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Malaria tolerance – for whom the cell tolls?

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Abstract

How is it that individuals exposed to intense malaria transmission can tolerate the presence of malaria parasites in their blood at levels that would produce fever in others? In light of evidence discounting a role for nitric oxide or antibodies to plasmodial glycosylphosphatidylinositols in maintaining this tolerant state, refractoriness to toxin-induced Toll-like receptor-mediated signalling has emerged as a likely explanation that links malarial and bacterial endotoxin tolerance. Understanding the mechanisms underlying tolerance and the potential for cross-tolerization has significant implications for understanding the potential for anti-toxic vaccine strategies, as well as interactions between different malaria species and between malaria and other human parasites.

Tolerance and the lexicon of antimalarial immunity

Conceptualizing immunity to malaria has proven difficult, yet it has long been observed that individuals exposed to intense malaria transmission commonly tolerate the presence of parasites in their blood that would render others symptomatic [1,2]. Indeed, this phenomenon arguably remains the best single indicator of natural immunity to malaria. Terms used to describe it include ‘clinical immunity’ and ‘antidisease immunity’ (a tautology that crept into the malaria literature ~15 years ago [3]). We prefer ‘tolerance’ and, ‘although bedevilled with many different and sometimes very restrictive definitions over the years’ [4], agree that it ‘should properly be applied to any endogenous mechanism by which a potentially injurious immune response is prevented, suppressed, or shifted to a non-injurious class of immune response’ [4].

It is likely that the mechanisms underlying malarial tolerance are multifactorial. Some could be described as ‘antitoxic’ and, although fairly said to ‘go through historical phases of popularity’ [5], the evolution of the long-established toxic paradigm in malaria [6] brings some legitimacy to this terminology. Antitoxic immunity has been conceived as comprising determinants that are predominantly ‘antifever’ (i.e. during the tolerant state), as well as others that are ‘antisevere disease’ (i.e. limiting severity in the face of symptomatic illness) [7]. Other responses that are not directly antitoxic appear to be important in limiting unchecked parasite growth during tolerance [8], and antibodies to nontoxic determinants such as erythrocyte surface adherence proteins [9] might contribute to limiting the severity of disease. These distinctions serve to clarify and highlight the complexities of antimalarial immunity. The purpose of this review is to draw attention to parallels between malaria tolerance and bacterial endotoxin tolerance, so the focus here is on mechanisms that mediate antifever responses rather than those limiting the severity of disease.

The toxic paradigm in malaria

The idea that the characteristic febrile paroxysms of malaria are induced by parasite products released at the time of schizont rupture originated in the 19th century [10]. In 1921, it was postulated that the 'toxic' features of malaria might result not from the plasmodial 'autonoxinogens' themselves but from the subsequent triggering of host-derived 'autonoxins' (Collier, W.A., quoted by Playfair *et al.* [3]). Subsequent development of the theory has proceeded along these lines, aided by the recognition that the clinical features and cytokine profiles of bacterial endotoxaemia and acute malaria share many similarities [6]. In both instances, the toxic paradigm is based on the assumption that the cascade of chemokine, cytokine and other soluble mediators released during acute disease can be ultimately traced back to the initial interaction between microbial toxin(s) and host 'immune' cells. The importance of pyrogenic cytokines such as tumour necrosis factor (TNF), interleukin (IL) 1 β and IL-6 in sustaining fever in malaria has been demonstrated by studies showing them to be elevated during clinical malaria [11], as well as therapeutic trials of monoclonal anti-TNF antibodies that led to abrogated febrile responses [12]. Recent studies with bacterial lipopolysaccharide (LPS) have challenged traditional notions regarding the very early (pre-cytokine) phases of fever induction [13] but it is unclear at this point what relevance this might have to malaria. In addition to fever, the resultant effects of toxin activation in malaria are probably wide ranging, including upregulation of endothelial cytoadherence receptors [14], anaemia [15] and the potential for various metabolic disturbances such as acidosis and hypoglycaemia [16].

