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Clinical and molecular aspects of malaria fever

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Although clinically benign, malaria fever is thought to have significant relevance in terms of parasite growth and survival and its virulence which in turn may alter the clinical course of illness. In this article, the historical literature is reviewed, providing some evolutionary perspective on the genesis and biological relevance of malaria fever, and the available molecular data on the febrile-temperature-inducible parasite factors that may contribute towards the regulation of parasite density and alteration of virulence in the host is also discussed. The potential molecular mechanisms that could be responsible for the induction and regulation of cyclical malaria fevers caused by different species of *Plasmodium* are also discussed.

Historically malaria fever has been synonymous with malaria disease

Febrile illness is a generalized initial response by humans to infectious agents, autoimmune diseases, cancers and other physical ailments including injury. However, the cyclical and predictable nature of malaria fever sets it apart from most other febrile manifestations and is indeed the most salient, yet poorly understood, feature of *Plasmodium* infection (Box 1).

The disease characterized by cyclical fever was well recognized before *Plasmodium* species were identified as the causative agents of malaria. Early records of fever that can be attributed to malaria can be found in Bronze Age texts from China, India and Greece that include the writings of Huang Ti, the Atharvaveda, and early Greek medical works. In these accounts, there are references to symptoms such as tertian and quartan fevers, enlarged spleen, and the association of the disease with monsoon weather [1–3]. Definitive accounts of malaria occur in classical Indic (Charaka and Sushruta) and Greek (Hippocrates) medical texts. These texts not only document the classification of malaria fevers but also the association of these fevers with residence in marshy places [4]. Paradoxically, for centuries, malaria fever has also been associated with clinical benefits against several diseases including epilepsy, gout, mania and autoimmune diseases. In fact, in the early twentieth century, febrile illness induced by malaria infection was routinely used for the treatment

of neurosyphilis (for which Wagner-Jauregg was awarded the Nobel Prize in 1927) until the availability of the antibiotic penicillin made this practice obsolete [5]. The genesis of malaria fever and its biological foundations and consequences are poorly understood. While a notable body of information is available on the host response to malaria fever, few studies have examined how malaria fever impacts the parasite. In this review, we discuss febrile illness in the context of information gleaned from clinical malariology and recent laboratory-based studies on the parasite. These studies have shed new light on how malaria fever may affect the survival and virulence of *Plasmodium* parasites and impact the course of pathogenesis and immunity in the host.

The universal mechanism of fever induction

Fever is caused by fever-inducing agents (pyrogens) that signal to the thermoregulatory regions of the brain to elevate core body temperature (Figure 1.) External stimuli (such as injuries or infections) or internal stimuli (such as autoimmune diseases or cancer) cause the release of endogenous pyrogens that elicit a febrile response through a cascading series of events [6]. Some of the known endogenous pyrogens include the inflammatory cytokines TNF$-\alpha$, IL-1β, and IL-6, as well as complement factor 5a (C5a) and prostaglandin E$_2$ (PGE$_2$) [7]. Phagocytic monocytes in peripheral tissues have been shown to be important sources of these endogenous pyrogens in response to the exogenous pyrogens derived from pathogenic organisms. Studies with bacterial lipopolysaccharide (LPS) have shown that endogenous pyrogens act in several pathways that can transmit fever inducing signals from the periphery to the brain [6]. The choice of pathway appears to depend on the specific pyrogen and its route of delivery, and it is likely that more than one signaling pathway is used to regulate the different phases of fever initiation and maintenance [7].

Experiments have shown that in some fever signaling pathways, endogenous and exogenous pyrogens can travel through the circulation to the brain where they are detected by endothelial and perivascular cells associated with the blood brain barrier [8]. The circumventricular organs, and in particular the vascular organ of the laminar terminalis, are likely to play a key role in this process since

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they are located close to the thermoregulatory regions of the hypothalamus, are comprised of both vascular and neuronal tissue, and contain receptors for endogenous and exogenous pyrogens. At these sites, peripheral signals may be actively transported across the endothelial barrier, or they may be transduced into downstream signaling molecules such as PGE_2. PGE_2 is an indispensible mediator of fever signaling, and cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 are two key components of PGE_2 synthesis in the brain and peripheral tissues. Within the hypothalamus, PGE_2 is detected by the EP3 prostaglandin E receptor on preoptic neurons which in turn contact the brain regions that drive thermogenesis and skin vasoconstriction.

