The silent path to thousands of merozoites: the *Plasmodium* liver stage

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Abstract | *Plasmodium* sporozoites are deposited in the skin of their vertebrate hosts through the bite of an infected female *Anopheles* mosquito. Most of these parasites find a blood vessel and travel in the peripheral blood circulation until they reach the liver sinusoids. Once there, the sporozoites cross the sinusoidal wall and migrate through several hepatocytes before they infect a final hepatocyte, with the formation of a parasitophorous vacuole, in which the intrahepatic form of the parasite grows and multiplies. During this period, each sporozoite generates thousands of merozoites. As the development of *Plasmodium* sporozoites inside hepatocytes is an obligatory step before the onset of disease, understanding the parasite's requirements during this period is crucial for the development of any form of early intervention. This Review summarizes our current knowledge on this stage of the *Plasmodium* life cycle.

Hemocele
The system of blood-containing spaces pervading the body in the mosquito.

Gliding motility
A form of substrate-dependent locomotion in which the parasite maintains a fixed shape.

Parasitophorous vacuole
A vacuole within the host cell in which the parasite resides.

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Despite the fact that several countries were able to eradicate malaria during the past century, it remains one of the most prevalent infectious diseases worldwide. Forty percent of the world's population is at risk of infection, and 500 million people become infected and up to 3 million children die every year[1]. Malaria also has devastating economic consequences as it drains as much as 2% of the gross domestic product of countries in sub-Saharan Africa.

Malaria infection is initiated when *Plasmodium* sporozoites enter the mammalian host through the bite of an infected female *Anopheles* mosquito. During a blood meal, an average of 15–123 sporozoites have been reported to be deposited under the skin of the host, which migrate to the liver, where they infect hepatocytes and begin to develop into merozoites[2–5]. Between 2 and 16 days later, depending on the *Plasmodium* species, thousands of merozoites per invading sporozoite are released into the bloodstream[6–8]. Each merozoite will invade an erythrocyte, initiating a replication cycle that ends with the release of new merozoites from the mature infected erythrocyte (schizont), which go on to infect other erythrocytes. Malaria-associated pathology only occurs during the blood stage of infection. The *Plasmodium* life cycle continues when some merozoites develop into the sexual parasite stages, the male and female gametocytes, which can be taken up by mosquitoes during blood meals. Gametocytes undergo fertilization and maturation in the mosquito midgut, forming an infective ookinete form that migrates through the mosquito midgut into the hemocele, developing into the oocyst in which sporozoites are formed. When fully matured, the oocysts burst and release sporozoites, which migrate into the mosquito's salivary glands, ready for the next transmission step.

An obligatory step during infection is the establishment and full development of *Plasmodium* sporozoites inside hepatocytes, which, although symptomatically silent, gives rise to thousands of merozoites in each hepatocyte. Similar to many of the invasive stages of other parasites in the phylum *Apicomplexa*, *Plasmodium* sporozoites have two interconnected characteristics that are crucial for the completion of their life cycle: gliding motility[9] and the capacity to migrate through host cells[10]. The infection of host cells by *Plasmodium* sporozoites requires sporozoite motility[11] and presumably involves invagination of the plasma membrane, with the formation of a parasitophorous vacuole around the parasite[12]. *Plasmodium* sporozoites can also enter and exit host cells by breaching the plasma membrane, a process that requires gliding motility and is used to migrate through host cells and tissues[10].

Four different species of *Plasmodium* can cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. *P. falciparum* is by far the deadliest of the four and is responsible for most of the mortality and morbidity associated with malaria. It is worth mentioning that *P. vivax* and *P. ovale* can exist in dormant forms in the liver, called hypnozoites. The

latent nature of this stage is responsible for the relapses that can occur when malaria is caused by either of these two species[13]. A significant amount of research involving the *Plasmodium* pre-erythrocytic stages has made use of *Plasmodium* spp. that infect rodents, specifically *Plasmodium berghei* and *Plasmodium yoelii*. These two species present differences in infectivity, which depend not only on the species but also on the clone and the genetic background of the rodent host[14–16]. *P. berghei* remains the most widely used rodent parasite because the technologies to enable transfection were developed earlier for *P. berghei*[17] than for *P. yoelii*[18]. These differences should always be taken into account when interpreting results.

