Role of host cell traversal by the malaria sporozoite during liver infection

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Malaria infection starts when the sporozoite stage of the Plasmodium parasite is injected into the skin by a mosquito. Sporozoites are known to traverse host cells before finally invading a hepatocyte and multiplying into erythrocyte-infecting forms, but how sporozoites reach hepatocytes in the liver and the role of host cell traversal (CT) remain unclear. We report the first quantitative imaging study of sporozoite liver infection in rodents. We show that sporozoites can cross the liver sinusoidal barrier by multiple mechanisms, targeting Kupffer cells (KC) or endothelial cells and associated or not with the parasite CT activity. We also show that the primary role of CT is to inhibit sporozoite clearance by KC during locomotion inside the sinusoid lumen, before crossing the barrier. By being involved in multiple steps of the sporozoite journey from the skin to the final hepatocyte, the parasite proteins mediating host CT emerge as ideal antibody targets for vaccination against the parasite.
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This appeared to strengthen the gateway model, suggesting that sporozoites crossed the sinusoidal barrier by KC traversal (Yuda and Ishino, 2004). Here, we investigate both the way sporozoites cross the sinusoidal barrier and the role of its CT capacity in the liver by intravital imaging of wild-type and CT-deficient sporozoites in rodents.

**RESULTS**

**KCs are not mandatory for sporozoite crossing the liver sinusoidal barrier**

To investigate the interactions of Plasmodium sporozoites with KCs and ECs in the liver sinusoids, the three cell types were differentially labeled and their dynamic interplay was examined in the liver of mice using intravital laser spinning-disk confocal microscopy. We used *P. berghei* sporozoites constitutively expressing RedStar fluorescent protein (RFP+; Sturm et al., 2009). ECs were visualized using *flk1*-gfp transgenic C57BL/6 mice (Xu et al., 2010), which express GFP in ECs including in the liver sinusoids. The fluorescence of the thin EC cytoplasmic processes sharply delineated the sinusoidal lumen, thus permitting us to define the exact sites and moments of sporozoite crossing (Fig. 1). KCs were labeled using Alexa Fluor 647–conjugated anti-F4/80 monoclonal antibody injected intravenously in the host cell (Mota et al., 2001). This cell traversal (CT) behavior was first observed with peritoneal macrophages (Vanderberg et al., 1990) and later with various other cell types, including hepatocytes (Mota et al., 2001; Amino et al., 2008). Work on sporozoite CT, also using *P. berghei*, has focused on sporozoite–hepatocyte interactions and has reported several roles of hepatocyte traversal. Hepatocyte traversal was found to render sporozoites competent for the final invasion event (Mota et al., 2002) and to facilitate parasite intravacuolar development via the activity of hepatocyte growth factor (HGF) released from traversed hepatocytes (Carrolo et al., 2003). Conversely, traversal of hepatocytes was also reported to limit the development of intracellular parasites by the expression of inducible NO synthase via MyD88-mediated NF-κB activation (Torgler et al., 2008). These hypotheses, however, are questioned by the phenotype of sporozoite mutants deficient in CT bearing null mutations in *SPECT* and *SPECT2*, which retain normal capacity to invade and develop inside hepatocytes in vitro (Ishino et al., 2004, 2005). In vivo, these mutant sporozoites were found to be poorly infective but to recover normal infectivity after KC depletion (Ishino et al., 2004, 2005).

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occurred at sites overlapping or closely adjacent to a F4/80-positive area, noted as KC+ events. At least $\geq 32\%$ (19/60) of the sporozoite crossing events occurred in F4/80-negative areas, i.e., without interaction with KCs at the crossing site, KC− (Fig. 1 C). We also observed that sporozoites of *P. yoelii*, another rodent-infective *Plasmodium* species, were able to cross the sinusoidal barrier in a zone lacking KCs (unpublished data). This indicated that *Plasmodium* sporozoites might cross the barrier not only through KCs but also through ECs.