There is also evidence that pyrogenic signals may be transmitted from the periphery to the brain along nerve impulses. For example, Kupffer cells in the liver rapidly produce C5a and PGE_2 in response to LPS, and it has been proposed that branches of the vagal nerve may convey these signals to the hypothalamus through a noradrenergic pathway [6]. Such neuronal mechanisms have the potential to respond more rapidly than humoral-based mechanisms that presumably rely on the sequential synthesis and transport of pyrogenic mediators. Since the early phases of LPS-induced fever can be observed before pyrogenic cytokines are detected in circulation, it is likely that alternative mechanisms such as neuronal signaling play significant roles in regulating the early phases of the febrile response [6]. Malaria fever is caused by toxins of parasite origin, and data from field studies suggest that a minimum parasite density is required to trigger a febrile response (Boxes 2 and 3). Figure 2 depicts the

characteristic episodes of malaria fever experienced during *Plasmodium falciparum* infection in a malaria-naïve host.
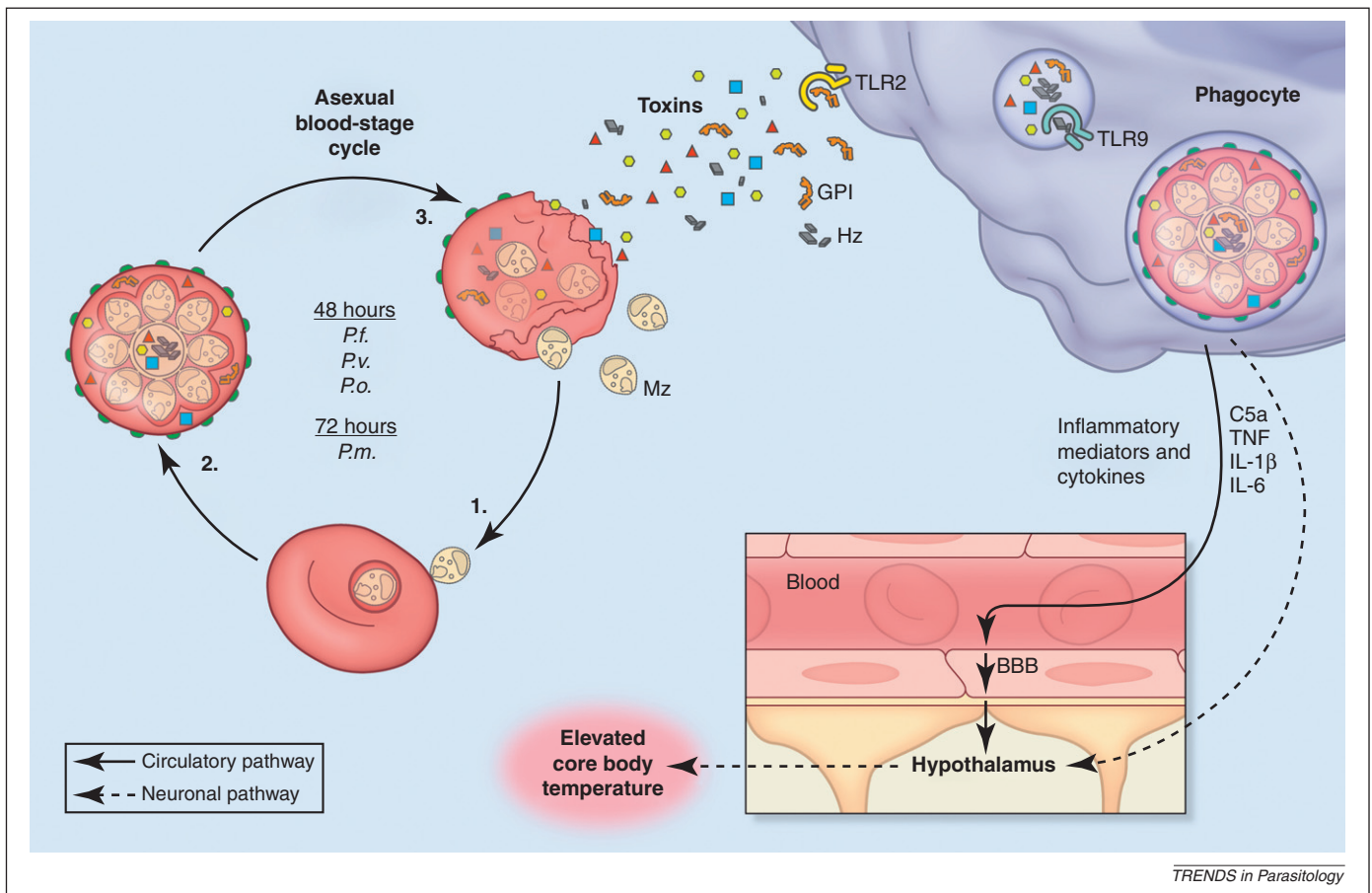
Potential sites of malaria fever signaling

