Migration through host cells activates *Plasmodium* sporozoites for infection

MARIA M. MOTA[1], JULIUS C.R. HAFALLA[2] & ANA RODRIGUEZ[2]

[1]*Department of Pathology,* [2]*Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, New York, USA*

Correspondence should be addressed to A.R.; email: rodria02@popmail.med.nyu.edu

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***Plasmodium* sporozoites, the infective stage of the malaria parasite transmitted by mosquitoes, migrate through several hepatocytes before infecting a final one. Migration through hepatocytes occurs by breaching their plasma membranes, and final infection takes place with the formation of a vacuole around the sporozoite[1]. Once in the liver, sporozoites have already reached their target cells, making migration through hepatocytes prior to infection seem unnecessary. Here we show that this migration is required for infection of hepatocytes. Migration through host cells, but not passive contact with hepatocytes, induces the exocytosis of sporozoite apical organelles, a prerequisite for infection with formation of a vacuole. Sporozoite activation induced by migration through host cells is an essential step of *Plasmodium* life cycle.**

Exocytosis of apical organelles is associated with apicomplexan invasion of host cells[2–4]. To investigate the importance of sporozoite migration through host cells in infection, we first analyzed whether apical exocytosis is required for the process of migration itself or is necessary only during invasion with formation of an internalization vacuole. *Plasmodium* sporozoites are motile, secreting proteins and lipids at the parasite apical end and releasing them from posterior end, leaving trails on the substrate[5] (Fig. 1*a*). Sporozoite migration through host cells[1] and infection of hepatocytes[6] require parasite motility. Another type of secretory activity occurs when sporozoites are incubated with host cells or with Ca^{2+} ionophores. Apical exocytosis is observed over one pole of the sporozoite as a 'cap'[7] containing circumsporozoite protein (data not shown), thrombospondin-related anonymous protein (TRAP) (Fig. 1*f*), and a protein recognized by antiserum specific for *P. falciparum* erythrocyte-binding antigen 175 (EBA175) (Fig. 1*g*). Secreted TRAP and EBA175-related protein can also be detected by western blotting in the supernatants of sporozoites incubated with host cells (data not shown).

To differentiate between these two types of secretory activities, we pretreated sporozoites with brefeldin-A (BFA), an inhibitor of constitutive exocytosis in eukaryotic cells and apicomplexan parasites[8–12]. BFA impeded parasite motility (Table 1), indicating that the secretory activity required for sporozoite motility resembles constitutive exocytosis in eukaryotic cells.

Plasmodium merozoites and the sporozoites and tachyzoites of other apicomplexan parasites, *Eimeria tenella* and *Toxoplasma gondii*, also present apical exocytosis triggered by incubation with host cells[2–4] or Ca^{2+} ionophores[3]. This secretory activity is inhibited with the protein-kinase inhibitor staurosporine[3]. Pretreatment of *Plasmodium* sporozoites with staurosporine resulted in the inhibition of exocytosis induced either by the Ca^{2+} ionophore ionomycin or by incubation with the mouse hepatoma cell line, Hepa1-6 (ref. 13); however, it did not affect sporozoite motility (Table 1). These results indicate that *Plasmodium* sporozoites present two types of secretory activities: a BFA-sensitive exocytosis that seems to be required for sporozoite motility, and a staurosporine-sensitive regulated exocytosis that is not necessary for motility.

We next studied the role of both types of exocytosis in migration through host cells and infection. Treatment with BFA resulted in inhibition of sporozoite migration through host cells and consequent infection (Table 1), most likely as a result of impaired motility. Conversely, inhibition of regulated exocytosis with staurosporine, which does not affect sporozoite motility, did not inhibit migration through host cells but did decrease sporozoite infectivity (Table 1). These results indicate that regulated exocytosis is not required for the process of migration through host cells, and they suggest that this type of exocytosis is necessary later during the process of hepatocyte infection.

Regulated exocytosis can be inhibited in *Plasmodium* sporozoites using intracellular Ca^{2+} chelators such as (1,2-bis (0-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra-(acetoxymethyl)ester) BAPTA-AM (ref. 7). Treatment of sporozoites with this drug resulted in complete inhibition of sporozoite motility and, subsequently, migration through host cells and infection (data not shown).

