Cell Traversal Activity Is Important for *Plasmodium falciparum* Liver Infection in Humanized Mice

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**Article**

http://dx.doi.org/10.1016/j.celrep.2017.03.017

**SUMMARY**

Malaria sporozoites are deposited into the skin by mosquitoes and infect hepatocytes. The molecular basis of how *Plasmodium falciparum* sporozoites migrate through host cells is poorly understood, and direct evidence of its importance in vivo is lacking. Here, we generated traversal-deficient sporozoites by genetic disruption of sporozoite microneme protein essential for cell traversal (PfSPECT) or perforin-like protein 1 (PIPLP1). Loss of either gene did not affect *P. falciparum* growth in erythrocytes, in contrast with a previous report that PIPLP1 is essential for merozoite egress. However, although traversal-deficient sporozoites could invade hepatocytes in vitro, they could not establish normal liver infection in humanized mice. This is in contrast with NF54 sporozoites, which infected the humanized mice and developed into exoerythrocytic forms. This study demonstrates that SPECT and perforin-like protein 1 (PLP1) are critical for transcellular migration by *P. falciparum* sporozoites and demonstrates the importance of cell traversal for liver infection by this human pathogen.

**INTRODUCTION**

*Plasmodium falciparum* is responsible for more than 220 million clinical malaria infections each year and causes severe morbidity and enormous loss of life (WHO, 2015). Malaria parasites are transmitted through the bite of sporozoite-infected female *Anopheles* mosquito. Once injected into the mammalian host, parasites migrate from the inoculation site to the liver, where they infect hepatocytes and develop into exoerythrocytic forms (EEFs). EEFs develop into many thousands of merozoites that exit the liver and infect erythrocytes, initiating clinical disease.

In rodents, migration of *Plasmodium berghei* and *Plasmodium yoelii* sporozoites from the inoculation site in the skin to the liver involves traversal of host cells (Mota et al., 2001). Sporozoites traverse different types of host cells at the dermis, including fibroblasts and phagocytes (Amino et al., 2008), and at the liver capillaries, including Kupffer cells (Frenert et al., 2007) and sinusoidal endothelial cells (Tavares et al., 2013), in order to gain access to hepatocytes. Sporozoites preferentially traverse cells with low-sulfated heparin sulfate proteoglycans (HSPGs) but preferentially invade cells with high-sulfated HSPGs, as is the case on hepatocytes in vitro (Coppi et al., 2007).

Cell traversal was first observed as non-phagocytic entry of *P. berghei* sporozoites into macrophages followed by “escape” from these cells (Vanderberg et al., 1990). The biochemical, biophysical, and stepwise processes of traversal are still unclear, but it has been suggested by electron microscopy that host cell rupture occurs upon entry and exit from the host cell (Mota et al., 2001). The use of cell-impermeant dyes such as propidium iodide (PI) confirmed that wounding of host membranes occurs during traversal (Tavares et al., 2013); however, the temporal delay of PI nuclear labeling made it impossible to distinguish whether wounding occurred during sporozoite entry into the cell, exit, or both. Recently it was shown that *P. yoelii* sporozoites can enter hepatocytes via a transient vacuole and that host membrane rupture occurs upon cell exit rather than cell entry (Risco-Castillo et al., 2015).

Interestingly, sporozoites also traverse hepatocytes before establishing a productive hepatocyte infection (Mota et al., 2001). Several possibilities emerged as to why this occurs. The first suggested that migration through hepatocytes primes parasites for invasion by activating apical exocytosis (Mota et al., 2002). The second suggested that traversal releases hepatocyte growth factor (HGF), making neighboring hepatocytes more

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Interestingly, sporozoites also traverse hepatocytes before establishing a productive hepatocyte infection (Mota et al., 2001). Several possibilities emerged as to why this occurs. The first suggested that migration through hepatocytes primes parasites for invasion by activating apical exocytosis (Mota et al., 2002). The second suggested that traversal releases hepatocyte growth factor (HGF), making neighboring hepatocytes more
susceptible to infection (Carrolo et al., 2003). Lastly, other studies suggest that it takes some time for sporozoites to switch off the machinery for traversal and activate invasion machinery (Amino et al., 2008; Coppi et al., 2007), and that traversal primarily functions to penetrate cell barriers and avoid phagocytosis en route to the liver (Amino et al., 2008; Coppi et al., 2007; Tavares et al., 2013).