The molecular events that culminate in a malarial febrile episode are initiated by the interaction between toxins released during schizont rupture and phagocytic cells. Given the sequestering nature of malaria parasites, the question arises whether there is a preferred site where signaling for malaria fever takes place. Circulating phagocytic cells may engulf the free malaria toxins in blood and initiate the febrile response. In children, the presence of circulating phagocytic cells containing hemozoin has been associated with severe malaria [9]. In addition, in severe malaria patients, hemozoin pigment is a marker of the presence of sequestered parasites and toxins in the liver, lungs, spleen, brain and placenta and in other vascularized tissues such as the intestines and kidneys [10–13]. Among these sites, the liver is likely to play a role in the febrile response to malaria due to its ability to filter and concentrate toxins from the blood and because it contains Kupffer cells, liver resident macrophages. Kupffer cells comprise the largest population of phagocytes in the body and are well poised to initiate fever signals. The lungs should also be considered favorable sites in the generation of malaria fever due to the presence of alveolar macrophages and the well characterized inflammatory response that leads to respiratory distress in severe malaria. In experimental fevers induced by LPS, early expression of fever signaling molecules has been detected in both the liver and lungs [7].

The biological basis of malaria fever remains poorly understood

Malaria glycosylphosphatidylinositol (GPI) and hemozoin, two well-recognized malaria toxins (Box 3), are *Plasmodium* pathogen associated molecular patterns (PAMPs) that

Figure 1. **A schematic diagram showing a proposed mechanism of malaria fever induction.** Cyclical malaria fever is linked to the parasite asexual blood-stage cycle which occurs inside red blood cells (RBCs) and causes the rupture of infected RBCs every 48 (*P. falciparum, P. vivax, P. ovale*) or 72 hours (*P. malariae*). 1: Erythrocyte Invasion; 2: Schizont; 3: Schizont Rupture and Release of Merozoites and Toxins. Lysis of infected RBCs releases parasite toxins that are recognized by pattern recognition receptors on phagocytes and other innate immune cells which then in turn produce pyrogenic inflammatory mediators and cytokines. Pyrogens induce the thermoregulatory regions of the hypothalamus to increase core body temperature (dashed bold line arrow), and may travel through the circulation to transduce signals across the blood brain barrier (solid line arrows), or alternatively they may stimulate the brain through sensory nerves (dashed thin line arrow). Abbreviations: Mz, merozoite; TLR, toll-like receptors; GPI, glycosylphosphatidylinositol; Hz, hemozoin; C5a, complement factor 5a; BBB, blood brain barrier.

Box 3. Toxins of parasite origin cause malaria fever

A large body of scientific data exists defining the molecular basis of fever induction and is based mainly on experimental studies of endotoxins such as lipopolysaccharide from Gram-negative bacteria. In comparison, our knowledge of *Plasmodium* toxins and how they induce malaria fever is still scant. Historical data indicate that pyrogenic toxins released by rupturing schizonts are the trigger for malaria fever. As early as 1894, Marchiafava and Bignami recognized that malaria fever is caused by parasite release of pyrogenic material in the blood [46]. Currently, hemozoin and glycosylphosphatidylinositol (GPI) are two candidate toxins with demonstrated proinflammatory properties (Figure 1), while other malaria toxins may remain to be discovered. Hemozoin, the first proposed malaria toxin, was initially implicated as a parasite factor responsible for the malaria paroxysm in 1912 by Wade H. Brown when intravenous injection of rabbits with hemozoin produced paroxysms characterized by a brief prodromal period, followed by chills and rising temperature, and a subsequent hot stage [47]. Hemozoin is formed during parasite degradation of host hemoglobin into amino acids as a source for protein synthesis; while globin is rapidly degraded to amino acids, ferrous heme is oxidized to a toxic ferric form that is subsequently detoxified by polymerization into hemozoin, an insoluble crystal formed in the food vacuole and released in the circulation upon schizont rupture [48]. The most studied malaria toxin candidate is GPI, a glycolipid that anchors proteins to the cell membrane. Although GPIs are expressed ubiquitously by eukaryotes, *Plasmodium* GPIs differ substantially from human GPIs in structure, abundance, and heterogeneity [14].