From the skin to the liver

When an infected mosquito bites a mammalian host, it probes for a blood source under the skin. During this process, the mosquito injects saliva containing vasodilators and anti-coagulants to facilitate the ingestion of blood[19,20]. If the mosquito is infected and contains *Plasmodium* sporozoites in its salivary glands, a small number of these are deposited in the skin during the blood meal[3] (FIG. 1). Most sporozoites are injected into the dermis and not directly into the circulation[2,21,22]. After injection into the skin, the sporozoites move through the dermis until they come into contact with a blood vessel and move into the circulatory system, which allows them to travel to the liver[2,22]. A proposed alternative route for the sporozoite journey to the liver is through the lymphatic system, possibly inside leukocytes[23,24]. Intravital microscopy work[22] using the *P. berghei* rodent model of malaria showed that sporozoites deposited into avascular dermal tissue use gliding motility to migrate through the skin and into dermal vessels, a process that can take over 30 minutes. It has been proposed that a sporozoite surface phospholipase (PbPL) is required to breach host cell membranes during migration in the skin, as parasites that are deficient in PbPL are impaired in their ability to cross epithelial cell monolayers, and their infectivity is greatly decreased when they are transmitted by mosquito bite[25]. During migration in the skin the parasite might be vulnerable to antibodies against *Plasmodium* surface proteins, which might function as the first line of the host's acquired immune response against the parasite[22].

Recent intravital microscopy observations showed that a significant proportion of mosquito-injected sporozoites remain in the dermis after gliding has stopped[2]. Of those that leave the area of the bite within 1 hour of injection, approximately 70% enter the blood vessels and the remaining 30% invade the lymphatic vessels. Most of those entering the lymphatic vessels do not reach the circulatory system, as had been previously assumed. Instead, they are trapped in the lymph nodes, where most are phagocytosed by dendritic cells. Some of these lymphatic sporozoites have been found to partially develop into small-sized exoerythrocytic forms, similar to the *Plasmodium* stage that develops inside hepatocytes, before eventually being degraded[2]. Whether the presence of parasites in an important organ of the immune system has any influence on the development of the anti-malarial immune response remains to be elucidated.

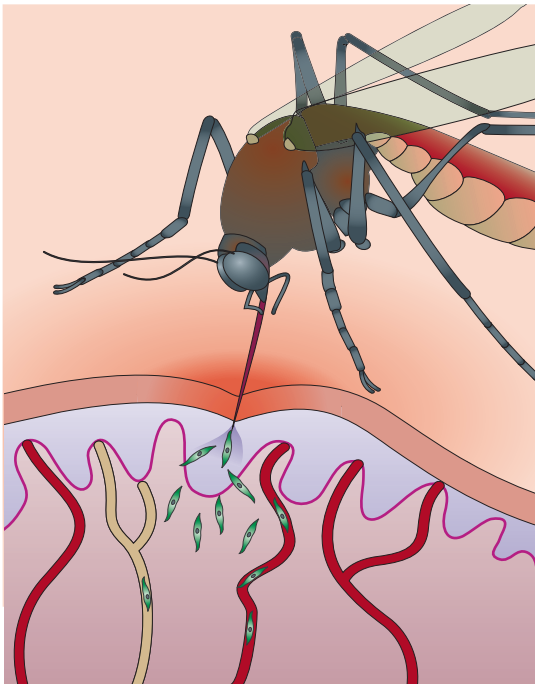


Figure 1 | **Entering the vertebrate host.** *Plasmodium* sporozoites (green) are deposited under the skin of the vertebrate host through the bite of an infected female *Anopheles* mosquito. After injection into the skin, the sporozoites move through the dermis until they contact blood vessels (red) and move into the circulatory system, which allows them to travel to the liver. A small proportion of sporozoites can enter the lymphatic system (yellow).

Arrest in the liver

Once inside the circulatory system, sporozoites rapidly reach the liver sinusoids (FIG. 2). Specific targeting of the liver by sporozoites is efficient, as they are found in hepatocytes as early as 2 minutes after intravenous injection into mice[26]. The selectivity of this process suggests that it involves specific interactions between parasite-encoded surface protein(s) and host molecule(s).

Both host and parasite molecules involved in sporozoite arrest have been extensively studied. A major *Plasmodium* sporozoite surface protein, the circumsporozoite protein (CSP), seems to have a crucial role in these processes by interacting with the heparan sulphate proteoglycans (HSPGs) of the liver cells. Initially, it was shown that recombinant CSP binds specifically to hepatocyte microvilli within the space of Disse, a space that separates the sinusoidal endothelium from hepatocytes[27–29]. Two domains in CSP, a thrombospondin-like cell-adhesive region II-plus at the carboxyl terminus and a positively charged motif upstream from conserved region I, seem crucial for this binding[28,30,31].

Intravital microscopy
The direct real-time imaging of biological phenomena in exposed tissues.

Dendritic cell
A 'professional' antigen-presenting cell that is found in the T-cell areas of lymphoid tissues and as a minor cellular component in most tissues. Dendritic cells have a branched or dendritic morphology and are the most potent stimulators of T-cell responses.