Although it has been shown that *P. falciparum* sporozoites traverse human cells (Behet et al., 2014; Cha et al., 2015; Dumoulin et al., 2015; van Schaijk et al., 2008), the molecular basis for this process is largely unstudied. Antibodies induced by chloroquine prophylaxis with sporozoites interfere with cell traversal, and these may also target CSP (Behet et al., 2014). Recently it was shown that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) on the parasite surface interacts with CD68 on Kupffer cells during traversal (Dumoulin et al., 2015), but this is due to inhibition of motility rather than a direct effect (Cha et al., 2015). Furthermore, antibodies induced by chloroquine prophylaxis with sporozoites interfere with cell traversal, and this is due to inhibition of motility rather than a direct effect (Cha et al., 2015).

In rodent malaria parasites such as *P. berghei*, two sporozoite microneme proteins have been identified that are essential for cell traversal (sporozoite microneme protein essential for cell traversal [SPECT; Ishino et al., 2004] and SPECT2 [Ishino et al., 2005], also called perforin-like protein 1 [PLP1] [Kaiser et al., 2004]). Even though genetic disruption of SPECT or SPECT2 rendered sporozoites unable to traverse murine cells, they still invaded hepatocytes in vitro (Ishino et al., 2004, 2005). When injected into rodents, sporozoites lacking SPECT or SPECT2 were impaired for liver infection, but a small number of sporozoites could still establish liver infection that resulted in subsequent latency. However, depletion of Kupffer cells allowed mutants to establish liver infection at levels comparable with wild-type parasites (Ishino et al., 2004, 2005). Thus, traversal by rodent-infecting sporozoites is important for navigating through the sinusoidal layer, but not for hepatocyte invasion, EEF development, or growth within erythrocytes (Ishino et al., 2004, 2005).

Recently, it was shown that the ortholog of SPECT2 in *P. yoelii*, PLP1, is essential for cell traversal. Although this protein is not required for hepatocyte entry, it plays an important role in egress from transient vacuoles during traversal (Risco-Castillo et al., 2015). Thus, sporozoites that infect rodents can traverse host cells by generating a vacuole at the entry step and use a perforin-like protein to escape from this compartment, and perhaps the host cell, during cell exit.

*P. falciparum* is among the most lethal parasites of humans. How sporozoites of this species navigate to the liver to infect hepatocytes in vivo is an important topic of investigation. Here, we define the kinetics and functional relevance of cell traversal by *P. falciparum* sporozoites during infection. We show that sporozoites require SPECT and PLP1 for cell traversal in vitro and that parasites lacking PLP1 cannot establish normal infection of human hepatocytes in humanized mice. This provides direct evidence that host cell traversal is an important feature of *P. falciparum* liver infection.

**RESULTS**

**SPECT and PLP1 Expression in *P. falciparum* Sporozoites**

To study SPECT and PLP1 in *P. falciparum* (Figure S1), we generated antibodies that specifically recognized each protein in sporozoites (Figure S2). To characterize the subcellular localization of PISPCT and PIPLP1 in sporozoites, we performed immunofluorescence microscopy. In non-permeabilized parasites, PISPCT and PIPLP1 labeling was seen in puncta on the surface and apical tip of sporozoites, and the signals partly colocalized with each other (Figure 1A). As expected, PICSP was observed circumferentially around the sporozoite on the surface of non-permeabilized parasites (Figure 1A). In permeabilized sporozoites, punctate labeling was observed within and on the parasite for both PISPCT and PIPLP1, as well as at the apical tip, where the two proteins partly co-localized (Figure 1B).

Thus, traversal by rodent-infecting sporozoites, punctate labeling was observed within and on the parasite for both PISPCT and PIPLP1, as well as at the apical tip, where the two proteins partly co-localized (Figure 1B). The similar overall localization pattern of SPECT and PLP1 to AMA1 suggested the former are also micronemal (see also Figure S2). Because some spots did not colocalize with each other (Figure 1B), it is possible that PISPCT and PIPLP1 are stored in different subsets of micronemes (Matthiesen et al., 2003) and secreted at different times, as described previously for micronemal proteins (Healer et al., 2002). In a subset of permeabilized sporozoites, PISPCT and PIPLP1 labeling had a circumferential pattern, consistent with distribution at the parasite membrane or surface (Figure 1C). Collectively, these results show that SPECT and PLP1 are secreted proteins that localize on the parasite surface, consistent with an interaction with host cells.