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have been shown to be recognized by toll-like receptors (TLRs), a class of animal-specific receptor proteins with variable extra-cellular leucine-rich repeats, expressed by cells of the innate immune system that stimulate a proinflammatory response when activated by non-self ligands [14] (Figure 1). Indeed, recognition of malaria GPI by cell surface TLR2 results in MyD88 dependent TNF$-\alpha$ production by macrophages [15]. Similarly, presentation of malaria DNA coated with hemozoin to intracellular TLR9 also elicits a potent inflammatory response [16–18]. The observation that malaria fever can be abrogated by administration of monoclonal anti-TNF antibody [19] indicates that TNF may be the primary pyrogenic cytokine that mediates malaria fever. While the pro-inflammatory cytokines triggered by malaria toxins are a recognized cause of malaria fever, such cytokine responses are also responsible for the more severe malaria symptoms such as cerebral malaria, anemia and in some cases multi-organ dysfunction [20]. Fittingly, efforts are underway to develop malaria-toxin-based anti-disease vaccines that would suppress the release of pro-inflammatory cytokines during acute infection [21].

While the universal mechanism of fever induction is likely to apply, differences in the number of infecting parasite broods, length of the erythrocytic stage cycle,
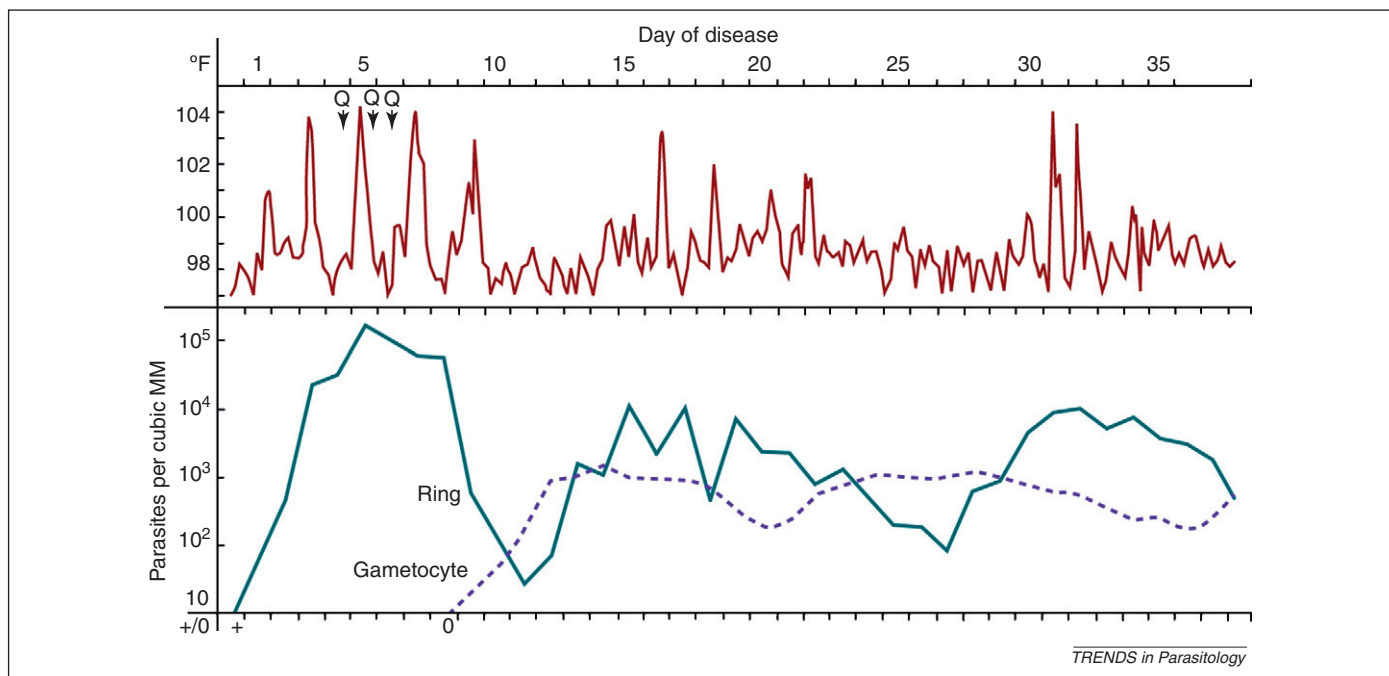
Figure 2. Episodes of tertiary fever observed during primary *P. falciparum* infection including mild remittance (caused by administration of quinine and denoted as Q) and two cycles of recrudescence. The solid line denotes ring form parasitemia while the broken line signifies gametocytes (modified from Ref. [49]).

and type and level of toxin moieties generated may account for the varying clinical patterns of malaria fever caused by different *Plasmodium* species (Box 1). Host genetic background and levels of anti-parasite immunity are also important yet poorly understood determinants of the severity of malaria febrile illness.

