

Looking under the skin: the first steps in malarial infection and immunity

Robert Ménard¹, Joana Tavares¹, Ian Cockburn², Miles Markus³, Fidel Zavala⁴ and Rogerio Amino¹

Abstract | Malaria, which is caused by *Plasmodium* spp., starts with an asymptomatic phase, during which sporozoites, the parasite form that is injected into the skin by a mosquito, develop into merozoites, the form that infects erythrocytes. This pre-erythrocytic phase is still the most enigmatic in the parasite life cycle, but has long been recognized as an attractive vaccination target. In this Review, we present what has been learned in recent years about the natural history of the pre-erythrocytic stages, mainly using intravital imaging in rodents. We also consider how this new knowledge is in turn changing our understanding of the immune response mounted by the host against the pre-erythrocytic forms.

Sterilizing immunity
Immunity resulting in parasite clearance from the host.

¹Institut Pasteur, Unité de Biologie et Génétique du Paludisme, 28 Rue du Dr Roux, 75724 Paris Cedex 15, France.

²Department of Pathogens and Immunity, John Curtin School of Medical Research, Australian National University, Canberra, ACT 0200, Australia.

³School of Animal, Plant and Environmental Sciences, Faculty of Science, and Wits Research Institute for Malaria, Faculty of Health Sciences, University of the Witwatersrand, Private Bag 3, Wits, Johannesburg, 2050, South Africa.

⁴Johns Hopkins Malaria Research Institute, Bloomberg School of Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, Maryland 21210, USA.

Correspondence to R.M.
e-mail:

robert.menard@pasteur.fr
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Malaria is the most deadly parasitic infection of humans. Although economic development and the implementation of control measures during the twentieth century have eliminated malaria from many areas of the world¹, the disease is still rampant in the tropics and in the poorest regions of the globe, affecting 3 billion people and killing up to 1 million annually². Sub-Saharan Africa pays the heaviest toll, mainly because of the efficiency of *Anopheles gambiae* mosquitoes (which live in this region) as hosts of *Plasmodium falciparum*, the *Plasmodium* species that is most lethal to humans, delivering up to 120 infective bites per person per year³.

The symptoms of malaria are caused by cycles of parasite multiplication inside host erythrocytes, and various complications, including cerebral malaria, result from cytoadherence of infected erythrocytes to endothelia. However, infection with *Plasmodium* spp. starts when parasites are injected into the skin by a mosquito (FIG. 1). The first, pre-erythrocytic (PE) phase of infection is clinically silent. During this phase, the few parasites that are inoculated into the skin by the mosquito, called sporozoites, reach the liver. They invade and multiply inside hepatocytes into new parasite forms, termed merozoites, which invade erythrocytes. This preclinical parasite metamorphosis in the liver lasts ~7–10 days in humans and ~2 days in rodents.

The PE stages (namely, the sporozoite and ensuing liver stages) are present in very small numbers in the host. Therefore, they constitute a transmission bottleneck and are ideal vaccination targets, offering the possibility of preventing progression of the parasite life cycle before clinical illness occurs. PE forms have been the

subject of intensive immunological research ever since the first demonstrations, in animal models and humans, that injection of attenuated parasites which do not cause blood infection confers protection against sporozoite challenge^{4–6}. Today, this vaccination method is still the most efficient at offering sterilizing immunity against *Plasmodium* spp. infection.

This Review gives a perspective on our understanding of the PE phase of natural infection in rodents and of host defence against the PE stages in both rodents and humans. We begin by describing recent insights into PE infection. Many approaches have been used to better define sporozoite fate in the host, including powerful intravital imaging techniques that have been adapted for use in laboratory rodents (BOX 1). Although numerous molecular insights into the basic biology of the sporozoite and liver stages have also been gained, we restrict our coverage here to a few details relevant to our specific focus on infection at the organismal level. We then deal with the immunobiology of the PE stages, with emphasis on the immune response induced by live attenuated parasites (LAP). Most notable in recent years has been the increasing recognition of the importance of the initial skin step, which affects both infection by, and immunity to, PE parasites.

An historical perspective on PE stages

Defining the PE phase of malaria has been a long and winding road. After the discoveries of *Plasmodium* sp. parasites in the blood of a patient in 1880 by Laveran⁷ and of the role of mosquitoes in *Plasmodium* spp. transmission in the late 1890s by Ross⁸ and Grassi⁹, studies