Sinusoid
A small, fenestrated blood vessel found in the periphery of the lobules of the liver. Sinusoids are lined with endothelial and Kupffer cells.

Heparan sulphate proteoglycan
(HSPG). A family of glycoproteins that function as co-receptors for growth factors and matrix proteins. They are present on the cell surfaces and in the extracellular matrix of most mammalian cells. In the liver, HSPGs are thought to either directly internalize bound lipoproteins by hepatocytes or to localize lipoproteins to the cell surface.

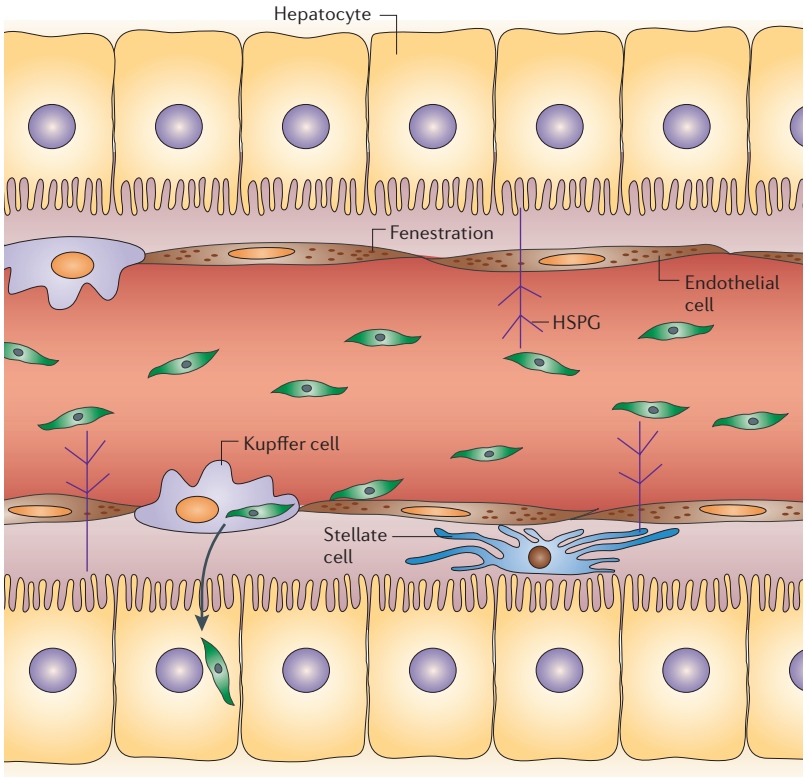


Figure 2 | **Sporozoite arrest in the liver.** Once the sporozoites (green) reach the liver sinusoids, they glide over the endothelium and interact with heparan sulphate proteoglycans (HSPGs) from hepatocyte and stellate cells. They then cross the sinusoidal layer, possibly through Kupffer cells as shown.

In the host, the HSPGs on the surface of hepatocytes are considered the main receptors for *Plasmodium* attachment in the liver sinusoids[32,33]. As liver endothelial cells have fenestrations, it has been proposed that hepatocyte or extracellular matrix HSPGs extending through endothelial fenestrations to the sinusoidal lumen can interact with CSP, leading to sporozoite sequestration[32]. Although HSPGs are present in most tissues, those associated with cells in the liver have a significantly greater degree of sulphation than those present in other tissues[34], which might account for the selective recognition of recombinant CSP and *Plasmodium* sporozoites in the liver. In particular, sulphation of the glycosaminoglycan chains at both the *N*- and *O*-positions is required for sporozoite adhesion to cells[35].

More recently, the proteoglycan species produced by different liver cell types have been characterized. CSP and thrombospondin-related anonymous protein (TRAP) recognize distinct cell-type-specific surface proteoglycans, not only on hepatocytes, but also on Kupffer cells and stellate cells. Moreover, because stellate cells synthesize eight times more sulphated proteoglycans than hepatocytes and incorporate twice the amount of sulphate into heparan sulphate, matrix proteoglycans that are produced by stellate cells and protrude through the endothelial fenestrations have been suggested to mediate the initial arrest of *Plasmodium* sporozoites in the liver sinusoids[36,37].

Kupffer cell
A macrophage, or monocytic cell, that is permanently located in the liver between sinusoidal endothelial cells. Kupffer cells form the largest population of macrophages in the body.

Stellate cell
A highly branched perisinusoidal cell, which is the principal producer of the extracellular matrix in the liver that contributes to hepatic inflammation through the secretion of chemokines and the recruitment of leukocytes.