Effect of malaria fever on *P. falciparum* survival and growth

P. falciparum can be cultured *in vitro* [22], and this enables studies on the effects of febrile temperature on parasite growth in the absence of factors such as host genetics and immunity. Several *in vitro* studies have demonstrated that elevated temperature adversely affects the growth of *P. falciparum* parasite cultures [23,24]. In one study of asynchronous cultures, parasite survival was reduced by 23%, 66%, and 100% following 2, 8, and 16 hours of cultivation at 41 °C, respectively [25]. Paradoxically, recurrent fever may actually promote intraerythrocytic parasite development [26]. In a study that measured parasite stage progression by flow cytometric analysis of DNA content, there was a noticeable decline in parasite count in cultures subjected to one heat shock. However, growth inhibition was surprisingly absent following exposure of parasites to two heat shocks separated by a 10 hour recovery phase at 37 °C. Furthermore, recurrent exposure to elevated temperature actually accelerated parasite development from the ring to trophozoite stage resulting in a fourfold increase in newly invaded ring stage parasites. These results suggest that the parasite may have developed mechanisms to respond actively to the continued temperature peaks seen in malaria fever. Exposure to febrile temperature also promotes synchronization of asexual stage *P. falciparum* parasites in cultures [23]. Hence, we may speculate that malaria fever might serve as a communication signal among the

parasites that allow them to maintain their synchronicity and the length of duration of the erythrocytic stages cycle *in vivo*. If correct, this phenomenon may have a wide array of implications for parasite biology and virulence including the periodic and regulated release of toxins by rupturing schizonts and synchronized invasion of erythrocytes by merozoites.

Limited data is available for the direct effect of malaria fever on parasite density in the host. In a retrospective study in individuals infected with *P. vivax*, very high fevers ($\geq 40.5^0$C) were associated with a lower parasite density [27]. Even less data is available on the effect of malaria fever on gametocytogenesis. In one study, malaria fever and gametocytemia coincided in *P. vivax* and *P. ovale* infections [28]. Field studies are needed to determine whether malaria fever serves as a trigger to initiate gametocyte production and thus aid parasite transmission. Future studies are also needed to identify the following: (i) molecular signatures of fever that are unique to each *Plasmodium* species; (ii) the molecular events responsible for the rise, maintenance and decline of core body temperature during malaria; and (iii) a unifying mechanism of malaria fever induction that is common to all *Plasmodium* species.

