

DOUTORAMENTO EM CIÊNCIAS FARMACÊUTICAS

MICROBIOLOGIA

Studying the homing of *Plasmodium* sporozoites to the liver

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D

2021





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Studying the homing of *Plasmodium* sporozoites to the liver

Ph.D. thesis of the 3rd cycle of studies leading to the
Doctor Degree in Pharmaceutical Sciences –
Microbiology Specialty

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July 2021

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE A DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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Author's declaration

Under the terms of the “Decreto-lei no 216/92, de 13 de Outubro”, it is hereby declared that the author made a major contribution to the conceptual design and technical execution of the work, interpretation of the results, and manuscript preparation of the published works (or in preparation) included in this dissertation.

Under the terms of the “Decreto-lei no 216/92, de 13 de Outubro”, it is hereby declared that the following original articles/communications were prepared in the scope of this dissertation or in related fields.

Scientific publications

Articles in international peer-reviewed journals

In the scope of this thesis

Costa DM, Sá M, Teixeira AR, Pérez-Cabezas B, Golba S, Formaglio P, Franke-Fayard B, Janse CJ, Amino R, Tavares J. Downregulation of the secreted protein with altered thrombospondin repeat (SPATR) impacts the infectivity of malaria sporozoites. In preparation.

Costa DM, Sá M, Teixeira AR, Loureiro I, Thouvenot C, Golba S, Amino R, Tavares J. TRSP is dispensable for the *Plasmodium* pre-erythrocytic phase. Scientific Reports. 2018 Dec 10;8(1):15101.

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Outside the scope of this thesis

Neves JV, Gomes AC, **Costa DM**, Barroso C, Vaulont S, Cordeiro-da-Silva A, Tavares J, Rodrigues PNS. A role for hepcidin in the anemia caused by *Trypanosoma brucei* infection. Haematologica 2020 Jan 9;106(3):806–18.

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Communications

Oral communications in the scope of this thesis

Costa DM*. Evaluating the role of malaria sporozoite surface molecules in the homing of the parasites to the liver. Young Scientists Seminar, i3S, Porto, Portugal (virtual), 2021 April 21.

Costa DM*, Sá M, Teixeira AR, Pérez-Cabezas B, Golba S, Franke-Fayard B, Janse CJ, Amino R, Cordeiro-da-Silva A, Tavares J. The infectivity of malaria sporozoites: players and bystanders. 1st Annual Meeting in Pharmaceutical Sciences, Coimbra, Portugal, 2019 November 15.

Costa DM*, Sá M, Teixeira AR, Pérez-Cabezas B, Golba S, Franke-Fayard B, Janse CJ, Amino R, Cordeiro-da-Silva A, Tavares J. Stage-specific downregulation of SPATR impacts *Plasmodium* sporozoite infectivity. 2nd PhDay at i3S – PhD countdown: a time for Science, Porto, Portugal, 2019 September 26.

Costa DM*, Amino R, Cordeiro-da-Silva A, Tavares J. Addressing the role of the *Plasmodium* thrombospondin-related sporozoite protein (TRSP) in the pre-erythrocytic phase. 5th i3S Annual Meeting, Póvoa de Varzim, Portugal, 2016 November 3-4.

* presenting author

Oral communications outside the scope of this thesis

Teixeira AR*, Pérez-Cabezas B, **Costa DM**, Sá M, Sá M, Golba S, Tsuji M, Boscardin S, Amino R, Cordeiro-da-Silva A, Tavares J. Immunization of mice with CSP and the RIG-I adjuvant 5'ppp-dsRNA induces sterile protection against *Plasmodium*. BioMalPar XVII: Biology and Pathology of the Malaria Parasite, EMBL, Heidelberg, Germany (virtual), 2021 May 25-27.

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* presenting author

Poster communications in the scope of this thesis

Costa DM*, Sá M, Teixeira AR, Pérez-Cabezas B, Golba S, Franke-Fayard B, Janse CJ, Cordeiro-da-Silva A, Amino R, Tavares J. Stage-specific downregulation of SPATR impacts malaria sporozoite motility and infectivity. Molecular Parasitology Meeting XXXI, Woods Hole, USA (virtual), 2020 September 21-24, pp. 72, ID: 286E.

Costa DM*, Amino R, Cordeiro-da-Silva A, Tavares J. Addressing the role of the *Plasmodium* thrombospondin-related sporozoite protein (TRSP) in the pre-erythrocytic phase. 5th i3S Annual Meeting, Póvoa de Varzim, Portugal, 2016 November 3-4, pp. 40, ID: 205.

* presenting author

Poster communications outside the scope of this thesis

Sá M*, **Costa DM**, Teixeira AR, Formaglio P, Pérez-Cabezas B, Golba S, Cordeiro-da-Silva A, Amino R, Tavares J. MAEBL is important for the adhesion of *Plasmodium* sporozoites to mammalian host tissues. BioMalPar XVII: Biology and Pathology of the Malaria Parasite, EMBL, Heidelberg, Germany (virtual), 2021 May 25-27.

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Sá M*, **Costa DM**, Teixeira AR, Golba S, Amino R, Cordeiro-da-Silva A, Tavares J. Exploring the role of thrombospondin-related anonymous protein (TRAP) in the homing of *Plasmodium* sporozoites to the liver. Day of the Microbiology Laboratory, Faculty of Pharmacy of the University of Porto, Portugal, 2019 November 15, pp. 25, ID: P-14.

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This work was performed at the Parasite Disease Group of the Instituto de Biologia Molecular e Celular (IBMC, University of Porto, Portugal), now part of the Instituto de Investigação e Inovação em Saúde (i3S, University of Porto, Portugal) in collaboration with the Malaria Infection and Immunity Unit at Institut Pasteur (Paris, France). The author has received financial support through an individual doctoral fellowship from the Portuguese Foundation for Science and Technology (FCT, Portugal) with the reference SFRH/BD/123734/2016. The experimental research performed in this work has received financial support from FCT/Ministério da Ciência, Tecnologia e Ensino Superior (MECTES), co-funded by FEDER (EXPL/JTAVARES-IF/00881/2012/CP0158/CT0005) under the Partnership agreement PT2020 (PTDC/SAU-PAR/31340/2017). The work received funds from project Norte-01-0145-FEDER-000012 – Structured program on bioengineered therapies for infectious diseases and tissue regeneration, supported by the North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through FEDER.



Acknowledgments

First and foremost, I would like to thank my supervisor Dr. Joana Tavares. Thank you for awarding me the opportunity to work with you and, most importantly, to learn from you. I am grateful for all the support and advice you provided me throughout the years and I am sorry for the headaches I may have caused you. Thank you for investing in me, pushing me forward and guiding me through this long and winding road.

I would then like to acknowledge both my co-supervisors for the guidance and support. To Prof. Dr. Anabela Cordeiro da Silva, thank you for letting me join the lab, for all the opportunities I received during my PhD, and for being there throughout the entire process. To Dr. Rogerio Amino, thank you for welcoming me to your lab, for crucial feedback, for all the interesting discussions, and for challenging me to think differently and approach problems from new perspectives.

I would like to thank every member of the PD group I met during this process, you all contributed to my development as a PhD student and as a person. In no particular order, I would like to acknowledge Nuno Santarém, Mónica (my benchwork buddy), Ana Rafaela, Begoña, Inês Costa, Sofia, Pedro, Joana Faria, Renata, Diana, Nuno Graça, Carla Lima, Inês Loureiro, Ana Pinto, Clara, Carla Carvalho, Ricardo, Sara, Catarina, Helena, Joana Teixeira, Luís, and all the other members who joined the lab through the years. I am also grateful to all the members of the i3S/IBMC community I encountered during my PhD and helped me in any way, especially to Dona Rosa, Paula and Tânia.

Now I would like to thank the rest of the IIP group – Pauline (you are a godsend), Jean-Michel, Eduardo, Manuela, and Maryse – for being so welcoming, for all the time you spent teaching me, and for being so nice and helpful. I hope I wasn't too heavy a burden. I apologize for upsetting the balance of the universe and causing COVID-19 spikes that shut down Paris twice (maybe you will find this thesis was worth it). Many thanks to the CEPIA team as well.

I am also very thankful to my family and friends, specially my mother, my father and my brother. A special mention to Tata Fernanda, Tonton Paulo and Andréa, who when out of their way to create a home for me in Paris. Thank you all for supporting my choice to do a PhD, for making the effort to care about my work (or at least pretend to) and for all the words that kept me afloat during the most trying times of this journey, even when I kept you oblivious to my struggles or if it felt like I did not appreciate your help. I really did.

Truthfully, thank you all.

Abstract

Malaria is a vector-borne disease caused by *Plasmodium* parasites and responsible for hundreds of thousands of deaths every year. *Plasmodium* sporozoites are deposited in the skin of the host via the bite of *Anopheles* mosquitoes. Sporozoites depart from the skin through the bloodstream and specifically target the liver, where they differentiate and multiply within hepatocytes, before initiating the symptomatic blood stage of infection. This phase of the infection also allows the transmission of the parasite back to the vector, where the parasite produces new sporozoites, which gain access to the salivary glands of the mosquito through the hemolymph, thus being ready for transmission.

No antimalarial vaccine currently boasts satisfactory efficacy. The steps of the life cycle that precede the blood stage in the mammalian host constitute attractive targets for vaccination. Thus, the study of the molecular mechanisms that support sporozoite infectivity could aid in the delineation of strategies for rational vaccine development. The arrest of sporozoites in the liver sinusoids is one of the critical steps of the life cycle whose molecular basis is not entirely understood. Therefore, to improve our knowledge of the mechanisms that govern this process we developed a bioluminescence-based and minimally invasive method for the evaluation of parasite distribution in mice shortly after intravenous injection. We determined that once in the bloodstream sporozoites rapidly and almost exclusively accumulate in the liver, unlike the erythrocyte-infective merozoites or other protozoans. Using this method, we also observed that the homing capacity of sporozoites increases during their maturation program. Thus, we selected two surface proteins bearing adhesive domains and displaying increased expression in mature sporozoites, and evaluated their roles in sporozoite homing to the liver as well as in overall infectivity, using reverse genetics in the rodent-infecting *Plasmodium berghei*. These proteins were the thrombospondin-related sporozoite protein (TRSP) and the secreted protein with altered thrombospondin repeat (SPATR).

The deletion of the *trsp* gene did not affect sporozoite homing, nor parasite development in the liver, the blood and the mosquito. The dispensability of TRSP for sporozoite infectivity is indicative of a redundant function and clashes with the previous report of its involvement in hepatocyte invasion. More studies are required to elucidate

the real function of TRSP and the discrepancies in the phenotypes of independently generated *P. berghei trsp* knockout lines.

Conversely, SPATR proved to be required for the blood stages, precluding the isolation of knockout parasites. Therefore, conditional knockdown lines were generated via a promoter swap approach. The downregulation of SPATR early during sporozoite development hampered salivary gland invasion and abrogated infectivity of sporozoites collected from the hemolymph to mice. This loss of infectivity likely stemmed from defective motility, homing capacity and/or hepatocyte invasion. The genetic complementation of the SPATR knockdown line partially restored salivary gland colonization. However, SPATR knockdown salivary gland sporozoites displayed a milder infectivity impairment. This suggests SPATR may also be involved in redundant processes in mature sporozoites.

In conclusion, we demonstrate sporozoites specifically home to the liver, yet the molecular mechanisms that control this process still remain poorly defined. Nonetheless, we generated tools and data that allow us to keep studying this behavior and could contribute to the design of malaria prevention strategies.

Keywords: Malaria; Sporozoite; Homing; TRSP; SPATR.

Resumo

A malária é uma doença transmitida por vetores causada por parasitas do género *Plasmodium* e responsável por milhares de mortes todos os anos. A infeção é iniciada por esporozoítos que são depositados na pele do hospedeiro através da picada de mosquitos do género *Anopheles*. Estes parasitas deixam a pele através da via sanguínea e infetam o fígado, onde se diferenciam e multiplicam dentro de hepatócitos, antes de iniciarem a fase sanguínea e sintomática da infeção. Esta fase também permite a transmissão do parasita ao vetor, onde o parasita produz esporozoítos, os quais invadem as glândulas salivares através da hemolinfa, ficando assim disponíveis para iniciar uma nova infeção.

Nenhuma vacina aprovada contra a malária apresenta eficácia satisfatória. As etapas do ciclo de vida que precedem a fase sanguínea da infeção representam um alvo atrativo para o desenvolvimento de vacinas. Assim, o estudo dos mecanismos moleculares requeridos para a infetividade do esporozoíto poderá auxiliar o desenvolvimento racional de vacinas. A retenção dos esporozoítos nos sinusóides hepáticos é uma das importantes etapas do ciclo de vida cujo mecanismo molecular não é completamente conhecido. Portanto, com o objetivo de elucidar os mecanismos envolvidos neste processo, desenvolvemos um método minimamente invasivo baseado em bioluminescência para a avaliação da distribuição do parasita em murganhos logo após a injeção por via intravenosa. Determinamos que, uma vez na corrente sanguínea, os esporozoítos acumulam-se de forma rápida e quase exclusiva no fígado, ao contrário das formas sanguíneas do parasita que infetam eritrócitos, os merozoítos, ou de outros protozoários. Utilizando este método, observamos que a eficiência da acumulação no fígado aumenta com a maturação dos esporozoítos. Por esta razão, selecionamos duas proteínas de superfície com domínios adesivos e mais abundantes em esporozoítos maduros, com o objetivo de avaliar os seus papéis na acumulação dos esporozoítos no fígado e para a infetividade do parasita, recorrendo a técnicas de genética reversa em *Plasmodium berghei*, o qual infeta roedores. Estas proteínas foram a *thrombospondin-related sporozoite protein* (TRSP) e a *secreted protein with altered thrombospondin repeat* (SPATR).

A deleção do gene *trsp* não afetou a acumulação dos esporozoítos no fígado, nem o desenvolvimento do parasita no fígado, no sangue e no mosquito. A dispensabilidade da TRSP para a infeciosidade do esporozoíto é sugestiva de redundância funcional e contradiz a potencial participação desta proteína na invasão de hepatócitos, como foi previamente sugerido. Portanto, mais estudos são necessários para elucidar tanto a função da TRSP como as discrepâncias entre os fenótipos de linhas de *P. berghei* geradas independentemente através da deleção do gene *trsp*.

Por outro lado, a SPATR demonstrou ser necessária para a fase sanguínea, impossibilitando a deleção do gene. Portanto, recorrendo à técnica de troca de promotor geramos linhas em que a expressão do gene foi condicionada e, portanto, diminuída no esporozoíto. A diminuição da expressão da SPATR reduziu a invasão das glândulas salivares e aboliu a infeciosidade de esporozoítos da hemolinfa *in vivo*. Esta redução da infeciosidade deveu-se possivelmente a defeitos na motilidade, na capacidade de acumulação no fígado (*homing*) e/ou na invasão de hepatócitos. A complementação genética de uma das linhas mutantes restaurou parcialmente a colonização das glândulas salivares pelo esporozoíto. No entanto, esporozoítos mutantes recolhidos das glândulas salivares exibiram um comprometimento da infetividade mais moderado, sugerindo que a função da SPATR em esporozoítos maduros pode também ser redundante.

Em conclusão, demonstramos o *homing* específico dos esporozoítos no fígado, mas os mecanismos moleculares que controlam este processo permanecem pouco definidos. No entanto, geramos ferramentas e dados que nos poderão ajudar a compreender este comportamento e, potencialmente, contribuir para criação de novas estratégias para a prevenção da malária.

Palavras-chave: Malaria; Esporozoíto; *Homing*; TRSP; SPATR.

Table of contents

Author's declaration	v
Acknowledgments	xi
Abstract	xiii
Resumo	xv
Index of figures.....	xix
Index of tables	xxiii
Abbreviations	xxv
Chapter I. Introduction.....	29
1. Malaria	31
1.1. Clinical features and epidemiology	31
1.2. The parasite.....	32
1.3. The vector.....	37
2. The life cycle of <i>Plasmodium</i> parasites	40
2.1. Development within the mosquito vector	41
2.1.1. In the mosquito midgut	41
2.1.2. Road to the salivary glands.....	43
2.2. The pre-erythrocytic phase.....	44
2.2.1. The skin stage	44
2.2.2. Homing of sporozoites to the liver	47
2.2.2.1. The role of CSP	48
2.2.2.2. The role of TRAP	51
2.2.3. Arrival in the liver parenchyma.....	54
2.2.4. Hepatocyte invasion	59
2.2.5. Liver stage development.....	62
2.3. The blood stage	63
2.3.1. Naturally acquired immunity and pathogenesis.....	67
3. Control strategies	69
3.1. Diagnosis.....	69

3.2.	Treatment	69
3.3.	Prevention	70
3.3.1.	Vector control.....	70
3.3.2.	Antimalarial vaccines	71
3.3.2.1.	Blood stage vaccines	71
3.3.2.2.	Transmission-blocking vaccines.....	72
3.3.2.3.	Pre-erythrocytic vaccines.....	72
3.3.2.4.	Multistage vaccines	74
4.	Analysis of gene function in sporozoites.....	75
4.1.	Conventional genetic modification	75
4.2.	Site-specific genome editing	76
4.3.	Conditional systems	78
4.4.	Extrachromosomal gene expression.....	82
5.	References	84
Chapter II. Objectives and results.....		135
1.	Thesis contextualization and objectives	137
1.1.	References	140
2.	Results	143
2.1.	<i>In vivo</i> imaging of pathogen homing to the host tissues	143
2.2.	TRSP is dispensable for the <i>Plasmodium</i> pre-erythrocytic phase.....	153
2.3.	Downregulation of the secreted protein with altered thrombospondin repeat (SPATR) impacts the infectivity of malaria sporozoites.....	169
Chapter III. Discussion		221
1.	Bioluminescence imaging in mice: a tool to investigate the molecular determinants of pathogen target tissue recognition.....	223
2.	TRSP and SPATR: proposed functions in sporozoites	224
3.	What mechanisms drive the arrest of sporozoites in the liver sinusoids?.....	232
4.	References	237

Index of figures

Figure 1. Global estimated numbers of malaria cases and deaths from 2000 to 2019. Data from WHO (10).	32
Figure 2. Incidence of <i>P. falciparum</i> (A) and <i>P. vivax</i> (B) infections per 1,000 people in 2019. Downloaded from The Malaria Atlas Project (19).	33
Figure 3. Structural features and length of the <i>Plasmodium</i> sporozoite and merozoite. The apical complex encompasses the conoidal and apical polar rings, and the polar secretory organelles (rhoptries and micronemes). The more distal of the two apical polar rings anchors the subpellicular microtubules. The positioning of the simplified conoid relative to the apical polar rings depends on the activation state of the zoite (31,32). The ookinete (not represented) constitutes the third <i>Plasmodium</i> zoite and lacks both the rhoptries and dense granules (36,37). Illustrations are not to scale and were constructed with BioRender.	35
Figure 4. The <i>Plasmodium</i> glideosome complex. Transmembrane adhesins present on the parasite plasma membrane (pPM) interact with a substrate, such as a host cell plasma membrane (hPM), through their ectodomains and indirectly with actin likely via linker such as the glideosome-associated connector (GAC), which interacts with the C-terminus of the adhesins. Several glideosome-associated proteins (GAPs) anchor the single-headed myosin A (MYOA) to the IMC, through the MYOA-tail interacting protein (MTIP). The head of MYOA suffers conformational changes with the hydrolysis of ATP, which moves the actin filaments rearwards (A). This results in the forward movement of the parasite, as other glideosome components present in the IMC such as GAPs with multiple membrane spans (GAPM) then link the IMC to the cytoskeleton of the zoite, while GAP45 spans across the space between the IMC and the pPM to ensure the structural integrity of the complex. The interaction with the substrate is then disengaged through the cleavage of the transmembrane domain of the adhesin via the action of proteases such as rhomboid protease 4 (ROM4; B). Adapted from Frénal <i>et al.</i> with permission (38).	37
Figure 5. Distribution of the dominant malaria vector species in 2010. Downloaded from The Malaria Atlas Project (19).	38
Figure 6. The life cycle of mammal-infecting <i>Plasmodium</i> species. Sporozoites inoculated into the skin by a female anopheline mosquito make their way to the liver through the bloodstream, where they generate thousands of erythrocyte-infecting merozoites.	

Merozoites either multiply or commit to sexual differentiation within erythrocytes to be transmitted to the mosquito. In the vector, male and female gametes fuse and form the zygote, which transforms into a motile ookinete that develops into a sporozoite-producing oocyst in the midgut wall of the mosquito. Newly formed sporozoites are released into the mosquito’s hemolymph and invade the salivary glands to start a new cycle. Adapted from Ménard *et al.* with permission (59). 40

Figure 7. Development of *P. berghei* in *Anopheles funestus* mosquitoes. (A) Mosquito midgut containing oocysts expressing a green fluorescent protein (GFP; e.g., arrows) at day 7 post-infectious blood meal. (B) Infected midgut at day 20 post-infection showing full (e.g., arrow) and ruptured (e.g., arrowhead) oocysts. (C) Imaging of fluorescent parasites through the cuticle of a live mosquito, showing parasite development in the midgut (MG) and salivary glands (SG) at day 20 post-infection. (D, E) Infected salivary glands at day 20 post-infection. Panel E shows the preferential accumulation of fluorescent sporozoites in the distal lateral (DL) lobes compared to the median (M) lobe of the salivary gland. Bars: A-C = 500 µm; D,E = 200 µm. Adapted from Xu *et al.*, with permission (72). 42

Figure 8. Fates of *Plasmodium* sporozoites deposited in the skin. Sporozoites need to find blood vessels to reach their next target organ, the liver. However, sporozoites may also invade the lymphatics, which constitutes a developmental dead end for the parasite. Most sporozoites that fail to exit the skin eventually die, although some invade skin cells and complete their intracellular replication. Adapted from Ménard *et al.* with permission (59). 46

Figure 9. Structure of the *P. falciparum* CSP. The structural elements of the CSP of *P. falciparum* 3D7 strain were obtained from available literature and databases (125,127,128) and the scheme was built with IBS online (129). The numbers represent the first and last residues of the protein. GPI: GPI anchor. NANP: asparagine-alanine-asparagine-proline major repeat. NPDP: asparagine-proline-aspartic acid-proline motif. NVDP: asparagine-valine-aspartic acid-proline repeat. SP: signal peptide. 48

Figure 10. CSP processing and conformational changes. The locations of epitopes of various CSP-specific monoclonal antibodies are shown at the bottom. The question mark indicates that the form and conformation of CSP in hemolymph sporozoites are speculative. Adapted from Herrera *et al.*, with permission (148). 50

Figure 11. Structural features of the *P. falciparum* TRAP. The structural elements of TRAP from the *P. falciparum* 3D7 strain were from were obtained from available literature and

databases (77,80,81,128) and the scheme was built with IBS online (129). The black bars within the I domain represent the invariable residues of the MIDAS, which span over several loops. The arginine-glycine-aspartic acid motif (RGD) is not conserved among *Plasmodium* species (162). TRAP also contains a proline-rich stalk between the RGD and the transmembrane domain (TMD) with repetitive sequences, which are also species-specific (128). The numbers represent the first and last residues of the protein. CTD: C-terminus domain. SP: signal peptide.52

Figure 12. Sporozoite homing to the liver parenchyma and completion of the pre-erythrocytic phase. Sporozoites arrested in the liver sinusoids cross the blood vessel through Kupffer and endothelial cells, traverse parenchyma cells and invade hepatocytes, where they differentiate and produce hepatic merozoites. Merozoite-filled vesicles called merosomes carry the parasites to the bloodstream so they can initiate the blood stage of the life cycle. Adapted from Ménard *et al.* with permission (59).55

Figure 13. Crossing of the hepatic sinusoidal barrier by sporozoites. Time lapses of sporozoites (SPZ) crossing Kupffer cells stained with an Alexa Fluor 647 anti-F4/80 antibody (F4/80-KC; A) or GFP-expressing endothelial cells of Flk1 mice (Flk1-GFP-EC; B). The loss of plasma membrane integrity is shown by the incorporation of propidium iodide (green arrowheads) or loss of cytosolic content (white arrowhead; B). Yellow arrowheads mark the crossing site and asterisks the anterior pole of the parasite. Bars: 10 μ m. Adapted from Tavares *et al.*, with permission (175).57

Figure 14. Hepatocyte invasion by *Plasmodium* sporozoites. Some of the known molecules expressed by sporozoites to be involved in the invasion process as well as their suggested or unknown receptors on the host cell are represented. The illustration was made with BioRender.59

Figure 15. Steps of erythrocyte invasion by merozoites. (A) Merozoite attachment to the erythrocyte. (B) Reorientation of the merozoite. (C) Formation of a tight junction. (D) Merozoite entry into the erythrocyte. (E) Sealing of the parasitophorous vacuole and host cell plasma membrane. Adapted from Koch and Baum, with permission (249).64

Figure 16. Blood stages of *Plasmodium* species infective to humans. Images of thin blood smears showing the different intraerythrocytic stages of 4 *Plasmodium* species that cause malaria in humans. Adapted from Delahunt *et al.*, with permission (268).66

Figure 17. Homologous recombination in *P. berghei*. (A) Disruption of a target gene by single crossover homologous recombination. The representation of the backbone of the donor

plasmid, which contains the selectable marker (SM) or reporter gene (RG), was abridged. (B) Gene deletion by double crossover homologous recombination. The scheme shows the integration of the linearized transfection vector into the target locus using two distinct homology regions (HRs).75

Figure 18. Methodologies for conditional depletion of proteins in *P. berghei* sporozoites. (A) Example of a Flp-FRT system for gene deletion (left) or inversion (right) in sporozoites (Spz). The inversion of the target sequence is reversible, while the excision is strongly favored. (B) Example of a promoter swap approach for the maintenance of gene expression in merozoites (Mz) and oocysts (Ooc) and knockdown in Spz. (C) Example of Cre-loxP system for gene deletion in oocysts (Ooc) and Spz. To avoid recombination between loxP sites in successive alterations to the genome of the parasite, loxN sites may also be used, which are incompatible with loxP sequences. 79

Figure 19. Homing of *P. berghei* sporozoites to the liver. Midgut (MG), hemolymph (HEMO) or salivary gland (SG) sporozoites (SPZ) were intravenously injected in C57BL/6 mice and the bioluminescent signal was acquired 7 min post-infection. The quantification of the signal in the liver is shown in percentage of total bioluminescence (left) and a representative photograph of infected mice to which the signal map was superimposed is shown next to the radiance heatmap scale (right). Data from Sá *et al.* (in preparation).138

Figure 20. Structural features of the *P. berghei* TRSP and SPATR. The structural elements were obtained from available literature and databases (9,11–13) and the schemes were built with IBS online (14). The numbers indicate the position of the first and last amino acid residues of the respective full-length proteins. CTD: C-terminus domain. EGF-like: epidermal growth factor-like domain. SP: signal peptide. TMD: transmembrane domain.139

Index of tables

Table 1. List of <i>Plasmodium</i> surface molecules studied in the scope of this thesis.	139
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Abbreviations

ACT	Artemisinin-based combination therapy
AMA1	Apical membrane antigen 1
ApoH	Apolipoprotein H
Cas9	CRISPR-associated protein 9
CeTOS	Cell traversal protein for ookinetes and sporozoites
CEP76	Centrosomal protein 76
CD	Cluster of differentiation
CRISPR	Clustered regularly interspaced short palindromic repeat
CRMP	Cysteine repeat modular protein
CSP	Circumsporozoite protein
CSPBP	CSP-binding protein
CTD	C-terminus domain
CTRP	Circumsporozoite- and TRAP-related protein
DBP1	Duffy-binding protein 1
DDT	Dichlorodiphenyltrichloroethane
EBA	Erythrocyte-binding antigen
EBL	Erythrocyte-binding-like
EEF	Exoerythrocytic form
EGF	Epidermal growth factor
EphA2	Ephrin receptor A2
EXP	Exported protein
Flp	Flippase
FlpL	Flippase with low activity at 37 °C
FRT	Flippase recombination target
GAC	Glideosome-associated connector
GAG	Glycosaminoglycan
GAMA	GPI-anchored micronemal antigen
GAP	Glideosome-associated protein
GFP	Green fluorescent protein
GIMO	Gene insertion/marker out
GOMO	Gene out/marker out
GPI	Glycosylphosphatidylinositol

HADO	Haloacid dehydrogenase domain ookinete protein
HBsAg	Hepatitis B virus surface antigen
hDHFR	Human dihydrofolate reductase
hPDGFR β	Human platelet-derived growth factor receptor β
HSP70	Heat shock protein 70
HSPG	Heparan sulfate proteoglycan
ICP	Inhibitor of cysteine proteases
IMC	Inner membrane complex
LSA1	Liver stage antigen-1
MAEBL	Membrane associated erythrocyte binding-like protein
MHC	Major histocompatibility complex
MIDAS	Metal ion-dependent adhesion site
MSP	Merozoite surface protein
MTRAP	Merozoite TRAP-like protein
PAC	<i>Plasmodium</i> artificial chromosome
PAM	Protospacer adjacent motif
PAT	Pantothenate transporter
PbDHFR/TS	<i>Plasmodium berghei</i> dihydrofolate reductase-thymidylate synthase
PCR	Polymerase chain reaction
PEXEL	<i>Plasmodium</i> export element
PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
PIR	<i>Plasmodium</i> interspersed repeat
PlasmoGEM	<i>Plasmodium</i> Genetic Modification Project
PLP1	Perforin-like protein 1
PTEX	<i>Plasmodium</i> translocon of exported proteins
PvRBP	<i>Plasmodium vivax</i> reticulocyte-binding protein
RBL	Reticulocyte-binding-like
RDT	Rapid diagnostic test
RIF	Repetitive interspersed family
RON	Rhoptry neck protein
sgRNA	Single-guide RNA
SPATR	Secreted protein with an altered thrombospondin repeat
SPECT	Sporozoite protein essential for cell traversal
SR-BI	Scavenger receptor type B class I
STEVOR	Subtelomeric variant open reading frame

TgDHFR/TS	<i>T. gondii</i> dihydrofolate reductase-thymidylate synthase
TLP	TRAP-like protein
TRAP	Thrombospondin-related anonymous protein
TREP	TRAP-related protein
TRP1	Thrombospondin-related protein 1
TRSP	Thrombospondin-related sporozoite protein
TSR	Thrombospondin type 1 repeat
TSS	Transcription start site
UIS	Upregulated in infective sporozoites
VWA	von Willebrand factor A
WHO	World Health Organization

Chapter I

Introduction

1. Malaria

1.1. Clinical features and epidemiology

Malaria has walked alongside mankind for several millennia, shaping our biology and history. This disease is caused by parasites belonging to the genus *Plasmodium*, which are transmitted to humans when female *Anopheles* mosquitoes probe the skin in search for blood. Although the links between the disease, the parasite and the vector were only made in the 20th century, there are records of fatal periodic fevers as well as other classic symptomatic features of malaria dating from as early as 2,700 BC (1). In fact, a comparative analysis of the genomes of several closely related *Plasmodium* species revealed that *Plasmodium falciparum*, the most predominant and deadly human-infective species, initiated speciation 40,000 to 60,000 years ago, although the acquisition of host specificity must have been a more recent event (2). The consequent evolutionary tug of war between the pathogen and its hosts provided one of the earliest proofs of human adaptation through natural selection. Some of the most notable examples are the reduced susceptibility to malaria of people carrying the mutations responsible for sickle cell anemia (3), α -thalassemia (4) or glucose-6-phosphate dehydrogenase deficiency (5), which resulted in the increased prevalence of these red blood cell disorders in malaria-endemic areas (6,7).

As was already alluded to, malaria is a life-threatening acute febrile sickness. The first symptoms are often mild and of unspecific nature, such as chills, high fever, fatigue, myalgia, headaches and sweating. These symptoms may be presented in successive and cyclical stages, which is termed the malarial paroxysm (8). Moreover, if left untreated, malaria caused by *P. falciparum* can rapidly evolve to severe illness, resulting in death. Signs and symptoms of severe malaria may include multiorgan failure, severe anemia and respiratory distress. Nonetheless, cerebral malaria is the most serious complication of this disease and is a neurological syndrome characterized by loss of consciousness, seizures, retinopathy and encephalitis. Symptoms can appear 6 to 15 days after infection and clinical deterioration, including neurological involvement, can develop as soon as 24 hours after the onset of fever, with small children being particularly susceptible (8–10).

It is likely that the human-infective and disease-causing *Plasmodium* species accumulated a death toll unrivalled by any other pathogen in the history of humanity. In the 20th century alone, malaria was estimated to have been responsible for 2 to 5% of all deaths worldwide (9). Yet, malaria is not a disease of the past. Despite considerable efforts lead by the World Health Organization (WHO) to gradually decrease the burden of the disease, with the end-goal of achieving largescale eradication, malaria remains one of the major global public health

problems and a substantial hurdle for socioeconomic development. The WHO estimates that in 2019 alone over 3.7 billion people, roughly half of the world's population, were at risk of developing malaria and there were 229 million cases that resulted in approximately 409,000 deaths (Fig. 1). Notably, children under the age of 5 accounted for two thirds of total fatalities caused by this illness (10).

While 21 countries have successfully eliminated malaria in the last 2 decades, this disease is still endemic to 87 nations in Africa, Southeast Asia, Central and South America, the Middle East, and Oceania, although most cases and deaths were reported in Sub-Saharan Africa (10). In actuality, the rapid population growth over the years in this African region meant that the number of cases per 1,000 population at risk decreased from 80 in 2000 to 58.6 in 2019. Even more encouraging was the steady decline of the mortality rate of malaria, which was more than halved in the same period. In fact, an estimated 1.5 billion cases were averted and 7.6 million lives were saved due to the global efforts to control the disease since the year 2000. Nonetheless, the targets set by the WHO for 2020 were not met. These milestones included a 5-year 40% reduction incidence and mortality, and the elimination of malaria from at least 10 countries in which malaria was still transmitted in 2015 (10).

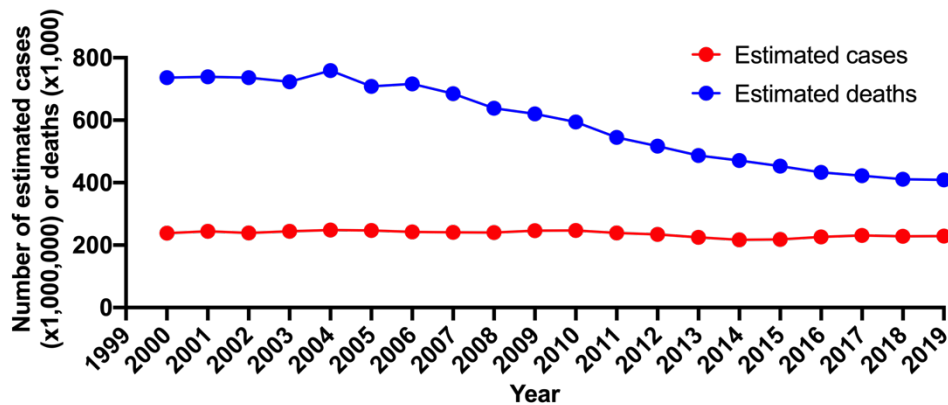


Figure 1. Global estimated numbers of malaria cases and deaths from 2000 to 2019. Data from WHO (10).

1.2. The parasite

Plasmodium spp. are obligate parasites belonging to the Apicomplexa phylum, which comprises a wide variety of protozoans including several other species that are pathogenic to humans, such as *Toxoplasma gondii* (11). Over 200 vertebrate-infective *Plasmodium* species have been identified and each displays a unique degree of host specificity (12). Of those, 5 species are widely accepted causative agents of malaria in humans: *P. falciparum*, *Plasmodium*

vivax, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. *P. falciparum* is responsible for most deaths, mostly in sub-Saharan Africa, although it also has significant clinical burden in most areas of the world endemic to malaria (Fig. 2A) (10,13). *P. vivax* is the second largest contributor to clinical malaria worldwide, the leading cause of recurring malaria, and the most prevalent species in South and Central America, as well as in Southeast Asia (Fig. 2B) (10,13,14). *P. malariae* and *P. ovale* infections are less prevalent and are thought to seldom cause severe complications, although their geographical distribution is widespread and they are common in coinfections with *P. falciparum* (15,16). *P. knowlesi* is the most recent addition to the list (17), as this macaque-infective species shares a lot of morphological similarities with *P. malariae* and was, consequently, historically misdiagnosed by microscopy in certain areas of Southeast Asia. *P. knowlesi* was, therefore, the first significant species proven to cause zoonotic malaria (18).

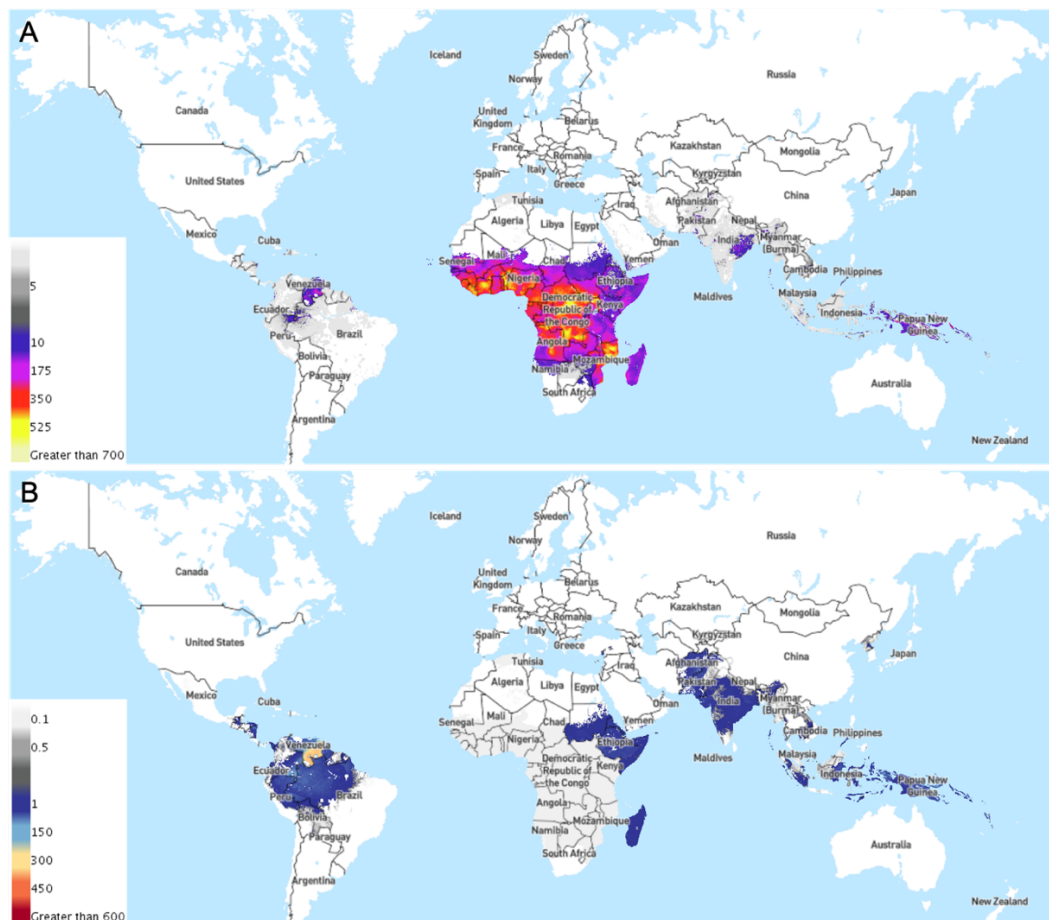


Figure 2. Incidence of *P. falciparum* (A) and *P. vivax* (B) infections per 1,000 people in 2019. Downloaded from The Malaria Atlas Project (19).

Similarly, other species that have claimed nonhuman primates as their natural hosts have been shown to be transmissible to humans either naturally or experimentally. These include *Plasmodium cynomolgi* (20–22), *Plasmodium inui* (23), and *Plasmodium simium* (24). The latter was recently proposed to be the second known etiological agent of zoonotic malaria, which had been overlooked once again due to morphological similarities with a human malaria species, in this case *P. vivax* (24). While it is possible that *P. simium* and *P. vivax* are the same species, a genome sequencing study revealed sufficient evidence that, at the very least, *P. simium* is undergoing speciation (25). On the other hand, *Plasmodium brasilianum* was also reported to be naturally transmitted to humans and to cause symptomatic infections, but most authors consider it to be the same species as *P. malariae* (26). Alongside the human-infective parasites, other *Plasmodium* species became experimentally relevant due to their phylogenetic proximity to the disease-causing species in humans or their usefulness in animal models of malaria. These include the nonhuman primate-infective species *Plasmodium reichenowi* and *Plasmodium coatneyi*, the rodent malaria species *Plasmodium berghei*, *Plasmodium yoelii* and *Plasmodium chabaudi*, and the avian malaria species *Plasmodium gallinaceum* (2,27,28). Despite significant biological diversity within the phylum, *Plasmodium* shares several important features with other apicomplexans. Among them is the differentiation into specialized invasive stages termed zoites (Fig. 3). These polarized cells possess the apical complex, a unique structure that defines their anterior pole and is composed of secretory organelles and apical polar rings (29). The apical complex of members of the coccidian subgroup of the Apicomplexa is also known to possess an elaborate conical structure composed of tubulin-rich fibers termed the conoid, which also partakes in host cell invasion (30). Fascinatingly, recent studies have demonstrated the conservation of conoid-associated proteins and conoidal structures in Apicomplexa, including *Plasmodium*, suggesting a wider distribution of canonical or divergent conoid complexes among the phylum's zoites (31,32). Amid the secretory organelles that sequentially discharge their contents through the apex of the parasite are the micronemes, which contain several proteins whose secretion is required for parasite motility, adhesion to and initiation of invasion of host cells; and the rhoptries, which are packed with proteins and lipids that are deployed during and after host cell invasion (33). In contrast, the secretion of the content of the dense granules, a third kind of secretory organelles, is thought to occur at a subapical level and by both regulated and constitutive mechanisms. Proteins stored in the dense granules have been proposed to play important roles in the maintenance of the parasitophorous vacuole, where the parasite develops once inside the host cell (34). Moreover, several Apicomplexa parasites also share a vestigial nonphotosynthetic plastid called apicoplast. This organelle contains a small circular genome

that encodes proteins required for plastid replication, and fatty acid and isoprenoid synthesis, among other less clear functions (35).

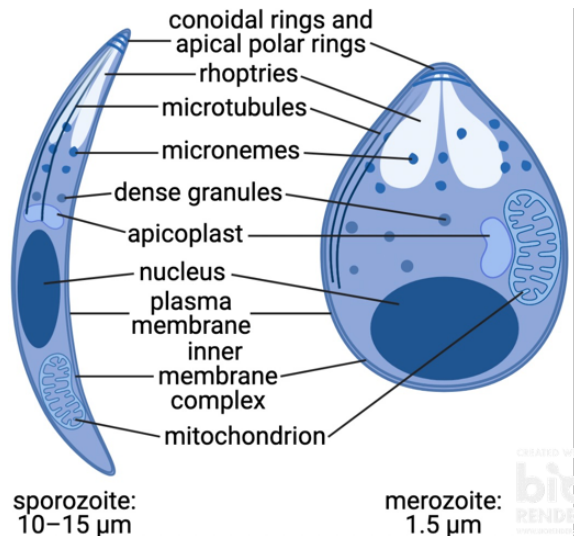


Figure 3. Structural features and length of the *Plasmodium* sporozoite and merozoite. The apical complex encompasses the conoidal and apical polar rings, and the polar secretory organelles (rhoptries and micronemes). The more distal of the two apical polar rings anchors the subpellicular microtubules. The positioning of the simplified conoid relative to the apical polar rings depends on the activation state of the zoite (31,32). The ookinete (not represented) constitutes the third *Plasmodium* zoite and lacks both the rhoptries and dense granules (36,37). Illustrations are not to scale and were constructed with BioRender.

Apicomplexan zoites, including *Plasmodium* sporozoites, merozoites and ookinetes, rely on an actomyosin-based motor to migrate through tissues and/or to enter or egress from host cells. This complex is also called the glideosome (Fig. 4). Most of the glideosome components are confined to the space between the plasma membrane and the inner membrane complex (IMC), which consists of flattened vesicles or alveoli visible by microscopy as double-membrane structures that are placed beneath the plasma membrane of the parasite and are interconnected to the cytoskeleton (38). Transmembrane adhesins secreted onto the plasma membrane of the parasite through the discharge of micronemes establish contact points with host cells and the extracellular matrix, bridging the actomyosin motor and the extracellular substrate (39). It has been proposed that *Plasmodium* parasites express these adhesins in a stage-specific manner, which include the thrombospondin-related anonymous protein (TRAP) in sporozoites, the circumsporozoite- and TRAP-related protein (CTRP) in ookinetes, and the merozoite TRAP-like protein (MTRAP) in the merozoite stage (40–42). Several glideosome-associated proteins (GAPs) and other components then anchor the complex to the IMC and help maintain the

structural integrity and functionality of the system (39). The tensile forces resulting from the interaction between the adhesins and the substrate are used to power the directional movement of the parasite, as myosin A pulls on short actin filaments through conformational changes that occur at the cost of ATP hydrolysis (Fig.4) (38). Thus, the speed at which sporozoites travel is dependent on the turnover of both the actin filaments and the focal adhesion sites formed by the adhesins, which are exported to the surface at the apical pole and translocated along the membrane toward the posterior end of the sporozoite, where they are shed likely through the action of a rhomboid protease (43–46). Moreover, this actomyosin motor is used by the parasite not only during migration but also to invade host cells. Indeed, it has also been shown that the phosphorylation state of myosin A also regulates force generation at the cost of speed. Thus, phosphorylated myosin A could allow motile forms of the parasite to move at high velocity, and when unphosphorylated it may provide the high force necessary for the parasites to enter host cells (47).

Gliding motility is, therefore, used by *Plasmodium* sporozoites and ookinetes during their migration through the tissues of their hosts. Additionally, these parasites can also move through host cells, in a process termed cell traversal. The capacity of these *Plasmodium* zoites to breach cellular barriers as they travel through host tissues is sustained by the release of molecules such as the aptly named sporozoite protein essential for cell traversal (SPECT) and the perforin-like protein 1 (PLP1), also called SPECT2, and the cell traversal protein for ookinetes and sporozoites (CelTOS), among others (48–53). Sporozoites have been shown to enter host cells by a process that is mechanistically different from productive invasion (described in more detail in section 2.2.4 of this chapter), with the formation of a transient vacuole that differs compositionally from the parasitophorous vacuole required for parasite development inside an invaded host cell. Subsequently, pore-forming molecules such as PLP1 are released upon lysosomal acidification, the vacuole is destroyed, and the parasite breaches the cell membrane on its way out (52). This facilitates the crossing of certain barriers the parasites may encounter on their way to their target tissues.

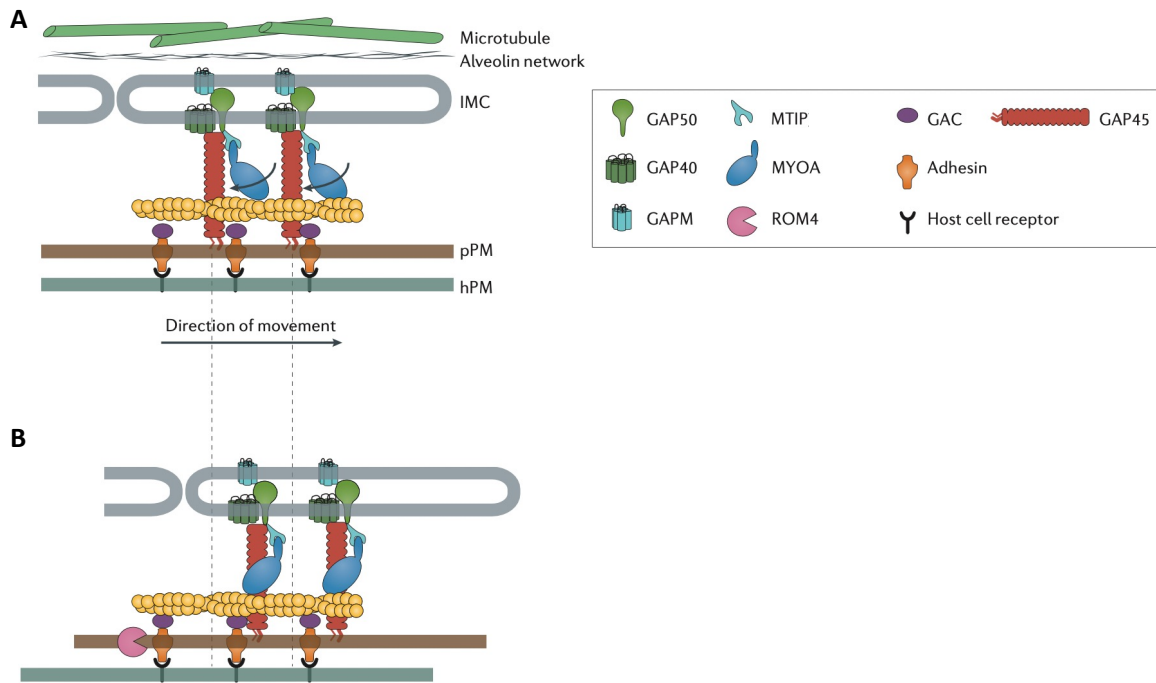


Figure 4. The *Plasmodium* glideosome complex. Transmembrane adhesins present on the parasite plasma membrane (pPM) interact with a substrate, such as a host cell plasma membrane (hPM), through their ectodomains and indirectly with actin likely via linker such as the glideosome-associated connector (GAC), which interacts with the C-terminus of the adhesins. Several glideosome-associated proteins (GAPs) anchor the single-headed myosin A (MYOA) to the IMC, through the MYOA-tail interacting protein (MTIP). The head of MYOA suffers conformational changes with the hydrolysis of ATP, which moves the actin filaments rearwards (A). This results in the forward movement of the parasite, as other glideosome components present in the IMC such as GAPs with multiple membrane spans (GAPM) then link the IMC to the cytoskeleton of the zoite, while GAP45 spans across the space between the IMC and the pPM to ensure the structural integrity of the complex. The interaction with the substrate is then disengaged through the cleavage of the transmembrane domain of the adhesin via the action of proteases such as rhomboid protease 4 (ROM4; B). Adapted from Frénal *et al.* with permission (38).