Hepatocyte invasion

After being sequestered in the sinusoids, sporozoites must reach and invade the hepatocytes (FIG. 2). They encounter two different cell types on the way: endothelial and Kupffer cells. Although liver endothelial cells have fenestrations, these are too small (about one tenth of the diameter of a sporozoite) to allow sporozoite passage[27]. As sporozoites can migrate through all nucleated cell types tested so far by disrupting their plasma membrane (M.M.M., unpublished results), it is plausible that sporozoites could traverse either endothelial or Kupffer cells. However, there is mounting evidence to indicate that sporozoites cross the sinusoidal layer primarily through Kupffer cells[38].

First, *in vitro*, in co-cultures of Kupffer and primary liver endothelial cells, sporozoites were found to preferentially invade the former[39]. Interestingly, invasion of Kupffer cells by sporozoites is reported to occur with the formation of a parasitophorous vacuole. However, because this vacuole does not fuse with lysosomes the authors suggested that this process does not involve phagocytosis[12,39]. Therefore, according to these results, crossing the sinusoidal layer occurs via Kupffer cells, not by traversing the cell by disrupting the plasma membrane but by the formation of a vacuole. Moreover, using intravital microscopy, all sporozoites arrested in the liver sinusoids were found to move towards the hepatocytes through Kupffer cells at a lower speed than the speed measured when sporozoites traverse cells using plasma-membrane disruption[40]. However, it has recently been reported that *P. berghei* parasites deficient in the SPECT (sporozoite microneme protein essential for cell traversal) and SPECT2 (also called *Plasmodium* perforin-like protein 1, PPLP1) proteins, which confers a defect in the ability to traverse cells, have low infectivity *in vivo*[41,42]. These results seem to indicate that the crossing of the sinusoidal layer can also occur by the disruption of cell plasma membranes. As the removal of Kupffer cells increased the infectivity of SPECT-deficient sporozoites, the authors also interpreted these results as a confirmation of the essential role of Kupffer cells in sporozoite infection of the liver. However, the treatment to deplete Kupffer cells might have left small discontinuities in the sinusoidal endothelial layer that would allow parasites to reach hepatocytes directly. Therefore, it is not clear from these results whether during normal infection, in the absence of discontinuities, Kupffer cells are the cells that are preferentially traversed. It is likely that sporozoites can use more than one cellular pathway to cross the sinusoidal endothelial layer, as a proportion of cell-traversal-deficient parasites can still infect the liver and propagate in mice[41,42].

After crossing the space of Disse in the liver, sporozoites migrate through several hepatocytes before invading a final hepatocyte in which a parasitophorous vacuole is formed[10,43] (FIG. 3). Using a cell-wounding assay, it has been shown both *in vitro* and *in vivo* that during migration through cells, *Plasmodium* spp. sporozoites breach the plasma membranes of several hepatocytes, which can rapidly be repaired[10]. Recently, sporozoite migration in the liver was confirmed by intravital microscopy[40].

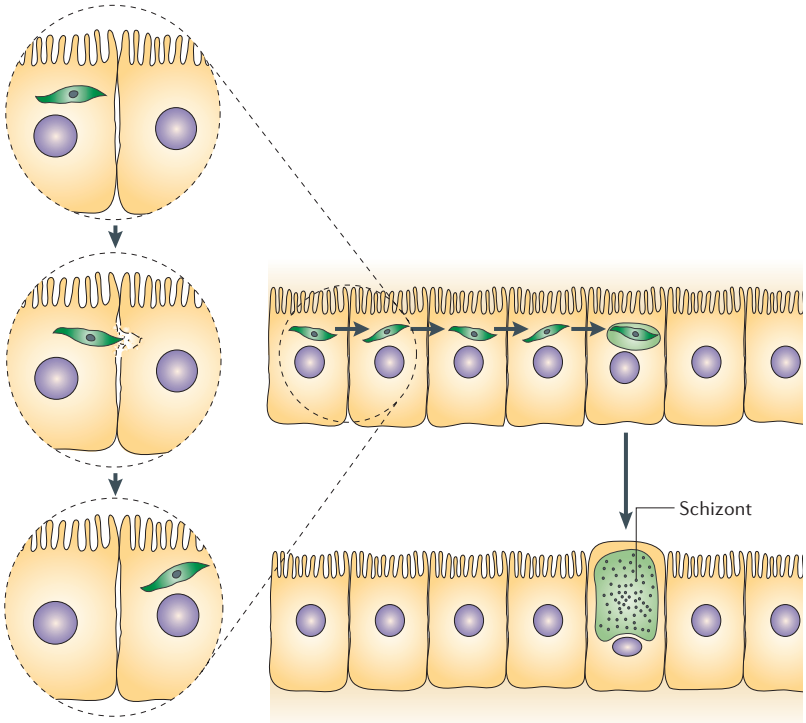


Figure 3 | **Maturation and replication in hepatocytes.** Once the sporozoite (green) has crossed the sinusoidal layer and entered a hepatocyte, it subsequently traverses several hepatocytes until it becomes established in one, in which a parasitophorous vacuole is formed. Each invading sporozoite develops and multiplies inside a hepatocyte, forming the schizont, which is made up of thousands of merozoites.