Induction of parasite apoptosis by febrile temperatures

The mechanism of parasite death at elevated temperature is of considerable importance to understand the biological basis for reduction in parasite survival during febrile illness. The presence of pyknotic and hyposegmented schizonts in *P. falciparum* cultures exposed to 40 °C has been previously reported [23]. These morphological features are generally associated with 'crisis form' parasites, a term used to describe malaria parasites undergoing cell death [29]. While the mechanism of induction of crisis form
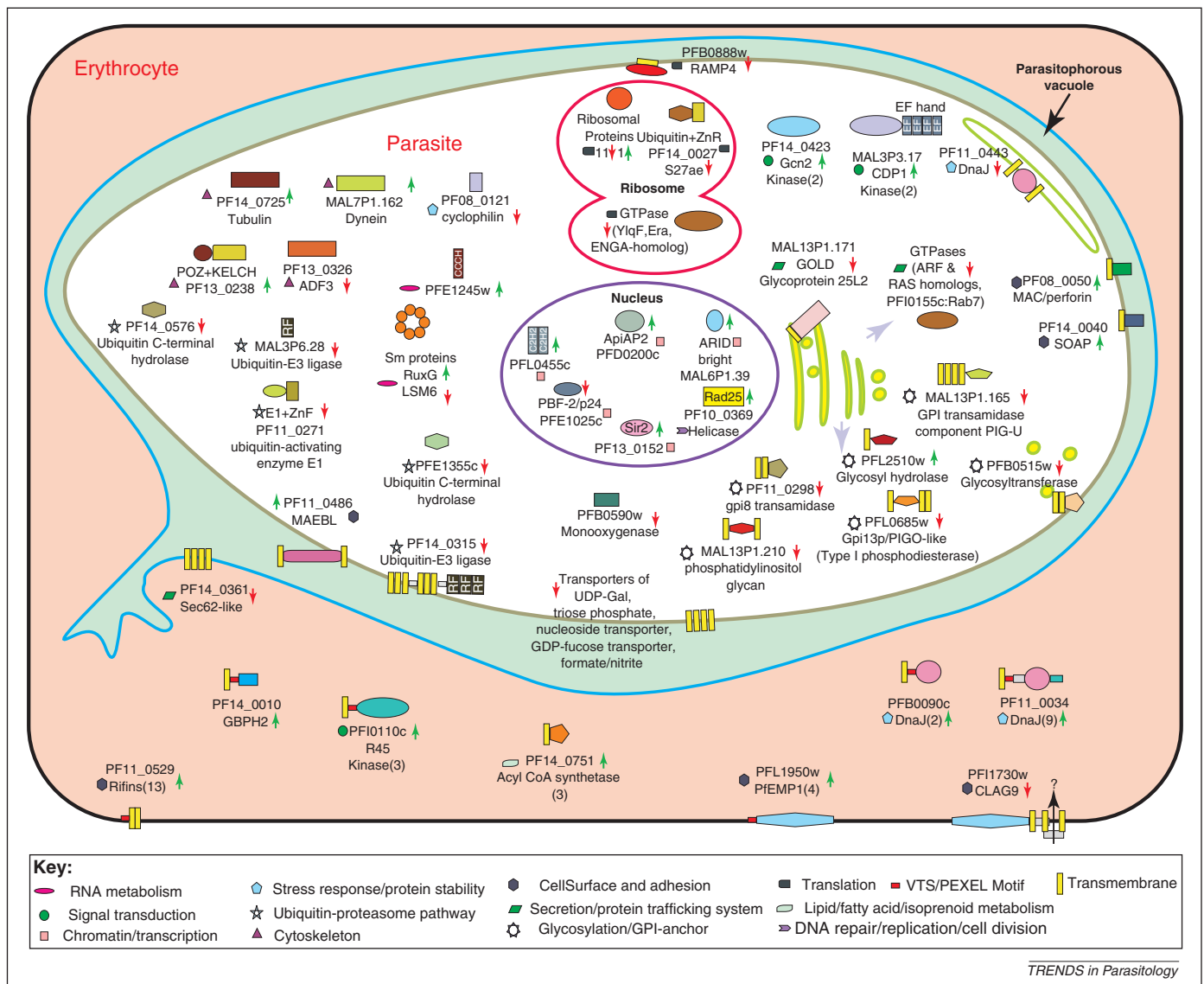
Figure 3. A cartoon depicting the predicted or known subcellular localization of various proteins whose genes show altered expression under febrile temperatures. The cells and organelles are schematically shown and are not drawn to scale. The membranous system depicted in the right side of the parasite cell is the Golgi and vesicular transport system. The alveolar membrane or endoplasmic reticulum system is shown to the extreme right. The proteins are depicted as domain architectures. The representative protein name is given and the number of lineage-specific paralogs showing similar expression changes, if any, is given in parentheses. Each protein is marked with a function symbol explained at the top of the figure. Green arrows pointing up indicate upregulation, while red arrows pointing down indicate downregulation. All the GPI anchor biosynthesis-related proteins are shown inside the parasite cell and are likely to colocalize with the Golgi and vesicular system involved in trafficking and maturation of proteins. Abbreviations: RF, ring finger; GBPH2, glycophorin-binding protein homolog 2; MAC, membrane attack complex; CoA, coenzyme A; ZnF, zinc finger domain. Figure modified from Ref. [25].

malaria parasites is not known, the possibility of febrile temperature inducing parasite apoptosis has been measured by the *in situ* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay that detects DNA fragmentation, which in several systems accompanies apoptosis. Cultivation of segmented *P. falciparum* schizonts for 2 hours at 41 °C resulted in TUNEL positivity of 60% of all infected red blood cells [25]. The thiol proteases known as metacaspases have been shown to be major regulators of apoptosis [30,31]. Although the *Plasmodium* genome encodes multiple metacaspases, a functional molecular pathway for apoptosis based on these proteins or other players has not yet been identified in these organisms. Hence, a key aspect in understanding malaria febrile illness would be the identification of components of the parasite apoptosis pathway.

