Published in final edited form as:

Parasitol Int. 2007 September ; 56(3): 171–178.

A Long and Winding Road: The *Plasmodium* Sporozoite's Journey in the Mammalian Host

Photini Sinnis* and Alida Coppi

Department of Medical Parasitology, New York University School of Medicine, 341 East 25th Street, New York, NY 10010

Abstract

The *Plasmodium* sporozoite, the infectious stage of the malaria parasite, makes a remarkable journey in its mammalian host. Here we review our current knowledge of the molecular and cellular basis of this journey, which begins in the skin and ends in the hepatocyte.

Introduction

Malaria infection occurs when an infected Anopheline mosquito injects sporozoites into the skin of a mammalian host while probing for a blood meal. These sporozoites enter the blood stream and are transported to the liver, where they invade hepatocytes and transform into exoerythrocytic forms (EEFs). After multiple rounds of replication, each EEF contains thousands of merozoites which are released into the blood stream and rapidly invade erythrocytes, thus initiating the erythrocytic stage of the life cycle which is responsible for the symptoms of the disease.

The pre-erythrocytic stages of the malaria parasite are clinically silent yet critical for establishing infection in the mammalian host. Until recently, our knowledge of the molecular interactions between host and parasite during this stage of infection was limited due to the small number of sporozoites and EEFs present in the mammalian host and to the lack of an in vitro system for growing sporozoites. With the sequencing of the human and rodent malaria parasite genomes [1,2] as well as advances in transfection [3-5] and intravital microscopy [6], our knowledge of these stages has increased dramatically. Here we summarize what is currently known about the interaction between *Plasmodium* sporozoites and their mammalian host, beginning with sporozoite injection into the dermis and ending with invasion of their target, the hepatocyte.

Gliding Motility

Plasmodium is a member of Apicomplexa, a phylum of obligate intracellular parasites whose invasive stages are motile. Motility of these zoites, or invasive stages, is not dependent upon flagella or a change in cell shape but is powered by a subpellicular actinmyosin motor that translocates proteins posteriorly resulting in the forward movement of the zoite [7,8]. Details of the motor and the precise mechanism by which gliding occurs have recently been summarized in several reviews [9,10] and will not be discussed in detail here. Nonetheless,

*corresponding author: Email: photini.sinnis@med.nyu.edu, Phone: 212 263 6818, Fax: 212 263 8116

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

gliding motility is central to the topics we will discuss here: It is required for zoite localization to its target cell as well as for the invasion process itself.

Into and Out of the Dermis

Plasmodium-infected mosquitoes inject saliva containing sporozoites as they probe for blood. Salivation stops when the mosquito locates and begins to imbibe blood, suggesting that most sporozoites are deposited into the dermis and not directly into the blood circulation. Recently, two groups showed experimentally that this was indeed the case by demonstrating that removal or heat-treatment of the injection site could prevent malaria infection in animals probed-upon by infected *Anopheles* mosquitoes [11,12]. Once it was known that sporozoites were primarily injected into the dermis of the mammalian host the size of the sporozoite inoculum could be determined experimentally. Previous studies had estimated inoculum size by counting the number of sporozoites ejected by salivating mosquitoes [13,14] or injected into membrane feeders by probing mosquitoes [15]. More recently, quantitative PCR analysis of the injection site after single mosquito feeds using the rodent malaria parasite *Plasmodium yoelii*, showed that the mean number of sporozoites injected by a single mosquito was 123, with a median of 18 and a range of 0 to 1,297 [16]. Remarkably, 20% of infected mosquitoes injected no sporozoites after 3 minutes of probing, a result also observed when salivating mosquitoes were analyzed [15]. These data demonstrate that the sporozoite inoculum is low and highly variable. After their injection, intravital microscopy studies show that sporozoites move randomly in the skin until they contact the endothelium of either the lymphatic or blood circulation [17, 18]. In vivo imaging of this event shows that sporozoites glide around and along these vessels until they enter and are carried away either rapidly, by the blood circulation, or slowly, in the lymphatic system [17].

It has always been assumed that sporozoite exit from the injection site is a rapid process, taking a maximum of 30 to 60 minutes and frequently occurring within minutes of injection. Although this had never been formally demonstrated, the waning infectivity of sporozoites dissected from mosquito salivary glands and kept at 37°C for 1 hr [19] combined with the fact that some sporozoites do enter the blood circulation within minutes of injection [12], led to the belief that if sporozoites had not left the injection site soon after their injection, they likely never would. Using a quantitative PCR approach to measure the kinetics with which the majority of sporozoites left the injection site, we found that when injected intradermally, either by needle or mosquito bite, about 90% of sporozoites remain at the injection site 1 hr post injection [20]. Indeed, if the injection site was removed 1 hr after sporozoite inoculation, parasite burden in the liver was reduced by 90 to 95%. Expanding this study to later time points we found that the majority of sporozoites leave the injection site between 1 to 3 hours after injection. Subinoculation experiments showed that the blood of mice inoculated intradermally with sporozoites was infectious, albeit with low numbers of sporozoites, to naïve mice for hours. Thus, sporozoites remain viable and infective for a prolonged period of time in the mammalian host and leave the injection site in a slow trickle [20].

Intravital microscopy of GFP-transgenic sporozoites at the injection site showed that approximately 20% of the inoculum left the injection site via the lymphatics and that some of these sporozoites began to develop in the draining lymph node [17]. Using a PCR approach we had remarkably concordant results with the finding that 20% of the inoculum was associated with the draining lymph node 3 hrs after intradermal inoculation of sporozoites [20]. Together these findings suggest that in natural infection, the draining lymph node may be the site at which the immune response to the sporozoite stage is initiated and indeed it has been recently demonstrated that a sporozoite-specific cytotoxic T-cell (CTL) response is evident in the draining lymph node just 2 days after intradermal immunization with irradiated sporozoites [Chakravarthy and Zavala, unpublished data]. When the draining lymph node was surgically

ablated to prevent the early local priming, the number of sporozoite-specific CTLs in the liver was significantly reduced, highlighting the importance of skin-draining lymph nodes in the initiation of the immune response to sporozoites during natural infection [Chakravarty and Zavala, unpublished data].