Breaching of the cell membranes by the *Plasmodium* parasite is likely to involve specific lipases, proteases and pore-forming proteins. Four distinct *P. berghei* proteins have been shown to have important roles during cell traversal: SPECT, SPECT2, cell traversal protein for ookinete and sporozoite (CelTOS) and the phospholipase PbPL[25,41,42,44]. At least two of these proteins, SPECT2 and PbPL, seem to be involved in pore formation[25,41], whereas CelTOS has been proposed to be required for movement through the host-cell cytosol[44].

It has been shown that migration through cells leads to regulated exocytosis of sporozoite secretory organelles, resulting in the exposure of membrane proteins from these organelles, such as TRAP, on the apical end of the parasite[45]. TRAP is normally visualized as a relatively faint staining distributed along the body of the parasite but, after incubation with host cells, high concentrations of TRAP are observed forming an apical 'cap' on the sporozoite surface[46]. This probably contributes to the tight interactions observed between the sporozoite and the hepatocyte cell membrane, which are believed to drive internalization[47]. Elevated cytosolic concentrations of Ca^{2+} in sporozoites induce exocytosis[45,46], suggesting that signalling cascades might be activated in the parasite during migration through cells. Another example of the ability of sporozoites to respond to host cells is provided by the cleavage of the surface sporozoite protein CSP, which is induced by contact with hepatocytes and is required for hepatocyte infection *in vitro* and *in vivo*[48].

It is generally accepted that sporozoites use gliding motility and their ability to migrate through cells in their journey from the skin into the liver, and only commit to the final infection after contact with hepatocytes[49,50]. What triggers the switch from sporozoite migration through cells to infection is still unknown. Disruption of the *spect* gene was shown to impair the ability of sporozoites to migrate through hepatocytes[42], but *spect*-deficient sporozoites can still infect hepatocytes[42]. Other genes were identified, the disruption of which led to a similar phenotype[41,44]. Therefore, the authors suggested that, although migration through cells is necessary to reach the hepatic parenchyma, migration through hepatocytes is not necessary for infection[42]. So, some data show that migration through host cells activates exocytosis, enhancing infection and that inhibition of exocytosis inhibits infection[45], whereas other data show that *spect*-deficient parasites, which are unable to migrate through cells, can infect hepatocytes *in vitro*[42]. These apparently conflicting results need further attention to be reconciled.

As sporozoite migration through cells occurs by the breaching of the plasma membrane, this leads to liver injury at the microenvironment level[10,40]. We have shown that host cell wounding by sporozoite migration induces the secretion of infection-susceptibility-inducing factors, which render hepatocytes more susceptible to infection[51]. One such factor is hepatocyte growth factor (HGF), which, by activating its receptor MET, leads to an increase in infection, not by functioning as a binding site for *Plasmodium* sporozoites, but as a mediator of signals that make the host cell more permissive to *Plasmodium* infection[51]. Again, however, the *spect* mutant phenotype questions the requirement of host factors released by traversed cells for infection.

Following migration through cells, *Plasmodium* sporozoites engage in a final invasion, with the formation of a parasitophorous vacuole. Both TRAP and CSP interact with hepatocytes. Whereas CSP seems to have an active role in sporozoite attachment rather than internalization[35], TRAP seems to contribute to sporozoite internalization and not attachment[52]. Recently, a protein termed apical membrane antigen 1 (AMA-1) was shown to be expressed in sporozoites and was implicated in the invasion of hepatocytes by *P. falciparum* parasites *in vitro*[53]. Two other *P. berghei* proteins, Pb36p and Pb36, which are specifically produced in liver-infective sporozoites and belong to the *Plasmodium* 6-Cys domain protein family, are also necessary for sporozoites to recognize hepatocytes and commit to infection[54].

HSPGs on the surface of hepatocytes seem to have an important role in binding and internalizing sporozoites. They interact with both CSP and TRAP, as mentioned earlier. The extracellular region of TRAP has also been shown to interact with the serum glycoprotein fetuin A on hepatocyte membranes, an interaction that enhances the parasite's ability to invade hepatocytes[55]. Another host molecule that seems to interact directly or indirectly with sporozoites is the tetraspanin CD81, a membrane protein that is expressed on the surface of hepatocytes and is a putative receptor for hepatitis C virus[56]. CD81

6-Cys domain protein family
A family of ten proteins characterized by a pattern of conserved cysteine residues that are unique to *Plasmodium* species.

