Malaria Parasite Liver Infection and Exoerythrocytic Biology

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In their infection cycle, malaria parasites undergo replication and population expansions within the vertebrate host and the mosquito vector. Host infection initiates with sporozoite invasion of hepatocytes, followed by a dramatic parasite amplification event during liver stage parasite growth and replication within hepatocytes. Each liver stage forms up to 90,000 exoerythrocytic merozoites, which are in turn capable of initiating a blood stage infection. Liver stages not only exploit host hepatocyte resources for nutritional needs but also endeavor to prevent hepatocyte cell death and detection by the host's immune system. Research over the past decade has identified numerous parasite factors that play a critical role during liver infection and has started to delineate a complex web of parasite–host interactions that sustain successful parasite colonization of the mammalian host. Targeting the parasites' obligatory infection of the liver as a gateway to the blood, with drugs and vaccines, constitutes the most effective strategy for malaria eradication, as it would prevent clinical disease and onward transmission of the parasite.

The genus *Plasmodium* consists of many different parasite species, each with a narrow host range and the causative agent of the disease malaria in its respective host. *Plasmodium* parasites have evolved exceedingly complex life cycles, using mosquitoes as the vehicle of transmission to infect reptiles, birds, and mammals, including humans. Human malaria parasite infection inflicts tremendous morbidity and significant mortality, mainly caused by two parasite species, *Plasmodium falciparum* and *Plasmodium vivax*. When the parasites' sporozoite stages are transmitted by a mosquito bite, these preerythrocytic forms establish the gateway to

host infection by invading host cells and initiating the first round of intracellular replication. Preerythrocytic infection precedes blood stage infection and, for all *Plasmodium* species that infect mammals, takes place in hepatocytes within the liver. Hence, the intrahepatocytic replication stages are called liver stages or exoerythrocytic forms (EEFs). Liver infection is completely asymptomatic; thus, it cannot be detected in humans and, in consequence, cannot be directly studied during natural infection. Fritz Schaudinn's infamous blunder at the turn of the 19th century, the alleged observation that sporozoites could directly infect red blood cells,

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held back research on preerythrocytic infection for decades, and it was only in the late 1940s that the liver stages of the parasite were discovered. This breakthrough was made possible by the discovery of rodent malaria parasites such as *Plasmodium yoelii* and *Plasmodium berghei*, and their introduction into laboratory research, which provided exceptionally powerful experimental tools for the direct analysis of liver infection. Indeed, most of the data concerning preerythrocytic infection have been generated with these models. Preerythrocytic stages of human malaria parasites remain exceedingly difficult to study. However, the development of in vitro platforms for the culture of human hepatocytes and the development of liver-humanized mice provide, for the first time, powerful preerythrocytic infection models, which are revolutionizing research on the initial stages of human malaria parasite infection. Here, we review the biology of liver infection by sporozoites and liver stage development, raise outstanding questions regarding the field, highlight opportunities for future research, and argue for the liver stages as the essential target for malaria eradication efforts.

HEPATOCYTE INVASION AND TRANSITION TO LIVER STAGE

After an infectious mosquito bite, sporozoites must leave the bite site and find their way to hepatocytes, where liver stage development occurs. The dermis-to-hepatocyte journey (reviewed in Ejigiri and Sinnis 2009) is complex and perilous for the sporozoites and their numbers are modest, yet a single infectious mosquito bite leads to liver infection, thereby setting the stage for successful host colonization. The sporozoite (Fig. 1A) is a unique invasive stage—unlike the parasite's ookinete stage that only traverses across the basal lamina of the mosquito midgut (Vinetz 2005) and the merozoites that only invade red blood cells (Cowman et al. 2012)—sporozoites are highly motile and must *traverse* and *invade* cells. They enter the bloodstream by traversing a dermal capillary, are transported to the hepatic capillary network (the sinusoids), and then traverse across

the endothelial barrier to enter the hepatic parenchyma. Here, sporozoites traverse multiple hepatocytes and then finally undergo a functional switch that allows them to invade a hepatocyte to take up residence. During and after invasion, the sporozoite releases the contents of a unique set of invasive organelles, the micronemes and rhoptries, whose constituent proteins mediate molecular interactions with the host cell (Fig. 1A–C). The molecular mechanisms and sequence of events in cell invasion have been extremely well dissected with regard to merozoite invasion of red blood cells, but much less is known about hepatocyte infection by sporozoites. Merozoite and sporozoite host cell invasion share commonalities, such as the parasite actin–myosin motor that powers active entry into the host cell (Fowler et al. 2004) and the host cell plasma membrane invagination during invasion that ensconces the intracellular parasite within a host membrane, known as the parasitophorous vacuole membrane (PVM) (Fig. 1D) (Meis et al. 1983). The PVM is extensively modified by the developing liver stage parasite and acts not only as a barrier to the host cell cytoplasm but also as a conduit for communication and nutrient acquisition (Figs. 1E and 2A,B). Research has revealed some of the key molecular players, both parasite and host, in the hepatocyte invasion process, many of which are unique to sporozoites.

The major sporozoite surface protein, circumsporozoite protein (CSP), is a key mediator of the sporozoites' interaction with the host during its journey to the liver and the infection of hepatocytes. CSP is composed of a central repeat region, diverse across *Plasmodium* species, flanked by two conserved domains—the amino-terminal region I and a cell-adhesive carboxy-terminal motif known as the type I thrombospondin repeat (TSR). Region I masks the adhesive TSR until the sporozoite reaches the liver at which time it is cleaved, allowing the TSR to mediate hepatocyte adhesion (Coppi et al. 2011). This begs the question, how does the sporozoite know it has reached the liver? Localized within the liver sinusoid are highly sulfated heparin sulfate proteoglycans (HSPGs), produced by stellate cells within the space of