Following the definition of TNF as a marker that would enable *in vitro* definition of specific toxins from extracts of malaria parasites [17], progressive experimentation culminated in two seminal reports that characterized the structure and activity of a likely candidate toxin for *Plasmodium falciparum* – glycosylphosphatidylinositol (GPI) [18,19]. Most of the steps in *P. falciparum* GPI biosynthesis have been described [20], and a fully lipidated version of the molecule has recently been chemically synthesized [21]. In parallel, several groups have independently shown native haemozoin to enhance inflammatory cytokine production in human monocytes in a similar manner to that proposed for GPIs [22-24]; haemozoin might also have immunosuppressive [25,26] and anti-inflammatory effects [27]. Further studies using fully synthetic versions of GPI and toxigenic determinants of haemozoin in the context of human (rather than murine) malaria should facilitate further advances in knowledge and will address doubts over whether results from previous studies (including our own [28,29]) might have been influenced by the impurity of preparations used.

Evidence for a pyrogenic threshold in malaria-endemic regions

A carefully conducted prospective study of children (aged 3–7 years) and adults by Miller in 1958 showed geometric mean (GM) parasite densities at the initiation of febrile episodes due to *P. falciparum* to be markedly higher in children (GM 17 277 μL^{-1}) than in adults (GM 881 mL^{-1}) [30]; indeed, the average daily parasite counts of asymptomatic children during the low transmission season exceeded the majority of these adults' 'clinical thresholds'. Miller concluded that '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' [30]. This age-associated pattern of disease and asymptomatic parasitaemia is common in regions of high transmission [31,32]. Indeed, estimation of a threshold level of parasitaemia at which a febrile child's illness could be attributed to malaria (the pyrogenic threshold) rather than another intercurrent infectious disease has been the goal of numerous studies conducted in endemic areas [33]. However, these studies have generally not focussed on determining the effect of

changes in age on the relationship between parasitaemia and fever (C.S. Boutlis, PhD thesis, Charles Darwin University, 2002; <http://www.menzies.edu.au/craigboutlis>).

Studies that have specifically examined the pyrogenic threshold as a function of age have shown that its level decreases as age increases in regions of intense malaria transmission [34,35]. For example, in Senegal it was found that an age-specific threshold of parasitaemia was definable from infancy through adulthood, and that this defined with remarkable precision the likelihood of fever; those with parasitaemias above this threshold had an odds ratio for fever of 44.4 (95% confidence interval 13.6–144.8) compared with those below [34]. This threshold decayed exponentially during early childhood, from a very high level in the first year of life to a low-level plateau in adulthood [34]. This pattern appears to be different in areas of lower endemicity, with less difference between children and adults and a more imprecise threshold separating febrile from afebrile that takes the form of an overlapping range rather than discrete values [36,37]. Notably, when it does occur, symptomatic malaria is both more frequent and severe in children resident in high transmission areas than in adults, whereas adults tend to experience more severe disease compared with children when both are transmigrated from an area of negligible to high endemicity [38]. Together, these observations are consistent with a model whereby antitoxic mechanisms mediating tolerance are more effectively inducible in children but where different acquired antitoxic mechanisms (i.e. including those that ameliorate severe disease; reviewed in Ref. [39]) are more evident in adults.

Falling from grace: proposed mediators of antifever immunity during tolerance

In 1999, our interest in the antitoxic mechanisms underlying malarial tolerance led us to investigate two competing explanations – high level production of the anti-inflammatory molecule nitric oxide (NO) by peripheral blood mononuclear cells (PBMCs) and production of antibodies to the putative GPI toxin. Others have examined antibodies to haemozoin [22] but not in the setting of tolerance. The NO theory had been developed on the basis of the following evidence: NO production was higher in asymptomatic malaria-exposed children compared with those with severe malaria [40]; thick film-positive asymptomatic children produced more NO than did thick film-negative children [41]; malaria-exposed children in Papua New Guinea (PNG) reportedly produced more NO than did adults [42]; and NO had been shown to decrease TNF production from mononuclear cells *in vitro* [43]. Further, a crucial role for NO in the development of bacterial endotoxin tolerance has been demonstrated in a murine model *in vivo* and in macrophages *ex vivo* [44]. An alternative proposition was that neutralizing antibodies to parasite toxin(s) could explain the sharply defined pyrogenic threshold noted in Senegal [34], a theory that was supported by available *in vitro* evidence using antibodies to inhibit malaria toxin-induced TNF production in mononuclear cells [45]. Inherent in both theories was the premise that these mechanisms would be more active in children than in adults, and that they would be inducible by, and presumably proportional to, the level of parasitaemia.