A previous study reported that PIPLP1 is expressed in blood stages using an anti-PIPLP1 antibody (Garg et al., 2013). That study showed that PIPLP1 mediates calcium-dependent egress of merozoites from erythrocytes, and that the PIPLP1 gene could not be genetically disrupted and is therefore essential (Garg et al., 2013). We examined whether PISPCT and PIPLP1 are expressed in blood-stage parasites. To this end, we generated NF54 parasites in which the PISPCT and PIPLP1 genes encoded triple hemagglutinin (HA) epitopes in-frame at the C terminus so that expression was still driven by the endogenous promoters (Figure 2A). The genotype of isogenic parasites was assessed by Southern blot and confirmed to be as expected (Figure S3). PISPCT-HA and PIPLP1-HA expression were examined every 4 hr throughout the asexual blood stage by immunoblot using antibodies specific to HA, which are highly specific for this epitope (Boddey et al., 2010). No expression of either protein was detected at any time point in the highly synchronous cultures, in contrast with control asexual parasites ectopically expressing PISPCT-HA or PIPLP1-HA under the control of the chloroquine resistance transporter (crt) promoter (crt-PISPCT-HA and crt-PIPLP1-HA, respectively) from a transfected plasmid as controls (Figure 2B). The artificial
expression of either protein from the crt promoter had no obvious effect on parasite growth (data not shown). Expression of epitope-tagged SPECT-HA was observed in salivary gland sporozoites (Figure S3C), confirming that the isogenic parasites were correctly tagged, as expected based on the Southern blots. Therefore, PfSPECT and PfPLP1 are expressed in sporozoites, but not in blood stages of P. falciparum.

Generation of P. falciparum Parasites Lacking SPECT and PLP1
To study the function of SPECT and PLP1 in P. falciparum, we generated NF54 parasites in which either gene was deleted by allelic exchange using double-crossover homologous recombination (Figure 3A). Two knockout clones of ΔPISPECT (C6 and E5) and ΔPPLP1 (D2 and D6) were successfully produced and confirmed by Southern blot analysis and PCR (Figure S4). The ability to obtain independent knockout parasite clones by targeting both PISPECT and PPLP1 genes demonstrated that neither is essential for asexual blood-stage growth. Nonetheless, the intraerythrocytic asexual growth rate of each knockout clone was evaluated in repeated experiments, but no difference was observed compared with the NF54 parent (Figure 3B). Together with the lack of protein expression in blood stages, these data indicate that neither PISPECT nor PPLP1 is essential during
the *P. falciparum* blood-stage cycle or involved in egress from erythrocytes, in contrast with a previous study reporting that PfPLP1 is essential for calcium-dependent egress of merozoites from erythrocytes (Garg et al., 2013).

**DPfSPECT** and **DPfPLP1** parasite clones were differentiated to gametocytes and fed to female *Anopheles stephensi* mosquitoes to determine whether PfSPECT and PfPLP1 are important in other life cycle stages of *P. falciparum*. No defect was seen in the number of developing gametocytes, midgut oocysts, or salivary gland sporozoites (Figures 3C–3E). Therefore, PfSPECT and PfPLP1 are not required for gametocyte development, ookinete infection of the mosquito midgut, sporozoite development in mosquitoes, or invasion of mosquito salivary glands.

**PfSPECT** and **PfPLP1** Are Essential for Cell Traversal Activity

We examined whether SPECT and PLP1 have functions in sporozoites. First, we assessed the kinetics of cell traversal activity by incubating wild-type NF54 sporozoites isolated from salivary glands with human cells in the presence of fluorescein isothiocyanate (FITC)-dextran and enumerating dextran-positive cells by flow cytometry (Dumoulin et al., 2015; Mota et al., 2001). We observed that NF54 sporozoites readily traversed human HC-04 hepatocytes, as well as mouse Hepa1-6 hepatocytes, although the latter with less efficiency (p = 0.0159; Figure 4A). Traversal of HC-04 hepatocytes could be identified clearly after 30 min of co-culture and increased steadily over the following 2 hr (Figure 4B). Traversal ceased after 2.5 hr, and this corresponded to 20%–30% of HC-04 cells having been traversed in this time (Figure 4B). We next studied whether SPECT or PLP1 was required for cell traversal activity. Incubation of hepatocytes with **DPfSPECT** and **DPfPLP1** sporozoites did not result in dextran uptake into the cells above background levels, illustrating that the mutants were unable to traverse the human cells (Figure 4C).

Subsequently, we investigated whether *P. falciparum* sporozoites could traverse primary human monocyte-derived macrophages (HMDMs) as a surrogate for liver-resident Kupffer cells. Kupffer cells are directly targeted by *P. berghei* and *P. yoelii* sporozoites to gain access to the liver parenchyma in vivo. NF54 sporozoites were incubated with HMDMs for 3 hr, and dextran uptake was clearly observed, indicating the sporozoites readily traversed HMDMs (Figure 4D). However, **DPfPLP1** sporozoites were completely defective for this trait (Figure 4D). Collectively, these experiments demonstrate that genetic inactivation of *Pfspect* and *Pfplp1* genes results in traversal-deficient sporozoites. Therefore, PfSPECT and PfPLP1 play essential roles in cell traversal activity by *P. falciparum*.

