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Interactions of the malaria parasite and its mammalian host

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A hallmark of *Plasmodium* development inside its mammalian victim is the remarkable restriction to the host species. Adaptation to an intracellular life style in specific target cells is determined by multiple parasite–host interactions. The first line of crosstalk occurs during intradermal sporozoite injection by an *Anopheles* mosquito. The following expansion in the liver is highly efficient and leads to successful establishment of the parasite population. During the periodic waves of fevers and chills the parasite destroys and re-infects red blood cells. Recent advances in experimental genetics and imaging techniques begin to expose the complex interactions at the changing parasite–host interfaces. Understanding the cellular and molecular mechanisms of target cell recognition, nutrient acquisition, and hijacking of cellular and immune functions may ultimately explain the elaborate biology of a medically important single cell eukaryote.

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Introduction

Malaria remains the most-important vector-borne infectious disease and probably kills more children than any other single pathogen. The complex disease, typically recognized by its cyclic patterns of fevers and chills, is caused exclusively during the rapid asexual multiplication phase of the *Plasmodium* parasite inside red blood cells [1•]. In order to get there the parasite first transforms and expand in an obligate, clinically silent, and uni-directional developmental phase in the liver [2]. The release of liver stage merosomes, packages of membrane-enclosed merozoites, marks the onset of malaria [3]. Individual merozoites use specialized surface proteins to propel themselves into erythrocytes [4].

Here, we will highlight some of the most recent insights into these parasite–host interactions with particular emphasis on their genetic basis. We will discuss how rodent *in vivo* models and new technologies, such as intravital imaging, influence our views of the parasite–host contacts underlying the successful persistence of the deadliest protozoan known to man.

Plasmodium sporozoites in the mammalian host: from skin stages to liver stages

Infection of the mammalian host with malaria is initiated when an *Anopheles* mosquito taking its blood meal injects *Plasmodium* sporozoites into the skin (Figure 1). Sporozoites subsequently travel from the dermis to the liver, invade and develop inside hepatocytes. Recent studies have established that after inoculation, sporozoites remain in the skin for extended periods of time [5,6], where they actively move in an apparently random fashion until they encounter a blood vessel and enter the blood circulation [7]. Not all injected sporozoites make it to the blood circulation and the liver. Some remain at the injection site in the skin, and are probably eliminated by recruited phagocytes [8•]. Others enter the lymphatic circulation and reach the draining lymph node, where they are eventually degraded [6]. These skin stages probably contribute to induction of protective immune responses against *Plasmodium*. In particular, it was shown that sporozoites injected in the dermis prime CD8+ T cell responses in the draining lymph node, which in turn act as effectors capable of eliminating parasites developing in the liver [9•].

In the skin, as previously proposed [10], sporozoites actively migrate through cells [8•], a process that involves disruption of the host cell plasma membrane [10]. At least three parasite proteins are involved during sporozoite cell traversal, sporozoite protein essential for cell traversal (SPECT)-1, SPECT-2 and a phospholipase [11–13]. Sporozoites lacking *SPECT-1* or *SPECT-2* are rapidly immobilized in the skin as a consequence of impaired cell traversal ability [8•].

Sporozoites that enter the blood circulation rapidly home to the liver, notably through interaction of the circumsporozoite protein (CSP), which covers the surface of sporozoites, with heparan sulfate proteoglycans (HSPGs) on liver cells. After crossing the sinusoidal cell layer, possibly through Kupffer cells [14], sporozoites switch from cell traversal to productive invasion (Figure 1). This later mode of invasion occurs without rupture of the host cell plasma membrane and results in the formation of a specialized compartment, the parasitophorous vacuole