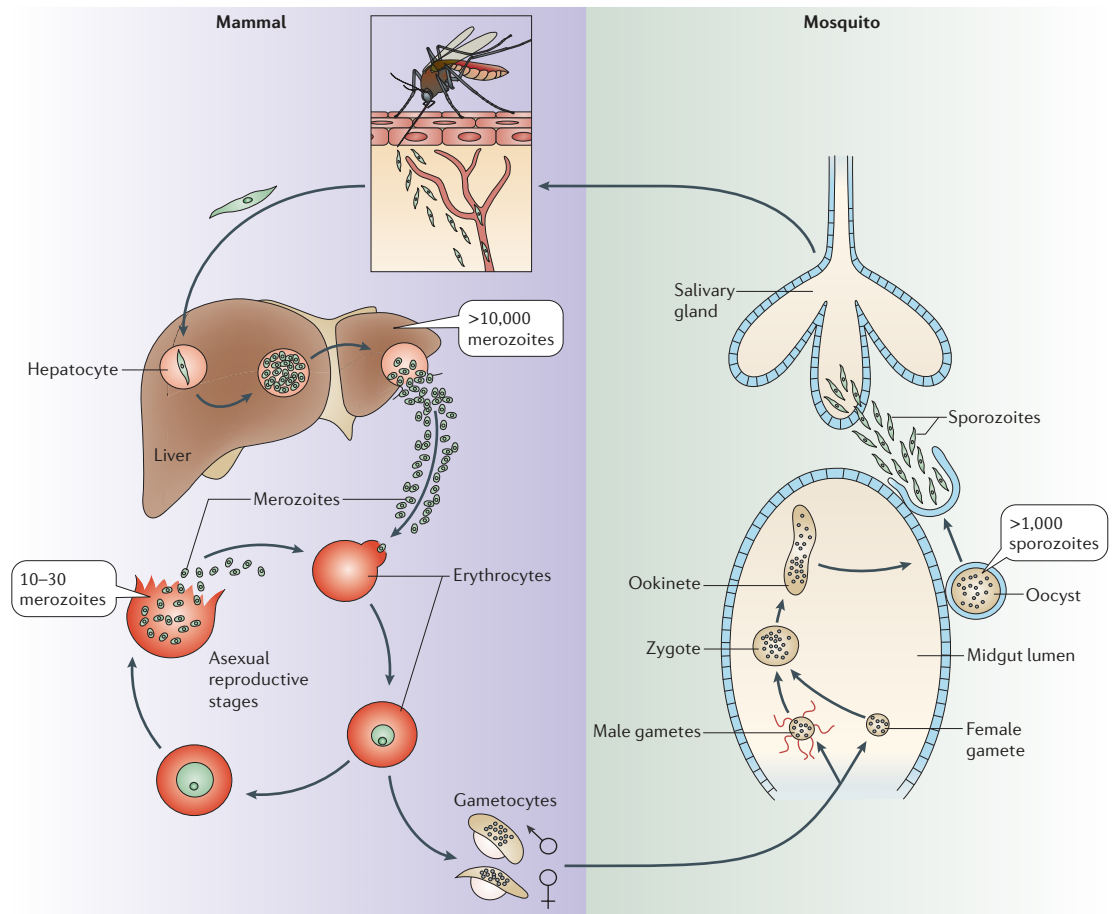


Figure 1 | The *Plasmodium* spp. life cycle. The life cycle of *Plasmodium* species that infect mammals. Symptoms of malaria are caused by cycles of parasite multiplication inside erythrocytes. One cycle is initiated as a result of erythrocyte invasion by a merozoite form and consists of 3–4 nuclear divisions that generate ~10–30 new merozoites; a cycle typically lasts ~24 hours and ~48–72 hours in species that infect rodents and humans, respectively. Some intra-erythrocytic parasites transform into male or female gametocytes, which are taken up by a mosquito. Gametocytes egress from erythrocytes, activate into gametes and fuse in the mosquito midgut lumen. The motile zygote, called an ookinete, crosses the gut epithelium to transform into an oocyst, in which thousands of sporozoites develop. Sporozoites are released into the mosquito body cavity and later pass through salivary gland cells to enter the salivary ducts. After transmission into the skin of the mammalian host during a bite by the mosquito, motile sporozoites reach the liver, where they invade hepatocytes. One intra-hepatocytic sporozoite generates tens of thousands of hepatocytic merozoites, which re-enter the bloodstream and invade erythrocytes. Numbers indicate parasite progeny after multiplication.

Schizonts

Multinucleate, intracellular parasite stages originating from a single organism that reproduces by schizogony. Schizonts contain many individual merozoites when mature.

Hypnozoites

Dormant, non-dividing, intrahepatocytic forms of certain *Plasmodium* species, including *Plasmodium vivax* and *Plasmodium ovale*, which infect humans.

Recurrences

New phases of parasite multiplication inside erythrocytes. These recurrences originate from intra-erythrocytic parasite forms (recrudescence) or hypnozoites (relapse).

on PE stages in the first half of the twentieth century were mainly carried out using avian models of infection. The pioneering work of Raffaele and others in the 1930s revealed the presence of exo-erythrocytic schizonts in macrophages at the inoculation site in the skin, as well as in reticuloendothelial cells in the liver and spleen of infected birds, giving rise to successive PE cycles of infection¹⁰. In mammals, evidence for a PE ‘tissue phase’ was first obtained in 1948: Shortt and colleagues found that when sporozoites of the simian parasite *Plasmodium cynomolgi* were transmitted by mosquitoes to rhesus monkeys, these parasites developed as schizonts in parenchymal cells, but not other cells, in the liver¹¹. Subsequent work showed that sporozoites of species that infect primates (including humans) undergo a single schizogonic cycle inside hepatocytes, although parasites

of some species are thought to also persist inside hepatocytes for weeks to years as singly occurring PE forms called hypnozoites^{12,13}. Recently, it has been speculated that at least some of the malarial recurrences which would conventionally be presumed to have a hypnozoite origin might actually have non-hypnozoite dormant stages as their source¹⁴.

The rodent-infecting *Plasmodium* species *Plasmodium berghei* was discovered in 1948 in *Grammomys surdaster* (an African tree rat)¹⁵, and in 1965, *P. berghei* sporozoites were shown to generate schizonts in the liver of laboratory rodents^{16,17}. Since then, *P. berghei* and its sibling species *Plasmodium yoelii*, both of which are easily amenable to molecular genetic research¹⁸, have become practical and powerful models for investigating the basic biology of the PE stages.

Box 1 | Imaging approaches

Recent advances in molecular biology and imaging technologies have allowed unprecedented opportunities to directly observe fluorescent and bioluminescent pathogenic microorganisms parasitizing their hosts *in vivo* and in real time.

Intravital fluorescence microscopy is an extremely powerful technique with which to study the very small numbers of parasites involved in the establishment of a pre-erythrocytic (PE) infection, permitting both a qualitative and a quantitative view of parasite population behaviour at the tissue and cellular levels. The use of fast microscopes is required to track the highly motile sporozoites at the correct spatiotemporal resolution. Usually, individual parasites are recorded in a three-dimensional volume of $\sim 300 \times 300 \times 50 \mu\text{m}^3$, using high-speed spinning-disk confocal microscopes. The use of high-speed two-photon microscopes with acousto-optical scanning technology can increase the depth of observation to $>500 \mu\text{m}$.

By contrast, at least several thousand luciferase-expressing sporozoites are required for detection in the skin by bioluminescence imaging. However, despite the low temporal and spatial resolution of the technique, bioluminescence imaging is useful for locating parasite populations in the animal body and quantifying parasite behaviour (mainly development within host tissues), as one sporozoite can generate thousands of merozoites inside an infected cell.

Intravital fluorescence microscopy can also be used to gain molecular and functional insights into key steps of infection. This approach relies on the analysis of parasite behaviour using loss-of-function mutant parasites and/or intravital markers that act as reporters of various biological activities or processes (for example, quenched fluorescent substrates to analyse proteolysis, calcium indicators to analyse signalling, fluorescent reporters to analyse transcriptional activation, and propidium iodide to analyse cell traversal). The combination of the two provides powerful means to evaluate the role of molecules or activities in the parasite life cycle, as exemplified by the identification of cell traversal activity of sporozoites in the liver sinusoids.

Infection by PE stages

The skin as a branch point. Sporozoites can move at high speed by gliding motility¹⁹ and can traverse host cells by piercing host membranes^{20,21}. They can also invade host cells inside a parasitophorous vacuole^{22,23}, where they typically differentiate into merozoites. Around 100 sporozoites are injected by a mosquito in an experimental situation^{24,25}, whereas in the wild, infected mosquitoes probably inoculate fewer than 50 sporozoites per bite on average²⁶. In agreement with this, a limited parasite release rate through the mosquito proboscis ($\sim 1\text{--}2.5$ sporozoites per second) was reported by counting fluorescent *P. berghei* sporozoites during *Anopheles stephensi* salivation²⁷ or transmission to mice²⁸. It has long been considered that sporozoites are injected directly into blood vessels by mosquitoes, and the notion that sporozoites are instead inoculated into the extracellular matrix of the skin received experimental support only recently from experiments using bite site removal²⁹ and interruption of mosquito feeding³⁰. Intravital fluorescence microscopy (BOX 1) confirmed that most sporozoites are injected into the skin when the mosquito proboscis probes the skin for blood and ejects saliva³¹. Sporozoites glide in the skin as fast as on glass slides ($\sim 1\text{--}2 \mu\text{m}$ per second in the first 30 minutes), display tortuous and apparently random three-dimensional movement patterns with no detectable tactism^{31–33} and can move for more than 1 hour, although their speed gradually decreases with time. Surprisingly, quantitative studies of *P. berghei* sporozoites injected by mosquitoes into the ear pinna of mice indicate that $\sim 25\%$ and $\sim 15\%$

of the sporozoites leave the skin by invading blood or lymphatic vessels, respectively, whereas $\sim 60\%$ remain at the bite site³² (FIG. 2). Quantitative PCR experiments show a similar tripartite fate for *P. yoelii* sporozoites inoculated into the skin³⁴. The exact proportions of parasites that follow each route is likely to be influenced by many factors, including the vessel density at the bite site and the parasite species.