1.3. The vector

Together with humans and the parasite, the mosquito vector completes the transmission triad, which was unveiled by the pioneering works of Charles Laveran in 1880, who discovered the parasite, and Ronald Ross in 1897, who first implicated mosquitoes in transmission (1). It is now believed that mosquitoes belonging to the *Anopheles* genus are solely responsible for the

transmission of the parasite. However, only around 70 of the nearly 500 recognized species of anophelines are known to be capable of successfully serving as transmission vectors (Fig 5), although only approximately 40 are considered vectors of major importance (54,55). This vectorial capacity of the anopheline mosquitoes has been closely associated to extensive genome introgression, or the flow of genes within closely related species (56). This phenomenon constantly shapes the boundaries between the *Anopheles* species and promotes adaptation between the parasite, the vector and the host (55,56). Therefore, the vectorial capacity and geographical distribution of *Anopheles* determine the global spread the different *Plasmodium* species. Together with mosquito anthropophilia, life span and abundance, these factors define the endemic areas of malaria (57).

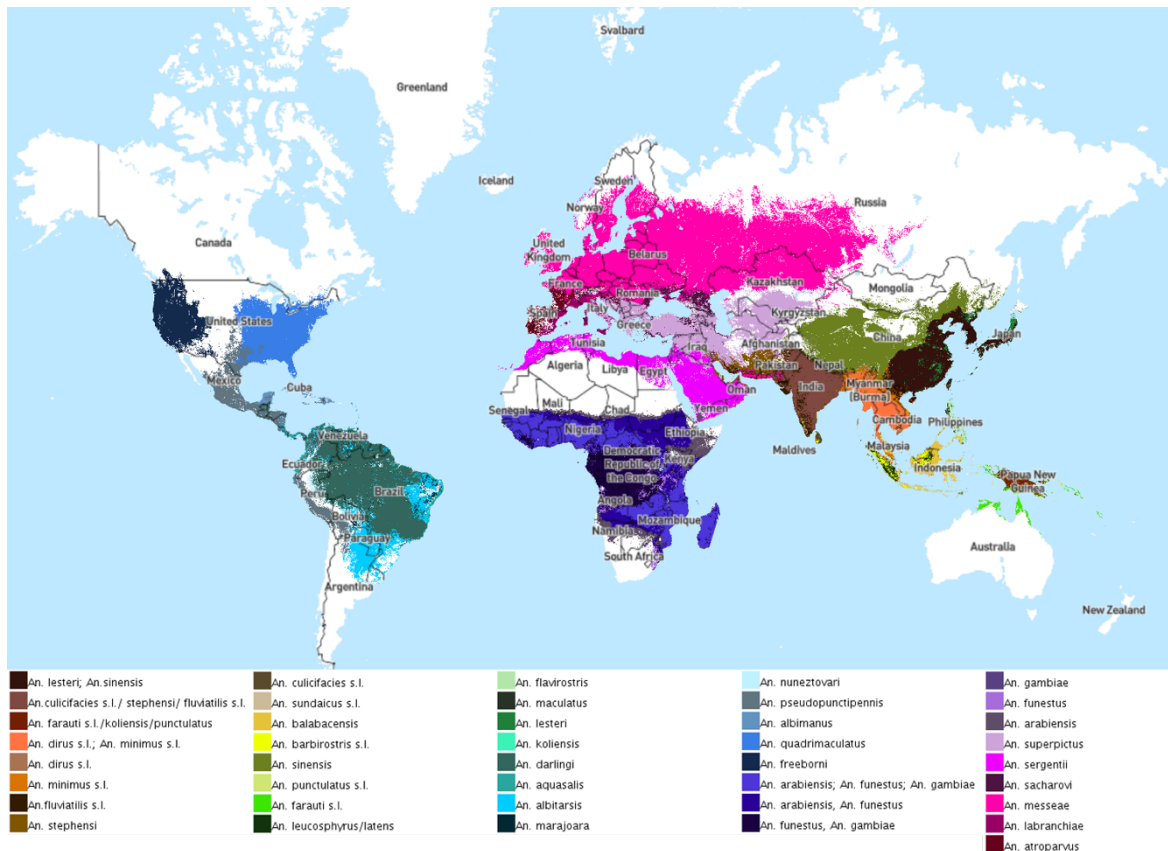


Figure 5. Distribution of the dominant malaria vector species in 2010. Downloaded from The Malaria Atlas Project (19).

The laboratory colonization of several *Anopheles* species allowed for the study of the entire life cycle of the parasite. Indeed, the broad vectorial capacity of laboratory-adapted anopheline mosquito species of major importance as malaria vectors, such as *Anopheles gambiae* and *Anopheles stephensi*, facilitated the study of parasite transmission and development in the

vector as they are also susceptible to infection by rodent-infecting *Plasmodium* species (58). The interactions between the parasite and its invertebrate host are discussed below.

2. The life cycle of *Plasmodium* parasites

The extremely intricate life cycle of *Plasmodium* parasites involves two distinct hosts, which harbor several developmental stages capable of specifically colonizing a variety of tissues (Fig. 6).

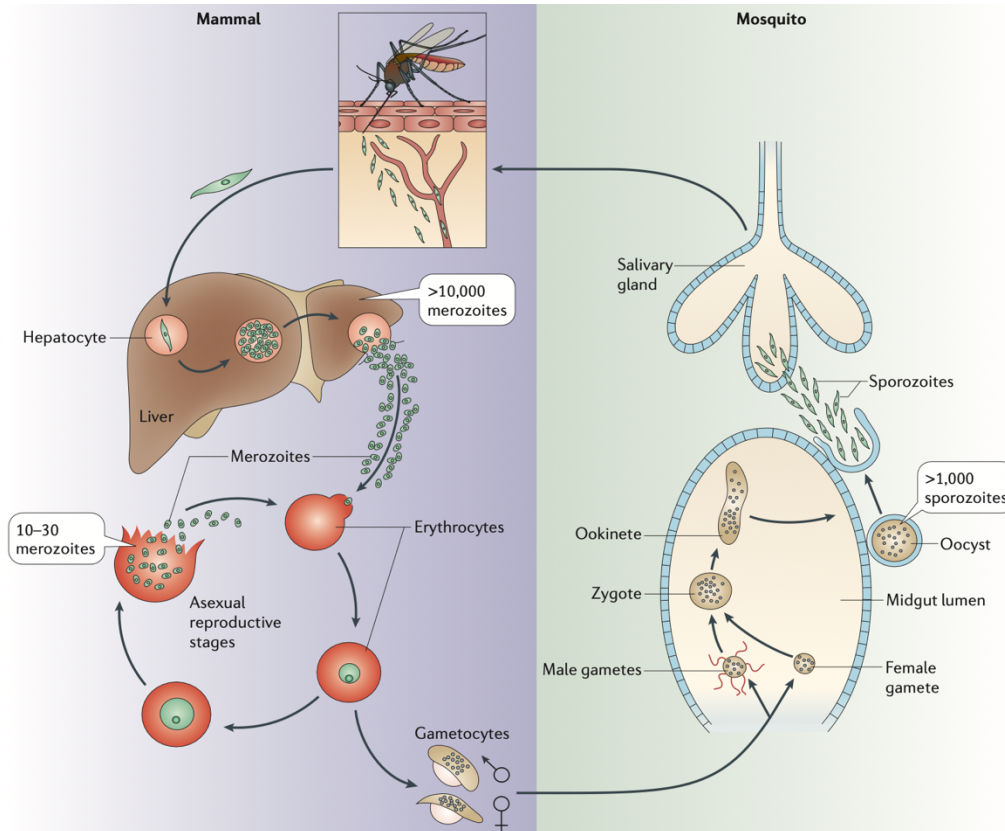


Figure 6. The life cycle of mammal-infecting *Plasmodium* species. Sporozoites inoculated into the skin by a female anopheline mosquito make their way to the liver through the bloodstream, where they generate thousands of erythrocyte-infecting merozoites. Merozoites either multiply or commit to sexual differentiation within erythrocytes, so the parasite can be transmitted to the mosquito. In the vector, male and female gametes fuse and form the zygote, which transforms into a motile ookinete that develops into a sporozoite-producing oocyst in the midgut wall of the mosquito. Newly formed sporozoites are released into the mosquito's hemolymph and invade the salivary glands to start a new cycle. Adapted from Ménard *et al.* with permission (59).

2.1. Development within the mosquito vector

The female *Anopheles* mosquito draws blood from a vertebrate host in order to support egg development. When this host is infected with *Plasmodium*, the mosquito may also ingest the circulating mature sexual forms of the parasite, the male and female gametocytes, also called microgametocytes and macrogametocytes, respectively. Fascinatingly, it has been shown that *Plasmodium* infection renders the host more attractive to female *Anopheles* mosquitoes, through the production of volatile signals. Thus, by modulating the mosquito's blood-seeking and feeding behaviors, the parasite can increase the odds of transmission (60–63).

2.1.1. In the mosquito midgut

Once in the gut of the mosquito, changes in temperature, pH and composition of the extracellular environment, namely the exposure to xanthurenic acid, quickly induce gametocyte activation (64). Activated macrogametocytes and microgametocytes egress from the host cell by proteolytic rupture of the parasitophorous vacuole (65) and the subsequent disintegration of the enveloping red blood cell membrane through the release of pore-forming molecules (66). During this process, female and male gametocytes convert into macrogametes and microgametes, respectively (Fig. 6). Microgametocytes undergo the most extreme metamorphosis, as they replicate their genome three times and mitotically generate eight flagellated and motile haploid microgametes, in a process termed exflagellation (67). Fertilization occurs when an individual microgamete finds and fuses with a macrogamete, originating a diploid zygote that within 2 hours duplicates its genome and becomes tetraploid. Since sexual reproduction occurs at this stage, the mosquito constitutes the primary host of the parasite (68).

In a process that can last less than a day, the zygote immediately starts to differentiate into an ookinete. In fact, it is during early ookinete differentiation that nuclear replication and two rounds of chromosome segregation by meiosis occur, resulting in four haploid genomes (69). The ookinete now also possesses an apical complex (without rhoptries) and the IMC, both of which play major roles in conferring invasive and motile properties to this form of the parasite, as previously mentioned (70). An imposing roster of adhesins, perforins and hydrolytic enzymes allow the ookinete to leave the blood meal and penetrate the midgut wall. Once the ookinete has invaded the midgut epithelium, it moves towards the basal lamina (64). To do this, the ookinete can modify its motile behavior, namely the pattern and speed of movement, in response to the different environments it encounters (71). It has been proposed that once the ookinete reaches the midgut epithelium, it switches from a circular motility pattern to a more

linear type of movement, allowing it to increase the displacement and reach the basal lamina, where the ookinete forms an oocyst that protrudes into the hemocoel of the mosquito (Fig. 7A-C) (71).

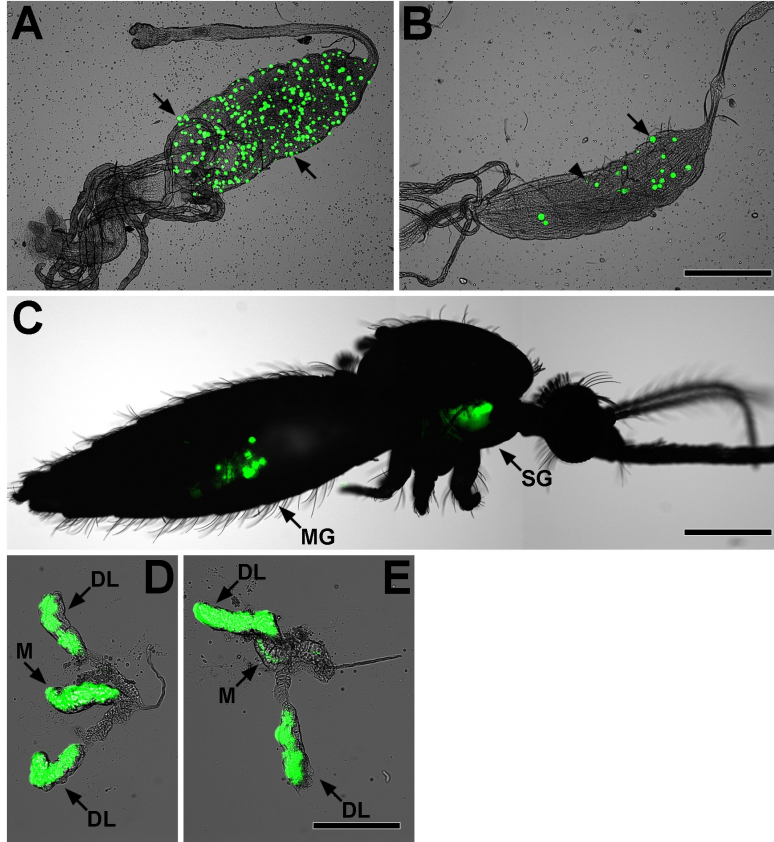


Figure 7. Development of *P. berghei* in *Anopheles funestus* mosquitoes. (A) Mosquito midgut containing oocysts expressing a green fluorescent protein (GFP; e.g., arrows) at day 7 post-infectious blood meal. (B) Infected midgut at day 20 post-infection showing full (e.g., arrow) and ruptured (e.g., arrowhead) oocysts. (C) Imaging of fluorescent parasites through the cuticle of a live mosquito, showing parasite development in the midgut (MG) and salivary glands (SG) at day 20 post-infection. (D, E) Infected salivary glands at day 20 post-infection. Panel E shows the preferential accumulation of fluorescent sporozoites in the distal lateral (DL) lobes compared to the median (M) lobe of the salivary gland. Bars: A-C = 500 μ m; D,E = 200 μ m. Adapted from Xu *et al.*, with permission (72).

Then, asexual replication by sporogony ensues inside the oocyst. The parasite starts to undergo multiple rounds of nuclear division by mitosis and grows. Simultaneously, invaginations from the plasma membrane of the oocysts form several sporoblasts, which are cytoplasmic islands that contain the replicating DNA. Haploid sporozoites, the next invasive and motile stage of the parasite, then start to bud from the sporoblast in successive waves as the IMC and microtubules

form beneath evaginations in the sporoblast membrane (73). This process eventually generates upwards of 1,000 individual sporozoites that completely fill the mature oocyst (74). Full oocyst maturation can take between 1 and 3 weeks depending on the human- or rodent-infective *Plasmodium* species (75,76).

2.1.2. Road to the salivary glands

Sporozoite egress from oocysts appears to come as a consequence of the induction of both sporozoite motility and degradation of the oocyst wall, and ultimately results in the release of the parasites into the hemocoel (77). Parasites are then passively transported by the hemolymph to the salivary glands. While sporozoites have been detected in most mosquito tissues due to their presence in the insect circulatory system, they preferentially accumulate inside the salivary glands (Fig. 7C-E), suggesting a receptor-mediated mechanism of salivary gland recognition, although this process may also be aided by the directionality and speed of hemolymph flow (78,79). Indeed, while the molecular mechanisms that regulate the binding to and invasion of the salivary glands by hemolymph sporozoites has not been fully elucidated, molecules from both the parasite and the mosquito have been implicated in these processes. Two of these sporozoite molecules are TRAP (40,43,80–82) and the circumsporozoite protein (CSP), which forms a dense coat on the surface of the sporozoite (83–89). On the host side, saglin has been shown to interact with TRAP and to be important for salivary gland invasion (90,91), although the spatial distribution of this protein in the salivary glands does not coincide with the preferential entry sites of the sporozoite, the distal lateral lobes of the salivary glands (92). Similarly, attempts to prove the existence of receptors for CSP in the salivary glands resulted in the identification of the mosquito CSP-binding protein (CSPBP), which is expressed in the distal region of the lateral and medial lobes of the salivary glands (88). The complete list of sporozoite proteins suggested to partake in salivary gland invasion to date is shown in Chapter II, section 2.3. These include the membrane associated erythrocyte binding-like protein (MAEBL), several rhoptry neck proteins (RONs) and additional adhesins containing a thrombospondin type 1 repeat (TSR), among other molecules.

Once the sporozoite has recognized a salivary gland, it crosses the basal lamina, travels to the space between this layer of extracellular matrix and the secretory cell, loses its thick coat and enters the host cell. Early studies indicated that this entry was made through a vacuole (93). Other authors, however, postulated that a halo of cytoplasmic material associates with the sticky coat of the sporozoite, until it is shed again, creating the illusion of the parasitophorous vacuole observed by electron microscopy (94). Next, the sporozoite binds to the cytoplasmic

side of the plasma membrane of the acinar cell and the parasite escapes the host cell. Free sporozoites, now covered with a thinner CSP coat, accumulate in the secretory cavities of the distal regions of the tri-lobed salivary glands (93). Here they can interact with each other, forming pairs or large bundles of dozens of sporozoites, often in spiraling arrangements analogous to those observed in mature oocysts (77,94). However, in contrast to the coordinated movement of sporozoites inside the oocyst observed just before egress, the motility of sporozoites within the salivary glands is weak and is only displayed by a fraction of the population (95). Indeed, salivary gland sporozoites have also been shown to exhibit different types of motility depending on their organization and spatial distribution, as most motile sporozoites reside outside of the parasite aggregates (95). Moreover, sporozoites seem to attain a transcriptional quiescence in the salivary glands of the mosquito, being awakened during salivation and subsequent transmission to the vertebrate host (96). While the importance of the interactions between sporozoites residing in the secretory cavities of the salivary glands and how they relate to transcriptional dormancy and motility are currently unclear, it is possible that they may constitute a regulatory mechanism of sporozoite fitness and infectivity. Additionally, exogenous factors such as saliva components have also been shown to modulate sporozoite motility and transmission (97,98).

These large clumps of parasites may also block the exit of the secretory cavity due to the architecture of the salivary glands and, consequently, limit the access to the salivary ducts and the number of sporozoites available for transmission (94). This could explain why only a small percentage of the total number of salivary gland sporozoites is ejected with the saliva and transmitted to the vertebrate host (95,99,100). Limiting the number of sporozoites available for transmission could constitute an advantage for the parasite as this would avoid the depletion of the sporozoite pool inside the glands, consequently expanding the number hosts that could potentially be infected.

2.2. The pre-erythrocytic phase

2.2.1. The skin stage

As the infected female *Anopheles* repeatedly probes the skin, it ruptures dermal blood vessels, creating hematomas from which it takes its blood meal (101). As it does so, the mosquito also ejects saliva, which contains a complex mixture of proteins that prevents hemostasis and inflammation (102). The few sporozoites present in the medial and proximal regions of the salivary apparatus take advantage of this process to find their next host, by using saliva as a transmission vehicle. This way, these parasites are deposited into the skin, both in the dermis

and the epidermis, as well as in the subcutaneous tissue (100,103,104). Interestingly, re-ingestion of sporozoites has been observed during the uptake of blood by the mosquito. Since this phenomenon is less likely to happen during the mere probing of the avascular tissue and most sporozoites are discharged before salivation stops, the quick localization of a blood source by the mosquito may result in a decrease in the number of sporozoites left in the skin (101,105). Most sporozoites, however, avoid re-ingestion and can then suffer different fates. They may remain in the skin or leave this organ through the lymphatic system or the bloodstream (Fig. 8). In order to exit the skin, *Plasmodium* sporozoites make use of their characteristic gliding motility. Sporozoites can travel large distances in the skin following irregular paths, at a speed of 1–2 $\mu\text{m/s}$ (100,101,106). This contrasts with the circular and faster motility (2–3 $\mu\text{m/s}$) observed on glass surfaces (107), or the low-speed locomotion ($\sim 0.1 \mu\text{m/s}$) displayed inside the salivary glands (95). Similarly to what was described for ookinete motility, sporozoite locomotion is also influenced by the stiffness of substrate (71), as well as the spacing between the adhesion sites (108). The directionality and efficiency of sporozoite motility has also been linked to the curvature of the parasite and the tilting of the polar rings, which creates a dorso-ventral polarity that skews the secretion of micronemes towards the substrate in a 2D system (109).

The second biological process sporozoites rely on to escape from the skin is host cell traversal. SPECT- and PLP1/SPECT2-deficient sporozoites were shown to be able to enter dermal cells, presumably by active invasion or phagocytosis, but failed to exit from the skin and thus remained in the inoculation site, indicating that host cell traversal is required to avoid the premature arrest of the parasites in this stage of the life cycle (106). In accordance with these results, sporozoites have been observed migrating through skin cells, most of which fail to reseal their membranes (110).

The extensive exodus of sporozoites from the skin within the first 30 minutes post-inoculation was demonstrated using rodent models and various experimental setups, although appreciable numbers keep entering blood vessels for up to 3 hours (100,103,106,111,112). Although the motility of sporozoites does not appear to be constrained by the species specificity of parasite-host interactions that occur during the invasion process (addressed later in this chapter), for example, the duration of migration and, subsequently, the timing of the departure from the skin varies among *Plasmodium* species (113).

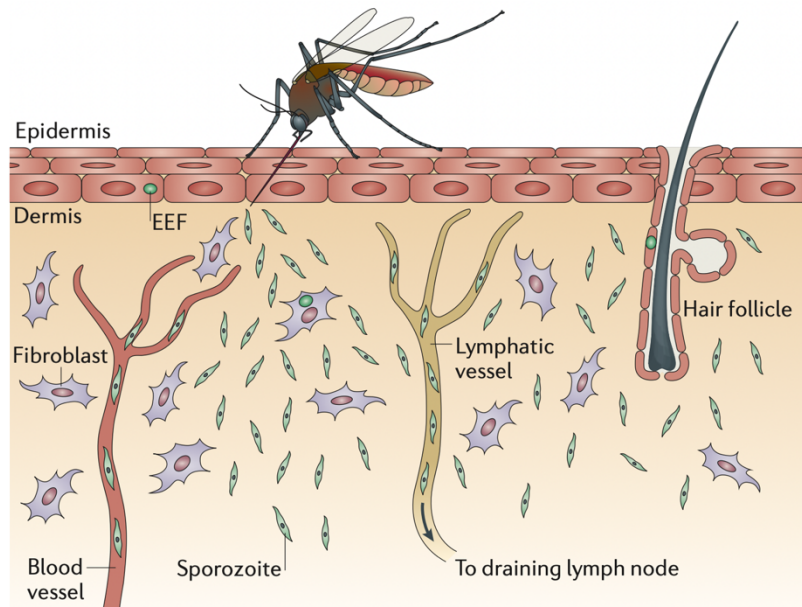


Figure 8. Fates of *Plasmodium* sporozoites deposited in the skin. Sporozoites need to find blood vessels to reach their next target organ, the liver. However, sporozoites may also invade the lymphatics, which constitutes a developmental dead end for the parasite. Most sporozoites that fail to exit the skin eventually die, although some invade skin cells and complete their intracellular replication. Adapted from Ménard *et al.* with permission (59).

Up to 20% of sporozoites inoculated in the skin are estimated to invade lymphatic vessels and reach the proximal draining lymph node (100,112). These sporozoites exhibit a decline in gliding motility over time, suffer morphological changes, and after 8 hours most are eliminated, most likely due to the action of lymph node-resident dendritic cells (100). The uptake of sporozoites by these immune cells enables antigen presentation to CD8+ T cells, which are important mediators of resistance to *Plasmodium* infection (114–117). Furthermore, it has been shown that CD8+ T cells primed in the skin-draining lymph nodes migrate to the liver and could be enough to induce sterile protection against sporozoite infection alone, without a significant contribution of T cells primed by infected hepatocytes (115,116). Remarkably, some sporozoites can infect lymphatic endothelial cells and convert into extra-hepatic exoerythrocytic forms (EEFs) (100). Yet, the skin-draining lymph nodes do not seem to be conducive to the proper development of the parasite, as these EEFs start to present signs of degeneration 24 hours post-infection and by the time the liver stages attain full maturity parasites can no longer be detected in these lymphoid organs (100).

A significant fraction of the sporozoites stay in the skin, where they can be detected for up to 7 hours post-injection (100,112,118). Moreover, sporozoites of the avian-infecting *P. gallinaceum* are known to invade and develop inside dermal macrophages (119). In fact, a small proportion

of rodent-infective *Plasmodium* sporozoites can infect skin cells, presumably fibroblasts and keratinocytes, and develop into small EEFs (59,106,118). Curiously, skin EEFs not only localize to the dermis and the epidermis but can also be found in association with the hair follicles, likely because these constitute immunoprivileged sites due to the production of immunosuppressive agents and the near absence of major histocompatibility complex (MHC) class I expression (Fig. 8) (118,120). Even more striking was the discovery that parasites that develop in the skin, contrarily to those that infect draining-lymph nodes and despite their smaller size in comparison to their hepatic counterparts, can generate merozoites fully capable of infecting erythrocytes (118,121). Be that as it may, the real contribution of these merozoites to the onset of the erythrocytic phase of infection is unlikely to be significant as they are outnumbered by the thousands of merozoites produced in the liver (118). Moreover, it remains to be determined if this process also occurs in species that cause human malaria. Nonetheless, if the skin is in fact an environment suitable for parasite multiplication, it may have some implications in the context of chemotherapy and vaccine development, as skin EEFs could evade immune responses induced by vaccines targeting the pre-erythrocytic stage and these parasites have been shown to be more resistant to treatment with primaquine, a drug known to be effective against the liver stages (118,121). Therefore, even though the skin stage is commonly seen as a transient step of the pre-erythrocytic phase of the life cycle, the existence of a bonafide replicative phase in the skin could serve as a parasite reservoir and a source of breakthrough parasites.

Finally, the larger part of the sporozoites that manage to depart from the skin do so through the bloodstream (105,112). Sporozoites have been observed decreasing their speed and gliding along or around blood vessels, often for significant periods of time, before quickly invading them (100,122). Of note, while cell traversal is required for sporozoites to find dermal vasculature, it appears to be dispensable for the crossing of both dermal lymphatic vessels and blood vessels (106).

2.2.2. Homing of sporozoites to the liver

Sporozoites accumulate in the liver following blood dissemination (123). However, the molecular mechanisms that allow circulating sporozoites to home to this organ and the dynamics of sporozoite retention in the liver sinusoids have not been completely clarified. Two adhesive proteins present on the surface of sporozoites – CSP and TRAP – have been proposed to specifically interact with molecules expressed by hepatic cells, but evidence of their importance for the selectivity of the interactions between sporozoites and the hepatic sinusoids

is lacking. The available data, limitations of existing studies and unanswered questions regarding the role these proteins on the arrest of sporozoites in the hepatic microvasculature are discussed below. Overall, the current model is based on *in vitro* experiments or indirect *in vivo* approaches often aiming to evaluate the outcome of the liver infection.

2.2.2.1. The role of CSP

CSP (Fig. 9) has been appointed as the main intermediary of the specific interaction between sporozoites and the hepatic microvasculature. CSP contains an adhesive TSR domain between its glycosylphosphatidylinositol (GPI) anchor at C-terminus and a long stretch of tandem repeats, whose composition varies according to the species of the parasite. It also possesses an N-terminal domain, which is flanked by a signal peptide and a small, conserved region termed RI (124). Within the TSR, which has been shown to be glycosylated and is located downstream of RIII, CSP harbors RII+ (124–126). RIII was proposed to fold over the TSR to form the α TSR, changing the adhesive properties of this domain (125).

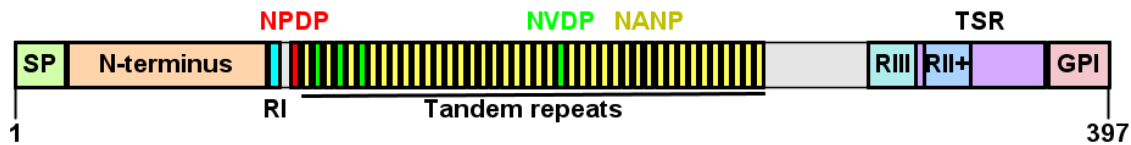


Figure 9. Structure of the *P. falciparum* CSP. The structural elements of the CSP of *P. falciparum* 3D7 strain were obtained from available literature and databases (125,127,128) and the scheme was built with IBS online (129). The numbers represent the first and last residues of the protein. GPI: GPI anchor. NANP: asparagine-alanine-asparagine-proline major repeat. NPDP: asparagine-proline-aspartic acid-proline motif. NVDP: asparagine-valine-aspartic acid-proline repeat. SP: signal peptide.

The first associations between the major sporozoite surface protein and the ability of sporozoites to target the liver were made based on the demonstration that recombinant *P. falciparum* CSP specifically bound to the sinusoids in liver sections and that RII+ was required for this interaction (130–132). Several attempts to pin down the molecular mechanisms behind the binding lead to the postulate that CSP interacts with the highly sulfated heparan sulfate proteoglycans (HSPGs) expressed by liver cells that protrude from the fenestrae of the sinusoidal endothelial cells (133–140). HSPGs are plasma membrane and extracellular matrix components comprised of a core protein containing one or more heparan sulfate glycosaminoglycan (GAG) chains (141). Although these macromolecules are ubiquitously expressed by mammalian cells, the chemical structure of their GAG chains can be extremely

diverse, conferring different binding properties to HSPGs (141). Consequently, the predilection of CSP for the liver sinusoids was linked to characteristically high degree of sulfation of the GAG chains of hepatic HSPGs (142–144). Indeed, *P. falciparum* and *P. yoelii* CSP as well as sporozoites of rodent malaria species were found to adhere to the surface of hepatocytes in a manner that is dependent on HSPG expression and sulfation (133,134,145), although sporozoites were still capable of invading cells that do not express GAGs, albeit less efficiently (146). Moreover, heparin, a naturally occurring highly sulfated GAG, was shown to compete with CSP and sporozoites for the binding to HSPGs and hepatocytes, particularly under flow conditions (133,134,136). Accordingly, it was initially proposed that the cluster of positively charged amino acids in the C-terminus of the RII+ portion of the TSR was responsible for the interaction with the highly sulfated and, thus, anionic GAG chains of the HSPGs (43,132,134,135).

However, a study using recombinant *P. falciparum* CSP fragments lacking different regions of the protein suggested that a major binding motif of CSP is located in its N-terminus and demonstrated that the deletion of the TSR did not negatively impact the interaction of the CSP segments to these receptors nor the invasion of liver cells by sporozoites in competition assays (138). It has equally been suggested that RI could be directly involved in this interaction as RI peptides of the *P. falciparum* CSP bound to hepatocytes, heparin and heparan sulphate, and prevented the binding of other CSP fragments to HSPGs (140,147). A cleavage site required for CSP processing, which results in the loss of most of the N-terminal sequence, was suggested to be positioned within or upstream of RI (87,148). This proteolytic cleavage was shown to be induced by contact with GAGs and was decreased by the deletion of RI itself, which also led to impaired sporozoite infectivity both *in vitro* and *in vivo* (149,150). Similarly, the use of a cysteine protease inhibitor also negatively affected the invasion of hepatocytes (149).

Intriguingly, it was then proposed that CSP already undergoes processing and, consequently, conformational changes during sporozoite development in the mosquito (Fig. 10) (148). This implies that sporozoites already possess a processed version of CSP when they reach the hepatic sinusoidal capillaries. Indeed, the generation of a line lacking the entire N-terminal sequence of the protein upstream of RI, constitutively exposing the TSR, exhibited increased infectivity *in vivo* when injected intravenously, which hinted once again at the dispensability N-terminus for the homing of sporozoites to the liver and host cell invasion. However, these mutant sporozoites failed to exit the skin when injected intradermally (150), revealing that the portion of the N-terminus not removed by proteolytic cleavage could hypothetically mask the TSR and, consequently, prevent it from sticking to the host tissues prior to the arrival in the liver, possibly through folding of the protein (148,150). These somewhat confounding data indicate that, in

the context of infection, the specificity and affinity of CSP binding to hepatic GAGs may involve the orchestration of post-translational modifications, conformational changes and interactions promoted by different regions of the protein.

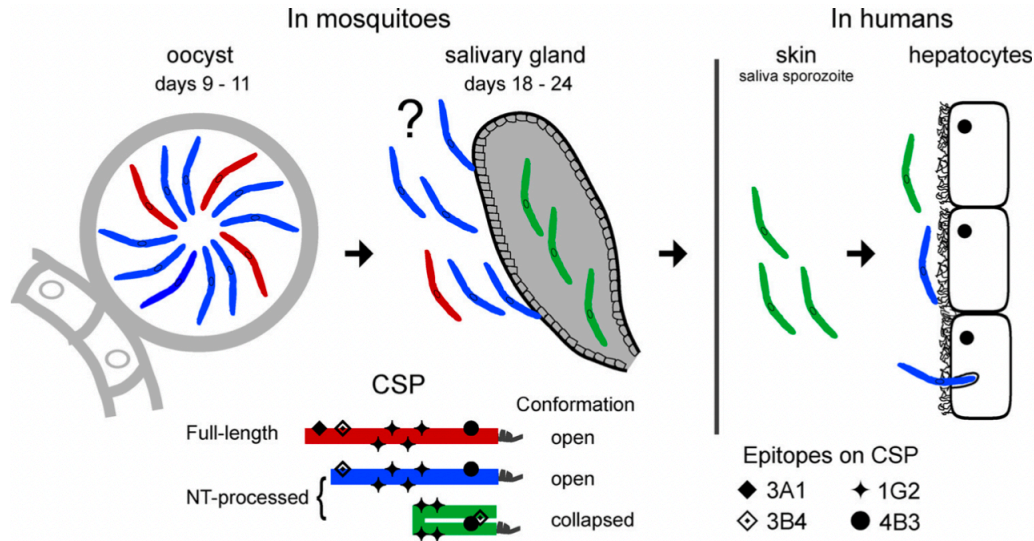


Figure 10. CSP processing and conformational changes. The locations of epitopes of various CSP-specific monoclonal antibodies are shown at the bottom. The question mark indicates that the form and conformation of CSP in hemolymph sporozoites are speculative. Adapted from Herrera *et al.*, with permission (148).

CSP was shown to bind not only to hepatocytes but also to stellate and Kupffer cells, although these interactions involved distinct types of proteoglycans presenting various degrees of sulfation (138). Interestingly, CSP did not bind to the endothelium (131,138), possibly due to the undersulfation of the HSPGs of endothelial cells (151). The fact that CSP accumulates in the subendothelial space of Disse (130), which holds extracellular matrix, and, more importantly, the basolateral surface of hepatocytes and stellate cells, is consistent with these observations. Thus, the highly sulfated HSPGs responsible for the binding to CSP are mainly produced by liver parenchyma cells. It has, therefore, been proposed that the fenestrations of the hepatic sinusoids grant circulating sporozoites partial access to the perisinusoidal space and, consequently, to the HSPGs needed for their arrest in the liver. Microvilli and cell projections can protrude through these small openings arranged in sieve plates and into the sinusoidal lumen (152,153), theoretically allowing sporozoites to interact with parenchyma cells, much like what has been described for lymphocytes (153). This is supported by the lack of accumulation of CSP around bigger blood vessels, which are not fenestrated (131). Moreover, the absence of fenestrations in other tissues may explain why sporozoites target the liver so efficiently, even

though CSP was able to bind to extravascular kidney tissues *ex vivo* (131,134) and the brain also presents particularly high sulfation levels (144).

However, several aspects of the hypothesis that CSP is the mediator of sporozoite accumulation in the liver still await clarification. Firstly, CSP has orthologues in *Plasmodium* species that do not display exclusive tropism for hepatocytes, such as the avian-infecting *P. gallinaceum* (154). The substitution of the *P. berghei* CSP by the *P. gallinaceum* orthologue resulted in a marked decrease in salivary gland invasion by sporozoites and infectivity to mice following intravenous injection. However, a chimeric version of CSP containing the RIII, TSR and C-terminus of the *P. gallinaceum* CSP and the repeats and N-terminus of the *P. berghei* protein partially regained infectivity to mice (87). Secondly, it has been proposed that the conformational changes that expose the TSR of CSP enable the interaction between this domain and the hepatic HSPGs. However, the TSR of oocyst sporozoites was proven to be accessible to antibodies (148,150) and yet these immature sporozoites are not retained in the liver sinusoids (Sá *et al.*, in preparation). Thus, CSP may not be the sole contributor to the liver-specificity of *Plasmodium* sporozoites.

2.2.2.2. The role of TRAP

TRAP (Fig. 11) is another protein long thought to be implicated in the preference of sporozoites for the liver. The deletion of the entirety or parts of the *trap* gene disclosed its importance for sporozoite motility, salivary gland invasion and infectivity to the mammalian host (40,80). This micronemal single-span transmembrane protein contains a TSR (155,156) that binds GAGs and sulfated glycoconjugates on the surface of hepatocytes (138,157,158). Additionally, TRAP possesses a second adhesive domain, the von Willebrand factor A (VWA) domain or simply A domain (80), which belongs to a subset VWA domains common in integrins (I domains) and is characterized by the presence of a metal ion-dependent adhesion site (MIDAS) necessary for ligand recognition (81,159). The I domain of TRAP was also shown to interact with GAGs *in vitro*, although the binding was proposed to occur through a secondary binding site as this interaction occurred independently of divalent cations (160,161). However, the relevance of such interactions was questioned by the observation that sporozoites expressing TRAP carrying a mutation in a residue associated to TSR function invaded sulfated proteoglycan-expressing or -deficient cells similarly, indicating that the invasion promoted by the I domain is independent of interactions with GAGs (81). This mutation did not impact oocyst sporozoite infectivity *in vivo* despite decreasing invasion of cells *in vitro*, suggesting that the TSR does not mediate sporozoite arrest in the liver (81). Similarly, the mutation of a sequence that encompasses several key residues of the TSR only lead to a small reduction of infectivity of

oocyst sporozoites to mice (80). Conversely, mutations in the MIDAS motif revealed that TSR-driven host cell invasion could be dependent on the presence of glycoconjugates. These lines exhibited severe impairments in the invasion of both the salivary glands and mammalian cells but not in adhesion, which also shows that interactions with HSPGs mediated by other hypothetical binding sites of the I domain do not appear to be responsible for the role of this region of the TRAP ectodomain in sporozoite infectivity (81). In conclusion, both the TSR and the I domain of TRAP seem to be required for invasion, albeit via different pathways, as the combination of mutations in the two domains completely abolished hepatocyte infection *in vitro* (81). The importance of these domains for sporozoite homing to the liver, however, remained unclear.

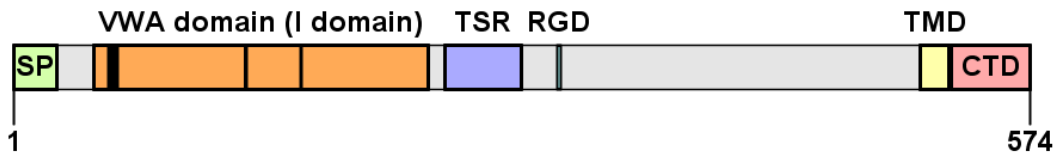


Figure 11. Structural features of the *P. falciparum* TRAP. The structural elements of TRAP from the *P. falciparum* 3D7 strain were obtained from available literature and databases (77,80,81,128) and the scheme was built with IBS online (129). The black bars within the I domain represent the invariable residues of the MIDAS, which span over several loops. The arginine-glycine-aspartic acid motif (RGD) is not conserved among *Plasmodium* species (162). TRAP also contains a proline-rich stalk between the RGD and the transmembrane domain (TMD) with repetitive sequences, which are also species-specific (128). The numbers represent the first and last residues of the protein. CTD: C-terminus domain. SP: signal peptide.

Both the TSR and I domains were also shown to bind to the human platelet-derived growth factor receptor β (hPDGFR β) *in vitro* (163). Despite being expressed by endothelial cells, hPDGFR β was not detected in the liver sinusoids and this binding was not conserved across human- or rodent-infective *Plasmodium* species (163). Therefore, this receptor is unlikely to dictate the arrest of sporozoites in the hepatic microvasculature. Likewise, the interaction between integrins and the *P. falciparum* TRAP, more specifically the I domain and the RGD motif, revealed a potential role for this receptor-ligand complex in the sporozoite migration through the skin rather than interaction with hepatocytes (162). Moreover, this binding was not observed using several TRAP orthologues, so this species-specific interaction cannot by itself explain the genus-spanning accumulation of sporozoites in the liver (162).

The cytosolic C-terminus domain (CTD) also proved to be required for TRAP function. The CTD contains a sorting motif necessary for the correct localization of TRAP to the micronemes and,

consequently, to the sporozoite surface (164). Furthermore, it has been proposed that the interaction between the CTD and intracellular aldolase allows TRAP to bridge the actin-myosin motor of the sporozoite and the extracellular substrate, providing traction for invasion and locomotion (165–168). However, the disruption of the interaction between aldolase and adhesins did not affect host cell invasion by *T. gondii* parasites, challenging this theory (169). Indeed, a more recently identified protein called glideosome-associated connector (GAC) has been shown to bind both actin and micronemal adhesins in *T. gondii*. The *T. gondii* GAC has orthologues in *Plasmodium* species, which display expression profile and localization consistent with a similar function (170). Truncation of the CTD, without affecting the sorting domain, also resulted in the failure to invade the mosquito salivary glands and hepatocytes both *in vitro* and *in vivo*, and the abrogation of productive motility (43), reproducing the phenotype of *trap* knockout parasites (40).

The central role of TRAP for productive gliding motility was showcased by the essentiality of this adhesin for the regulation of the constant formation and rupture of adhesion sites (45). The parts played by specific regions of the ectodomain of TRAP in motility, however, are not as clear-cut. While the mutation of the TSR did not have any overtly evident effect on motility, alterations of the MIDAS motif in the I domain lead to a drastic decrease in the percentage of salivary gland sporozoites capable of displaying their characteristic circular gliding motility *in vitro* (81). However, the sporozoites that did glide presented a motile pattern comparable to that of wildtype parasites, which lead the authors to hypothesize that this decrease in the proportion of motile parasites could have been a consequence of the drastic reduction of sporozoite numbers in the salivary glands rather than the loss of TRAP functionality (81). Nevertheless, mutant sporozoites expressing a version of TRAP lacking the I domain or in which the charge of residues surrounding the MIDAS motif was inverted showed severely hampered motility in a 2D system. The replacement of the I domain of TRAP by homologous regions of proteins from other species also resulted in defective motility (82). This indicates that the I domain of TRAP is tailored to generate the necessary forces to support the motility of *Plasmodium* sporozoites. Indeed, TRAP has been suggested to adopt distinct conformations in the presence or absence of an immobilized ligand (171). When the ligand binds through the MIDAS motif, TRAP adopts an elongated conformation due to the tension caused by the anchoring of the CTD to the actomyosin apparatus of the sporozoite (171). The release of energy resulting from the turnover of the adhesion sites formed by TRAP through the I domain is therefore thought to be responsible for the directed movement of the sporozoite (45).

Strikingly, the charge reversion of the I domain of TRAP or its substitution by the I domain of the *Toxoplasma gondii* TRAP orthologue, the micronemal protein 2 (MIC2), did not affect the

invasion of the salivary glands and resulted in moderate to no defects in infectivity to mice injected intravenously (82). Although clear defects in motility and infectivity were observed *in vitro* with these lines, these resulted in milder defects *in vivo* when the skin stage was bypassed, suggesting that *in vitro* systems might overestimate the biological impact of these I domain alterations (82). This could also mean that TRAP takes on independent roles in motility and invasion in the primary and secondary hosts of the parasite. More importantly, the unimpaired *in vivo* infectivity of parasites expressing TRAP containing the I domain from MIC2 indicates that this domain is probably not responsible for the homing of *Plasmodium* sporozoites. Indeed, it is unlikely that TRAP is the sole contributor to the specificity of the sporozoite-liver interactions, as TRAP has been shown to bind to a multitude of tissues and ligands in two different hosts, including saglin in the mosquito salivary glands (91), as previously stated, and widely expressed mammalian integrins (162), and even to inorganic surfaces (45). Moreover, TRAP has orthologues in avian-infecting species and in other apicomplexans, which do not exhibit this preference for liver colonization (154). Nonetheless, it is possible that TRAP could be required for the homing of sporozoites to the liver by playing a role downstream of the initial attachment to the vasculature. Moreover, the role of motility in this process also awaits further clarification.

2.2.3. Arrival in the liver parenchyma

Following their retention in the hepatic microvasculature, sporozoites must cross the sinusoidal barrier in order to gain access to the parenchyma, where their target cells reside (Fig. 12). The endothelial fenestrations are too small to allow the passage of sporozoites and the frequency of larger gaps resulting from the coalescence of these fenestrations in a healthy sinusoidal endothelium is probably too low to explain the highly efficient liver colonization by *Plasmodium* sporozoites (152,172).

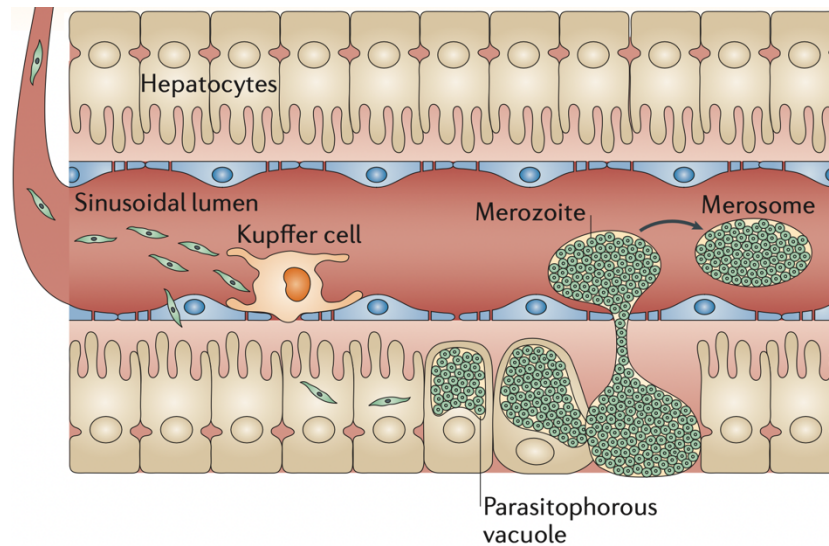


Figure 12. Sporozoite homing to the liver parenchyma and completion of the pre-erythrocytic phase. Sporozoites arrested in the liver sinusoids cross the blood vessel through Kupffer and endothelial cells, traverse parenchyma cells and invade hepatocytes, where they differentiate and produce hepatic merozoites. Merozoite-filled vesicles called merosomes carry the parasites to the bloodstream so they can initiate the blood stage of the life cycle. Adapted from Ménard *et al.* with permission (59).

The first theory on the crossing of the sinusoidal barrier by *Plasmodium* sporozoites to gain major recognition was known as the “gateway model” and proposed the liver resident macrophages, the Kupffer cells, acted as mandatory portals to the liver parenchyma. Sporozoites glide along the luminal surface of the endothelium at a similar speed to that of sporozoites migrating in the skin, which is significantly lower than the speed attained by parasites passively carried by the blood. Sporozoites then pause as they encounter a Kupffer cell, which they cross inside a vacuole that avoids fusion with lysosomes, in order to reach the space of Disse (123,173–175). This supported the earlier demonstrations of sporozoite interaction with and uptake by the liver resident macrophages, and that some of the internalized parasites were able to reach adjacent hepatocytes through Kupffer cell projections (173,174). Moreover, the cluster of differentiation (CD) 68 molecule on the surface of the Kupffer cells was proposed to be the receptor that enables the recognition and invasion of these cells by the sporozoites (176). Another study reinforced this theory by showing that the parasite burden in the liver was markedly reduced in mutant mice that possessed low numbers of Kupffer cells. The depletion of the liver resident macrophages using liposomal clodronate, however, lead to the reverse result, which was suggested to be a consequence of the extravasation of sporozoites through temporary discontinuities formed in the endothelium due

to the death and clearance of the phagocytic cells (177). Furthermore, the depletion of Kupffer cells also reverted the reduced infectivity of cell traversal-deficient sporozoites injected intravenously, which further cemented the role of these phagocytes as the entry point to the parenchyma and implicated host cell traversal in this process (48,49). Still, potential gaps in the sinusoidal endothelium resulting from the loss of the Kupffer cells could once again explain this result, although it remains to be demonstrated that such openings in the sinusoidal lining can be large enough to enable the passage of sporozoites.

Later, the use of intravital confocal microscopy techniques with higher resolution than those utilized before allowed for the exhaustive dissection of the processes that lead up to the arrival of sporozoites in the hepatic parenchyma. It was then made clear that most crossing events were, in fact, dependent on cell traversal activity and occurred in association with Kupffer cells (Fig. 13A) (175). Nonetheless, about a third of crossing events did not require interaction with these phagocytic cells, showing that Kupffer cells are not mandatory for sporozoite passage through the sinusoidal barrier (Fig. 13B). Indeed, sporozoites were seen traversing endothelial cells *in vivo* and *in vitro*. Moreover, a sizable proportion of parasites crossed the sinusoids in a way that did not result in the loss of host cell membrane integrity (175). This phenomenon could correspond to a transcellular crossing mechanism that does not wound the plasma membrane or to the passage of sporozoites via a paracellular route, that is, through junctions between sinusoidal cells. These potential cell wounding-independent pathways could also account for the few liver stages formed *in vivo* using sporozoites with a defective cell traversal activity (48–50).