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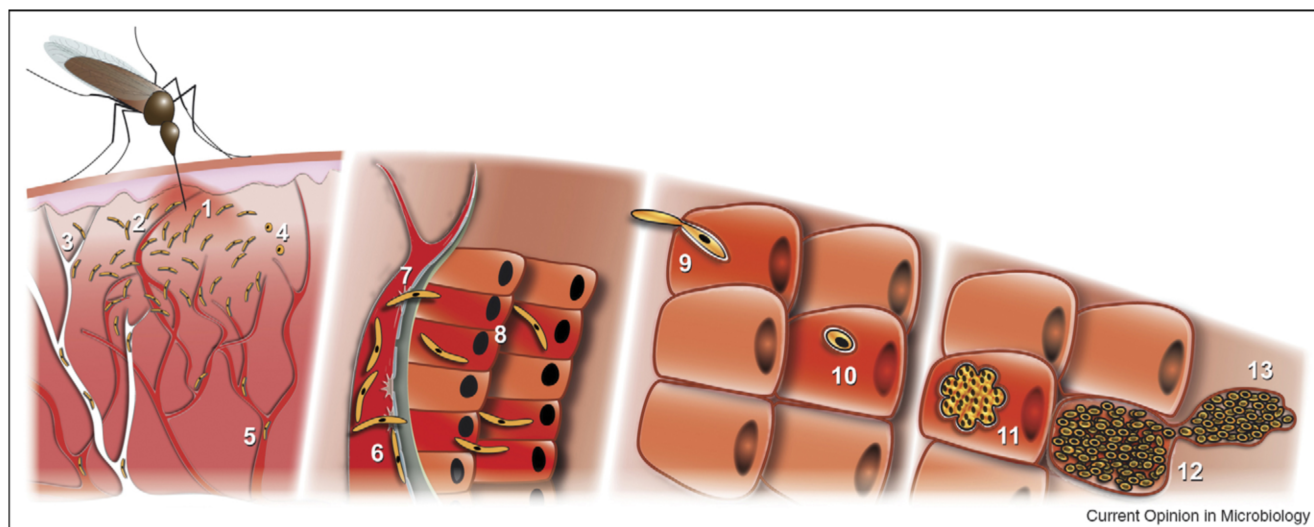
Figure 1



Plasmodium developmental program from transmission to egress of liver stage merozoites. (Far left) Malaria transmission. Sporozoite injection occurs during a blood meal of an infected female *Anopheles* mosquito. Sporozoites are injected intradermally and commence vivid migration in the dermis (1), resulting in either recognition of the basal side of a blood capillary (2), removal by the lymphatic system to the draining lymph node (3), or incomplete sporozoite transformation in the skin (4). In the skin, sporozoites actively traverse cells by breaching their plasma membranes. Once inside the blood vessel (5) sporozoites are rapidly distributed through the blood circulation. (Center left) Liver entry. When sporozoites pass the liver sinusoids they abruptly adhere to endothelial cells and start gliding locomotion (6). Crossing the sinusoidal barrier has been proposed to occur by transmigration through Kupffer cells, liver-resident macrophages (7). In the liver parenchyma, sporozoites actively traverse numerous hepatocytes (8). (Center right) Switch to productive invasion. Once a sporozoite reaches its final destination, a suitable hepatocyte, it actively enters under simultaneous formation of a tight junction and a nascent parasitophorous vacuole (PV) (9). Inside the PV, sporozoites rapidly transform into round early liver stages (10). (Far right) Maturation into liver stage-merozoites. Intrahepatic parasites commence cell division resulting in merozoite formation in a process called schizogony (11). Infectious merozoites are released as membrane-shielded merosomes (12). Merosomes are transported away (13) and eventually rupture in the lung microvasculature.

(PV). This switch from migration to productive invasion appears to be progressive since sporozoites continue to migrate through several hepatocytes before forming a PV in a final one [10,15]. The role of migration through hepatocytes during sporozoite infection is still debated [16], but recent results from Torgler *et al.* [17] indicate that it may have rather detrimental effects on parasite liver stage development through induction of inflammatory responses depending on NF-κB activation.

The molecular mechanisms underlying the transition from migration to productive invasion are not fully understood. *SPECT* mutants, which do not transmigrate but still infect cells by forming a PV [11], invade more rapidly than normal sporozoites [8•]. This observation suggests that migration may retard infection, and needs to be switched off to allow entry by PV formation. Coppi *et al.* [18•] found that the highly sulfated HSPGs in the liver provide signals promoting the switch to infection. Intracellular components encountered by sporozoites during cell traversal, including potassium and uracil derivatives, may also contribute to their activation [19,20]. Sporozoite activation results in apical regulated exocytosis and exposure of surface adhesive proteins [21]. These ligands may interact with cellular receptors to form a tight junction allowing the internalization of the sporozoite through an invagination of the hepatocyte plasma membrane [8•], leading to the formation of the PV.