Entering the liver. Most of the sporozoites injected into the bloodstream reach and remain in the liver³⁵. Extensive biochemical work in the 1990s implicated circumsporozoite protein (CSP), the ‘coat-forming’ protein of the sporozoite³⁶, in this process. CSP binds the particularly highly sulphated glycosaminoglycan chains in liver heparan sulphate proteoglycans (HSPGs) produced by hepatocytes and stellate cells³⁷. Which domain of CSP, the amino-terminal domain^{38,39} or the carboxy-terminal thrombospondin type I repeat (TSR) domain^{40–42} (both of which display HSPG-binding capacity), is involved in CSP binding to liver HSPGs and, thus, in sporozoite homing to the liver has been a long-debated question. A recent genetic study showed that only the N terminus of CSP is exposed on free sporozoites, and the TSR is masked; cleavage of the N terminus was found to expose the TSR and trigger sporozoite invasion⁴³. However, as intravenously injected sporozoites lacking the CSP N terminus infect the liver normally⁴³, it is possible that neither the N terminus (dispensable) nor the TSR (masked) is important for sporozoite arrest in the liver.

Insights into the life of the sporozoite in the liver have come from studies of its aggressive cell traversal (CT) behaviour. Following the first description of sporozoite traversal of host cells²⁰, which had been seen using macrophages *in vitro*, work focused on traversal of hepatocytes, which was observed both *in vitro*²¹ and *in vivo*^{21,44}. A wild-type sporozoite traverses several hepatocytes before invading a final hepatocyte inside a parasitophorous vacuole, where it differentiates. Initial studies using wild-type sporozoites concluded that hepatocyte CT activates parasite invasion of hepatocytes⁴⁵, as well as intracellular development⁴⁶, via the activity of hepatocyte growth factor (HGF) released from the traversed cells. More recently, a 1.5-fold increase in the number of parasites developing inside cultured hepatocytes in the presence of excess HGF was observed for *P. berghei* but not for *P. yoelii*, leading to the conclusion that the role of HGF is crucial and species specific⁴⁷. By contrast, studies using CT-deficient sporozoites paint a different picture, in which CT is important because it allows traversal of cell types other than hepatocytes^{48,52}. CT-deficient sporozoites were initially shown to be highly impaired in liver infection *in vivo*, but to invade and develop inside hepatocytes normally *in vitro*^{48–50}. The *in vivo* contributions of CT (FIG. 3) were later revealed by imaging of sporozoites lacking sporozoite protein essential for cell traversal 2 (SPECT2)⁴⁹, a protein containing a membrane attack complex/perforin-like domain. CT allows sporozoite locomotion and access to blood capillaries in the skin, as $\sim 80\%$ of SPECT2-null sporozoites

Gliding

A substrate-dependent type of unicellular motility defined by the lack of cell deformation during movement.

Parasitophorous vacuole

The vacuole inside which the parasite resides on host cell entry and throughout intracellular development.

Stellate cells

Pericytes that are located between a hepatocyte and a sinusoidal endothelial cell; also called Ito cells.