Tetraspanin
The tetraspanin protein family contains proteins that span the membrane four times with two exoplasmic loops, and that can be found at the cell surface.

seems to have an essential role in the invasion of mouse hepatocytes by *P. yoelii* and human hepatocytes by *P. falciparum*[43], which it has been proposed is linked to the fact that CD81 is localized in tetraspanin-enriched microdomains[57]. By contrast, infection of HepG2 cells by *P. berghei* seems to be independent of CD81 expression[43]. Despite efforts to identify a *Plasmodium* molecule that interacts with CD81, no proof of a direct interaction has been obtained[43]. Therefore, the authors proposed that CD81 might regulate the activity of another host molecule that has an essential role in sporozoite invasion, which is in agreement with the reported ability of tetraspanins to associate with, and regulate the function of, molecular partners[43].

In the search for the host receptors that are required for invasion, several host molecules have been shown to not be essential for sporozoite infection. Mice that are deficient in intercellular adhesion molecule 1 (ICAM-1) and ICAM-2 showed similar levels of infection with *P. yoelii* sporozoites as wild-type mice[58]. Similar results were also obtained for mice deficient in CD36 (a scavenger receptor expressed by many cell types including Kupffer and endothelial cells) and syndecan-1 (a major transmembrane HSPG expressed by many cell types, including the sinusoidal, basolateral side of hepatocytes)[59,60]. However, given that CSP binds to the glycosaminoglycan portion, and not to the core protein, of the various syndecans; that cells typically express several syndecan species; and that a given type of cell normally links the same species of glycosaminoglycan chains to all of the syndecans it produces, the lack of effect of deleting the core protein of one of the syndecans, syndecan-1, does not necessarily mean that these molecules are not involved in sporozoite infection. Mice deficient for both SR-AI and SR-AII (scavenger receptors expressed by Kupffer and liver endothelial cells) and wild-type mice are also equally susceptible to malaria infection by *P. berghei* sporozoites[61]. Many pathogens can invade host cells using multiple, alternative pathways, with significant redundancy. This is true, for example, for red-blood-cell invasion by *P. falciparum* merozoites[62]. Therefore, it is plausible that, for hepatocyte invasion, other players exist that might be difficult to identify owing to redundancy. Whether any of the receptors mentioned above do have specific functions in malaria infection remains to be elucidated.

Developing in the hepatocyte

After the final invasion, each *Plasmodium* sporozoite develops and multiplies inside the hepatocyte, thereby generating thousands of new parasites (merozoites). However, relatively little is known about the cellular and molecular interactions in either the parasite or the host during this part of the life cycle. Recently, three distinct *P. berghei* proteins, the removal of which leads to an impairment of parasite development in hepatocytes, have been identified. These are UIS3 and UIS4 (UIS stands for upregulated in infective sporozoites) and Pb36p (REFS 63–65). As mentioned earlier, Pb36p-deficient parasites have also been reported to have a deficiency in terms of hepatocyte invasion[54]. Van Dijk *et al.*[65] suggested that this protein is involved in parasite development because Pb36p-deficient sporozoites are mainly found inside cells that do not show signs of wounding and, therefore, have presumably entered the cell with the formation of a parasitophorous vacuole. The mutant parasite deficient for Pb36p obtained by Ishino *et al.*[54] can also be found inside non-wounded cells, but in a much smaller proportion than that observed for wild-type parasites (approximately 10% for Pb36p-deficient parasites compared with 60% for wild-type parasites). Therefore, it seems that most of these mutant sporozoites cannot invade host cells. It is likely that Pb36p-deficient parasites are impaired in both invasion of the host cell and intracellular development. Interestingly, in the Pb36p mutant obtained by Ishino *et al.*, the lack of infectivity leads to the continuous traversal of hepatocytes, which results in a 5–6-fold increase in the proportion of wounded cells, a phenotype not observed in the Pb36p-deficient parasites obtained by van Dijk *et al.*[65] The reasons for these differences are presently unknown, and the role of these parasite molecules during *Plasmodium* development in hepatocytes is still unclear.

Immunization with any of these mutant parasites (deficient in UIS3, UIS4 or Pb36p) confers full protection against a subsequent challenge with fully infective *P. berghei* sporozoites[63–65]. Therefore, genetically attenuated sporozoites, as has previously been observed for radiation-attenuated sporozoites[66], can induce protective immune responses and might be useful in a future vaccination strategy (BOX 1).