Taken together this group of studies demonstrates that there are likely significant interactions between sporozoites and their mammalian hosts at the injection site. These data suggest that there is indeed a “skin stage” of malaria infection and have spurred new lines of research on the sporozoite stage.

Migration in Apicomplexan Parasites

Most invasive stages of Apicomplexan parasites are released in close proximity to their target and are not required to move large distances. *Plasmodium* sporozoites differ from other zoites in this regard and in the mammalian host, they must make their way from the dermis to the hepatocyte. Although they make use of the blood and possibly the lymphatic circulation for their travels, they must localize to and cross cell barriers in both the skin and the liver sinusoid in order to reach the hepatocyte. That motility is required for these barrier crossings is clear, however, as outlined below, other processes are also necessary.

Recently it was shown that *Plasmodium* sporozoites migrate through cells, wounding the cell in the process [21]. This interaction with cells does not result in productive infection and in fact appears to be qualitatively different [21]. *Plasmodium* sporozoites are not the only Apicomplexans that can migrate through cells, although they may have perfected the process. The ookinete stage of *Plasmodium* [22] as well as sporozoite stages of *Eimeria* [23] and *Toxoplasma* [24] are also capable of cell traversal though these zoites appear to cause more damage to the traversed cell.

Cell traversal is a complex event that remains poorly understood. Although we are still far from a molecular understanding of this process, four proteins involved in cell traversal recently have been identified. Three of these, SPECT 1 (sporozoite microneme protein essential for cell traversal) and SPECT 2 (also called perforin-like protein 1; PLP1) and CelTOS (cell traversal protein for ookinetes and sporozoites) were identified using EST databases [25-28] while the fourth, a phospholipase (PL) was found using an RNA subtraction screen that identified genes up-regulated in salivary gland sporozoites [29,30]. Expression of SPECT 1, SPECT 2 and PL are specific to salivary gland sporozoites whereas CelTOS is expressed by both ookinetes and salivary gland sporozoites. Sporozoites in which one of the SPECT proteins or CelTOS was deleted using gene transfection technology show similar phenotypes: In vitro they lack the ability to traverse cells but when placed directly on hepatocytes they invade and develop normally. In vivo these mutants lack infectivity for the mammalian host when injected intravenously, a phenotype that can be reversed by pretreating mice with liposome-encapsulated dichloromethylene diphosphonate (CL) which depletes the liver of Kupffer cells and leaves gaps in the sinusoidal barrier thus allowing sporozoites direct access to hepatocytes [25,27,28]. It is likely that these proteins are also required for sporozoite exit from the dermis and in the case of the SPECT 1 knockout this has been recently directly demonstrated by intravital microscopy [Amino and Menard, unpublished data]. The fourth protein, a phospholipase, has a carboxy-terminal domain with significant similarity to mammalian LCATs (lecithin:cholesterol acyl transferases) and when expressed as a recombinant protein has lipase and membrane lytic activity [30]. Mutant sporozoites in which PL is deleted or its catalytic site altered, are impaired in reaching the liver when injected intradermally but have normal infectivity when injected intravenously. In vitro, these mutants have reduced cell traversal activity. Interestingly, PL appears to be required only for crossing cell barriers in the skin suggesting that the mechanism by which sporozoites traverse cells in the skin and liver

may differ. Precisely how these proteins function in cell traversal is not known, however, both PL, with its lipase activity, and SPECT2 (or PLP1) which contains a membrane attack complex/perforin-like domain [26], have similarity to mammalian proteins that lyse or make holes in membranes. SPECT 1 and CelTOS, however, have no similarity to known proteins and how they function in cell traversal remains a mystery.

As sporozoites migrate through tissues to reach the liver there must be a mechanism by which they know they are in the skin or blood circulation, rather than the liver, and modulate their behavior accordingly. To investigate whether sporozoites behave differently in the presence of different cell types we developed a quantitative migration assay and compared sporozoite migratory activity in dermal fibroblasts, endothelial cells and hepatocytes [Coppi and Sinnis, unpublished data]. We found that the migratory activity of sporozoites was increased 5-fold in non-hepatic cells compared to hepatocytes and the frequency with which sporozoites productively invaded different cell lines was inversely correlated to their migratory activity. Because the only host cell molecules thus far shown to bind sporozoites are highly sulfated heparan sulfate proteoglycans (HSPGs; reviewed in [31]), we hypothesized that sporozoites differentially recognize different cell types according to the sulfation level of their cell surface HSPGs. Testing this hypothesis, we found that indeed sporozoites tend to migrate through cells with undersulfated HSPGs and productively invade cells with highly sulfated HSPGs. In hepatocytes, pretreatment with chlorate, a metabolic inhibitor of sulfation, completely altered the nature of the interaction between target cell and parasite; sporozoites now migrated through these cells rather than invaded them. Because in the mammalian host, hepatocytes express highly sulfated HSPGs whereas dermal fibroblasts and endothelial cells express undersulfated HSPGs, these data suggest that sporozoites use the sulfation level of HSPGs as a type of “GPS” to know where they are and optimize their infectivity for an organ that is a significant distance from the skin, where their journey begins.