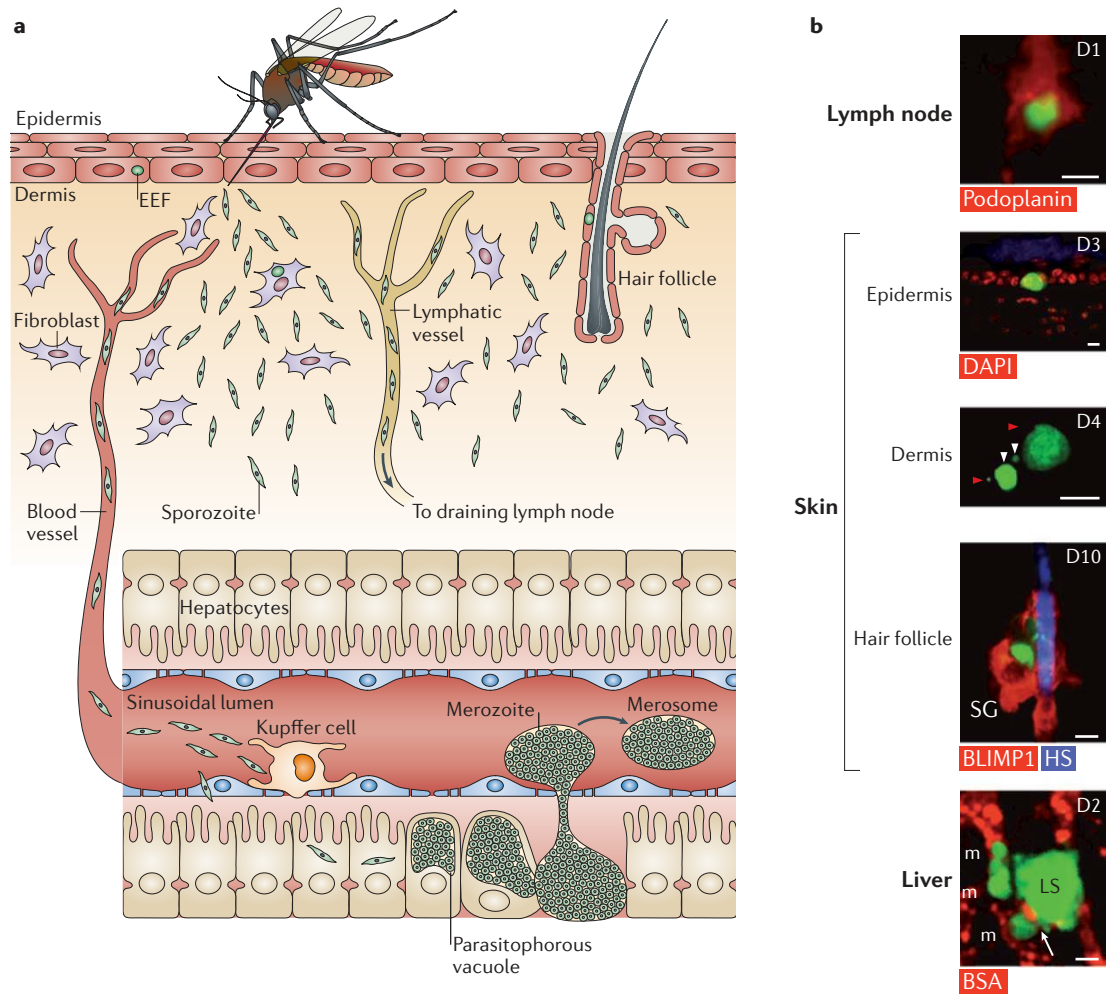


Figure 2 | The pre-erythrocytic phase in rodents. **a** | Motile sporozoites injected into the skin of rodents during a mosquito bite can leave the bite site via blood or lymphatic vessels, or stay in the skin. Sporozoites drained by lymphatic vessels can invade host cells in the proximal lymph node. Skin sporozoites can invade cells in the epidermis, dermis or associated with the hair follicle (exo-erythrocytic forms (EEFs)). Sporozoites that leave the bite site via the bloodstream stop in the liver, glide in the sinusoids (which are lined by endothelial cells and harbour Kupffer cells), wound Kupffer cells, cross the sinusoidal barrier, traverse several hepatocytes and invade a final hepatocyte within a parasitophorous vacuole. Merozoites, the erythrocyte-infecting parasite form, can be formed inside hepatocytes and skin cells. **b** | Confocal microscopy images from tissue cryosections (lymph node and epidermis) or intravital imaging (dermis, hair follicle and liver) show EEFs of the parasite (green). In the lymph node, the parasite is seen associated with a podoplanin-expressing cell. In the epidermis, the parasite is seen in the basal layer (cells stained with DAPI). In the dermis, an infected cell (right) releases merozoites (white arrowheads) and merozoites (red arrowheads). In a hair follicle of a *Blimp1-GFP* mouse, the parasite is shown associated with *Blimp1-GFP*-expressing cells in the sebaceous glands (SG); the hair shaft (HS) is visible because of autofluorescence. In the liver, a mature liver stage releases several merozoites (m) into the blood circulation, indicated by bovine serum albumin (BSA); the sinusoidal barrier, through which the merozoites are released, is indicated (arrow). Days post-infection are shown; scale bars represent 10 μ m. Lymph node image is reproduced, with permission, from REF. 32 © (2006) Macmillan Publishers Ltd. All rights reserved. Dermis image is reproduced and hair follicle image is modified, with permission, from REF. 72 © (2010) National Academy of Sciences USA. Liver image is reproduced, with permission, from REF. 68 © (2006) American Association for the Advancement of Science.

were rapidly immobilized in the skin⁵¹. CT then ensures sporozoite survival in the liver sinusoids, because >90% of intravenously injected SPECT2-null sporozoites were rapidly phagocytosed by Kupffer cells⁵². Furthermore, CT facilitates sporozoite translocation across the liver sinusoidal barrier into the parenchyma, as 75% of the crossing events by wild-type sporozoites occurred by traversal of endothelial cells and/or Kupffer cells⁵². Intravital imaging showed that wild-type sporozoites use multiple pathways

to cross the liver sinusoidal barrier⁵² (FIG. 3), contradicting the long-held view that sporozoites translocate across the barrier exclusively through Kupffer cells, a concept known as the gateway model^{44,53,54}.

CT is therefore important for the progression of sporozoites through host tissues and for sporozoite resistance to host innate immunity en route to hepatocytes, but hepatocyte traversal does not seem to play a significant part in the establishment of infection. An

Kupffer cell
A resident macrophage in the liver; these cells mostly double-line the endothelial cell wall inside the sinusoid lumen.

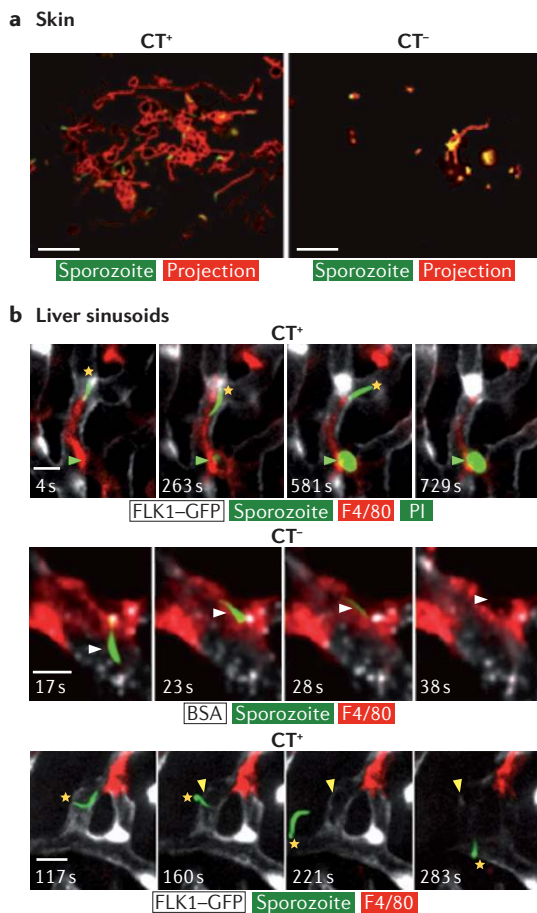


Figure 3 | Host cell traversal by sporozoites. Images obtained by intravital confocal imaging of cell traversal (CT) protein-expressing (CT⁺) or CT-deficient (CT⁻) *Plasmodium berghei* sporozoites. **a** | CT in the skin. Maximal intensity projections of CT⁺ (left) or CT⁻ (right) sporozoite trajectories in the mouse ear over a 10-minute period. Immobilized CT⁺ sporozoites are shown in yellow. Scale bars represent 50 μ m. **b** | CT in the liver sinusoids. Time-lapse microscopy of sporozoites (anterior tip indicated by a yellow star) in the liver of a mouse expressing fetal liver kinase fused to GFP (FLK1-GFP) (labelling endothelial cells) (upper and lower panels); Kupffer cells are labelled with F4/80-specific antibody; fluorescent bovine serum albumin (BSA) is used to label the liver sinusoids (middle panel). In the upper panel, a CT⁺ sporozoite wounding a Kupffer cell is seen by the incorporation of propidium iodide (PI; arrowheads) into the nucleus of the wounded Kupffer cell. In the middle panel, a CT⁻ sporozoite durably interacts with, and is cleared by, a Kupffer cell (arrowheads). In the lower panel, a CT⁺ sporozoite extravasates (yellow arrowhead) by traversing an endothelial cell, visualized by fading of the fluorescence of the traversed endothelial cell (arrowheads). Times of imaging are shown. Scale bars represent 10 μ m. Part **b** images are reproduced, with permission, from Ref. 52 © (2013) The Rockefeller University Press.