Figure 1. Sporozoite invasion of host hepatocytes. Graphic representation of the invasion steps that culminate in the productive infection of the hepatocyte host cell. (A) The surface of the motile sporozoite is coated with circumsporozoite protein (CSP) (shown in *inset*), which engages heparin sulfate proteoglycans (HSPGs) on the hepatocyte surface. The apical end of the sporozoite contains organelles whose contents are essential for hepatocyte invasion: rhoptries (Rh, pink), micronemes (Mc, orange), and likely dense granules (DG, blue), although the latter have not been unequivocally identified. (B,C) Contact with an invasion-permissive hepatocyte (brown) triggers CSP processing, which, via an unknown mechanism, triggers the release of invasion-essential proteins from the micronemes (*inset*). These proteins include thrombospondin-related adhesive protein (TRAP), which binds to HSPGs on the hepatocyte cell surface and to the sporozoite internal glideosome complex (not shown) via its cytoplasmic domain to provide traction for the invading sporozoite at the moving junction (also known as the tight junction, shown as a gray ring). The microneme proteins P52 and P36 are also involved in the invasion process and may interact with each other as well as interacting with the hepatocyte Ephrin A2 receptor (EphA2). The P52/P36/EphA2 axis appears to be critical for the formation of the parasitophorous vacuole (PV). The hepatocyte receptor CD81 is also important for the invasion process and PV formation, but it is not clear whether the sporozoite directly interacts with it. The inner membrane complex (IMC) (in yellow) anchors the internal glideosome complex, allowing for sporozoite movement into the hepatocyte. Hepatocyte invasion occurs through the moving junction at the point of entry and is accompanied by the polymerization of host F-actin in association with Arp2/3. Invasion results in the invagination of the hepatocyte plasma membrane and the release of rhoptry proteins, including ROP2, ROP4, and RAP2/3. (D) Successful invasion results in the sporozoite residing within a PV surrounded by a parasitophorous vacuole membrane (PVM) of hepatocyte origin. The PVM is extensively modified by the parasite and the putative dense granule proteins UIS3 and UIS4 are released and trafficked to the PVM, as is EXP-1 (*inset*). During sporozoite dedifferentiation, the IMC (dashed yellow line) and the apical organelles are broken down. (E) The nascent liver stage trophozoite resides within a PV, has its own parasite plasma membrane still coated by CSP (green), and is surrounded by a PVM (blue).

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Figure 2. Liver stage development. Graphic representation of liver stage maturation that leads to the formation of exoerythrocytic merozoites. (A) The tubulovesicular network (TVN) (blue)—membrane-bound extensions and whorls that emanate from the parasitophorous vacuole membrane (PVM)—interacts with the hepatocyte's autophagosomes (which express Atg8/LC3) likely for nutrient uptake. Parasite proteins expressed on the PVM/TVN include IBIS1, EXP1, UIS4, and UIS3, which has been shown to interact with host L-FABP. The parasite plasma membrane (PPM)-associated protein B9 is known to be important for liver stage development. (B) As the liver stage parasite matures, multiple invaginations of the PPM occur (cytomere formation), and this is accompanied by the expression of MSP1 and ZIPCO. Additionally, LISP1 and LISP2 expression occur on the PVM. (C) Toward the end of liver stage development, individual exoerythrocytic merozoites begin to form, the PVM breaks down (a process that relies on LISP1), and LISP2 is released into the host hepatocyte. (D) Merozoites, merozoites surrounded by hepatocyte plasma membrane, are released into the bloodstream through the liver sinusoid, which is demarcated by epithelial cells (red) and liver resident macrophages, the Kupffer cells (yellow).

Disse, and these HSPGs are recognized by the sporozoite via CSP (Fig. 1B) (Frevert et al. 1993). This might lead to a signaling event that switches the sporozoite to an invasive phenotype (Coppi et al. 2007), resulting in the aforementioned cleavage of region I, a process that is catalyzed by a sporozoite protease (Coppi et al. 2005).

CSP cleavage appears to be a prerequisite for productive infection, but what other molecules are involved in the process? A number of sporozoite micronemal proteins are known to be crucial and they include thrombospondin-

related anonymous protein (TRAP) and the 6-cys domain proteins P52 and P36 (Fig. 1B). TRAP is a type 1 transmembrane protein, and like CSP, it contains a TSR domain but also an integrin-like domain. TRAP is released onto the sporozoite surface from the micronemes during invasion and, during this process, TRAP binds to the sporozoite actin-myosin motor through its carboxy-terminal cytoplasmic domain and binds to HSPGs through its extracellular domain (Kappe et al. 1999). During invasion, TRAP is translocated from the anterior to the posterior of the sporozoite and is subsequently

shed from the sporozoite by cleavage within its transmembrane domain, likely by the rhomboid protease ROM4, which allows for hepatocyte invasion, because the cleavage releases the TRAP–HSPG bond (Baker et al. 2006; Ejigiri et al. 2012) (Fig. 1B). The micronemal proteins P52, a predicted GPI-anchored protein, and P36, a predicted secreted protein, are members of the 6-cys protein family (Templeton and Kaslow 1999), and they are critical for hepatocyte infection (Fig. 1B,C). Other members of the 6-cys family that are expressed in different parasite stages also have roles in parasite–parasite or host–parasite interactions (Pradel 2007; van Dijk et al. 2010; Molina-Cruz et al. 2013; Sala et al. 2015). The 6-cys domains are likely involved in protein–protein interaction, but evidence for this has been scarce. P52 is released from the micronemes to the sporozoite surface before invasion and disruption of either *P52* or *P36* in *P. berghei* (Ishino et al. 2005; van Schaijk et al. 2008), and *P52* in *P. falciparum* (van Schaijk et al. 2008) results in sporozoites that are defective in hepatocyte invasion. In *P. yoelii*, the simultaneous deletion of *P36* and *P52* renders sporozoites unable to form or maintain a PVM, resulting in early abortion of liver stage development (Labaied et al. 2007). Similarly, *P. falciparum* sporozoites lacking *P36* and *P52* cannot develop after hepatocyte entry (VanBuskirk et al. 2009). Recent work revealed the hepatocyte receptor that directly interacts with these proteins: the transmembrane receptor Ephrin A2 (EphA2) (Fig. 1B). EphA2 is essential for sporozoite invasion and concomitant PVM formation in rodent and human malaria parasites and antibodies to EphA2 block sporozoite infection (Kaushansky et al. 2015). Intriguingly, its natural ligand EphrinA1 shares structural similarity with the 6-cys fold and there is strong evidence that EphA2 directly interacts with P36, thus constituting the first bona fide host receptor–parasite ligand pair with a critical role in hepatocyte infection (Fig. 1B).