**Sporozoite crossing by EC traversal**

When we analyzed the GFP+ ECs matching the site of sporozoite crossing during events in the KC− group, we frequently observed a rapid, specific, and significant decrease in fluorescence intensity, a phenotype we called EC fading (ECfad+), which occurred during or just before the complete passage of the sporozoite through the EC (Fig. 2 A). To determine whether the ECfad+ phenotype resulted from sporozoite traversal of ECs, we characterized the phenotype in vitro using flk1-gfp mouse 30 min before sporozoite injection (Fig. 1 A, left). The F4/80 specificity was confirmed in vivo by depleting KCs with clodronate (Van Rooijen and Sanders, 1994), which completely abolished KC staining in the sinusoids (Fig. 1 A, middle). F4/80 labeling also colocalized with fluorescent beads taken up by phagocytic cells (Fig. 1 A, right) and with weakly GFP+ myelomonocytic cells in the liver of lys-gfp transgenic mice (Fig. 1 B). Importantly, neither the anti-F4/80 antibody nor GFP expression in ECs impaired *P. berghei* sporozoite infectivity (not depicted), and thus presumably did not alter sporozoite, EC, or KC behaviors in vivo.

After intravenous injection of $\sim 3 \times 10^5$ RFP+ sporozoites in F4/80-labeled flk1-gfp mice, individual sporozoites were imaged in the left liver lobe in a volume of 125 × 125 × 40 µm³ (8–10 confocal Z-stacks) until a crossing event was observed, i.e., a sporozoite (pseudo-colored in green) translocating from the sinusoidal lumen delineated by the GFP fluorescence (pseudo-colored in white) into the black parenchyma (Fig. 1 C). 60 such crossing events were recorded, of which 68% (41/60) occurred at sites overlapping or closely adjacent to a F4/80-positive area, noted as KC+ events. At least $\sim 32\%$ (19/60) of the sporozoite crossing events occurred in F4/80-negative areas, i.e., without interaction with KCs at the crossing site, KC− (Fig. 1 C). We also observed that sporozoites of *P. yoelii*, another rodent-infective *Plasmodium* species, were able to cross the sinusoidal barrier in a zone lacking KCs (unpublished data). This indicated that *Plasmodium* sporozoites might cross the barrier not only through KCs but also through ECs.

**Figure 2.** Crossing the liver sinusoidal barrier by EC traversal. (A–C) Time-lapse intravital confocal microscopy of RFP+ sporozoites (green) crossing the liver sinusoidal barrier through (white) ECs (yellow arrowheads) in *flk1-gfp* mice harboring (red) F4/80-labeled KCs. The white arrowheads indicate the decrease of EC-GFP fluorescence after sporozoite crossing. Images (left) are Z-projections of three contiguous pictures separated by 5 µm. Asterisks indicate the anterior poles of sporozoites. Bars, 10 µm. Graphs (right) show the quantification of normalized GFP mean intensity of ECs at the crossing site (white triangles) and in at least seven ECs from the same field and focal plane that did not interact with the parasite (green triangles, mean ± SD). The red lines represent the lower tolerance limit for 95% of the population. Dark triangles, black bars, and letters [a–d] represent the respective time-lapse images. (D and E) Quantification of crossing events by sporozoites injected via intravenous injection (D) or mosquito bite (E), classified by KC association and EC fading phenotypes. Sporozoites were delivered by intravenous injection (A, B, and D; n = 60 events from the same 25 independent experiments analyzed in Fig. 1 C) or mosquito bite (C and E; n = 8 from 15 independent experiments).
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Dynamic microscopy. SPECT2 is a parasite protein that contains a perforin-like domain and is essential for the sporozoite CT activity (Ishino et al., 2005). Although control sporozoites caused the fading of up to 30% of ECs, with a linear and direct relationship between the frequency of faded ECs and the ratio of sporozoite to ECs (Fig. 3 C, yellow arrowheads and graph), the GFP+ SPECT2− sporozoites were unable to induce any detectable EC fading in the same conditions (Fig. 3 C). We conclude that the ECfad+ phenotype is a consequence of the sporozoite membrane wounding ability and that, in vivo, the ECfad+ phenotype is a signature of sporozoite traversal of ECs.