Once sporozoites contact hepatocytes, however, they continue in cell traversal mode for a short period of time. This has been observed both in vitro [32] and using intravital microscopy, in vivo [33]. One possibility is that activation for productive invasion is a progressive process that takes some time to occur once the sporozoite has contacted the correct target cell. Another possibility is that continued cell traversal activity further activates the sporozoite for invasion [32]. This continued migration in the liver also leads to the release of hepatocyte growth factor from wounded hepatocytes, making the surrounding hepatocytes more resistant to apoptosis and therefore better host cells for sporozoite development [34].

To the Liver

Once in the blood circulation, sporozoites are rapidly arrested in the liver. Several lines of evidence suggest that this arrest is mediated by the interaction between the major surface protein of sporozoites, the circumsporozoite protein (CSP) and the highly sulfated HSPGs of hepatocytes [35-39]. Recombinant CSP binds specifically to hepatic HSPGs and in vivo, the physiologic ligands for hepatic HSPGs, namely lactoferrin and lipoprotein remnants, delay CSP clearance from the circulation and inhibit sporozoite infection of hepatocytes [40]. Two distinct regions of CSP have been shown to bind to HSPGs, the cell-adhesive sequence in the carboxy-terminus of the protein as well as sequences in the NH₂-terminus of the protein [41, 42], however, whether these two regions bind to HSPGs cooperatively or sequentially is not yet known.

HSPGs are ubiquitous molecules found on the surface of most mammalian cells, raising the question of how CSP binding to HSPGs can account for the specific arrest of sporozoites in the liver. The glycosaminoglycan chains (GAGs) of HSPGs are made of repeating disaccharide units of N-acetyl-glucosamine and glucuronic acid which can undergo a series of

modifications, giving rise to a large amount of structural heterogeneity. The addition of sulfate moieties to HSPG GAGs is one of these modifications and as stated above, liver HSPGs are more highly sulfated compared to HSPGs from other organs [43], suggesting that the degree of GAG chain sulfation could account for the selective targeting of sporozoites to the liver. Indeed it has been found that CSP binds preferentially to cells expressing HSPGs with highly sulfated GAGs [38,39] and that sporozoite attachment to cells decreases as the degree of GAG chain sulfation decreases [38]. One confounding issue is that these highly sulfated HSPGs are found behind the endothelial cell barrier. The endothelial cells of the liver sinusoids, however, have open fenestrations that allow for direct contact between the blood circulation and the underlying loose basement membrane known as the space of Disse. Recently it has been suggested that the majority of the highly sulfated HSPGs that “capture” sporozoites are located in the space of Disse and not on the hepatocyte surface which is some distance behind the endothelial cell barrier [39]. These HSPGs are primarily produced by stellate cells [44], a unique dendritic-shaped cell found in this location [45] and like the HSPGs of hepatocytes, they are highly sulfated and bind to recombinant CSP and sporozoites [39], lending support to the idea that it is HSPGs in the space of Disse that arrest circulating sporozoites.

How sporozoites cross the sinusoidal barrier after their arrest is matter of some debate. The liver sinusoid is composed of a single layer of fenestrated endothelial cells along which Kupffer cells, the resident macrophages of the liver, are dispersed. To cross the sinusoid, sporozoites may migrate through or between the endothelial cells [46] or they may use Kupffer cells as a gateway to the underlying hepatocyte, a hypothesis put forward after sporozoites were observed inside Kupffer cells at early time points after injection [47]. More recent in vitro observations of the interaction between sporozoites and sinusoidal cells found that sporozoites preferentially associate with Kupffer cells and are capable of going through Kupffer cells to productively enter hepatocytes [48]. In addition, op/op mice which have reduced numbers of Kupffer cells have somewhat lower parasite burdens when inoculated with sporozoites by mosquito bite [49]. However, treating mice with CL, which as stated earlier eliminates Kupffer cells and creates large gaps in the sinusoidal barrier [49], leads to enhanced liver infection with wild type sporozoites and restores the infectivity of SPECT 1, SPECT 2 and CeITOS knockout sporozoites [25,27,28] indicating that Kupffer cell passage is not obligatory for hepatocyte infection. In the normal hepatic sinusoid, however, the majority of sporozoites probably gain access to the hepatic parenchyma by crossing Kupffer cells. Controversy remains as to how Kupffer cells are crossed, whether it is by a unique mechanism involving parasitophorous vacuole formation [48] or whether they traverse these cells in the same way they traverse other cells, a hypothesis suggested by the inability of SPECT 1, SPECT 2 and CeITOS knockout sporozoites to cross this barrier.

Into the Hepatocyte

Invasion by Apicomplexan Zites in General and *Plasmodium* Sporozoites in Particular

Target cell entry by Apicomplexan protists differs from mechanisms used by bacterial and viral pathogens in that they actively move into their host cell, rather than being taken up by host cells. Gliding motility, therefore, is a requirement for invasion and zoites that cannot move, either because of drug treatment or mutations in their motility machinery are not capable of invading target cells [50,51]. In the case of sporozoites, this was shown when a key protein in the motility machinery of the sporozoite was deleted [52]. This protein, TRAP (thrombospondin-related-anonymous protein), is a transmembrane protein that forms the link between the actomyosin motor and the extracellular substrate upon which the sporozoite glides, thus allowing the force of the motor to result in the forward movement of the sporozoite (reviewed in [9]).