In addition to EphA2, other hepatocyte surface proteins are critical for successful sporozoite infection. The tetraspanin membrane protein “cluster of differentiation 81” (CD81) (Fig. 1B) is required on hepatocytes for *P. yoelii*

and *P. falciparum* sporozoite invasion with PVM formation but interestingly not for *P. berghei*. *P. yoelii* sporozoites are unable to infect CD81-deficient mouse hepatocytes, and antibodies against mouse and human CD81 inhibit the in vitro hepatocyte infection by *P. yoelii* and *P. falciparum* sporozoites (Silvie et al. 2003). Yet, a sporozoite ligand for CD81 has not been identified. Cholesterol is involved in the assembly of CD81-rich microdomains on the cell surface, and this assembly is necessary for sporozoite infection (Silvie et al. 2006), indicating that it is the organization and composition of microdomains, rather than a direct interaction with CD81, that enable successful sporozoite infection of hepatocytes. A further hepatocyte membrane receptor, scavenger receptor BI (SR-BI), which mediates the selective uptake of cholesteryl esters from both high- and low-density lipoproteins, plays a role in hepatocyte infection. This role appears rather indirect because SR-BI deficiency causes a decreased expression of CD81 on the hepatocyte surface (Yalaoui et al. 2008). Antibodies to SR-BI do not block sporozoite invasion (Foquet et al. 2015), adding credence to the idea that SR-BI expression is necessary for CD81 microdomain formation but not a receptor for the sporozoite. Recently, an important link between the initial interaction of the sporozoite with the hepatocyte surface and the signaling to trigger processes within sporozoites has been revealed—the presence of CD81 on hepatocytes appears to be critical for the subsequent discharge of the rhoptries (Risco-Castillo et al. 2014). Research thus indicates an orchestrated sequence of events whereby sporozoite interaction with the hepatocyte promotes downstream signaling that triggers the secretion of proteins necessary for the formation and modification of the PVM.

Once the sporozoite lies completely ensconced in the PVM (Fig. 1D), it must metamorphose into a highly metabolically active intracellular replication machine, a process that requires jettisoning or disassembling its invasive organelles and inner membrane complex (IMC) (Fig. 1D). IMC breakdown is accompanied by spherical plasma membrane expansion in the center of the still elongated parasite and

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the contraction of its distal ends (Jayabalasingham et al. 2010). This shape conversion is accompanied by the clearance of the IMC and invasion-associated organelles, which are discharged as large exocytic vesicles or possibly undergo autophagy. However, the presence of autophagy in *Plasmodium* is controversial because the key autophagy marker protein Atg8/LC3 localizes to the parasite apicoplast and does not relocalize to autophagosome-like vesicles or vacuoles (Eickel et al. 2013; Jayabalasingham et al. 2014). The nascent liver stage preferentially develops in the host juxtanuclear region and the PVM appears to form an association with the host endoplasmic reticulum (ER) (Bano et al. 2007). Parasite modification of the PVM allows the passage of molecules of up to 855 Da from the host cytosol to the PV through open channels, and it is possible that host-derived cholesterol that accumulates at the PVM may modulate channel activity (Bano et al. 2007). These channels ensure that molecules from the nutrient-rich hepatocyte can feed the growing liver stage, although a tubulovesicular network, described below, likely allows for the uptake of larger molecules. Although the PVM is vital for successful liver stage development, the initial metamorphosis from sporozoite to trophozoite can occur outside a host cell. This indicates that the intracellular environment does not provide specific signals to trigger this process but rather that more general changes that occur with the sporozoites transition into a mammalian host, such as an elevation in temperature and the presence of serum components (Kaiser et al. 2003).

As described above, the initiation of liver stage development is a complex process that is initiated when the sporozoite detects a suitable hepatocyte for infection. Once the sporozoite has invaded its host cell, it must then dedifferentiate from an invasive extracellular parasite into an intracellular trophozoite contained within a PVM. During this process, signaling events prevent the parasites' destruction by the host cell. Finally, the trophozoite transitions into a highly replicative schizont that relies on nutrient uptake from its host as well as its own metabolic processes to ensure its

eventual goal of exoerythrocytic merozoite release.

It must be noted that the transition to schizogony, which always occurs in *P. falciparum* liver stage development, does not always occur in *P. vivax*. The *P. vivax* sporozoite, once it has entered a host hepatocyte, dedifferentiates and can then become a dormant trophozoite, known as the hypnozoite. The hypnozoite can lie dormant for months and even years and then reactivate and fully develop, leading to *P. vivax* malaria relapses. The processes that lead to hypnozoite formation and reactivation are very poorly understood, and malaria elimination must address the radical cure of the dormant hypnozoite.

LIVER STAGE DEVELOPMENT

The liver stage undergoes schizogony—nuclear division without cell division—during its maturation and only in the final phase of this process do invasive exoerythrocytic merozoites form. Liver stage schizogony constitutes one of the most rapid eukaryotic replication events—in a little >2 d, rodent malaria sporozoites can transform into a mature ellipsoid liver stage containing up to 29,000 exoerythrocytic merozoites (Baer et al. 2007). Liver stage development in *P. falciparum* takes longer, ~6.5 d, with estimates of up to 90,000 exoerythrocytic merozoites packaged in the fully mature parasite (Vaughan et al. 2012). Liver stage schizogony is characterized by the rapid branching and growth of the parasite's organelles such as the mitochondria and the relict plastid organelle, the apicoplast (Stanway et al. 2009), as well as massive DNA replication. Studies utilizing parasite and host transgenesis have shed light on the important pathways that allow for parasite growth, and, not surprisingly, a number of parasite proteins involved in PVM formation and modification are essential for liver stage formation and development.