**PfSPECT-** and **PfPLP1-Deficient Sporozoites Invade Human Hepatocytes**

To further investigate the function of SPECT and PLP1 in sporozoites, we incubated parasites with hepatocytes and quantified internalization using an invasion assay reported previously, which employs differential surface labeling of extracellular and intracellular parasites (Rénia et al., 1988; Tao et al., 2014; van Schaijk et al., 2008). We quantified intracellular parasites 3 hr post-infection, once traversal by wild-type parasites had ceased (see above). The same number of intracellular NF54, **DPfSPECT**, and **DPfPLP1** sporozoites was observed (Figure 4E).
and ΔPIPLP1 parasites was observed across repeated experiments (p > 0.3410; Figure 5). Therefore, traversal-deficient ΔPISPECT and ΔPIPLP1 parasites can still invade hepatocytes, and SPECT and PLP1 are not essential for this function, at least in vitro.

Cell Traversal Activity Is Important for Liver Infection in Humanized Mice

The relevance of cell traversal activity for in vivo infection by P. falciparum sporozoites has not been definitively proven. Homozygous urokinase plasminogen activator-severe combined...
immunodeficiency (uPA-/-SCID) mice transplanted with primary human hepatocytes to regenerate chimeric human livers (Mercer et al., 2001; Sacci et al., 2006; VanBuskirk et al., 2009) were therefore intravenously injected with $7.0 \times 10^5$ NF54 or traversal-deficient ΔPfPLP1 sporozoites. The parasites were provided a further 6 days to differentiate into EEFs before parasite liver load was measured in each mouse by qPCR. We followed the same protocol published previously, which reported the detection of one infected hepatocyte in 25% of whole liver (Foquet et al., 2013). Consistent with the observation that P. falciparum parasites can traverse human and mouse cells (see Figure 2A), NF54 sporozoites readily infected the livers of humanized mice and developed exponentially into EEFs, similar to parasite levels reported previously (Foquet et al., 2013; Sacci et al., 2006; Vaughan et al., 2012); however, no parasite DNA could be detected in the livers from mice infected with ΔPfPLP1 parasites (Figure 6A). For qualitative assessment, immunofluorescence microscopy was performed on liver sections from the mice using antibodies against heat shock protein 70 (PHSHP70) and exported protein 2 (PIEXP2). The regular presence of between one and four EEFs measuring up to 65 μm in diameter was observed under 1,000× magnification per liver section from mice infected with wild-type parasites (Figure 6B), confirming that they underwent extensive replication in the chimeric human livers, as previously reported (Foquet et al., 2013; Sacci et al., 2006; Vaughan et al., 2012). In contrast, no ΔPfPLP1 parasites could be identified micrographically in the liver sections observed from mice infected with these mutants, in agreement with the qPCR results. All mice had similar levels of human chimerism (Figure 6C), consistent with previously published levels in these mice (Mercer et al., 2001; Sacci et al., 2006), confirming that the lack of ΔPfPLP1 parasites was not specifically due to low engraftment of human cells. Taken together, these experiments demonstrate that cell traversal is an important process that facilitates P. falciparum liver infection in vivo.

**DISCUSSION**

The molecular basis of liver infection by P. falciparum remains understudied and poorly understood. This is due in part to technical challenges associated with genetic manipulation of P. falciparum and a lack of widely accessible in vivo models to study human malaria parasites. This study demonstrates that SPECT and PLP1 are essential for P. falciparum sporozoites to traverse human cells and definitively proves that cell traversal is important for establishing infection of human hepatocytes in humanized mice. This provides a clear basis to characterize the biochemical and biophysical events that occur during the traversal process, because this is currently not well understood for any malaria parasite species.

Recently it was shown that GAPDH is an important sporozoite protein for traversal of Kupffer cells by P. falciparum and P. berghei sporozoites, because it binds the receptor CD68 (Cha et al., 2016). Our study demonstrates that SPECT and PLP1 are required for traversal of both macrophages and hepatocytes. The hepatocyte receptor for traversal has not yet been identified, although Ephrin A2 receptor is involved in formation of the parasitophorous vacuole during sporozoite invasion of hepatocytes (Kaushansky et al., 2015).