In addition to gliding motility, cell invasion by Apicomplexan zoites requires the coordinated and ordered release of proteins, and likely other molecules, from specialized secretory organelles called micronemes and rhoptries found at the apical end of the zoite [53]. Exocytosis of these organelles is stimulated by the mobilization of parasite intracellular calcium [54]. These events have been elucidated primarily using *Toxoplasma* tachyzoites (reviewed in [55, 56]), however, a few studies have shown that when *Plasmodium* sporozoites contact hepatocytes, there is an upregulation of microneme secretion through the mobilization of intracellular calcium [32,57]. Many of the proteins found in micronemes contain cell-adhesive domains that function in zoite-host cell interactions required for invasion (reviewed in [56]). Most of these adhesins are proteolytically processed, either intracellularly (on their way to or within micronemes) or extracellularly after their secretion onto the zoite surface. Processing is required to release parasite-adhesin/host-cell-receptor complexes from the zoite surface and likely also controls the exposure of adhesive domains required for host-cell interactions (reviewed in [58-60]). Although the number of microneme and rhoptry proteins identified thus far in sporozoites is low, those characterized thus far are clear orthologues of *Toxoplasma* microneme proteins or the same as those found in *Plasmodium* merozoites.

Morphological studies of cell invasion have demonstrated that this process occurs in distinct stages, beginning with initial reversible attachment of the zoite to the target cell followed by irreversible attachment and the formation of a tight junction between the parasite and the target cell membrane (reviewed in [61,62]). The parasite's actomyosin motor translocates this junction posteriorly along its surface resulting in the forward movement of the zoite into the cell. When the parasite is completely within the host cell, the junction, which is now at the posterior end of the parasite, is sealed off and the intracellular zoite is in a parasitophorous vacuole. The majority of the work on this process has been performed using *Plasmodium* merozoites and *Toxoplasma* tachyzoites because sufficient numbers of synchronized zoites can be obtained to perform these studies. Although the sporozoite stage of *Plasmodium* shares a similar overall structure, it is not yet known if sporozoite invasion of hepatocytes proceeds in the same way. Importantly, no one has documented the formation of a tight junction during sporozoite invasion. This may be due to the difficulties in obtaining sufficient quantities of synchronized sporozoites, however it is also possible that there are differences in invasion strategies among different zoites.

Sporozoite and Hepatocyte Proteins Involved in the Invasion Process

The major surface protein of the sporozoite, CSP, forms a dense coat on the parasite's surface. While it has been known that CSP is critical for sporozoite localization to the liver, it has not been known if CSP – hepatocyte interactions function during the invasion process itself. Recently, however, we found that CSP was proteolytically cleaved by a parasite cysteine protease upon contact with hepatocytes [63]. Inhibition of cleavage using cysteine protease inhibitors results in inhibition of sporozoite invasion but not migration through cells, indicating that CSP cleavage is specifically associated with productive invasion [63]. CS proteins from all species of *Plasmodium* have a similar overall structure composed of a central species-specific repeat region, a highly conserved cell adhesive sequence in the carboxy-terminus of the protein with similarity to the type I thrombospondin repeat (TSR) and a highly conserved 5 amino acid sequence called region I, just upstream of the repeats. We have recently found that region I contains the cleavage site and that sporozoites whose CSP has been genetically engineered to lack region I, cleave CSP poorly and invade hepatocytes with low efficiency [Coppi, Natarajan and Sinnis, unpublished data]. The function of CSP cleavage is still not clear although recent findings in our laboratory indicate that removal of the NH₂-terminal third of the protein may expose the carboxy-terminal TSR. The identity of the protease that cleaves CSP is not known, however our data clearly show that CSP is cleaved extracellularly and is significantly upregulated after sporozoites contact hepatocytes, suggesting that the protease is

found in apical organelles or is on the sporozoite surface and activated only after contacting hepatocytes.

The genome projects [1,2] in conjunction with RNA subtraction studies [29] have led to the identification of many more microneme and rhoptry proteins of sporozoites. Two of these, TRAP (thrombospondin-related anonymous protein; [64]) and AMA-1 (apical membrane antigen 1; [65]), have been characterized in some detail and several lines of evidence support a role for these proteins in hepatocyte invasion.

TRAP is a transmembrane protein whose extracellular portion has two cell-adhesive sequences, an A-domain (or I-domain of integrins) and a type I thrombospondin motif (TSR) (reviewed in [66]). Additionally, it has a cytoplasmic tail that links the surface of the sporozoite to the motility machinery of the parasite [51]. TRAP is normally present in small amounts on the sporozoite surface, however, contact with hepatocytes triggers the release of large amounts onto the zoite surface, suggesting a role in hepatocyte invasion [57]. This has been definitively shown with the generation of sporozoites expressing TRAP with alterations in the A-domain or TSR that would be predicted to abolish the adhesive interactions of the respective domain [67,68]. These mutants have normal gliding motility but significantly decreased infectivity for hepatocytes. Additionally, when these mutations are combined to make sporozoites expressing TRAP with alterations in both adhesive domains, infectivity is completely abolished, indicating that the adhesive properties of TRAP are required for hepatocyte invasion. Like its ortholog in *Toxoplasma*, MIC2, TRAP is proteolytically cleaved in its NH₂-terminus and then released from the sporozoite surface via a COOH-terminal cleavage [69]. Likely this latter cleavage event is required to release the adhesive ectodomains from the sporozoite surface. It has been shown that the TSR of TRAP binds to hepatic HSPGs associated with the space of Disse [70, 71] and the A-domain binds to heparin [72]. More work is required to demonstrate the role of these binding events during cell invasion and whether there are other TRAP-binding proteins on the hepatocyte.

AMA-1, originally shown to be involved in *Plasmodium* merozoite invasion of erythrocytes, has recently been shown to be also expressed in sporozoites [69,73]. It is primarily found intracellularly and like TRAP, is secreted onto the sporozoite surface in the presence of hepatocytes [69]. Antibodies to AMA-1 can inhibit sporozoite invasion of hepatocytes, albeit only at high concentrations. This is likely because the controlled release of AMA-1 results in a high local concentration of the protein, making it difficult for antibodies to compete with AMA-1 binding to its receptor or binding partner, which has yet to be identified. A similar situation is observed with anti-TRAP antibodies which poorly inhibit hepatocyte invasion despite the fact that TRAP is clearly essential for this process [57]. AMA-1 is proteolytically processed similarly in sporozoites and merozoites [69,74]; with a loss of an NH₂-terminal fragment prior to secretion onto the surface and a carboxy-terminal cleavage, sensitive to a subset of serine protease inhibitors, that sheds the extracellular domain from the sporozoite surface.