The liver stage PVM proteins UIS3 and UIS4 are encoded by genes belonging to the up-regulated in infective sporozoites (UIS) group (Figs. 1D–E, 2A, 3A–C,F). These genes are highly transcribed in sporozoites when they

Figure 3. Immunofluorescence images of liver stage development. (A–E) *Plasmodium yoelii* liver stage development in a mouse liver. (A) The spherical, early liver stage parasite, 12 h after sporozoite invasion, is significantly smaller than the host hepatocyte nucleus (blue). The liver stage parasitophorous vacuole (PVM) is shown in red (UIS4 expression), the endoplasmic reticulum (ER) is shown in green (BiP expression), and the parasite contains a single nuclear center (blue). (B) By 24 h, the *P. yoelii* liver stage parasite (the UIS4-positive PVM is shown in red) has entered schizogony (multiple centers of nuclear replication [blue]). (C) At 30 h of development, the *P. yoelii* liver stage (the UIS4 PVM is shown in green) has undergone multiple rounds of nuclear replication (blue) and contains a highly branched apicoplast (acyl carrier protein expression, shown in red). (D) Late in *P. yoelii* liver stage development (48 h), the parasite plasma membrane (PPM) undergoes extensive invagination resulting in cytomere formation (MSP1, green). LISP2 (red) is expressed on the PVM as well as the hepatocyte membrane (white arrows). The nuclei (blue) of the individual parasites can be seen within the liver stage parasite. (E) At the end of liver stage development (52 h), individual exoerythrocytic merozoites (MSP1 expression in green, upper panel, delineates the merozoite membrane) can be seen within the mature liver stage parasite (differential interference contrast, lower panel). (F) Image of a *P. vivax* hypnozoite in a humanized mouse liver. The hypnozoite remains latent, is small, and does not replicate its DNA, and its PVM has a unique UIS4-positive prominence (green). The hypnozoite mitochondrion (HSP60 expression) is shown in red and is branched. Scale bars, 10 μ m.

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still reside in the mosquito salivary glands but some are translationally repressed with protein production peaking only after hepatocyte invasion. This “just in time” protein expression from stored mRNAs exemplifies one of the approaches the sporozoite has evolved to ready itself for the rapid transition to liver stage replication and indicates that proteins such as UIS3 and UIS4 must be playing an important role starting very early in liver stage development. Indeed, deletion of either *UIS3* or *UIS4* gene causes arrest of early liver stage development (Mueller et al. 2005a,b; Jobe et al. 2007). The carboxyl termini of both UIS3 and UIS4 face the hepatocyte cytoplasm and the amino termini the PV lumen, suggestive of roles in parasite–hepatocyte interactions. Indeed, UIS3 interacts with liver fatty acid protein (L-FABP) (Fig. 2A) (Mikolajczak et al. 2007), suggesting an interaction that could serve as a conduit for the transport of lipid into the liver stage to support growth. Lipid transport within and between eukaryotic cells can occur through membrane-bound ATP-binding cassette (ABC) transporters, and the ABCC class of transporters is expressed by both *P. berghei* and *P. falciparum* liver stages. Interestingly, the deletion of *ABCC2* (also known as *MRP2*) in both *P. berghei* and *P. falciparum* leads to liver stage arrest, demonstrating the importance of parasite ABC transporters in liver stage development and hinting to their role in lipid transport (Rijpmma et al. 2016). To date, UIS4 function has remained elusive, as has the function of a second PVM-resident protein exported protein 1 (EXP1, also known as Hep17 in rodent malaria parasites) (Fig. 2A). Later in liver stage development, two further PVM-associated proteins are expressed, LISP1 and LISP2 (Figs. 2B,C and 3D) (Ishino et al. 2009; Orito et al. 2013). LISP1 is involved in PVM breakdown and subsequent merozoite release, whereas LISP2 (which contains a modified 6-cys domain) is localized to the PV and PVM and is also transported to the cytoplasm and plasma membrane of infected hepatocytes (Figs. 2B,C and 3D). Although the functions of these two proteins are not fully understood, gene deletion studies in *P. berghei* have indicated their importance in late liver

stage growth because parasites that lack LISP1 or LISP2 do not mature effectively. Further critical proteins expressed by liver stage parasites include the 6-cys-like protein, B9, expressed on the parasite plasma membrane (PPM) (Fig. 2A) (Annoura et al. 2014) and ZIPCO, an iron transporter expressed on the late liver stage PPM (Fig. 2B) (Sahu et al. 2014). Although an increasing number of PV and PVM proteins have been identified that play important roles in liver stage development, an important future goal is the comprehensive identification of all liver stage PVM, PV, and PPM proteins that will supersede the current identification of important players using candidate approaches. Importantly, research needs to go beyond the assembly of “parts lists” and ought to delineate the relationship and interaction among liver stage PVM and PV proteins and their interaction with host proteins to establish a network of important liver stage–hepatocyte interactions.

Although the liver stage clearly parasitizes the extremely metabolically active host hepatocyte, the massive increase in parasite biomass during liver stage development likely requires the participation of parasite anabolic pathways. Indeed, combined transcriptome and proteome analysis of liver stage development showed that the parasite *de novo* type II fatty acid synthesis pathway (FAS II) is highly active (Tarun et al. 2008). To assess the importance of FAS II, which is localized to the parasite apicoplast, gene knockouts in rodent malaras have been performed and showed that FAS II is critical for late liver stage development (Yu et al. 2008; Vaughan et al. 2009; Pei et al. 2010; Falkard et al. 2013; Lindner et al. 2014). These reports show that the liver stage requires its own fatty acid synthesis, but it is not currently understood why the fatty acids are synthesized—they might be incorporated into exoerythrocytic merozoite membranes, but they could also be incorporated into the growing hepatocyte plasma membrane or the PVM. As mentioned above, the UIS3/L-FABP interaction likely allows for lipid acquisition from the hepatocyte. The cholesterol component of this lipid can be derived from low-density lipoprotein internalization by the hepatocyte as well as *de novo* synthesized hepa-

toocyte cholesterol (Labaied et al. 2011). Further research has shown that hepatocyte phosphatidylcholine, the major membrane phospholipid, is taken up by liver stages, and preventing the rate-limiting steps in the synthesis of hepatocyte phosphatidylcholine affects liver stage development (Itoe et al. 2014). All of these studies show that liver stage parasites critically depend on both lipid uptake from the host hepatocyte and endogenous lipid synthesis to support their dramatic growth and differentiation.

During growth, liver stage mass increases so much that the infected hepatocyte must significantly expand to accommodate the replicating parasite. However, the liver stage parasites remain relatively undifferentiated until shortly before completion of liver stage maturation. Late in development, the PPM undergoes rapid expansion and extensive invagination (called cytomere formation) (Figs. 2B and 3D), which dramatically increases parasite surface area and enables rapid formation of individual exoerythrocytic merozoites (Figs. 2C and 3E). This process also requires coordinated nuclear and organellar fission to ensure partitioning of nuclei and organelles into nascent merozoites (Stanway et al. 2011). This coordinated fission likely relies on both DNA- and RNA-binding proteins, and a liver stage-specific RNA-binding protein, PlasMei2, appears to be essential for the partitioning of nuclear DNA. The deletion of *PlasMei2* in *P. yoelii*, without affecting liver stage growth, prevents the partitioning of nuclear DNA, and this in turn prevents the formation of exoerythrocytic merozoites (Dankwa et al. 2016). Once merozoites have fully formed, the PVM must break down (Fig. 2C) before they can be released, and this process is a result of changes in PVM permeability (Sturm et al. 2009) and relies on a parasite phospholipase, which localizes to the PVM (Burda et al. 2015).