Hepatocyte receptors mediating sporozoite entry have not been identified yet, but a recent study suggests that several pathways might be involved [22], one at least depending on the tetraspanin CD81 and cholesterol-enriched microdomains [23]. Two sporozoite proteins containing 6-cystein domains, P36 and P36p/P52, have been proposed to play a role in the establishment of the PV [24–26], but it is not known whether these molecules bind to host cell receptors or instead mediate signals promoting the switch to productive invasion. It is clear that additional yet uncharacterized sporozoite molecules are involved during invasion of hepatocytes, and may constitute potential targets for malaria vaccines.

Liver stage development: quiet seize of a perfect host cell

Following productive invasion, *Plasmodium* sporozoites develop and multiply into thousands of newly formed merozoites, which are contained within the PV membrane (PVM) until shortly before they are released from the hepatocytes into the bloodstream (Figure 1)

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(reviewed in [2]). However, only recently have the molecular events that take place during the hepatic stage of infection begun to be elucidated and very little is known about the interactions that occur between *Plasmodium* liver stages (LS) and host cell molecules. It seems likely, however, that LS have developed multiple strategies to exploit the rich hepatocyte's resources whilst ensuring their own survival in a potentially hostile environment. The demonstration that the parasite's CSP plays an important role in creating favorable conditions for parasite development, by enhancing its growth and downregulating the expression of inflammatory genes, constitutes a striking example of such a strategy [27].

In the last few years, several *Plasmodium* proteins were identified that seem essential for the parasite's normal developmental process. Parasites lacking *UIS* (upregulated in infective sporozoites) gene 3, *UIS4*, and *Pb36p* [25,28,29] display arrested intrahepatocytic development and, more importantly, are able to confer long-lasting, sterile protection against re-infections. These observations led to a surge in the investigation of the potential use of such genetically attenuated parasites (GAPs) as part of a whole-organism vaccine strategy (reviewed in [30]).

UIS3 was recently shown to interact with the liver-fatty acid binding protein (L-FABP) *in vitro* [31]. Although a direct interaction between UIS3 and L-FABP could not be demonstrated in hepatocytes, the authors note that downregulation of L-FABP leads to a reduction of parasite development. UIS3 localizes to the PVM that constitutes the interface between host cell cytoplasm and the parasite [31]. The reported interaction of UIS3 and L-FABP suggests that the latter may dock to UIS3 to deliver fatty acids to the LS. Whether or not this is the case, it seems evident that lipid delivery is an important requirement for LS development, as the downregulation of the expression of the lipoprotein receptor scavenger receptor type B class I (SR-BI) was also shown to inhibit parasite growth *in vitro* [32].

At the end of their developmental process in the liver, *Plasmodium* parasites differentiate into merozoites, which are contained inside host cell derived vesicles called merosomes (Figure 1) [3]. Cystein proteases are thought to mediate the release of merozoites from hepatocytes, a process also known as egress (reviewed in [33]). A class of potential cysteine proteases, termed serine repeat antigens (SERAs), is upregulated in late LS [34], suggesting a potential involvement in the liberation of merozoites from merosomes. Intravital microscopy analysis of *Plasmodium yoelii*-infected rodents has revealed that most merosomes exit the liver intact, presumably thus protecting the hepatic merozoites from phagocytosis by Kupffer cells, the resident macrophages in the liver [3,35•]. Interestingly, merozoites are eventually released in the lung capillaries, where they reach the bloodstream and initiate the symptomatic blood-stage of infection [35•].

A recent study combining transcriptome and proteome analysis identified approximately 2000 active genes and 800 proteins throughout the development of *P. yoelii* LS [36•]. A complementary, but more restricted, study employed expression profiling of subtracted cDNAs during late LS development and identified a few annotated genes that are differentially upregulated as compared to trophozoite development [37]. The emerging picture is that during maturation of liver stage and blood stage merozoites the parasite utilizes stage-specific parasite factors to generate otherwise identical invasive stages. Nevertheless, it seems clear that these, as well as other recently gathered data, will require further investigation before one can gain a more thorough insight into the molecular mechanisms that regulate the silent, yet obligatory, stage of the *Plasmodium* life cycle.