As was mentioned above, HGF/MET signalling increases hepatocyte infection[51]. The mechanism behind this is not fully understood but it seems to involve

Box 1 | The silent stages as targets for malaria control

The hepatic stage of a *Plasmodium* infection constitutes an appealing target for the development of an anti-malarial vaccine or prophylatic drug, as they would function before the onset of pathology. Until now, the only demonstrably effective vaccine shown to confer a sterile and lasting protection both in mice[66] and in humans[74–77] is the inoculation of γ-radiation-attenuated sporozoites (RAS), which can invade but not fully mature inside the hepatocyte. Appealing as it might seem, an RAS-based large-scale vaccination effort would be difficult for logistical and safety reasons, including the necessity of using extremely precise and reproducible radiation doses (reviewed in REFS 78–80).

For these reasons, during the past three decades efforts have concentrated on constructing a subunit vaccine based on *Plasmodium* sporozoite and/or liver-stage antigen(s). This approach has achieved some success, with the most promising candidate being the RTS, S vaccine that is currently undergoing field trials[81]. Recent reports by different groups have shown that sporozoites deficient in certain genes (*uis3*, *uis4* and *pb36p*) can confer long-lasting protection against malaria in rodents[63–65]. These findings created renewed hopes for a whole-organism vaccine against malaria based on genetically attenuated *Plasmodium* sporozoites (GAS).

The mechanisms of protection by GAS are not fully understood and their use as a human vaccine poses a few safety problems that cannot be ignored, such as the possibility of breakthrough infections[63,65], especially in immunocompromised individuals. Moreover, like RAS, GAS can only be generated by using infected mosquitoes, which raises practical issues concerning the number of parasites required for large-scale vaccinations, their purity and the high production costs. Therefore, despite the fact that GAS opens an avenue in the quest for an effective malaria vaccine that merits further exploration, efforts towards obtaining drug-based treatments and prophylactics against this devastating disease must not be overlooked[82].

protecting the host cell from apoptosis[67] and, potentially, rearrangement of the host-cell cytoskeleton[51]. In fact, infection of host cells by *P. berghei* sporozoites confers a significant level of protection against apoptosis[67,68], which decreases when hepatocytes are infected with irradiated sporozoites[69]. Indeed, the uptake of apoptotic infected hepatocytes by macrophages and dendritic cells in the liver was observed and has been discussed as a possible pathway for the presentation of sporozoite antigens[69,70]. At an early stage of development, HGF/MET signalling seems to have an important role in inducing this protective effect. However, additional ways of inhibiting host-cell apoptosis during parasite development probably exist and complement the effects of HGF.

Another host molecule that seems to have an important role in intrahepatocyte development is ApoA1, which localizes at the parasitophorous vacuole 24 hours post-infection, and seems to interact with the parasite UIS4 protein (A.-K. Mueller and K. Matuschewski, personal communication). One problem that any intracellular pathogen faces is how to create enough space for replication, with considerable expansion of the parasitophorous vacuole required to allow intra-vacuolar replication of malaria sporozoites. Such an increase in vacuole size requires the synthesis of large amounts of additional membrane and it is possible that ApoA1 might have a role in this process.

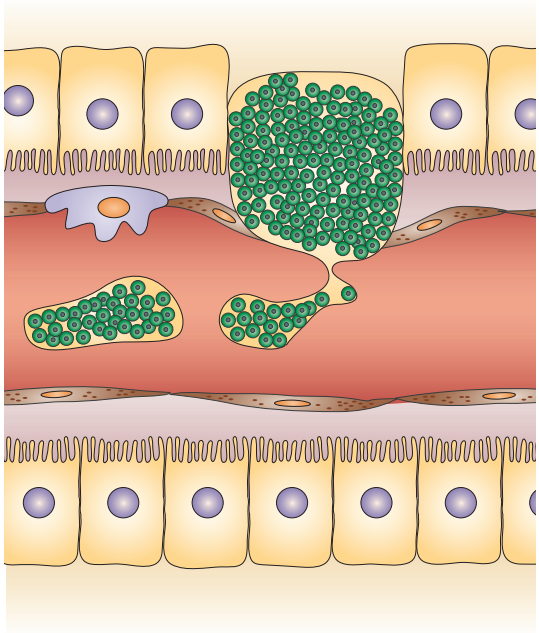
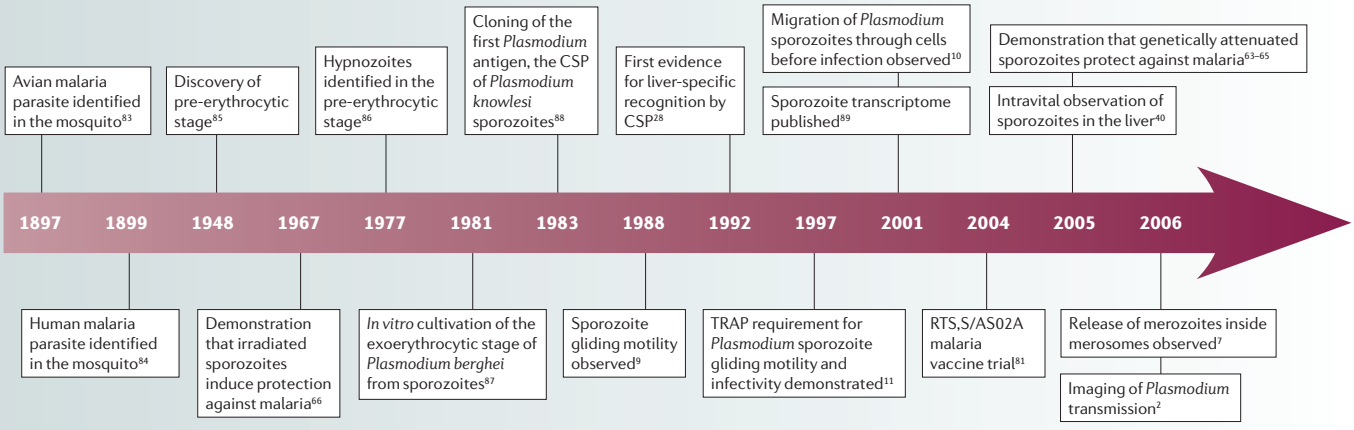