Intravital microscopic observations of live-fluorescent *P. yoelii* and *P. berghei* mature liver stages in mice have shown that they induce the detachment of their host hepatocyte, leading to the budding of merosomes (merozoite-filled vesicles) into the sinusoidal lumen (Fig. 2D) (Sturm et al. 2006; Tarun et al. 2006). The mer-

osome membrane derives from the host hepatocyte plasma membrane (Graewe et al. 2011), allowing the merosome to hide from the immune system and thereby avoid uptake and destruction by the numerous liver-resident macrophages (the Kupffer cells), on its way to the bloodstream. Merosomes have also been observed in mature *P. falciparum* liver stages (Vaughan et al. 2012), confirming their existence in human malaria parasite liver stages. Most *P. yoelii* merosomes exit the liver intact and adapt to a relatively uniform size, within which 100 to 200 merozoites reside (Baer et al. 2007). Merosomes eventually break up inside pulmonary capillaries, resulting in merozoite liberation and red blood cell infection.

PARASITE PERTURBATIONS OF HOST HEPATOCYTES

So far, we have discussed parasite proteins and some host molecules that are necessary for sporozoite invasion and liver stage development, but to acquire nutrients and grow, the parasite must not simply reside in the hepatocyte and exploit it but actively manipulate and subvert it to avoid its demise. How does this happen? Already at the point of invasion, the parasite subverts its host. As the sporozoite invades, it induces the formation of a ring-shaped structure in the hepatocyte composed of de novo polymerized host F-actin (Fig. 1C) (Gonzalez et al. 2009). The hepatocyte Arp2/3 complex, an actin-nucleating factor, is recruited to this ring structure and is necessary for parasite invasion (Fig. 1C). The PVM is essential for liver stage development, and the formation of a productive PVM is a prerequisite for the parasite's ability to render its host hepatocyte resistant to apoptosis (van de Sand et al. 2005; van Dijk et al. 2005; Kaushansky et al. 2013a). Although the sensitivity to apoptosis is dependent on the Bcl-2 family of mitochondrial proteins that the parasite subverts (Kaushansky et al. 2013a), the parasite molecules that interfere with hepatocyte apoptosis are not known. A line of defense used by many host cells against pathogen infection is the synthesis of stress granules, thereby shutting down protein synthe-

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sis. However, there is no evidence that hepatocyte infection causes stress granule formation, which implies that the parasite has evolved to prevent this response from occurring in the hepatocyte (Hanson and Mair 2014). In a surprising twist on parasite–host manipulation, liver stage survival actually benefits from the host ER stress response pathway (Inacio et al. 2015), which typically shuts down cellular activity. Proteins and transcripts that act in the unfolded protein response leading to ER stress were found to be elevated in hepatocytes in response to infection, but why the parasite perturbs its host in this way is not clear. It is possible that ER stress supports parasite growth by regulating lipid metabolism, vital for parasite growth (Itoe et al. 2014), or diminishing antigen presentation so the liver stage can remain hidden from the immune system—a trick used by hepatitis C (Tardif and Siddiqui 2003).

The perturbations of the host hepatocyte have been explored by analyzing changes in hepatocyte transcription and protein expression early during liver stage infection (Albuquerque et al. 2009; Kaushansky et al. 2013b). Transcriptome data showed that parasite infection initially causes a global stress response, which then morphs into an engagement of host metabolic processes and the maintenance of cell viability to ensure parasite and host cell survival (Albuquerque et al. 2009). Using cell lysate arrays to simultaneously monitor many protein perturbations, a subset of hepatocyte protein levels and accompanying posttranslational modifications were measured 24 h postinfection with rodent malaria parasites and compared with uninfected hepatocytes (Kaushansky et al. 2013b). This revealed a signaling network aimed at preventing host cell death but also determined that the tumor suppressor P53 is substantially dampened in infected hepatocytes. This is of functional significance because increasing P53 levels whether chemically or genetically nearly eliminates liver stage infection and development and mice lacking P53 are hypersusceptible to infection (Kaushansky et al. 2013b).

During liver stage development, a tubulovesicular network (TVN) forms, which includes membranous extensions of the PVM as well

as the creation of autonomous vesicular structures within the infected hepatocyte (Fig. 2A) (Mueller et al. 2005a). The TVN likely functions in hepatocyte interactions and in accessing nutrients from the hepatocyte, but these functions need to be experimentally verified. Fluorescent tagging of two *P. berghei* PVM proteins, UIS4 and IBIS1, and the use of correlative light-electron microscopy have shown that the TVN membranes extend throughout the host cell and include dynamic vesicles as well as long tubules that extend and contract from the PVM (Fig. 2A) (Grutzke et al. 2014). Additionally, labeling of host hepatocyte compartments revealed an association of late endosomes and lysosomes with the TVN-associated elongated membrane clusters. Furthermore, the host autophagosome protein Atg8/LC3 colocalizes with UIS4 at the PVM and TVN (Fig. 2A). These data suggest that the intimate association between the TVN and host endosomes/lysosomes allows for parasite–hepatocyte signaling as well as nutrient uptake. Further studies have shown that complete parasite development actually correlates with the gradual loss of autophagy marker proteins and associated lysosomes from the PVM even though other autophagic events such as nonselective canonical autophagy continue in the host cell (Prado et al. 2015). Thus, it is possible that the parasite continues to benefit from nonselective canonical autophagy for its growth.