Apicoplast
A relict, non-photosynthetic plastid-like organelle of red-algal origin, inherited from secondary endosymbiosis.

important question is how the sporozoite switches from CT to invasion. As mentioned above, cell invasion is associated with cleavage of CSP and exposure of the TSR domain⁴³, possibly corresponding to cell contact-dependent activation of sporozoite invasion. Turning

off CT and membrane wounding, to avoid lysis of the parasitophorous vacuole membrane, is also obligatory for cell invasion, and indeed CT-deficient sporozoites readily invade cells⁵¹. A related question is, what triggers the switch? One study identified the particularly high levels of sulphation of liver HSPGs as the factor that induces the switch⁵⁵. This conclusion, however, gives rise to the question of whether HSPGs can both cause arrest of sporozoites in the liver sinusoids⁴⁰⁻⁴² and activate sporozoites to invade the final hepatocyte⁵⁵. In addition, cleavage of glycosaminoglycan chains from HepG2 cell surfaces does not affect sporozoite invasion⁵⁶. More work is needed to discover what triggers sporozoites to stop traversing cells, and how the switch to invasion operates.

Egressing from hepatocytes. Inside the parasitophorous vacuole, the parasite undergoes a spectacular development involving metamorphosis of the slender sporozoite into a large spherical liver stage, followed by schizogony to generate tens of thousands of merozoites in ~2 days⁵⁷. Molecular insights have been gained into the composition of the parasitophorous vacuole membrane, the strategies for scavenging vital compounds from the host cell, and the contribution of the apicoplast, and these subjects have been reviewed elsewhere⁵⁸⁻⁶⁰. Another aspect of cellular parasitism that has been studied is the way the parasite controls host hepatocyte survival. Initially, host cell apoptosis is blocked⁶¹, possibly via inhibitor of cysteine proteases (ICP; called falstatin in *P. falciparum*)⁶², which has been suggested to inhibit cathepsin L-like host cell proteases but not cathepsin B-type parasite proteases. The *P. yoelii* liver stage has been shown to modify the levels of several hepatocyte proteins, including p53, which is suppressed to enhance survival of the infected cell⁶³. Later in development, the parasitophorous vacuole membrane is lysed⁶⁴, probably after activation of cysteine proteases belonging to the serine repeat antigen (SERA) family⁶⁵, and the merozoites are released into the host cell cytoplasm. The parasite then controls a unique form of host cell death that has some features of apoptosis, such as mitochondrial disintegration and nuclear condensation, but lacks others, such as caspase activation; crucially, this type of cell death preserves the integrity of the host cell membrane⁶⁶.

The final merozoite egress, which was long assumed to occur by mechanical bursting of the host cell⁶⁷, is instead a sophisticated process of host cell membrane manipulation⁶⁸. Real-time *in vivo* imaging has shown that hundreds of merozoites are packaged together inside vesicles called merozoites, surrounded by the hepatocyte membrane (FIG. 2b). These structures bud off and detach from the cell into the sinusoidal lumen^{68,69} and have also been seen during *P. falciparum* infection in immunocompromised mice engrafted with human hepatocytes⁷⁰. Merozoites are solid enough to pass through the heart and reach the lung circulation, where they have been observed liberating infectious merozoites *ex vivo*⁷¹. Merozoites manipulate the merozoite membrane by impeding the exposure of phosphatidylserine

on the outer leaflet^{64,68} (a normal 'eat-me' signal of dead cells), so merozoites are immunologically silent carriers. Therefore, whereas the sporozoite relies on its motile and membrane-wounding capacities to evade host phagocytes, the immotile merozoite has evolved a membrane-wrapping strategy.

A skin-draining lymph node step in PE malaria. The sporozoites that leave the skin inoculation site by invading lymphatic vessels remain in the proximal draining lymph node³². Although sporozoites are still motile on arrival in the subcapsular sinuses of the lymph node, most are quickly taken up by CD11c⁺ dendritic cells (DCs). However, a few parasites invade cells expressing podoplanin (a membrane protein found in lymphatic endothelial cells) (FIG. 2b), and the parasites in these cells do not complete exo-erythrocytic development³³. The lymphatic route thus seems to be a dead end in terms of the parasite life cycle.

Surprisingly, the skin itself is a suitable niche for parasite development^{72,73}. Intravital imaging of *P. berghei* sporozoites inoculated into the skin by mosquitoes revealed that up to 10% of the sporozoites develop in the dermis, the epidermis and in association with hair follicles⁷² (FIG. 2b). Although most skin parasites die before completing their intracellular development, both *P. berghei* and *P. yoelii* sporozoites can multiply to form merozoites inside skin cells (probably in dermal fibroblasts). *Ex vivo* experiments have shown that skin merozoites can infect erythrocytes⁷². However, whether skin merozoites can initiate a blood infection *in vivo* could not be decisively demonstrated^{72,73}, although the observation of merozoites detaching from skin cells and moving within the skin^{72,73} favours this hypothesis. Sporozoites expressing CSP with a constitutively exposed TSR induce blood infection without leaving the skin⁴³, a finding which further suggests that erythrocyte infection can be initiated by skin merozoites. Another striking revelation was the persistence of a small proportion of parasites for weeks in association with hair follicles⁷², an immunoprivileged site in the mammalian body⁷⁴, raising the possibility that parasites associated with hair follicles are a source of infection recurrences in mammalian malaria.

The traditional view that *Plasmodium* spp. sporozoites travel only from the skin to the liver in mammals should thus be revisited. At least in rodents, after mosquito delivery, a large proportion of sporozoites (>50% in the ear) stay in the skin, where multiplication can occur, whereas other sporozoites end up in the draining lymph node until they are eventually cleared (FIG. 2). Whether the same picture holds for human malaria is still unknown. Nonetheless, the development of avian *Plasmodium* spp. in the skin⁷⁵, the similarity between host cell invasion by *P. yoelii* and *P. falciparum* sporozoites⁷⁶, and an early description of *Plasmodium vivax* in a human lymph node⁷⁷ all point to the possibility that a skin-draining lymph node component in the PE phase of the *Plasmodium* spp. life cycle is more conserved than previously appreciated.

Protection against PE stages

The PE stages are attractive targets for immunological intervention. During their journey to the liver, sporozoites are exposed to antibodies in tissue fluids and the bloodstream. Moreover, developing PE parasites in the skin and liver are the only parasite forms that are present in a nucleated cell and so capable of presenting antigens in association with major histocompatibility complex (MHC) class I molecules. There is, however, little evidence that natural exposure to parasites induces even partially protective immunity against the PE stages⁷⁸. By contrast, immunization with large doses of LAP, which are incapable of completing PE development and causing blood infection, has long been known to induce protection against sporozoite challenge in animal models^{4,5} as well as in humans^{6,79} (FIG. 4). Although LAP were initially radiation-attenuated sporozoites (RAS), it has been shown that parasites attenuated by genetic engineering^{80–91}, drug treatment^{92–99} or chemical modification¹⁰⁰ also act as potent vaccines in rodents and humans.