Intravital imaging revealed that 53% of the sporozoite crossing events (32/60) were ECfad+, i.e., ultimately depended on EC traversal. Of these, 16.7% were ECfad+ KC− (Fig. 2, A and D), corresponding to crossing events by direct EC traversal, whereas 36.6% were ECfad+ KC+ (Fig. 2, B and D), corresponding to crossing events by EC traversal after interactions with a KC. Finally, because prior observations were made after intravenous injection of sporozoites, primary liver sinusoid flk1-gfp (GFP+) ECs and RFP+ sporozoites by dynamic microscopy. When a primary liver sinusoidal EC monolayer was incubated with sporozoites, a specific fading of EC fluorescence was frequently observed, triggered by sporozoite contact with, and presumably traversal of, ECs (Fig. 3 A), similar to the ECfad+ phenotype observed in vivo. To test whether the ECfad+ phenotype correlated with a loss of host cell membrane integrity and GFP leakage, we imaged the interactions in the presence of PI, a membrane-impermeant molecule that only highly fluoresces after entry into cells and binding to nucleic acids. As shown in Fig. 3 B, the EC fading induced by sporozoites was concomitant with the increase in PI fluorescence in the EC nucleus. This suggested that sporozoites were breaching the EC plasma membrane, allowing the simultaneous entry of PI into and leakage of GFP from, traversed ECs. To further confirm this, the EC monolayer was incubated with CT-deficient GFP+ SPECT2− sporozoites (Amino et al., 2008) or control GFP+ sporozoites (Ishino et al., 2006), and sporozoite–EC interactions were imaged over 90 min by dynamic microscopy. SPECT2 is a parasite protein that contains a perforin-like domain and is essential for the sporozoite CT activity (Ishino et al., 2005). Although control sporozoites caused the fading of up to 30% of ECs, with a linear and direct relationship between the frequency of faded ECs and the ratio of sporozoite to ECs (Fig. 3 C, yellow arrowheads and graph), the GFP+ SPECT2− sporozoites were unable to induce any detectable EC fading in the same conditions (Fig. 3 C). We conclude that the ECfad+ phenotype is a consequence of the sporozoite membrane wounding ability and that, in vivo, the ECfad+ phenotype is a signature of sporozoite traversal of ECs.

Intravital imaging revealed that ~53% of the sporozoite crossing events (32/60) were ECfad+, i.e., ultimately depended on EC traversal. Of these, 16.7% were ECfad+ KC− (Fig. 2, A and D), corresponding to crossing events by direct EC traversal, whereas 36.6% were ECfad+ KC+ (Fig. 2, B and D), corresponding to crossing events by EC traversal after interactions with a KC. Finally, because prior observations were made after intravenous injection of sporozoites,
To monitor KCs during such ECfad− KC+ crossing events, we established a PI wounding assay (Tavares et al., 2013) for in vivo detection of KC wounding. We first validated the use of PI as a marker of cell wounding in vivo using ECs as target cells. PI was injected subcutaneously in a flk1-gfp mouse, RFP+ sporozoites and anti-F4/80 antibody were injected intravenously, and the proportions of crossing events causing the ECfad+ phenotype and the incorporation of PI in the EC nucleus, called ECPI+ phenotype, were compared. As exemplified in Fig. 4 A, most ECfad+ events were also ECPI+ (16/17), which validated PI incorporation as a marker of cell wounding in vivo. We then used the PI intravital assay to quantify the proportion of ECfad− KC+ crossing events that wounded KCs and caused PI incorporation in its nucleus, noted as KCPI+ phenotype. The majority (~75%) of the ECfad− KC+ events were associated with a KCPI+ phenotype (Fig. 4, B and D). These ECfad− KC+ KCPI+ events were also ECPI− (Fig. 4 B), confirming the absence of EC traversal. These events were thus compatible with sporozoites crossing the barrier by KC traversal.