Nitric oxide

In a cross-sectional study that explored the relationship between parasitaemia and NO production in West Papuan adults, we found that asymptomatic subjects who were thick film-positive for *P. falciparum* had significantly higher serum levels of NO metabolites than aparasitaemic healthy controls [46]. By contrast, a larger subsequent longitudinal study in Madang, PNG (an area of more intense transmission) found no relationship between NO metabolite levels and parasitaemia, either cross-sectionally at baseline or in comparing the same subjects during parasitaemic and nonparasitaemic episodes [32]. Importantly, the

expected pattern of decline in NO production with age was not observed. In neither study was PBMC NO synthase (NOS)-2 expression or activity related to parasitaemia or age and nor did NOS2 expression or activity correlate with NO metabolite levels. In fact, there was remarkable intra-individual stability in NO production and NOS2 expression over time, regardless of changes in parasitaemic status (whether owing to *P. falciparum* or *Plasmodium vivax* [32]). However, in both studies [32,46], NO metabolites and NOS2 expression were significantly higher in malaria-exposed children and adults than in Western adult controls. These findings suggest that the West Papuan and PNG populations were exposed to numerous infective stimuli that were capable of inducing NO from a variety of cellular sources, including malaria, but also helminths and other enteric infections. These findings also provide some of the most compelling evidence to date for the ability of human mononuclear cells to express NOS2 at high levels *in vivo*. Reconciling the differences in findings relating NO to parasitaemia between the two study sites is not straightforward but these could result from statistical chance, differences in the epidemiology of nonmalarial infections influencing NO, and/or genetic polymorphisms in NOS2 or other regulatory cytokine genes. However, no evidence was found of commonly reported NOS2 polymorphisms in the PNG cohort [47]. The lack of an apparent role for NO in mediating tolerance does not preclude an antitoxic and anticytoadherence role for NO in protecting from severe disease, for which there is evidence *in vivo* [40].

Antibodies to GPIs

The proposition that T cell-independent IgM antibodies to malaria toxin(s) could mediate the antitoxic immune responses underlying tolerance [34] was plausible, given that such antibodies could be induced in mice in response to vaccination with proposed toxins [48]. Similarly, it had been reported that patients with *P. falciparum* and *P. vivax* produced IgM antibodies that neutralized toxin-induced TNF production *in vitro* and that these antibodies bound specifically to liposomes containing phosphatidylinositol [49]. An underlying assumption in this theory was that the youngest children would be capable of mounting a vigorous antitoxic IgM antibody response but that adults would not. Antibody responses were measured to purified *P. falciparum* GPIs in the PNG population of children and adults in whom NO had been studied [32], and exactly the opposite was found – that the anti-GPI antibody response was characteristic of many anti-peptide antibody responses to malaria [50,51]: predominantly IgG rather than IgM, and limited in early childhood but increasing with advancing age [28]. Importantly, many asymptomatic children were observed with relatively high parasitaemias who had no detectable antibodies at all [28]. These findings are consistent with those from other studies [18,52-54]; in addition, the anti-GPI antibody response might be relatively short-lived because of a predominance of short half-life IgG3 antibodies, especially in children [29]. Although antibodies raised to a synthetic subcomponent of *P. falciparum* GPI ameliorate some toxic manifestations of murine malaria [19], evidence for a clinical effect in human studies so far has been less than convincing (reviewed in Ref. [55]).

Parallels between malarial tolerance and endotoxin tolerance

Bacterial endotoxin tolerance

Inferences of tolerance to the fever-producing effects of bacterial pyrogens date back almost 100 years (reviewed in Ref. [56]), with diminished febrile responses demonstrated to repeated injection of purified typhoidal toxin into humans >60 years ago [57]. Subsequent characterization and purification of the dominant Gram-negative bacterial endotoxin, LPS, and the ease with which it can be studied *in vitro* and in humans *in vivo* [58], has led to a substantial understanding of the events involved in endotoxin-induced cellular signalling and endotoxin tolerance (reviewed in Ref. [59]). In brief, LPS activates mononuclear