The basis of protection induced by RAS has been studied extensively in rodents. Protection efficiency seems to depend on the host–parasite combination, varying according to both the susceptibility of the host to infection and the ability of the host to mount effective immune responses^{101,102}. Work in the 1980s first revealed an important role for CSP-specific antibodies, which immobilize sporozoites, in RAS-induced protection^{103,104}. It was subsequently acknowledged that CD8⁺ T cells, which recognize parasite epitopes associated with MHC class I on the surface of infected hepatocytes, play a major part in killing infected hepatocytes^{105–108}, although CD4⁺ T cells have also been implicated^{109,110}. How these protective CD8⁺ T cells kill liver-stage parasites is still unclear. These cells use various cytotoxic effectors, such as perforin, FAS ligand, granzymes, interferon- γ (IFN γ) and TNF, which are expressed in response to inducible nitric oxide synthase (iNOS), but parasite-specific CD8⁺ T cells lacking IFN γ , perforin and FAS ligand can still eliminate liver-stage parasites^{111,112}. The recent direct observation of CD8⁺ T cells killing *P. yoelii* liver-stage parasites *in vivo* showed that parasite-specific CD8⁺ T cells recruit both specific and nonspecific T cells to the infected hepatocyte, leading to multiple phenotypes of parasite death¹¹³. Therefore, liver-stage elimination seems to depend on the cooperative activity of multiple CD8⁺ T cells and probably also other cell types, and to involve diverse and functionally redundant cytotoxic effectors.

Historically, LAP have mainly served as models to guide the development of subunit vaccination approaches. However, the subunit vaccines developed so far have not matched the protective efficacy of LAP; this is also true for the most advanced vaccine, RTS,S, which is composed of B cell and T cell epitopes of *P. falciparum* CSP in hepatitis B virus-like particles¹¹⁴. The idea that protective immunity against PE stages would require the induction of immune responses to a broad range of parasite antigens has consequently become increasingly accepted, and it might be that only whole-parasite vaccines will

Immunoprivileged sites

Sites in the mammalian body that are characterized by the lack of major histocompatibility complex expression, the absence of phagocytic and antigen-presenting cells, and a potent immunosuppressant microenvironment.

Subunit vaccination

Vaccination using material that is distinct from live parasites (typically, recombinant proteins or virus-vectored antigens).

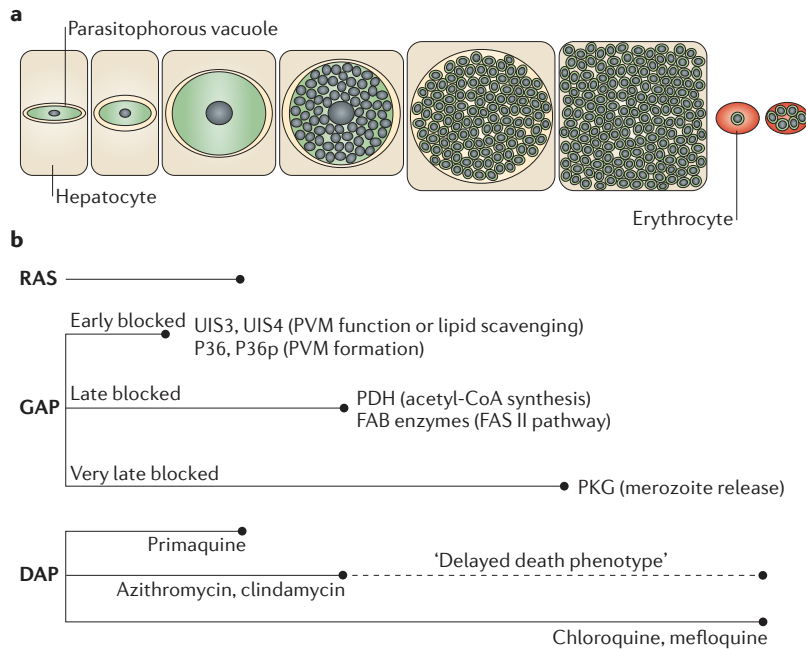


Figure 4 | Liver-stage maturation and live attenuated parasite-based vaccine strategies. **a** | Sporozoite transformation into merozoites inside a hepatocyte. Within a parasitophorous vacuole, the sporozoite transforms into a spherical liver stage that progressively enlarges and undergoes karyokinesis (involving the formation of up to 30,000 nuclei in ~30 hours) without cytokinesis, resulting in a multinucleate mother cell. Schizogony ends by the budding off of uninucleate daughter merozoites inside the vacuole. Merozoites egress from hepatocytes and invade erythrocytes. **b** | Stages of growth arrest of various live attenuated parasites (LAP). Growth of radiation-attenuated sporozoites (RAS) is arrested when DNA replication starts (at ~20–24 hours after infection, midway through development), although depending on the irradiation dose, some nuclear division might continue. For genetically attenuated parasites (GAP), depletion of P36 and/or P36p (called P52 in some *Plasmodium* species) prevents the formation and/or maintenance of the parasitophorous vacuole membrane (PVM), causing very early liver-stage arrest, whereas depletion of UIS3 or UIS4 (which are lipid scavengers inserted in the PVM) causes rapid liver-stage elimination. Depletion of the apicoplast-localized pyruvate dehydrogenase (PDH; an enzyme that catalyses the conversion of pyruvate to acetyl-CoA) or FAB enzymes (type II fatty acid biosynthesis enzymes; that is, FABB/F, FABG, FABZ/A and FABI) causes mid-to-late liver-stage arrest, before merozoite formation and merozoite surface protein 1 (MSP1) expression. Conditional depletion of cyclic GMP-dependent protein kinase (PKG) in liver stages results in a very late liver-stage block, preventing the egress of fully formed merozoites. For drug-arrested parasites (DAP), the antifolate pyrimethamine and the 8-aminoquinoline primaquine both avert nuclear division of liver stages, causing mid liver-stage arrest. Azithromycin and clindamycin, which are macrolide and lincosamide antibiotics, respectively, inhibit apicoplast biogenesis in the liver stage; development is not arrested, but gives rise to non-infectious merozoites, blocked midway during their intra-erythrocytic growth (called the ‘delayed death phenotype’). Chloroquine, a 4-aminoquinoline, and mefloquine, a 4-methanolquinoline, do not affect liver-stage growth and specifically inhibit haemozoin formation in infected erythrocytes, preventing the growth of intra-erythrocytic asexual parasites.

exposed to just 15 infectious mosquito bites on each of three occasions and kept under chloroquine cover (see below) were protected for a prolonged period^{98,99}. This compares somewhat favourably with RTS,S, which gives 32–85% protection under similar laboratory challenge conditions^{118–120}. No LAP have yet been tested under stringent field conditions, whereas RTS,S has been reproducibly shown to confer protection against clinical disease, although it has not exhibited any ability to block infection by parasites^{121–123}.