To investigate how regulated exocytosis is induced, we tested whether passive contact between sporozoites and host cells was sufficient to trigger exocytosis, as described for other apicomplexan parasites[2–4]. We used the myosin inhibitor butanedione-monoxime (BDM), which inhibits the motility of *Plasmodium* sporozoites[14] and *Toxoplasma* tachyzoites[15]. Both migration through host cells and infection were inhibited by BDM treatment (Table 1), most likely because of the effect on motility. BDM did not inhibit ionomycin-induced apical exocytosis in *Toxoplasma* tachyzoites[3] or *P. yoelii* sporozoites (Table 1), indicating that BDM treatment does not affect regulated apical exocytosis machinery in sporozoites. In contrast, treatment of *P. yoelii* sporozoites with BDM inhibited regulated apical exocytosis induced by incubation with host cells. Similar effects on ionomycin-induced and cell-induced apical exocytosis were observed when sporozoite motility was inhibited by incubation with BFA (Table 1). These results suggest that passive contact of sporozoites with host cells is not sufficient to trigger regulated apical exocytosis. Furthermore, similar to migration through cells[1], the induction of apical regulated exocytosis is not restricted to hepa-

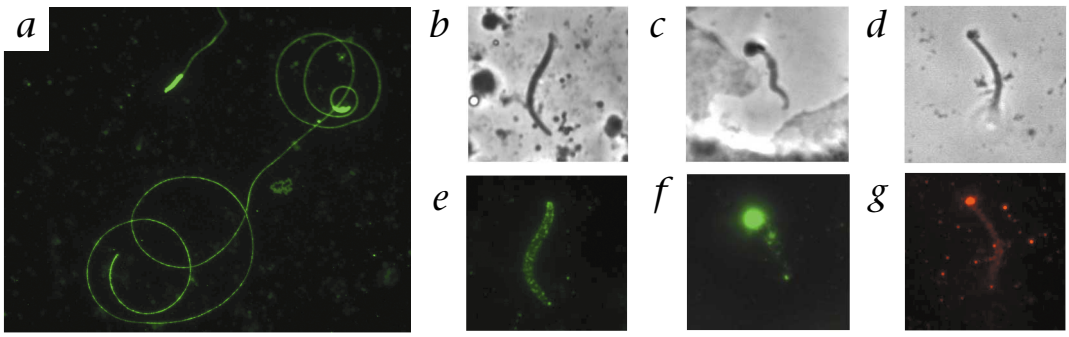

Fig. 1 Sporozoites present two types of exocytosis. ***a***, Trails left by *Plasmodium* sporozoites on a glass coverslip stained with antibodies to CS. ***b***–***g***, *Plasmodium* sporozoites on glass coverslips, incubated with (*c*, *d*, *f* and *g*) or without (*b* and *e*) a monolayer of Hepa1-6 cells. Surface staining with monoclonal antibody against TRAP (*e* and *f*) and antiserum against EBA175 (*g*). Upper panels show the same microscope field in phase contrast. Apical regulated exocytosis is observed as a 'cap' in one end of the sporozoite.

tocytes, and it can be induced by incubation of sporozoites with other cell types (data not shown). The requirement of sporozoite motility for cell-induced apical exocytosis and the lack of cell-type specificity suggest that migration through host cells may trigger this exocytosis.

To investigate this possibility, we independently examined sporozoites that had migrated through host cells and sporozoites that had only been in contact with host cells. We added *P. yoelii* sporozoites to Hepa1-6 hepatoma cells cultured to confluency on filters, and collected sporozoites that traversed the Hepa1-6 cells on coverslips placed underneath the filters. Regulated apical exocytosis was induced in most sporozoites that had migrated through host cells, but not in those that remained in contact with cells in the filters (Fig. 2*a*). These results indicate that sporozoite-regulated apical exocytosis is induced by the process of migration through host cells. We also observed that incubation of *P. yoelii* sporozoites with a lysate of Hepa1-6 cells activated exocytosis in the parasite (Fig. 2*b*), suggesting that this process may be triggered by a cytosolic component of host cells that sporozoites contact during migration through these cells.