Previous studies with P. berghei have shown that SPECT and SPECT2/PLP1 are sporozoite micronemal proteins required for cell traversal and passage through different murine cells in the dermis and liver sinusoids (Amino et al., 2008; Ishino et al., 2004, 2005). Interestingly, the traversal-deficient sporozoites could still infect the murine liver at low levels and displayed a 1-day delay to patency (Ishino et al., 2004, 2005). Our study with traversal-deficient P. falciparum sporozoites also showed a strong defect in liver infection. We did not detect any EEFs, suggesting either that the level of liver infection was low, below the sensitivity of qPCR detection, or that the traversal-deficient sporozoites could not access human hepatocytes in the humanized mice. Although we cannot exclude that this may involve species restriction of receptor-ligand interactions in PLP1-deficient parasites, we note that wild-type NF54 sporozoites infected the chimeric livers of humanized mice successfully and traversed mouse hepatocytes in vitro. We also cannot exclude that traversal-deficient sporozoites are more readily phagocytosed by macrophages in the liver environment. Nonetheless, both possibilities support our conclusions that traversal is an important mechanism for hepatocyte infection by P. falciparum in vivo.

Previously it was reported that PLP1 in P. falciparum is essential for calcium-dependent egress of merozoites from erythrocytes (Garg et al., 2013). We did not detect expression of PLP1 in asexual parasites in our study and were able to successfully delete the Piplp1 locus in independent NF54 clones without affecting asexual blood-stage growth or gametocyte development. This demonstrates that PLP1 is not essential for merozoite egress from erythrocytes in P. falciparum. Our results are in agreement with the lack of expression of PLP1/SPECT2 by...
P. berghei in murine erythrocytes and with PLP1/SPECT2 mutants having normal blood-stage growth in mice (Ishino et al., 2005). The discordant results obtained previously with P. falciparum (Garg et al., 2013) were due, first, to the inability of the previous study to disrupt the PPlp1 locus. This may be because of poor transfection efficiency without sufficient genomic integration controls to correctly interrogate the locus, such as the controls reported previously (Russo et al., 2010). Second, this may be caused by cross-reactive anti-PPlp1 antibodies.

The precise function of SPECT in cell traversal is unknown and requires further investigation. This is also the case for PLP1; however, this protein contains a conserved MAC/perforin-like domain. Similar domains are associated with membrane destabilization and pore formation in other proteins that mediate vacuolar escape and lysis of host cell membranes (Hybsiske and Stephens, 2015; Rosado et al., 2008; Wade and Tweten, 2015). Imaging of sporozoite traversal in the presence of fluorescent dextran or cell-impermeant dyes has previously confirmed that the host cell membrane is indeed compromised (Formaglio et al., 2014; Mota et al., 2001), but the timing of this event, i.e., upon cell entry or exit, is still not fully resolved. Some studies report that lysis occurs upon entry and exit (Formaglio et al., 2014; Mota et al., 2001), whereas others describe host membrane rupture occurring during cell exit (Risco-Castillo et al., 2015). Indeed, many protozoan and bacterial pathogens use MAC/perforin-like proteins after entering cells to escape from membrane compartments such as vacuoles in order to access the cytoplasm (Friedrich et al., 2012; Rosado et al., 2008). This includes the perforin-like protein 2 in Toxoplasma gondii, which is also involved in egress from the host cell (Kafasck et al., 2009). By analogy, it is possible that Plasmodium sporozoites use PLP1 to escape a vacuole and/or the host cell membrane during traversal, but this has not yet been definitively proven.

Recently, an important piece of the puzzle was identified, when it was shown that P. yoelii sporozoites can traverse hepatocytes by generating a transient vacuole upon cell entry (Risco-Castillo et al., 2015). This raises the question as to how sporozoites control membrane dynamics in order to synthesize and then disrupt the vacuole membrane. Initial evidence suggests that sporozoites remodel the protein composition of vacuole membranes differently, such that those produced during productive invasion, i.e., the parasitophorous vacuole, are resistant to lysosomal fusion compared with transient vacuoles that are sensitive to this fusion event. Live imaging studies of PLP1-deficient sporozoites could not detect parasites egressing from vacuoles, suggesting that PLP1 is required for exiting the vacuole (Risco-Castillo et al., 2015). It will be important to determine whether this is also the case in P. falciparum and, if so, how the proteins involved are regulated and how they biophysically mediate membrane lysis. Our attempts to visualize P. falciparum traversal by live imaging have so far been unsuccessful. Clearly, overcoming this challenge will be an important advance that should help to further delineate the mechanisms governing traversal by this pernicious pathogen.

**EXPERIMENTAL PROCEDURES**

**Parasite Maintenance**

P. falciparum NF54 asexual stages were maintained in human type O-positive erythrocytes (Melbourne Red Cross) in RPMI-HEPES supplemented with 10% heat-inactivated human serum (Melbourne Red Cross) at 37°C. Gametocytes for transmission to mosquitoes were generated using the “crash” method (Saliba and Jacobs-Lorena, 2013) with daily media changes.