Crossing events often resulted in the death of Kupffer cells, as indicated by the irreversible loss of the ability of host cells to repair their plasma membranes and the consequent incorporation of a membrane-impermeable dye 1 hour after the crossing event (175). Additionally, it has also been demonstrated that sporozoites are able to render Kupffer cells insensitive to pro-inflammatory stimuli and to ultimately induce apoptosis, which might further hamper the antiparasitic responses in subsequent interactions with other sporozoites (175,178). Lastly, a big part of cell traversal-deficient sporozoites are taken up by Kupffer cells and killed. These data provide the evidence that host cell traversal also prevents sporozoite clearance by resident macrophages in the liver in addition to facilitating their migration through the skin (175).

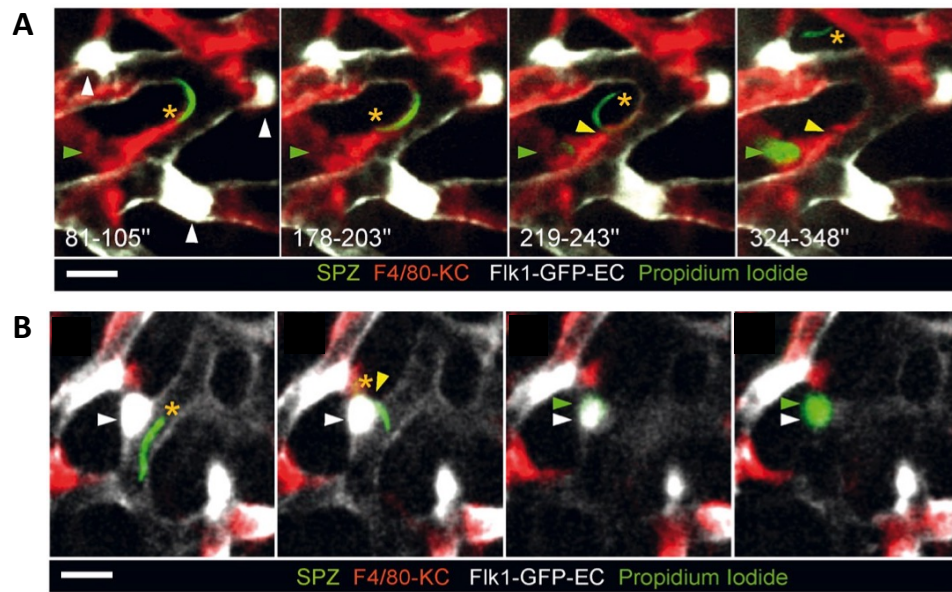


Figure 13. Crossing of the hepatic sinusoidal barrier by sporozoites. Time lapses of sporozoites (SPZ) crossing Kupffer cells stained with an Alexa Fluor 647 anti-F4/80 antibody (F4/80-KC; A) or GFP-expressing endothelial cells of Flk1 mice (Flk1-GFP-EC; B). The loss of plasma membrane integrity is shown by the incorporation of propidium iodide (green arrowheads) or loss of cytosolic content (white arrowhead; B). Yellow arrowheads mark the crossing site and asterisks the anterior pole of the parasite. Bars: 10 μ m. Adapted from Tavares *et al.*, with permission (175).

The importance of host cell traversal once the parasites reach the liver parenchyma has also been under scrutiny. Although the ability of *Plasmodium* sporozoites to actively traverse host cells had already been observed *in vitro* (179), the first demonstration that this process occurred *in vivo* was made in the context of the liver stage in the beginning of this century (180).

Once in the parenchyma, sporozoites migrate extensively and can traverse several hepatocytes, fatally wounding some of these cells in the process (106,123,180). The exposure of sporozoites to intracellular levels of K^+ was suggested to heighten their infectivity (181) and a soluble molecule released by traversed cells, the hepatocyte growth factor, was shown to initiate a signaling cascade that resulted in an increase in the susceptibility of hepatocytes to infection, by promoting the early intracellular development of the parasite (182). These phenomena were proposed to be essential for the activation of an invasive mode of the sporozoite, that is, the commitment of the parasite to the invasion of a single hepatocyte where it will undergo multiplication.

On the other hand, the demonstration that only *P. berghei* but not in *P. yoelii* or *P. falciparum* sporozoites can induce the aforementioned signaling cascade (183), and that parasites displaying deficient cell traversal activity still retain the ability to invade hepatocytes and develop into liver stages suggests otherwise (48–50,184). Furthermore, the inflammation resulting from the release of endogenous material after hepatocyte crossing proved to be unfavorable to parasite development inside neighboring hepatocytes (185). In fact, rather than promoting hepatocyte invasion, host cell traversal activity appears to postpone it, as *P. berghei* PLP1/SPECT2 knockout sporozoites were proven to invade primary hepatocytes more rapidly than control parasites (106).

However, in a different study *P. yoelii* sporozoites lacking PLP1 were shown to accumulate inside vacuoles within nonpermissive HepG2 hepatoma cells (52). Therefore, these likely corresponded to nonreplicative vacuoles, raising the question of whether this phenomenon was misinterpreted as the precocious productive invasion of *P. berghei* PLP1 knockout sporozoites (186). Nonetheless, the entry of *P. berghei* PLP1-deficient sporozoites leads to parasite development and was associated with a tight junction, which is a hallmark of productive invasion as it is currently thought to not occur during the formation of transient vacuoles, although this subject requires further investigation (52,106,187). *P. falciparum* sporozoites lacking PLP1 were also capable of infecting human hepatocytes, although no early time points were evaluated, precluding direct comparisons with the *P. berghei* data (53). *In vivo*, these parasites were not capable of infecting mice with humanized livers, supporting the role of cell traversal in the ability of sporozoites to reach the parenchyma previously reported using *P. berghei* PLP1 knockout sporozoites in the presence of Kupffer cells (48,49,53).

The aforementioned *P. berghei* data, along with the increased detection of PLP1-null sporozoites inside skin cells, suggests that cell traversal activity is important for migration in the skin and escape from phagocytic cells in the hepatic sinusoids, rather than activation of a putative invasive state of the sporozoite (106,175). Therefore, the migratory phenotype of sporozoites in the liver parenchyma might just be a consequence of the residual activity of the cell traversal machinery, which needs to be exhausted for the parasite to permanently settle inside a hepatocyte. Additionally, if the overturning of the migratory mode is a timed process, the traversal of host cells may also be a way for the sporozoite to safely buy some time and escape host immune responses until it is ready to invade a hepatocyte and replicate. Yet, it is still possible that both intrinsic and environmental factors could contribute to the attainment of a state necessary for the commitment to invasion.

2.2.4. Hepatocyte invasion

The remarkable voyage of the sporozoite comes to an end once it commits to invasion (Fig. 14). The sporozoite interacts with a hepatocyte and a tight junction at the surface of the host cell is likely formed, with the local reorganization of host cell actin (187). A parasitophorous vacuole derived from the invagination of the host cell plasma membrane starts to fully engulf the sporozoite, which draws on the same actomyosin motor that fuels its locomotion to force its way into the cell, through the tight junction. The cell then seals the entry point on the plasma membrane, which remains intact and disconnected from the parasitophorous vacuole membrane (188).

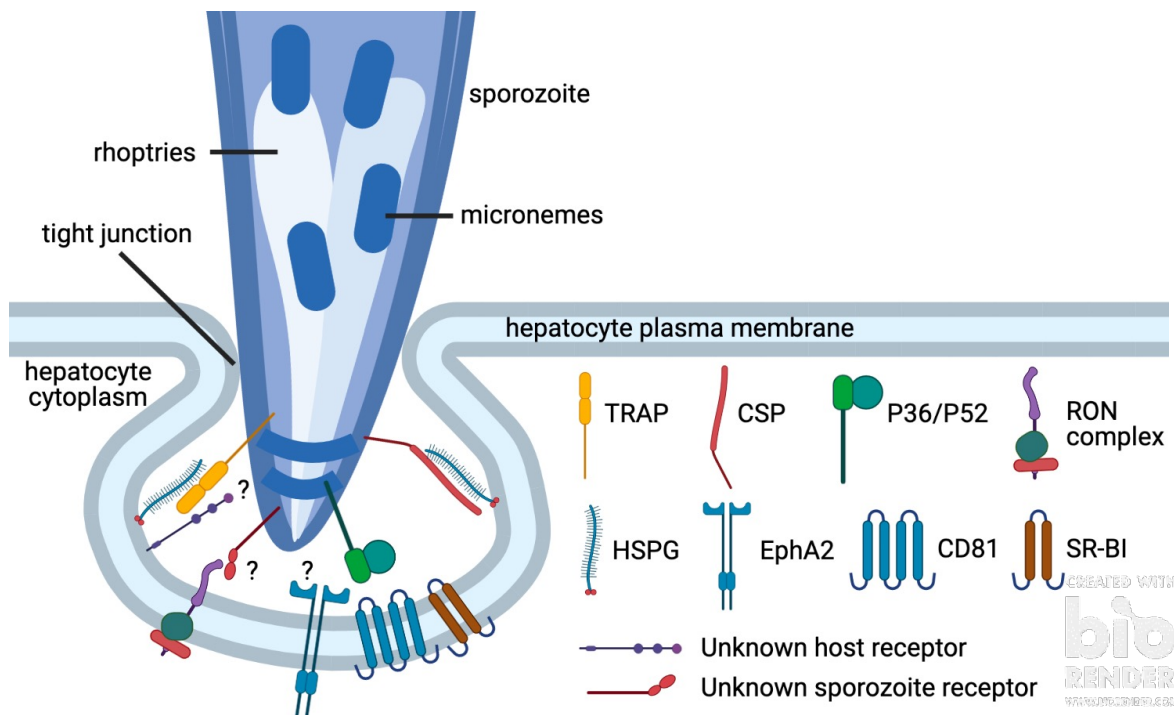


Figure 14. Hepatocyte invasion by *Plasmodium* sporozoites. Some of the known molecules expressed by sporozoites to be involved in the invasion process as well as their suggested or unknown receptors on the host cell are represented. The illustration was made with BioRender.

Most of the insights into the invasion mechanisms employed by *Plasmodium* parasites have been gleaned through the study of the interaction between merozoites and erythrocytes (discussed in this chapter, in section 2.3). This is due to the challenges associated with hepatocyte invasion research, which include the difficulty of the examination of the pre-erythrocytic phase of *P. falciparum* *in vivo* and *in vitro*, as immortalized cell lines do not fully support infection by this species, although great advances have been made using humanized mice with chimeric livers (189,190). Nonhuman primates are an alternative to rodent models

but face many ethical, financial, and logistical hurdles that restrict their use. The comparative abundance of tools to study the blood stage of *P. falciparum* *in vitro* also make this phase of the life cycle more attractive for the dissection of the invasion process. Moreover, the molecular basis of hepatocyte invasion is highly species-dependent, as will be made evident below, and studies that take advantage of the easier to use rodent-infecting *Plasmodium* species often fail to uncouple all the steps that lead up to the establishment of the parasite inside the host cell, particularly *in vivo*, since these happen in quick succession and are mechanistically intertwined. Therefore, the invasion process in the liver remains poorly understood at the molecular level, although part of the mechanisms that govern host cell invasion are likely shared between the two phases of the life cycle, such as the involvement of the actomyosin motor.

Nonetheless, some host molecules appear to be specifically required for the invasion of hepatocytes by sporozoites. As was previously mentioned, TRAP and CSP are thought to mediate the adhesion to hepatocytes, the first step of the invasion process, and CSP can also be detected in developing liver stages (81,82,133,134,138,148–150,191,192). Two members of the 6-cys protein family, P36 and P52, have also been shown to be required for hepatocyte invasion and liver stage development, as they are critical to hepatocyte recognition or to the formation and/or maintenance of the parasitophorous vacuole (193–197). In fact, the hepatocyte ephrin receptor A2 (EphA2) was appointed as the binding partner of P36. Productive invasion was found to be correlated with EphA2 expression, and low levels of this receptor in host cells phenocopied the double knockout of P36 and P52 (198). However, the direct downregulation of EphA2 or the blocking of this receptor via antibodies did not reveal any association between EphA2 availability and hepatocyte invasion by sporozoites (199).

Two additional molecules on the host side have been suggested to partake in species-specific hepatocyte invasion pathways, namely CD81 and the scavenger receptor type B class I (SR-BI). CD81 is a widely expressed tetraspanin with well-known roles in cell adhesion and signal transduction. It was shown to be essential for hepatocyte invasion in *P. falciparum* and *P. yoelii*, although *P. vivax* and *P. berghei* sporozoites were also capable of resorting to CD81-independent routes of cell infection (197,200–203). It has also been suggested that CD81 is indirectly involved in the invasion process through the assembly of microdomains on the hepatocyte surface, which facilitate the entry of the parasite (202). SR-BI also seems to be needed for the formation of the CD81-rich microdomains, which stimulate the discharge of the rhoptries of *P. yoelii* sporozoites (204–206). SR-BI was also found to be involved in both host cell recognition/entry by sporozoites in the absence of CD81 and in the development of the parasite inside the parasitophorous vacuole (197,205). Interestingly, it was suggested that P36

may interact directly or indirectly with CD81 or SR-BI, dictating which pathway is used depending on the parasite species and availability of the host receptor (197).

The use of antibodies against MAEBL or the apical membrane antigen 1 (AMA1), two structurally related proteins, and a peptide capable of binding to AMA1 and inhibiting its function reduced hepatocyte invasion by *P. falciparum* or *P. yoelii* sporozoites (190,207–209). While a functional study using a conditional knockdown of AMA1 in *P. berghei* did not support these results (210), *P. falciparum* MAEBL knockout hemolymph sporozoites also showed reduced invasive capabilities (190). Additional parasite lines with reduced capacity to invade hepatocytes include deletion mutants for proteins also required for sporozoite motility, such as the inhibitor of cysteine proteases (ICP), the TRAP-related protein (TREP) and LIMP (211–213).

Another interesting group of molecules are the rhoptry proteins, as these play well-described roles in the invasion of erythrocytes by merozoites. Indeed, several of these rhoptry neck and bulb proteins have been detected in the rhoptries of sporozoites (214,215). Among these is the rhoptry neck protein (RON) 2, which forms a complex with AMA1 at the merozoites-erythrocyte interface, whose key role in the invasion of red blood cells (216) is discussed in more detail later in this chapter. A RON2 conditional knockdown showed that this protein is vital to sporozoite infectivity both *in vivo* and *in vitro* (217), although a potential association with AMA1 is unlikely to be required since, as was previously mentioned, the depletion of this protein did not have a negative effect on the invasion of hepatocytes (210). Although this RON2 mutant line also displayed impaired motility (217), which could affect the ability of the parasites to invade host cells, the tagging of this rhoptry molecules with a fluorescent reporter proved that this protein is discharged during invasion (206). Similarly, RON4 also became undetectable following the entry of the sporozoite in the host cell and its conditional depletion reduced hepatocyte invasion (206,210). In contrast, the rhoptry-associated protein 2/3 (RAP2/3) appears to serve a different function as it localized to the nascent parasitophorous vacuole (206). A comparable phenotype to the one caused by the loss of RON2 was obtained when RON11 was downregulated, with a decrease in motility and hepatocyte invasion (218). Finally, sporozoites lacking the thrombospondin-related sporozoite protein (TRSP), which was suggested to localize to the rhoptries and shown to be one of the main surface molecules of sporozoites, were reported to remain stuck in the hepatocyte membrane during cell entry (126,219,220).

2.2.5. Liver stage development

By the end of the invasion process, the sporozoite is completely concealed inside a parasitophorous vacuole within the hepatocyte. Now, the parasite enters a new developmental phase that entails the metamorphosis into a factory fully dedicated to merozoite production (Fig. 12). Initially, the parasite suffers morphological and metabolic changes to prepare for the generation of thousands of merozoites from a single infected cell. The parasite gets rid of the now unnecessary migratory and invasive machinery and breaks down its IMC, which results in the loss of the sporozoite's characteristic shape and rounding of the cell (221). Meanwhile, the differentiating parasite also exports proteins to the parasitophorous vacuole membrane, such as the proteins encoded by the genes upregulated in infective sporozoites (UIS) *uis4* and *uis3*. While these genes are transcribed in mature sporozoites, their translation is repressed until the start of liver stage development, where UIS4 and UIS3 are crucial for the early growth of the hepatic EEFs (219,222–225). While the function of UIS4 has been associated with the recruitment of host late endocytic vesicles for cholesterol procurement by the parasite (226), UIS3 has been suggested to play a specific role in fatty acid acquisition in *P. yoelii* (224). Moreover, UIS3 is capable of sequestering molecules involved in autophagy, thus protecting the parasite from lysosomal degradation (227,228). It is important to note that, while some autophagy-related responses can function as an innate immune mechanism that allow the elimination of the parasite by the hepatocyte, liver stages may also seize canonical autophagy pathways to improve their access to nutrients (229–232).

The *Plasmodium* translocon of exported proteins (PTEX) allows proteins containing a specific motif downstream of the signal peptide, the *Plasmodium* export element (PEXEL), to cross the parasitophorous vacuole membrane and gain access to the host cytoplasm during intraerythrocytic development (233,234). The exported protein (EXP) 2 is a pore-forming molecule that constitutes the channel of the PTEX complex (233,235). This protein was also deemed essential for the development of the liver stages, although the PTEX of the hepatic EEFs was shown to be compositionally different from the complex expressed by the blood stages. Moreover, it might also not have the same function, since it was not able to translocate a fluorescent reporter fused to the PEXEL across the parasitophorous vacuole membrane (235). Instead, the role of the PTEX during liver stage development might be related with nutrient uptake, for a second function in nutrient acquisition has been attributed to EXP2 in the blood stage (236). Indeed, another parasitophorous vacuole resident exported protein, EXP1, appears to be required for the correct localization of EXP2 and its loss leads to the abrogation of the nutrient-permeable channel activity in parasites infecting red blood cells (237). Accordingly, in

the liver stages, EXP1, also known as Hep17 in rodent-infecting *Plasmodium* species, has been demonstrated to interact with the host apolipoprotein H (ApoH), potentially mediating the uptake of ApoH or ApoH-associated nutrients (238). Nonetheless, EXP2 has also been shown to be secreted during invasion, with a possible role in the repair of the hepatocyte membrane, which suggests this pore-forming molecule may have additional translocon-independent functions (239).

In the initial stages of development within the hepatocyte, the EEF, called trophozoite at this stage, appears as a small and round cell. However, the trophozoite rapidly increases its size and transforms into a schizont as it starts to divide its nucleus. By the time the parasite reaches the late developmental stage, it takes up much of the space inside the swollen host cell and has already undergone 12 to 14 rounds of exponential nuclear replication, generating a multinuclear syncytium with thousands of nuclei. Then, the plasma membrane of the schizont, which already contain the merozoite surface protein (MSP) 1, expands and invaginates to originate individual hepatic merozoites (240). This process is accompanied by the formation and segregation of the merozoite organelles into the individual nascent cells (178). The parasitophorous membrane is then lysed as a consequence of both phospholipase and protease activity (241,242), cell death and detachment are induced, and merozoites are delivered into the lumen liver sinusoids in large host-derived vesicles, called merosomes, each containing hundreds of parasites (Fig. 12) (243,244). Free merozoites are subsequently released into the bloodstream once merosomes reach the lungs circulation, and promptly invade erythrocytes (244).

The duration of the developmental process depends on the *Plasmodium* species. While *P. falciparum* takes 6 to 7 days to initiate the blood stage, the development of rodent-infecting species in the liver takes just 2 to 3 days (188,245). However, *P. vivax*, and potentially *P. ovale*, has the ability to form dormant trophozoites, designated hypnozoites, which can reactivate and resume liver stage development following an extended period of quiescence (246). This is the reason *P. vivax* constitutes the main cause of malaria relapses.

2.3. The blood stage

Free merozoites only remain infectious for a few minutes (247,248). However, this does not constitute an issue for these parasites as their target host cells are easily accessible and the invasion of erythrocytes consists of an exceptionally efficient series of tightly coordinated steps (Fig. 15). Merozoites waste no time finding red blood cells and, *in vitro*, the initial recognition of the host cell happens within the first minute after the release of the parasites (247). This

attachment is the first step of the invasion process and involves the binding of MSPs to receptors on the host cell (249). MSPs are exported to the surface during merozoite development, allowing free parasites to immediately seek and bind to neighboring red blood cells. MSP1 is the most abundant member of this group of proteins, forms multiple complexes likely to be at least in part functionally redundant, and binds to various surface-bound erythrocytic molecules (250–253). Although merozoites are often regarded as immotile invasive forms of the parasite, it has been demonstrated that they are indeed capable of using gliding motility to disperse following the egress from the host cell *in vitro* and that this behavior is maintained during the merozoite infectivity window (254). If and how merozoites use their minute displacement capacity in the bloodstream remains to be elucidated.

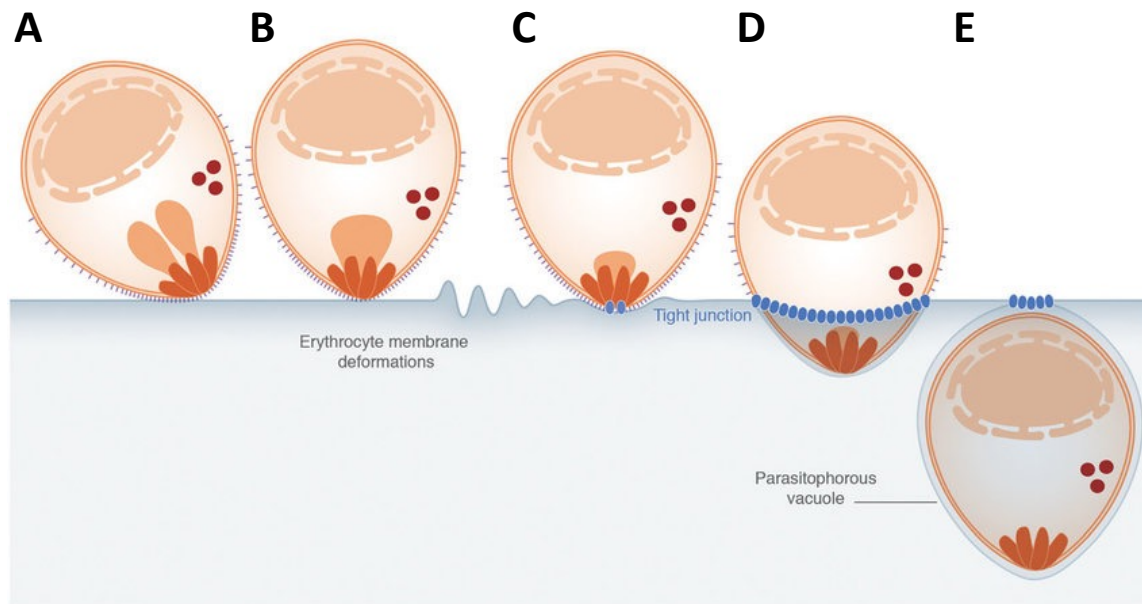


Figure 15. Steps of erythrocyte invasion by merozoites. (A) Merozoite attachment to the erythrocyte. (B) Reorientation of the merozoite. (C) Formation of a tight junction. (D) Merozoite entry into the erythrocyte. (E) Sealing of the parasitophorous vacuole and host cell plasma membrane. Adapted from Koch and Baum, with permission (249).

Reorientation of the merozoite and repositioning of the apical pole towards the plasma membrane of the erythrocyte ensues. The high density of micronemal adhesins with high affinity for host cell receptors at the apex of the merozoite is thought to be responsible for the reorientation of the parasite, which may also prompt structural alterations to the plasma membrane of the red blood cell (247,255). The micronemal AMA1, which is partially released prior to attachment, has been shown to be essential for this process (256,257). Then, the merozoite commits to invasion with the secretion of the contents of the rhoptries. In a manner

analogous to that of hepatocyte infection by sporozoites, a tight junction is formed, the cytoskeleton of the erythrocyte is remodeled, the merozoite forces its way into the host cell in a plasma membrane-derived parasitophorous vacuole using its actomyosin motor, and the host cell membrane is resealed. In contrast to the discharge of the apical secretory organelles, the secretion of the dense granules appears to be a post-invasion event (257). Remarkably, AMA1 is not only necessary for the reorientation of the parasite but also for the commitment to invasion. It forms a junctional complex with RONS through the binding to RON2, which functions as a parasite-derived receptor on the host cell as it is secreted and inserted into the plasma membrane of the erythrocyte during the initiation of the invasion process (216,258–262).

Other micronemal molecules involved in the establishment of receptor-ligand interactions required for the invasion process include erythrocyte-binding-like (EBL) proteins or erythrocyte-binding antigens (EBAs). The reticulocyte-binding-like (RBL) or Rh rhoptry proteins are also known to control this process. All these proteins define several redundant pathways for invasion, which confer extreme plasticity to the invasive prowess of the merozoite (249,262). Altogether, the invasion process lasts less than a minute (247).

Once inside the red blood cell, the *P. falciparum* merozoite undergoes a string of morphological transformations (Fig. 16). The merozoite first turns into a small ring stage, designated this way due to its ring-like biconcave discoidal shape, and then develops into a more globular-shaped form of the parasite, called the trophozoite, as it grows (263). Nuclear replication by schizogony is initiated, which does not necessarily follow the geometric expansion commonly associated with canonical binary divisions. This is due to the asynchrony of genome replication in schizonts, which, after cytokinesis, typically results in the generation of 10 to 32 individual merozoites per intraerythrocytic schizont in a process that takes 24 to 72 hours in species infective to humans or rodents (59,264–266). Finally, the rupture of the parasitophorous vacuole membrane and the breakdown of the host cell cytoskeleton and plasma membrane permit the release of mature merozoites, which can now infect new host cells (267). Successive intraerythrocytic cycles therefore result in the exponential growth of the parasites in the blood of the host.

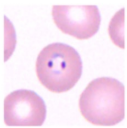
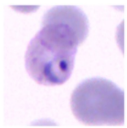
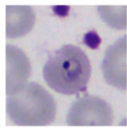
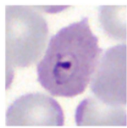
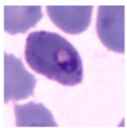
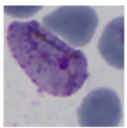
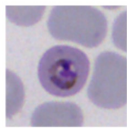
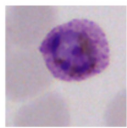
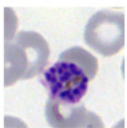
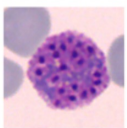
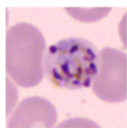
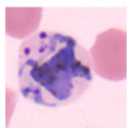
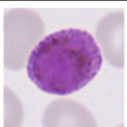
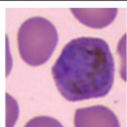
		Species			
		<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. malariae</i>	<i>P. ovale</i>
Stage	Ring				
	Trophozoite				
	Schizont				
	Gametocyte				

Figure 16. Blood stages of *Plasmodium* species infective to humans. Images of thin blood smears showing the different intraerythrocytic stages of 4 *Plasmodium* species that cause malaria in humans. Adapted from Delahunt *et al.*, with permission (268).

The synchronicity of the development of some human-infective *Plasmodia* within red blood cells has been appointed as the cause of the classical malarial paroxysm, which is sometimes presented by patients, and is characterized by a febrile state that is preceded by a cold stage with shivering and is followed by profuse sweating as the body temperature drops rapidly (8). These cyclical bouts of illness typically alternate with symptom-free periods, coincide with the synchronous rupture of the schizonts, and are more irregular or nonexistent in *P. falciparum* malaria. Thus, when present, the periodicity of the fever, which is caused by host responses to the release of parasite material to the bloodstream, depends on the duration of the intraerythrocytic development of the *Plasmodium* species (8,269). It is noteworthy that the intraerythrocytic cycle of rodent malaria parasites *P. berghei* and *P. yoelii* is asynchronous and all the characteristic blood stage forms can be found simultaneously in the blood on infected animals (270).

Other key differences between species in the blood stage include the host cells that are preferentially infected. Whereas most clinically or experimentally relevant *Plasmodium* species can infect mature red blood cells, *P. vivax* merozoites exhibit exclusive tropism to reticulocytes (271,272). *P. vivax* merozoites interact with the Duffy blood group antigen through the Duffy-binding protein 1 (DBP1), accounting for the resistance of Duffy-null individuals to *P. vivax* infection (273,274). However, infection of Duffy-negative people has been described, which

could be explained by low affinity interactions mediated by DBP1 (275–277). Moreover, since the Duffy blood group antigen is also expressed on the erythrocyte surface, the restriction to reticulocyte infection must be mediated by other receptor-ligand pairs. Indeed, *P. vivax* binds to receptors lost during the maturation of reticulocytes into erythrocytes via complexes formed by the species-specific *P. vivax* reticulocyte-binding proteins (PvRBPs) (278). This predilection for reticulocytes restricts parasite loads and implicates the bone marrow as the preferred location for the invasion of host cells and for the initial stages of *P. vivax* intraerythrocytic development (271,272,279).

Species pertaining to the *Plasmodium* genus also express different variable surface antigen gene families, such as the *P. falciparum* var, subtelomeric variant open reading frame (*stevor*) and repetitive interspersed family (*rif*) multigene families, or the *Plasmodium* interspersed repeat (*pir*) gene family in species infective to rodents (280). The proteins encoded by these genes are exported to the surface of the merozoite or to the plasma membrane of the infected erythrocyte, as they contain PEXEL motifs, and have been implicated in host cell invasion, immune evasion, and/or pathogenesis, as will be discussed in more detail in the next section (281–283).

Finally, a part of the parasite population must commit to the generation of sexual progeny in order to be transmitted to the mosquito, a process that can take between 1 and 12 days depending on the species of the parasite (266). The transcription factor AP2-G has been designated the master regulator of gametocytogenesis due to its ability to induce the expression of a host of early gametocyte-associated genes (284). In *P. falciparum*, these transcriptional changes allow sexually committed parasites to go through 5 distinct morphological phases, ultimately resulting in the formation of the characteristic crescent-shaped male and female gametocytes (Fig. 16) (266). It is thought that sexually committed merozoites invade red blood cells either in the blood stream or in the bone marrow and gametocyte maturation progresses extravascularly in this tissue (285). Fully developed gametocytes migrate back into the vasculature and can await for the next blood meal for several days in circulation, maximizing their chance of transmission and, consequently, completion of the life cycle (266,285).

2.3.1. Naturally acquired immunity and pathogenesis

Naturally acquired immunity to malaria is attained over several years through continued exposure to the parasite, it increases with age, and results in protection against the disease without total parasite clearance (286–290). This incomplete protection is ascribed to the development of a partial immunity to the blood stage, which limits parasite growth and,

therefore, averts the symptomatology (291–296). In parallel, the host more readily develops independent mechanisms that confer tolerance to high parasite densities without developing fever (290,296). However, since clinical immunity does not imply sterile protection, it contributes to the transmission of the parasite, as asymptomatic individuals constitute a parasite reservoir and are less likely to seek and adhere to treatment (297–300).

Naturally acquired immunity has been shown to be mediated by antibodies targeting infected red blood cells or free merozoites (301). More specifically, a humoral response is mounted against the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variants and several invasins (291–293,302–308). PfEMP1 is exported to the surface of parasitized erythrocytes and mediates adhesion to a variety of host receptors, leading to the sequestration of these erythrocytes in the vascular endothelium, which consequently avoid splenic passage and elimination (294,309–312). Rather than a single protein, PfEMP1 is a family of proteins encoded by around 60 *var* genes expressed in a mutually exclusive manner (313,314). While the differential expression of *var* genes results in the trafficking of a single PfEMP1 variant to the erythrocyte surface, several members of this multigene family may be expressed within a parasite population (315–318). This clonal antigenic variation limits the efficacy of immune responses against PfEMP1 and constitutes the main immune evasion mechanism employed by the *P. falciparum* blood stages. The ability of the parasite to switch from one PfEMP1 variant to another during successive replication cycles allows infected red blood cells to avert host responses mounted against immunologically distinct variants, so parasites survive and perpetuate the infection (283,319). Thus, as the immune responses against PfEMP1, the immunodominant blood stage antigen, are closely related to the generation of clinical immunity and survivability of the host, they may also increase the odds of transmission (320,321). Additionally, PfEMP1 has also been implicated in rosette formation, a process by which parasite-harboring and uninfected erythrocytes aggregate, which further promotes the tissue sequestration of infected erythrocytes and reduces accessibility to PfEMP1-targeting antibodies (311,320). Cytoadherence and rosette formation therefore represent two added mechanisms of immune evasion exploited by *Plasmodium* in the blood stage. Similar functions have been attributed to the proteins encoded by the *stevor* and *rif* multigene families (281,282,322). Moreover, these phenomena lead to the obstruction of the cerebral microvasculature, leading to endothelial dysfunction. Together with the immune responses to infection, cytoadherence and rosetting have been proposed to be responsible for the pathogenesis of cerebral malaria (323,324).

3. Control strategies

The outcomes of disease control and elimination programs are reliant not only on the efficacy of the interventions but also on the allocation of adequate funding necessary to tackle an undertaking of this magnitude. Although malaria is no longer considered a neglected tropical disease (325), the WHO reported that public and private expenditure on malaria research and programs was not only insufficient but the difference between the resources provided and the amount required expanded over the years (10). Nevertheless, the stronger reduction of malaria mortality in comparison to incidence likely reflects the contribution of the improved access to early diagnosis and treatment.

3.1. Diagnosis

The diagnosis of clinical malaria is traditionally symptom-based, followed by parasitological confirmation. The microscopical observation of parasites in Giemsa-stained blood films remains the gold standard diagnosis method as it allows for accurate detection of the parasite and identification of the species, especially at high parasitemia. However, quality microscopy-based diagnosis requires skilled laboratory technicians, infrastructure, instrumentation, and reagents, which are not always available (326). While the detection of parasitic DNA by polymerase chain reaction (PCR) is associated to enhanced sensitivity (327,328), it is heavily reliant on laboratory accessibility and, subsequently, has limited utility in the field. On the other hand, rapid diagnostic tests (RDTs) have gained increasing popularity over the last decades. RDTs are lateral flow immunochromatography devices that detect circulating parasite antigens and enable a quick diagnosis without requiring technical expertise, which can be extremely advantageous in areas with limited access to proper microscopic diagnosis (326). The downfalls of some of these tests are their lack of sensitivity and the emergence of strains carrying deletions of the genes encoding the target antigens (328–330). This originates false-negative results and, consequently, potential misdiagnoses, which can cause fatal outcomes. Therefore, the choice of the diagnostic method depends on the availability of quality diagnostic services and is critical for disease management.

3.2. Treatment

Due to the severity and often quick progression of severe malaria, a rapid and accurate diagnosis is essential for the success of treatment. Artemisinin-based combination therapies (ACTs) consist of the combination of an artemisinin derivative with a longer-acting antimalarial and are currently the preferred treatments against malaria caused by *P. falciparum* (331).

Parenteral artesunate is used to treat severe malaria until patients can tolerate an oral ACT and artemether is another parenteral alternative. Second-line oral therapies include quinoline drugs, such as chloroquine and quinine, which are also used to treat uncomplicated malaria and pregnant women in the first trimester. Primaquine is used to prevent relapses in infections caused by *Plasmodium vivax* due to its efficacy against the liver stages (331).

The emergence of drug resistance threatens to compromise the vital role of effective therapies in global disease control strategies. Resistance to antimalarials previously employed as monotherapies, such as pyrimethamine and chloroquine, arose in different endemic areas around the globe, leading to the relegation of these medications to second-line treatments, chemoprophylaxis, or ACTs (331–334). In fact, ACTs were implemented in an attempt to combat drug resistance by combining molecules with different mechanisms of action, which alleviates the selective pressure that gives rise to resistant strains of the parasite (331,335). Worryingly, multi-resistant strains of *P. falciparum*, including resistance to artemisinin and its derivatives, have already been reported in Southeast Asia (336,337), justifying the need to continuously feed the drug development pipeline in hopes of finding new effective drugs or combinations of drugs that are less prone to cross-resistance. A few antimalarial candidates with novel modes of action have been developed from compounds identified through high-throughput screenings, some of which have entered clinical trials in recent years (338–340).

3.3. Prevention

3.3.1. Vector control

The final cornerstone of programs aiming to achieve a malaria-free world are the preventive measures. In fact, we owe much of the progress made towards the reduction of malaria burden to insecticide-based vector control strategies (341). Dichlorodiphenyltrichloroethane, commonly known as DDT, was developed in the 1940s and saw widespread use and significant success in the fight against malaria and other insect-borne diseases. Impressively, the use of DDT resulted in the elimination of malaria in several regions of the world (342). However, as DDT's efficacy declined due to the emergence of resistance (343) and its negative impact on the environment and health started to be unveiled, its usage dwindled, and it was eventually banned (344). DDT was recently brought back, although its current usage is limited to indoor residual spraying, as an alternative to other insecticides, namely pyrethroids, organophosphates, carbamates, and clothianidin (342,345). This strategy consists in spraying insecticides on surfaces where mosquitoes might rest and can significantly contribute to the success of vector control strategies if thoroughly applied (342,345).

Long-lasting insecticide-treated mosquito nets constitute the second insecticide-based method to limit exposure to the vector still in use. It has proven to be a cost-effective intervention (341,346) that was estimated to have been responsible for 68% of all averted malaria cases from 2000 to 2015 (347). In fact, mathematical models predicted that even low levels of resistance to long-lasting pyrethroids in mosquito nets would significantly increase the incidence of malaria, such is the impact of this preventive measure (348). Alternative tools for mosquito population suppression such as genetic control by release of sterile mosquitoes into wild populations (349–353), and different methods for larval source management (354–356) and biological control (355,357–359) have also been trialed with varying degrees of success (359).

3.3.2. Antimalarial vaccines

Despite the significant contribution of the outlined strategies, these have proven to be insufficient, as the progress toward malaria elimination seems to be reaching a plateau. Indeed, there is a key element missing from the prevention programs to expedite worldwide malaria eradication: a protective vaccine.

3.3.2.1. Blood stage vaccines

Immunity against highly variable blood stage antigens might be a difficult concept to translate into a protective vaccine for the general population (310,360,361). Consequently, vaccine development has also had blood stage antigens other than PfEMP1 in consideration, namely proteins involved in erythrocyte invasion, as blocking this process would curtail parasite multiplication. In line with this hypothesis, several merozoite invasins have been proposed to partake in the acquisition of natural immunity. These include AMA1, MSP1, MSP3 and EBA-175 (303–308,362). However, these vaccine candidates failed to show substantial efficacy in dozens of clinical trials over the past two decades (361). Overall, blood stage vaccines face considerable challenges that arise from the nature of both the antigens and the immune responses in the blood stage: high antigen polymorphism (363), the redundancy of the invasion pathways (364), and the high antibody titers and quality that may be needed to neutralize elevated parasite numbers, which also remain extracellular for short periods. For these reasons, the number of trials for blood stage vaccines has been decreasing over the years (361). Nonetheless, new candidates have been undergoing pre-clinical studies. Rh5 has appeared as a more promising blood stage antigen (365–369), as it is characterized by limited polymorphism and antibodies raised against Rh5 block erythrocyte invasion by merozoites from heterologous

P. falciparum strains (365,367,370–373). A new clinical trial to assess the immunogenicity and safety of an Rh5-based vaccine in endemic areas is currently underway (338).

3.3.2.2. Transmission-blocking vaccines

Alternatively, transmission-blocking vaccines aim to kill the parasite in the mosquito by including antigens of the transmission stages, namely the gametes, the zygote and the ookinete. Such antigens include the *P. falciparum* surface molecules Pfs230, Pfs48/45, Pfs25 and Pfs28 (338,361). Although stopping the infection of the vector was not a novel idea (374–378), the failure of more favored approaches justified the increased interest in transmission-blocking vaccines seen in recent years. In fact, several of these vaccine candidates recently entered clinical trials or preclinical development (338,361). Since they do not protect against the infection or the symptoms in humans, however, transmission blocking vaccines potentially used in the field would have to be highly efficient and would heavily depend on complementary strategies to prevent and manage the disease before herd immunity was achieved.

3.3.2.3. Pre-erythrocytic vaccines

Vaccines can also be directed at the initial stages of infection in humans. The pre-erythrocytic phase represents an attractive target for malaria vaccines because: it precedes the symptomatic phase disease and transmission of the pathogen; it represents a numerical bottleneck of the parasite's life cycle; sporozoites are not antigenically variant and remain extracellular for longer periods than merozoites, which increases accessibility to neutralizing antibodies; and hepatocytes are active participants in host immune responses.

The subunit vaccine approach has been extensively pursued over the past decades, with a special focus being placed on CSP (379). This venture resulted in the development of the RTS,S/ASo1 vaccine (Mosquirix™, GlaxoSmithKline), the only anti-malarial vaccine to date to be approved for use by the European Medicines Agency (380). RTS,S is a chimeric protein comprised of the last 19 NANP repeats of the central region and the C-terminus of the *P. falciparum* CSP, excluding the GPI anchor, fused with the hepatitis B virus surface antigen (HBsAg), which spontaneously self-assembles with free HBsAg in a 1:3 ratio of fusion protein to HBsAg monomers and acts as carriers for the truncated CSP (381,382). RTS,S is then combined with the liposomal Adjuvant System ASo1 (383,384).

The safety of RTS,S/ASo1 was considered acceptable, and vaccination was able to reduce the incidence of clinical and severe malaria in 6- to 12-week-old infants and in children 5 to 17 months old (385–387). However, the efficacy of the vaccine a year after vaccination was modest

and dwindled over time (387). This protection was correlated with the titers of antibodies against the tandem repeats of CSP (383,388,389). Thus, the short duration of protection against clinical malaria was attributed to the waning of anti-CSP antibodies titers, which was quicker in high transmission areas, denoting the exceptionally high anti-CSP antibody levels required to maintain protection (389). Nonetheless, new generation CSP-based vaccines try to improve on RTS,S/A01 efficacy by: including CSP epitopes in, for example, chimeric proteins, self-assembling protein nanoparticles or synthetic peptides (390–392); utilizing the nearly full-length protein (393–399); increasing the proportion of CSP versus HBsAg in the vaccine, in a next-generation version of RTS,S known as R21 (400); and/or using novel virus-like particles (397,401,402).

Sporozoites attenuated via radiation, chemically or genetically do not initiate the blood stage while still eliciting protective immune responses (403–408). This protective effect was initially linked to anti-CSP humoral immunity (409–412), which were later shown to immobilize and kill sporozoites in the skin (101,413–415). However, since then CD8⁺ T cells have been shown to be the main mediators of protection conferred by whole-sporozoite vaccines (114,115,416–431). Numerous efforts were made to optimize the production, purification, and cryopreservation of sporozoites, which allowed for some of these vaccines to be tested in clinical trials (432,433). The protection engendered by the most advanced whole-sporozoite vaccines varied substantively, weaned with time and required intravenous administration (424,427,430,431,434–438). Therefore, the main conceivable drawbacks of these vaccines are the parenteral administration, the level and duration of protection, their cost and the logistical challenges associated with the delivery of the vaccine in the field. These might prove to be unsurmountable hindrances to the incorporation of these vaccines in preventive strategies, considering the geographical and socioeconomical factors that affect the accessibility to vaccines in certain endemic areas.

Experiments using B-cell-deficient and CSP-tolerant transgenic mice revealed that other pre-erythrocytic antigens can sterile protect against infection following immunization with radiation-attenuated sporozoites, mainly through CD8⁺-T-cell-mediated responses (439). This suggests that while CSP is the immunodominant surface antigen of sporozoites, it is not the only antigen responsible for the protection conferred by whole sporozoite vaccines. These data are further supported by the sterile protection obtained in mice immunized with irradiated *P. berghei* sporozoites but challenged with a strain of the parasite expressing the *P. falciparum* CSP instead of the CSP of *P. berghei*, despite the low similarity between the B-cell and T-cell epitopes of the two orthologues (440). Indeed, although the protective properties of pre-erythrocytic antigens other than CSP are poorly characterized, several *Plasmodium falciparum*

proteins have been shown to elicit humoral responses in individuals immunized with radiation-attenuated sporozoites (441). This prompted the development of subunit vaccines based on individual antigens such as the liver stage antigen-1 (LSA1) (442,443) and CelTOS (444), or multi-antigen vaccines such as ME-TRAP (445–448), MuStDO5 (449) or MVA-L3SEPTL (450), all of which contain TRAP epitopes among other antigens. However, except for ME-TRAP-based formulations, which are still being tested in clinical trials, these vaccines exhibited substandard efficacy in humans (338).

3.3.2.4. Multistage vaccines

Subunit vaccines can include antigens required for discrete steps of the life cycle, including different forms of the parasite, thus eliciting cross-stage immunity (451). Alternatively, it should be possible to take advantage of the additive or synergistic effects of the combination of individual components in multiantigen, multistage vaccines. Indeed, we have witnessed a rise in the number of multicomponent vaccines acting on different stages of the life cycle in development in recent years. Some vaccines are directed at both the pre-erythrocytic phase and the blood stage (338,452–458), while others target either the pre-erythrocytic stages or asexual blood forms and the transmission stages of the parasite (459–463), or all the above simultaneously (338,464–467). However, finetuning the component-specific efficacies of complex multiantigen vaccines and driving the appropriate immune responses in stages with different immunological requirements to achieve protection might prove to be arduous. Furthermore, the increment in the number of components might also raise the cost and risk of adverse effects of these vaccine candidates. These limitations might have been reflected in the disappointing results of the multistage vaccines that have reached early clinical trial phases so far (338,453–455).

Altogether, the immunology and vaccinology data gathered over the past 6 decades emphasize the complexity of the immune responses that lead to protection from *Plasmodium* infection. This is unsurprising considering the intricate life cycle of these parasites and their extensive adaptation to the vertebrate hosts. It is then understandable that, despite the substantial investment in vaccine research and development, we are yet to attain our ambition of creating a protective vaccine that might turn the tide in our battle against malaria. Nonetheless, great strides have already been made and the critical status of malaria as a public health issue still demands our relentless effort in the search for the holy grail of malaria prevention strategies.

4. Analysis of gene function in sporozoites

4.1. Conventional genetic modification

Many of the insights into *Plasmodium* biology have been provided by reverse genetics. Conventional genetic manipulation techniques allow the deletion, disruption, mutation or insertion of genomic sequences. These take advantage of the ability of the parasite to repair damaged DNA via recombination events in the presence of a homologous DNA template. Parasites are usually transfected with DNA vectors containing cassettes bearing selectable markers for positive and/or negative selection or fluorescent reporter genes for the selection of mutant parasites; the homology regions required for the integration of the DNA template into the target locus; and other sequences that may be necessary to achieve the desired genetic alteration, such as parts of genes containing point mutations, for example. These plasmids have to be linearized in order to be integrated into the genomes of *P. berghei* and *P. yoelii*, while *P. falciparum* utilizes circular constructs as donor templates (468,469).

The insertion of genetic material into a chromosome of the parasite by single crossover homologous recombination only requires one homology sequence and results in the integration of the plasmid backbone in the target locus, duplicating the homology region. Nonetheless, genetic alterations to the genomes of the rodent malaria species using this approach require the linearization of plasmid in the targeting sequences, originating a short and a long homology arm (Fig. 17A). On the other hand, double crossover homologous recombination requires two homology regions and substitutes the sequence flanked by these two targeting regions in the chromosome for the sequence present in the provided vector (Fig. 17B) (468,469).

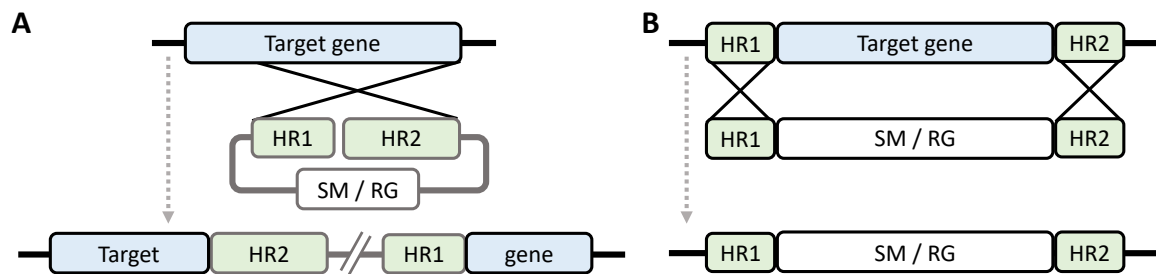


Figure 17. Homologous recombination in *P. berghei*. (A) Disruption of a target gene by single crossover homologous recombination. The representation of the backbone of the donor plasmid, which contains the selectable marker (SM) or reporter gene (RG), was abridged. (B) Gene deletion by double crossover homologous recombination. The scheme shows the integration of the linearized transfection vector into the target locus using two distinct homology regions (HRs).

The minimal length of the homology regions required for the efficient insertion of the construct into the correct location of the genome of the parasite may vary depending on the targeted locus, although sequences of at least 250 base pairs are commonly included in targeting vectors (468). However, since the frequency of integration increases with the length of the homologous sequences, transfection vectors bearing large targeting sequences have been created by the *Plasmodium* Genetic Modification Project (*PlasmoGEM*). These vectors for double crossover homologous recombination in *P. berghei* carry gene specific barcodes to identify individual mutants, which enables the simultaneous transfection of several plasmids, and cassettes for both positive and negative selections, which allows the recycling of the selectable marker, which is also the goal of the “gene insertion/marker out” (GIMO) strategy (470,471). The analogous “gene out/marker out” (GOMO) method adds fluorescent reporter genes to the positive and negative selection steps to simplify the isolation of marker-free recombinant parasites (472). The usefulness of drug-selectable marker recycling is evident if we consider the paucity of genes for positive selection available for *P. berghei* genetic manipulation, which limits the number of successive modifications to the genome. In fact, only three selectable markers can be used for positive selection in this species, namely the genes encoding the *T. gondii* dihydrofolate reductase-thymidylate synthase (TgDHFR/TS), which confers resistance to pyrimethamine; an equivalent pyrimethamine-resistance version of the *P. berghei* DHFR/TS; and the human dihydrofolate reductase (hDHFR), which confers resistance to both pyrimethamine and WR99210. Moreover, mutations that naturally arise in the endogenous *P. berghei* DHFR/TS (PbDHFR/TS) complicate the selection of transfectants following the transfection (473). The roster of selectable markers available for *P. falciparum* is considerably more diversified, although the *hdhfr* still sees more use due to its robustness. Other options include the genes encoding the blasticidin S deaminase (selected with blasticidin), the neomycin phosphotransferase II (selected with G418, a neomycin analogue), the puromycin-N-acetyltransferase (selected with puromycin), and the yeast dihydroorotate dehydrogenase (selected with atovaquone or the inhibitor DSM1) (473).