Supporting the hypothesis that the endosomal system supports liver stage growth, the growth of the parasite is dependent on phosphoinositide 5-kinase (PIKfyve), which converts phosphatidylinositol 3-phosphate into phosphatidylinositol 3,5-bisphosphate (PI (3,5) P₂) within the endosomal system—an enzymatic reaction essential for late endocytic membrane fusion (Thieleke-Matos et al. 2014). Indeed, the PI (3,5) P₂ effector protein TRPML1, involved in late endocytic membrane fusion, was shown to be present in vesicles in close contact with the PVM and PIKfyve inhibition delayed parasite growth. Additionally, contents of late endocytic vesicles have been found within the parasite cytoplasm (Lopes da Silva et al. 2012). We are only just beginning to un-

derstand how the parasite manipulates its host, and many questions remain unanswered. Most notably, there is a complete lack of understanding of how the liver stage, during its own massive growth, also enables the growth of the PVM and expansion of the host hepatocyte to accommodate its heft—cells do not normally dramatically increase in size, but the mature liver stage-infected hepatocyte can expand to 200 times its normal volume.

The developing liver stage parasite, as well as interacting with its host hepatocyte to ensure its survival, must also evade the host's immune system to ensure merozoite maturation and release. As noted above, the autophagy marker Atg8/LC3 colocalizes with UIS4 at the PVM and TVN, and aids in liver stage growth. Conversely, autophagy has been identified as a downstream pathway that is activated in response to interferon γ (IFN- γ) in the control of intracellular infections (Deretic et al. 2013). Indeed, IFN- γ , a master regulator of immune functions, has been known for many years to induce the elimination of liver stage parasites in vivo (Ferreira et al. 1986). Thus, the parasite needs to precisely coordinate autophagy for its benefit. Research on *P. vivax* liver stages has shown that IFN- γ -mediated restriction of liver stage *P. vivax* depends on autophagy-related proteins including Beclin-1, PI3K, and Atg5 and enhanced the recruitment of LC3 to the PVM. In this case, the LC3 decoration of the PVM led to a noncanonical autophagy pathway resembling that of LC3-associated phagocytosis, promoting the fusion of *P. vivax* compartments with lysosomes and subsequent killing of the pathogen (Boonhok et al. 2016). Thus, research has uncovered positive and negative aspects of autophagy on parasite survival, and it is clear that although the parasite can take advantage of host cell autophagy, it can also be killed by the pathway and thus must carefully control this intracellular process for its own gain.

IFN- γ production in response to liver stage parasite detection is part of a programmed innate immune response to liver stage infection (Liehl and Mota 2012). This innate immune response to infection is pronounced and can actually lead to the effective killing of a second-

ary liver stage infection (Miller et al. 2014). This response is in part initiated by liver-resident cells and driven by *Plasmodium* RNA, which acts as a pathogen-associated molecular pattern capable of activating the type I IFN response via the cytosolic pattern recognition receptor Mda5 (Liehl et al. 2014). Furthermore, the response is abrogated in mice deficient in IFN- γ , as well as the type I IFN- α/β receptor (IFNAR), demonstrating the important role of IFNAR in driving the innate immune response (Liehl et al. 2014; Miller et al. 2014). The cell type linked to the secretion of IFN- γ was shown to be CD49b⁺CD3⁺ natural killer T (NKT) cells and an increase in CD1d-restricted NKT cells was critical in reducing liver stage burden of a secondary infection (Miller et al. 2014). In turn, the lack of IFNAR signaling abrogated the increase in NKT cell numbers in the liver, showing a link between type I IFN signaling, cell recruitment, and parasite elimination (Miller et al. 2014).

The above highlights the extent of our current knowledge on how the liver stage parasite influences both the host hepatocyte and the host innate immune system. Far more research is necessary and will help in the design and implementation of drugs to target the liver stage parasite and effective vaccines that engender appropriate immune responses to liver stage parasites. For these advances to take place, we need better models for liver stage development and liver stage isolation.

ADVANCES IN MODEL DEVELOPMENT FOR HUMAN MALARIA PARASITE LIVER INFECTION

A natural sporozoite infection typically results in the productive invasion of only a small number of hepatocytes. Because of this bottleneck, the liver stage parasite has been extremely difficult to study. However, such studies are essential to uncover pathways that are necessary for parasite survival and replication. These pathways can then be perturbed to prevent development and ultimately to thwart life cycle progression to blood stage disease. To this end, parasite transgenesis has enabled the creation of fluorescent parasites and this led to the sorting of murine

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hepatocytes infected with *P. yoelii* fluorescent liver stages, resulting in the first extensive liver stage transcriptomes and proteomes (Tarun et al. 2008; Albuquerque et al. 2009). However, extensive -omic analysis of human malaria liver stages has still not been completed and needs to be performed. Such analysis is important for the reason that numerous studies have shown significant biological differences between rodent and human malaria parasites. Nevertheless, platforms for the growth of human malaria parasite liver stages are not easily established, because of the limitation that *P. falciparum* and *P. vivax* preerythrocytic stages only infect human and some nonhuman primate hepatocytes and do not grow well in hepatoma cells. Additionally, the *P. vivax* sporozoite, unlike the model rodent malaria sporozoite, can develop into either a liver stage schizont or a liver stage hypnozoite (Krotoski et al. 1982)—a dormant liver stage form (Fig. 3F) that can activate and cause malaria relapse infections in affected individuals.

In vitro development of *P. vivax* liver stages in primary human hepatocytes was first documented in 1984 (Mazier et al. 1984) and for *P. falciparum* in 1985 (Mazier et al. 1985), and both parasites were later shown to mature in the human hepatocyte cell line HC-04 (Sattabongkot et al. 2006), albeit poorly. Rapid advances in cryopreservation methods for primary human hepatocytes make this cell type more easily available, and investigators no longer depend on cells fresh from liver surgery. Recently, an improved in vitro system using primary human hepatocytes in a microscale human liver platform was shown to support *P. falciparum* and *P. vivax* liver stages development including *P. vivax* hypnozoite formation (March et al. 2013). An additional promising platform uses induced pluripotent stem cell-derived hepatocyte-like cells and liver stage development of both *P. falciparum* and *P. vivax* appears to mirror that observed in primary human hepatocytes (Ng et al. 2015). Thus, this platform is an attractive alternative because it enables a potential unlimited production of hepatocytes.

Although in vitro models for infection have greatly improved over the past years, robust

in vivo infection models for preerythrocytic stages of human malaria parasites are highly desirable. Fortunately, human-liver chimeric mouse models that were originally developed for drug metabolism studies and hepatitis research are promising as animal models for human malaria liver infection. These models are all built around some form of immunocompromised background plus genetically encoded liver injury that selectively ablates mouse hepatocytes, thereby creating niches in the liver that can then be repopulated with primary human hepatocytes. An initial study using the SCID Alb-uPA mouse transplanted with human hepatocytes showed that *P. falciparum* liver stages developed after intravenous sporozoite injection (Sacci et al. 2006).