In addition to TRAP and AMA-1, there are other proteins, about which we know much less but nevertheless have been identified in sporozoites and likely have roles in hepatocyte invasion. P36p, a member of a small family of *Plasmodium* proteins that have 6 conserved cysteine residues has recently been shown in the rodent malaria parasite *Plasmodium berghei* to be important for early stages of hepatocyte infection, although some controversy exists as to whether it is required for the invasion process itself or early development in the hepatocyte [75,76]. The Py235 rhoptry proteins, first described in *Plasmodium yoelii* but with clear homologues in other rodent and human malaria species, are encoded by a multigene family with differential expression during the life cycle (reviewed in [77]). They function during merozoite invasion of erythrocytes [78,79] and antibody inhibition studies show that

they are also involved in sporozoite invasion of hepatocytes [80]. Other proteins for which a role in hepatocyte invasion is suggested either by their localization in the sporozoite or the fact that antibodies directed against them can decrease sporozoite infectivity include SPATR [81], EBA 175 [82], STARP [83] and PfEMP3 [84].

Although there are undoubtedly many host cell molecules that the parasite uses and likely requires to enter cells, few have been described to date. As discussed above, HSPGs containing highly sulfated GAGs are involved in localization to the liver and may be involved in invasion. More recently, the tetraspanin CD81 was identified as an important hepatocyte molecule required for invasion by both the rodent malaria parasite *Plasmodium yoelii* and the human malaria parasite *Plasmodium falciparum* [85], whereas the rodent parasite *Plasmodium berghei* can use both CD81-dependent and CD81-independent pathways to enter the hepatocyte [86]. Tetraspanins are proteins that associate extensively with one another as well as with other proteins to form membrane microdomains that are distinct from lipid rafts. To date, the precise role of CD81 remains elusive as attempts to identify a sporozoite ligand for CD81 have not yet revealed a binding partner suggesting that CD81 may not function as a receptor but instead have another role [85]. One possibility is that a CD81-dependent protein in the tetraspanin web is the actual sporozoite receptor. Another possibility is that CD81 functions during the early stages of parasitophorous vacuole formation. This intriguing possibility is suggested by the finding that the small proportion of *P. yoelii* sporozoites that do invade CD81-negative cells are found without a vacuole, developing in the nucleus [87].

We Have Not Reached the End of the Road

Although much remains unknown, there have been significant advances in our understanding of the molecular mechanisms by which *Plasmodium* sporozoites establish infection in the mammalian host. The findings discussed in this review point to the next set of compelling questions that need answering. Among them are: What are the interactions between sporozoite and host in the dermis and in what way, if any, do these interactions impact on the ensuing infection? What are the molecular mechanisms by which sporozoites migrate through cells? What is the role of CD81 in sporozoite invasion of hepatocytes? What are the other microneme and rhoptry proteins involved in sporozoite invasion of hepatocytes? As we continue working towards a better understanding of the sporozoite in the mammalian host, it is likely that the knowledge we gain will help us in our effort to control malaria infection in humans.

Acknowledgements

P.S. would like to acknowledge the support of the National Institutes of Health (R01 AI056840) and Brandy Bennet for critically reviewing the manuscript.

References

1. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 2002;419:498–511. [PubMed: 12368864]
2. Carlton JM, Angiuoli SV, Suh BB, Kooij TW, Pertea M, Silva JC, et al. Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* 2002;419:512–519. [PubMed: 12368865]
3. van Dijk MR, Waters AP, Janse CJ. Stable transfection of malaria parasite blood stages. *Science* 1995;268:1358–1362. [PubMed: 7761856]
4. Wu Y, Kirkman LA, Wellems TE. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. *Proc Natl Acad Sci USA* 1996;93:1130–1134. [PubMed: 8577727]
5. Carvalho TG, Thiberge S, Sakamoto H, Menard R. Conditional mutagenesis using site-specific recombination in *Plasmodium berghei*. *Proc Natl Acad Sci USA* 2004;101:14931–6. [PubMed: 15465918]