4.2. Site-specific genome editing

One of the main disadvantages of the standard methods for the alteration of the *Plasmodium* genome is the time needed for the isolation of pure mutant parasite populations. This shortcoming stems in part from the low frequency of the recombination events, since these methods rely on the natural formation of double strand breaks in the vicinity of the target

region. Therefore, several new techniques for site-specific and, therefore, rapid gene editing have been adapted to malaria models in recent years.

Bxb1 is an integrase that can be transiently expressed in *Plasmodium* parasites and catalyzes the recombination between an attP site that is present on a plasmid and attB element that previously inserted in the genome. This system enables the insertion of large constructs in the target site and has little off-target effects. However, since it has only been developed for *P. falciparum*, its utility in the study of sporozoite biology is limited. Moreover, the insertion location of attB site on the genome must be carefully considered and this step is performed by conventional crossover homologous recombination (474,475).

Zinc finger nuclease-based systems produce double strand breaks at the desired locus and trigger DNA repair by homologous recombination if a donor template is provided, which greatly improves the efficiency of the genetic modification. This technique has been successfully employed in *P. falciparum*, *P. berghei* and even *P. vivax in vivo* (476–478). The expression of the zinc finger nucleases can also be stage specific by choosing different well-characterized promoters, therefore allowing stage-specific genetic alterations (476,478). However, this system is prone to off-target effects and the customization and testing of the target-directed zinc finger nucleases can be costly and time-consuming (473,475).

The advent of clustered, regularly interspaced, short palindromic repeat (CRISPR)/ CRISPR-associated protein 9 (Cas9) systems (CRISPR/Cas9) revolutionized gene editing in a multitude of living organisms. These systems were readily applied to the genetic modification of *P. falciparum* (479–481) and *P. yoelii* (482,483), but only recently to *P. berghei* (484). CRISPR/Cas9 requires 3 elements: the Cas9 nuclease, which can be expressed by knock-in parasites or from an episome; the donor template DNA, which can be circular or linear, depending on the *Plasmodium* species and the system; and a single-guide RNA (sgRNA), which may be delivered in its own plasmid or incorporated into the circular donor template or Cas9 plasmids. The sgRNA contains a customizable 20 nucleotide sequence that pairs with the target sequence, which has to be located directly upstream a NGG protospacer adjacent motif (PAM). The sgRNA and Cas9 form a complex that induces a double strand break in the target locus, a few nucleotides upstream of PAM (485). Thus, the main advantages of CRISPR/Cas9 are the drastically improved efficiency of the genetic modifications, the reduction in the time needed to obtain mutant lines, and its ease of use, although potential off-target cleavage is still a major shortcoming of this type of genome editing approach (486). Therefore, careful design of the sgRNA is required, which may not be simple considering the restriction to PAM-adjacent targeting sites. Commercially available sgRNA and recombinant Cas9 have also been shown to

allow the editing of *P. falciparum* genome, adding a more cost-effective option to the list of CRISPR/Cas9 (487).

4.3. Conditional systems

The transfection of *Plasmodium* parasites is performed in merozoites, or in rings stages in the case of *P. falciparum*, as large numbers of parasites can be collected, which increases the chance of selecting mutant parasites as the electroporation procedure causes the death of many cells and the efficiency of vector integration in the genome is relatively low (488). As the genome is haploid in this form of the parasite, only one round transfection is normally necessary to achieve the intended genetic alteration. However, this also makes the study of genes essential for the asexual blood stages considerably more challenging.

Therefore, numerous conditional systems have been adapted to the study of the function of essential genes in the *P. falciparum* blood stages. These include systems for the conditional deletion of genes or regulatory sequences (489–491), inducible downregulation of transcription (492,493), and regulatable translation or degradation of transcripts (494–499), and conditional degradation or mislocalization of proteins (500–503). In contrast, the downregulation of gene expression in *P. berghei* sporozoites is substantially more challenging as, for example, the activity of some systems is optimal at 37 °C, and the delivery of DNA templates for homologous recombination and of some exogenous molecules required for the activation of many inducible systems cannot be performed in the mosquito. Sporozoites collected from infected mosquitoes may be pre-treated with drugs, but the long duration of these incubations needed to achieve protein degradation (502), for example, may decrease sporozoite infectivity. Thus, only two methodologies have been successfully used in multiple studies to achieve conditional gene knockout or knockdown in *P. berghei* sporozoites.

The first is the flippase (Flp)-flippase recombination target (FRT) system. This method is based on the expression of Flp, which is a yeast recombinase that recognizes 34-base-pair-long FRT sequences and excises or inverts the genomic region flanked by two of these sites, depending on their orientation (Fig. 18A) (504,505). Different promoters can drive the expression of Flp at specific stages of the life cycle, ensuring stage-specific gene deletion. For instance, the promoter of *uis4* has been used to drive the expression of Flp in mature sporozoites, while the promoter of *trap* enables an earlier excision of the target sequence (504). The efficiency of excision is further controlled by the temperature at which mosquitoes are incubated, due to the optimal enzymatic activity of the recombinase variants at specific ranges of temperature. The activity of Flp is increased in the 23–30 °C range and near optimal at 30 °C. On the other hand,

the use of the thermolabile variant FlpL (low activity at 37 °C) avoids premature excision during the blood stages while permitting the deletion of target genes at 20–25 °C, although it is more active at the upper limit of the thermal range (505).

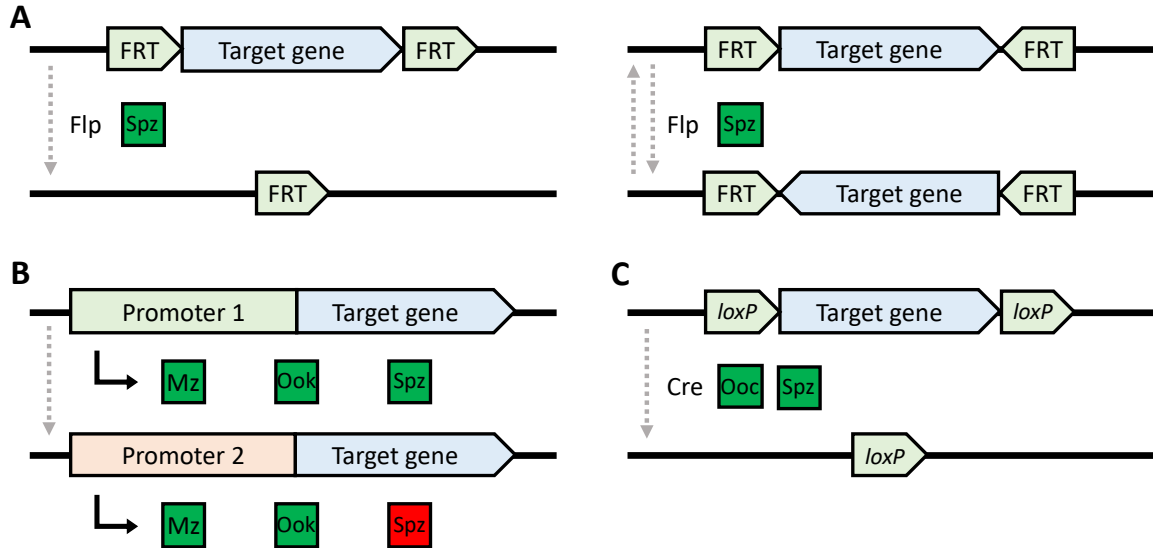


Figure 18. Methodologies for conditional depletion of proteins in *P. berghei* sporozoites. (A) Example of a Flp-FRT system for gene deletion (left) or inversion (right) in sporozoites (Spz). The inversion of the target sequence is reversible, while the excision is strongly favored. (B) Example of a promoter swap approach for the maintenance of gene expression in merozoites (Mz) and oocysts (Ooc) and knockdown in Spz. (C) Example of Cre-loxP system for gene deletion in oocysts (Ooc) and Spz. To avoid recombination between loxP sites in successive alterations to the genome of the parasite, loxN sites may also be used, which are incompatible with loxP sequences.

P. berghei “deleter clones” have been created by integrating the genes encoding Flp variants into the genome. Flp and FlpL have been paired with the *uis4* and *trap* promoters, respectively, although different combinations are possible (504,505). Therefore, although the promoter of *trap* initiates the excision of target sequences in the oocysts, maximum recombination efficiency is only achieved once mosquitoes are incubated at 25 °C. This temperature switch is typically performed from day 16 post-infection onwards to allow the sporozoites to colonize the salivary glands of the mosquito, as mosquito mortality increases at higher temperatures and, more importantly, the invasion of the salivary glands is markedly reduced at 25 °C, in comparison to 21 °C (505). Therefore, efficient excision during early sporozoite development using Flp-FRT systems is not always achievable. Moreover, the insertion of the FRT sites needs to be carefully considered to avoid alterations of gene expression, and gene excision within a

sporozoite population may be incomplete, which can impact the study of genes whose deletion only leads to mild defects (235,241,504,506). Indeed, a direct comparison between Flp-FRT and Cre-*loxP* systems in the blood stages of *P. falciparum* showed that the latter, in which the Cre recombinase excises a region flanked by *loxP* sites, was far more efficient even at the optimal temperature of Flp (507).

Alternatively, target genes can also be placed directly under transcriptional control of stage-specific promoters as to ensure expression in the replicative blood stages and the timely downregulation of expression in the desired stage(s) of the life cycle (Fig. 18B). This approach has been effectively used to study parasite fertility and transmission (508–510). More recently, it was also shown to enable the specific gene knockdown in sporozoites, revealing roles of proteins essential for the asexual blood stages in salivary gland invasion, motility and infectivity (218,511,512).

In theory, the promoter exchange methodology is a fully customizable approach to the study of gene function, allowing not only for knockdown but also upregulation of transcription in one of multiple steps of the life cycle of the parasite. However, due to the complexity of the expression profiles of certain genes, the promoter selection may not always be straightforward and needs to be tailored to the goal of the study. Therefore, the versatility of this method for the modulation of gene expression is a double-edged sword. Potential promoters often need to be validated since the number of well-characterized promoters is limited. Nonetheless, efforts have been made to characterize several stage-specific promoters or to design synthetic promoters with well-defined expression profiles (513–515). The lack of knowledge regarding the promoter elements and transcription factors that drive the stage-specific gene expression in *Plasmodium* also complicates the identification the correct regulatory regions, limiting both the selection of the new promoters and the identification of the endogenous promoter. However, promoter mapping can be performed by defining the minimal length of the regions upstream of a coding sequence required to drive the expression of a fluorescent reporter, for example (515).

The identification of the transcription start sites (TSSs) has also been used to guide the selection of putative promoter regions (516–518). The occupancy of nucleosomes, which are composed of a histone octamer, defines regions of chromatin accessibility that can be modulated by epigenic mechanisms (519). *P. falciparum* blood stages display a nucleosome-depleted region upstream of the TSS, which allows the assembly of the preinitiation complex of transcription on the core promoter, while an increased nucleosome occupancy in promoter regions hinders the initiation of transcription (519,520). It has also been shown that the TSS usually resides within 1 kb upstream of the start codon in *P. falciparum* blood stages (521). Yet, promoters

have more commonly been discovered by empirically selecting ~1.5-2 kb portions of the genomic sequences upstream of the coding sequence, although smaller sequences have also been tested (504,513,515). Nonetheless, the validation of promoters using these strategies is still a process of trial and error and, consequently, may require testing of several putative promoters. Thus, regardless of the chosen approach, promoter validation can be a laborious and time-consuming process.

Several other factors condition promoter swap strategies. The promoter of choice needs to not only secure appropriate transcript levels but also the precise timing of expression, as this has been shown to be a potentially critical factor for the correct subcellular localization and, consequently, the function of the encoded protein (522,523). Moreover, the various mechanisms for transcriptional and translational control employed by *Plasmodium* parasites may complicate the interpretation of the expression profiles (519). For instance, the fact that the sexual stages and sporozoites can use translational repression to control the timing of protein synthesis implies that transcripts for a certain gene could be detected in developmental stages where the respective promoter is not active (37,524,525). Genes can also be turned on and off through the regulation of three-dimensional genome organization, as well as chromatin structure and accessibility (520,526,527). Despite the lack of evidence of a generalized role of enhancer, silencer or insulator elements in *Plasmodium* gene expression regulation, long-range intra- or interchromosomal interactions have been shown to play an important part in transcriptional control (527–530). Importantly, this has been shown to affect the expression of genes involved in processes necessary for sporozoite infectivity (527). Therefore, promoter exchange strategies may need to consider genome architecture and the localization of the target gene locus within the chromosome.

Unlike trypanosomes such as *Leishmania* spp. and *Trypanosoma* spp., for example, *Plasmodium* is not known to possess mechanisms for polycistronic transcription of the chromosomal genome (519,531). However, the presence of bicistronic mRNAs in *P. falciparum* has been documented, indicating that the parasite is capable of using promoters to drive the expression of multiple genes, even if it is a rare occurrence (532,533). In fact, despite their mostly monocistronic transcription, by controlling chromatin accessibility and transcription, *P. falciparum* can use both genetic and epigenetic mechanisms to form regulatory units, in which two or more genes are co-expressed in specific stages of the life cycle (534,535). The presence of regions of open chromatin, which are associated with active transcription, has also been shown to often correlate with the expression of genes separated by more than one coding sequence, which could indicate that the target gene of a regulatory element may not be limited to the nearest gene (535). And finally, although transcription in *Plasmodium* is tightly

controlled, promoter leakiness may still be an issue, specifically for proteins naturally present in low abundance. All these factors should be taken into account when delineating a promoter swap strategy. Despite these limitations, this approach still offers several advantages over the Flp-FRT systems, namely its flexibility and robustness once the right promoters are chosen, thus constituting a valuable tool for the study of gene function in specific or multiple stages of the parasite.

Lastly, it was recently shown that an inducible Cre recombinase-based system could not only be used to excise genes in the *P. berghei* blood stages *in vivo*, but also in the mosquito stages by administering rapamycin to the vector in sugar solutions (Fig. 18C) (536). Despite the optimal activity of the Cre enzyme at 37 °C, recombination could be induced in the mosquito stages from day 5 post-infection without the increase in the mosquito incubation temperature, which is an advantage over the Flp-FRT systems. Thus, the versatility of these methodologies allows the conditional deletion of genes across the life cycle of the parasite. However, parasite loads in the salivary glands of rapamycin-treated mosquitoes were reduced and nonexcised parasites were still clearly detectable in both the midguts and the salivary glands of treated mosquitoes, which were distinguishable from excised parasites due to the differential expression of fluorescent reporters (536). Therefore, it remains to be seen if this method will be widely used to manipulate the genome of sporozoites.

4.4. Extrachromosomal gene expression

The transfection of *Plasmodium* parasites with episomes has limited use to the study of sporozoite biology as plasmids do not segregate evenly during parasite multiplication and are therefore lost without selective pressure. Furthermore, drug pressure is only easily achievable during the blood stage and may also induce the increase of plasmid copy numbers and, consequently, the expression of the gene it carries (537). To circumvent these shortcomings, *Plasmodium* artificial chromosomes (PACs) and centromeric plasmids have been engineered. PACs are linear constructs bearing telomeres and a centromere region and, thus, behave like *Plasmodium* chromosomes (538,539). Centromeric plasmids are circular versions of these constructs, therefore lacking the telomeric sequences while maintaining the core centromere region that increases the fidelity of plasmid segregation among daughter cells during mitosis and limits episome copy numbers per cell without selective pressure (538). However, studies using a centromeric plasmids in *P. berghei*, *P. yoelii* and *P. vivax* showed that the percentage of parasites carrying these expression vectors may still decrease during the multiple rounds of cell division during the blood stage (540,541). The cycling of the parasites through mosquitoes

and back into mice might increase the loss of the episomes as parasites multiply by sporogony or schizogony in the two hosts (541). Additionally, the choice of promoter to drive the expression of the gene in PACs and centromeric plasmids may also affect protein levels, localization and function, as previously mentioned.

Nonetheless, extrachromosomal expression vectors disregard any unspecific phenotypes that may arise, for example, from the alterations of gene organization and locus structure rather than from modifications made to the target gene. Moreover, as no integration into the genome is required, the selection of transfected parasites is significantly improved. Therefore these are valuable tools for the complementation of knockout or knockdown lines, the study of the effect of overexpression of endogenous proteins, the heterologous expression of transgenes, and localization studies using tagged proteins.

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Chapter II

Objectives and results

1. Thesis contextualization and objectives

The efficiency of infection by *Plasmodium* parasites accentuates the remarkable adaptation of these organisms to distinct environments. The dissection of the molecular mechanisms underlying key processes required for the completion of the life cycle could add new molecules to the roster of antigens that can be exploited by antimalarial vaccines. It is also instrumental to the selection of the life cycle steps to target, which, in turn, is of paramount significance to the delineation of rational vaccine development strategies. One of the understudied steps of the journey of these parasites in their mammalian hosts is the arrest of sporozoites in the liver sinusoids. Blocking this crucial point of the life cycle would prevent the infection, especially if combined with other approaches to simultaneously tackle distinct steps of the migration and development of the parasite in their vertebrate hosts.

The current model that explains the homing of sporozoites to the liver relies mostly on *in vitro* data or *in vivo* experiments where the outcome of a liver infection was evaluated, as discussed in the previous chapter. The possibility to perform live imaging in animal models has been critical for the advance of our knowledge of critical steps of the parasite's life cycle. Indeed, when combined with genetically modified parasites and/or animal models, live imaging techniques have been key for groundbreaking discoveries in malaria (1). In this project, we started by exploring the use of whole-body bioluminescence imaging to visualize and quantify the homing of the parasites to the liver following bloodstream dissemination.

The increased infectivity of parasites residing in the mosquito salivary glands in comparison to sporozoites in earlier stages of development has been extensively documented (2–4). This difference unquestionably comes from cumulative defects of the immature sporozoites in discrete biological process required for the completion of the pre-erythrocytic phase, such as migration and invasion. However, a direct link between sporozoite maturation and their capacity to home to the liver has not been established. Using whole-body bioluminescence imaging, we observed that the homing of sporozoites to the liver was directly correlated with their maturation (Fig. 19; Sá *et al.*, in preparation). This finding was somehow unexpected as immature (oocyst) and mature (salivary gland) sporozoites express comparable levels of CSP (5,6), one of the main candidates thought to be involved in this process. Moreover, the conformation CSP adopts in oocyst sporozoites also exposes its adhesive TSR domain (7,8), as previously mentioned.

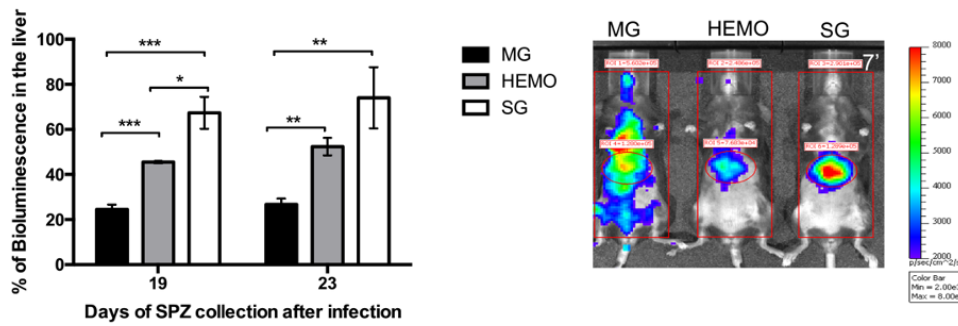


Figure 19. Homing of *P. berghei* sporozoites to the liver. Midgut (MG), hemolymph (HEMO) or salivary gland (SG) sporozoites (SPZ) were intravenously injected in C57BL/6 mice and the bioluminescent signal was acquired 7 min post-infection. The quantification of the signal in the liver is shown in percentage of total bioluminescence (left) and a representative photograph of infected mice to which the signal map was superimposed is shown next to the radiance heatmap scale (right). Data from Sá *et al.* (in preparation).

Therefore, we hypothesized that the molecule(s) required for the homing of sporozoites to the liver are acquired during the maturation program of the parasite in the mosquito. Alternatively, sporozoites could gradually suffer post-translational modifications to constitutively expressed surface molecules, such as the proteolytic cleavage of CSP, which would allow them to recognize their cognate receptor(s) in the hepatic sinusoids more efficiently.

To test the first theory, we selected candidate molecules using the following core criteria: 1) increase in protein abundance in salivary gland sporozoites in comparison to oocyst sporozoites, with more than 1 unique peptide per protein detected in salivary gland sporozoite samples (5); 2) evidence of surface localization, namely the presence of a predicted signal peptide, transmembrane domains or a GPI anchor, or previously published localization studies; 3) existence of a *P. berghei* orthologue, as this rodent model of malaria would be used to evaluate the role of these surface proteins in the arrest of sporozoites in the liver, based on the assumption that molecules responsible for this process are conserved among species that develop in this organ; 4) no evidence of functions in developmental stages other than the sporozoite. This final criterium was applied in order to maximize the chances of identifying proteins specifically involved in this process, although we could not preclude that molecules necessary for this sporozoite-specific behavior could also perform other functions in discrete stages of the life cycle. Additionally, we also prioritized molecules containing predicted adhesive domains. In the context of this thesis, we studied the candidates shown in Table 1 and Fig. 20.

Table 1. List of *Plasmodium* surface molecules studied in the scope of this thesis.

Protein	Accession number		Surface localization				NUP/P (5)	
	<i>P. falciparum</i>	<i>P. berghei</i>	SP	TM	GPI	Ref.	Ooc	SG
SPATR	PF3D7_0212600	PBANKA_0309500	X			(9,10)	0	5
TRSP	PF3D7_0104000	PBANKA_0209100	X	X		(11)	1	3

GPI: GPI anchor. NUP/P: number of unique peptides per protein. Ooc: oocyst sporozoites. Ref.: references. SG: salivary gland sporozoites. SP: signal peptide. SPATR: secreted protein with an altered thrombospondin repeat domain. TM: transmembrane domain. TRSP: thrombospondin-related sporozoite protein.

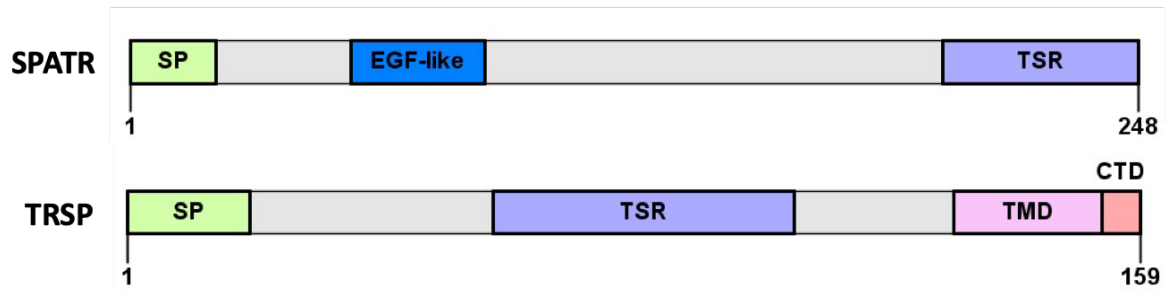


Figure 20. Structural features of the *P. berghei* TRSP and SPATR. The structural elements were obtained from available literature and databases (9,11–13) and the schemes were built with IBS online (14). The numbers indicate the position of the first and last amino acid residues of the respective full-length proteins. CTD: C-terminus domain. EGF-like: epidermal growth factor-like domain. SP: signal peptide. TMD: transmembrane domain.

TRAP is also among the candidates we have been evaluating in the laboratory. Although I also had the opportunity to contribute to this project during my PhD, since the work falls in the scope of another PhD thesis (Sá *et al.*, in preparation), the results will not be discussed herein.

In conclusion, the specific objectives of this thesis were:

1. Establishment of a method for the direct visualization and quantification of sporozoite accumulation in the liver.
2. Generation of TRSP and SPATR knockout or knockdown lines of bioluminescent and fluorescent *P. berghei* parasites.
3. Evaluation of the homing of mutant sporozoites to the liver using live imaging.
4. Study of the mutant phenotypes in other key steps of the life cycle of the parasite.

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2. Results

2.1. *In vivo* imaging of pathogen homing to the host tissues

Hematogenous dissemination followed by tissue tropism is a characteristic of the infectious process of many pathogens including those transmitted by blood-feeding vectors. After entering into the blood circulation, these pathogens must arrest in the target organ before they infect a specific tissue. Here, we describe a non-invasive method to visualize and quantify the homing of pathogens to the host tissues. By using *in vivo* bioluminescence imaging we quantify the accumulation of luciferase-expressing parasites in the host organs during the first minutes following their intravascular inoculation in mice. Using this technique we show that in the malarial infection, once in the blood circulation, most of bioluminescent *Plasmodium berghei* sporozoites, the parasite stage transmitted to the host skin by a mosquito bite, rapidly home to the liver where they invade and develop inside hepatocytes. This homing is specific to this developmental stage since blood stage parasites do not accumulate in the liver, as well as extracellular *Trypanosoma brucei* bloodstream forms and liver-infecting *Leishmania infantum* amastigotes. Finally, this method can be used to study the dynamics of tissue tropism of parasites, dissect the molecular and cellular basis of their increased arrest in organs and to evaluate immune interventions designed to block this targeted interaction.

Reprinted from Methods 2017 Aug 15;127:37-44. doi:10.1016/j.ymeth.2017.05.008.

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Methods xxx (2017) xxx–xxx



Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth*In vivo* imaging of pathogen homing to the host tissuesJoana Tavares^{a,b,*}, David Mendes Costa^{a,b}, Ana Rafaela Teixeira^{a,b}, Anabela Cordeiro-da-Silva^{a,b,c},
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ARTICLE INFO

Article history:

Received 2 March 2017

Received in revised form 19 April 2017

Accepted 10 May 2017

Available online xxxx

Keywords:

Live imaging

Arrest

Tropism

Plasmodium

Leishmania

Trypanosoma

ABSTRACT

Hematogenous dissemination followed by tissue tropism is a characteristic of the infectious process of many pathogens including those transmitted by blood-feeding vectors. After entering into the blood circulation, these pathogens must arrest in the target organ before they infect a specific tissue. Here, we describe a non-invasive method to visualize and quantify the homing of pathogens to the host tissues. By using *in vivo* bioluminescence imaging we quantify the accumulation of luciferase-expressing parasites in the host organs during the first minutes following their intravascular inoculation in mice. Using this technique we show that in the malarial infection, once in the blood circulation, most of bioluminescent *Plasmodium berghei* sporozoites, the parasite stage transmitted to the host skin by a mosquito bite, rapidly home to the liver where they invade and develop inside hepatocytes. This homing is specific to this developmental stage since blood stage parasites do not accumulate in the liver, as well as extracellular *Trypanosoma brucei* bloodstream forms and liver-infecting *Leishmania infantum* amastigotes. Finally, this method can be used to study the dynamics of tissue tropism of parasites, dissect the molecular and cellular basis of their increased arrest in organs and to evaluate immune interventions designed to block this targeted interaction.

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1. Introduction

The use of the host circulatory system to disseminate and infect specific tissues is a recurrent characteristic observed in the lifecycle of innumerable viruses, bacteria and parasites. This tissue tropism is frequently associated with the mode of transmission of these microorganisms, as observed for pathogens that invade salivary glands of arthropod vectors and are transmitted into the host skin with the vector saliva during an infectious bite [1,2]. Tissue tropism is also associated with the pathology caused by these microorganisms as known, for example, for the hepatitis or encephalitis-causing viruses [3]. In some cases this targeted infection can also be asymptomatic as observed for *Plasmodium* sporozoites, the infective malaria parasite stage inoculated in the skin during a mosquito bite. Following deposition in the skin tissue, sporozoites

invade blood vessels [4,5] and enter into the blood circulation to specifically and transiently infect the liver [6–8].

Since mammals have a closed circulatory system, the use of the blood circulation to reach specific tissues requires the arrest of the pathogen in the target organ and the ensuing crossing of the endothelial barrier, which physically separates the blood compartment from the organ parenchyma. Some organs, such as the liver, spleen and adrenal glands, are directly exposed to blood-borne pathogens due to the presence of fenestrated vessels, allowing a direct extravasation of small pathogens (e.g., fenestra diameter of hepatic sinusoids: ~100 nm [9]), or a local open circulation. Consequently, the arrest of pathogens can be expected to occur through their binding to molecules on the surface of either tissue-specific endothelial cells or resident immune cells, or to molecules of extravascular cells exposed to the blood circulation in the case of fenestrated sinusoids and in the open splenic circulation.

The dissection of the mechanisms used by pathogens to specifically infect a tissue is hampered by the lack of an easy and quantitative method to assess this first step of arrest in the host organs following blood dissemination. The localization and quantification of pathogens in tissues usually requires the dissection of organs

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<http://dx.doi.org/10.1016/j.ymeth.2017.05.008>

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Please cite this article in press as: J. Tavares et al., Methods (2017), <http://dx.doi.org/10.1016/j.ymeth.2017.05.008>

and the measurement of pathogen load in the excised samples by PCR, radioactive labeling, subinoculation or *in vitro* culture. On the other hand, bioluminescence imaging can reveal the distribution of living luciferase-expressing cells in a non-invasive and quantitative manner in the whole body of small hosts, such as mice. This technique, which is based on an enzymatic reaction that generates photons following the oxidation of a substrate, has been frequently applied to study *Plasmodium* behaviour [10] in the blood [11,12], liver [13–15] and skin of rodents [8,16]. The firefly luciferase has been preferentially used for *in vivo* studies when compared to the NanoLuc and Gaussia luciferase, which despite emitting more light *in vitro*, seem to be less efficient *in vivo* [17]. This is in part due to the higher absorption and scatter by the host tissues of the blue light emitted by their substrates [18]. Accordingly, increased *in vivo* sensitivity has been successfully achieved by the red-shifted variant of firefly luciferase [19,20]. Here, using bioluminescence imaging we demonstrate the specific homing of firefly luciferase-expressing *Plasmodium berghei* sporozoites to the liver following the first minutes of their hematogenous dissemination.

2. Material and methods

2.1. Mice

All experiments were carried out in accordance with the IBMC, INEB Animal Ethics Committees and the Portuguese National Authorities for Animal Health guidelines or in accordance with the Animal Care and Use Committee of Institut Pasteur both according to the statements on the directive 2010/63/EU of the European Parliament and Council. Four to six weeks old C57BL/6, Swiss, NMRI or BALB/c mice were purchased from Elevage Janvier or Charles River.

2.2. Parasites

Throughout the experiments parasites used include *Plasmodium berghei* ANKA strain clone 676c11 expressing a GFP-Luciferase fusion gene via the pbef1 α promoter [21], *Trypanosoma brucei brucei* Lister 427 expressing the redshifted luciferase gene (PpyRE9h) flanked by 5'VSG/3'tubulin [20,22], a cloned line of *Leishmania infantum* (MHOM/MA/67/ITMAP-263) expressing luciferase under the control of the intergenic region of α -tubulin [23].

To obtain *P. berghei* (Pb) sporozoites, female *Anopheles stephensi* mosquitoes (SDA 500 strain) reared in the Center for the Production and Infection of Anopheles at the Pasteur Institute were infected 3–4 days after emergence and kept as previously described [24]. Sporozoites were collected from salivary glands 21–28 days after the infectious blood meal and kept on ice in PBS until inoculated into mice. Merozoites were obtained by culturing infected mouse blood in RPMI 1640 (Lonza) supplemented with 20% heat inactivated fetal bovine serum (Biowest) and 50 μ g/ml neomycin (Sigma) and flushed 90 s at 1.5–2 bar pressure with a gas mixture of 5% CO₂, 5% O₂ and 90% N₂ for 16 h at 37 °C and under shaking. Mature schizonts were separated using a Nycodenz (Axis Shield) gradient and merozoites were isolated by filtration of schizonts through a 1.2 μ m filter [25].

The bloodstream forms of *T. b. brucei* (Tb) Lister 427 were grown in HMI-9 medium (Sigma) supplemented with 10% heat inactivated fetal bovine serum (Biowest) and 100 IU/ml of penicillin/streptomycin (Lonza) at 37 °C, 5% CO₂ in a humidified atmosphere [26].

L. infantum (Li) axenic amastigotes were cultured at 37 °C, and 5% CO₂ in a cell-free medium called MAA/20 (medium for axenic amastigotes growth) as described previously [27]. Briefly,

MAA/20 consisted of modified medium 199 (Invitrogen) with Hank's Balanced Salt Solution supplemented with 0.5% tryptocasein (Oxoid), 15 mM D-glucose (Sigma), 5 mM glutamine (Lonza), 4 mM NaHCO₃, 0.023 mM bovine hemin (Fluka) and 25 mM HEPES (Lonza) to a final pH of 6.5 and supplemented with 20% heat inactivated fetal bovine serum (Biowest).

2.3. Bioluminescence imaging

Parasite loads of mice infected with luciferase-expressing parasites were assessed by bioluminescence imaging using the IVIS Lumina LT system (Perkin Elmer). Prior to infection, animals had their ventral fur shaved with an appropriate clipper. In all the models, mice were infected following the intravenous injection into the tail vein of 100 μ l of parasite suspension.

For the experiments with Pb, C57BL/6 mice were typically infected with either $\sim 1.5 \times 10^5$ salivary gland sporozoites or ~ 2 to 6×10^7 blood merozoites. Infections with Tb bloodstream forms or with Li axenic amastigotes were performed in BALB/c mice following the inoculation of respectively 1×10^6 or 1 to 2×10^8 parasites. Immediately after parasite inoculation, mice were anesthetized with 2.5% isoflurane and four minutes after the infection, injected subcutaneously with D-luciferin (2.4 mg, Perkin Elmer). Mice were then transferred to the stage of an intensified charge-coupled device photon-counting video camera box where anaesthesia was maintained with 2.5% isoflurane. After a three minute incubation allowing the distribution of the substrate in the body of the anesthetized animals, a five minute signal acquisition controlled by the Living Image software (Perkin Elmer) was initiated. At the end of this period animals returned to their cage and recovered from the anaesthesia. Measurement of infection by bioluminescence one day later was performed as described above.

The detection of the bioluminescence signals by the system resulted in the generation of signal maps automatically superimposed to the grey-scale photograph of the mice. The quantifications were performed using the Living Image software (Perkin Elmer). Bioluminescent signals on the regions of interest (ROI) encompassing most of the ventral view of the animal body, thorax, liver, spleen and lower abdomen were manually defined as described below and applied to all animals. The total flux (photons/s) and average radiance (photons/s/cm²/steradian) within these ROIs was automatically calculated. ROI background signal was obtained from imaging non-infected mice that received D-luciferin as described above. The total flux or the average radiance background signal of the respective ROIs were subtracted to the respective total flux or average radiance measured on infected animals. The percentage of bioluminescence in the defined ROIs (thorax, liver, spleen and lower abdomen) was calculated by dividing the total flux of the respective ROI by the total flux of the ventral animal body ROI.

2.4. Statistical analysis

All the statistical analysis and graphical representation of the data were performed using GraphPad Prism 6 software. Students *t*-test or a two-way analysis of variance (ANOVA) with Sidak's multiple comparisons test was performed and statistical significance was asserted whenever the value of *p* was lower than 0.05 by *(*p* < 0.05), **(*p* < 0.01), ***(*p* < 0.001) and ****(*p* < 0.0001).

3. Results and discussion

In this study we applied 2D bioluminescence imaging to investigate the accumulation of blood-circulating Pb bioluminescent sporozoites in the liver of living mice during the first minutes fol-

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J. Tavares et al. / Methods xxx (2017) xxx–xxx

3

lowing their intravascular inoculation, herein defined as homing. The pattern of parasite homing was also further compared to the successful establishment of infection as measured by the distribution of the parasite bioluminescence signal one day later.

To assess the unambiguous accumulation of sporozoites in the liver, we use different firefly luciferase-expressing blood-borne parasites with relatively similar sizes and with different specificities and capacities of invasion.

As shown in the Fig. 1A, once in the blood circulation, Pb sporozoites and merozoites invade and develop, respectively, inside hepatocytes and erythrocytes [28]; Li amastigotes are known to survive and multiply inside phagocytic cells, such as macrophages and dendritic cells of the liver, spleen and bone marrow [29,30], and extracellular replicative Tb bloodstream forms live in the blood, lymphatic system and interstitial space of organs [31]. Therefore, the parasites used in this study include pathogens that infect the liver (Pb sporozoites and Li amastigotes), the spleen (Li amastigotes) and the blood of the host (Pb merozoites and Tb bloodstream forms).

Taking advantage of the Living Image software that can superimpose the bioluminescence signal of parasites and the grey-scale photograph of mice, the ventrally exposed body of the host, placed in the dorsal position, was equally divided from the nostril to the perineal body in four rectangular areas, delineating the head, thorax, upper abdomen and lower abdomen (Fig. 1B). Importantly, the middle line of this zone, laying between the thorax and the upper abdominal area, coincided with the sternum, and therefore served as a robust fiducial mark. The total area of these four rectangular regions of interest (ROIs) was used to determine the total bioluminescence signal emitted by the parasites in the ventral view of the animal body (ventral ROI). Inside these rectangular ROIs and for quantification purposes, elliptical ROIs encompassing organs well known to be infected by some of the parasites listed in

the Fig. 1A were defined using the Living Image software and used to quantify the relative distribution of parasite load by bioluminescence at early (homing) and late (infection) time points.

In the upper abdomen, the ROI for quantifying parasite load in the liver was defined by infecting mice with luciferase-expressing Pb sporozoites. Two days after sporozoite inoculation in the host, when parasites reach the maximum of their intracellular development inside hepatocytes, we averaged the bioluminescence signal of three infected mice and determined the corresponding ROI of the infected liver (Fig. 1B, hepatic infection, left panel). As expected, the liver was the only visceral organ to emit photons and the hepatic ROI was placed just below the sternum matching the model illustrated in the IVIS mouse atlas model (Fig. 1B, right panel). The position of the liver using bioluminescence was confirmed using luciferase-expressing Li amastigotes. Accordingly, the hepatic ROI obtained using amastigote infection corresponded to the bioluminescence signal of sporozoite infection (Fig. 1B, left and middle panel). In addition, amastigotes also infected a region adjacent to and distinct from the hepatic ROI, which according to its anatomical position and the tissue tropism of amastigotes should correspond to the ventral extremity of the spleen (Fig. 1B, splenic infection, middle panel). To quantify the relative load of parasites in the thorax, which contains the lungs, thymus and heart, and in the lower abdomen, which contains the intestine and bladder among other organs, the elliptical area of the hepatic ROI defined using the Pb sporozoite infection was transposed to the thorax and lower abdomen rectangular areas, and was defined as thoracic and lower abdominal ROIs, respectively (Fig. 1B, right panel).

The homing and infectivity of the different parasites were determined by measuring the intensity of the parasite bioluminescence throughout the anatomical regions defined above (thoracic, hepatic, splenic and lower abdominal ROIs) relative to that of the

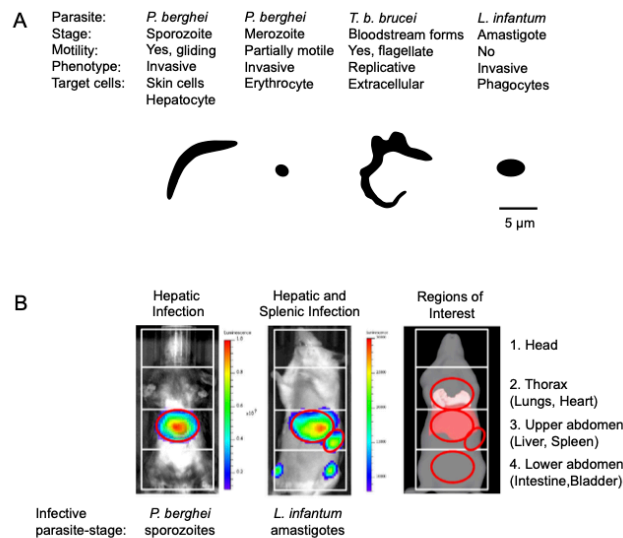


Fig. 1. Defining pathogens and regions of interest in the host. (A) Schematic representation of parasite species and stages used in this work to evaluate by ventral body *in vivo* bioluminescence imaging the parasite homing and infectivity to the host organs. (B) Defining the Regions of Interest (ROIs) used for quantitative analysis. The ventral view of the animal body was divided into four main rectangular regions to delineate the head, thorax, upper abdomen and lower abdomen. Inside these regions and for quantification purposes elliptical ROIs were drawn with Living Image software. The liver and spleen ROIs in the upper abdomen were defined based on the bioluminescence signal of mice infected with *P. berghei* sporozoites (day 2; left) and *L. infantum* amastigotes (day 2; middle). The schematic mice model (right) was obtained from the IVIS digital atlas.

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4

J. Tavares et al./Methods xxx (2017) xxx–xxx

ventral view of the animal body (ventral ROI). For that, the total flux (photons/s) of the respective ROI was divided by the total flux of the ventral ROI, previously subtracted by the background values calculated from non-infected animals. In addition, the parasite development in the tissues was evaluated by comparing the average radiance (photons/s/cm²/steradian) of ROIs at early (homing) and late (infection) time points.

Once in the blood circulation, *Plasmodium* sporozoites are known to transform and multiply into merozoites, the red blood cells infective forms, exclusively inside hepatocytes [6–8]. Sporozoites can be detected in the liver a few minutes after intravascular inoculation [16,32]. After two hours, most of injected sporozoites accumulate in the liver [33]. Therefore, prior to invading hepatocytes, sporozoites passively transported in the blood must arrest in the liver sinusoids. This arrest has been proposed to be due to both a slower blood circulation speed in the sinusoids and the interaction of the most abundant sporozoite surface protein, the circumsporozoite protein (CSP) with the highly sulphated proteoglycans that protrude through the fenestra of endothelial cells [28,34]. Noteworthy, the current model explaining this crucial step

is based on indirect experiments interpreting the binding of recombinant proteins to hepatocytes or the outcome of a liver infection by sporozoites. When and how sporozoites arrest in the liver is still an unsettled issue.

To evaluate whether *Plasmodium* sporozoites specifically home to the liver in the first minutes following their intravascular inoculation in C57BL/6 mice, the distribution and relative intensity of the bioluminescence signal emitted by luciferase-expressing sporozoites was calculated in the previously defined ROIs and compared to that obtained after injection of luciferase-expressing blood merozoites. Using this method, ~70% of the total bioluminescence signal detected 7 min after the intravenous injection of sporozoites was found in the liver ROI, contrasting with only ~20% after injection of merozoites (Fig. 2A and B). While sporozoite bioluminescence is clearly concentrated in the liver, the bioluminescence of merozoites is seen all over the body suggesting parasites are in circulation. Indeed, free merozoites recognize, attach, and invade red blood cells and this process occurs rapidly, in the order of minutes, as the antigens present on the parasite surface are susceptible to immune attack [35]. Inside red blood cells,

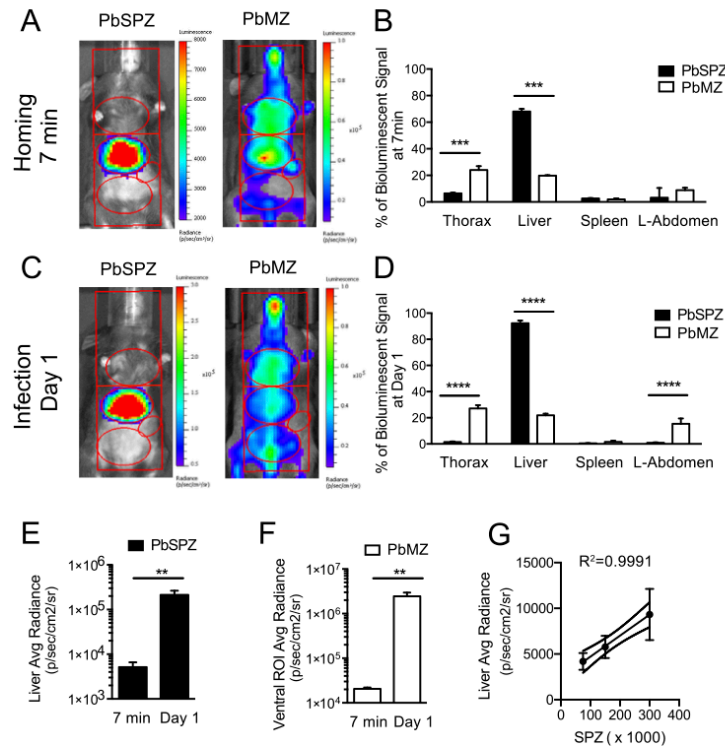


Fig. 2. *P. berghei* salivary gland sporozoites home to the liver immediately after intravascular inoculation. C57BL/6 mice were injected intravenously with luciferase expressing salivary gland sporozoites (PbSPZ) or blood merozoites (PbMZ), and the distribution of parasites was evaluated by bioluminescence imaging at 7 min (A-B) and day 1 (C-D). (B;D) The percentage of the bioluminescence signal in the thorax, liver, spleen and lower-abdomen was calculated dividing the total flux (photons/s) of the respective ROIs by that of the ventral ROI previously subtracted by the background values calculated from non-infected mice. The graphs show the mean + standard deviation (n = 3) and are representative of at least 2 independent experiments. Statistical significance between sporozoites and merozoites was determined using 2-way ANOVA with Sidak's multiple comparisons test. (E) Quantification at 7 min and day 1 of liver infection in average radiance (photons/s/cm²/steradian) following sporozoite injection. (F) Quantification at 7 min and day 1 of whole body infection in average radiance (photons/s/cm²/steradian) following merozoite injection. (E-F) Statistical significance calculated by *Students t*-test. **p < 0.01; ***p < 0.001; ****p < 0.0001. (G) Quantification of the liver average radiance (photons/s/cm²/steradian) at 7 min following intravenous injection of different numbers of sporozoites. The graph shows the linear regression of the bioluminescent signal with the 95% confidence bands (R² = 0.99) Symbols represent mean ± standard deviation (n = 3).

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they develop through the ring, trophozoite, and schizont stages, replicating to produce between 16 and 32 daughter merozoites. After egressing from the infected cell and invading another red blood cell, these merozoites initiate a new cycle of multiplication. This cyclic process is responsible for the clinical manifestations of malaria and lasts nearly 24 h in rodents [35]. Therefore, one day after merozoite injection, the bioluminescence signal remained distributed all over the animal body while concentrated in the liver of mice infected with sporozoites. Indeed, in the latter ~90% of the total bioluminescent signal was detected in the liver versus 20% in mice inoculated with merozoites (Fig. 2C and D). Notably, the pattern of parasite distribution in the ROIs, concentrated in the liver after sporozoite injection and disseminated through the body after merozoite injection, did not change substantially from the homing and infection time points. In the first case, this indicates that sporozoites rapidly accumulate in the liver to establish the infection of hepatocytes. In the second, this similarity suggests that merozoites and infected red blood cells are principally circulating in the vasculature. The exponential multiplication of parasites in the liver (Fig. 2E) or in the blood (Fig. 2F) was assessed by quantifying the average radiance of the bioluminescence signal respectively, in the hepatic and ventral ROIs.

In the range of 75,000–300,000 injected sporozoites, a linear relationship is observed between the number of injected parasites and the average bioluminescence quantified in the hepatic ROI in the first minutes post-inoculation (Fig. 2G, $R^2 = 0.99$). Therefore, this method can be used to quantify not only changes in the parasite relative distribution (homing pattern) but also in the sporozoite load, early during the hepatic infection.

To confirm the specificity of sporozoite homing to the liver, another blood circulating bioluminescent parasite, the extracellular replicative monomorphic Tb was intravenously injected into BALB/c mice. The relative distribution of the bioluminescence signal of Tb bloodstream forms was quantified using the pre-defined ROIs (Fig. 3) and compared to that obtained using *Plasmodium* parasites (Fig. 2). Immediately after intravascular inoculation, the bioluminescence signal was detected all over the body as observed following Pb merozoite injection. Similar percentages of bioluminescence in the hepatic (~20%) and in the other ROIs strongly indicate that both Pb merozoites and Tb bloodstream forms are circulating with the same pattern in the blood, presumably without any detectable specific arrest in the organs (Fig. 3A and B). Consequently, the values obtained in the ROIs should correspond approximately to the percentual blood volume of these regions. Indeed, the value of 20% of total bioluminescence in the liver is in the range (14–30%) of the total blood percentage found in the liver [36,37]. The distribution of Tb bloodstream forms in the ROIs did not also change substantially at the homing and infection time points, except for the splenic ROI. Owing to parasite multiplication the average radiance in the thorax, liver and lower abdomen increased ~15-fold from the first minutes after infection to day 1, contrasting with the 90-fold observed in the splenic ROI (Fig. 3C).