Figure 4. KC-related crossing of the liver sinusoidal barrier. (A–C) RFP+ sporozoites were intravenously injected in flk1-gfp mice labeled with anti-F4/80 antibody and imaged in liver sinusoids in the presence of PI in 20 independent experiments. (A) Time lapse of a sporozoite (green) crossing the barrier (yellow arrowhead) through EC traversal, with simultaneous EC fading (white arrowheads) and PI incorporation (green arrowheads, ECPI+). The graph shows the simultaneous loss of the normalized GFP+ EC fluorescence intensity and the increase of the PI fluorescence intensity in the EC nucleus. Dark triangles and letters (a–d) represent the respective time-lapse images in the graph. (B) Time lapse of a sporozoite (green) crossing the barrier through KC traversal (yellow arrowheads) visualized by the nuclear PI incorporation in the traversed KC (green arrowheads, KCPI+). (C) Time lapse of a KC+ crossing event (yellow arrowheads) without PI incorporation in the KC (green arrowheads, KCPI−). In B and C, white arrowheads indicate EC lining the site of crossing that did not fade or become PI+ during crossing. Images are maximal Z-projections of three contiguous planes separated by 5 µm. T-projections are indicated at the panel bottom (white time interval). Asterisks mark the anterior pole of sporozoites. Bars, 10 µm. (D) Quantification of the crossing events through KC traversal, represented by the phenotype KCPI+, ECfad−/ECPI− (green bar).
CT-independent sporozoite crossing

The remainder (~25%) of the ECfad− KC+ events, representing ~8% of the total crossing events, did not cause PI incorporation in the contacted KC, KCPI− (Fig. 4, C and D), or in ECs (ECPI−). These crossing events are compatible with the invasion-transcytosis variant of the gateway model but could also correspond to parasites crossing the barrier between adjacent KCs and ECs.

Finally, ~15% of the total crossing events were associated with an ECfad− KC− phenotype (Fig. 5 A), indicating sporozoites crossing at a distance of KC without traversing an EC. This phenotype is compatible with a paracellular pathway, i.e., between two ECs, or with a transcellular pathway, i.e., through ECs but without loss of membrane integrity. The existence of CT-independent crossing paths is in agreement with the findings that CT-deficient sporozoites, e.g., SPECT−, SPECT2−, and CelTOS−, which are all defective in CT activity (Ishino et al., 2004; 2005; Kariu et al., 2006), can all induce a blood infection, albeit with reduced efficiency. To further investigate CT-independent crossing, we imaged GFP+ SPECT2− sporozoites in KC-depleted mice, in which SPECT2− sporozoites recover normal infectivity after intravenous injection (Ishino et al., 2005; Fig. 5 B). GFP+ SPECT2− or GFP+ control sporozoites were injected intravenously into mice and the numbers of sporozoites present in the liver parenchyma 2 h after injection were compared by intravital imaging (Fig. 5 C). Similar numbers of control and SPECT2− sporozoites had reached the parenchyma 2 h after injection, confirming that the CT activity is not essential for sporozoite crossing the sinusoidal barrier and that CT-independent crossing mechanisms are operational in sporozoites. We also imaged crossing events by GFP+ SPECT2− sporozoites in KC-depleted (Fig. 5, D and E) as well as KC-harboring (lys-gfp, not depicted) mice, which revealed sporozoites translocating into the parenchyma 2 h after injection.