Merozoite entry into the erythrocyte: multiple choices

Plasmodium and related apicomplexan parasites, such as *Babesia* and *Theileria*, have the unusual capacity to rapidly invade red blood cells (RBC). Erythrocyte invasion by merozoites occurs in seconds and follows several steps, each involving multiple receptor–ligand interactions (Figure 2) (reviewed in [4]). Our view of the underlying molecular events is largely influenced by biochemical and structural data (reviewed in [38]), primarily because experimental genetics are not feasible in erythrocytes and limited to redundant genes in the malaria parasite. Initial attachment occurs at any orientation and is mediated by the major merozoite surface proteins (MSPs), mainly the interaction of MSP1 with band 3 on the erythrocyte surface [39]. Studies with inhibitory antibodies indicated that the transmembrane protein apical membrane antigen 1 (AMA-1) mediates the next event, reorientation of the apical end of merozoite towards the erythrocyte surface [40]. Two distinct and largely redundant parasite transmembrane protein families drive merozoite penetration, under simultaneous formation of the parasitophorous vacuole. The erythrocyte binding antigens (EBAs), for example EBA140, EBA175, and EBA181, bind to glycophorin C, glycophorin A and a yet unknown receptor, respectively. The *Plasmodium falciparum* reticulocyte-binding homologs (*Pf*Rh), for example *Pf*RH1, *Pf*RH2a, *Pf*RH2b, and *Pf*RH4, engage unknown receptors (reviewed in [4]).

Hierarchic organization of *Pf*RHs and EBAs rather than a switch in gene expression permits efficient entry while maintaining the possibility to instantaneously opt for a whole range of invasion pathways [41]. A recent study in Kenya showed that these alternative invasion pathways are apparently extensively used by wild-type parasites [42•]. Inhibitory antibodies from malaria-exposed individuals

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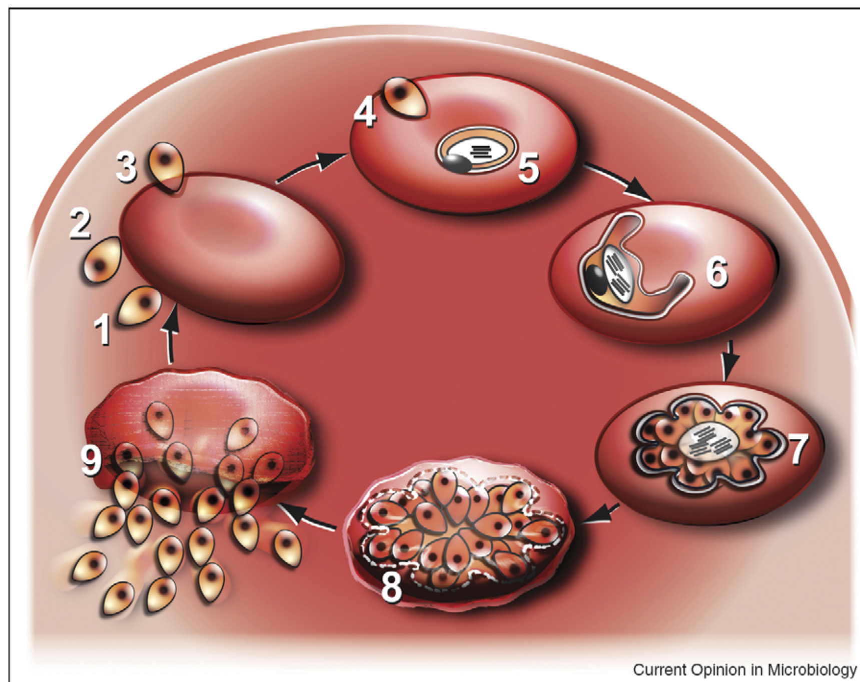
Figure 2



Cycles of asexual parasite replication inside red blood cells. (1) Extracellular merozoites attach randomly to the erythrocyte surface. (2) Initial attachment is re-inforced by orientation of the apical end of merozoite towards the erythrocyte surface. (3) Tight junction formation and active merozoite entry into the erythrocyte. (4) Merozoite invasion occurs by simultaneous formation of a parasitophorous vacuole. After completion of invasion the merozoite transforms into a ring stage (5). This stage is characterized by a large digestive vacuole, where hemoglobin digestion results into formation of the malaria pigment, hemozoin. (6) The expanding parasite during its intraerythrocytic growth phase is termed trophozoite. Hemozoin accumulates in the digestive vacuole. (7) DNA replication precedes cell budding, a process termed schizogony. Merozoites bud off the central syncytium. (8) Merozoites secrete exonemes to initiate exit from the parasitophorous vacuole and host erythrocyte. (9) Merozoite egress. Free merozoites adhere to an adjacent erythrocyte within seconds (1), initiating a new erythrocytic cycle.