Protective forms of LAP. A crucial issue is to determine which forms of the parasite are responsible for inducing LAP-mediated protection. Early evidence indicated a role for the liver stages rather than sporozoites, when it was discovered that RAS invade hepatocytes normally but stop developing at the onset of nuclear division¹²⁴, roughly midway through liver-stage development (FIG. 4). Reports that protection depends on the sporozoite irradiation dose^{125,126}, with the most protective dose allowing several nuclear divisions, further suggested that late arrest of liver-stage maturation confers better protection than earlier arrest. LAP models based on drug-arrested parasites (DAP) recently confirmed this idea. Intravenous injection of normal sporozoites into mice treated with chloroquine^{92–94} or mefloquine⁹⁵, which have no effect on parasites in the liver but kill erythrocytic stages, can protect the host; compared with the number of RAS required for protection, fewer of these fully developing DAP are required for protection against subsequent challenge. Similar results have been obtained for treatment with clindamycin or azithromycin^{96,97}, which interfere with the development of liver-stage parasites and render hepatic merozoites non-infective. As mentioned above, protection can be induced by just 45 immunizing mosquito bites delivering infectious sporozoites to humans under chloroquine cover^{98,99}, whereas >1,000 bites delivering RAS alone were required for protection⁷⁹. Remarkably, protection in normal sporozoite-treated volunteers is long-lasting, with four out of six treated volunteers still protected after 2 years, and this protection seems to be correlated with parasite-specific, multifunctional T effector memory cells⁹⁹. Although after immunization with wild-type sporozoites under chloroquine cover the parasites eventually reach erythrocytes, which might induce blood-stage immunity¹²⁷, it was recently shown that this DAP model essentially confers PE immunity, as it protects against sporozoite, but not blood-stage, challenge¹²⁸.

The direct demonstration that late-blocked liver stages induce better protection than RAS in the absence of any blood-stage contribution came from studies on genetically attenuated parasites (GAP) in mice (FIG. 4). The first-generation GAP lack genes crucial to the biogenesis or maintenance of the parasitophorous vacuole membrane^{80–86}. These parasites are blocked soon after sporozoite invasion and provide protection similar to or lower than that conferred by RAS. The discovery of the essential role of the apicoplast-resident fatty acid synthesis type II (FAS II)-dependent pathway during late liver-stage development¹²⁹ inspired the creation of the second-generation GAP,

be able to achieve this¹¹⁵. In recent years, various LAP have entered small-scale human clinical studies, and an important goal is now to establish whether it is possible to formulate LAP for large-scale vaccination^{116,117}. In early research with RAS in humans, 14 volunteers were immunized via bites from more than 1,000 irradiated infected mosquitoes over more than ten occasions, and 13 of these volunteers were found to be protected against sporozoite challenge⁷⁹. More encouragingly, volunteers

which are deficient in the FAS II pathway^{87–90}. *P. yoelii* GAP lacking FABB/F, an enzyme involved in the FAS II pathway, are blocked later than RAS but before merozoite formation⁹⁰. After intravenous immunization, they induce broader and more protective CD8⁺ T cell responses than RAS, involving a larger fraction of effector memory CD8⁺ T cells, as well as some degree of cross-stage protection against challenge with blood-stage parasites⁹⁰. This superior protection could be due to the expression of antigens specific to late liver-stage parasites, directing stronger anti-liver-stage protection, or due to the expression of antigens shared with erythrocytic merozoites, potentially providing protection against both blood-stage and liver-stage parasites (FIG. 4). A further block in GAP development has been achieved by disruption of the cyclic GMP-dependent protein kinase, specifically preventing merozoite release from the infected hepatocyte⁹¹. Given that liver-stage development follows large transcriptomic changes in the parasite, GAP arrested at different developmental stages express different and unique sets of genes; for example, ~480 genes are co-expressed by sporozoites and merozoites¹³⁰, whereas ~770 transcripts are differentially expressed in mid (24 hour) versus mid-to-late (40–50 hour) *P. yoelii* liver-stage infection¹³¹ (schematically corresponding to RAS and the FABB/F-null GAP, respectively).

LAP delivery into the skin. Studies involving late-arresting parasites have highlighted a role for late liver-stage antigens in protection after intravenous injection of LAP; however, work using RAS has shown that immune responses following inoculation into the skin are largely induced in skin-draining lymph nodes^{132,133}. The development of a protective CD8⁺ T cell response after RAS delivery into the skin was dissected using transgenic CD8⁺ T cells specific for an epitope of *P. yoelii* CSP (CSP-Tg)¹³⁴. After RAS injection into the ear via syringe or mosquito, CSP-Tg CD8⁺ T cells are primed in the draining lymph node of the ear and, to a lesser extent, in the spleen, before migrating to the liver and other lymphoid organs, but are minimally activated in the draining lymph nodes of the liver¹³². Moreover, surgical ablation of the lymph node draining the injection site drastically decreases the number of activated CD8⁺ T cells in the liver¹³². It was

also shown that in the draining lymph node, sporozoite antigen is cross-presented by DCs to naive CD8⁺ T cells to induce the protective T cell response^{135,136}. Interestingly, antigen continues to be presented for up to 2 months following immunization via the skin with RAS or GAP, and this persistent antigen presentation is crucial for the complete development and expansion of protective CD8⁺ T cell responses^{90,137}.

Which parasite forms induce protective responses in the skin-draining lymph node remains unclear. Because dead (heat-inactivated) sporozoites left in the skin are not protective¹³⁸, the protective material might be sporozoites that actively reach the draining lymph node of the skin³² or those that develop inside skin cells⁷², and proteins that are continuously shed by gliding and cell-traversing sporozoites in the skin and/or draining lymph node⁵¹ might also play a part. Importantly, if the immunogenic forms are those sporozoites that reach the draining lymph node and develop there to only a limited extent³², then late-blocked GAP might not be more protective than early-blocked GAP when injected into the skin.

Another question concerns the protective efficacy of LAP injection into the skin. As mentioned above, all LAP studies carried out so far in humans have delivered the LAP to the skin via mosquitoes⁷⁹. One exception has been the use of cryopreserved RAS, which did not protect after intradermal injection¹¹⁶, but provided protection after intravenous injection¹³⁹. Recent work in rodents has yielded contradictory results. One study reported lower protective efficacy after intradermal compared with intravenous immunization with *P. berghei* RAS or DAP¹⁴⁰. However, other studies using *P. yoelii* DAP⁹⁵ and RAS¹⁴¹ have recorded similar protection levels for intradermal and intravenous immunization. Some researchers who used *P. yoelii*⁹⁵ or *P. berghei*^{140,142} have found the parasite load in the liver to be lower after intradermal versus intravenous injection of RAS, suggesting that protective efficacy is related to the ability of sporozoites to reach the liver^{116,34}. By contrast, others using *P. yoelii* sporozoites have noted that the parasite load in the liver does not depend on the injection route³⁴.