To determine if the capacity to infect the liver is associated with the homing of parasites to this organ as seen for sporozoites, we assess the distribution pattern of bioluminescent Li amastigotes known to infect macrophages including those of the liver, as depicted in the Fig. 1B. Shortly after intravenous injection of amastigotes into BALB/c mice, bioluminescence is mostly observed in the thorax, presumably in the right and left lungs given the anatomical position and shape of the signal, as well as, in the spleen and in the liver (Fig. 4A). The spleen and the liver are known to harbour resident macrophages, which can contact the blood and clear particulate material from the circulation [38]. Interestingly, the bioluminescence signal of amastigotes in the liver, ~20% of the total signal, did not statistically differ from the percentage observed using injection of blood-circulating Tb bloodstream

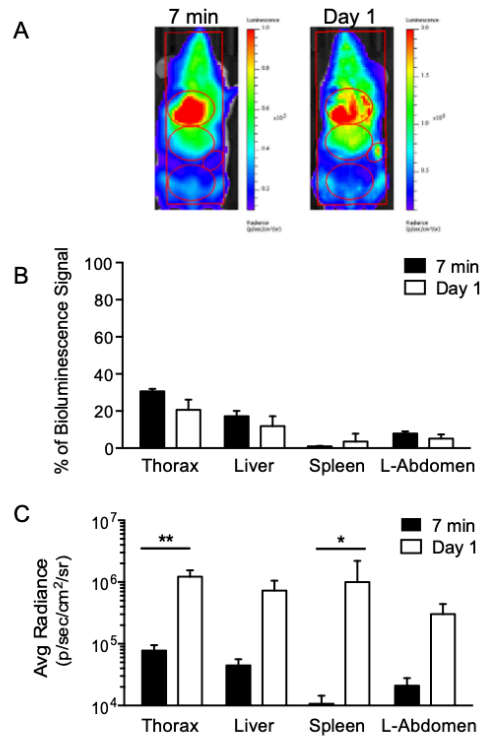


Fig. 3. Relative distribution and replication of *T. b. brucei* bloodstream forms. (A–C) BALB/c mice were injected intravenously with red-shifted luciferase expressing Tb bloodstream forms and the distribution of parasites evaluated by bioluminescence imaging at 7 min and day 1. (B) The percentage of the bioluminescence signal in the thorax, liver, spleen and lower-abdomen was calculated dividing the total flux (photons/s) of the respective ROIs by that of the ventral ROI previously subtracted by the background values calculated from non-infected mice. (C) Quantification of the bioluminescence signal in average radiance (photons/s/cm²/steradian) in the thorax, liver, spleen and lower abdomen. (B–C) The graphs show the mean + standard deviation ($n = 4$) and are representative of 2 independent experiments. Statistical significance between 7 min and day 1 for the several regions of interest was determined using 2-way ANOVA with Sidak's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$.

forms and Pb merozoites (Fig. 5A), in clear contrast with the 70% of Pb sporozoites that accumulated in the liver. These results show that despite infecting the liver, Li amastigotes do not significantly accumulate in this organ shortly after intravascular inoculation. On the other hand, the accumulation of amastigotes in the spleen was significantly higher than those of Pb merozoites and Tb bloodstream forms, indicating a preference of Li amastigotes to accumulate in this organ. Regarding the distribution of amastigotes in the thoracic region, despite the distinctive pulmonary pattern, the percentage of bioluminescence was not statistically different from Tb injection but differed from Pb merozoites, complicating the interpretation of the data (Fig. 5A). Importantly, the percentage of the total bioluminescence detected in the thorax decreased from ~40 to 5% when comparing the first minutes after Li amastigotes inoculation to day 1 (Fig. 4B), while it increased in the liver and spleen from ~20 to ~40% and from ~20 to 35%, respectively (Fig. 5). Analysis of the average radiance shows a significant reduction in the

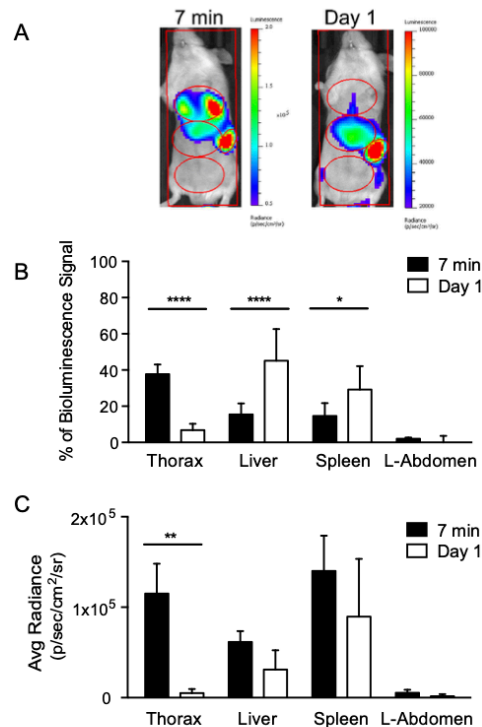


Fig. 4. Homing and infection of *L. infantum* amastigotes. (A–C) BALB/c mice were injected intravenously with luciferase expressing axenic Li amastigotes and the distribution of parasites evaluated by bioluminescence imaging at 7 min and at day 1. (B) The percentage of the bioluminescence signal in the thorax, liver, spleen and lower-abdomen was calculated dividing the total flux (photons/s) of the respective ROIs by that of the ventral ROI previously subtracted by the background values calculated from non-infected mice. (C) Quantification of the bioluminescence signal in average radiance (photons/s/cm²/steradian) in the thorax, liver, spleen and lower abdomen. (B–C) The graphs show the mean + standard deviation (n = 3–4) and are representative of 2 independent experiments. Statistical significance between 7 min and day 1 for the several regions of interest was determined using 2-way ANOVA with Sidak's multiple comparisons test. *p < 0.05; **p < 0.01; ***p < 0.001.

signal detected in the thorax at day 1 comparing to 7 min post-inoculation indicating that the parasites initially observed in the thorax were cleared in the lungs in the case of a possible arrest in this region or they were simply removed from the circulation by phagocytes of the liver, spleen, bone marrow or blood (Fig. 4C).

The liver can rapidly clear microorganisms from the blood circulation [39] through the scavenging activity of Kupffer [40,41] and endothelial cells [42]. This clearance can be extremely fast, mediated by the direct recognition of pathogens [41], or relatively slower, mediated by the recognition of host molecules bound to the opsonized pathogens [43]. However, some pathogens, such as Li amastigotes, can survive to this clearing activity and exploit this scavenging function to arrest and specifically infect the liver. *Plasmodium* sporozoites are known to be captured by KCs in the lumen of liver sinusoids [44], presumably by the interaction of CD68 with the parasite GAPDH [45,46]. After a transitory interaction with KCs, most sporozoites escape from a phagocytic death by traversing these cells and either return to the blood circulation or invade

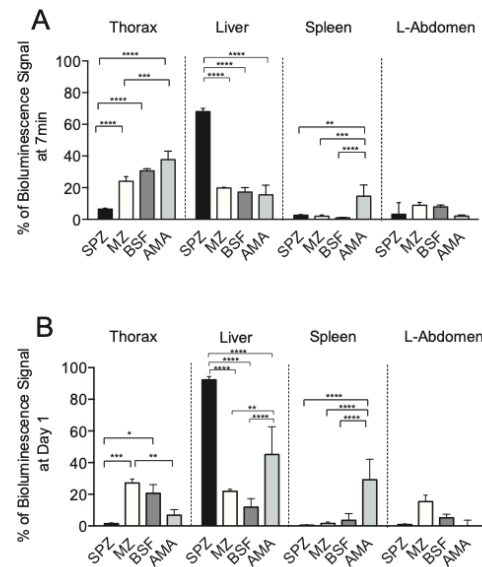


Fig. 5. Summary of homing and infection of parasites. Comparative analysis of the distribution of the bioluminescence signal (%) in the first minutes (A) and at day 1 (B) following intravenous injection of *P. berghei* sporozoites (SPZ) or merozoites (MZ), *T. b. brucei* bloodstream forms (BSF) or *L. infantum* amastigotes (AMA). (A–B) The graphs show the mean + standard deviation (n = 3–4) and are representative of at least 2 independent experiments. Statistical significance between the different parasite stages was evaluated using 2-way ANOVA with Sidak's multiple comparisons test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

the hepatic parenchyma [44]. However, KCs do not seem to be directly involved in the homing of sporozoites to the liver, since their depletion by liposome-encapsulated clodronate, rather increases the hepatic infection [47,48]. *In vivo* imaging indeed indicates that sporozoites bind to endothelial cells prior to the crossing of the sinusoidal barrier after interacting, or not, with KCs [44,49]. The lack of the intercellular adhesion molecule (ICAM) 1 and 2, complement factors C3 and C4 [50], LDL receptor related protein [51], CD36 [52] or macrophage scavenger receptors (SR) AI and AII [53] does not change sporozoite infectivity in mice, indicating that these molecules are also unlikely to play a role in the sporozoite homing. On the other hand, the sulfated polysaccharides, dextran sulfate and fucoidan, can inhibit sporozoite infectivity *in vivo* [54], while the role of SR-BI still remains controversial [55–57]. In all these cases, sporozoite infectivity is assessed by the measurement of parasite load in the liver two days post-infection, rendering impossible the discrimination of an effect of these molecules on sporozoite homing from an inhibitory effect on the invasion and/or development inside hepatocytes. This novel homing method can therefore be used to reassess the impact of these molecules and cells in the specific arrest of sporozoites in the liver. For example, combining this method with the use of mice lacking CD68 [58], stellate cells [59] or KCs [48,49], or alternatively, with the targeting of putative arrest/invasion receptors, such as CD81 [60], SR-BI [55–57] and EphA2 [61], will allow the elucidation of the role played by these factors in the sporozoite homing. Complementary to this approach, mutant sporozoites lacking adhesion molecules or neutralizing antibodies can be used to determine the impact of parasite proteins on the specific accumulation of sporozoites in the

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J. Tavares et al. / Methods xxx (2017) xxx–xxx

7

liver. Finally, this method can be adapted for the quantification of the homing of bioluminescent human-infecting sporozoites to the liver of humanized mice harboring human hepatocytes [15,57].

4. Conclusion

A complete picture of increased arrest and subsequent development of parasites in organs, defined here respectively, as homing and infection is shown in the Fig. 5. The increased arrest leading to parasite accumulation in organs was defined by statistical comparison with the distribution of blood-circulating parasites, Pb merozoites and Tb bloodstream forms. Based on this parameter, we conclude that Pb sporozoites specifically home to the liver in contrast to Pb merozoites, Tb bloodstream forms and even liver-infecting Li amastigotes. Interestingly, using this methodology, we observe the splenic and hepatic infection of amastigotes, but only the parasite accumulation in the spleen statistically differs from the distribution of both blood-circulating control parasites. These results allow us to clearly discriminate at an early time point of analysis the increased arrest (homing step) from the regular arrest of parasite in organs, both leading to a specific tissue infection (tissue tropism).

The combination of this non-invasive and quantitative imaging method with genetically modified pathogens and transgenic mouse strains lacking surface molecules on putative target cells will be key to dissect the molecular mechanisms involved in the homing of microorganisms to tissues following hematogenous dissemination. Finally the use of hosts immunized against pathogen molecules involved in this arrest step certainly will assist in the design of better strategies to prevent infection.

Funding

This work was supported by funds from the Fundação para a Ciência e Tecnologia (FCT)/Ministério da Educação e Ciência (MEC) co-funded by FEDER (EXPL/JTAVARES-IF/00881/2012/C P0158/CT0005, EXPL/IMI-MIC/1331/2013) under the Partnership agreement PT2020, through the Research Unit No.4293, the Institut Paris – Paris, the French National Research Agency (Grant No. ANR-10-JCJC-1302-PlasmoPEP), the French Government's Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" (Grant No. ANR-10-LABX-62-IBED). JT is an Investigator FCT funded by National funds through FCT and co-funded through European Social Fund within the Human Potential Operating Programme. DC is funded by FCT (SFRH/BD/123734/2016).

Acknowledgements

We would like to acknowledge the team of the Center of Production and Infection of Anopheles (CEPIA), Institut Pasteur, in particular Marek Szatanik, Catherine Thouvenot and Sylvain Golba for providing the *Anopheles stephensi* female mosquitoes; the team of the Platform of Dynamic Imaging, Institut Pasteur, in particular Spencer Shorte and Marie-Anne Nicola for the help with bioluminescence imaging; Martin Taylor from the London School of Hygiene and Tropical Medicine for kindly providing the construct used to generate the *T. b. brucei* Lister 427 strain expressing the red-shifted luciferase.

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J. Tavares et al./Methods xxx (2017) xxx–xxx

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2.2. TRSP is dispensable for the *Plasmodium* pre-erythrocytic phase

Plasmodium sporozoites deposited in the skin following a mosquito bite must migrate and invade blood vessels to complete their development in the liver. Once in the bloodstream, sporozoites arrest in the liver sinusoids, but the molecular determinants that mediate this specific homing are not yet genetically defined. Here we investigate the involvement of the thrombospondin-related sporozoite protein (TRSP) in this process using knockout *Plasmodium berghei* parasites and *in vivo* bioluminescence imaging in mice. Resorting to a homing assay, *trsp* knockout sporozoites were found to arrest in the liver similar to control parasites. Moreover, we found no defects in the establishment of infection in mice following inoculation of *trsp* knockout sporozoites via intravenous and cutaneous injection or mosquito bite. Accordingly, mutant sporozoites were also able to successfully invade hepatocytes *in vitro*. Altogether, these results suggest TRSP may have a redundant role in the completion of the pre-erythrocytic phase of the malaria parasite. Nonetheless, identifying molecules with paramount roles in this phase could aid in the search for new antigens needed for the design of a protective vaccine against malaria.

Reprinted from Scientific Reports 2018 Oct 10;8(1):15101. doi: 10.1038/s41598-018-33398-8.

SCIENTIFIC REPORTS

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TRSP is dispensable for the *Plasmodium* pre-erythrocytic phase

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Received: 16 July 2018

Accepted: 26 September 2018

Published online: 10 October 2018

Plasmodium sporozoites deposited in the skin following a mosquito bite must migrate and invade blood vessels to complete their development in the liver. Once in the bloodstream, sporozoites arrest in the liver sinusoids, but the molecular determinants that mediate this specific homing are not yet genetically defined. Here we investigate the involvement of the thrombospondin-related sporozoite protein (TRSP) in this process using knockout *Plasmodium berghei* parasites and *in vivo* bioluminescence imaging in mice. Resorting to a homing assay, *trsp* knockout sporozoites were found to arrest in the liver similar to control parasites. Moreover, we found no defects in the establishment of infection in mice following inoculation of *trsp* knockout sporozoites via intravenous and cutaneous injection or mosquito bite. Accordingly, mutant sporozoites were also able to successfully invade hepatocytes *in vitro*. Altogether, these results suggest TRSP may have a redundant role in the completion of the pre-erythrocytic phase of the malaria parasite. Nonetheless, identifying molecules with paramount roles in this phase could aid in the search for new antigens needed for the design of a protective vaccine against malaria.

Malaria is a vector-borne infectious disease caused by apicomplexan parasites belonging to the genus *Plasmodium*. It remains one of the most concerning public health problems with 216 million cases reported in 2016 leading to nearly half a million deaths, most of which were children under the age of 5 in Sub-Saharan Africa¹.

A considerable effort has been made to generate a vaccine against malaria. In fact, several projects are currently underway² and, most notably, the circumsporozoite protein (CSP)-targeting RTS,S/AS01 has completed Phase 3 clinical trials³. Nonetheless, the protection conferred by RTS,S/AS01 against malaria in young children is only partial and, therefore, more effective options are required.

The pre-erythrocytic phase of malaria is initiated when an infected female anopheline mosquito injects sporozoites in the skin of a mammalian host while taking a blood meal. Only a few sporozoites eventually leave the skin and travel through the bloodstream to reach the liver. Inside hepatocytes they differentiate and multiply into merozoites, the red blood cell-infective forms. These are subsequently released into the bloodstream to initiate the symptomatic erythrocytic phase⁴.

Prior to hepatocyte invasion, sporozoites passively transported in the blood must arrest in the liver sinusoids, a crucial step for the ensuing liver infection. This arrest has been proposed to be enabled by both slower blood circulation speed in the sinusoids and the interaction of sporozoite surface molecules such as CSP and the thrombospondin-related anonymous protein (TRAP) with the highly-sulphated heparan sulphate proteoglycans (HSPGs) of liver cells^{5–8}. It is noteworthy that the current model explaining this critical step is based on the interpretation of experiments where the binding of recombinant proteins to hepatocytes was tested or the outcome of a liver infection by sporozoites was assessed. Using *in vivo* bioluminescence imaging, we have recently demonstrated that *Plasmodium* sporozoites home to the liver in the first minutes following their intravascular inoculation in mice⁹. Thus, the identification of the parasite determinants involved in this stage-specific behaviour could be possible by generating transgenic parasites lacking candidate surface molecules and following their capacity to home to the liver by direct imaging *in vivo*.

The thrombospondin-related sporozoite protein (TRSP), a protein found on the parasite surface and previously implicated in the pre-erythrocytic phase, is among those candidates^{10,11}. TRSP possesses an adhesive thrombospondin type 1 repeat (TSR) domain that is conserved throughout *Plasmodium* species and shows great

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similarity to those of TRAP and CSP^{10,12}. TSRs are present in a diverse group of proteins, promote cell-cell and cell-matrix interactions in a wide array of organisms, and can bind to proteoglycans^{13–15}. It has been proposed that TRAP and CSP TSRs bind specifically to HSPGs in the liver^{5,7,16,17}. In the absence of TRSP, *Plasmodium berghei* sporozoites have been shown to present compromised hepatocyte entry *in vitro*, which correlated with lower parasite burden in the liver¹⁰. However, the role of this protein in mediating the arrest of sporozoites in the liver, a step that precedes hepatocyte infection, has never been addressed.

In this work, we have engineered luciferase-expressing *P. berghei* sporozoites lacking TRSP and evaluated their capacity to home to the liver using bioluminescence imaging. Moreover, we found no defects in the infectivity of *trsp* knockout sporozoites suggesting this molecule has a redundant role in the pre-erythrocytic phase of malaria.

Results

***trsp* knockout sporozoites efficiently target the liver.** In order to investigate the potential role of TRSP in the homing of salivary gland sporozoites to the liver, we resorted to a homing assay using live imaging⁹. Briefly, making use of 2D bioluminescence imaging in mice it is possible to quantify the specific accumulation of luciferase-expressing sporozoites in the liver in the first minutes following their intravascular inoculation⁹. Thus, we generated a *trsp* knockout line on a bioluminescent background via targeted gene deletion by double cross-over homologous recombination (Supplementary Fig. 1A). Transfer populations were cloned to select isogenic lines hereby designated TRSP[−]. The two clonal parasite lines used throughout this work, TRSP[−] I21 and I26, were confirmed to have integrated the selectable marker to replace the *trsp* locus via PCR (Supplementary Fig. 1B) and Southern blot (Supplementary Fig. 1C), and shown to lack *trsp* mRNA by RT-PCR (Supplementary Fig. 1D).

In the homing assay, we detected no difference in the bioluminescent signal in the liver 7 minutes after mice being inoculated with control versus TRSP[−] sporozoites either in percentage (Fig. 1A,E) or absolute values (Fig. 1B,F). This indicates that sporozoites lacking TRSP efficiently home to the liver. In addition to the short 7-minute time point, bioluminescence was also quantified 2 to 4 hours post-infection and also no significant differences in the bioluminescent signal either in percentage (Fig. 1C,G) or absolute values (Fig. 1D,H) among the different groups were observed. Altogether, these observations demonstrate that *trsp* knockout parasites are able to successfully reach the liver.

TRSP is dispensable for the completion of the *P. berghei* pre-erythrocytic phase. To evaluate the capacity of intravenously-injected sporozoites to infect hepatocytes, bioluminescence in the liver was quantified at days 1 and 2 post-infection (Fig. 2A,C) and the percentage of infected red blood cells was calculated on the following days (Fig. 2B,D). No differences in bioluminescent signal in the liver or in the percentage of infected red blood cells were observed between mutant and control parasites. Since intravenous inoculation bypasses the critical skin stage of the sporozoite journey to the mammalian liver, mice were also infected intradermally (Fig. 2E–H). Similarly, TRSP[−] parasites did not show any defects in the liver (Fig. 2E,G) and blood (Fig. 2F,H) infection. In conclusion, loss of TRSP does not impact the ability of sporozoites to reach and infect the liver, regardless of the infection route.

Although intravenous and intradermal infections are helpful experiments to scrutinize the behaviour of the parasites in specific stages of the pre-erythrocytic phase, these circumvent the deposition of sporozoites and mosquito saliva into the skin through the mosquito proboscis. To study the importance of TRSP in the context of natural transmission, infected mosquitoes were allowed to probe on naive mice and the bioluminescent signal in the liver was measured two days after infection (Fig. 3A,C). Data obtained on day 1 were not considered because infections following mosquito bite typically lead to low parasite burdens in the liver when compared to intravenous (Fig. 2A,C) or intradermal (Fig. 2E,G) infections at this time point. No impairment of liver infection was discernible on day 2 (Fig. 3A,C). Inspection of parasitaemia levels on later days also did not reveal differences (Fig. 3B,D).

Overall, these data demonstrate that TRSP[−] are as capable as control sporozoites of accomplishing the pre-erythrocytic phase of their life cycle. Furthermore, mutant parasite growth during the blood stage was indistinguishable from that of control parasites (Figs 2B,D,F,H and 3B,D). In addition, comparable numbers of salivary gland sporozoites were observed (Supplementary Fig. 2), revealing that no problems in transmission to or infection of the mosquito occurred. This is further evidence that TRSP is not necessary for the *P. berghei* life cycle in standard conditions.

TRSP[−] sporozoites exhibit no impairment in hepatocyte entry. A particular phenotype relating to defective sporozoite entry into hepatocytes was previously described *in vitro* for *trsp* knockout parasites¹⁰. We inspected the ability of the TRSP[−] clonal lines generated in this study to invade the HepG2 hepatoma cell line using an automated counting method to examine anti-CSP double staining, which distinguishes between intracellular and extracellular sporozoites¹⁸. Images were acquired using an IN Cell Analyzer 2000 and the numbers of host cell nuclei, total and intracellular sporozoites were counted either manually or using the IN Cell Developer Toolbox v.1.9.2 software (Fig. 4A–E). The two counting methods yielded indistinguishable results (Fig. 4B–E), validating the use of the automated method to determine the percentage of infected cells (Fig. 4F). No statistically significant differences were observed between TRSP[−] and control sporozoites, indicating TRSP[−] sporozoites have no major defects in host cell entry. These results also differ from the former study on the *P. berghei trsp* knockouts, where the authors described a failure in sporozoite entry into HepG2 cells that culminated in an incomplete entry phenotype¹⁰. We failed to observe this phenotype with our TRSP[−] lines (Fig. 4G–I). In fact, the partial entry of sporozoites was an extremely rare event in both control and mutant parasites (Fig. 4G). At most, only approximately 0.1% of host cells exhibited such events using any of the parasite lines (Fig. 4H) and these events did not constitute more than 2.1% of total intracellular parasites (Fig. 4I) in the selected representative experiments.

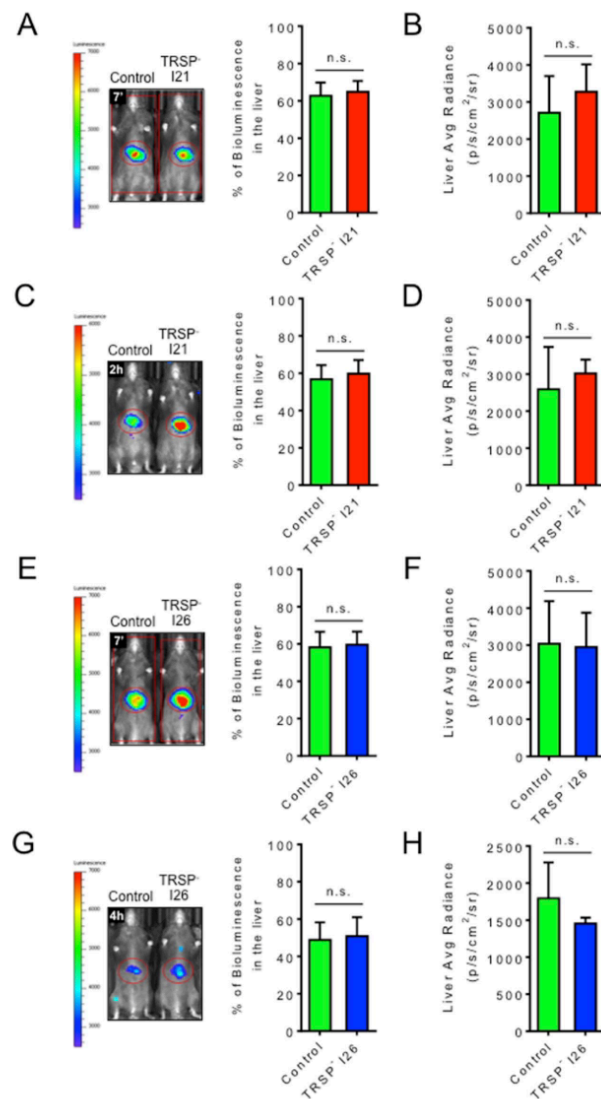


Figure 1. Homing of *trsp* knockout sporozoites to the liver. (A,C,E,G) Representative pictures of C57BL/6 mice injected intravenously with 1.5×10^5 luciferase-expressing control or TRSP I21 (A,C) or I26 (E,G) sporozoites collected from mosquito salivary glands. Quantification of the signal in percentage of total flux (photons/s) in the liver versus total body signal (mean $\pm$ SD; $n = 3$), determined 7 minutes (A,E) or 2–4 hours (C,G) after sporozoite injection. (B,D,F,H) Bioluminescent signal in the liver plotted as average radiance (photons/s/cm²/sr; mean $\pm$ SD; $n = 3$) of the mice infected with TRSP I21 (B,D) or I26 (F,H) sporozoites 7 minutes (B,F) or 2–4 hours (D,H) post-infection. Data shown is representative of two independent experiments. Statistical analysis was performed using the Mann Whitney test; n.s., non-significant.

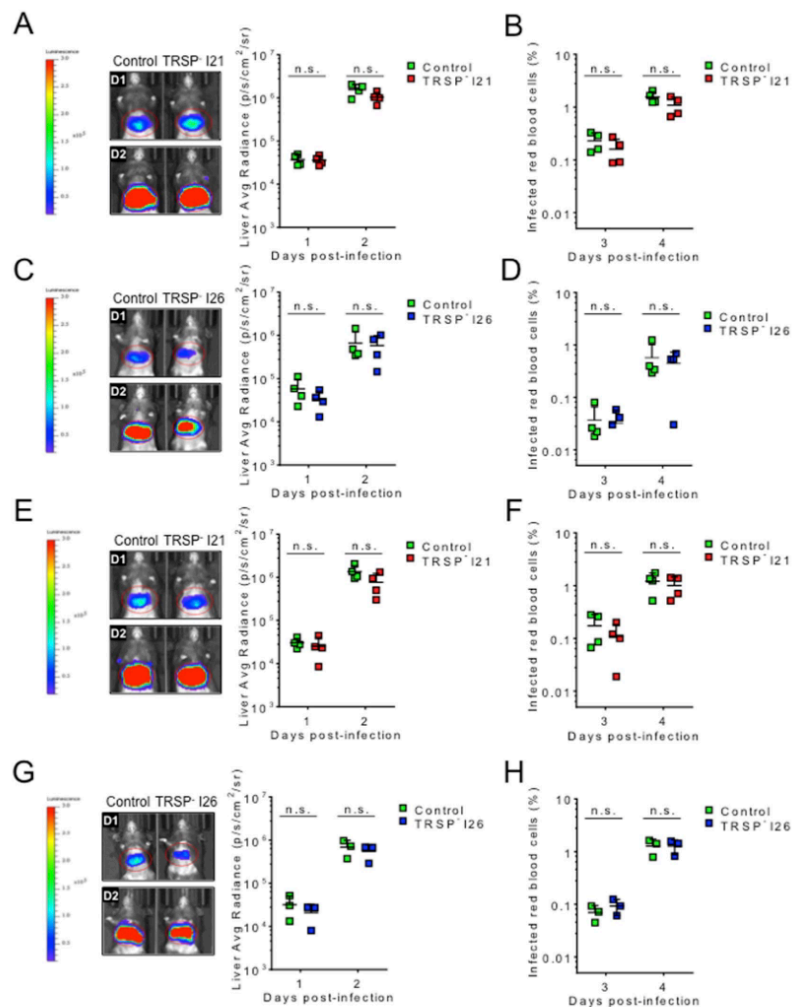


Figure 2. Infectivity of *trsp* knockout sporozoites in the mammalian host following intravenous or intradermal infection. Infectivity of TRSP (clones I21 or I26) and control sporozoites following infection by intravenous (A–D) or intradermal (E–H) routes. In these representative experiments, intravenous infections were carried out using 1×10^4 sporozoites, while intradermal inoculations were performed with $1.5\text{--}2 \times 10^4$ sporozoites. Parasite burden in the liver ($n = 3$ or 4) was assessed by live imaging on days 1 (D1) and 2 (D2) post-infection using IVIS Lumina LT. The bioluminescent signal as average radiance (photons/s/cm²/sr) of individual mice (symbols) and the average $\pm$ SD (bars) of groups were plotted (A,C,E,G). Starting on day 3 post-infection, parasitaemia of infected animals was determined daily by a Giemsa-stained blood smear. Parasitaemia of individual mice (symbols) and the average $\pm$ SD (bars) of groups are represented (B,D,F,H). Statistical analysis was performed using the Mann Whitney test: n.s., non-significant.

Discussion

This is the first study where the importance of a sporozoite surface protein in the homing of *Plasmodium* parasites to the liver was investigated using live imaging techniques. We generated TRSP bioluminescent clonal lines and made use of a homing assay to dissect the role of this protein in the specific retention of sporozoites in the liver.

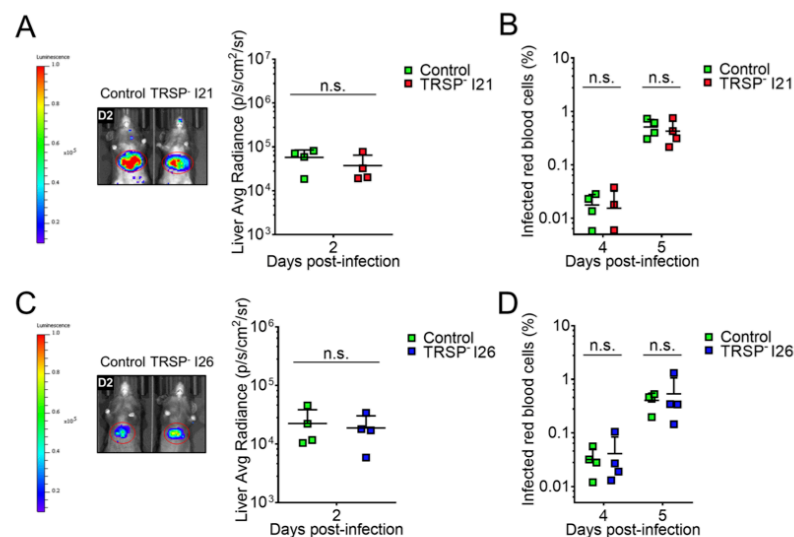


Figure 3. Infectivity of TRSP sporozoites through mosquito bite. Mosquitoes infected with TRSP I21 (A,B) or I26 (C,D) and control parasites were used in bite experiments. Five to six mosquitos/mouse were allowed to probe for a total of 25 consecutive minutes. Parasite burden in the liver was assessed by live imaging on day 2 (D2) post-infection using IVIS Lumina LT. Bioluminescent signal was quantified and plotted as average radiance (photons/s/cm²/sr) (A,C). Starting on day 3 post-infection, parasitaemia of infected animals was determined daily by a Giemsa-stained blood smear (B,D). Values for individual mice (symbols) and the average +SD (bars) of groups are represented. Statistical analysis was performed using the Mann Whitney test: n.s., non-significant.

Strikingly, we found that TRSP not only is not required for the homing of sporozoites to the liver, but is also dispensable for the establishment of a successful infection in the mammalian host. These results challenge the findings of a previous study that advocated an involvement of TRSP in hepatocyte entry and *P. berghei* infectivity¹⁰. The paucity of the *in vitro* partial entry events could, to an extent, explain the disagreeing results. Nonetheless, the infrequency of incomplete hepatocyte entry events in our experiments suggests this phenomenon is most likely not biologically relevant. Moreover, different methods were used in both studies to evaluate the liver infectivity of the TRSP-deficient sporozoites, as in the previous study parasite burdens in the liver were assessed using RT-PCR¹⁰. We cannot exclude that the measurement of parasite burden using bioluminescence imaging is less sensitive than RT-PCR. However, no differences in the emergence of the blood infection following inoculation of TRSP or control sporozoites were found in our study, supporting a similar capacity for both parasites to infect the liver. Once again, this contrasts with the one-day delay in the pre-patent period reported in the former work¹⁰. Noteworthy, in this study we used the *P. berghei* ANKA strain rather than the NK65 strain¹⁰. Despite the high similarity between the genome sequences of several *P. berghei* laboratory lines¹⁹ we cannot preclude that such phenotypic discrepancies may come from the fact that the *trsp* knockouts were engineered in distinct strains.

The processes in which TRSP may participate remain therefore undisclosed. The *trsp* gene in *P. berghei* codes for a relatively small 159 amino-acid protein with a signal peptide, a transmembrane domain and a short cytoplasmic tail, in addition to the adhesive TSR¹⁰. TRSP has been demonstrated to have apical localization in *Plasmodium yoelii*¹² and has also been readily detected on the surface of purified and untreated *P. falciparum* and *P. yoelii* salivary gland sporozoites^{11,20}. The TRSP exact subcellular localization and the mechanism that regulates its trafficking to the sporozoite surface remain to be elucidated. *trsp* transcripts are sporozoite-specific and are enriched in salivary gland sporozoites in comparison to their midgut-associated counterparts^{12,21}. Hence, TRSP would be predicted to play a part in the initial stages of the *Plasmodium* life cycle in the mammalian host, in processes such as motility, adhesion or invasion^{22,23}. On the other hand, the similarities shared between TRSP and other sporozoite surface proteins, namely those belonging to the TRAP family and other TSR-containing proteins, lead us to speculate that functional redundancy among these molecules may exist. This phenomenon has already been reported in similar contexts for other proteins^{24,25}. It is possible that other adhesins compensate for the loss of TRSP and allow for the parasite to complete its life cycle without any major impairment.

Finally, the fact that TRSP appears to be dispensable for the parasite to successfully infect the mammalian host dampens the hope that this surface molecule could become an attractive target for neutralizing antibodies¹¹. Nonetheless, the identification of sporozoite surface proteins with functions that are vital to the completion of the

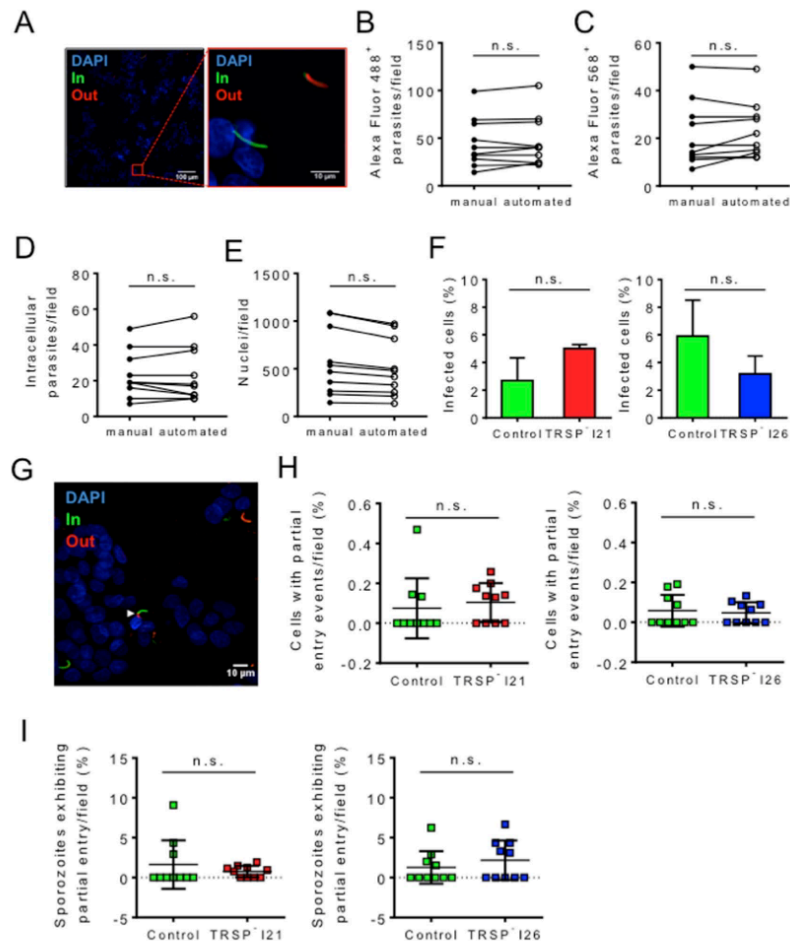


Figure 4. TRSP sporozoite entry in hepatocytes. (A) Representative immunofluorescence image of an anti-CSP double staining using control sporozoites in an *in vitro* entry assay. Intracellular sporozoites (In) are labelled in green whilst extracellular sporozoites (Out) appear in red and green. DAPI-stained nuclei appear in blue. The image on the right represents a magnified section of the image on the left. (B–E) Comparison of the number of sporozoites per field stained with anti-CSP antibodies and Alexa Fluor 488-conjugated (B) or Alexa Fluor 568-conjugated (C) secondary antibodies; intracellular sporozoites (D), corresponding to the number of Alexa Fluor 568⁺ parasites subtracted from the number of Alexa Fluor 488⁺ parasites; and nuclei (E) in a representative anti-CSP double staining, determined manually or by automated analysis. (F) Percentage of HepG2 cells infected by control and TRSP I21 (graph on the left) or I26 (graph on the right) sporozoites, in 25 fields from each replicate well. (G) Partial entry events were defined as Alexa 488⁺ sporozoites stained with Alexa 568 secondary antibody up to roughly 50% of its length in an anti-CSP double staining. A partial entry event of control sporozoites in an immunofluorescence image is identified with a white arrowhead. DAPI-stained nuclei appear in blue (H) Percentage of cells exhibiting partial entry events using TRSP I21 (graph on the left) or I26 (graph on the right) sporozoites. Ten fields of a representative assay were analysed and events were counted manually. (I) Percentage of sporozoites partially inserted into the hepatocyte membrane using TRSP I21 (graph on the left) or I26 (graph on the right) sporozoites. This parameter represents the number of partial entry events out of the total number of intracellular parasites in a field, represented as a percentage. Means $\pm$ SD (F) or means $\pm$ SD (H,I) are represented. Four independent experiments per clone were performed. Statistical analysis was performed using the Mann Whitney test: n.s., non-significant.

sporozoite journey in the mammalian host remains a promising approach to design a novel vaccine. Stimulating a humoral response against these antigens would not only directly target the parasites and facilitate their elimination but also block crucial steps of the life cycle and, subsequently, impede the natural progression of infection.

Materials and Methods

Ethics statement. All experiments carried out on mice were approved by the IBMC/INEB Animal Ethics Committees and the project was licenced by the Portuguese National Authority for Animal Health, in accordance to the statements on the directive 2010/63/EU of the European Parliament and Council.

Mice, parasites and mosquitoes. Four- to six-week-old C57BL/6 or NMRI mice used in this study were purchased from Charles River or the IBMC/i3S animal facility.

P. berghei ANKA strain clone 676cl1 expressing a GFP-luciferase fusion gene via the pbef1 promoter (GFP:Luc)²⁶ was used to generate mutant lines and was included in experiments the parental control.

Anopheles stephensi mosquitoes (Sda500 strain) were reared in the Centre for Production and Infection of Anopheles (CEPIA) at the Pasteur Institute using standard procedures. Female mosquitoes were fed on infected NMRI mice as previously described²⁷ and from 21 to 28 days after the infectious blood meal these were either dissected for collection of salivary gland sporozoites or used in transmission experiments. In the latter scenario, starvation by sucrose deprivation 1 day prior to experimentation was performed to enhance the rate of mosquito bite.

Generation of TRSP⁺ clonal lines. Targeted deletion of *trsp* by double crossover homologous recombination was achieved using a previously described strategy¹⁰, with modifications. To this end, the 3' and 5' UTR sequences used as homology regions were amplified from genomic DNA using a Taq DNA polymerase with proofreading activity (Takara) and primer pairs P1 + P2 and P3 + P4 (Supplementary Table 1), respectively. Amplicons were cloned into pGEM-T Easy Vector (Promega), sequenced and later subcloned into the KpnI/HindIII or EcoRI/BamHI sites of pL0001 vector (MR4; 5' or 3' homology regions, respectively). The linearized targeting vector resulting from KpnI/BamHI digestion was used to transfect schizonts with the Nucleofector[®] device (Amaxa)²⁸. Briefly, electroporated merozoites were injected intravenously into two mice (parental populations) and selected by pyrimethamine treatment at 0.07 mg/L in drinking water, started the day following infection. Once parasitaemia was above 1%, blood from each animal was transferred to two naive mice (transfer populations) for a second round of selection. Parasites were then cloned by limiting dilution.

Genetic validation by PCR and Southern blot analysis. *Genomic DNA extraction.* Blood collected from infected animals was filtered with a Plasmodipur leukocyte filter (EuroProxima), followed by erythrocyte lysis with saponin (0.15%). DNA was then extracted and purified from the resulting parasite pellet using the QIAamp DNA Blood Mini Kit (Qiagen).

PCR. Absence of the *trsp* open reading frame (ORF) and integration of the transfection construct at the correct locus were confirmed by standard PCR analysis with primer pairs P5 + P6 and P7 + P8 (Supplementary Table 1), respectively.

Southern blot. For Southern blot analysis, 5 µg of genomic DNA were digested with EcoRV, separated on a 0.8% agarose gel and transferred to a Hybond-N + nylon membrane (Amersham). The DNA sequence amplified by PCR using primer pair P1 + P2 (Supplementary Table 1) was used as a probe. Labelling of the probe and signal generation and detection were performed resorting to the AlkPhos Direct Labeling and Detection System with CDP-Star chemiluminescent detection reagent (Amersham).

Evaluation of gene expression by reverse transcription PCR (RT-PCR). Total RNA was isolated from salivary gland sporozoites using the Nucleospin RNA II kit (Macherey-Nagel) and reverse transcription for cDNA production was achieved with the NZY First-Strand cDNA Synthesis Kit (NZYtech). Primer pair P5 + P9 (Supplementary Table 1) was utilised to detect *trsp* cDNA by PCR. Tubulin beta chain gene cDNA was used as a control, employing primer pair P10 + P11 (Supplementary Table 1).

Hepatocyte entry assays. *Immunofluorescence assays.* The assays were conducted by seeding 7.5×10^4 to 1×10^5 HepG2 cells (ATCC HB-8065) in an 8-well Lab-Tek chamber slide (Thermo Fisher Scientific) in duplicates or triplicates. Cells were cultured at 37 °C in DMEM 10% FBS, left to adhere overnight and infected with 1×10^4 – 5×10^4 sporozoites. Preparations were then fixed with 4% paraformaldehyde 1 hour post-infection.

The ability of sporozoites to enter HepG2 cells *in vitro* was evaluated via an anti-CSP double staining. Preparations were blocked with 5% FBS in PBS, incubated with an α-CSP 3D11 mouse monoclonal antibody (~2 µg/ml; MR4), and then with the goat α-mouse Alexa Fluor 568 antibody (1:500; Invitrogen). Permeabilization of the host cell membrane with 1% Triton 100 in PBS ensued, followed by incubation with the primary antibody. Finally, the goat α-mouse Alexa Fluor 488 (1:500; Invitrogen) antibody was used to stain all parasites. This allowed for the discrimination between intracellular (Alexa Fluor 488⁺/Alexa Fluor 568⁻) and extracellular (Alexa Fluor 488⁺/Alexa Fluor 568⁺) parasites. Nuclei were stained with DAPI (1:5000; Invitrogen).

Automated analysis. Image acquisition was performed using IN Cell Analyzer 2000 (GE Healthcare). HepG2 nuclei as well as green and red sporozoites were counted using the IN Cell Developer Toolbox v.1.9.2 software (GE Healthcare). In hepatocyte entry assays, 25 images corresponding to 0.57 mm² fields were taken per well. Automated slide analysis was validated in all experiments by comparison of values obtained with the developed protocol and manual counts using ImageJ (National Institutes of Health) for randomly selected images.

Mouse infections. For homing experiments⁹, 1.5×10^5 sporozoites were injected intravenously in C57BL/6 mice. In the other experiments, C57BL/6 mice were infected with 1×10^4 – 1.5×10^4 or 1.5×10^4 – 2×10^4 sporozoites injected intravenously or intradermally, respectively. To assess the role of TRSP in the context of natural transmission, approximately 25–40 mosquitoes were allowed to probe mice anaesthetized with ketamine (125 mg/kg) and xylazine (12.5 mg/kg) in sessions of 1 minute with rotation of the animals, for a total of 25 consecutive minutes.

Bioluminescence imaging. Liver parasite loads in C57BL/6 mice infected with luciferase-expressing sporozoites were assessed by bioluminescence imaging using the IVIS Lumina LT system (Perkin Elmer). Prior to image acquisition, animals had their ventral fur shaved with an appropriate clipper. Infected mice were anaesthetized with 2.5% isoflurane (O_2 flow of 1 L/min) and injected subcutaneously with D-luciferin potassium salt (2.4 mg, Perkin Elmer) five minutes before image acquisition. Mice were then transferred to the stage of an intensified charge-coupled device photon-counting video camera box where anaesthesia was maintained with 2.5% isoflurane (O_2 flow of 0.3 L/min). Signal acquisition was controlled by the Living Image software (Perkin Elmer). In the end, animals returned to their cage and recovered from the anaesthesia. The detection of bioluminescent signals by the system resulted in the generation of signal maps automatically superimposed to the grey-scale photograph of the mice. The quantifications were performed using the Living Image software (Perkin Elmer). The regions of interest (ROI) encompassing the liver and, in homing assays, the whole ventral views of the animal body were manually defined⁹. The total flux (photons/second) and average radiance (photons/second/cm²/steradian) within these ROIs were automatically calculated. In the homing assays, the percentage of bioluminescent signal in the liver ROI was calculated by dividing the total flux of the respective ROI by the total flux of the ROI of the animal body in the ventral position.

Parasitaemia. The percentage of infected red blood cells was evaluated by Giemsa-stained thin blood smear starting from the third day after sporozoite inoculation. At least 40 microscopic fields (~20,000 red blood cells; detection limit 0.005%) were analysed.

Statistical analysis. Mann-Whitney test was performed with GraphPad Prism Software (version 6.0). Statistical significance was found for values of $p < 0.05$.

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Acknowledgements

We would like to thank: Prof. Anabela Cordeiro da Silva from the IBMC/i3S for the exceptional hosting conditions; Dr. Ana Xavier Carvalho from the IBMC/i3S for the critical reading of the manuscript; the team of the CEPIA from Institut Pasteur, Paris, for providing the *Anopheles stephensi* female mosquitos. The following reagents were obtained through BEI Resources, NIAID, NIH: (a) Plasmid pL0001, for Transfection in *Plasmodium berghei*, MRA-770, contributed by Andrew P. Waters; (b) Hybridoma 3D11 Anti-*Plasmodium berghei* 44-Kilodalton Sporozoite Surface Protein (Pb44), MRA-100, contributed by Victor Nussenzweig. This work was supported by funds from project Norte-01-0145-FEDER-000012 - Structured program on bioengineered therapies for infectious diseases and tissue regeneration, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through FEDER. This work also received funds from the Fundação para a Ciência e Tecnologia (FCT)/Ministério da Educação e Ciência (MEC) co-funded by FEDER (EXPL/JTAVARES-IF/00881/2012/CP0158/CT0005, EXPL/IMI-MIC/1331/2013) under the Partnership agreement PT2020, through the Research Unit No. 4293. J.T. is an Investigator FCT funded by National funds through FCT and co-funded through European Social Fund within the Human Potential Operating Programme. D.M.C., M.S. and A.R.T. are funded by FCT individual fellowships SFRH/BD/123734/2016, SFRH/BD/133485/2017 and SFRH/BD/133276/2017 respectively). The authors acknowledge the support of the BioSciences Screening i3S Scientific Platform, member of the PPBI (PPBI-POCI-01-0145-FEDER-022122).

Author Contributions

J.T., R.A. and D.M.C. conceived the experiments, D.M.C., J.T., M.S., A.R.T., and I.L. conducted the experiments and analysed the data; C.T. and S.G. reared the mosquitoes; J.T. and R.A. supervised the work; D.M.C., J.T. and R.A. prepared the manuscript; all authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-33398-8>.

Competing Interests: The authors declare no competing interests.