Figure 5. CT-independent crossing of the liver sinusoidal barrier. (A) Time-lapse intravital confocal microscopy of sporozoites (green) crossing the barrier via (white) ECs in a flk1-gfp mouse harboring (red) F4/80-labeled KCs. White and blue arrowheads show the EC+ GFP fluorescence after sporozoite crossing (yellow arrowheads). Red arrowheads show a PI positive KC. Images are Z-projections of three contiguous pictures separated by 5 µm. Bar, 10 µm. The graph shows the quantification of normalized GFP mean intensity of the two ECs in the vicinity of the crossing site (white and blue triangles) and in seven ECs from the same field and focal plane that did not interact with the parasite (green triangles, mean ± SD). The red line represents the lower tolerance limit of 95% of the population. (B) SPECT2− sporozoite infectivity in C57BL/6 mice after depletion of KCs by intravenous injection of clodronate liposomes 2 or 5 d before infection. Control mice received PBS. Groups of mice (n = 4) were infected intravenously with 2 × 10^4 GFP+ control or GFP+ SPECT2− sporozoites and parasitemia was quantified by FACS analysis at day 5 after infection. A representative experiment from three independent experiments is shown. The bars and error bars represent the mean parasitemia and one SD, respectively. (C) Percentage of GFP− control and GFP− SPECT2− sporozoites in the liver parenchyma and sinusoidal lumen. 2 h after intravenous injection of parasites in C57BL/6 mice depleted of phagocytic cells, sporozoites were counted by intravital confocal microscopy in 30 fields of 125 × 125 × 40 µm^3 each. (D and E) Time lapses representative of CT-deficient sporozoites (green, GFP− SPECT2−) crossing the liver sinusoidal barrier (red, Alexa Fluor 555-BSA) of C57BL/6 mice in which phagocytic cells were depleted by clodronate treatment. Images are maximal Z-projections of three contiguous planes separated by 5 µm. Asterisks indicate the anterior pole of sporozoites and yellow and white arrowheads indicate the sporozoite crossing site and the dynamics of BSA+ endocytic vesicles, respectively. The area delineated by dotted lines is the lumen of the sinusoid. Bars, 10 µm.
apparently through ECs characterized by the intracellular movement of endocytic vesicles containing BSA. However, in the absence of appropriate intercellular junction markers, we could not discriminate between paracellular and transcellular routes.

**Sporozoites gliding in the liver sinusoids wound and kill KCs**

Using the intravital PI assay, we imaged F4/80-labeled KCs interacting with RFP⁺ sporozoites gliding inside sinusoids of *flk1-gfp* mice. First, we evaluated the percentage of KC wounding, indicated by the appearance of a KCPI⁺ phenotype after a transient interaction with a gliding sporozoite. Transient interactions were defined by the superposition of KCs and sporozoite fluorescent signals or by KC extensions surrounding the parasite for at least 2 min (Fig. 6, A and B). The majority (~60%) of such short-lived interactions caused a KCPI⁺ phenotype (Fig. 6, A and C). The 40% of KCs that remained PI⁻ were indeed not wounded rather than not stained as a result of low PI concentration because other cells were frequently observed becoming PI⁺ in the same microscopic field (Fig. 6 B). When CT-deficient GFP⁺ SPECT2⁻ sporozoites were imaged in the same conditions, PI incorporation into the nuclei of interacting KCs was not observed (Fig. 6 D). This showed that while gliding in the sinusoids, and irrespective of barrier crossing, sporozoites transiently interact with, and frequently wound KCs.

We then evaluated the fate of KCs traversed by sporozoites using a double wounding assay. To detect KC traversal, we injected GFP⁺ sporozoites and PI in F4/80-labeled C57BL/6 mice and scored sporozoite–KC interactions that resulted in the KCPI⁺ phenotype. After 1 h, we injected SYTOX green and assessed its incorporation in the nuclei of traversed KCs (KCI⁺, Fig. 6 E). SYTOX is a fluorescent dye frequently used to detect dead cells; it does not penetrate live cells and its fluorescence increases upon nucleic acid binding. Of the nine traversed KCs identified by PI incorporation, seven incorporated SYTOX-Green after 1 h (n = 9 from five independent experiments). Bars, 10 µm.
CT prevents sporozoite clearance by KCs

The ~2.5-fold increase in parasitemia that was reproducibly obtained after injection of sporozoites into clodronate-treated compared with untreated mice (Fig. 5 B, P < 0.05) suggested a negative impact of KCs on parasite infectivity. Because KCs play an important role in removing microorganisms from the blood circulation (Helmy et al., 2006), we tested whether sporozoite CT activity could play a role in resisting clearance by KCs.