Molecular alterations in *P. falciparum* induced by malaria fever

Microarray studies performed on *in vitro* parasite cultures that had been incubated at 37 °C or 41 °C resulted in the identification of 336 novel parasite biomarkers of malaria fever (Figure 3) [25]. Not surprisingly, these studies revealed a potent heat shock response that was characterized by the transcriptional overexpression of genes encoding two chaperone proteins and nine DnaJ domain proteins which are co-chaperones of HSP70. Most of these overexpressed DnaJ proteins belong to a remarkable lineage-specific expansion that has occurred in *P. falciparum*, suggesting that a heat-shock response mediated by these proteins might have a key role in survival at febrile temperature. Ten genes encoding parasite ubiquitin (Ub) system proteins were also down-regulated, resulting in

significant inhibition of ubiquitination of parasite proteins. This result is somewhat paradoxical, but might indicate that the parasite downregulates the Ub-mediated systems for protein degradation to temporarily slow down the growth process.

Indeed, computational analysis of microarray expression data also presents other direct molecular evidence for diminished growth, replication, and development of the parasite at febrile temperature. For example, *P. falciparum* replication factor C subunit 5 and replication factor A are downregulated, suggesting that parasite DNA replication may be hindered by elevated temperature. Likewise, pervasive downregulation of genes encoding ribosomal proteins is also observed, suggesting that growth is also reduced at the level of translation. This translational control might tie in with suppression of the Ub-system, as the decreased rate of protein synthesis might not support rapid protein turnover through Ub-targeted degradation.

The parasite's ability to sequester in the vasculature of host organs plays a critical role in malaria virulence, and it also facilitates immune evasion and avoidance of splenic clearance by the parasite. In *P. falciparum*, sequestration is caused by the cytoadherence of infected erythrocytes to host endothelial cells through the interaction of parasite erythrocyte membrane 1 (EMP1) expressed on the erythrocyte surface and host endothelial receptors such as ICAM1, CD36, and chondroitin sulfate A. *In vitro* studies have shown that febrile temperature augments the cytoadherence of young parasites to the CD36 and ICAM1 receptors [32]. Furthermore, elevated temperature induces transcriptional expression of five of the 60 members of the *var* gene family which encode the antigenically diverse EMP1 proteins. Similarly, the transcriptional regulatory protein Sir2a, which epigenetically regulates *var* gene switching, is also upregulated by elevated temperature. These data indicate that malaria fever may influence cytoadherence by altering the type and level of *var* antigen expression.

Malaria pathogenesis is also determined by the parasite's capacity to induce a potent proinflammatory response which is mediated by malaria PAMPs such as GPI. Genes for malaria GPI biosynthesis have been identified [33], and five key enzymes of this pathway are transcriptionally repressed by febrile temperature, indicating that fever may negatively regulate malaria GPI synthesis. This negative regulation of the parasite GPI biosynthetic pathway might reduce proinflammatory cytokine levels, consequently moderating fever, and might have a role in establishment of its cyclical pattern. Thus, it appears that parasite responses to febrile temperature could have simultaneous opposite effects on disease severity: an augmentative role for the parasite via increased cytoadherence, and a protective role for the host via downregulation of parasite PAMPs such as GPI.

There is also molecular evidence for significant remodeling of the host erythrocyte by the parasite in response to elevated temperature. The presence of a *Plasmodium* export element (PEXEL) [34] /vacuolar transport signal (VTS) [35] is essential for parasite proteins destined for transport into the cytoplasm or cell surface of the host erythrocyte (termed the malaria secretome). Approximately 22% (75 genes) of temperature altered genes encode proteins with a potential PEXEL motif and 72% (54 of 75) of these genes are overexpressed, indicating that elevated temperature may induce a major extrusion of parasite proteins to the host erythrocyte [25]. While several components of this temperature altered secretome have a role in cytoadherence and virulence (such as the *var* and *rif* genes), others might have a broader biological significance. Of these, the DnaJ domain proteins might help stabilize the host cell architecture at elevated temperatures. The R45 family of kinases, three of which are upregulated under febrile conditions, might play a role in modifying host proteins as part of coping with the heat shock. Lastly, members of the fatty acyl coenzyme A synthetase family, some of which have been demonstrated to be exported in specific vesicular structures to the host cell [36], are also overexpressed under febrile conditions, suggesting that alteration of the host lipids or membranes might be an important facet of parasite behavior in this situation. The biochemistry of these enzymes as well as the host-targeted, lineage-specifically expanded *Plasmodium* RESA N-terminal (PRESAN) or *Plasmodium* helical interspersed subtelomeric (PHIST) family are aspects of the malaria secretome that are likely to provide key insights in future investigations.