**Transgenic Parasites**

P. falciparum NF54 was used to generate all transgenic parasites. Primers for amplification of PISPECT (PlasmoDB: PF3D7_1342500) flanks were: forward primer 5′-GAAGTTCTTTGTTATTGTC-3′ and reverse 5′-CTCATATTCTTCTGGAATTAG-3′ (5′ flank); forward primer 5′-GTTAATTACGCTTCTGAAAT-3′ (3′ flank). Primers for amplification of PPlp1 (PlasmoDB: PF3D7_0408700) flanks were: forward primer 5′-CTTTAATGTTTCTGAAAT-3′ (5′ flank); reverse primer 5′-CTTGTTCTTCTGAAAT-3′ (3′ flank). Flanks were cloned into pC1 via SacII/SpeI (5′ flank) and EcoRI/AviRI (3′ flank), generating knockout constructs. Purified plasmid DNA (80 μg) was
transfected into NF54 and selected using 5 mM WR92210 followed by negative selection with 5-fluorocytosine (5-FC) (Duraisingh et al., 2002). Lines were cloned by limiting dilution and genotypes assessed by Southern blot using a digoxigenin kit (DIG; Roche) according to manufacturer’s instructions.

For HA tagging, primers for amplification of the 3’ flank were: forward primer 5’-TGGTTGATGATTTTAGTGATG-3’ and reverse 5’-GATTTAAACATGGCCTATCA-3’ (PfSPECT); forward primer 5’-TAGTTAACGAATGCCGATA-3’ and reverse 5’-ATGCTTTGCAAAAATAATAAA-3’ (PfPLP1). Flanks were cloned into p1.2-SHA (Boddey et al., 2010) via BglII/PstI. Transfection, selection, and confirmation of successful genetic manipulation were as described earlier. Clonal lines were used in all experiments.

**Blood-Stage Growth Assay**

Sorbitol-synchronized ring-stage parasites in erythrocytes were diluted to 0.5% parasitemia, 2% hematocrit (confirmed by fluorescence-activated cell sorting [FACS]; FACSCalibur; BD) using ethidium bromide (10 μg/ml; Bio-Rad) (Sleebs et al., 2014). Final parasitemia was determined 48 hr later by FACS. For each line, triplicate samples of 50,000 cells were counted in each
of the three independent experiments. Growth was expressed as a percentage of the parasitemia achieved by NF54.

Mosquito Infection and Analysis of Parasite Development

Five- to 7-day-old female Anopheles stephensi mosquitoes were fed on asynchronous gametocytes, diluted to 0.3%–0.6% stage V gametocytemia. Mosquitoes were sugar starved for 2 days after blood meal to select for blood-fed mosquitoes. Surviving mosquitoes were provided 5% glucose ad libitum via filter paper wicks or sugar cubes. Oocyst numbers were obtained from midguts dissected from cold-anesthetized and ethanol-killed mosquitoes 7 days post-infection and stained with 0.1% mercuric chloride. Salivary glands were dissected from mosquitoes (days 16–20 after blood meal), crushed using pestle, and then glass wool filtered to obtain sporozoites used in all subsequent assays.

Antibody Generation

For PISCPECT, DNA encoding residues 23–245 of PISCPECT1-6His (lacking the signal peptide) was cloned into a modified tobacco etch virus protease-cleavable pGEX-4T-1 plasmid (GE Healthcare) and expressed in BL21-CodonPlus(DE3)-RIL cells (Stratagene) by isopropyl β-D-thiogalactoside (IPTG) induction at 18 C. PISCPECT-6His was purified by standard techniques including glutathione Sepharose 4B affinity chromatography, tobacco etch virus protease digestion to release the N-terminal glutathione S-transferase (GST)-tag, and a final stage of size exclusion chromatography. Purified protein was injected into mice for a total of four boosts. Serum was collected and m1432 used at 1:1,000 for immunoblots and 1:100 for immunofluorescence assay (IFA). For PIPLP1, affinity-purified rabbit antibodies (at 1 mg/mL) were raised to synthetic peptide fragment EQDDVMDNDQNDKK of PIPLP1 by GenScript and used in immunoblots (1:500) and IFAs (1:100).