6. Amino R, Menard R, Frischknecht F. In vivo imaging of malaria parasites--recent advances and future directions. *Curr Opin Microbiol* 2005;8:407–414. [PubMed: 16019254]
7. King CA. Cell motility of sporozoan protozoa. *Parasitol Today* 1988;4:315–319. [PubMed: 15463014]
8. Russell DG, Sinden RE. The role of the cytoskeleton in the motility of coccidian sporozoites. *J Cell Sci* 1981;50:345–359. [PubMed: 7033252]
9. Kappe SH, Buscaglia CA, Bergman LW, Coppens I, Nussenzweig V. Apicomplexan gliding motility and host cell invasion: overhauling the motor model. *Trends Parasitol* 2004;20:13–16. [PubMed: 14700584]
10. Keeley A, Soldati D. The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa. *Trends Cell Biol* 2004;14:528–532. [PubMed: 15450974]
11. Matsuoka H, Yoshida S, Hirai M, Ishii A. A rodent malaria, *Plasmodium berghei*, is experimentally transmitted to mice by merely probing of infective mosquito, *Anopheles stephensi*. *Parasitol Internat* 2002;51:17–23.
12. Sidjanski S, Vanderberg JP. Delayed migration of *Plasmodium* sporozoites from the mosquito bite site to the blood. *Am J Trop Med Hyg* 1997;57:426–429. [PubMed: 9347958]
13. Beier J, Davis J, Vaughan J, Noden B, Beier M. Quantitation of *Plasmodium falciparum* sporozoites transmitted in vitro by experimentally infected *Anopheles gambiae* and *Anopheles stephensi*. *Am J Trop Med Hyg* 1991;44:564–570. [PubMed: 2063960]
14. Rosenberg R, Wirtz RA, Schneider I, Burge R. An estimation of the number of malaria sporozoites ejected by a feeding mosquito. *Trans Roy Soc Trop Med Hyg* 1990;84:209–212. [PubMed: 2202101]
15. Ponnudurai T, Lensen AHW, van Gemert GJA, Bolmer MG, Meuwissen JHET. Feeding behavior and sporozoite ejection by infected *Anopheles stephensi*. *Trans Roy Soc Trop Med Hyg* 1991;85:175–180.
16. Medica DL, Sinnis P. Quantitative dynamics of *Plasmodium yoelii* sporozoite transmission by infected Anopheline mosquitoes feeding on vertebrate hosts. *Infect Immun* 2005;73:4363–4369. [PubMed: 15972531]
17. Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, et al. Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat Med* 2006;12:220–224. [PubMed: 16429144]
18. Vanderberg J, Frevert U. Intravital microscopy demonstrating antibody-mediated immobilisation of *Plasmodium berghei* sporozoites injected into skin by mosquitoes. *Int J Parasitol* 2004;34:991–996. [PubMed: 15313126]
19. Vanderberg JP. Development of infectivity by the *Plasmodium berghei* sporozoite. *J Parasitol* 1975;61:43–50. [PubMed: 1090717]
20. Yamauchi LM, Coppi A, Snounou G, Sinnis P. *Plasmodium* sporozoites trickle out of the injection site. *Cell Micro*. 2007In Press
21. Mota M, Pradel G, Vanderberg JP, Hafalla JCR, Frevert U, Nussenzweig RS, et al. Migration of *Plasmodium* sporozoites through cells before infection. *Science* 2001;291:141–144. [PubMed: 11141568]
22. Han YS, Thompson J, Kafatos FC, Barillas-Mury C. Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: The time bomb theory of ookinete invasion of mosquitoes. *EMBO J* 2000;19:6030–6040. [PubMed: 11080150]
23. Danforth HD, Entzeroth R, Chobotar B. Scanning and transmission electron microscopy of host cell pathology associated with penetration by *Eimeria papillata* sporozoites. *Parasitol Res* 1992;78:570–573. [PubMed: 1438148]
24. Speer CA, Dubey JP, Blixt JA, Prokop K. Time lapse video microscopy and ultrastructure of penetrating sporozoites, types 1 and 2 parasitophorous vacuoles, and the transformation of sporozoites to tachyzoites of the VEG strain of *Toxoplasma gondii*. *J Parasitol* 1997;83:565–574. [PubMed: 9267394]
25. Ishino T, Chinzei Y, Yuda M. A *Plasmodium* sporozoite protein with a membrane attack complex domain is required for breaching the liver sinusoidal cell layer prior to hepatocyte infection. *Cell Microbiol* 2005;7:199–208. [PubMed: 15659064]
26. Kaiser K, Camargo N, Coppens I, Morrissey JM, Vaidya AB, Kappe SH. A member of a conserved *Plasmodium* protein family with membrane-attack complex/perforin (MACPF)-like domains