Because migration through host cells induces sporozoite-regulated exocytosis—seemingly required for hepatocyte infection—migration through host cells should be essential for infection. To test this, we analyzed sporozoites before and after the process of migration through host cells. *P. yoelii* sporozoites were added to empty filters or to filters containing confluent Hepa1-6 cells. Sporozoites that traversed the filters encountered Hepa1-6 cells again on coverslips placed underneath. In this way, we could distinguish between sporozoites that migrated through cells (Hepa1-6 cells filters) or did not (empty filters) before encountering the cells on the coverslip. Sporozoites that traversed filters with cells migrated through less cells before infection in the coverslips when compared with sporozoites that migrated through empty filters (Fig. 2*c*). Sporozoites that migrated through cells in the filters appear ready to infect host cells in the coverslips underneath without need for further migration, whereas sporozoites that did not encounter cells in the filters still required migration through cells in the coverslips to be infective.

As an alternative way to analyze sporozoite infectivity before and after the process of migration through host cells, we incubated *P. yoelii* sporozoites with or without Hepa1-6 cells for 30 min, before transferring them to new Hepa1-6 cell monolayers. Sporozoites that were pre-incubated with Hepa1-6 cells migrated through fewer cells before infection when they contact cell monolayers the second time (Fig. 2*d*). Together, these results suggest that sporozoite migration through host cells is a necessary step that precedes infection.

Our results indicate that migration through host cells induces the infection requirement of sporozoite apical-regulated exocytosis. Therefore, stimulation of exocytosis by other means should overcome the need for migration through host cells before infection. To test this, we incubated *P. yoelii* sporozoites with different concentrations of ionomycin or lysates of Hepa1-6 cells to induce regulated exocytosis before incubation with intact Hepa1-6 cells. The number of infected cells was increased in a concentration-dependent manner, indicating that stimulation of regulated exocytosis in sporozoites increased their infectivity and confirming the need for this process in infection. In addition, activation of sporozoite exocytosis using ionomycin or cell lysates reduced sporozoite migration through host cells, indicating that such migration is no longer necessary when exocytosis is induced by an alternate way (Fig. 3*a* and *b*). Similar results were found using sporozoites from the rodent malaria parasite *P. berghei* (data not shown). These results indicate that migration through host cells is an essential step that activates sporozoites for infection.

To verify whether a similar mechanism also occurs in a natural infection, we induced apical exocytosis of sporozoites by incubation with ionomycin before injection into mice. We observed that when *P. berghei* sporozoites were activated with ionomycin, sporozoite migration through hepatocytes in the liver was greatly

Table 1 Effect of different inhibitors on sporozoite motility, migration through host cells, exocytosis and infection

Inhibitor	Gliding motility	Regulated exocytosis induced		Migration through cells	Infectivity
		by cells	by ionomycin		
BFA	85.7 ± 7.1	70.1 ± 10.1	28.7 ± 6.3	92.5 ± 8.2	71.4 ± 2.5
Staurosporine	0 ± 0.4	91.6 ± 6.2	95.1 ± 9.3	3.0 ± 5.1	91.4 ± 9.8
BDM	93.2 ± 6.8	94.3 ± 9.4	5.0 ± 8.5	90.7 ± 13.8	96.4 ± 11.4

Results are expressed as percentage of inhibition after treatment with each drug compared with untreated controls. Gliding motility was determined as number of circles in the trail of each sporozoite and migration through host cells as percentage of dextran$^+$ Hepa1-6 cells. Regulated exocytosis was calculated as the percentage of *P. yoelii* sporozoites showing a 'cap'. These results were confirmed using western blots to detect TRAP in the incubation media of each experiment (data not shown). Infectivity was determined as number of EEFs per 1×10^5 sporozoites after 24 h of development.