Immunofluorescence Assays

Salivary gland sporozoites were either fixed in solution or as spots on slides with 4% paraformaldehyde for 20 min at room temperature (RT). Samples were blocked in 3% BSA (Sigma Aldrich) in PBS for 1 hr before primary antibodies were diluted in 3% BSA/PBS and were used for 1 hr as follows: mouse anti-CSP (1:2,000), mouse anti-SPECT (m1432, 1:100), rabbit anti-PIPLP1 (1:100), and mouse α-PiA1A1 (2H4: 1:100, Yap et al., 2014). Labeling of non-permeabilized sporozoites was performed in solution with centrifugation in between steps, whereas permeabilized samples were treated on slides for 5 min in ice-cold methanol before blocking and antibody labeling as above. After washing samples three times with PBS, secondary Alexa 488 and 594 antibodies (1:1,000; Invitrogen) were incubated with samples for 1 hr before washing three times in PBS, staining with DAPI, air-drying, and being mounted beneath cover glasses with Vectashield (Vector Labs). For imaging EEFs in humanized mice, see later Measuring Exoerythrocytic Development in Humanized Mice section. All micrographs were acquired on a DeltaVision Elite microscope (Applied Precision) using an Olympus 100×/1.42 PlanApoN objective equipped with a CoolSnap HQ2 CCD camera as 2 stacks. Images were deconvolved and are presented as maximum intensity projections, which were processed using Fiji (ImageJ).

Immunoblotting

Proteins solubilized in Laemmli buffer were separated through 10% Bis-Tris SDS-polyacrylamide gels (for sporozoites) and 4%–12% Bis-Tris polyacrylamide gels (for anti-HA time course) (Invitrogen). Proteins were transferred onto nitrocellulose membrane, and blots were blocked in 10% skim milk, 1% BSA, 1% Tween 20 (30 min, RT) without brake, and the peripheral blood mononuclear cell (PBMC)/buffy layer was removed. PBMCs were washed three times in ice-cold PBS at 250 × g (10 min at 4°C). PBMCs were resuspended in RPMI (supplemented with 7.5% autologous heat-inactivated plasma), and monocytes were adsorbed onto six-well tissue culture plates (2 hr, 3°C, 5% CO2). Wells were washed twice in warm RPMI to remove non-adherent cells. Monocytes were differentiated into macrophages over 7 days in Macrophage–SFM ( Gibco) (supplemented with 1% autologous heat-inactivated plasma, 10 ng/mL GM-CSF [PeproTech], 1x penicillin/streptomycin [pen/strep]). Media were replaced twice during this period to remove apoptotic cells and debris.

Cell Traversal Assays

The traversal activity of sporozoites was measured using a standard cell-wounding assay as described previously (Dumoulin et al., 2015). In brief, 5 × 103 HC-04, Hepa1-6, or HMDM cells (Volunteer Blood Donor Registry, Melbourne) were seeded into each well of a 48-well plate (Corning, Sigma-Aldrich) coated with rat tail collagen. The following days, wells were seeded with 5 × 104 sporozoites (MOI 1:1) for the indicated times (generally 2.5 hr unless stated otherwise) in the presence of 1 mg/mL FITC-labeled dextran (10,000 molecular weight [MW]; Sigma-Aldrich). Cells were trypsinized to obtain a single-cell suspension. For each condition, triplicate samples of 10,000 cells were counted by FACS in each of the three independent experiments.

Hepatocyte Invasion Assays

HC-04 cells (5 × 104) were seeded onto rat tail collagen-coated coverslips in 24-well plates using DMEM without glucose (11966-025; Life Technologies), supplemented with 1 mM sodium pyruvate (11360-070; Life Technologies); 1% FBS (35-010-CV; Cellgro); 1× Pen/Strep (30-001-CI; Corning); 1× modified Eagle’s medium (MEM) non-essential amino acids without L-glutamine (M5550; Sigma-Aldrich), and 1:500 dilution of Lipid Mixture 1, Chemically Defined (L-0288; Sigma-Aldrich). Sporozoites (5 × 103) were added to the cells 12 hr later and incubated for 3 hr. Coverslips were fixed in 4% paraformaldehyde for 20 min at RT and then processed as described previously (Flena et al., 1989). Sporozoites were detected by immunofluorescence staining using mouse monoclonal antibodies against CSP (1:2000) and anti-mouse Alexa 488 (1:1,000). Multiple images were taken at 200× magnification (DeltaVision system). A minimum of 270 fields with approximately 10,000 HC-04 cells were counted, and the percentage of cells with intracellular sporozoites was calculated from this dataset. For each condition, duplicate samples were manually counted in each of two independent experiments.