Figure 4 | **The exit from the liver.** The final step involves the release of merozoites (green) into the bloodstream. The signal(s) that trigger the release remain unknown. *Plasmodium* merozoites are released by the formation of merozoite-filled vesicles (merosomes), which bud off from the infected hepatocytes into the sinusoidal lumen.

Leaving the hepatocyte

The final important step in the lifecycle of intracellular pathogens or pathogens with intracellular stages is the exit from the host cell after replication, but the molecular mechanisms involved in this process are still poorly understood. The release of *Plasmodium* merozoites from hepatocytes is usually referred to as occurring after hepatocyte rupture, but this has never been directly observed and the signal(s) that trigger the exit remain unknown. During erythrocyte infection, the rupture of the vacuole containing *Plasmodium* merozoites occurs before the rupture of the erythrocyte plasma membrane[71], and distinct proteases are involved in each of these steps[71,72]. Recently, it has been reported that *P. berghei* merozoites are not released by the rupture of the hepatocyte but by the formation of merozoite-filled vesicles (merosomes), which bud off from the infected hepatocytes into the lumen of the liver sinusoids[7] (FIG. 4). Initially, merozoites are released from the parasitophorous vacuole membrane and mix freely with the host-cell cytoplasm[7,73]. Interestingly, although the merozoite-containing host cell has apoptotic features, the amount of phosphatidylserine on its surface does not increase. The authors

Timeline | **Selected milestones in research on the biology of the pre-erythrocytic stages of *Plasmodium* species**



suggest that, in this way, the parasite manipulates the host cell to prevent infected hepatocytes from being phagocytosed. Moreover, the merosomes, the plasma membranes of which are of host-cell origin and therefore should not be recognized by dendritic cells or Kupffer cells, guide the merozoites safely into the bloodstream[7]. The molecular details of this process are not yet fully understood but it has been shown that proteases mediate the liberation of merozoites from the parasitophorous vacuole and the formation of the merosomes[7]. Merosome-like structures released into the sinusoids have also recently been reported for *P. yoelii*[8]. Whether similar features exist in *P. falciparum* remains unknown.

Conclusions

The biological events that occur between the bite of a malaria-infected mosquito and the release of *Plasmodium* merozoites into the host bloodstream are obligatory steps in the establishment of a malaria infection. Recently, our knowledge of the initial steps of the infection process after the injection of *Plasmodium* sporozoites into the skin has increased. Also, we now have a greater understanding of how sporozoites traverse the liver sinusoids and reach the hepatocytes (TIMELINE), and it is likely that *Plasmodium* sporozoites have developed multiple ways to achieve this goal. Detailed information is still lacking on the host and parasite molecules responsible for *Plasmodium* development within hepatocytes and the exit of merozoites from these cells.

As sporozoite multiplication in the liver is not associated with pathology, this stage of malaria infection is not a target for therapy. However, it is the most appealing stage for vaccine and prophylaxis strategies as, if infection is blocked at this stage, there will be no pathology and therefore no disease. In terms of prophylaxis, the main concerns regarding this strategy are drug resistance if the drug target is parasite-derived, and toxicity if the drug target is host-derived. With increasing knowledge of the molecular mechanisms underlying parasite development in the liver, it might become possible to design drugs that do not interfere with normal liver functions but do prevent infection.

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Competing interests statement
The authors declare no competing financial interests.

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