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1 **TRSP is dispensable for the *Plasmodium* pre-erythrocytic phase**

2

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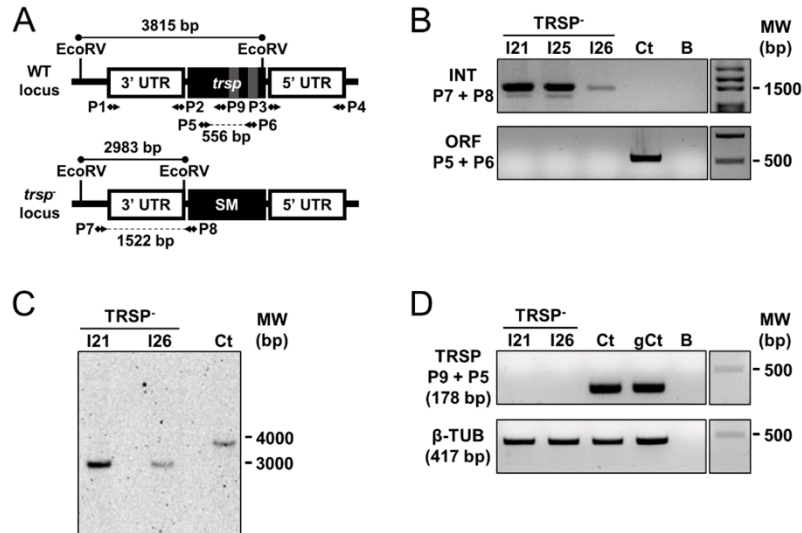
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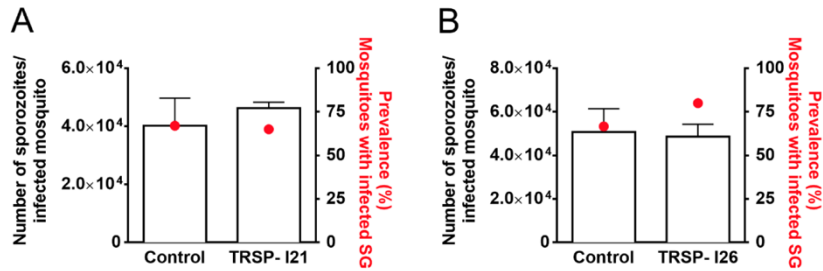
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Supplementary Figure 1. Targeted gene replacement of *trsp* in GFP:LUC parasites. (A) Schematic representation of the *trsp* replacement strategy by homologous recombination in GFP:LUC parasites. The targeting vector used to generate the *trsp* knockout (*trsp*⁻) locus was comprised of the selectable marker (SM), the *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase gene, flanked by 5' and 3' untranslated regions (UTRs) amplified with primers pairs P3 + P4 and P1 + P2 (Supplementary Table 1), respectively, which functioned as homology arms. Solid black or gray boxes in the wild type (WT) locus represent exons or introns, respectively. (B,C) Genetic analysis of TRSP⁻ clones and control (Ct) parasites by PCR (B) and Southern blot (C). Primers used in the reactions, the restriction sites and the expected fragment (solid lines) or amplicon (dashed lines) length are specified in panel A. The sequence that served as the homology region downstream of the gene was used as the probe in C. (D) mRNA expression of TRSP in TRSP⁻ I21 and I26 and control salivary gland sporozoites evaluated by RT-PCR. The predicted size of the amplicons is indicated to the left of the gels.

29 Genomic DNA extracted from control parasites (gCt) was used as a control PCR. The DNA
30 ladder in B and D was run on the same gel as the PCR products on the left but the corresponding
31 lanes were cropped from the respective images to facilitate visualization. B, blank (PCR without
32 DNA). bp, base pairs. INT, integration PCR. ORF, open reading frame PCR. MW, molecular
33 weight. TUB, tubulin beta chain.



34

35 **Supplementary Figure 2.** Mosquito infectivity by TRSP⁺ parasites. Percentage of mosquitos
 36 infected with control or either TRSP⁺ I21 (A) or I26 (B) parasites determined by counting the
 37 number of GFP fluorescent salivary glands (SG) and total numbers of salivary gland sporozoites
 38 per infected mosquito (means + SD of two independent determinations). These data correspond
 39 to representative experiments.

40 **Supplementary Table 1.** Oligonucleotide sequences

Primer	Sequence
1	5' TAATGGATCCGCAAAATATAGTTACACCCATA 3'
2	5' TAATGAATTCGATAATAGTTTGGCTTTTCG 3'
3	5' TAATAAGCTTCATATAAACTATGAACG 3'
4	5' TAATGGT <u>ACCG</u> TTTGTGCACCTTAATCCC 3'
5	5' TCCCTTCAGAATTGTCAGGAC 3'
6	5' GTGCTCAAATAATCAACCTGTTG 3'
7	5' TGAGTGCACATAAGCAATTC 3'
8	5' CCCATTGTGAACATCCTCAAC 3'
9	5' AAACGAGTTGCGTAATTGTG 3'
10	5' TGGAGCGGAAATAACTGGG 3'
11	5' ACCTGACATAGCGGCTGAAA 3'

41 Introduced restriction sites are underlined.

2.3. Downregulation of the secreted protein with altered thrombospondin repeat (SPATR) impacts the infectivity of malaria sporozoites

No vaccine against human malaria currently boasts satisfactory efficacy. The identification of *Plasmodium* proteins important for the establishment of the pre-erythrocytic phase could aid in the development of a protective vaccine. Thus, we set out to disclose the involvement of the secreted protein with an altered thrombospondin region (SPATR), previously suggested to only be required for the blood stage, in the mosquito and pre-erythrocytic phases of the *Plasmodium berghei* life cycle. We resorted to a promoter swap approach to achieve specific downregulation of transcription in oocysts and sporozoites, and revealed SPATR is required for the successful colonization of the mosquito salivary glands. Moreover, SPATR knockdown hemolymph sporozoites displayed impaired motility and infectivity to mice. In conclusion, we exposed a role for SPATR in sporozoite infectivity that other approaches did not detect, highlighting the usefulness of complementary methods to study discrete aspects of sporozoite biology.

Manuscript in preparation.

Downregulation of the secreted protein with altered thrombospondin repeat (SPATR) impacts the infectivity of malaria sporozoites

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Abstract

No vaccine against human malaria currently boasts satisfactory efficacy. The identification of *Plasmodium* proteins important for the establishment of the pre-erythrocytic phase could aid in the development of a protective vaccine. Thus, we set out to disclose the involvement of the secreted protein with an altered thrombospondin region (SPATR), previously suggested to only be required for the blood stage, in the mosquito and pre-erythrocytic phases of the *Plasmodium berghei* life cycle. We resorted to a promoter swap approach to achieve specific downregulation of transcription in oocysts and sporozoites, and revealed SPATR is required for the successful colonization of the mosquito salivary glands. Moreover, SPATR knockdown hemolymph sporozoites displayed impaired motility and infectivity to mice. In conclusion, we exposed a role for SPATR in sporozoite infectivity that other approaches did not detect, highlighting the usefulness of complementary methods to study discrete aspects of sporozoite biology.

Introduction

Plasmodium sporozoites are motile and invasive stages of the malaria parasites that are formed inside oocysts in the midgut of the mosquito vector. Fully developed sporozoites egress from the oocysts and are carried by the mosquito's circulatory system, the hemolymph, to the salivary glands, which invade to ensure transmission (1,2). Once deposited into the skin of the mammalian host by a mosquito bite, sporozoites actively migrate and invade blood vessels (3). Sporozoites passively transported by the bloodstream then specifically home to the liver and cross the sinusoidal barrier to get access to hepatocytes, where they multiply (4–6). Thus, sporozoites and the ensuing liver stages mediate the pre-erythrocytic phase of infection, and constitute a transmission bottleneck, as well as ideal vaccination targets (7).

The most advanced malaria vaccine, RTS,S/AS01, targets the circumsporozoite protein (CSP), the major sporozoite surface antigen. Anti-CSP antibody titers were shown to be surrogates of protection, but the vaccine had an efficacy of merely 50% or 36% in its target age group against the clinical episodes caused by *Plasmodium falciparum*, the most dangerous malaria parasite to humans, over 1 or 4 years, respectively (8–10). The development of multivalent subunit vaccines is one of several approaches to improve vaccine efficacy (11). Accomplishing this goal, however, will require greater knowledge of the biology of the parasite. This includes the identification of sporozoite molecules for targeted immune interventions that interfere with the timely completion of the journey of the parasite in the mammalian host. To this end, significant advances have been made to define the *Plasmodium* proteome, including the surface proteome of infectious sporozoites (12–15).

The secreted protein with an altered thrombospondin region (SPATR) contains two adhesive domains: an atypical thrombospondin type-1 repeat (TSR) and a Type II epidermal growth factor (EGF)-like domain (16–18). It is conserved across *Plasmodium* species and has several orthologues among the Apicomplexa phylum (19). Although its gene is transcribed in all invasive stages (sporozoites, merozoites and ookinetes) (20,21), SPATR levels are upregulated in mature sporozoites of both human and rodent infecting *Plasmodium* species (12,15). Moreover, immunolocalization studies detected the protein at the surface of sporozoites of several *Plasmodium* species (16,18,19), despite the lack of predicted transmembrane domains or a glycosylphosphatidylinositol anchor. Antibodies against the SPATR of *P. falciparum* also blocked hepatocyte invasion by sporozoites (18).

The failed attempt to generate a *spatr* knockout (KO) in blood-stage parasites, the stage where genetic manipulation of *Plasmodium* parasites is performed, suggest an essential role for SPATR in the blood stage of the rodent malaria parasite *Plasmodium berghei* (19). This is in

agreement with a previous genome-wide essentiality screen (22). The stage-specific KO of *spatr* using a flippase-flippase recombination target (Flp-FRT) system (23), which has been used to study gene function in sporozoites (24), consolidated the indispensability of SPATR for the *P. berghei* blood stage (19). However, no defects in sporozoite infectivity to the mosquito or hepatocytes were detected using this conditional KO line (19). In fact, upon hepatocyte infection with sporozoites, merozoites were formed but failed to infect red blood cells (19).

The excision of the desired genetic sequence through the stage-specific expression of the Flp recombinase is frequently incomplete at the population level, which may hinder the phenotyping of mutant parasites (19,23,24). Additionally, the translational repression that occurs during the maturation of sporozoites adds another limitation to this Flp-FRT system (15). Indeed, the gene excision achieved using the thermolabile variant of Flp (FlpL) under transcriptional control of the regulatory sequences of the thrombospondin-related anonymous protein (TRAP) gene mostly occurs once sporozoites have already invaded the salivary glands. Due to these limitations, distinct strategies might be required to ascertain the involvement of SPATR in various steps of the life cycle.

In this study, using *P. berghei*, we confirmed the essentiality of SPATR for the asexual blood stages and we employed a promoter swap approach to specifically downregulate *spatr* expression early during oocyst development and sporozoite maturation in an attempt to disclose its importance for sporozoite infectivity.

Materials and methods

Animals and parasites

Three- to six-week-old C57BL/6, BALB/c, NMRI, and SWISS mice were purchased from Elevage Janvier, Charles River France or the i3S animal facility. Procedures were conducted in accordance with the guidelines of the Animal Welfare and Ethics Review Body at i3S and the Portuguese National Authorities for Animal Health guidelines, or the Animal Care and Use Committee of Institut Pasteur, following the directive 2010/63/EU of the European Parliament and Council.

All experiments were performed using the *Plasmodium berghei* ANKA strain clone 676cl1 expressing a GFP-Luciferase fusion protein (25). Female *Anopheles stephensi* SDA-500 mosquitoes reared in the Center for the Production and Infection of *Anopheles* at the Pasteur Institute in Paris, were fed on infected SWISS or NMRI mice 3–5 days after emergence and kept at 21 ± 0.5 °C and 70% humidity. A feeding using naïve mice was performed 6–7 days after the infectious feeding (26).

Oocyst and salivary gland sporozoites were obtained by mechanical disruption of mosquito midguts or salivary glands, respectively, and collected into Dulbecco's Phosphate Buffered Saline (DPBS; Gibco). Oocyst sporozoite suspensions were filtered using a 35 µm cell strainer cap (Falcon). To analyze oocysts, midguts of infected mosquitoes were collected at different time points post-infection. To recover hemolymph sporozoites, the haemocoel of mosquitoes was perfused with approximately 15–30 µl of DPBS. The thorax and abdomen of mosquitoes were recovered after the removal of the digestive system, salivary glands and hemolymph. Mosquito material and sporozoites were kept on ice until further processing. Blood schizont purification was performed using a Nycodenz (Axis Shield) gradient following the culture of infected mouse blood in RPMI 1640 (Lonza) supplemented with 50 µg/ml neomycin (Sigma) and 20% heat-inactivated fetal bovine serum (FBS; Biowest) in hypoxic conditions (5% CO₂, 5% O₂ and 90% N₂), for 16 to 18 h at 37 °C and under gentle agitation (27). Free merozoites were isolated via resuspension of mature schizonts using a micropipette or insulin syringe and filtration through a 1.2 µm Acrodisc® filter (Pall Corporation).

Oligonucleotides

The sequences of all primers (Sigma or Eurofins Genomics) used in this study are shown in Table S1.

Generation of *P. berghei* transgenic lines

Deletion of the majority of the *spatr* gene (PBANKA_0309500) by double cross-over homologous recombination was attempted using the NotI-linearized PbGEM-270402 vector (*PlasmoGEM*, Sanger) (28,29). A second construct for total gene deletion was generated by cloning the 5' (843 bp) and 3' (292 bp) homology regions amplified by PCR using a Taq DNA polymerase with proofreading activity (Takara) and primers P12 + P13 and P14 + P15 into the pLOO01 vector (BEI Resources) via the KpnI/ClaI and EcoRI/BamHI cloning sites, respectively. The linearized constructs were used to transfect synchronized schizonts with the Nucleofector® device (Amaxa). Electroporated parasites were injected intravenously into two mice (parental populations) and positive selection of transfectants was initiated the following day with 70 mg/L pyrimethamine (Sigma) in drinking water. When resistant populations arose, blood was transferred to naïve mice (transfer populations) for a second round of selection with pyrimethamine. Isolation of isogenic lines was performed by limiting dilution *in vivo*, as previously described (27).

To generate *spatr* knockdown (SPATR KD) lines we resorted to a two-step strategy to: 1) introduction of a second copy of the *spatr* gene under transcriptional control of the intergenic regions upstream of the genes encoding the haloacid dehydrogenase domain ookinete protein (*hado*; PBANKA_0603900) or the putative centrosomal protein 76 (*cep76*; PBANKA_0102600) into a silent intergenic locus in chromosome 6 (30); 2) deletion of the endogenous copy of *spatr*, performed using the PbGEM-270402 vector (*PlasmoGEM*, Sanger). The first step required the insertion of the 5' (490 bp) and 3' (474 bp) homology regions amplified by PCR with primer pairs P16 + P17 and P18 + P2 into the KpnI/ClaI and SpeI/SacII cloning sites, respectively, of the pLOO01 vector. The genomic region comprised of the open reading frame (1,083 bp) and 396 bp of the downstream sequence were cloned into the construct using the primer pair P19 + P20 and the BamHI and SpeI restriction enzymes. The 5' intergenic regions of *hado* (2,414 bp) and the putative *cep76* (845 bp) were amplified with primer pairs P21 + P22 and P23 + P24, respectively, and inserted into the plasmid using the EcoRV/BamHI restriction sites. Transfections were performed as before, and selection of transfectants was achieved using pyrimethamine as previously described (step 1) or 10–16 mg/kg/day of WR99210 (Jacobus Pharmaceutical Company) injected subcutaneously for 3 consecutive days (step 2). Parasites were then cloned by limiting dilution. Two isogenic lines (SPATR KD^{V1} and SPATR KD^{S16}) were used in this study.

To generate a parasite line expressing a c-Myc-tagged version of SPATR, the WT *spatr* open reading frame was replaced by a copy of *spatr* fused with a sequence encoding a 2x c-Myc tag at the C-terminus, by single cross-over homologous recombination. Since the linearization of

the transfection vector was restricted to the *AdhI* restriction site within the *spatr* gene, modifications were made to pL0033 (BEI Resources) in order to remove the *AdhI* restriction site originally present in the plasmid. The pL0033 fragment containing the ampicillin resistance cassette and the *AdhI* site that resulted from *SapI* and *NcoI* digestion was swapped by the fragment containing the kanamycin marker obtained by digestion of pET-28a(+) (Novagen) with the same restriction enzymes, generating pL0033_Kan. The genomic region encompassing 439 base pairs of the 5' intergenic region and the open reading frame of *spatr* excluding the termination codon was then amplified by PCR using primers P25 + P26 and cloned into pL0033_Kan using the *NotI* and *NcoI* restriction sites. The 3' regulatory sequence of *spatr* (358 bp) amplified with P27 + P28 was inserted downstream of the sequence encoding the 2x c-Myc tag using the *FseI*/*NdeI* restriction sites. A point mutation was inserted in the start codon to prevent the translation of the full-length untagged SPATR because of the duplication of part of the locus resulting from the genetic recombination event. To do this, the region containing the first 13 nucleotides of the coding sequence and the entire 5' intergenic region previously inserted in the construct was swapped for the sequence amplified using primers P25 + P29 and using the *NotI*/*AhdI* restriction sites. Parasites were transfected using the *AhdI*-linearized construct. Transfectant selection with pyrimethamine and cloning was performed as described above.

To genetically complement the SPATR KD^{V1} line, we transfected parasites with constructs generated by modifications of a plasmid containing the centromere sequence of the *P. berghei* chromosome 5 (31–33). The sequence comprised of the open reading frame of *spatr* and the 3' intergenic region (1,491 bp) was amplified with primers P30 + P31 and cloned into the episome using *SacII* and *XhoI*. The gene was put under transcriptional control of the 5' intergenic region of *spatr* (1,000 bp) or the promoter of the gene encoding the thrombospondin related anonymous protein (*trap*; PBANKA_1349800; 1,501 bp) (23), which were amplified using primer pairs P32 + P33 or P34 + P35, respectively, and inserted into the *PstI*/*SacII*-digested transfection vector. This plasmid also contained the promoter of the eukaryotic elongation factor 1 α gene (*eef1 α* ; PBANKA_1133400) (34) constitutively driving the expression of the RedStar red fluorescent protein (RFP) reporter. This allowed for the enrichment of episome-carrying blood stages by fluorescence-activated cell sorting (FACS) using a FACS Aria II machine (Benton Dickson) (35). Blood was diluted in DPBS containing 2% FBS, and 30 to 1x10⁴ GFP+/RFP+ erythrocytes were sorted at room temperature, collected in 100-150 μ l of DPBS containing 10% FBS and immediately injected intravenously in recipient mice. Several

successive enrichment steps were performed to increase the percentage of RFP+ parasites within the populations. FACS data was analyzed using the FlowJo software version 10.

All PCR products were primarily cloned into the pGEM-T Easy vector (Promega) and sequenced (LightRun, Eurofins Genomics) before being subcloned into the respective transfection vector. All restriction enzymes used in this study were purchased from New England Biolabs, with the exception of AdhI (Fermentas).

Genotyping of transgenic parasites

Blood collected from infected animals was filtered with a Plasmodipur leukocyte filter (EuroProxima) and erythrocytes were lysed with saponin (Fluka) at 0.15%. Genomic DNA was extracted and purified from the resulting parasite pellet using the QIAamp DNA Blood Mini Kit (Qiagen). Integration of the constructs in the correct loci and absence of the WT loci were then confirmed by PCR using the Phusion DNA Polymerase (New England Biolabs).

For Southern blot, 5 µg of genomic DNA were digested with EcoRI and EcoRV, separated on a 0.8% agarose (NZYTech) gel and transferred to a Hybond-N⁺ nylon membrane (Amersham). The intergenic sequence at 3' or *spatr* amplified using primer pair P14 + P15 was used as a probe. Labelling of the probe and signal generation and detection were performed with the AlkPhos Direct Labeling and Detection System with CDP-Star chemiluminescent detection reagent (Amersham). Signal detection was performed using a ChemiDoc Imaging System (Bio-Rad).

Analysis of blood stages

The growth of asexual blood stages was assessed by infecting NMRI mice at a parasitaemia of 0.01% and counting infected red blood cells in blood smears stained with Hemacolor (Merck). The percentage of total gametocytes was also determined by counting in blood smears. To assess microgamete exflagellation, a drop of blood was collected from an infected mouse and placed on a glass slide. Exflagellation was induced by breathing on a coverslip, placing it on top of the drop of blood and incubating for 10 min at room temperature. Exflagellation centers were counted in 5 fields using a widefield microscope at 40X magnification.

Reverse transcriptase quantitative PCR (RT-qPCR)

Total RNA from merozoites, oocysts and sporozoites was extracted using TRIzol LS Reagent (Invitrogen) and Phase Lock Gel Heavy tubes (5PRIME), followed by the removal of contaminating DNA with TURBO DNase (Invitrogen). Superscript® II Reverse Transcriptase (Invitrogen) was used to synthesize cDNA and the determination of *spatr* (primers P36 + P37),

hado (primers P38 + P39), *cep76* (primers 40 + 41) and *trap* (primers 42 + 43) transcript levels by RT-qPCR was performed using iQ or iTaq™ Universal SYBR Green supermix (Bio-Rad) and the iQ5 or CFX96 real-time PCR detection systems (Bio-Rad). We used *hsp70* as the reference gene (primers P44 + P45) and the $2^{-\Delta CT}$ method was used to determine the relative gene expression. Quantification of sporozoites associated with the abdomen and thorax following the removal of the digestive tracts, salivary glands and hemolymph was performed on day 15 post infection by detection of 18S ribosomal RNA (primers P46 + P47) and interpolation from a standard curve constructed with salivary gland sporozoites processed the same way as the mosquito carcasses. Amplification efficiencies were between 90 and 110%.

Analysis of oocyst development

Mosquito midguts were collected from day 6 to day 15 post-infection. To calculate oocyst number and size, midguts were collected into an 18-well μ -slide (ibidi) containing DPBS and images were taken using a Axio Observer Z1 fluorescence microscope (Zeiss) at 5X magnification. From day 11 post-infection, midguts were crushed, and the hemolymph and salivary glands were collected for sporozoite quantification in all compartments.

Sporozoite viability

Hemolymph sporozoites were diluted in DPBS containing propidium iodide (PI; Invitrogen) at a final concentration of 5 μ g/ml, transferred to an 18-well μ -slide (ibidi), and centrifuged for 5 min at room temperature and 500 x g. Photos were taken using an IN Cell Analyzer 2000 (GE Healthcare) and PI incorporation was evaluated with the ImageJ software.

Production of a recombinant protein

The coding sequence of *spatr* lacking the 22 amino acids of the predicted signal peptide (SignalP-4.1 server) was amplified from salivary gland sporozoite cDNA using primer pair P48 + P49 and cloned in frame into the expression plasmid pET-28a(+) using NcoI and XhoI restriction sites. A recombinant SPATR (rSPATR) fused to a polyhistidine tag (His-tag) was produced in BL21-CodonPlus-RIL competent cells (Invitrogen) and protein purification was performed under denaturing conditions using Ni-NTA Agarose (Qiagen) following the instructions of the manufacturer. Desalting and concentration of rSPATR was performed using a Vivaspin 20 3,000 MWCO PES (Sartorius) centrifugal concentrator and DPBS. To remove endotoxins, the recombinant protein was briefly vortexed in the presence of 1% Triton X-114 (Sigma), incubated on ice for 15 min, vortexed again, and incubated at 37 °C for 10 min (36). The mixture was centrifuged at room temperature and at 2,000 x g for 10 min and the top phase

was collected. The procedure was performed a total of 3 times and endotoxin levels were determined using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific).

Production of polyclonal antibodies

A mouse polyclonal antibody was generated by immunizing 6-week-old BALB/c mice intraperitoneally 3 times with 25 µg of rSPATR adjuvanted with 50 µg of Poly(I:C) (polyinosinic-polycytidylic acid) as an adjuvant, followed by a fourth booster dose of 50 µg of rSPATR and 50 µg of Poly(I:C). All doses were administered 2 weeks apart. Serum was collected 12 days after the final dose.

SDS-Page and Western blot

Purified merozoites and sporozoites were lysed in loading buffer at 95 °C for 10 min. Sample volumes containing $1.5\text{--}4 \times 10^6$ merozoites, $1\text{--}4 \times 10^5$ sporozoites or 100 to 5,000 ng of rSPATR were separated on a 12% acrylamide gel by SDS-PAGE. SDS-PAGE gels were stained with Coomassie blue and scanned using a GS-800 densitometer (Bio-Rad). For Western blot, the transfer to a PVDF membrane was performed with a Trans-blot Turbo Transfer System (Bio-Rad). The membranes were blocked using DPBS with 0.1% Tween 20 (Sigma) and 5% skim milk for at least 1 h at room temperature. Incubations with primary mouse α -rSPATR polyclonal antibodies (1:200 dilution), rabbit α -His-tag antibodies (0.1 µg/ml; MicroMol), mouse α -c-Myc antibodies clone 4A6 (2.5 to 5 µg/ml; Merck), or mouse α -CSP 3D11 monoclonal antibodies (6,37) (0.3 µg/ml; BEI resources), diluted in the blocking solution, were performed overnight at 4 °C (α -rSPATR) or for 1 h at room temperature (α -His-tag and α -CSP 3D11). The membranes were washed and incubated with the secondary HRP-conjugated anti-rabbit (1:10,000) or anti-mouse (1:5,000; SouthernBiotech) antibody for 1 h at room temperature. Signal detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and a ChemiDoc Imaging System (Bio-Rad).

Immunofluorescence assays and fluorescence microscopy of live sporozoites

Hemolymph sporozoites were centrifuged for 5 min at 450 x g and 4 °C in an 8-well Lab-Tek chamber slide (Nunc) or a 384-well plate (Greiner), fixed with 4% formaldehyde (Merck), washed 5 times with DPBS, and blocked with DPBS containing 5% FBS for at least 30 min at room temperature. Incubation with α -CSP 3D11 mouse monoclonal antibodies (2.3 µg/ml) was performed for 1 h at room temperature. Incubation with α -TRAP rabbit polyclonal antibodies (1:10,000) was performed overnight at 4 °C. The secondary antibodies, either goat α -mouse or

α -rabbit Alexa Fluor 568 antibodies (2 μ g/ml; Invitrogen), were added and incubation for 30 min at room temperature ensued. Finally, nuclei were stained with DAPI (1:5,000) for 10 min at room temperature and the slides mounted with a 90% glycerol (Alfa Aesar), 0.5% (w/v) n-propyl gallate (Sigma), 20 mM Tris-HCl (Sigma) pH 8.0 antifade solution. All stainings were performed using antibodies diluted in the blocking solution and were followed by 5 washes with DPBS. Live sporozoites were centrifuged for 3 min at 450 x g in a 18-well μ -slide (ibidi), at room temperature. Sporozoites were then analyzed using a DMI6000 FFW microscope (Leica Microsystems) and the ImageJ software.

Transmission electron microscopy

For the ultrastructure analysis, salivary glands collected from infected mosquitoes at day 27 post-infection were fixed in a solution of 2.5% glutaraldehyde (Electron Microscopy sciences) with 2% formaldehyde (Electron Microscopy sciences) in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h, at room temperature. After washing in buffer and two hours in post-fixating 2% osmium tetroxide (Electron Microscopy sciences) in 0.1M sodium cacodylate (Electron Microscopy sciences) buffer (pH 7.4), tissues were washed in water and then incubated with 1% uranyl acetate (Electron Microscopy sciences) overnight. Samples were dehydrated through graded series of ethanol, and embedded in EPON resin (Electron Microscopy sciences). Ultra-thin sections (50 nm thickness) were cut on a RMC Ultramicrotome (PowerTome) using Diatome diamond knives, mounted on mesh copper grids (Electron Microscopy Sciences), and stained with uranyl acetate substitute (Electron Microscopy Sciences) and lead citrate (Electron Microscopy Sciences) for 5 min each. Samples were viewed on a JEOL JEM 1400 transmission electron microscope and images were digitally recorded using an ORIUS 1100W CCD digital camera.

***In vitro* invasion and development assays**

Between 7×10^4 and 1.2×10^5 HepG2 cells (ATCC) per well were seeded in an 8-well Lab-Tek chamber slide (Nunc), previously coated with rat tail collagen type I (Sigma) at 200 μ g/ml and incubated at 37 °C overnight in DMEM (Gibco or Lonza) supplemented with 10% FBS, in a humidified incubator with 5% CO₂. Hemolymph from mosquitoes containing GFP-expressing oocysts in their midguts was collected by perfusion with DPBS at 4 °C. Sporozoites were counted, diluted in an equal volume of DMEM containing 20% FBS and 200 U/ml of penicillin/streptomycin (Lonza), and $\sim 1.5 \times 10^4$ hemolymph sporozoites were added per well. The slides were centrifuged at 450 x g for 5 min at room temperature to promote contact between sporozoites and HepG2 cells, and incubated at 37 °C. To evaluate the capacity of

sporozoites to infect host cells, the preparations were fixed with 4% formaldehyde (Merck) 1.5–2 h after the addition of the sporozoites. The medium in slides prepared to assess liver stage development was changed daily until the fixation time point (24 or 48 h). Intracellular and extracellular sporozoites were discriminated via an anti-CSP double staining. Preparations were blocked with DPBS containing 5% FBS for 30 min at room temperature, incubated with α -CSP 3D11 mouse monoclonal antibodies (2.3 $\mu\text{g}/\text{ml}$) for 1 h at room temperature, washed with DPBS, incubated with goat α -mouse Alexa Fluor 568 antibodies (2 $\mu\text{g}/\text{ml}$; Invitrogen) for 1 h at room temperature, and washed again. The incubation with the primary α -CSP antibodies was repeated after permeabilization with 1% Triton X100 (Sigma) for 4 min and washing. After the washing steps, incubation with goat α -mouse Alexa Fluor 488 antibodies (2 $\mu\text{g}/\text{ml}$; Invitrogen) was performed for 1 h at room temperature. Nuclei were then stained with DAPI diluted 1:5,000 in DPBS for 10 min at room temperature. To facilitate the detection of weakly-fluorescent liver stages, cells were permeabilized as described above and sequentially stained with primary rabbit α -GFP antibodies (1:250; MBL) and secondary goat α -rabbit Alexa Fluor 488 antibodies (2 $\mu\text{g}/\text{ml}$; Invitrogen) for 1 h at room temperature. Finally, the wells were washed and the slides mounted with antifade solution. Image acquisition and analysis was performed as previously described (26). Briefly, 25 images per well, corresponding to a total area of 0.57 mm², were taken using IN Cell Analyzer 2000 (GE Healthcare). HepG2 nuclei as well intracellular and extracellular sporozoites were counted using the IN Cell Developer Toolbox v.1.9.2 software (GE Healthcare). The area of liver stages was also determined using this software.

***In vitro* cell wounding assay**

Cell wounding assays were performed using a previously described protocol (38), with modifications. Briefly, HepG2 cells (ATCC) were seeded at 2×10^4 cells per well on a collagen-coated 96-well plate (Orange Scientific) and incubated at 37 °C overnight in DMEM containing 10% FBS. Hemolymph and salivary gland sporozoites collected in DPBS were diluted in an equal volume of DMEM containing 20% FBS, 200 U/ml of penicillin/streptomycin and PI at 10 $\mu\text{g}/\text{ml}$, and 2.7×10^4 to 4×10^4 sporozoites were added per well. Controls without PI and/or parasites were also included. The plate was centrifuged at 500 x g for 5 min at room temperature and incubated for 1 h at 37 °C in a humidified incubator with 5% CO₂. The medium was removed, and the cells were washed and digested with trypsin (Gibco) for 10 min at 37 °C. DMEM supplemented with 10% FBS was added, cells were centrifuged at 500 x g for 5 min at room temperature and resuspended in DPBS containing 2% FBS. The percentage of PI-positive

cells was determined by flow cytometry analysis using a FACSCanto II (BD Biosciences) and FlowJo software version 10.

***In vivo* infections with sporozoites**

To evaluate the infectivity of the mutant lines, 1.1×10^4 to 6×10^4 hemolymph or 200 to 2,500 salivary gland sporozoites were injected intravenously into C57BL/6 mice. Parasite burdens in the liver were evaluated by bioluminescence imaging at day 1 and/or 2 post-infection. Isoflurane-anaesthetized mice were shaved and injected subcutaneously with 2.4 mg of D-luciferin potassium salt (Perkin Elmer) dissolved in DPBS. Animals were transferred to the IVIS Lumina LT system (Perkin Elmer) stage and kept under anesthesia during signal acquisition, which was initiated 5 min after the administration of the substrate. Parasitemias were calculated from day 3 post-infection by counting infected red blood cells in blood smears stained with Hemacolor (Merck).

The targeting of the liver by hemolymph sporozoites in the first minutes post-infection was assessed using whole-mouse bioluminescence imaging (6). Mice were pre-treated with 10 μ l/g of clodronate liposomes (Liposoma BV) 2 to 4 days prior to infection to deplete Kupffer cells (4). C57BL/6 mice were previously shaved and infected with 9×10^4 to 1.5×10^5 hemolymph sporozoites by intravenous injection and anesthetized, and D-luciferin was administered 4 min after infection. Signal detection was initiated 7 min post-infection and performed for 5 min. Living Image software (Perkin Elmer) was used to control the acquisition process, to quantify the bioluminescent signal in regions of interest (ROIs) and to generate the photograph and signal map overlays.

Gliding assays

The evaluation of sporozoite motility *in vitro* was performed by live microscopy on 18-well μ -slides (ibidi). Sporozoites were collected from the hemolymph or the salivary glands of infected mosquitoes on days 18–19 or 24–25 post-infectious feeding, respectively. Salivary glands were mechanically disrupted using a pestle and filtered using a 35 μ m cell strainer cap (Falcon). Sporozoite suspensions prepared in DPBS and kept on ice were diluted in equal parts of DMEM (4.5 g/l glucose) with 10% FBS and transferred to the slide at a final concentration of approximately 5,000 parasites per well. Centrifugation at 400 x g, 4 °C, for 3 min ensued, to allow the parasites to attach to the bottom of the well. The slide was then transferred to the inverted phase contrast microscope (Axio Observer Z1) and incubated at 37 °C, 5% CO₂, for 3 min. Up to 3 videos of 3 min were sequentially recorded in different fields of each well using the 20X objective, and the trajectories and speed of individual parasites were analyzed using

the ImageJ software. Motile behaviors were classified as follows: motile ≥ 1 circle – motile sporozoites capable of completing at least a full circle; motile < 1 – motile sporozoites that did not complete a full circle; waving – sporozoites that at any point attached to the bottom of the well, often through one of the poles, without displacement; drifting – parasites that did not at any point attach to the bottom of the well; immotile – sporozoites that remained stationary during the duration of the video.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism Software (version 8.0). Statistical significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

Results

Knockdown of *spatr* in *P. berghei* sporozoites

We attempted to knock out the *spatr* gene by double crossover homologous recombination, using two different strategies (Fig. S1). We first made use of a *PlasmoGEM* (Sanger) targeting vector (Fig. S1A), which contains long homology regions that increase the efficiency of integration of the construct at the target locus (29,39). While integration at the correct locus was confirmed in transfectant populations, isogenic transgenic parasites could not be isolated in several independent attempts (Fig. S1B). To exclude the possibility that the long homology regions that span over neighboring genes could have also led to alterations in the expression of these genes, affecting the viability of mutant parasites, we constructed another transfection vector that restricted the homology regions to the intergenic sequences flanking the *spatr* gene (Fig. S1C). However, we were unable to detect by PCR the presence of transgenic parasites within the parental populations that arose after the transfection (data not shown). These results support other reports of SPATR essentiality for *P. berghei* replicative blood stages (19,22).

Thus, to ascertain the potential involvement of SPATR in sporozoite infectivity to the mosquito and mammalian hosts, we devised a promoter swap strategy to attain the knockdown of *spatr* early during sporozoite development. Available RNA-seq datasets showed higher abundance of *spatr* transcripts in blood schizonts, ookinetes and salivary gland sporozoites (20,21) (Fig. 1A). Thus, we searched for genes that mirrored the overall expression profile of *spatr*, but were turned off in sporozoites. We selected the genes that encode the haloacid dehydrogenase domain ookinete protein (HADO) and the putative centrosomal protein 76 (CEP76) based on these criteria (Fig. 1A and S2A). While the absence of *hado* transcripts in oocysts had been previously reported (40), no such data was available for the *cep76* gene, although we hypothesized this gene would be expressed during sporogony due to its expected role in cell division (41), serving as a control for translational repression. To confirm this, we evaluated the transcriptional profiles of *spatr* and the selected genes by RT-qPCR (Fig. 1B and S2B). These data show that unlike *spatr*, whose transcription upregulation within oocysts appeared to roughly coincide with the start of sporozoite formation after day 8 days after the infectious blood meal (42) and peaked in hemolymph sporozoites, *hado* was lowly expressed throughout oocyst development and sporozoite maturation (Fig. 1B). On the other hand, *cep76* transcripts were detected in oocysts and were drastically reduced in hemolymph and salivary gland sporozoites (Fig. S2B).

The exact localization of the promoter elements required to drive the expression of *spatr* in the different stages of the life cycle remains unknown. Therefore, swapping the short intergenic sequence that precedes the *spatr* gene by the selected putative promoters would likely affect regulatory elements of the upstream gene. Moreover, neighboring *Plasmodium* genes can form co-expressed units, which may be controlled by a single promoter (43), and bicistronic transcripts have been reported in *P. berghei* (44). Additionally, *Plasmodium* utilizes several layers of tight transcriptional control, some of which are promoter-independent (45). Thus, keeping the *spatr* gene in its native locus could still lead to transcription in the mosquito stages regardless of the stage specificity of the new promoters. For these reasons, we opted for a conservative promoter exchange approach that involved the insertion of a second copy of *spatr* under transcriptional control of the *hado* or *cep76* putative promoters in a previously reported silent locus in chromosome 6 (30), and the subsequent deletion of the endogenous gene located in chromosome 3 (Fig. 1C and S2C). The intermediate lines that were generated were named SPATR double-expressers (SPATR DE), as they were expected to possess two active copies of the gene in select developmental stages. While the expression of *spatr* induced by the 5' intergenic region of *hado* (2,414 bp; Fig. 1C) in the blood stage allowed the deletion of the endogenous copy of the gene, generating knockdown (KD) lines (Fig. 1D), we failed to knock out the *spatr* gene in the line containing the exogenous copy of the gene under transcriptional control of the 5' intergenic region of *cep76* (845 bp; Fig. S2D). This may have been due to insufficient or ill-timed expression of *spatr* in merozoites. Indeed, *spatr* transcripts were approximately two times more abundant than *cep76* mRNA in merozoites, although the difference was not statistically significant (Fig. S2B). Alternatively, we cannot exclude that the selected genomic sequence does not contain the promoter elements that drive the expression of the *cep76* gene. Therefore, references to SPATR DE and SPATR KD parasites will henceforth only refer to the isogenic lines obtained using the strategy that resorted to the *hado* promoter.

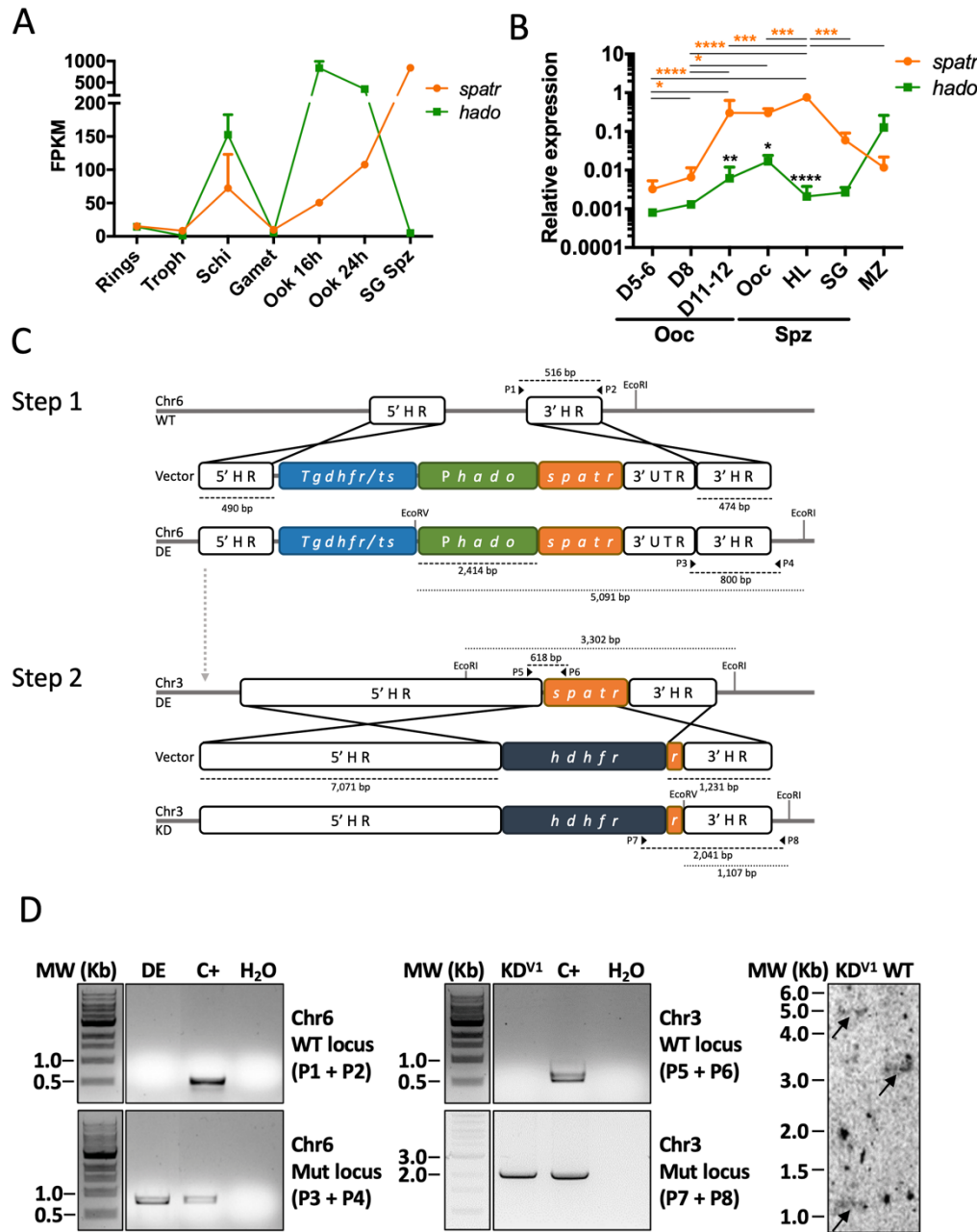


Fig 1. Generation of *spatr* knockdown (KD) lines. (A) RNA-seq data of *spatr* and *hado* genes in different stages of the *P. berghei* life cycle. Data from published datasets (20,21). (B) Expression of *spatr* and *hado* relative to *hsp70* in different stages and days post-infection, assessed by RT-qPCR (mean values + standard deviation of at least two biological replicates). Statistical analyses of differences between genes (black asterisks) or developmental stages (colored asterisks) were performed using two-way ANOVA with Sidak's multiple comparison test or two-way ANOVA with Turkey's multiple comparison test, respectively. (C) Schematic representation of the two-step promoter exchange strategy using the promoter of *hado*. The sizes of genomic regions are shown next to dashed line. Sequences are not to scale. (D) Genotyping of transfected populations by PCR (left and center) and Southern blot (right). Restriction sites and primers used for the genotypic analyses by Southern blot or PCR,

respectively, are depicted in panel C. Amplicon sizes and sequences detected by Southern blot are shown next to dashed or dotted lines, respectively.

bp: base pairs. C+: positive control (WT or transfer population on the top or bottom gels, respectively). Chr: chromosome. DE: SPATR double-expressor. Gamet: gametocytes. FPKM: fragments per kilobase of transcript per million mapped reads. *hdhfr*: human dihydrofolate reductase. HL: hemolymph. HR: homology region. Kb: kilobases. KD^{V1}: SPATR knockdown V1 clone. Mut: mutant. MW: molecular weight. Ooc: oocyst(s). Ook: ookinetes. Schi: schizonts. SG: salivary gland. Spz: sporozoites. *Tgdhfr/ts*: *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase. Troph: trophozoites. UTR: untranslated region.

SPATR is important for the colonization of the mosquito salivary glands

SPATR KD blood stages displayed normal growth (Fig. 2A) and successfully differentiated into functional gametocytes (Fig. 2B), as evidenced by the formation of microgametes (Fig. 2C) and their ability to infect mosquitoes (Fig. 2D). While the SPATR DE line showed a prevalence of infected mosquitoes (Fig. 2D) and sporozoite numbers (Fig. 2E) comparable to the WT, SPATR KD sporozoites did not invade the salivary glands as efficiently as the control lines (Fig. 2E–F). Interestingly, the blockage of salivary gland invasion did not result in the accumulation of sporozoites in the hemolymph (Fig. 2E). With no evident structural modifications, a few SPATR KD sporozoites were detected within the salivary glands by transmission electron microscopy (Fig. 2F). This indicates that some parasites were indeed capable of infecting the mosquito salivary glands, rather than remaining associated to the basal lining of the glands. Although we only observed a very small number of SPATR KD sporozoites inside the salivary glands, no parasites were seen within the secretory cavities. Conversely, high numbers of WT sporozoites were present both in the parenchyma cells and in the secretory cavities of the salivary glands (Fig. 2F). Altogether, these data suggest a less efficient salivary gland recognition and/or invasion by SPATR KD sporozoites.

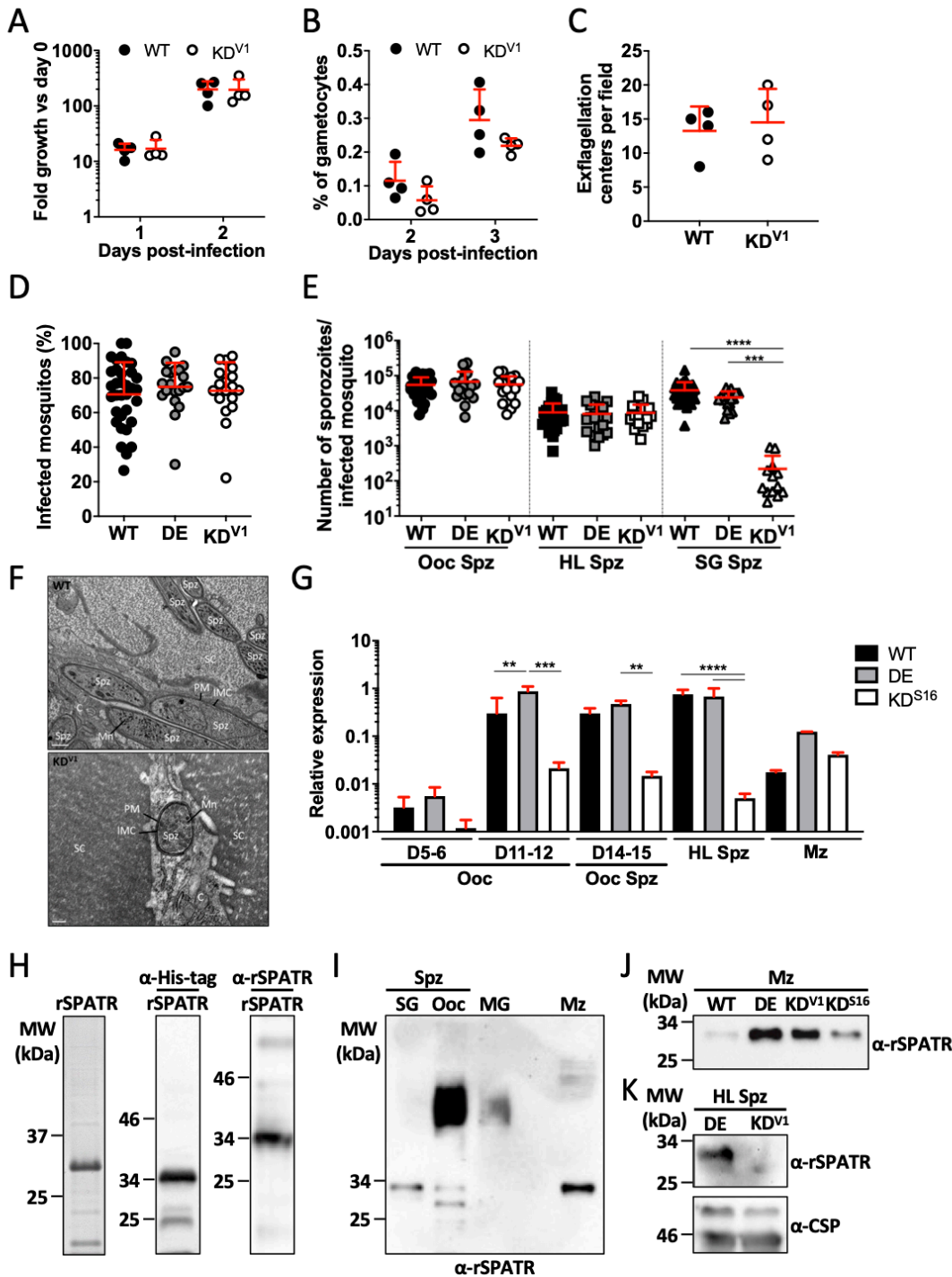


Fig 2. Infectivity of SPATR KD parasites in the blood and mosquito and evaluation of *spatr* expression. (A) Fold- growth of blood stages versus parasitemia on infection day. (B) Gametocytemia. (C) Microgamete exflagellation centers. (D) Prevalence of infected mosquitoes in independent experiments. (E) Numbers of oocyst, hemolymph and salivary gland sporozoites per mosquito in independent experiments on days 14 to 28 post-infection. (F) Ultrastructure and localization of sporozoites within the salivary glands. Scale bars: 500 nm (top) or 200 nm (bottom). (G) Expression of *spatr* relative to *hsp70* in WT, SPATR DE and SPATR KD parasites determined by RT-qPCR using at least two biological replicates. (H) SDS-

PAGE gel showing 5 µg of purified recombinant SPATR (rSPATR; left) and reactivity of an α-His-tag antibody (center) and the mouse α-SPATR polyclonal antibodies (right) against 100 ng of rSPATR. (I) Detection of SPATR by Western blot in samples prepared with 1.25×10^4 WT salivary gland and oocyst sporozoites or 1.5×10^6 WT merozoites. An amount of uninfected midguts (MG) equivalent to that loaded in the oocyst sporozoite lane was used to test the reactivity of the polyclonal antibodies against mosquito-derived material. (J) Western blot analysis of merozoite extracts prepared with 4×10^6 merozoites of the different lines. (K) SPATR levels in 1.5×10^4 SPATR DE or SPATR KD hemolymph sporozoites determined by Western blot. CSP levels evaluated with mouse anti-CSP monoclonal antibody 3D11 were used as a loading control.