immune cells through an initial association that mainly involves recognition by cell surface Toll-like receptor (TLR) 4 receptors [60]. Intracellular signalling proceeds via several different pathways that ultimately result in the induction of transcription factors such as nuclear factor- κ B (NF- κ B), which increases the expression of inflammatory cytokine genes that are also common to malaria, including TNF, IL-1 β and IL-6 [6,61]. In general, the 'tolerant' state induced by repeated stimulation of mononuclear cells with LPS *in vitro* involves downregulation of proinflammatory responses (similar to those mentioned above), along with upregulation of several predominantly anti-inflammatory responses, such as the production of IL-10 and prostaglandin E2 [59]. This mirrors the change in cytokine profiles observed in human volunteers following repeated injections of LPS [58]. The molecular mechanisms of endotoxin tolerance are yet to be defined fully but the available evidence has been comprehensively reviewed [62]. To summarize, the bulk of this evidence suggests that the LPS-tolerant phenotype results from perturbations of intracellular signalling involving upregulation or downregulation of particular signalling proteins, rather than alterations in receptor expression or the effects of autocrine and/or paracrine mediators. Recent evidence also implicates widespread suppression at the transcriptional level of mitochondrial energy production and protein synthesis machinery as possibly having a role, which has been observed in human volunteers to whom endotoxin was administered [63], and in acute clinical malaria (Boutlis *et al.*, unpublished).

Cross-tolerance between malaria and endotoxin

The obvious parallels between endotoxin tolerance and mechanisms operative during malarial tolerance were drawn as long ago as 1949, when it was observed that neurosyphilitic patients had a diminished febrile response to the normally pyrogenic typhoid vaccine in the days following a course of malaria therapy [64]. Subsequently, it was shown that experimental inoculation of human volunteers with *P. vivax* resulted in cross-tolerance to the febrile response induced by injection of purified bacterial endotoxin [65]. Evidence that this cross-tolerance was underpinned by parallel signalling in mononuclear cells by malarial toxins and LPS converged at the same time that we, and others [66], were occupied refuting our earlier theories. Unlike LPS, but in common with Gram-positive bacterial lipoteichoic acid [62], *P. falciparum* GPIs have been shown to activate mouse macrophages and human-derived monocytes predominantly via TLR2 [67]. A lesser contribution was attributed to TLR4 activation, and a potential minor role demonstrated for other pathways not dependent on the TLR2 and TLR4 shared adapter protein MyD88. Subsequent intracellular signalling involved the second messengers ERK1 and ERK2, JNK and p38, which ultimately activated NF- κ B. The pattern of TNF, IL-6, IL-12 and NO production by GPI-stimulated mouse macrophages was shown to depend on differential activation of these signalling molecules, with a variable requirement for co-stimulation by interferon- γ [68]. Similarly, the GPI-anchored mucin of *Trypanosoma cruzi* appears to be recognized by both TLR2 and TLR4 [69,70]. Malarial haemozoin has additionally been shown to activate TLR9 in murine dendritic cells [71], and we have found expression of TLR5 and TLR7 to be increased in human PBMCs during acute malaria (Boutlis *et al.*, unpublished); however, it is presently unclear what relevance these observations might have to malarial tolerance.

The clinical phenomenon of cross-tolerance described above is consistent with several *in vitro* studies that have demonstrated the induction of cross-tolerance by LPS and TLR2 ligands [72,73], including between *T. cruzi* GPI-anchored mucin and *Escherichia coli* LPS [74]. Also, there is emerging evidence to suggest that haemozoin might inactivate signal transduction downstream of NF- κ B via haemozoin-linked production of 15(*S*)-hydroxyeicosatetraenoic acid, a peroxisome proliferator-activated receptor- γ ligand [26]. There have been other recent examples of cross-tolerance with potential relevance to malaria, involving interspecies interactions with helminths. Schistosomal TLR2 ligands have

been shown to induce IL-8, IL-10, IL-6 and TNF responses in PBMCs *ex vivo*, with reduced responses demonstrated to IL-8 and TNF in particular in cells taken from children infected with *Schistosoma haematobium* relative to controls [75]. Responses to the TLR4 ligand LPS followed a similar pattern. Similarly, *S. mansoni* antigens have been shown to suppress LPS-induced activation of murine macrophage inflammatory cytokine production *in vitro* [76]. Crosstalk between TLR-initiated pathways (and probably others, reviewed in Ref. [77]) might explain several interspecies interactions ranging from the classic observations of the Murray family involving the interrelationship between malaria and ascariasis [78], to models of nematode–malaria coinfection in mice [79] and studies showing (sometimes contradictory) effects of helminth infections on human malaria [80,81].