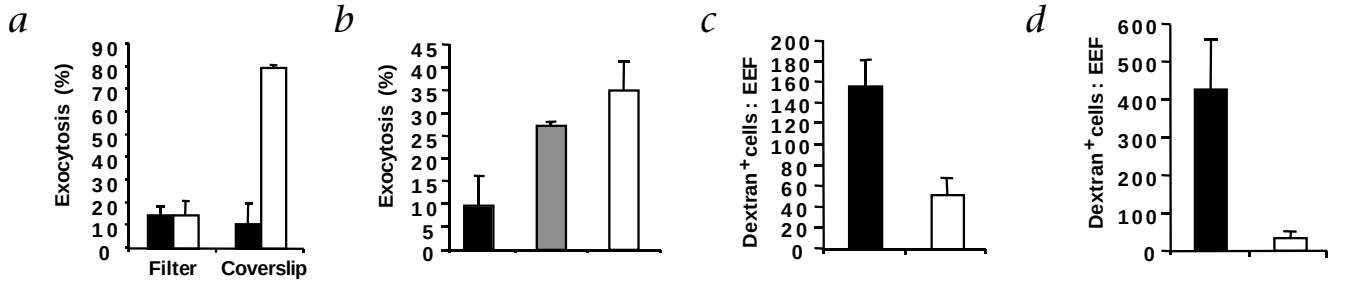

Fig. 2 Migration through host cells induces sporozoite apical-regulated exocytosis. ***a***, *P. yoelii* sporozoites were added to filter insets with (□) or without (■) Hepa1-6 cells. Sporozoites were collected on empty coverslips placed underneath the filters in the lower chamber. Percentage of sporozoites in filters and coverslips showing apical-regulated exocytosis is shown. ***b***, Percentage of *P. yoelii* sporozoites showing apical-regulated exocytosis after incubation with Hepa1-6 cell monolayers or with a lysate of Hepa1-6 cells. ■, control; ■, intact cells; □, cell lysate. ***c***, Hepa1-6 cells were cultivated on filters and coverslips with the same cells were placed underneath the filters in the lower chamber. *P. yoelii* sporozoites were added to the filter insets. As a control, sporozoites were added to filters containing no cells. The ratio of dextran$^+$ cells to infected cells (EEFs) is shown for coverslips placed under filters with (□) or without (■) Hepa1-6 cells. ***d***, *P. yoelii* sporozoites were incubated with medium alone (■) or with monolayers of Hepa1-6 cells (□), before transfer of the supernatants containing sporozoites to new Hepa1-6 monolayers. The ratio of dextran$^+$ cells to infected cells (EEFs) is shown for each condition.

reduced, indicating that sporozoites also skip the step of migration through host cells *in vivo* when activation is induced by alternative means (Fig. 3*c*). The level of infectivity of *P. berghei* sporozoites treated with ionomycin was significantly increased (Fig. 3*d*). These results indicate that, as observed *in vitro*, sporozoite migration through host hepatocytes is required for infection *in vivo* and that this step is no longer necessary if activation is induced by other means.

When these experiments were performed with *P. yoelii*, a parasite that is better adapted for liver infection in mice than *P. berghei*[16], we observed that migration through host hepatocytes was also reduced when sporozoites were activated with ionomycin before injection (data not shown). Infectivity was not enhanced by this treatment, probably because maximum activation of sporozoites is achieved during an infection *in vivo* and cannot be further enhanced by ionomycin.

Our observations show that *Plasmodium* sporozoites require activation before becoming competent for infection. This activation is acquired during the process of migration through host cells, probably mediated by the contact with specific molecules in the cytosol of these cells that trigger Ca^{2+}-mediated signaling pathways within the sporozoite necessary to induce exocytosis. This exocytosis delivers molecules that are probably required for formation of the internalization vacuole and hepatocyte infection (Fig. 3*e*). Migration through host cells is therefore an essential step for the establishment of a malaria infection because it activates sporozoites, making them competent for hepatocyte invasion.

Parasite-host cell contact is sufficient to trigger apical regulated exocytosis in *Plasmodium* merozoites and in *Toxoplasma* tachyzoites[2,3], but *Plasmodium* sporozoites seem to have a different mechanism of activation that requires parasite migration through host cells. The sporozoite stage of other apicomplexan parasites may also have the ability to traverse through host cells before infection[17], as sporozoites from *Toxoplasma* and *Eimeria* enter and exit cells in culture[18–20].