Mean values + standard deviation are shown in panels A–E and G. Statistical analyses of were performed using the Mann-Whitney test (A–C) or the Kruskal-Wallis test with Dunn's multiple comparisons test (D, E, G).

C: salivary gland cell. D: day post-infection. DE: SPATR double-expressor. HL: hemolymph. IMC: inner membrane complex. KD^{S16}: SPATR knockdown S16 clone. KD^{V1}: SPATR knockdown V1 clone. MW: molecular weight. Mn: microneme. Mz: merozoites. Ooc: oocyst(s). PM: plasma membrane. SC: secretory cavity. SG: salivary gland. Spz: sporozoites.

To confirm the decreased transcription of *spatr* in SPATR KD sporozoites, we analyzed oocysts at different time points after the infectious blood meal, sporozoites collected from mechanically disrupted oocysts, hemolymph sporozoites and purified merozoites by RT- qPCR (Fig. 2G). Transcripts were found to be significantly less abundant in SPATR KD oocysts and sporozoites than in the control lines starting from days 11–12 post-infection. On the other hand, the SPATR DE line had similar levels of *spatr* transcripts in comparison to the WT line in all sporozoite stages, although transcript levels were around 7 times higher in SPATR DE merozoites (Fig. 2G). This is indicative that, as expected, both copies of the *spatr* gene are being transcribed in the merozoite stage (Fig. 2G). Mouse polyclonal antibodies against a His-tagged recombinant SPATR (rSPATR; Fig. 2H) detected bands of the approximate predicted size of SPATR (29 or 26 kDa with or without the signal peptide, respectively) in extracts of WT sporozoites collected from the salivary glands and oocysts, as well as in merozoites (Fig. 2I). As anticipated, SPATR was also expressed in SPATR DE and SPATR KD merozoites (Fig. 2J), but no signal was detected in SPATR KD hemolymph sporozoites (Fig. 2K). The detection of the protein in WT oocyst-derived sporozoites was unexpected, as previous proteomics studies performed in *Plasmodium falciparum* and *Plasmodium yoelii* had only detected SPATR in salivary gland sporozoites, whereas transcripts were present in sporozoites collected from both the oocysts and the salivary glands, which was suggestive of translational repression^{12,15}. Whether this was due to differences in the parasite species, the technical approaches, or the age of oocyst sporozoites – these were collected from day 19 post-infection onwards in this work, as opposed to days 10–14 in the *P. falciparum* and *P. yoelii* proteomics studies – remains to be elucidated. Nonetheless, it is possible that the expression of SPATR can also be regulated by intrinsic

temporal mechanisms rather than just the migration of sporozoites through the mosquito. Similar band patterns were obtained by Western blot using extracts of sporozoites expressing a c-Myc-tagged SPATR at the C-terminus (Fig. S3). Moreover, the tagging of the protein was not detrimental to the parasite as SPATR-myc sporozoites were present in similar numbers compared to controls in oocysts and in the salivary glands (Fig. S3D) and no overt defects were observed during blood stage growth in any of the infections resulting from the generation and testing of the SPATR-myc isogenic lines (data not shown).

Analysis of the kinetics of SPATR KD sporozoite development

In an attempt to understand why the blockage of salivary gland infection did not result in the accumulation of SPATR KD sporozoites in the hemolymph, we evaluated oocyst and sporozoite development from early time points post-infection (Fig. 3A–E). Neither the number (Fig. 3A) nor the size (Fig. 3B) of the oocysts revealed any clear developmental impairments. However, whereas the number of sporozoites per oocyst did not vary significantly in the WT and SPATR DE lines from day 11 until day 15 post-infection, a significant increase in the number of sporozoites per oocyst was observed in both SPATR KD isogenic lines on day 15 (Fig. 3C). When the same ratio was applied to hemolymph sporozoites, all lines exhibited an overall similar increase in numbers over time (Fig. 3D). The lower numbers of sporozoites inside oocysts on days 11–13 post-infection could be met with a transient accumulation of the few parasites released from oocysts in the hemolymph, resulting in similar sporozoite numbers in the circulatory system of the mosquito. This hypothesis is supported by the observation that the mortality of SPATR KD hemolymph sporozoites was not superior to that of WT parasites (Fig. 3F) and that sporozoites were not being sequestered in other tissues of the mosquito (Fig. 3G). Indeed, SPATR KD oocysts also contained more sporozoites than the control lines from day 24 post-infectious blood meal (Fig. 3H). Altogether, these data could be suggestive of an alteration in the egress of sporozoites from oocysts and/or a delay in development. Finally, we performed a qualitative analysis of CSP and TRAP expression in SPATR KD hemolymph sporozoites, as both these proteins have been previously implicated in the invasion of the salivary glands (46–59). No overt differences in expression and localization of CSP were detected, which also indicated aberrant morphology was not the cause of the low numbers of SPATR KD salivary gland sporozoites (Fig. S4A). The typical localization of TRAP showed that the biogenesis, subcellular localization and discharge of TRAP-containing micronemes remained unaffected (Fig. S4B).

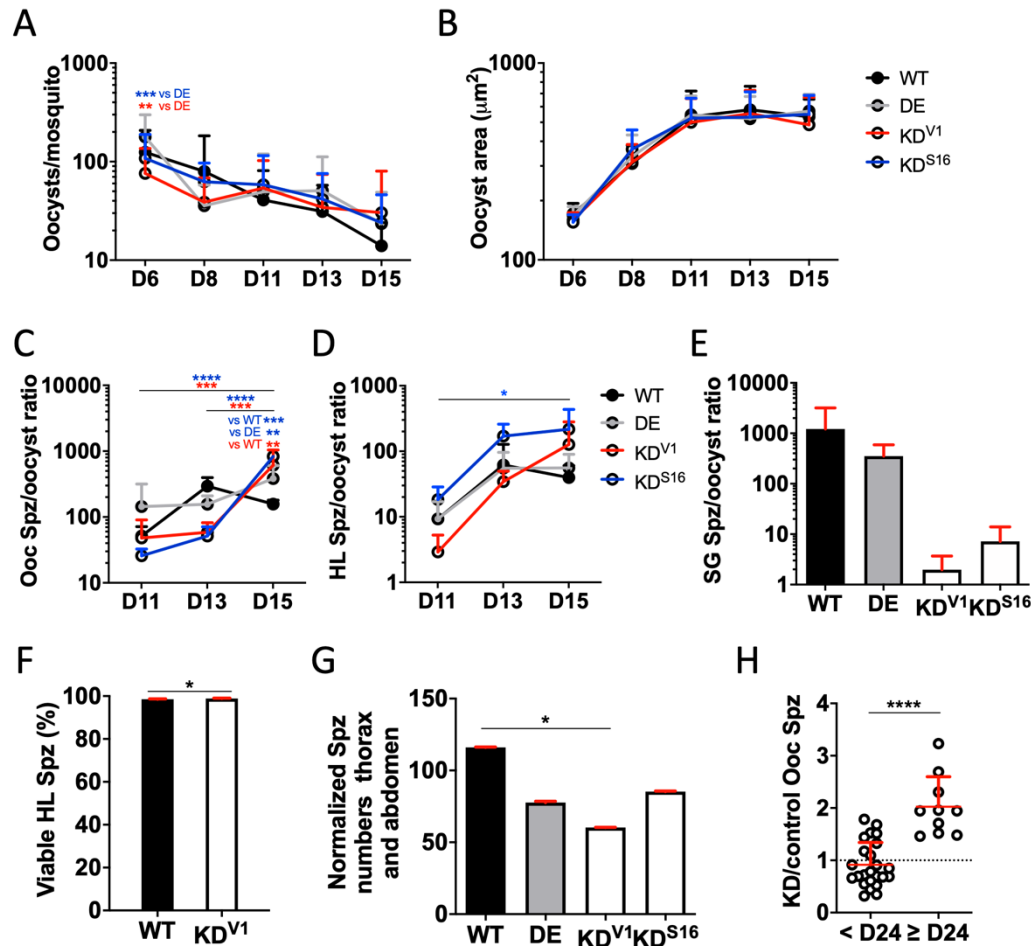


Fig 3. Development and localization of SPATR KD parasites in the mosquito. (A–E) Kinetics of SPATR KD sporozoite development. Oocyst numbers (A), oocyst area (B), and numbers of oocyst (C), hemolymph (D) and salivary gland (E) sporozoites normalized by the number of oocysts are shown. (F) Viability of hemolymph sporozoites represented by the percentage of sporozoites displaying PI incorporation. (G) Numbers of sporozoites recovered from the thorax and abdomen per mosquito, normalized by oocyst numbers, determined by quantification of 18S ribosomal RNA by RT-qPCR, via interpolation from a standard curve. (H) Ratio of the number of oocyst sporozoites detected in mosquitoes infected with either SPATR KD clone (KD) to the number of oocyst sporozoites obtained with the WT or SPATR DE control lines prior to and from day 24 post infection.

Mean values + standard deviation are represented. Statistical analyses were performed using the Mann-Whitney test (F, H), Kruskal-Wallis test with Dunn's multiple comparisons test (E, G), or two-way ANOVA with Turkey's multiple comparison test (A–D). In panels A–D, red or blue asterisks refer to statistical significance associated with SPATR KD^{V1} or SPATR KD^{S16}, respectively, in comparisons between different time points (over black bars; only performed in panels C and D) or between lines (over the symbols at the appropriate time point).

DE: SPATR double-expressor. HL: hemolymph. KD^{S16}: SPATR knockdown S16 clone. KD^{V1}: SPATR knockdown V1 clone. Ooc: oocyst. SG: salivary gland. Spz: sporozoites.

The establishment of liver infection in mice by SPATR KD sporozoites is impaired

Sporozoites collected from the hemolymph were injected intravenously in mice. While DE and WT parasites developed similarly in the liver and in the blood, SPATR KD hemolymph sporozoites failed to produce a detectable bioluminescent signal in the liver stage and were rarely able to establish a blood infection (Fig. 4A and Table S2). An attenuated phenotype was observed when we used the few SPATR KD sporozoites we could collect from salivary glands, with reduced parasite burdens in the liver that culminated in an extension of the prepatent period of approximately 1 day (Fig. 4B and Table S2). Conversely, infections with SPATR DE sporozoites consistently yielded similar burdens in both the liver and blood to those obtained with the WT line (Fig. 4A–B and Table S2).

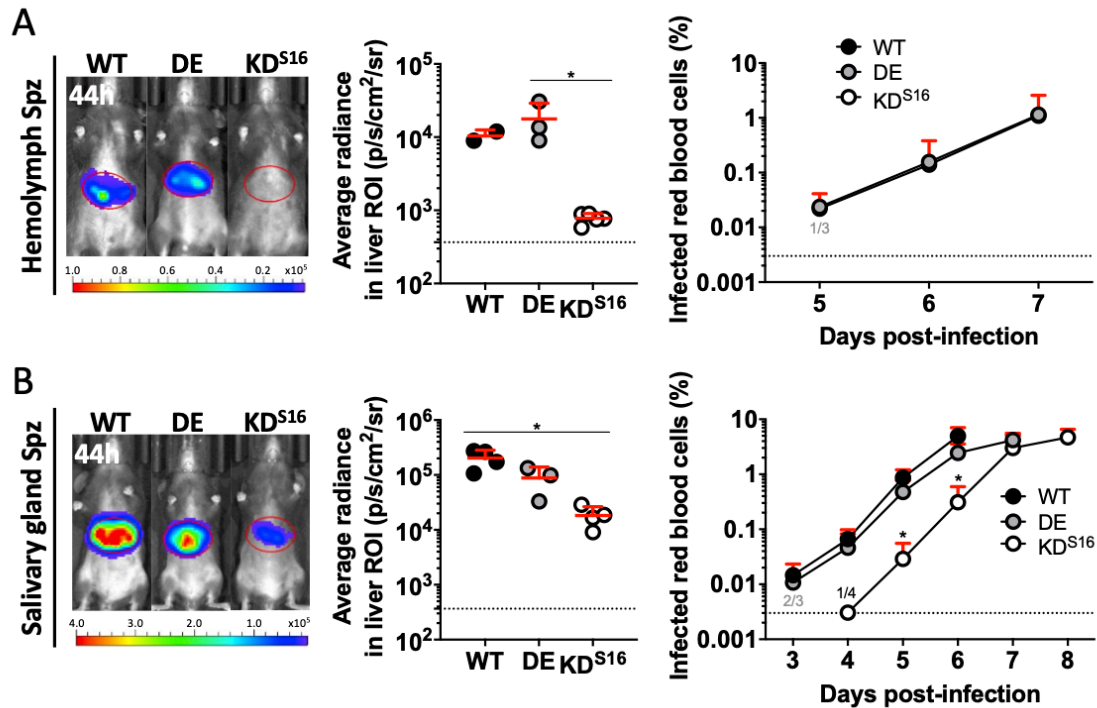


Fig 4. Infectivity of SPATR KD sporozoites. (A, B) Intravenous infection of C57BL/6 mice with 2×10^4 hemolymph (A) or 2,500 salivary gland sporozoites (B). A representative image resulting superimposition of the bioluminescence signal map and a grey-scale photograph of the mice at 44 h post-infection (left), the quantification of the signal in the defined liver ROI (center) and the parasitemias of infected animals (right) are shown. The fractions of animals with parasitemia above detection limit are shown in time points where not all animals had detectable parasitemia. In the plot on the right of panel B, statistical significance was found when comparing SPATR KD and WT lines. Dotted lines on graphs represent the detection limit of the respective technique.

The mean values + standard deviations in representative experiments are shown in all panels. Statistical analyses were performed using the Kruskal-Wallis test with Dunn's multiple comparisons test (A, B).

DE: SPATR double-expressor. KD^{S16}: SPATR knockdown S16 clone. Spz: sporozoite.

SPATR knockdown sporozoites exhibit defects in hepatocyte invasion, migration and homing to the liver

A decrease in the parasite burdens in the liver two days after intravenous injection of SPATR KD sporozoites can be attributable to impairments in parasite arrest in the liver sinusoids, crossing of the sinusoidal barrier, recognition and invasion of hepatocytes, or development within host cells (5,7). Thus, we first evaluated the capacity of SPATR KD parasites to invade and infect hepatocytes *in vitro* using the HepG2 hepatoma cell line. DE hemolymph sporozoites did not display any infectivity or developmental defects *in vitro* (Fig. 5A–C). Conversely, invasion assays performed with SPATR KD hemolymph sporozoites yielded at least 5 times fewer intracellular sporozoites than with the WT line (Fig. 5A), which also resulted in lower numbers of SPATR KD exoerythrocytic forms (Fig. 5B). While differences in the size of liver stages were occasionally observed (Fig. 5C), which could indicate a developmental impairment, the small numbers of SPATR KD parasites analyzed preclude the definite assignment of a defect in liver stage development to SPATR KD parasites.

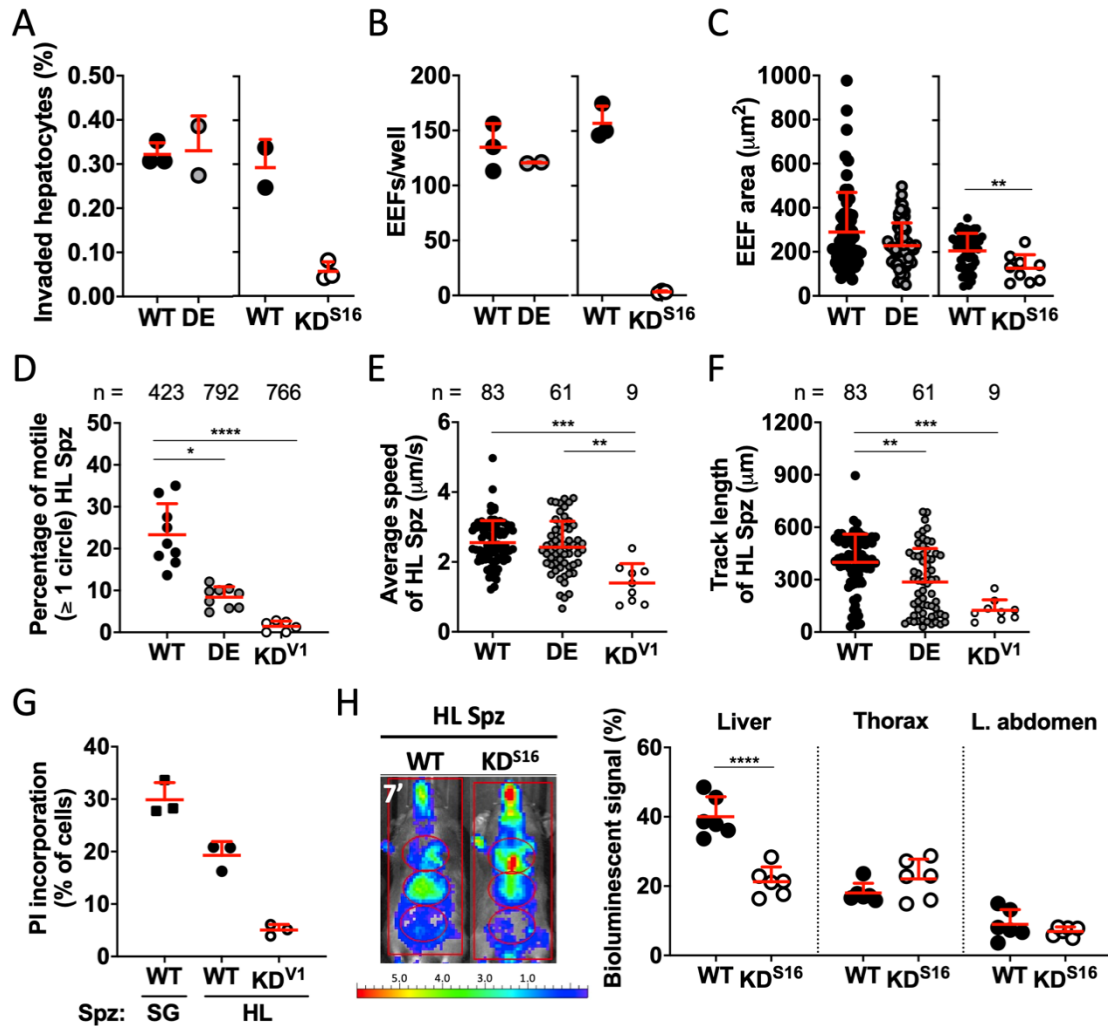


Fig 5. *In vitro* and *in vivo* studies with SPATR KD hemolymph sporozoites. (A) Invasion of HepG2 cells by hemolymph sporozoites. (B, C) Numbers (B) and area (C) of exoerythrocytic forms (EEFs) following the infection of HepG2 cells with hemolymph sporozoites (D–F). Percentage of motile hemolymph sporozoites (D), and their average speed (E) and track length (F). Numbers above the plots indicate the total number of sporozoites analyzed. The symbols in panel D represent the percentages obtained in different videos, for several sporozoites. (G) Determination of PI incorporation by HepG2 cells by flow cytometry analysis following incubation with sporozoites. The average percentage of PI+ cells only incubated with PI (no sporozoites) was subtracted to the percentage of PI+ cells obtained in each experimental condition. WT salivary gland sporozoites were used as a positive control. (H) Homing of hemolymph sporozoites from 7 minutes post-infection. A representative superimposed image is shown on the (left) next to the percentage of total photons detected in the several anatomical regions of C57BL/6 mice (right). The color scale represents radiance values (photons/s/cm²/sr). Data from two independent experiments were pooled. Mean values + standard deviations are represented and representative experiments are shown (except in panel H). Statistical analyses were performed using the Mann-Whitney test (A–C, H) or the Kruskal-Wallis test with Dunn's multiple comparisons test (D–F). All panels show data from representative experiments. DE: SPATR double-expressor. HL: hemolymph. KD^{S16}: SPATR knockdown S16 clone. KD^{V1}: SPATR knockdown V1 clone. SG: salivary gland. Spz: sporozoites.

Next, we tested the ability of SPATR KD hemolymph sporozoites to wound and traverse cells, which was measured by the incorporation of propidium iodide, a membrane-impermeable intercalating agent, by hepatocytes following incubation with the parasites. SPATR KD hemolymph sporozoites tendentially wounded less cells than WT hemolymph sporozoites, which in turn showed a less efficient cell wounding activity than WT parasites collected from the salivary glands (Fig. 5G). The modest reduction (1.4- to 3.6-fold, in two independent experiments) of cell wounding activity in SPATR KD hemolymph sporozoites indicates that cell traversal should play a minor role in the severely impaired infectivity of this mutant line in mice.

Finally, to dissect the capacity of SPATR KD sporozoites to home to the liver, we infected mice with hemolymph sporozoites and quantified the bioluminescent signal shortly after the intravascular injection. Importantly, it has been shown that Kupffer cells function as facultative entry points to the liver parenchyma for salivary gland sporozoites (4,60). However, hemolymph sporozoites do not possess a fully matured cell traversal machinery (61) and may be retained by Kupffer cells, as was observed for the cell traversal-deficient sporozoites lacking the sporozoite protein essential for cell traversal (SPECT2) (4). Therefore, homing experiments were performed in clodronate-treated mice for Kupffer cell depletion to ensure the detected signal more accurately reflected parasite capacity to be arrested in the liver sinusoids. In contrast to the partial accumulation of WT hemolymph sporozoites in the anatomical area corresponding to the liver (Fig. 5H), the percentage of bioluminescent signal in the liver of

animals infected with SPATR KD hemolymph sporozoites (~21%; Fig. 5H) was similar to those obtained in mice infected with circulating parasites such as *Trypanosoma brucei* bloodstreams forms or *P. berghei* merozoites (6). These data show that SPATR KD hemolymph sporozoites fail to accumulate in their target organ in this short time frame.

In conclusion, these cumulative impairments explain the incapacity of these sporozoites to productively infect the liver.

Genetic complementation partially reverts the SPATR KD phenotype

In an attempt to correlate the loss of infectivity observed in SPATR KD sporozoites to the decrease in SPATR levels, we genetically complemented the SPATR KD V1 isogenic line with an episome carrying part of the *spatr* locus (Fig. 6A). Since the nature and location of the promoter elements required for the transcription of *spatr* have not yet been identified, we also constructed a plasmid containing the *spatr* open reading frame downstream of the promoter of *trap* (Fig. 6A), which has been shown to drive the expression of genes in ookinetes and sporozoites starting from the oocyst stage (23,62). Moreover, we confirmed that the transcriptional profile of *trap* in selected stages of the parasite was similar to that of *spatr* (Fig. 6B). The plasmid also contained the centromeric region of chromosome 5 of *P. berghei*, the PbCEN5-core, to enhance segregation stability during cell division (31–33). Lastly, the RedStar RFP reporter gene present in the centromeric plasmid allowed us to perform the enrichment RFP-positive blood stage parasites by flow cytometry (Fig. 6C). While erythrocytes harboring parasites complemented with the episome containing the *spatr* gene under transcriptional control of the promoter of *trap* (SPATR COMP^{Ptrap}) represented approximately half of the total infected erythrocyte population after several enrichment steps, the percentage of erythrocytes carrying parasites transfected with the version of the plasmid bearing the putative promoter of *spatr* (SPATR COMP^{Pspatr}) never surpassed 10% (Fig. 6C). The overall lower than expected proportion of RFP-positive to RFP-negative parasites after several consecutive steps of enrichment might be a consequence of asymmetrical daughter cell segregation during schizogony and plasmid loss, despite the inclusion of the PbCEN5-core.

For these reasons, we only subjected the mixed populations containing SPATR COMP^{Ptrap} parasites to a mosquito passage. Overall, the percentage of RFP-positive oocysts (Fig. 6D) was lower than the percentage of RFP-positive blood stages (Fig. 6C), which implies further loss of the episome during schizogony prior to the infectious blood feeding or during multiplication within the oocysts. Nonetheless, the genetic complementation resulted in the increase of sporozoite numbers inside the salivary glands in mosquitoes infected with SPATR COMP^{Ptrap} parasites, as demonstrated by the reduced ratio between the numbers of oocyst and salivary

gland sporozoites in comparison to the SPATR KD line (Fig. 6E). Despite the fact that only nearly 1 in 4 SPATR COMP^{P_{trap}} oocysts contained the plasmid (Fig. 6D), which could also explain why we did not attain full reversion of the defective salivary gland invasion phenotype (Fig. 6E), almost all SPATR COMP^{P_{trap}} sporozoites present in the salivary glands had detectable RFP signal (Fig. 6F–I). Moreover, the proportion of sporozoites carrying the plasmid also increased from the oocyst and hemolymph sporozoite populations to the parasite population present in the salivary glands (Fig. 6I). This demonstrate that parasites carrying the episome specifically accumulate in the salivary glands and links the low numbers of SPATR KD salivary gland sporozoites to the downregulation of *spatr* expression.

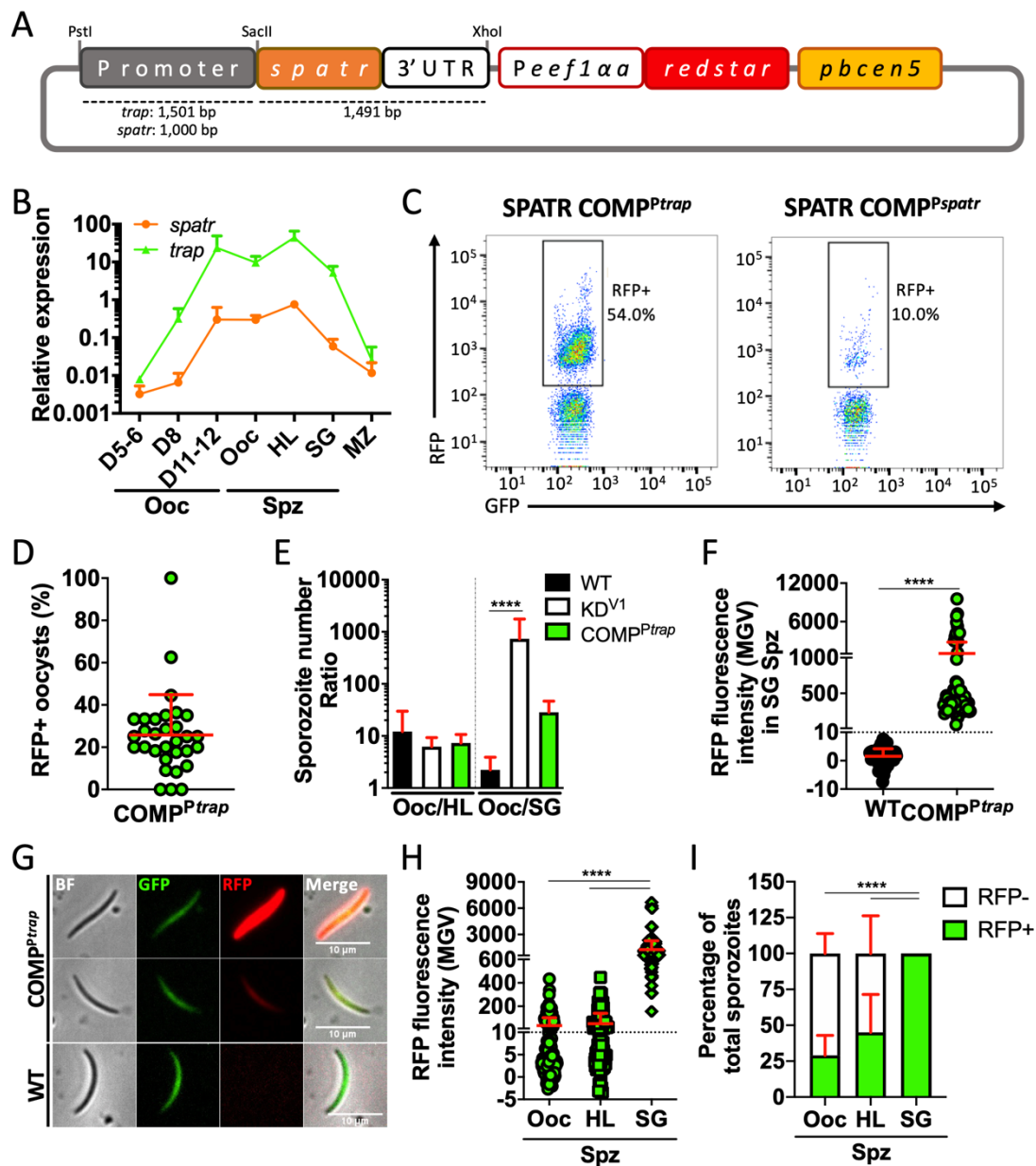


Fig 6. Genetic complementation of SPATR KD parasites. (A) Schematic representation of the centromeric episome used to complement the SPATR KD^{V1} line. Restriction enzymes and size of the sequences (not to scale) used to construct the plasmid are indicated. (B) Expression of *trap* relative to *hsp70* in different stages and days post-infection, assessed by RT-qPCR using at least two biological replicates. The transcription data of *spatr* shown in Fig. 1B were plotted here to facilitate comparison of the transcriptional profiles. (C) FACS analysis of blood collected from mice infected with either SPATR COMP^{P_{trap}} (left) or SPATR COMP^{P_{spatr}} (right) populations. The percentages of parasites harboring the episome (RFP+ events gated in the GFP+ population) are shown. (D) Percentage of RFP-expressing oocysts at day 15 post-infection, determined by fluorescence microscopy. Each symbol represents an individual midgut. (E) Ratios between oocyst and either hemolymph or salivary gland sporozoite numbers from days 14 to 26 post-infection. (F) Fluorescence intensity of live salivary gland sporozoites collected at day 26 post-infection. The dotted line represents the maximum fluorescence intensity value obtained for WT salivary gland sporozoites with the same image acquisition settings. (G) Representative immunofluorescence images of salivary gland sporozoites at day 26 post-infection. (H) RFP fluorescence intensity of live oocyst, hemolymph and salivary gland SPATR COMP^{P_{spatr}} sporozoites on day 19 post-infection. The dotted line represents the maximum fluorescence intensity value obtained for WT salivary gland sporozoites with the same image acquisition settings (F). (I) Percentage of RFP-expressing SPATR COMP^{P_{spatr}} sporozoites, using the cutoff established on panels F and H. Representative experiments are shown and mean values + standard deviation are represented. Statistical analyses were performed using the Mann-Whitney test (F) or the Kruskal-Wallis with Dunn's multiple comparisons test (E, H, I). BF: brightfield. bp: base pairs. HL: hemolymph. MG: mean grey values. MZ: merozoite. Ooc: oocyst(s). *pbcen5*: PbCEN5-core. *Peef1αa*: promoter of the *eef1αa* gene. SG: salivary gland. Spz: sporozoites. UTR: untranslated region.

We then attempted to determine if the genetic complementation of the SPATR KD parasites restored sporozoite infectivity to mice. The injection of high numbers of SPATR COMP^{P_{trap}} hemolymph sporozoites in 2 mice resulted in the detection of parasites in the blood of both animals, while none of the 5 mice injected with the same inoculum of SPATR KD became infected (Table S2). This is consistent with a partial recovery of hemolymph sporozoite infectivity, especially if we consider less than half of COMP^{P_{trap}} hemolymph parasites carry the episome (Fig. 6I). Interestingly, the percentage of RFP-positive blood stages in the populations arising from these infections, as well as infection initiated with COMP^{P_{trap}} salivary gland sporozoites (Table S2), ranged from 0-10%. Since SPATR KD hemolymph sporozoites rarely managed to infect mice (Table S2) and close to 100% of SPATR COMP^{P_{trap}} salivary gland sporozoites were determined to express RFP (Fig. 6I), it is likely that these infections were initiated with RFP-expressing sporozoites, but the plasmid was lost during schizogony, supporting our previous conclusions.

Discussion

SPATR is a conserved protein with adhesive domains expressed in all invasive stages of *Plasmodium*. Despite the upregulation of transcription in infectious sporozoites, we found that SPATR is, as previously described, essential for the asexual blood stages (12,15,19). Using a promoter swap approach to specifically downregulate *spatr* expression early during sporozoite development, we established that SPATR is required for the invasion of the mosquito salivary glands by sporozoites. Moreover, the ability of SPATR KD sporozoites collected from the hemolymph to infect the murine liver was severely hindered. These sporozoites also displayed impaired infectivity *in vitro* and motility in coverslips, as well as moderately reduced cell wounding capacity, which could explain their inability to effectively initiate liver infections. Furthermore, we observed that, unlike WT sporozoites, SPATR KD hemolymph sporozoites were incapable of homing to the liver in the first instances following blood dissemination in Kupffer cell-depleted mice, indicating parasite arrest in the sinusoids was also compromised. While the defective migratory and infective capacity of SPATR KD hemolymph sporozoites may have been a direct consequence of the downregulation of SPATR, it could have also stemmed from an increase in their permanence time in the vector circulatory system. In general, the known *Plasmodium* mutant lines likely to present a defect in salivary gland invasion also exhibit reduced infectivity and/or motility (Table S3). The fact that all these lines share analogous phenotypes is indicative of one of two options: sporozoites either rely on a diverse roster of proteins whose functions might be intricately related and simultaneously affect a multitude of biological processes, from motility to invasion of the salivary glands and infection of the host; or impediments to salivary gland invasion induce changes to hemolymph sporozoites, which result in a decrease in overall fitness and infectivity. These alterations could be intrinsic, meaning that sporozoites may need to timely cross checkpoints to retain their infective qualities, or exogenous, such as the potentially increased contact with antimicrobial peptides and other hemolymph components, although we did not detect a decrease of hemolymph sporozoite viability in a SPATR KD line. Nonetheless, the magnitude of the liver infectivity defects varies between the mutant lines (Table S3), indicating that the defective phenotypes may in fact arise from a role of the studied molecules in this process. Indeed, we are studying several *P. berghei* mutant lines whose sporozoites exhibit defective salivary gland invasion and the degree of the liver infectivity impairment using hemolymph sporozoites is dependent on the line (data not shown).

A milder defective phenotype was obtained when we infected mice with SPATR KD salivary gland sporozoites, as these parasites were capable of infecting mice, unlike their hemolymph

counterparts, albeit with a small extension of the prepatent period in comparison to the WT line (0.73 ± 0.57 days, across 7 independent experiments using either SPATR KD clone; Table S2). The minute numbers of sporozoites within the salivary glands made it technically unfeasible to reliably evaluate the motility and infectivity of SPATR KD salivary gland sporozoites *in vitro*, as well as the downregulation of *spatr* transcription in this stage. However, the *in vivo* data indicate that either small amounts of SPATR that may be produced by SPATR KD salivary gland sporozoites are sufficient to secure a certain degree of functionality, or that fully matured sporozoites acquired compensation mechanisms that allowed them to partially rescue the phenotype of SPATR KD hemolymph sporozoites, which would be suggestive of functional redundancy. Alternatively, the reduced capacity to invade the salivary glands and, as a consequence, the lower parasite density achieved by these mutant lines in the glands could result in decreased sporozoite fitness. Additionally, it was also shown that saliva components that affect sporozoite functionality are upregulated upon infection (63), which could be dependent on parasite loads. Thus, it is plausible that there is a density threshold within the salivary glands required for the maximization of sporozoite fitness and infectivity. Indeed, SPATR conditional knockout sporozoites generated using the Flp-FRT system were capable of invading the salivary glands and showed no liver stage defects (19). Nevertheless, it is important to note that these recombinase-based systems often fail to achieve full deletion of the target sequence within a population (64,65), which could be of particular significance in the context of secreted proteins. Moreover, maximum excision of the target locus is only achieved upon a temperature switch, which is usually performed once sporozoites are already present in the salivary glands, as the rise in temperature leads to the blockage of salivary gland colonization (64). Since *spatr* starts being transcribed in oocysts and is then subjected to translational repression (15), this creates a temporal window for sporozoites to produce and store mRNA that can be translated even after the recombination event. Therefore, the study of the importance of genes for the mosquito phase of the life cycle of the parasite using the Flp-FRT system may have additional limitations.

We were unable to generate knockdown lines using the strategy that resorted to the intergenic region upstream of the putative *cep76* gene, indicating that the promoter elements that drive the expression of this gene in blood stages are not present in the selected genomic region, the levels of *spatr* transcripts were insufficient to sustain parasite survival or the timing of transcription was not appropriate. On the other hand, the insertion of a second copy of *spatr* under transcriptional regulation of the *hado* promoter in a phenotypically silent locus of chromosome 6 enabled the deletion of the endogenous copy of the gene, originating SPATR KD lines. These lines showed lower levels of *spatr* mRNA throughout sporozoite development and

protein levels in hemolymph sporozoites were undetectable by western blot, unlike in SPATR DE parasites. This demonstrated that SPATR is expressed by WT sporozoites prior to salivary gland colonization and that the use of the 2.4 Kb genomic region upstream of the gene that encodes HADO in a promoter exchange system enables the downregulation of highly expressed sporozoite proteins, while sustaining expression in merozoites and, presumably, in ookinetes. The genetic complementation of a SPATR KD line with an episomally-expressed copy of *spatr* partially rescued the marked reduction of sporozoite numbers in the salivary glands, which confirmed SPATR is required for the invasion of the salivary glands. Remarkably, this infectivity defect of the SPATR KD lines in the mosquito was not accompanied by the expected accumulation of sporozoites in the hemolymph. This could come as a result of the balance between the salivary gland invasion impairment and other factors, such as a potential mild defect in sporozoite egress from oocysts, as suggested by the increased numbers of sporozoites still residing inside oocysts at late time points post-infection and the greater rise in of fully formed SPATR KD sporozoite numbers within oocysts from days 11–13 to day 15, in comparison to the control lines. The similar numbers of hemolymph sporozoites in the first few days after the egress of sporozoites from oocysts is expected to be initiated (day 10 to 11 post-infection) also point towards the attainment of a balance state early on, considering sporozoites residing in the hemolymph did not appear to accumulate in other mosquito tissues and did not exhibit increased mortality.

In conclusion, the loss of SPATR lead to defects in several biological processes necessary for infectivity of hemolymph sporozoites to both the vertebrate and the invertebrate hosts. The function of SPATR in salivary gland colonization and infectivity to the mammalian host might be more crucial in sporozoites that have not yet completed their maturation process (i.e. hemolymph sporozoites) if proteins more highly expressed by salivary gland sporozoites, namely other adhesins, are capable of fulling the same role. This was only made evident upon the early knockdown of *spatr* in the mosquito stages, which also showcases the importance of the selection of the appropriate methods to study discrete aspects of sporozoite biology.

Acknowledgements

We would like to thank Prof. Anabela Cordeiro da Silva from the IBMC/i3S for the hosting conditions and CEPIA from Institut Pasteur, Paris, for providing the mosquitoes. We would like to thank the Sanger Institute for providing the *PlasmoGEM* construct PbGEM-270402. The following reagents were obtained through BEI Resources, NIAID, NIH: (a) Plasmid pL0001, for Transfection in *P. berghei*, MRA-770, contributed by Andrew P. Waters; (b) Plasmid pL0033, for Transfection in *Plasmodium berghei*, MRA-802, contributed by Andrew P. Waters; (c) Hybridoma 3D11 Anti-*Plasmodium berghei* 44-Kilodalton Sporozoite Surface Protein (Pb44), MRA-100, contributed by Victor Nussenzweig.

This work was supported by funds from project Norte-01-0145-FEDER-000012 - Structured program on bioengineered therapies for infectious diseases and tissue regeneration, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through FEDER. This work also received funds from the Fundação para a Ciência e Tecnologia (FCT)/Ministério da Ciência, Tecnologia e Ensino Superior (MECTES) co-funded by FEDER (PTDC/SAU-PAR/31340/2017) under the Partnership agreement PT2020, through the Research Unit No. 4293. J.T. is an Investigator funded by National funds through FCT and co-funded through European Social Fund within the Human Potential Operating Programme (CEECIND/02362/2017). D.M.C., M.S. and A.R.T. are funded by FCT individual fellowships SFRH/BD/123734/2016, SFRH/BD/133485/2017 and SFRH/BD/133276/2017 respectively). We also acknowledge the support of the i3S Advanced Light Microscopy and Histology and Electron Microscopy Scientific Platforms, members of the PPBI (PPBI-POCI-01-0145-FEDER-022122).

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Supplementary information

Table S1. Oligonucleotides.

Primer	Sequence
1	5' GTGTCTATCCGTTCCCTCAAG 3'
2	5' AACCGCGGAACGCACAAATTTGTTAAAAAG 3'
3	5' TTAAATGCGTAGGCAATCTC 3'
4	5' CGAATACGCAATTTCTCAAAC 3'
5	5' TGTGTCTGCACCAACATCAGGT 3'
6	5' CGCTTACAACACTTGCAAAACTGGA 3'
7	5' CATACTAGCCATTTTATGTG 3'
8	5' AACTAATGGCATTAAATGAGATATATGA 3'
9	5' TATTTACCATTTTGAGGCCC 3'
10	5' CTTATTAACTTCTGTTCCAGATCC 3'
11	5' CCCATTGTGAACATCCTCAAC 3'
12	5' TTGGTACCTTACCTGCTTGGTTCTGG 3'
13	5' GCATCGATGGGAATTACACATTTGGTTG 3'
14	5' CGGAATTCGTTATACGAAAGATGAAG 3'
15	5' CCGGATCCTAGTAATAATTGTGATAAC 3'
16	5' CTGGTACCTATCATGAATTTCTATTCGC 3'
17	5' TCATCGATAAAATTTATGTATATGCTC 3'
18	5' CGACTAGTGTTTCAGGAACTTTTGTGTG 3'
19	5' TAGGATCCATGACAAACCGTCGAATAGTG 3'
20	5' CAACTAGTCTTTACTTGCACACACAACGGG 3'
21	5' TTGATATCCACTTTATGTGGGAAATATTG 3'
22	5' TTGGATCCTTTTTTCACTTTTATTTTCACC 3'
23	5' TTGATATCTATCCAAGTACAAGAATAAC 3'
24	5' TTGGATCCTTTTTTACATTAAAATATTAAG 3'
25	5' TGCGGCGGCCTTACAACACTTGCAAACTG 3'
26	5' CGCCATGGTTTCACACGATCGAGTTTCC 3'
27	5' AGGCCGGCCAGTATAAGTTTTTTTTTTTG 3'
28	5' GCATATGATTACATTTTTCTTGATTTATGC 3'
29	5' GACGGTTTGTCTCgTCACATTAAAATAATA 3'
30	5' CCGCGGATGACAAACCGTCGAATAGTG 3'

31	5' <u>CTGCAGT</u> AATATGGGTAGGGGAAATTG 3'
32	5' <u>CTGCAGCC</u> AGAAGTGATCGTTTAAAGCC 3'
33	5' G <u>CCCGCGG</u> CACATTAAAATAATAAAAGAAA 3'
34	5' <u>CTGCAGT</u> AATATGGGTAGGGGAAATTG 3'
35	5' <u>CCGCGG</u> ATTTCTTTTATAAGGGAAAGGG 3'
36	5' GAGGCTCATATATTAAACC 3'
37	5' CAGTAACATCTTCTTCAC 3'
38	5' TCTGCCCCGAGATACATTAGTT 3'
39	5' CAAGCATTTTCGTTTCATCATTCC 3'
40	5' CCGATGAGAATTATGAGGATT 3'
41	5' CTTGGAAGTGGAGGGATTAC 3'
42	5' GGTAGTTGGGATCAAGCC 3'
43	5' CAGTCATCTCTCCAGCAC 3'
44	5' AGTTAAGGCAACAGCAGGTGA 3'
45	5' TGCTCTTTTCGCATTGTGTTC 3'
46	5' GGAGATTGGTTTTGACGTTTATGTG 3'
47	5' AAGCATTAAATAAAGCGAATACATCCTTAC 3'
48	5' T <u>ACCATGG</u> GCCAATATAATAGAGATCATTC 3'
49	5' TT <u>CTCGAGT</u> TCACACGATCGAGTTTC 3'

Restriction sites are underlined.

Bases in italic were introduced to ensure in-frame cloning.

Lower case letters represent point mutations.

Table S2. Initiation of blood stage infection by hemolymph and salivary gland sporozoites.

Line	SG Spz		HL Spz	
	Prepatency increase (days)	% of infected mice	Prepatency increase (days)	% of infected mice
WT	n.a.	27/29 (93%)	n.a.	16/16 (100%)
DE	0.44 ± 0.10	11/12 (92%)	0.32 ± 0.34	11/13 (85%)
KD ^{V1/S16}	0.73 ± 0.57	18/21 (86%)	1 [†]	1/24 (4%) [†]
COMP ^{Ptrap}	0.34 ± 0.57	6/7 (86%)	n.a. [#]	2/3 (67%) [#]

The average prepatency delay versus mice infected with WT salivary gland (SG) or hemolymph (HL) sporozoites (Spz) in independent infections carried out with inocula. SG Spz inocula ranged from 200 to 2,500 SG sporozoites is shown.

[†]: Infections with WT and KD lines were performed with 15,000 to 60,000 HL Spz. Only one mouse injected with 20,000 presented parasitemia.

[#]: one mouse was injected with 11,000 hemolymph sporozoites (HL Spz) and did not show parasitemia and the two infected mice were injected with 60,000 HL Spz. No infections with WT were performed simultaneously.

COMP^{Ptrap}: SPATR COMP^{Ptrap}. DE: SPATR DE. n.a.: not applicable. KD^{V1/S16}: SPATR KD^{V1/S16}.

Table S3. Phenotypic classification of mutant *Plasmodium* spp. lines displaying defective salivary gland invasion.

Deleted, downregulated or mutated protein (one or several mutants)	SG Spz numbers	Accumulation in the hemolymph	<i>In vitro</i> invasion		<i>In vivo</i> infectivity		Motility		References
			HL Spz	SG Spz	HL Spz	SG Spz	HL Spz	SG Spz	
SPATR	+	No	+	N.a.	-	++	+	N.a.	This work
MAEBL (membrane associated erythrocyte binding-like protein)	+	Yes	++	N.a.	+	N.a.	+++	N.a.	(66,67)
TRAP	+	Yes	-	-	-	-/+	-	-	(48,51,59,68)
RON2 (rhoptry neck protein 2)	+	No	++	N.a.	++	N.a.	++	N.a.	(69)
RON4 (rhoptry neck protein 4)	+	No	N.a.	N.a.	N.a.	N.a.	++	N.a.	(70)
RON5 (rhoptry neck protein 5)	+	No	N.a.	N.a.	N.a.	N.a.	++	N.a.	(70)
RON11 (rhoptry neck protein 11)	+	No	-	N.a.	+	N.a.	-	N.a.	(71)
LIMP	++	No (less oocysts)	N.a.	+	N.a.	-	+	+	(72)
CRMP1 (cysteine repeat modular protein 1)	-	Yes	N.a.	N.a.	++	N.a.	N.a.	N.a.	(73)
CRMP2 (cysteine repeat modular protein 2)	-	Yes	N.a.	N.a.	+	N.a.	N.a.	N.a.	(73)
ICP (inhibitor of cysteine proteases)	+	No	-	N.a.	-	N.a.	+	N.a.	(74)
PAT (pantothenate transporter)	++	No	N.a.	N.a.	N.a.	++	-	N.a.	(75)
TREP (TRAP-related protein)	+	Yes	+	N.a.	++/+++	+/++	+	N.a.	(76–78)
Coronin	++	N.a.	N.a.	N.a.	N.a.	++	N.a.	+	(79)
α 1-tubulin	+ / ++	N.a.	N.a.	N.a.	N.a.	N.a. / ++	N.a.	++ / +++	(80)
CSP [#]	- / ++	No	N.a.	- / ++	N.a.	- / +++	N.a.	- / +++	(46,47,50,52–58)
TRP1 (thrombospondin-related protein 1)	-	No	N.a.	N.a.	N.a.	N.a.	+++	N.a.	(2)
Myosin A	+	Yes	N.a.	N.a.	N.a.	++	+	++	(81)

-: abrogation of the process; +: severe defect; ++: mild defect; +++: no defect; #: includes studies where antibodies and peptides were used to study salivary gland invasion; HL: hemolymph. N.a.: not applicable or assessed; SG: salivary gland; Spz: sporozoite.