Importance of chromatin-level changes in the expression of the parasite secretome is illustrated by the observation that about 26% (90) of the genes showing a change in transcription in response to febrile conditions map to the subtelomeric gene arrays that encode members of the PRESAN, *var*, Rifin, and DnaJ families, in addition to several other proteins. This observation indicates a statistically significant bias in the preferential regulation of genes associated with chromosome ends, and points to special chromatin-related changes in the subtelomeric regions. At least 70% of these subtelomeric genes detected in the transcriptome analysis were found to be overexpressed, suggesting that these chromatin changes are likely to result in increased accessibility of particular regions of subtelomeric chromatin to allow the enhanced transcription of these genes [25]. Figure 3 summarizes the known and predicted sub-cellular localization of *P. falciparum* proteins based on microarray analysis of genes in parasites under febrile temperature.

Evolutionary considerations

The malaria fever presents several features of interest from an evolutionary standpoint. Indeed, some of its more puzzling aspects might become clear when the molecular aspects derived from expression studies are analyzed from the life history perspective of the parasite and host. Natural history and phylogenetic studies indicate that *Plasmodium* infects all the major lineages of amniote vertebrates, namely mammals, archosaurs (thus far seen only in birds and not crocodiles) and lepidosaurs (lizards) and is likely to have been ancestrally transmitted by a culicid mosquito [37]. It is likely that *Plasmodium* and related groups such as *Haemoproteus* and *Leucocytozoon* have been established in amniotes from at least the Middle Jurassic period (176-161 million years ago) when the culicomorph flies first

radiated. While saurian and avian malarias present symptoms comparable to mammalian malarias (e.g. decreased hemoglobin levels and rupture of erythrocytes), the former are not accompanied by periodic fevers [38]. However, both birds and lizards have been shown to be capable of a fever response (innate and behavioral, respectively). This suggests that fever as a host response to malaria has emerged more recently only in mammals. This is probably because modulation of parasite burden by elevated temperature reduces the prospect of a fatal outcome prior to development of an effective immune response. Hence, this symptom in mammals may be compared to the clinical benefit of fever, which has been reported in several infectious diseases and to the fact that anti-pyretic therapy results in poorer outcomes in several non-life-threatening infections [39].

As the spread of malaria is entirely dependent on the vector, it would aid transmission if the host is immobilized to allow unhindered blood-feeding by the mosquito. High fevers can result in such immobility; hence, it appears that the mammalian *Plasmodium* has exploited the host febrile response to favor its own transmission by fostering the development of debilitating fevers [40]. Indeed, the parasite-derived origin of the pyrogenic toxins supports this idea. On the other hand, a runaway febrile response could not just immobilize the host but also kill it, thereby nullifying the parasite's fitness. Hence, it appears that the parasite has evolved multiple mechanisms to regulate the febrile response. First, the achievement of synchrony in schizogony might allow a periodic febrile response allowing the host a period of recovery. The presumed parasite apoptosis and reduced growth and proliferation (evidenced from transcriptome analysis) might also contribute to control a runaway febrile response and reinforce its periodicity. This might also explain the fact that the fever-induced transcriptional changes in the parasite have opposing effects in terms of downregulating proinflammatory PAMPs (i.e. the GPI biosynthesis genes) while actively expressing cytoadherence genes that might facilitate parasite sexual development in sequestered sites for future transmission. Simultaneously, the parasite has also evolved means to mount a robust heat shock response that also protects the host cell from thermal effects. The possible deployment of DnaJ proteins and potential lipid-metabolism or membrane-remodeling proteins into the host cell during febrile illness might be key factors that help protect the host cell, shielding the parasite from thermal damage.

Concluding remarks

Evolutionary evidence and laboratory data suggest that malaria fever has a far reaching biological significance that confers survival benefits for both the parasite and the host. Recent studies indicate that exposure to febrile temperature induces cell death in *P. falciparum* parasites by an as yet undefined mechanism and thus helps regulate the parasite density in the host. At the molecular level, exposure to febrile temperature results in significant alteration in the *P. falciparum* transcriptome; the most notable changes include the molecules involved in growth and proliferation, the ubiquitin system, virulence, and the secretome of the parasite. Additional in-depth studies will be needed to understand the full impact of the effect of malaria fever on parasite biology and host pathogenesis and immunity.

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