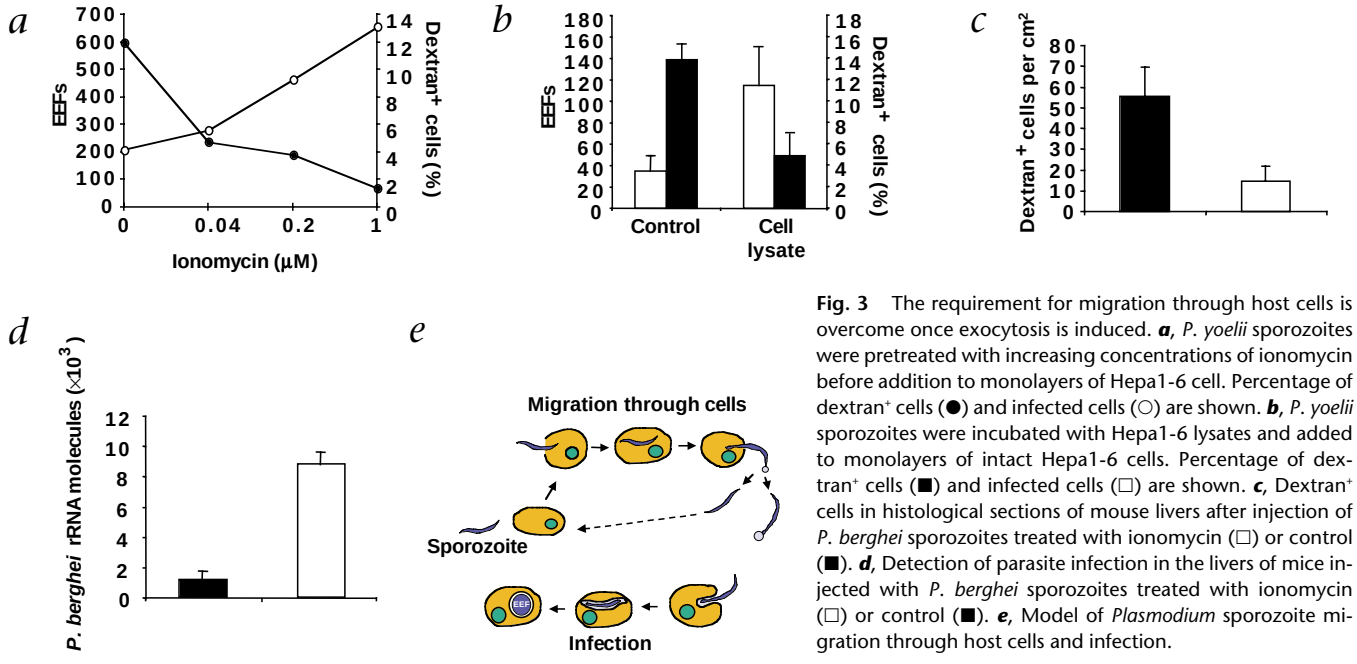

Fig. 3 The requirement for migration through host cells is overcome once exocytosis is induced. ***a***, *P. yoelii* sporozoites were pretreated with increasing concentrations of ionomycin before addition to monolayers of Hepa1-6 cell. Percentage of dextran$^+$ cells (●) and infected cells (○) are shown. ***b***, *P. yoelii* sporozoites were incubated with Hepa1-6 lysates and added to monolayers of intact Hepa1-6 cells. Percentage of dextran$^+$ cells (■) and infected cells (□) are shown. ***c***, Dextran$^+$ cells in histological sections of mouse livers after injection of *P. berghei* sporozoites treated with ionomycin (□) or control (■). ***d***, Detection of parasite infection in the livers of mice injected with *P. berghei* sporozoites treated with ionomycin (□) or control (■). ***e***, Model of *Plasmodium* sporozoite migration through host cells and infection.

The sequential steps of activation acquired by sporozoites while migrating through host cells likely represent an essential feature for a parasite with highly restricted host-cell specificity. Although *Plasmodium* sporozoites probably migrate through different types of host cells during a natural infection, from the skin to the liver, using a sequential mechanism of activation might ensure that the final step of activation required for infection occurs only after the parasite reaches the liver.

Methods

Cells and parasites. Hepa1-6 cells (ATCC CRL-1830) were maintained in DMEM 10% FCS. *P. berghei* (parasite line NK65) and *P. yoelii* (parasite line 17 XNL) sporozoites were obtained from dissection of infected *Anopheles stephensi* mosquito salivary glands.

Drug treatments. Sporozoites were pretreated at room temperature with 20 μg/ml BFA for 1 h, 1 μM staurosporine for 10 min, or 30 mM BDM for 15 min. Treatment with 1 μM ionomycin was performed for 30 s at room temperature before spinning sporozoites on coverslips placed in 24-well dishes containing 1 ml culture medium per well. This results in a 10-fold dilution of BFA, staurosporine and ionomycin concentrations. BDM concentration was kept at 30 mM during the time of incubation with Hepa1-6 cells because its effect is highly reversible. In the experiment where ionomycin-treated sporozoites were added to cells, the same final concentrations (diluted 10-fold) of ionomycin were added to the cells before addition of sporozoites. No effect was observed on either migration through host cells or infection. The motility of ionomycin-treated sporozoites was not affected (data not shown).