A second liver-chimeric mouse model, the FRG huHep mouse (Azuma et al. 2007), can yield up to 95% human hepatocyte chimerism. After intravenous injection of *P. falciparum* sporozoites into FRG huHep mice, complete liver stage development occurred, resulting in the creation of schizonts packed with tens of thousands of exoerythrocytic merozoites that were able to transition in vivo to red blood cell infection, which was then continuously maintained in vitro (Vaughan et al. 2012). This enabled the creation of *P. falciparum* genetic crosses, which were previously possible only in splenectomized chimpanzees (Vaughan et al. 2015). The FRG huHep mouse has, in addition, been used to show the efficacy of antibodies against sporozoite proteins to reduce or prevent *P. falciparum* liver infection (Sack et al. 2014). This has also been achieved in SCID Alb-uPA mice (Behet et al. 2014; Foquet et al. 2014). Together, the work establishes liver-chimeric mice as an important preclinical tool to assess interventions against human malaria parasite pre-erythrocytic stages and further refinement could ascertain their use as a new stepping-stone toward clinical testing of interventions in controlled human malaria infection trials.

Intriguingly, the FRG huHep mouse was also recently used for infections with *P. vivax* (Mikolajczak et al. 2015). In this study, robust liver infection with *P. vivax* sporozoites was observed that culminated in complete liver stage

development and exoerythrocytic merozoite release 9.5 d after infection and infectious merozoites could be captured by the injection of human reticulocytes. Alongside liver stage development, the establishment and persistence of hypnozoites was observed, enabling, for the first time, a detailed characterization of this unique latent EEF. Although DNA replication did indeed not occur in hypnozoites as previously thought, in contrast to previously held assumptions, latent hypnozoites were not “dormant” but metabolically active, showing limited organelle replication and modest growth over time. Hypnozoites also exhibited a unique thickening of the PVM, called a “prominence,” that appears to distinguish them from replicating liver stages (Fig. 3F). The study also showed that the FRG huHep mouse will be useful to model hypnozoite activation, which leads to the production of second- and third-generation exoerythrocytic merozoites and relapsing blood stage infection, which is a distinguishing clinical feature of *P. vivax* infection. Thus, the FRG huHep model might prove useful to test the effectiveness of drugs that can eliminate hypnozoites and thus prevent relapsing infection with *P. vivax*.

VACCINATION WITH ATTENUATED PREERYTHROCYTIC PARASITES

Following earlier research on bird malaria (which do not have a liver stage), a study in 1967 showed that mice inoculated with irradiation-attenuated *P. berghei* sporozoites induced protection from a subsequent infection with wild-type sporozoites (Nussenzweig et al. 1967) and with this, the concept of an attenuated, whole sporozoite malaria vaccine was born. Similar studies in humans with irradiated *P. falciparum* sporozoites showed equal promise (Clyde et al. 1973; Rieckmann et al. 1974). Subsequent work showed that attenuated sporozoites have to be infectious and invade hepatocytes to unfold their protective potential likely by virtue of expressing new antigens before the cessation of liver stage development. Live sporozoite immunization elicits both protective humoral responses and cellular responses (Doll and

Harty 2014). In the 1970s, irradiation-attenuated sporozoites were not considered a practical vaccine because of the issues with irradiation, mass production, preservation, and delivery. However, increasing success in producing and storing *P. falciparum* sporozoites, irradiated sporozoites gained a resurgent interest in the 2000s and intravenous inoculation of humans with cryopreserved, irradiated *P. falciparum* sporozoites recently showed complete protection from infectious sporozoite challenge (Seder et al. 2013).

The discovery of genes that are essential for liver stage development, discussed earlier, gave rise to the idea that attenuation of the parasite could be accomplished through engineered gene deletion within the parasite’s complex genome, thus rendering irradiation obsolete. In this effort, vaccine development meets parasite biology and synergizes effectively to create a biologically informed malaria vaccine strategy. Today, numerous genes essential for liver stage development have been identified, mostly in rodent malaria models (Table 1). Gene deletions arrest the parasite at distinct points during liver stage development, and studies have shown that late liver stage–arresting parasites afford superior immunity when compared with both an early arresting, genetically engineered parasite and irradiation-attenuated sporozoites. This is due to the significant increase in antigen load and breadth, which engenders a broader and diversified immune response (Butler et al. 2011; Sack et al. 2015). Engineering of *P. falciparum* liver stage–attenuated parasites has been undertaken (VanBuskirk et al. 2009); the parasites have undergone initial human clinical trial testing and are now undergoing improvements in attenuation (Table 1) (Mikolajczak et al. 2014; van Schaijk et al. 2014b). Ultimately, this will likely yield an engineered parasite that can replace irradiated sporozoites and such a vaccine will play a crucial role in malaria elimination.

FUTURE PERSPECTIVES

A call for malaria eradication by 2040 is perceived to be an achievable goal (endmalaria

Table 1. *Plasmodium* gene knockouts that lead to liver stage arrest

Gene knockout	Function	Temporal/spatial expression	Knockout phenotype			References
			Pb	Py	Pf	
B9	Unknown	PPM	Late arrest/ partial attenuation	Late arrest/ partial attenuation	–	Annoura et al. 2014
β -Ketoacyl-acyl-carrier protein synthase I/II (Fab B/F)	Fatty acid synthesis	Apicoplast throughout development	–	Late arrest/ complete attenuation	No sporozoites	Vaughan et al. 2009; van Schaijk et al. 2014a
cGMP-dependent protein kinase (PKG) (conditional knockout)	Protein phosphorylation	Throughout development	Late arrest/ complete attenuation	–	–	Falae et al. 2010
Glycerol 3-phosphate acyltransferase (G3PAT)	Phosphatidic acid synthesis	Apicoplast throughout development	–	Late arrest/ complete attenuation	–	Lindner et al. 2014
Glycerol 3-phosphate dehydrogenase (G3PDH)	Phosphatidic acid synthesis	Apicoplast throughout development	–	Late arrest/ complete attenuation	–	Lindner et al. 2014
Lipoic acid protein ligase B (LipB)	Fatty acid synthesis	Apicoplast throughout development	Late arrest/ partial attenuation	–	–	Falkard et al. 2013
Liver stage antigen 1 (LSA1)	Unknown	PV late in development	–	–	Aberrant maturation	Mikolajczak et al. 2011
Liver-specific protein 1 (LISP1)	Unknown	PVM late in development	Late arrest/ partial attenuation	–	–	Ishino et al. 2009
Liver-specific protein 1 (LISP2)	Unknown	PVM late in development/host hepatocyte cytoplasm	Late arrest/ partial attenuation	–	–	Orito et al. 2013; Annoura et al. 2014
Macrophage migration inhibitory factor (MIF)	Unknown	–	–	Decreased size/ partial attenuation	–	Miller et al. 2012