block an increasing number of specific invasion pathways as individuals grow older. While providing a fascinating example for coevolution of parasite–host cell receptor–ligand interactions, this finding highlights the challenges for potential vaccine strategies that need to account for multiple alternative ligands.

The molecular events that permit cytolysis and efficient egress out of the erythrocytes are less well understood. Yeoh *et al.* [43••] showed that *Plasmodium* compartimentalizes the molecules that function in egress in specialized organelles, termed exonemes. Among others, they contain the subtilisin-like serine protease subtilase 1 (SUB1) that proteolytically activates abundant SERAs, which in turn may process cellular substrates.

Development inside red blood cells: a life-threatening niche

Mature red blood cells are terminally differentiated cells that lack standard biosynthetic pathways and intracellular organelles. Of considerable immunological advantage for a persisting pathogen, erythrocytes do not display antigens in the context of the major histocompatibility complexes on their surfaces. However, the absence of endocytic and secretory pathways poses a potential obstacle for a fast-growing intracellular parasite that typically recruits host organelles for nutrient acquisition rather than relying on cellular diffusion processes. The solution for the growing ring stages is dietary restriction to the abundant hemoglobin and refurbishment of their new home by dramatic expansion of their surface area through formation of a tubovesicular network (TVN) and by considerable export of a range of remodelling and virulence factors (reviewed in [44,45]). Protein export into the host RBC is mediated through a specific targeting sequence, termed *Plasmodium* export element (PEXEL) or host targeting (HT) signal. A recent elegant study by Chang *et al.* [46•] established that this motif functions as a classical cleavage and *N*-acetylation site. This short signature is present in several hundred parasite proteins, half of which belong to families of variable antigens that are exported to the erythrocyte surface, including the variant antigens (VARs), subtelomeric variable open reading frames (STEVORs), and repetitive interspersed family (RIFINs). Functional analysis of the dozens of hypothetical proteins will expand our current list of documented

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interactions of parasite protein with the erythrocyte cytoskeleton that is extensively modulated during intracellular expansion (reviewed in [45]).

An example of parasite-encoded effector proteins is a class of kinases, FIKK/TSTK, which is expanded in the human pathogen *P. falciparum* [47,48]. Individual kinases appear to be differentially expressed and are exported via the PEXEL/HT signature to the Maurer's clefts [47], *P. falciparum*-induced lamellar structures where virulence proteins accumulate en route to the erythrocyte surface [49]. Systematic studies of the exported proteins are expected to ultimately explain the evolvement of host restriction and coevolution of parasite effector and host cell target proteins [50]. Intriguingly, a similar mechanism is employed by oomycetes, plant pathogens that translocate effector proteins in order to establish infection [51]. This shared mechanism is a striking indication for a phylogenetic relationship of otherwise distant eukaryotic pathogens.

From a cell biological perspective, infected RBCs are an ideal model system to study the minimal host cell repertoire required for intracellular development of a pathogen. Two recent proteome studies identified 592 and 644 membrane-associated and soluble proteins in (non-infected) human and mouse RBCs, respectively [52,53]. These studies revealed that RBC physiology and their transformation from reticulocytes to aging erythrocytes are highly dynamic processes that involve many more proteins than anticipated previously. The in-depth knowledge of the protein make-up paves the way for future systematic biochemical studies of RBC host factors that are central for *Plasmodium* development.

Refinement of our understanding of the molecular interactions at the parasite–host interface crucially depends on studies that address the composition of the PVM. Spielmann *et al.* [54•] recently performed the first in-depth analysis of a class of abundant PVM-resident proteins, termed early transcribed membrane proteins (ETRAMPs). These small, highly charged transmembrane proteins locate to the PVM of the growing parasite [55]. *ETRAMP* expression is developmentally regulated and some members are expressed exclusively in ring stages [55], whereas others are liver-stage specific [27,28]. *In vivo* cross-linking and heterologous expression studies showed that ETRAMPs form distinct oligomers that localize to microdomains, indicating polarity of the parasite–host interface [54•].