For whom the cell tolls

The LPS-tolerant phenotype has been regarded as having ‘evolved as a complex orchestrated counter regulatory response to inflammation’ [62]. This complexity underlies an otherwise basic aspect of innate immunity that appears to be active from a very young age – an inbuilt refractoriness of mononuclear cells to produce inflammatory cytokines in the face of repeated stimulation by a variety of microbial toxins. The putative malarial toxin GPI has been shown to stimulate inflammatory cytokine production primarily via activation of the evolutionarily conserved TLR2, using intracellular pathways parallel to those involved in LPS activation of TLR4. Repeated stimulation of these receptors (as might occur in areas of high malaria endemicity) has been shown to invoke changes characteristic of mononuclear cell tolerance. This tolerance probably hinges on alterations in TLR-initiated intracellular signalling, although it is possible (but by no means necessary) that changes in receptor expression and/or autocrine mediators have a contributory role. Cross-tolerance secondary to ligation of different TLRs has been demonstrated *in vitro*, which provides a possible explanation for the fascinating observation of reduced febrile responses to LPS challenge that was demonstrated following *P. vivax* infection almost 40 years ago. Taken together, these studies not only provide a framework within which to understand the potential for cross-tolerization between different species of malaria [82], but also to appreciate the importance of interactions between infection with malaria and other human parasites.

To demonstrate our point (summarized in Box 1), we have focused on antifever mechanisms operative during tolerance of parasitaemia in asymptomatic malaria-infected individuals that are mediated by GPI-initiated activation of TLR signalling in PBMCs. Other TLR-mediated processes, toxins and signalling pathways might also be involved in maintaining this tolerant state. Additional mechanisms that limit unchecked expansion of parasitaemia (such as density-dependent regulation [8]) must be operative during tolerance, and these mechanisms must be both highly effective in children and primarily antiparasitic in nature. Likewise, the array of immune responses that explain differences in malaria severity between individuals might include antiparasitic and antitoxic components, and these could have different age relationships to those operative during tolerance. Separating intrinsic and acquired differences in antimalarial immune responses between children and adults has proven challenging, especially in populations exposed to malaria since birth. Studies of Indonesians transmi-grated from regions of negligible to high malaria endemicity have been helpful in this regard, suggesting that children might initially enjoy some ‘innate’ protection from severe disease relative to adults [38]. Also, age-dependent intrinsic differences in levels of TLR responsiveness to malarial antigens might exist between children and adults, for which there is some precedent in the case of TLR4 and LPS [83].

Box 1**Main concepts**

- Individuals exposed to intense malaria transmission often harbour levels of parasitaemia in their blood that would commonly be associated with fever in malaria-naïve individuals, a state known as ‘malarial tolerance’.
- In keeping with the toxic paradigm of malarial immunopathology, it has long been supposed that antitoxic immune responses exist that prevent or suppress injurious toxin-inducible events such as fever in these individuals.
- Diminution of febrile responses has also been well described in humans following repetitive challenge with bacterial endotoxin, a phenomenon known as ‘endotoxin tolerance’.
- Cross-tolerance between experimentally induced malaria infection and inoculation with endotoxin was described over 40 years ago, and recent evidence suggests that endotoxin and putative malaria toxins such as GPIs share similar cell signalling pathways in inducing fever.
- Activation of PBMCs by these toxins appears initially to involve recognition by evolutionarily conserved TLRs, and cross-tolerance has been demonstrated *in vitro* between TLR4 (activated by endotoxin) and TLR2 (a major receptor for GPIs). We contend that repetitive stimulation of immune effector cells by malarial toxins is responsible for the antifever responses characteristic of malarial tolerance, via a process akin to that of bacterial endotoxin tolerance.
- Understanding the molecular changes underlying malarial tolerance has implications for malaria vaccine strategies, as well as providing a framework for understanding interactions between different species of malaria and between malaria and other human parasitic infections.

Concluding remarks

Many questions remain unanswered. Among them: what happens at the transition point between tolerance of malaria parasitaemia and disease? Of the age-related differences in antimalarial immunity between children and adults in malaria-endemic areas, which are innately determined versus those that are acquired? To what extent does crosstalk between TLR-mediated pathways explain *P. falciparum*–*P. vivax* and other interspecies interactions? Answering these questions will require targeted studies of specific immune effector cells that preferably utilize synthetic candidate malaria toxins in comparing cytokine production and expression of mediators along TLR-activated (and other [77]) signalling pathways. It will be particularly important to compare responses in children and adults, both from malaria-naïve populations (to examine age-dependent changes independently of stimulation by malaria and other parasites) and populations exposed to intense malaria transmission that have a discrete pyrogenic threshold.

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