For this, we analyzed the fate of CT-deficient GFP+ SPECT2− sporozoites interacting with KCs. GFP+ SPECT2− and GFP+ control sporozoites were injected intravenously into C57BL/6 mice harboring F4/80-labeled KCs and imaged in the liver for 150 min. After this time, most control sporozoites had left the microscopic field (~48%) or invaded the parenchyma (~45%). In contrast, only ~14 and 3% of the GFP+ SPECT2− sporozoites had left the observation field and invaded the parenchyma, respectively (Fig. 7 A). As expected, most (~83%) of the CT-deficient sporozoites were trapped in lasting interactions with KCs; importantly, these interactions started from the beginning of the imaging period (Fig. 7 B). In contrast, only ~10% of control sporozoites displayed similar lasting interactions with KCs, which only started to occur ~30 min after injection, possibly involving sporozoites that had exhausted their CT capacity (Fig. 7, A and B). For both populations of early KC interactions with SPECT2− and late KC interactions with control sporozoites, sporozoite fluorescence gradually decreased with time until undetectable levels (Fig. 7, C and D). Therefore sporozoite CT helps evading clearance by KCs.

Lastly, we compared the fate of GFP+ SPECT2− and GFP+ control sporozoites inside primary KCs in vitro. Primary KCs were purified, incubated with control or SPECT2− sporozoites for 1 h, and samples were stained using a double labeling assay to discriminate internalized and extracellular zoites (Fig. 7, E and F). More than 90% of the SPECT2− and ~30% of control sporozoites were located inside cells (P < 0.0001, Fig. 7 E). Samples were also stained with antibodies to the lysosome marker LAMP1 (Fig. 7, F and G). Almost all intracellular SPECT2− sporozoites colocalized with LAMP1 staining, suggesting they were inside phagolysosomes, and frequently displayed dim green fluorescence. In contrast, only half of control, internalized sporozoites were associated with LAMP1 staining, confirming that WT sporozoites can also be phagocytosed by KCs. Presumably, the other half corresponds to traversing sporozoites or sporozoites internalized inside a parasitophorous vacuole. We conclude that sporozoite CT confers resistance to phagocytosis by KCs and likely other phagocytic cells.

DISCUSSION

This study provides the first comprehensive picture of how malaria sporozoites infect the liver and reach their final destination inside a hepatocyte. Our spinning-disk confocal imaging approach using flk1-gfp mouse harboring F4/80-labeled KC allowed a clear delineation of the sinusoids and a precise definition of the sporozoite crossing sites. It demonstrated that KCs do not constitute a mandatory gateway for Plasmodium sporozoites and revealed the multiplicity of P. berghei sporozoite...
crossing mechanisms. Most crossing events (~77%, n = 60) were associated with CT activity. Of these, the majority occurred through ECs (53%), sometimes also involving a KC presumably double lining the traversed EC, whereas some CT events also occurred specifically through KCs (~24%). Approximately a quarter (23%) of the crossing events were CT-independent, presumably accounting for the residual capacity of CT-deficient mutants to cross normal sinusoidal barriers (Ishino et al., 2004, 2005; Kariu et al., 2006). These CT-independent crossing events might correspond to a paracellular pathway, between two ECs or between an EC and a KC, as has been suggested for Toxoplasma tachyzoite transmigration of cultured cell monolayers (Barragan et al., 2005). Alternatively, sporozoites could use a transcellular pathway through the formation of channels inside ECs, like those generated by extravasating leukocytes (Carman and Springer, 2004), or use and manipulate the fenestrations of liver sinusoidal ECs (Warren et al., 2007).