- localizes to the micronemes of sporozoites. *Mol Biochem Parasitol* 2004;133:15–26. [PubMed: 14668008]
27. Ishino T, Yano K, Chinzei Y, Yuda M. Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. *PLoS Biol* 2004;2:77–84.
  28. Kariu T, Ishino T, Yano K, Chinzei Y, Yuda M. CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. *Mol Microbiol* 2006;59:1369–1379. [PubMed: 16468982]
  29. Matuschewski K, Ross J, Brown SM, Kaiser K, Nussenzweig V, Kappe SH. Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage. *J Biol Chem* 2002;277:41948–53. [PubMed: 12177071]
  30. Bhanot P, Schauer K, Coppens I, Nussenzweig V. A surface phospholipase is involved in the migration of *Plasmodium* sporozoites through cells. *J Biol Chem* 2005;280:6752–6760. [PubMed: 15590623]
  31. Sinnis P, Nardin E. Sporozoite Antigens: Biology and Immunology of the Circumsporozoite Protein and Thrombospondin Related Anonymous Protein. In: Perlmann, P.; Troye-Blomberg, M., editors. *Malaria Immunology*. Basel, Switzerland: S. Karger Press; 2002. p. 70–96.
  32. Mota MM, Hafalla JCR, Rodriguez A. Migration through host cells activates *Plasmodium* sporozoites for infection. *Nat Med* 2002;8:1318–1322. [PubMed: 12379848]
  33. Frevert U, Engelmann S, Zougbede S, Stange J, Ng B, Matuschewski K, et al. Intravital observation of *Plasmodium berghei* sporozoite infection of the liver. *PLoS Biol* 2005;3:e192. [PubMed: 15901208]
  34. Carrolo M, Giordano S, Cabrita-Santos L, Corso S, Vigario A, Silva S, et al. Hepatocyte growth factor and its receptor are required for malaria infection. *Nat Med* 2003;9:1363–1369. [PubMed: 14556002]
  35. Cerami C, Frevert U, Sinnis P, Takacs B, Clavijo P, Santos MJ, et al. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. *Cell* 1992;70:1021–1035. [PubMed: 1326407]
  36. Cerami C, Frevert U, Sinnis P, Takacs B, Nussenzweig V. Rapid clearance of malaria circumsporozoite protein (CS) by hepatocytes. *J Exp Med* 1994;179:695–701. [PubMed: 8294876]
  37. Frevert U, Sinnis P, Cerami C, Shreffler W, Takacs B, Nussenzweig V. Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J Exp Med* 1993;177:1287–1298. [PubMed: 8478608]
  38. Pinzon-Ortiz C, Friedman J, Esko J, Sinnis P. The binding of the circumsporozoite protein to cell surface heparan sulfate proteoglycans is required for *Plasmodium* sporozoite attachment to cells. *J Biol Chem* 2001;276:26784–26791. [PubMed: 11352923]
  39. Pradel G, Garapaty S, Frevert U. Proteoglycans mediate malaria sporozoite targeting to the liver. *Mol Micro* 2002;45:637–651.
  40. Sinnis P, Willnow TE, Briones MRS, Herz J, Nussenzweig V. Remnant lipoproteins inhibit malaria sporozoite invasion of hepatocytes. *J Exp Med* 1996;184:945–954. [PubMed: 9064354]
  41. Sinnis P, Clavijo P, Fenyo D, Chait B, Cerami C, Nussenzweig V. Structural and functional properties of region II-plus of the malaria circumsporozoite protein. *J Exp Med* 1994;180:297–306. [PubMed: 8006589]
  42. Rathore D, Sacci JB, de la Vega P, McCutchan TF. Binding and invasion of liver cells by *Plasmodium falciparum* sporozoites. *J Biol Chem* 2002;277:7092–7098. [PubMed: 11751898]
  43. Lyon M, Deakin JA, Gallagher JT. Liver heparan sulfate structure. *J Biol Chem* 1994;269:11208–11215. [PubMed: 8157650]
  44. Gressner AM, Schafer S. Comparison of sulfated glycosaminoglycan and hyaluronate synthesis and secretion in cultured hepatocytes, fat storing cells and Kupffer cells. *J Clin Chem Clin Biochem* 1989;27:141–149. [PubMed: 2708943]
  45. Wake K. Perisinusoidal stellate cells, their related structure in and around the liver sinusoids and vitamin A-storing cells in extrahepatic organs. *Int Rev Cytol* 1980;66:303–353. [PubMed: 6993411]
  46. Vanderberg JP. Clearance of malaria sporozoites. *Parasitol Today* 1995;11:24.
  47. Meis JFGM, Verhave JP, Jap PHK, Neuwissen JHET. An ultrastructural study on the role of Kupffer cells in the process of infection by *Plasmodium berghei* sporozoites in rats. *Parasitol* 1983;86:231–242.

48. Pradel G, Frevert U. Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocytes invasion. *Hepatology* 2001;33:1154–1165.
49. Baer K, Roosevelt M, Clarkson AB, van Rooijen N, Schnieder T, Frevert U. Kupffer cells are obligatory for *Plasmodium yoelii* sporozoite infection of the liver. *Cell Micro* 2007;9:397–412.
50. Dobrowolski JM, Sibley LD. *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* 1996;84:933–939. [PubMed: 8601316]
51. Kappe S, Bruderer T, Gantt S, Fujioka H, Nussenzweig V, Menard R. Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. *J Cell Biol* 1999;147:937–943. [PubMed: 10579715]
52. Sultan AA, Thathy V, Frevert U, Robson KJH, Crisanti A, Nussenzweig V, et al. TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. *Cell* 1997;90:511–522. [PubMed: 9267031]
53. Carruthers VB, Sibley LD. Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur J Cell Biol* 1997;73:114–123. [PubMed: 9208224]
54. Carruthers VB, Sibley LD. Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol Micro* 1999;31:421–428.
55. Carruthers VB. Armed and dangerous: *Toxoplasma gondii* uses an arsenal of secretory proteins to infect host cells. *Parasitol Int* 1999;48:1–10. [PubMed: 11269320]
56. Soldati D, Dubremetz JF, Lebrun M. Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. *Int J Parasitol* 2001;31:1293–1302. [PubMed: 11566297]
57. Gantt S, Persson C, Rose K, Birkett AJ, Abagyan R, Nussenzweig V. Antibodies against thrombospondin-related anonymous protein do not inhibit *Plasmodium* sporozoite infectivity in vivo. *Infect Immun* 2000;68:3667–73. [PubMed: 10816526]
58. Binder EM, Kim K. Location, location, location: trafficking and function of secreted proteases of *Toxoplasma* and *Plasmodium*. *Traffic* 2004;5:914–924. [PubMed: 15522094]
59. Dowse T, Soldati D. Host cell invasion by the Apicomplexans: the significance of microneme protein proteolysis. *Curr Opin Microbiol* 2004;7:388–396. [PubMed: 15358257]
60. Carruthers VB, Blackman MJ. A new release on life: emerging concepts in proteolysis and parasite invasion. *Mol Microbiol* 2005;55:1617–1630. [PubMed: 15752188]
61. Bannister LH, Dluzewski AR. The ultrastructure of red cell invasion in malaria interactions: a review. *Blood Cells* 1990;16:257–292. [PubMed: 2257315]
62. Sinnis P, Sim BKL. Cell invasion by the vertebrate stages of *Plasmodium*. *Trends Microbiol* 1997;5:52–58. [PubMed: 9108930]
63. Coppi A, Pinzon-Ortiz C, Hutter C, Sinnis P. Proteolytic processing of the *Plasmodium* circumsporozoite protein is required for cell invasion. *J Exp Med* 2005;201:27–33. [PubMed: 15630135]
64. Robson KJH, Hall JRS, Jennings MW, Harris TJR, Marsh K, Newbold CI, et al. A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. *Nature* 1988;335:79–82. [PubMed: 3045563]
65. Peterson MG, Marshall VM, Smythe JA, Crewther PE, Lew A, Silva A, et al. Integral membrane protein located in the apical complex of *Plasmodium falciparum*. *Mol Cell Biol* 1989;9:3151–4. [PubMed: 2701947]
66. Menard R. The journey of the malaria sporozoite through its hosts: two parasite proteins lead the way. *Microb Infect* 2000;2:633–642.
67. Wengelnik K, Spaccapelo R, Naitza S, Robson KJH, Janse CJ, Bistoni F, Waters AP, Crisanti A. The A-domain and the thrombospondin-related motif of *Plasmodium falciparum* TRAP are implicated in the invasion process of mosquito salivary glands. *EMBO J* 1999;18:5195–5204. [PubMed: 10508153]
68. Matuschewski K, Nunes AC, Nussenzweig V, Menard R. *Plasmodium* sporozoite invasion into insect and mammalian cells is directed by the same dual binding system. *EMBO J* 2002;21:1597–1606. [PubMed: 11927544]