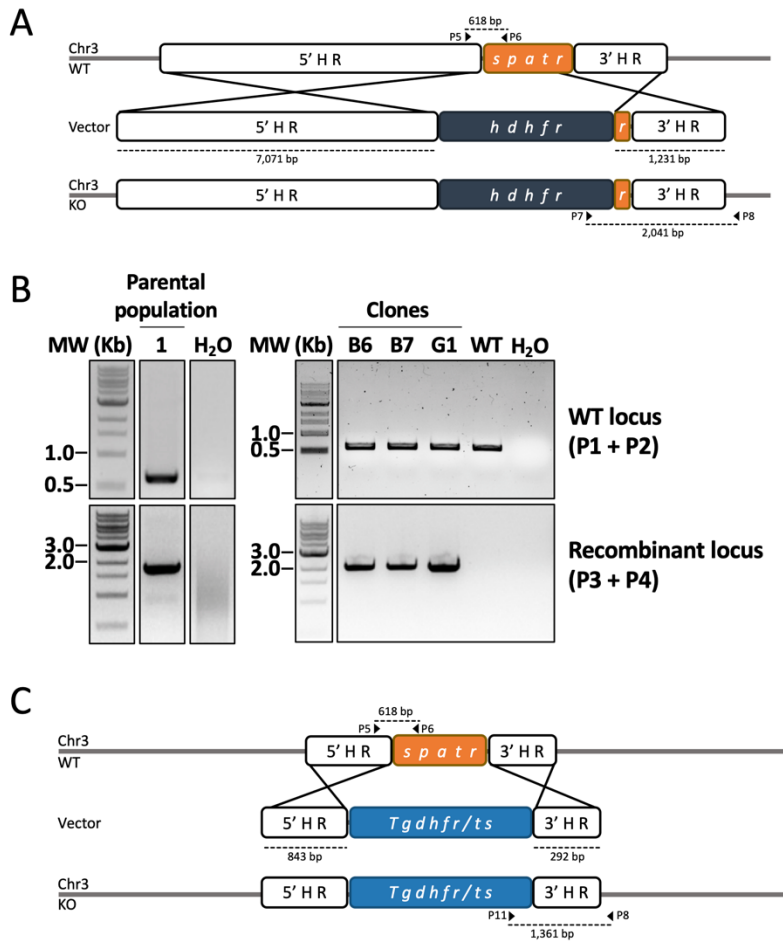


Fig S1. Attempts to knockout *spatr* by targeted gene deletion. (A) Schematic representation of the gene deletion strategy using the PbGEM-270402 vector. The sizes of genomic regions are shown next to dashed lines. Sequences are not to scale. (B) Genetic analysis of transfected populations by PCR. The primers used for the genetic analysis and the amplicon sizes are depicted in panel A. (C) Schematic representation of the alternative knockout strategy using the modified pLOO01 vector. The sizes of the homology regions, as well as the primers used for genetics analysis (data not shown) and amplicon sizes are shown. Sequences are not to scale.

bp: base pairs. Chr: chromosome. *hdhfr*: human dihydrofolate reductase. HR: homology region. Kb: kilobases. KO: knockout. MW: molecular weight. *Tgdhfr/ts*: *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase.

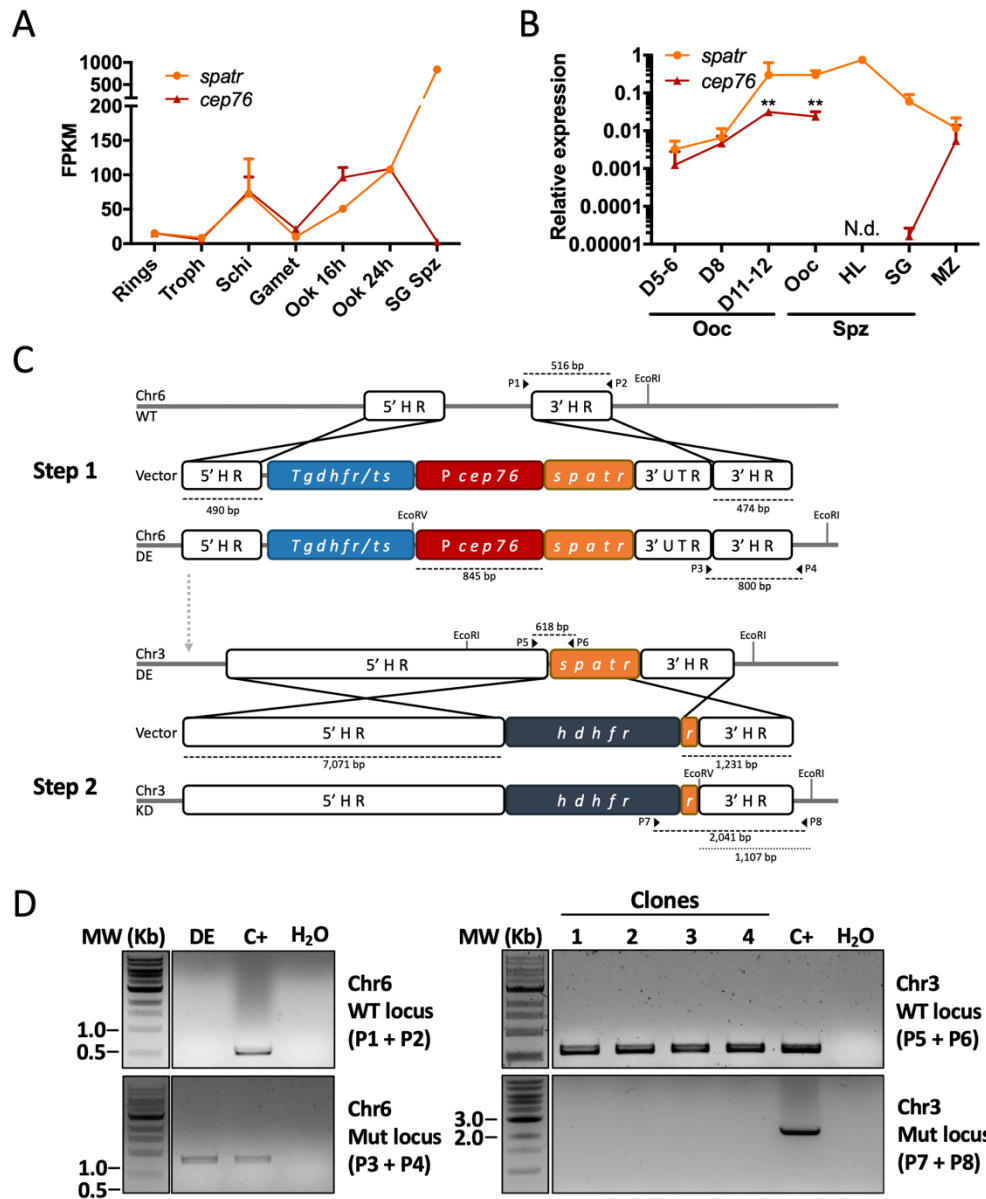


Fig S2. Promoter swap using the intergenic region upstream of the putative *cep76*. (A) RNA-seq data of the putative *cep76* gene in different stages of the *P. berghei* life cycle. Data from published datasets (20,21). (B) Expression of the putative *cep76* gene relative to *hsp70* in different stages and days post-infection, assessed by RT-qPCR using independently prepared RNA samples. Statistical analyses of differences between genes was performed using the two-way ANOVA with Sidak's multiple comparison. The *spatr* transcription data shown in Fig. 1A and 1B were plotted in panels A and B, respectively, to facilitate comparison the of the transcriptional profiles. Mean values + standard deviation are represented in both panels and statistical analysis in panel B was performed using the Kruskal-Wallis test with Dunn's multiple comparisons test. (C) Stepwise schematic representation of the promoter exchange strategy using the promoter of the putative *cep76* gene. The sizes of genomic regions are shown next to dashed line. Sequences are not to scale. (D) Genetic analysis of transfected populations by PCR. Primers used for the genotyping and amplicon sizes are depicted in panel C.

bp: base pairs. C+: positive control (WT or transfer population on the top or bottom gels, respectively). Chr: chromosome. DE: SPATR double-expressor. FPKM: fragments per kilobase of transcript per million mapped reads. Gamet: gametocytes. *hdhfr*: human dihydrofolate reductase. HL: hemolymph. HR: homology region. Kb: kilobases. Mut: mutant. MW: molecular weight. N.d: not detected. Ooc: oocysts. Ook: ookinetes. Ring: ring stages. Schi: schizonts. SG: salivary gland. Spz: sporozoites. *Tgdhfr/ts*: *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase. TP: transfer population. Troph: trophozoites. UTR: untranslated region.

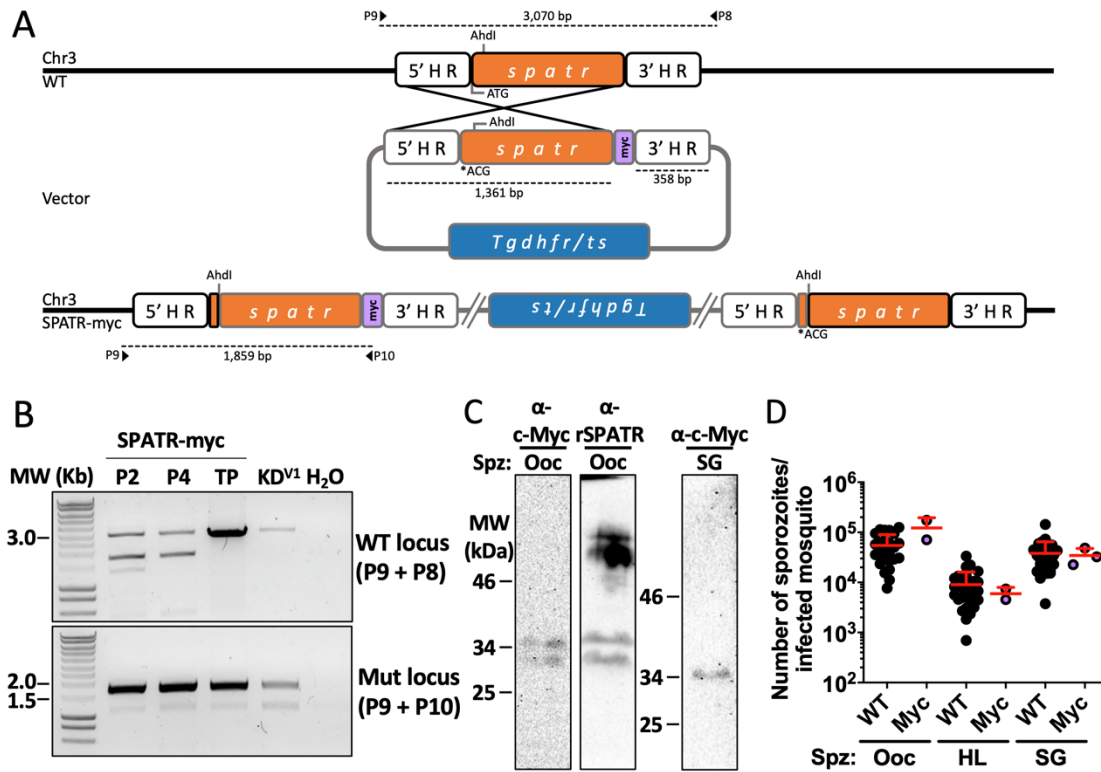


Fig S3. Generation of a line expressing a c-myc-tagged SPATR. (A) Schematic representation of the generation of the SPATR-myc line, which expressed SPATR fused to two c-myc sequences (myc) at C-terminal. The duplicated copy of the gene at 3' contains a mutated start codon (ACG) to prevent the expression of the full length and untagged SPATR. The sizes of the homology regions are shown below dashed lines, under the vector scheme. Sequences are not to scale. (B) Genetic analysis of two clonal populations by PCR. A transfer population (TP) and SPATR KD^{V1} were used as positive and negative controls, respectively. The primers used for the genetic analysis and the amplicon sizes are depicted in panel A. (C) Reactivity of α-c-Myc and α-rSPATR antibodies to extracts prepared with 4×10^5 SPATR-myc oocyst sporozoites or 1.5×10^5 SPATR-myc salivary gland sporozoites, evaluated by western blot. (D) Numbers of SPATR-myc (Myc) oocyst, hemolymph and salivary gland sporozoites per mosquito. WT values are also shown in Fig. 2E. Mean values + standard deviation are represented and statistical analysis was performed using the Mann-Whitney test. bp: base pairs. Chr3: chromosome 3. HL: hemolymph sporozoites. HR: homology region. Mut: mutant. MW: molecular weight. SPATR Myc Ooc: oocyst. SG: salivary gland. Spz: sporozoites. *Tgdhfr/ts*: *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase.

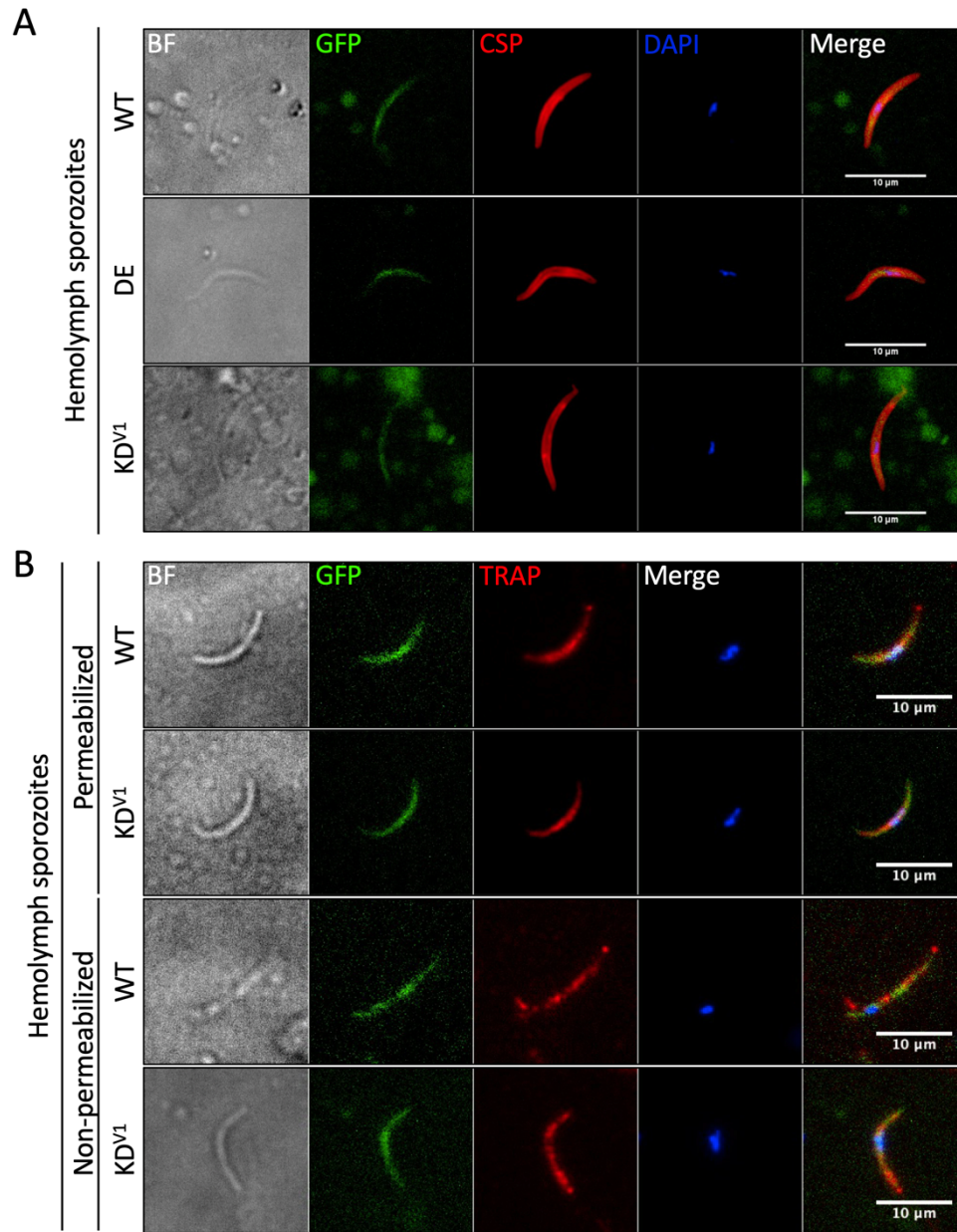


Fig S4. Morphology of KD sporozoites and expression of surface molecules. (A, B) Expression and localization of CSP (A) and TRAP (B) in hemolymph sporozoites, evaluated by fluorescence microscopy.

BF: brightfield. DE: SPATR double-expressor. KD^{V1}: SPATR knockdown V1 clone.

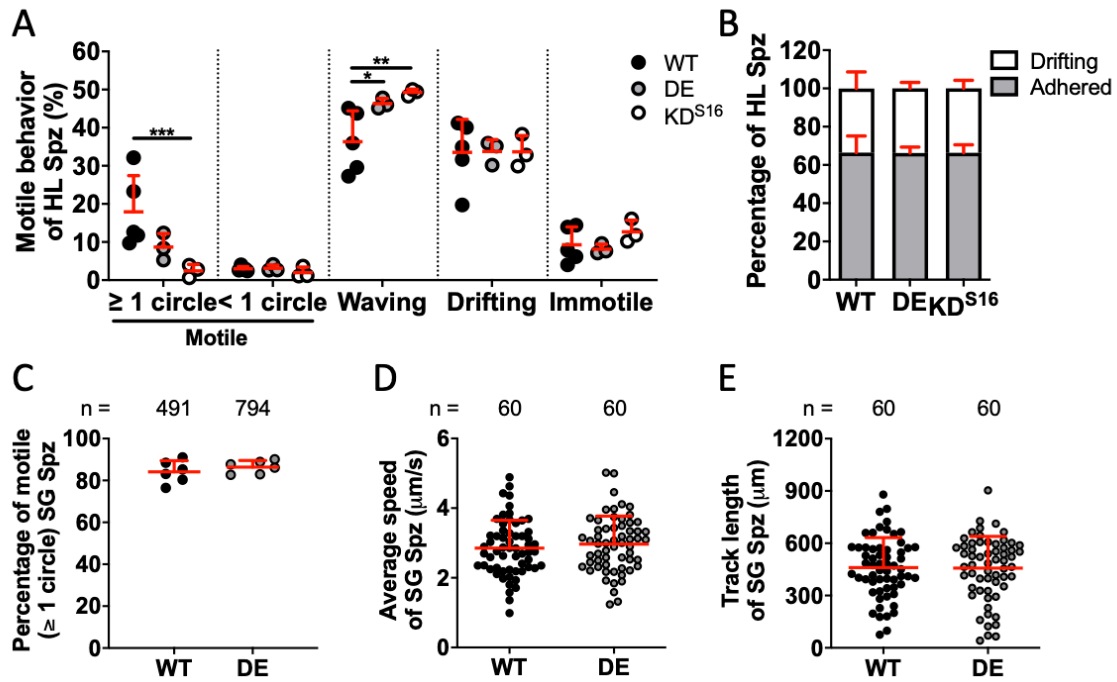


Fig S5. Motile behaviors of SPATR KD and SPATR DE sporozoites. (A) Percentage of hemolymph sporozoites exhibiting distinct motility-associated behaviors. Each symbol represents the mean value of an independent experiment and a total of at least 1223 sporozoites per line was analyzed. (B) Percentage of drifting or adhered hemolymph sporozoites. The percentage of adhered sporozoites was calculated by adding the percentages of motile, waving and immotile parasites in each experiment. Mean values + standard deviation are represented in both panels. (C–E) Percentage of motile salivary gland sporozoites (C), and their average speed (D) and track length (E). Numbers above the plots indicate the total number of sporozoites analyzed. The symbols in panel C represent the percentages obtained in different videos, rather than individual sporozoites analyzed.

Statistical analyses were performed using the Kruskal-Wallis with Dunn's multiple comparisons test.

DE: SPATR double-expressor. HL: hemolymph. KD^{S16}: SPATR knockdown S16 clone. SG: salivary gland. Spz: hemolymph sporozoites.

Chapter III

Discussion

1. Bioluminescence imaging in mice: a tool to investigate the molecular determinants of pathogen target tissue recognition

We developed a bioluminescence-based method to visualize the distribution of parasites *in vivo* shortly after blood dissemination. We showed that *Plasmodium* sporozoites collected from the salivary glands rapidly and efficiently accumulate in the liver in the first minutes after intravenous injection. Furthermore, the almost exclusive retention of parasites in this organ was specific to *Plasmodium* sporozoites, as neither merozoites nor other protozoan parasites displayed similar distributions (1). The latter included the liver-infecting *L. infantum*, thus revealing clear differences between the kinetics of parasite accumulation in the respective target organs. Additionally, the accumulation of *L. infantum* axenic amastigotes in the lungs in the first minutes post-intravenous injection and the subsequent drop in the bioluminescent signal the following day deserves further investigation. Understanding which mechanisms allow the elimination of *Leishmania* parasites from the lungs may contribute to important advances in the field.

The high numbers of sporozoites needed to study the homing of *Plasmodium* sporozoites constitutes the main limitation of this method. This factor may hinder the study of sporozoites collected from the hemolymph, as is required for mutant lines with severe defects in salivary gland colonization, for example. The bioluminescent *Plasmodium* parasites we used express the firefly luciferase under transcriptional control of the promoter of the elongation factor 1 α gene (2). Whether red-shifted firefly luciferase constructs or luciferase expression governed by stronger promoters such as that of the heat shock protein 70 (*hsp70*) gene would contribute to improved signal generation or detection and, consequently, reduce the inoculum remains to be investigated.

The main advantage of this method, however, lies on the possibility to directly evaluate the role of a particular molecule in the homing of sporozoites using genetically modified parasites and/or mice. Therefore, this method also served as the basis for the evaluation of the homing ability of *P. berghei* mutant lines, with the aim of answering one of the aforementioned interrogations: what are the molecular mechanisms that mediate sporozoite target tissue recognition?

2. TRSP and SPATR: proposed functions in sporozoites

The first surface protein whose role in sporozoite homing to the liver warranted investigation was TRSP, a protein exclusively expressed by members of the *Plasmodium* genus. The generation of a TRSP knockout in the *P. berghei* NK65 strain had previously revealed that this TSR-containing protein that possibly localizes to the rhoptries may partake in the hepatocyte invasion process, which would explain the reduced infectivity of these knockout sporozoites in a rodent model (3). However, we did not obtain similar results using the *P. berghei* ANKA strain and otherwise comparable experimental setups. In fact, we demonstrated that TRSP was not required for sporozoite infectivity irrespective of the infection route nor for the completion of the rest of the life cycle, indicating that this molecule is dispensable for the infectivity of the parasite.

Despite their complicated life cycles, *Plasmodium* parasites possess compact genomes with a particularly high proportion of genes required for normal parasite development and growth (4,5). The fact that many of these genes have proven stage-specific functions showcases the extensive optimization of genome complexity and of functional redundancy of the gene products. Therefore, TRSP is likely to fulfil a relevant function in the pre-erythrocytic phase of infection, if we consider it is one of the most abundant surface proteins of salivary gland sporozoites and is highly conserved among *Plasmodium* species (6,7). So what could its purpose be? Although the previously proposed role in host cell entry could not be confirmed using a different strain of *P. berghei*, TRSP could indeed function as an adhesive molecule in processes required for infectivity. The first hint that this might be the case is the exclusive detection of *trsp* transcripts in schizonts, ookinetes and sporozoites (8,9). Moreover, TRSP has been shown to be part of the sporozoite and merozoite proteomes (6,10–12). This suggests that TRSP could be used by the invasive stages of the parasite, including the weakly motile merozoite.

The subcellular localization of TRSP could be an indicator of a role in host cell binding or entry. As was already described, rhoptry proteins are functionally tied to the invasion process and the discharge of these secretory organelles has been shown to be induced by host cell recognition promoted by micronemal proteins, in a sequential series of steps. It is important to note, however, that the potential rhoptry localization was strictly inferred from the apical distribution of the protein, which differed from that of TRAP, a micronemal marker (13). Therefore, colocalization studies with rhoptry markers would be helpful to the discrimination of the functions TRSP performs in the sporozoite stage.

Secondly, the adhesive TSR of TRSP suggests this transmembrane protein, found on the surface of the sporozoite (6), may be involved in the establishment of interactions with other proteins, either in isolation or as a part of a complex. Some apicomplexan zoites are known to utilize alternative pathways to get access to the host cells, which also rely on several functionally redundant proteins, including molecules secreted by the rhoptries (14–17). This indicates that the plasticity of pathways associated to specific processes such as invasion constitute an important strategy for parasite survival in their natural environment, as the shrinking genomes *Plasmodium* must have kept mostly genes that are advantageous for the parasite under the right conditions.

One of the main differences between laboratory and field conditions is the antigenic diversity of the hosts. As was shown in the context of CD81 requirement for hepatocyte invasion by sporozoites, different *Plasmodium* species favor certain pathways for cell entry (15,17). It is thus possible that different *P. berghei* strains could also prioritize particular invasion pathways, which may be more or less reliant on TRSP. This would explain why the *P. berghei* NK65 strain presented incomplete invasion of a human hepatoma cell line in the absence of TRSP, while the ANKA strain did not. Humans, however, are not suitable hosts for *P. berghei* despite their ability to multiply within human hepatocytes (18). Nevertheless, this species was originally isolated from the African woodland thick-knee rat (*Grammomys surdaster*) but can also infect several additional rodents, including rats (*Rattus* spp.), tree rats (*Thamnomys surdaster*), mice, hamsters and gerbils, at least experimentally (19–21). Therefore, the redundancy of the invasion pathways could be a tactic *P. berghei* employs to adapt to different hosts. Other processes such as gliding motility and cell traversal are less likely to depend on specific interactions between host and parasite molecules and, thus, do not need to rely on such adaptative measures. In contrast, *T. gondii* possesses a much lower percentage of genes essential for parasite survival, suggesting a much stronger role for functional redundancy, which could be necessary to support the impressive ability of this parasite to multiply in a wide range of tissues in virtually all warm-blooded animals (22,23).

The same concept could be applied to human-infecting *Plasmodium* species, as, for example, *P. knowlesi* and *P. malariae* are known to commonly infect nonhuman primates in the wild and gorillas have also been shown to be rare natural hosts of *P. falciparum* (24–26). Moreover, the diversity of environments and host cells the parasite might encounter is not restricted to the comparison between different host species. Indeed, interindividual variability among the same host species is often mitigated by the use of genetically defined animal models in

laboratory settings but potentially affects all *Plasmodium* species, even those with stricter host preferences, such as *P. falciparum*. Genetic polymorphism or even the absence of host ligands in certain individuals within the same population may have driven the acquisition of separate molecular pathways associated with vital biological processes, such as host cell recognition and entry. Additionally, structurally related molecules may be able to functionally compensate for the loss of a particular parasite-host interaction. In this sense, adhesins exposed on the surface of the parasite, including other TSR-bearing molecules, could take over the role of TRSP in its absence. This functional overlap has already been proposed for the TRAP-like protein (TLP), which, as its name indicates, shares many structural similarities with TRAP, including a TSR domain (27). In fact, the *tlp* gene is also expressed in all invasive stages of the parasite (8,9,27) and could partake in the functional complementation of TRSP knockout sporozoites the *P. berghei* ANKA strain. However, TLP has been suggested to be required for cell traversal and force-generation during sporozoite motility, which means it is unlikely to possess a specific role in productive invasion (28–30).

Therefore, the elucidation of the real part played by TRSP during the lifetime of the sporozoite could potentially be achieved by testing a wide range of experimental conditions, via the identification of potential binding partners, or through the sequential deletion of other surface molecules, to disclose the identity of the molecules responsible for the suggested functional redundancy. Alternatively, changes in the sporozoite transcriptome and/or proteome in the absence of TRSP may also reveal potential compensatory mechanisms that may have been set in motion in these mutant parasites.

Interestingly, another protein that could account for the lack of phenotype of the TRSP knockout line in the *P. berghei* ANKA strain is SPATR. These two proteins share a few features, namely the predicted export to the surface of the sporozoite, the presence of a TSR, and the suggested rhoptry localization (6,13,31–33). While the rhoptry localization of SPATR was only shown in merozoites (31,33), it is intuitive to assume that the protein is trafficked to the same organelles in both stages and that its localization on the surface of salivary gland sporozoites results from the secretion of the contents of the rhoptries. Moreover, the *spatr* gene is also expressed in all invasive stages of the parasite and has even been shown to be important for the initiation of the blood stage (4,8,9,33). Thus, we can speculate that its function is related to host cell recognition/entry or even the formation/maintenance of the parasitophorous vacuole, which are typical functions of rhoptry proteins. Consequently, the fact that the function of SPATR was only initially apparent in the blood stage could be due to the higher abundance of

adhesins or invasins capable of performing the same tasks as SPATR in sporozoites. Hence, it would be interesting to determine if the concomitant depletion of both SPATR or TRSP would result in synergistic effects on parasite fitness or infectivity.

We also uncovered the requirement for SPATR during the residency of sporozoites in their insect host. The downregulation of *spatr* transcription during sporozoite formation and maturation resulted in a drastic reduction of the number of sporozoites detected inside the salivary glands of the mosquito. These results could indicate that the function of SPATR might not be limited or even related to host cell invasion, since while sporozoites enter acinar cells, these only constitute a gateway to the secretory cavity. However, this raises new questions: could rhoptry proteins, which are traditionally associated with the process of host cell invasion, also be necessary for the colonization of the salivary glands? Could the molecular mechanisms used by sporozoites to transiently infiltrate the salivary gland cells more closely resemble those employed during the invasion of mammalian cells, in comparison to, for instance, cell traversal?

In fact, SPATR is not the only protein suggested to localize to the rhoptries to be required for the efficient invasion of the salivary glands. RON2, RON4, RON5 and RON11 have all been implicated in this process (34–36). The knockdown of these proteins in sporozoites resulted in overall phenotypes comparable to that obtained with the equally stage-specific knockdown of SPATR (see Table S3 in chapter II, section 2.3), which is suggestive of similar functions being performed by these molecules. As was already discussed, RON2 has a well-defined role in erythrocyte invasion and has been shown to be discharged from the rhoptries during invasion of CD81-expressing hepatocytes (37,38). Moreover, RON4 is also important for hepatocyte invasion, while AMA1, the binding partner of RON2 in merozoites, is not (39). Despite the fact that RON2, RON4 and RON5 appear to form a complex in both sporozoites and merozoites (36), the requirement for each individual component and the identity of potential binding partners might vary depending on the developmental stage of the parasite. Moreover, there is no clear evidence that sporozoites enter the salivary gland cells via a tight junction. Therefore, while these knockdown lines showed limited adhesion *in vitro*, suggesting that this RON complex allows the attachment to the basal lamina of the salivary glands (34–36), it is unclear what the exact function of these proteins during the invasion of the salivary glands is and what the potential contribution of SPATR might be. Since SPATR does not possess any predicted membrane-anchoring domains, it must be exported to the surface as a complex or secreted onto

the extracellular milieu and then associate to surface proteins. If this putative complex includes the mentioned RONs or any other protein remains to be determined.

The defective attachment of sporozoites could also explain the motility and hepatocyte invasion defects ascribed to the RON mutant lines. However, SPATR may have a distinct function since we did not find evidences of such impairments in our SPATR KD lines, despite comparable reductions in infectivity and migratory capacity of hemolymph sporozoites. The evaluation of the attachment of SPATR KD sporozoites to host cells or different substrates could help us understand if SPATR is indeed required for the initial binding. Nonetheless, a direct role in hepatocyte invasion would be consistent with the reduced infectivity of SPATR KD hemolymph sporozoites and the hypothesis that SPATR could have a general part to play in the invasion process. This role would most likely be dispensable for *P. berghei* salivary gland sporozoites as the conditional knockout of the *spatr* gene in the final stage of the sporozoite maturation program did not result in any infectivity impairments during the pre-erythrocytic phase (33). If this theory is correct, it would also mean that hemolymph sporozoites lack the proteins whose expression is likely upregulated in fully mature sporozoites and that compensate for the loss of SPATR during the invasion process. Therefore, parasites collected from the hemolymph could be perceived as simplified or incomplete versions of the sporozoite and may, therefore, allow for a better discrimination of the importance of proteins involved in redundant pathways related to sporozoite infectivity.

Adding to the uncertainty surrounding the role of SPATR during the pre-erythrocytic phase, there are few data regarding what molecules SPATR may bind on the salivary glands or on the surface of the host cells. An interaction between SPATR and CD163 was recently identified in an *in vitro* screen for interactions between ectodomains of *P. falciparum* sporozoite and human hepatocyte surface proteins (40). However, this hemoglobin-haptoglobin complex scavenger receptor has been shown to be expressed mostly by macrophages, including Kupffer cells, so more studies are necessary to validate its biological significance in the context of hepatocyte invasion (41,42). Nonetheless, it was reported that a recombinant *P. falciparum* SPATR bound to hepatocytes and antibodies targeting this protein inhibited invasion of hepatocytes by sporozoites (31). Furthermore, the *T. gondii* SPATR has also been implicated in the invasion process. However, the defective infectivity of the *T. gondii* SPATR knockout tachyzoites was not reverted upon complementation with the *P. falciparum* SPATR, perhaps due to the failure to exit the secretory organelles or association with membrane components (43). This could be related to the potentially different subcellular organization of SPATR in the two Apicomplexa

species, as this protein is stored in the micronemes of *T. gondii* parasites (43). Assuming SPATR does in fact localize to the rhoptries in sporozoites, these data could suggest that SPATR function and trafficking might have suffered alterations through speciation that lead to the adaptation of the parasites to the distinct environments and requirements for invasion of the different apicomplexan species. For example, whereas *Plasmodium* parasites invade specific cells at different points of its life cycle, the more promiscuous *T. gondii* parasites are capable of infecting any nucleated cells belonging to a multitude of host species. Moreover, SPATR orthologues are also present in other Apicomplexan organisms, most notably in several coccidian genera besides *Toxoplasma*, which include *Eimeria* and *Neospora* (31,33). The widespread distribution of SPATR among Apicomplexa is another factor that alludes to an important yet general role of this protein in such different life cycles. Unlike the homing to the liver, which is genus-specific, mandatory phases of intracellular development is something all these protozoans have in common.

It is also difficult to justify the involvement of rhoptry proteins in motility due to the lack of evidence of rhoptry discharge during sporozoite locomotion. Yet, something most mutant lines with compromised salivary gland invasion share is, indeed, reduced motility *in vitro* (see Table S3 in chapter II, section 2.3). This is especially intriguing considering the diversity of the proteins responsible for these defects in salivary gland colonization. The list includes molecules containing a TSR (TRAP, TREP, TRP1 and CSP) or other adhesive domains (MAEBL, CRMP1 and CRMP2), enzymes potentially involved in the processing or trafficking of some of these adhesive molecules (ICP, PAT), proteins necessary for the maintenance of the cytoskeleton or actin-myosin motor of the sporozoite (α 1-tubulin or myosin A and coronin, respectively), and the RONS mentioned previously. Therefore, it is plausible that the occupation of the mosquito salivary glands by sporozoites involves the orchestration of linked processes involving a wide array of functionally distinct molecules.

Alternatively, the defects displayed by some of these lines could be a downstream effect of the disruption of a single process. For instance, motility is required for parasite migration through different tissues and for the crossing of biological barriers, so impeding this process could affect many steps of the life cycle. However, evidence linking motility and salivary gland invasion is lacking. In fact, the ability of sporozoites that express versions of TRAP containing altered I domains to successfully invade the salivary glands, despite their mildly to severely impaired gliding motility *in vitro* in 2D systems, indicates that motility is not required for this step of the sporozoite's journey in the mosquito (44,45). On the other hand, problems in adhesion could

affect all of the mentioned biological processes. Although we did not obtain any evidence that SPATR KD sporozoites do not attach properly to the a coverslip *in vitro*, it is possible that SPATR depletion could have a bigger impact on attachment to substracts *in vivo*, particularly if these putative SPATR-mediated interactions are restricted to certain tissues.

In a similar fashion, the high degree of commonality in the phenotypes of hemolymph sporozoites of these mutant lines could be caused by the blockage of salivary gland invasion. Recent single-cell transcriptomics studies revealed extensive heterogeneity among sporozoites in all stages of maturation, with substantial overlap of the respective expression profiles (46–48). It is not known if the transcriptional changes that occur during sporozoite maturation are a consequence or the cause of the migration of sporozoites through the mosquito. However, these transcriptional changes are likely tied to the increase in infectivity that accompanies the maturation process (49,50). Therefore, the heterogeneity of the different sporozoite populations may explain why different proportions of parasites collected from oocysts and the hemolymph can infect the vertebrate host. If the normal progression of sporozoite through the mosquito anatomy is required for the acquisition of infectivity through undetermined mechanisms, the forced stop in the hemolymph could alter the capacity of sporozoites to migrate and infect the mammalian host. In experiments using the SPATR KD lines, we did not observe increased hemolymph sporozoite death in the mutant line, indicating that immune responses from the anopheline host are unlikely to be responsible for these changes, although it is still possible that host-induced alterations to sporozoite infectivity may not be reflected by cell viability. We did not evaluate the contribution of phagocytosis to the elimination of sporozoites from the hemolymph either, but this should have little impact on the infectivity of free sporozoites.

It is also unknown how long sporozoites can remain in the hemolymph after they egress from the oocyst before entering the salivary glands. Previous studies demonstrated that purified oocyst sporozoites can occupy the salivary glands of uninfected mosquitoes when injected into the hemolymph, unlike salivary gland sporozoites, which indicates that sporozoites suffer irreversible changes to their ability to invade the glands of the mosquito (50). Moreover, it was shown that oocyst sporozoites were rapidly eliminated from the hemolymph by mechanisms that involve but are not restricted to phagocytosis by patrolling hemocytes (51–55). However, the handling and purification process of the sporozoites used in these experiments could have affected the survivability of the parasites. Additionally, the sporozoites were injected into naïve mosquitoes, which disregards potential alterations to the host induced by infection that might

make it more permissive to sporozoite survival in the hemolymph. Finally, while these studies demonstrated that a fraction of the sporozoites collected from oocysts can enter the salivary glands, these parasites might behave differently from bonafide hemolymph sporozoites that matured and egressed naturally from the oocysts. Therefore, the potentially increased permanence time in the hemolymph, which is supported by the accumulation of sporozoites in the hemolymph in some mutant lines, could by itself lead to developmental and/or infectivity impairments.

The normal infectivity of the SPATR conditional knockout salivary gland sporozoites reported by Gupta *et al.* (33) may suggest that the abrogation of the homing capacity of hemolymph sporozoites in the SPATR KD line is indeed a nonspecific defect. However, several other explanations can be proposed. The simplest explanation is that sporozoites acquire functional redundancy once they reach the salivary glands, which is in line with the only marginally reduced infectivity of SPATR KD salivary gland sporozoites. The hypothetical promoter leakiness in salivary gland sporozoites is possible but not supported by the transcriptional profile of the *hado* gene and the expected transcriptional inertness of the locus in which the gene was inserted. On the other hand, the knockout of the *spatr* gene driven by the recombinase system used in the referred study should have mostly occurred after SPATR transcription was initiated, which would explain why no defect in salivary gland invasion impairment was detected by the authors. Moreover, if in the majority of the parasites the gene was excised after they invaded the salivary glands due to the timing of the temperature switch and the increased activity of the enzyme at higher temperature, sporozoites may have had the chance to produce and store *spatr* transcripts. Sporozoites could then use this RNA to synthesize the protein only after transmission, explaining why the protein was not detected in salivary gland sporozoites and these parasites did not display any defect in the pre-erythrocytic phase. The lack of SPATR would then only impact the ability of hepatic merozoites to invade red blood cells. Therefore, it would be important to further investigate both SPATR-depleted lines in order to ascertain if SPATR is in fact involved in sporozoite arrest in the sinusoids and overall infectivity.

Testing the homing of more lines exhibiting hampered salivary gland colonization to the liver would be equally informative and could confirm if our results did, in fact, reveal a role for SPATR in the arrest of sporozoites in the liver sinusoids or if this phenotype was simply a downstream manifestation of other alterations. Indeed, all lines from this list we have tested so far, including multiple TRAP mutants and a MAEBL knockout, showed curtailed accumulation of hemolymph sporozoites in the liver in the absence of Kupffer cells (Sá *et al.*, in preparation).

Provided that this defect is also generalized, it would be interesting to determine if these phenotypes are accompanied by a signature transcriptional profile. The comparison to circulating parasites with unrestricted access to the salivary glands could help us understand if, for example, sporozoite maturation is stunted, if the parasites are degenerating, or if they attained a dormant/exhausted state. Similarly, we could also block salivary gland invasion by downregulating the expression of mosquito molecules potentially required for salivary gland invasion (51–55) and evaluate the infective and migratory phenotypes of hemolymph sporozoites collected from these mosquitoes. These data could provide invaluable information regarding the mechanisms that control both salivary gland invasion and the homing of sporozoites to the liver.

3. What mechanisms drive the arrest of sporozoites in the liver sinusoids?

The study of TRSP and SPATR was prioritized since, in addition to fitting the remaining core selection criteria, at the time these studies were initiated there was little information regarding the function of SPATR in *Plasmodium* parasites or if the defect in the liver burden of TRSP knockout sporozoites previously reported could be, at least in part, due to an incomplete arrest in the liver sinusoids. However, the list of molecules that we obtained using the candidate selection procedure previously described included several additional proteins, which were then excluded in a case-by-case basis.

Indeed, three 6-cys proteins also met some of the criteria: P36, P52 and P38. While the participation of P36 and P52 in hepatocyte invasion and parasitophorous vacuole formation/maintenance was already mentioned, P38 was proven to form complexes on the surface of merozoites but was dispensable for both the blood stage and sporozoite production (56–59). Although the involvement of these proteins in the stop of sporozoites in the hepatic microvasculature was not ruled out, these candidates were left out of this thesis. Again, the fact that other functions have been assigned to these proteins decrease the chance that they are involved in the homing of sporozoites to the liver. The GPI-anchored micronemal antigen (GAMA) was in a similar position, but this protein did not contain any predicted adhesive domains and the deletion of the *gama* gene also resulted in a severe reduction of salivary gland sporozoite numbers due to its involvement in ookinete infectivity and sporozoite egress from the oocyst (60). Thus, GAMA was not included in this work either. Nonetheless, the homing capacity of sporozoites following the depletion of such molecules could still be quantified if we

consider the likelihood of this phenomenon being dependent on a series of tightly webbed processes and, thus, could involve proteins with different functions.

Improvements to the candidate selection criteria could also be made by taking into account transcriptomics data. Reports of the recently described translation repression in several stages of sporozoite maturation indicate that proteins for critical processes for the early steps of the pre-erythrocytic phase might be synthesized after the parasites are transmitted to the vertebrate host (11,44). Therefore, protein and transcript levels do not always correlate and including genes whose transcription is upregulated in salivary gland sporozoites could expand the list of candidates. However, whereas sporozoites transmitted through the natural route may take their transient residence in the skin as a chance to produce the molecular repertoire they require for the successful migration towards the liver, the intravenous injection of the parasites in the homing assay greatly reduces the time window for protein synthesis. This means sporozoites would have to produce these proteins in just a few minutes while in the bloodstream. Alternatively, restricting the selection to proteins expressed by mammal-infecting species could help narrow down the list to more relevant candidates.

So the main question remains: how do sporozoites exclusively home to the liver? A hypothesis that was previously alluded to but not addressed in this work is the structural modification of CSP, which could allow the sporozoite to modulate its adhesive properties as it travels through the mammalian host. Recombinant CSP injected intravenously in mice homes to the space of Disse and is also captured by Kupffer cells (61). The heterologous expression of CSP by cells from other organisms, including circulating parasites such as *Trypanosoma brucei* or immortalized cell lines, or the coupling of this protein to beads could help clarify if CSP can promote the accumulation of larger structures in the liver. Indeed, such approaches have been pursued but proved to be technically challenging and remain, for now, inconclusive (unpublished data from the Tavares team). A simpler strategy could be to evaluate the ability of noncytotoxic antibodies or, preferably, antibody fragments or nanobodies targeting different regions of CSP to block the retention of sporozoites in the liver using the homing assay described herein. In fact, an analogous strategy could also be employed to identify other surface molecules involved in this process.

Lastly, since the conditional depletion of CSP from the surface of the sporozoite is most likely unfeasible, important data could be gleaned by making use of both the homing assay and parasite lines expressing mutant versions of CSP lacking selected domains. Indeed, mutant sporozoites lacking the N-terminus and RI of CSP produced increased liver burdens in

comparison to control parasites when injected intravenously, which was attributed to an enhanced invasion capacity linked to the exposure of the TSR (62). It is also possible that these mutants adhered better to the hepatic sinusoids, although we demonstrated that most salivary gland sporozoites were already retained in the liver within 7 to 12 minutes post-infection, so the arrest in the sinusoids does not appear to be a limiting step for liver colonization (1). Conversely, the deletion of RI, which hampered the cleavage of CSP, caused an increase in the prepatent period (62). Therefore, testing luciferase-expressing versions of these lines could help us understand the role of CSP in sporozoite homing to the liver. If confirmed, this would further bolster the status of CSP as the jack-of-all-trades of the sporozoite.

Another previously identified candidate is TRAP, which also emerged from the candidate selection screen. The role of this protein and motility in the processes that occur in the liver vasculature is under investigation in a separate study and was, therefore, not addressed herein. However, the presence of TRAP on the sporozoite surface proved to be essential for the arrest of the parasite in the sinusoids and the deletion of the cytosolic domain that links host ligands that bind to the ectodomain of TRAP to the actomyosin motor phenocopied the knockout line (Sá *et al.*, in preparation). Moreover, the change of the charge of the I domain, which did not impact salivary gland invasion but greatly reduced sporozoite motility *in vitro* (63), also decreased the accumulation of salivary gland sporozoites in the liver (Sá *et al.*, in preparation). These results suggest motility could be a fundamental component of sporozoite retention in the liver. Indeed, although endothelial cells have been shown to be unfavorable substrates for sporozoite locomotion (64), sporozoite gliding motility inside the liver sinusoids was previously reported (65,66). The contribution of motility could explain why the capacity to home to the liver increases with sporozoite maturation, since the migratory capacity of sporozoites also increases during the maturation process. It could equally explain the circulatory distribution of SPATR KD hemolymph sporozoites and wildtype parasites collected from oocysts when injected intravenously (Sá *et al.*, in preparation). Nevertheless, the importance of motility in this critical step of the life cycle still awaits further clarification.

The tensile forces generated by the interaction with the substrate due to the anchoring to the actomyosin motor through the CTD of TRAP may also be required for the strengthening of the adhesion itself, meaning TRAP could be important for attachment in addition to or instead of motility. The substitution of the TRAP I domain by the corresponding region of the *T. gondii* MIC2 did not alter the capacity of salivary gland sporozoites to reach and infect the liver, indicating that this domain is not the source of the *Plasmodium*-specific accumulation of

parasites in this organ (63). Nonetheless, TRAP also contains a TSR that has been shown to be important for the interaction with hepatocytes *in vitro*, potentially via a GAG-dependent manner. However, the mutation of conserved residues of the TSR did not affect sporozoite infectivity by intravenous injection in mice, despite having an additive or synergistic effect in infectivity in combination with modifications made to the I domain (67). This indicates that the TSR is not single-handedly responsible for the initial recognition of HSPGs in the sinusoids, but might rather be involved in the invasion process. On the other hand, a recombinant TRAP I domain was shown to bind to heparin independently of the presence of divalent cations, which is indicative of lack of involvement of the MIDAS (68). Yet, it is improbable that the I domain of MIC2 also possesses this MIDAS-independent ability to specifically interact with the liver HSPGs, since *T. gondii* tachyzoites can also disseminate extracellularly via the bloodstream but do not home to the liver (69). TRAP could then mediate the strengthening of adhesion and/or engagement of motility through less tissue-specific interactions through the I domain, following the initial attachment to the blood vessels.

The TRAP of *P. falciparum* was shown to also interact with integrins containing an α V chain through the I domain and a region containing the RGD motif, although this interaction was not conserved among *Plasmodium* species (70). However, it is possible that TRAP binds to other integrins exposed to the lumen of the hepatic sinusoids. Interestingly, leukocytes also require the binding to integrins to consolidate their attachment to the endothelium, in a process called rolling adhesion. The first bonds involving selectins are formed and broken as the leukocyte rolls along the microvascular endothelium and slows down. The cell then stops in a post-adhesion step by integrin-dependent adhesion strengthening (71,72). A similar process is thought to cause the PfEMP1-mediated cytoadherence of *P. falciparum*-infected erythrocytes. In both cases there is the formation catch-slip bonds, in which the strength and lifetime of the bond initially increases with shear stress before decreasing at higher forces. These forces are created by the blood flow and have been shown to enhance the interactions mediated by both selectins and PfEMP1 (71).

We could then speculate that *Plasmodium* sporozoites use similar mechanisms to stop in the hepatic microvasculature. Indeed, TRAP has been shown to adopt a different conformation following the binding to an immobilized ligand through the MIDAS in the I domain. The tensile forces exerted due to the anchoring of TRAP to the actomyosin apparatus elongate the ectodomain and an extended open conformation, with putative high-affinity, is favored (73). This is consistent with the catch bonding that also regulates the adhesion of leukocytes and

infected red blood cells to the microvasculature. Without the cytosolic domain of TRAP, these forces may not be generated, so sporozoites can never fully adhere to the sinusoids. Then, the blood flow that in normal conditions enhances adhesion – initially by reorienting the parasite and increasing contact points with the substrate (29) and then by potentially inducing the correct conformation of TRAP for stronger binding – washes away the parasites. This could also explain why the deletion of the CTD abrogated the ability of parasites carried by the hemolymph to invade the salivary glands of the mosquito, although the abrogation of the migratory properties of sporozoites could by itself be sufficient to prevent sporozoite accumulation in the secretory cavities (74). Therefore, while TRAP does not appear to be required for adhesion *in vitro* (67) and, as was described, might not be responsible for the first interactions that take place in the liver microvasculature, it may be important for subsequent binding. In fact, the initial binding to the substrate *in vitro* is independent of actin dynamics (29). Due to the close relationship between TRAP and the actomyosin motor, this may also mean that TRAP is not involved in the initial interactions with the substrate. Thus, the CTD and I domain of TRAP could be required for both motility and strengthening of adhesion to blood vessels in the liver.

In conclusion, it is likely that the arrest of *Plasmodium* sporozoites in the hepatic sinusoids is a multifactorial process that depends on biophysical characteristics of both the liver sinusoids and the parasite. Instead of being mediated by a single dedicated sporozoite-host protein pair, this phenomenon may be a consequence of the combination of interactions between several surface molecules, potentially in a sequential manner, the hydrodynamic properties of blood flow, blood vessel composition and structure, the shape and motility of the parasite, and various other factors. Nonetheless, even though uncoupling these variables might prove to be challenging, the identification of the parasitic and host components of the system that allows sporozoites to specifically halt their circulatory dissemination in the liver would still be an invaluable contribution to our knowledge of *Plasmodium* biology and, therefore, aid us in the design of novel strategies for the control of malaria. Moreover, the tools and data generated in this thesis enable us to keep asking questions and perhaps find the answers in the future.

4. References

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