Another unresolved question was the role of the CT activity of the sporozoite during liver infection. Much emphasis has been put on the positive impact of hepatocyte traversal on parasite infection via the release of HGF from traversed hepatocytes, which was proposed to act on neighboring invaded cells and to facilitate parasite development (Mota and Rodriguez, 2004). Conversely, it was also proposed that WT, but not CT-deficient sporozoites, induced the expression of iNOS via activation of NF-kB, causing an approximately twofold decrease in parasite development inside hepatocytes (Torgler et al., 2008). In any event, these opposed effects in artificial in vitro conditions were minor and cannot account for the >20-fold and >100-fold reduction in the in vivo infectivity of P. berghei CT-deficient mutants after intravenous and subcutaneous infection, respectively (Ishino et al., 2004, 2005; Kariu et al., 2006; Amino et al., 2008). Therefore, the primary role of CT in the liver appears to be unrelated to hepatocyte traversal.

Here, we imaged a dual function of sporozoite CT in the liver. First, WT sporozoites wounded and, presumably, traversed KCs while gliding in the sinusoids, whereas the vast majority of the CT-deficient sporozoites were rapidly trapped and subsequently degraded inside KCs. This vital function of CT in avoiding parasite clearance is also likely to help the sporozoite resist attacks from other phagocytic cells than KCs, as visualized for granulocytes in hpz-gfp mice (unpublished data). Second, CT was associated with ~80% of the crossing events through ECs and/or KCs. However, the contribution to sporozoite infectivity of CT-dependent crossing of the sinusoidal barrier is more difficult to assess. CT-deficient sporozoites reached the parenchyma as efficiently as controls in the absence of KCs, showing that CT-independent mechanisms are sufficient to allow efficient barrier crossing. Nonetheless, in the presence of KCs the availability of CT-dependent crossing mechanisms might offer an advantage by reducing the time spent in the sinusoids and decreasing the risk of clearance by phagocytic cells. We also reported previously an important role of sporozoite CT in the skin after sporozoite deposition during natural mosquito transmission, in allowing normal sporozoite progression through the dermis and access to skin vessels (Amino et al., 2008). Together, these intravital imaging data suggest a dual role of the CT activity of the sporozoite, in allowing progression through cellular barriers at multiple steps of the sporozoite journey from the skin to the liver parenchyma and escape from host cell phagocytic activity along the journey. In comparison, hepatocyte traversal appears to add little to parasite infectivity. That sporozoites first traverse several hepatocytes before invading the final one might simply result from the time necessary to repress the CT activity, which would otherwise rupture the parasitophorous vacuole membrane and prevent successful invasion.

Sporozoite CT might have additional effects, for example on adaptive immunity. Indeed sporozoites traverse hepatocytes, KCs, and ECs, which can efficiently present antigens (Ebrahimkhani et al., 2011). The shedding of parasite surface antigens in these traversed cells might induce host tolerance or immunity to the corresponding antigens, possibly presented directly by traversed cells if they recover from CT, or via other antigen-presenting cells if they die. Our data indicate that most KC wounding/traversal events result in permanent alteration of KC plasma membrane integrity, and thus likely in KC death, which might affect mounting of a protective immune response. The repeated KC traversal events during the extensive sporozoite gliding in the liver sinusoids might also liberate antiinflammatory mediators such as IL-10 and TGF-β from traversed KCs (Klotz and Frevert, 2008), which might negatively impact antiparasite immunity.

The notion that the CT activity is important to the sporozoite at many steps of its progression from the skin to the final hepatocyte makes the proteins involved in the process attractive targets for antibody-mediated inhibition strategies. High levels of antibodies against SPECT2 correlate with protection from malaria episodes caused by P. falciparum in children (Crompton et al., 2010). Moreover, immunization using CelTOS, another important protein involved in CT, protects rodents from challenge with sporozoites by a combination of antibody and cell-mediated effector responses (Bergmann-Leitner et al., 2011). Many parasite proteins have been described as being involved in the CT phenotype, e.g., SPECT (Ishino et al., 2004), PL (Bhanot et al., 2005), TLP (Moreira et al., 2008), and GEST (Talman et al., 2011), in addition to CelTOS (Kariu et al., 2006) and the perforin-like SPECT2 (Ishino et al., 2005). Along with antibodies to the CS protein, which inhibit sporozoite motility in the dermis (Vanderberg and Frevert, 2004) and presumably elsewhere, antibodies targeting the CT-associated factors would likely add in preventing sporozoites from reaching their final destination in the liver.