Humanized Mice Production, Infection, and Processing

uPA+/-–SCID mice were housed in a virus- and antigen-free facility supported by the Health Sciences Laboratory Animal Services at the University of Alberta and were cared for in accordance with the Canadian Council on Animal Care guidelines. All experimental protocols involving mice were reviewed and approved by the University of Alberta Health Sciences Animal Welfare Committee, as well as the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. uPA+/-–SCID mice at 5–14 days old received 106 human hepatocytes (cryopreserved human hepatocytes were obtained from a commercial source; Bioreclamation/VT) by intrasplenic injection, and engraftment was confirmed 8 weeks post-transplantation by analysis of serum human albumin (Mercer et al., 2001). Thirteen-week-old humanized mice received 7.0 × 106 P. falciparum NF54 sporozoites (mouse: one female, two male) or JPIPLP1 sporozoites (mouse: two female, two male) freshly dissected from mosquito salivary glands by intravenous tail injection. Livers were obtained 6 days post-infection from CO2-anesthetized mice, and individual lobes were cut as described previously (Foquet et al., 2013). Lobes were either pooled and emulsified into a single-cell suspension and flash-frozen in liquid nitrogen for subsequent genomeic DNA (gDNA) extraction or embedded in Tissue-Tek O.C.T. (Miles Scientific) in an isopentane–liquid nitrogen bath, and 9 μm tissue sections were prepared using a Microm HM 525 Cryostat, mounted onto slides, and stored at ~ 80°C for future use.
Measuring Exoerythrocytic Development in Humanized Mice

To sensitively quantify parasite load in the chimeric livers, we isolated gDNA from the single-cell liver suspensions, and TaqMan probe-based qPCRs were performed as previously described (Foquet et al., 2013). Oligonucleotides specific for Pf18S (containing 6-carboxy-fluorescein [FAM] as a reporter and 6-carboxy-tetramethylrhodamine [TAMRA] as a quencher) and mouse and human Prostaglandin E receptor 2 (PTGER2) (containing FAM and Cy5 probes enabling us to quantify copy number of mouse and human PTGER2 in duplex) were used to quantify genome copy numbers of parasite, human, and mouse genomes in liver samples using known DNA standards for each species to derive control standard curves.

To qualitatively measure in vivo P. falciparum infections micrographically, we thawed liver sections from ~80°C storage, fixed them in ice-cold methanol for 10 min, blocked them in 3% BSA in PBS for 30 min at RT, and probed with mouse anti-HSP70 (1:500) and rabbit anti-EXP2 antibodies (1:400) at 37°C in a humidity chamber for 30 min before three washes in PBS and labeling with Alexa 488- and 594-conjugated secondary antibodies (1:1,000; Thermo Fisher Scientific) and a further three PBS washes. Samples were treated with DAPI solubilized in Vectashield (Vector Labs), sealed with coverslips, and micrographs were captured on a DeltaVision Elite microscope as described earlier.

Statistics

The sample sizes for studies with very limited humanized mice (n = 7) make testing for statistical significance difficult, although the effects between wild-type and ΔPPlp1 parasites were extreme. All statistical comparisons of means between groups in this study were compared using the Mann-Whitney test with Prism 6.

Ethics Statement

All experimental protocols involving mice were conducted in strict accordance with the recommendations in the National Statement on Ethical Conduct in Animal Research of the National Health and Medical Research Council and were reviewed and approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee (AEC2014.030). Human blood collected for this study from the Volunteer Blood Donor Registry, Melbourne was approved by the Walter and Eliza Hall Institute of Medical Research Human Research Ethics Committee (HREC15/16), and all donors provided written informed consent for the use of human blood obtained for this study.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.017.

AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

We thank the Melbourne Red Cross for human erythrocytes, Marcello Jacobs-Lorena for the Johns Hopkins University strain of Anopheles stephensi, the US Naval Medical Research Center for HC-04 cells, Ian Cockburn for Hepa1-6 cells, and Fidel Zavala, Alan Cowman, and Jana McBride for CSP, AMA1, and EXP2 antibodies, respectively. We sincerely thank Jelena Levitskaya, Stephanie Trop, Peter Dumoulin, Jinxia Ma, John Sacci, Jr., Julie Healer, and Melissa Hobbs for sharing technical expertise or providing valuable technical assistance, and Alan Cowman and Marc Pellegrini for stimulating discussions. This work was supported by the National Health and Medical Research Council of Australia (Project Grant 1049811), Human Frontiers Science Program (Young Investigator Grant RGY0073/2012), Ramaciotti Foundation (Establishment Grant 3197/2010), and Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS. A.S.P.Y. was supported by an Australian Postgraduate Award, and J.A.B. was supported by an Australian Research Council Queen Elizabeth II Fellowship (DP111015395). The funders had no role in study design or the decision to publish.

Received: September 10, 2016

Revised: February 6, 2017

Accepted: March 2, 2017

Published: March 28, 2017

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