Santos de Macedo et al. 2001). Further potential exists for targeting other pathways intricately linked to early GPI biosynthesis, such as the supply of PI, activated sugars and lipid chains (Eisenhaber et al. 2003).

The apparent importance of the side-chain Man_4 of *P. falciparum* GPIs to cell signalling and its likely requirement for protein anchoring (Naik et al. 2000a) makes the process of its addition an attractive therapeutic target. This is particularly so given that the process of cellular activation is currently thought to be novel, as it does not appear to involve membrane insertion or endocytosis (Gowda 2002). The fatty acyl requirements for cellular activation are still somewhat unclear, although it appears that features common to *P. falciparum* but absent in mammalian GPIs are important for activity (Gowda 2002). Although it was recently shown that Man_4-GPIs are much more common in humans than previously realised, it was also demonstrated that protein transfer to human GPIs occurs irrespective of the presence of Man_4 or Man_3-GPI precursors (Taron et al. 2004). Prior addition of Man_4 is mandatory for GPI protein anchoring in *Saccharomyces cerevisiae* (Grimme et al. 2001) and is likely to be a requirement in *P. falciparum*, given that all GPI protein anchors discovered to date possess a fourth mannose (Naik et al. 2000a). Together, these findings suggest that differences in the specificities of human, yeast and *P. falciparum* transamidase complexes for Man_3/Man_4 GPI precursors can be expected and potentially exploited, perhaps even in a tissue-specific manner (Taron et al. 2004). Further definition of the genes and proteins involved in

P. falciparum GPI synthesis, and how their substrate specificities differ from humans, will help to determine whether anti-GPI pharmacological therapy can indeed become a reality.

6
Prospects for Anti-GPI Vaccination

If the primary goal of active malaria vaccination is to protect malaria-exposed children from severe disease, then inhibiting toxin-induced pathophysiological responses is a potentially useful strategy. Immunisation of mice with deacylated synthetic *P. falciparum* GPI glycan conjugated to keyhole limpet haemocyanin reduced the early mortality of *P. berghei* (ANKA) challenge from 100% to 25% (Schofield et al. 2002). The glycan GPI analogue induced IgG antibodies in immunised mice that bound to intact intra-erythrocytic trophozoites and schizonts but did not cross-react with uninfected erythrocytes (which express endogenous GPIs on their surface). Serum from immunised mice completely neutralised production of TNF-α by mouse macrophages in response to stimulation by crude *P. falciparum* schizont extracts, suggesting that GPI alone is both sufficient and necessary for the induction of this inflammatory response. However, the early protection noted was independent of any reduction in parasitaemia, and immunised rodents eventually succumbed to haemolytic anaemia accompanied by massive parasitaemia. Other investigators have thus urged caution with GPI vaccination studies, especially given that *P. falciparum* GPIs are implicated in cell-mediated host-defence responses in addition to pathogenesis (Clark et al. 2004).

Vaccination specifically targeted at GPIs in humans raises concerns that vaccinated individuals with malaria would feel less sick and thus present to hospital later and with extremely high levels of parasitaemia, with the attendant risks of severe anaemia and organ failure. Inhibition of GPI-mediated early pro-inflammatory cytokine responses that limit parasite replication may also favour rapid rises in parasitaemia in vaccinated individuals (Kwiatkowski 1995). These concerns may be offset if anti-toxic vaccine antigen(s) were to be combined with epitopes primarily directed at generating an anti-parasitic response that limited exponential expansion in parasitaemia. However, this would require at least partial anti-parasitic efficacy in 100% of subjects receiving a combination vaccine—a target that would be very difficult to achieve. Moreover, the anti-parasitic immune responses induced by such a vaccine combination would need to be of longer duration than that of the anti-GPI immune response— again a difficult target. Active vaccination against GPI antigens may also theoretically interfere with any protective effect of NO

against malarial disease severity (Anstey et al. 1996; Hobbs et al. 2002), given that *P. falciparum* GPIs have been shown to induce NO production in vitro (Tachado et al. 1996). Passive immunisation of individuals with severe malaria by administration of monoclonal or polyclonal anti-GPI antibodies is an alternative adjunctive therapeutic strategy that may avoid interfering with early immune responses, and be transient enough to avoid long-term problems. In support of this approach, polyclonal antibody preparations that recognise bacterial super-antigens have been reported to improve clinical outcomes in streptococcal toxic shock syndrome (Kaul et al. 1999), which shares several features of the Th1 cytokine-dominated inflammatory response to severe malaria (Norrby-Teglund et al. 1997).

Field studies highlight the challenges involved in generating a sustained anti-GPI antibody response in children less than 5 years of age, which is the highest priority for vaccination in malaria-endemic areas. Understanding whether the events involved in GPI antigen presentation and processing (Sect. 4.3) can be modified by adjuvants (including other malarial antigens) or immuno-modulators may help to improve vaccine immunogenicity in this age group. Finally, given the conservation in the core structure of GPIs across eukaryotes, it would be important for anti-GPI vaccination to avoid inducing auto-immune responses. The finding that spleen cells primed with parasite-derived or mammalian Thy-1 derived GPI later responded to both homologous and heterologous antigenic challenge (Schofield et al. 1999) highlights these concerns.

7 Conclusion

At present, although data from model systems are encouraging, there is very little firm evidence to suggest that antibody-mediated immune responses to *P. falciparum* GPIs play a significant role in either mediating tolerance or reducing disease severity in human malaria. Concerns over the purity of GPI preparations derived from *P. falciparum*, in relation to the specific induction of both pathological and immune responses, can be effectively dispelled if results can be repeated using fully synthetic preparations. It is exciting to think that the availability of synthetic GPIs may enable detailed and direct examination of the human fever responses in much the same way as human volunteers have contributed to our understanding of the modes of action of bacterial lipopolysaccharides (van der Poll and van Deventer 1999). A theoretical, but real, concern that must be addressed prior to such human studies involves determining the likelihood of cross-reactive, and potentially auto-

immune, antibody responses. Should the pathological significance of GPIs be confirmed by further studies of pathophysiology and/or natural immunity, then strategies to address the potential for unchecked parasite replication in the face of vaccination must be considered. Such concerns are less relevant for the development of selective pharmacological inhibitors of *P. falciparum* GPI biosynthesis; although confirmation that GPIs are essential to ongoing parasite replication is a pre-requisite if this approach is to be successful.

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