MATERIALS AND METHODS

Parasites, mice, and mosquitoes. We used P. berghei ANKA clones expressing the GFP under the control of the hpz regulatory regions (GFP-Control; Ishino et al., 2006), the RFP under the control of elf1-α regulatory regions (line L733; Sturm et al., 2009), and the CT-deficient parasite SPECT2−, expressing the GFP under the control of the hpz regulatory regions (GFP-SPECT2−; Amino et al., 2008).

C57BL/6 and Swiss mice were purchased from Elevage Janvier. FRET-gfp C57BL/6 transgenic mice (Xu et al., 2010) were maintained by breeding heterozygous males with C57BL/6 females. All animal experiments were approved.
by the Animal Care and Use committee of Institut Pasteur and were performed in accordance with European guidelines and regulations.

Anopheles stephensi mosquitoes (SaI/900 strain) were reared in the Centre for Production and Infection of Anopheles (CEPIA) at the Pasteur Institute using standard procedures. Mosquitoes were fed on infected mice 1–2 d after emergence and kept as previously described (Amino et al., 2007). Infected mosquitoes used for transmission experiments (18–25 d after the infectious blood meal) were deprived of sucrose for 1 d before experimentation to enhance the rate of mosquito bite. For intravenous injections and for in vitro experiments, sporozoites were isolated from infected salivary glands 18–25 d after the infectious blood meal.

Spinning disk confocal intravital microscopy and image analysis. Mice were anesthetized with a mixture of ketamine and xylazine (ketamine: 125 mg/kg body weight; xylazine: 12.5 mg/kg body weight). Animals were prepared for intravital microscopy of the liver as described previously (Thiberge et al., 2007). In brief, once deeply anesthetized, a small left-ventral abdominal incision was made to expose the left liver lobe. The exposed lobe was moistened with a drop of phosphate buffer saline and immobilized on a coverslip with cyanoacrylate glue. The animal was placed on a motorized stage of an inverted Axiovert 200 microscope (Carl Zeiss) and covered with a heating blanket at 37°C. Images were acquired in the Imaging Platform at the Pasteur Institute (PFID) using a spinning disk confocal system (UltraView; Perkin Elmer). Usually continuous images were acquired using an LCI Plan-Neofluar 25×/0.8 Imm Corr DIC objective (Carl Zeiss) for up to 10 min and covered with a heating blanket at 37°C, 5% CO2. Imaging wavelengths: 405, 488, 561, and 647 nm) controlled by Volocity (Perkin Elmer). In brief, once deeply anesthetized, a small left-ventral abdominal incision was made to expose the left liver lobe. The exposed lobe was moistened with a drop of phosphate buffer saline and immobilized on a coverslip with cyanoacrylate glue. The animal was placed on a motorized stage of an inverted Axiovert 200 microscope (Carl Zeiss) and covered with a heating blanket at 37°C. Images were acquired in the Imaging Platform at the Pasteur Institute (PFID) using a spinning disk confocal system (UltraView; Perkin Elmer). Usually continuous images were acquired using an LCI Plan-Neofluar 25×/0.8 Imm Corr DIC objective (Carl Zeiss) for up to 10 min and covered with a heating blanket at 37°C, 5% CO2. Imaging wavelengths: 405, 488, 561, and 647 nm) controlled by Volocity (Perkin Elmer).

Values below the tolerance limit were considered statistically significant. Categorical variables were compared using two-tailed Fisher’s exact test using GraphPad. Differences in the parasitemia and in the distribution of lasting interaction were analyzed using two-tailed Student’s t-test and Wilcoxon rank-sum test, respectively. P values <0.05 were considered statistically significant.

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