69. Silvie O, Franetich J, Charrin S, Mueller MS, Siau A, Bodescot M, et al. A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites. *J Biol Chem* 2004;279:9490–6. [PubMed: 14676185]
70. Muller HM, Reckman I, Hollingdale MR, Bujard H, Robson KJH, Crisanti A. Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. *EMBO J* 1993;12:2881–2889. [PubMed: 8392935]
71. Robson KJH, Frevert U, Reckmann I, Cowan G, Beier J, Scragg IG, et al. Thrombospondin-related adhesive protein (TRAP) of *Plasmodium falciparum*: expression during sporozoite ontogeny and binding to human hepatocytes. *EMBO J* 1995;14:3883–3894. [PubMed: 7664729]
72. McCormick CJ, Tuckwell DS, Crisanti A, Humphries MJ, Hollingdale MR. Identification of heparin as a ligand for the A-domain of *Plasmodium falciparum* thrombospondin-related adhesion protein. *Mol Biochem Parasitol* 1999;100:111–124. [PubMed: 10376999]
73. Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* 2002;419:520–526. [PubMed: 12368866]
74. Howell SA, Withers-Martinez C, Kocken CHM, Thomas AW, Blackman MJ. Proteolytic processing and primary structure of *Plasmodium falciparum* apical membrane antigen-1. *J Biol Chem* 2001;276:31311–20. [PubMed: 11399764]
75. Ishino T, Chinzei Y, Yuda M. Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. *Mol Micro* 2005;58:1264–75.
76. van Dijk MR, Douradinha B, Franke-Fayard B, Heussler V, van Dooren MW, van Schaijk B, et al. Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. *Proc Natl Acad Sci USA* 2005;102:12194–99. [PubMed: 16103357]
77. Gruner AC, Snounou G, Fuller K, Jarra W, Renia L, Preiser PR. The Py235 proteins: glimpses into the versatility of a malaria multigene family. *Microb Infect* 2004;6:864–73.
78. Freeman RR, Trejdosiewicz AJ, Cross GA. Protective monoclonal antibodies recognizing stage-specific merozoite antigens of a rodent malaria parasite. *Nature* 1980;284:366–368. [PubMed: 7360274]
79. Holder AA, Freeman RR. Immunization against blood-stage rodent malaria using purified parasite antigens. *Nature* 1981;294:361–364. [PubMed: 7312033]
80. Preiser PR, Khan S, Costa FT, Jarra W, Belnoue E, Ogun S, et al. Stage-specific transcription of distinct repertoires of a multigene family during *Plasmodium* life cycle. *Science* 2002;295:342–345. [PubMed: 11786645]
81. Chattopadhyay R, Rathore D, Fujioka H, Kumar S, de la Vega P, Haynes D, et al. PfSPATR, a *Plasmodium falciparum* protein containing an altered thrombospondin type I repeat domain is expressed at several stages of the parasite life cycle and is the target of inhibitory antibodies. *J Biol Chem* 2003;278:25977–81. [PubMed: 12716913]
82. Gruner AC, Brahimi K, Letourneur F, Renia L, Eling W, Snounou G, et al. Expression of the erythrocyte-binding antigen 175 in sporozoites and in liver stages of *Plasmodium falciparum*. *J Infect Dis* 2001;184:892–7. [PubMed: 11528591]
83. Pasquetto V, Fidock DA, Gras H, Badell E, Eling W, Ballou WR, et al. *Plasmodium falciparum* sporozoite invasion is inhibited by naturally acquired or experimentally induced polyclonal antibodies to the STARP antigen. *Eur J Immunol* 1997;27:2502–13. [PubMed: 9368603]
84. Gruner AC, Brahimi K, Eling W, Konings R, Meis J, Aikawa M, et al. The *Plasmodium falciparum* knob-associated PfEMP3 antigen is also expressed at pre-erythrocytic stages and induces antibodies which inhibit sporozoite invasion. *Mol Biochem Parasitol* 2001;253–61. [PubMed: 11223132]
85. Silvie O, Rubinstein E, Franetich J, Prenant M, Belnoue E, Renia L, et al. Hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity. *Nat Med* 2003;9:93–96. [PubMed: 12483205]
86. Silvie O, Franetich JF, Boucheix C, Rubinstein E, Mazier D. Alternative invasion pathways for *Plasmodium berghei* sporozoites. *Int J Parasitol* 2007;37:173–82. [PubMed: 17112526]

87. Silvie O, Greco C, Franetich JF, Dubart-Kupperschmitt A, Hannoun L, van Gemert GJ, et al. Expression of human CD81 differently affects host cell susceptibility to malaria sporozoites depending on the *Plasmodium* species. *Cell Microbiol* 2006;8:1134–46. [PubMed: 16819966]