Intraerythrocytic development results in the initial rapid expansion and sustained cycling of the parasite population in the infected host. We are only beginning to understand the level of complexity of parasite–host factor interactions that permit growth in this unusual host cell.

Malaria pathology: responses to a persisting pathogen

Clinical manifestations of malaria cover a wide range of symptoms. Although most infected individuals will only have a relatively benign febrile illness, 1–3 million deaths per year occur from severe malaria, mainly in non-immune children. This comprises several syndromes such as severe anaemia, acute respiratory distress or cerebral malaria [1•]. The basic pathological mechanisms behind all these forms of severe disease are still not fully elucidated.

The use of rodent models of malaria pathology has been quite useful to help understanding the mechanisms behind the onset of severe malaria. Experimental infection of mice with *Plasmodium berghei* ANKA has provided the community with a powerful model to define genetic determinants that regulate the development of cerebral malaria (CM), and the actual picture is already quite complex. Like in *P. falciparum*, the major host receptor for sequestration of *P. berghei*-parasitized erythrocytes from the circulating blood is the scavenger receptor CD36 [56,57]. However, the onset and progression of CM in mice is CD36-independent. In recent years, it has been shown that the development of CM in *P. berghei* ANKA-infected mice requires the host complement cascades [58], histamine-mediated signalling [59], chemokine receptors in the brain [60–62], and dendritic cells and T cell subsets [63]. Recent studies from different laboratories have also opened several controversies, especially around the involvement of TLRs [64–66] and regulatory T cells [67–69] in the outcome of CM in mice, which hopefully will be solved soon.

Interestingly, it has also been shown that the host's rate-limiting enzyme in the catabolism of free heme, heme oxygenase-1 (HO-1), which degrades heme to generate biliverdin, iron and CO, dictates the susceptibility to CM in mice infected with *P. berghei* ANKA. HO-1 was found to be upregulated to a lesser extent in infected C57BL/6 mice, all of which succumb to CM, than in infected BALB/c mice, which do not develop signs of CM. Moreover, deletion of *Hmox1* or inhibition of HO activity in BALB/c mice increased CM incidence [70•]. Interestingly, NO, which induces HO-1, was also shown to protect mice from early CM [71•]. It has been also shown that exposure to inhaled CO protects mice against CM. While HO-1 and CO did not affect parasitemia, both prevented the disruption of the blood–brain barrier, brain microvasculature congestion and neuroinflammation, including $CD8^+$ T-cell brain sequestration [70•].

It has been shown that HO-1 and CO play a major role in preventing neuroinflammation in CM. The protective mechanism of CO seems to be mediated by the binding of CO to hemoglobin, preventing its oxidation and the generation of free heme, a molecule shown to contribute

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to the development of early CM in mice [70•]. Interestingly, the reported protective mechanism of NO in CM appears to operate by a similar mechanism, the binding of NO to hemoglobin to prevent the generation of free heme [71•].

More recently, an unexpected role for HO-1 during the initial liver stage of infection was also demonstrated. Infection of mouse liver by *Plasmodium* sporozoites leads to an upregulation of HO-1 in hepatocytes and macrophages/leukocytes. *Hmox1* deletion as well as HO-1 downmodulation using siRNA leads to complete abrogation of infection, when this is initiated with low numbers of parasites, due to an increase in the number and size of liver infiltrates and production of pro-inflammatory cytokines [72]. Thus, HO-1 is a host molecule that controls both the establishment of the *Plasmodium* liver stage of infection and the development of pathology during the blood stage of a malaria infection.

Indeed, one should keep in mind that the blood and liver stages of infection usually coexist in populations living in malaria-endemic areas. Therefore, the final outcome of the host–*Plasmodium* interactions is subject to an intricate control by many host molecules, some of which may play distinct roles in different tissues at different stages of the *Plasmodium* life cycle. While this has been shown unequivocally for HO-1 in the context of early CM and the liver stage of infection, one cannot exclude that other host factors might act in concerted ways to ensure the success of *Plasmodium*–host interactions.

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