Gliding motility. 3×10^4 *P. berghei* sporozoites were spun for 5 min at 1,800*g* on glass coverslips coated with anti-CS protein monoclonal antibody (3D11, ref. 21), and incubated for 1 h at 37 °C. Coverslips were fixed with 4% paraformaldehyde and stained with the same antibody coupled to FITC.

Regulated exocytosis. After drug treatments, 1×10^5 *P. yoelii* or *P. berghei* sporozoites were spun down for 5 min at 1,800*g* on glass coverslips with or without a monolayer of 2×10^5 Hepa1-6 cells. After 1 h incubation at 37 °C for sporozoites spun on coverslips with cells or 10 min incubation at 37 °C for sporozoites treated with 10 μM ionomycin on empty coverslips, sporozoites were fixed with 1% paraformaldehyde for 10 min before staining with anti-TRAP mAb (F3B5) or anti-*P. falciparum* EBA175 rabbit antiserum. To confirm that the observed effect was due to the action of the drugs on the parasite and not on Hepa1-6 cells, the same final concentrations of BFA, staurosporine and ionomycin were added to the cells before addition of sporozoites. No effect was observed (data not shown). Incubation media from each coverslip was collected and spun at 10,500*g* for 10 min to remove remaining sporozoites. Samples were probed in western blots with anti-TRAP mAb (F3B5) or anti-*P. falciparum* EBA175 rabbit antiserum.

Migration through cells and infection *in vitro*. Sporozoite migration through cells can be quantified by detection of parasite-wounded hepatocytes using a cell-impermeant fluorescent tracer macromolecule[1]. *P. yoelii* or *P. berghei* sporozoites (1×10^5 sporozoites per coverslip) were added to monolayers of 2×10^5 Hepa1-6 cells for 1 h in the presence of 1 mg/ml of rhodamine-dextran lysine fixable, 10,000 kD. Cells were washed and incubated for another 24 h before fixation and staining with anti-HSP70 mAb (2E6, ref. 22). Migration through host cells is quantified as percentage of dextran$^+$ cells. Infection was quantified as the number of EEFs per coverslip. On a different set of experiments, *P. yoelii* or *P. berghei* sporozoites (1×10^5 sporozoites per coverslip) were added to monolayers of 2×10^5 Hepa1-6 cells for 30 min. Sporozoites were then transferred to a new monolayer of Hepa1-6 cells and incubated for an additional 30 min in the presence of the tracer dextran.

Hepa1-6 cell lysates. Hepa1-6 cells (4×10^5 cells per ml) resuspended in culture medium were repeatedly passed through a 28G syringe until more than 95% of the cells were lysed, as determined by Trypan blue staining.

Transwell filter assays. Hepa1-6 cells (5×10^5 per filter) were cultivated on 3-μm pore diameter Transwell filters (Costar, Corning, New York) until they form a continuous monolayer. Empty coverslips or coverslips containing Hepa1-6 cells monolayers (2×10^5 Hepa1-6) were placed underneath the filters. *P. yoelii* sporozoites (2×10^5) were added to filter insets containing or not Hepa1-6 cells. Filters and coverslips were fixed after 2 h of incubation with sporozoites, before staining for surface TRAP. To determine migration through host cells, rhodamine-dextran (1 mg/ml) was added before addition of sporozoites. Coverslips were washed after 2 h of incubation with sporozoites and further incubated for 24 h before fixation, staining and quantification of dextran$^+$ cells and developing parasites with anti-HSP70.

Migration through host cells and infection *in vivo*. BALB/c mice were injected intravenously with 8 mg of FITC-dextran and 2×10^5 *P. yoelii* or 0.5×10^5 *P. berghei* sporozoites pretreated or not for 30 s with 1 μM ionomycin. After 45 min, mice were anesthetized, followed by liver perfusion with 20 ml of PBS and fixation with 20 ml of 8% paraformaldehyde[23]. Livers were frozen and sectioned before quantification of dextran$^+$ cells in histological sections. Liver infection was quantified by real-time PCR 40 h after injection[24] of 2×10^5 *P. yoelii* or 0.5×10^5 *P. berghei* sporozoites pretreated or not for 30 s with 1 μM ionomycin. Experiments using mice were approved by the NYU School of Medicine Institutional Animal Care and Use Committee.

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Competing interests statement

The authors declare that they have no competing financial interests.

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