Continued

Table 1. *Continued*

Gene knockout	Function	Temporal/spatial expression	Knockout phenotype				References
			Pb	Py	Pf		
MSP1 (conditional knockout)	Invasion	PPM late in development	Late arrest/ complete attenuation	–	–		Combe et al. 2009
MRP2 (ABCC2)	Unknown	Liver stage	Mid arrest/ complete attenuation	–	Mid-arrest		Rijpmma et al. 2016
P52	Invasion	Sporozoite microneme/PPM during hepatocyte invasion	Early arrest/ partial attenuation	–	Early arrest		van Dijk et al. 2005; Douradinha et al. 2007; van Schaijk et al. 2008
<i>Plasmodium</i> -specific apicoplast protein for liver merozoite formation (PALM)	Unknown	Apicoplast late in development	Late arrest/ partial attenuation	–	–		Haussig et al. 2011
Pyruvate dehydrogenase subunit E1 α (PDH E1 α)	Acetyl CoA synthesis	Apicoplast throughout development	Late arrest/ partial attenuation	Late arrest/ complete attenuation	No sporozoites		Pei et al. 2010; Cobbold et al. 2013; Nagel et al. 2013
Plasmei2	RNA binding	Mid-to-late liver stage cytoplasm	–	Late arrest/ complete attenuation	–		Dankwa et al. 2016
Pyruvate dehydrogenase subunit E3 (PDH E3)	Acetyl CoA synthesis	Apicoplast throughout development	–	Late arrest/ complete attenuation	–		Pei et al. 2010
Sporozoite asparagine-rich protein 1 (SAP1)/sporozoite and liver stage asparagine-rich protein (SLARP)	mRNA stability	Sporozoite cytoplasm	Early arrest/ complete attenuation	Early arrest/ complete attenuation	–		Aly et al. 2008; Silvie et al. 2008
<i>trans</i> -2-Enoyl-acyl carrier protein reductase (Fab I)	Fatty acid synthesis	Apicoplast throughout development	Late arrest/ partial attenuation	–	No sporozoites		Yu et al. 2008; van Schaijk et al. 2014a

Continued

Table 1. *Continued*

Knockout phenotype						
Gene knockout	Function	Temporal/spatial expression	Pb	Py	Pf	References
Up-regulated in infective sporozoites gene 3 (UIS3)	Lipid uptake	Sporozoite microneme then PVM throughout development	Early arrest/complete attenuation	Early arrest/complete attenuation	Gene deletion unsuccessful	Mueller et al. 2005b; Tarun et al. 2007
Up-regulated in infective sporozoites gene 4 (UIS4)	Unknown	Sporozoite microneme then PVM throughout development	Early arrest/partial attenuation	Early arrest/complete attenuation	Gene deletion unsuccessful	Mueller et al. 2005a; Tarun et al. 2007
Zrt-, Irt-like protein domain-containing protein (ZIPCO)	Iron uptake	PPM late in development	Late arrest/partial attenuation	–	–	Sahu et al. 2014
B9/SAP1 (SLARP)	–	–	Early arrest/complete attenuation	–	Early arrest	van Schaijk et al. 2014b
P52/P36	–	–	Early arrest/partial attenuation	Early arrest/partial attenuation	Early arrest/partial attenuation	Labaied et al. 2007; VanBuskirk et al. 2009; Ploemen et al. 2012; Spring et al. 2013
P52/P36/SAP1 (SLARP) UIS3/UIS4	–	–	–	–	Early arrest	Mikolajczak et al. 2014
	–	–	Early arrest/complete attenuation	–	–	Jobe et al. 2007

2040.org). However, *P. falciparum* malaria will not be eradicated globally without an effective preerythrocytic vaccine that will prevent the onset of disease-causing blood stage infection and onward transmission through the mosquito vector. In addition, the elusive *P. vivax* preerythrocytic hypnozoite forms must be targeted by radical cure drugs to ensure full malaria eradication, and thus drug development ought to place more emphasis on finding compounds with activity against liver stages. A robust understanding of hypnozoite biology might well be a prerequisite to develop new drugs that afford radical cure. A further critical research question in this context is whether preerythrocytic vaccination can be used to provide an “immunological radical cure” and as such should be a major aspect of *P. vivax* vaccine research. Overall, we must be continuously guided by the principle that novel and innovative intervention strategies will emerge only from critical research of parasite biology and the immunology of infection and vaccination.

CONCLUDING REMARKS

The past decade has seen an unprecedented progress in research on liver stage malaria. This has brought forth the identification of numerous parasite and host factors that are of critical importance to hepatocyte infection and intra-hepatocytic parasite development. Without doubt, research in the coming years will identify additional important factors. However, the study of parasite–hepatocyte interaction must now enter the next phase, by not only identifying critical host–pathogen interactions but also assembling these interactions into a temporal and spatial cascading network that fully delineates parasite infection and development within hepatocytes at the molecular level, from the point of invasion to the release of merozoites. These studies will be likely performed with rodent malaria models, but the increased maturity of in vitro and in vivo hepatocyte infection models of human malaria parasites gives hope that at least some of this work can be achieved with medically relevant parasite species. This is particularly important for the

study of *P. vivax* hypnozoites in gaining insights into the molecular mechanisms of their latency, their activation, and their interaction with hepatocytes during long periods of persistence.

Finally, identification of parasite molecules that are critical for liver stage development has transcended basic biology in that it enabled the development and use of genetically engineered, attenuated parasite strains for vaccination. It is hoped that the continued biological interrogation of liver stage malaria will carry on to bear fruit for parasite immunology and vaccine development, thereby potentiating its role in helping to develop new tools for the eradication of human malaria infection.

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