These discrepancies are likely to be due, at least in part, to different intradermal injection procedures and to the volumes used, as sporozoites display reduced

Box 2 | Immunobiology of *Plasmodium* spp. pre-erythrocytic stages: outstanding questions

- What are the parasite and host molecules that mediate sporozoite homing to, and arrest in, the liver, and can these steps in the life cycle be inhibited by antibodies?
- How do sporozoites coordinate motility, cell traversal and intravacuolar cell invasion?
- What is the basis of sporozoite transformation into a hypnozoite, does the process occur in cells other than hepatocytes, and can hypnozoite-containing cells be eliminated by leukocytes?
- Do human-infecting *Plasmodium* spp. develop in the skin?
- Can injection of live attenuated parasites (LAP) into the skin be used as a mass vaccination method for humans?
- Which parasite forms trigger immunity after injection of LAP into the skin?
- How are parasitized hepatocytes killed by host cells?
- Which T cell subset is most efficient at killing infected hepatocytes in humans?
- What are the relative contributions of antibodies and T cells for protection in humans?
- How can new protective pre-erythrocytic-stage antigens be identified to fuel the development of subunit vaccines?

Box 3 | Live attenuated parasites as vaccines for humans

Between 1972 and 1999, studies involving a total of ~30 volunteers showed that an average of 1,000 bites by irradiated *Plasmodium falciparum*-infected mosquitoes, divided into 8–10 sessions over the course of an ~10-month immunization schedule, resulted in protection in 90% of individuals on exposure to the bites of five infected mosquitoes ~10 weeks after the last immunization, and for up to 10 months when secondary immunizations were carried out⁷⁹. Recently, volunteers were also found to be protected by drug-arrested parasites (DAP) — that is, normal sporozoites delivered under chloroquine cover by mosquitoes^{98,99}. The company Sanaria Inc. was created in 2003 to manufacture cryopreserved *P. falciparum* radiation-attenuated sporozoites (RAS) for human vaccination against malaria¹¹⁵.

Efficiency

Experimental evidence suggests that live attenuated parasite (LAP)-mediated protection is directed at multiple antigens and could therefore be strain transcendent, which would allow host genetic restriction to be overcome. Strain transcendence remains to be demonstrated, as all challenges in human studies to date have used the same *P. falciparum* strain as the immunizing strain.

Administration route

Historically, LAP vaccination in humans has been achieved only via mosquito bites, which cannot be envisaged as a reliable mass-vaccination system. Sporozoites must therefore be injected by syringe via a clinically approved administration route (subcutaneous, intradermal or intramuscular); the intravenous route is least likely to be approved for mass vaccination. However, protection of humans has not yet been demonstrated by non-mosquito delivery of LAP in skin or muscle. Recently, it was found that five intravenous injections of >10⁵ cryopreserved RAS protected all six tested patients against a challenge by bites of five mosquitoes 3 weeks after the last immunization dose, whereas four intravenous immunizations protected only six of nine patients¹³⁹.

Safety

LAP doses must be devoid of both 'breakthrough' infection-causing parasites (100% attenuation) — an achievable goal for genetically attenuated parasites (GAP) — and viral and bacterial contaminants after parasite cultivation in human erythrocytes and mosquitoes.

Large-scale use

Sporozoites are generated only in mosquitoes, and their collection requires microdissection of mosquito salivary glands, a procedure that cannot easily be scaled up to the levels required for immunization in the field. A mosquito-free, tissue culture-based sporozoite production system would greatly boost the prospects of producing LAP for use in humans, but attempts have so far yielded only non-infectious sporozoites. Currently, live sporozoites can be stored only by cryopreservation at ultra-low temperature in liquid nitrogen, a process that kills most sporozoites, at least those of *Plasmodium yoelii*. Therefore, at the present time, LAP vaccines can be envisaged only for restricted human populations in the developed world, such as military personnel and travellers.

motility in, and reduced capacity to leave, flooded skin. Therefore, more work is needed in experimental models to optimize LAP immunogenicity after inoculation into the skin (BOX 2).

Protective antigens. To be protective, vaccine-induced CD8⁺ T cells must eliminate liver-stage parasites that are developing inside hepatocytes. Liver stages express a distinct, though overlapping, repertoire of genes compared with sporozoites¹³¹. Thus, RAS might fail to elicit responses to all the potential protective antigens and, at the same time, might induce responses to irrelevant antigens that are not present in infected hepatocytes. Little is known about parasite epitope presentation by hepatocytes. CSP presentation is proteasome- and TAP-dependent^{136,143}; that is, the antigen must be in the hepatocyte cytosol to be presented. This indicates that

proteins which are secreted across the parasitophorous vacuole membrane by developing parasites might be crucial protective antigens, and proteins that are deposited by traversing sporozoites might also be presented by cells that recover from the traversal event. Mechanisms of protein export across the parasitophorous vacuole membrane in the infected erythrocyte are becoming increasingly well defined^{144,145}. By contrast, protein secretion in the hepatocyte cytosol has received less attention. CSP bears a so-called PEXEL motif, which is present in many exported proteins in the erythrocyte; however, although this motif was found to be important for CSP translocation into the hepatocyte cytosol¹⁴⁶, it has no effect on the ability of CSP-specific CD8⁺ T cells to recognize and kill parasites¹³⁶. The basis of liver-stage protein translocation into the hepatocyte cytosol and entry into the MHC class I presentation pathway are clearly important areas for future investigation.

Conclusions and perspectives

Recent research into the *in vivo* fate of *Plasmodium* spp. sporozoites in mice has changed our understanding of the PE phase of mammalian malaria. In mice, the skin injection site and its draining lymph node, where the majority of parasites remain, are integral components of the PE phase and the site of the first parasite encounter with the host immune system. The most pressing question is whether the same tripartite picture of the initial PE phase — in which sporozoites can remain in the skin or exit via the draining lymph node or the bloodstream — holds true in humans. There are many unknown factors concerning sporozoite infection in the rodent host, including the molecular basis of the tripartite fate of sporozoites inoculated into the skin and of sporozoite retention in the liver, the functional importance (if any) of epidermal and hair follicle-associated parasite subpopulations, and the fate and possible subversion of traversed host cells.

Much remains to be done if we are to understand the host responses that are generated by injection of sporozoites into the skin (BOX 2). Research in this area will be facilitated by the experimental accessibility of the skin and draining lymph nodes, particularly in respect of the powerful immuno-imaging approaches that are now available, as well as by recent progress in understanding skin immunity¹⁴⁷. The initial aims are the identification of the host cell subset (or subsets) involved in antigen capture and in priming the protective CD8⁺ T cells in the draining lymph node, as well as the determination of which LAP forms, whether actively reaching the draining lymph node or remaining in the skin, cause protection. It will also be important to investigate the interaction with, and potential subversion of, regulatory T cells. These cells are abundant in the epidermis of human skin, and sporozoites glide extensively in the epidermis in rodents. A recent meta-analysis of >1,900 immunization studies led to the prediction that live sporozoites in the skin induce the expansion of antigen-specific regulatory T cells that ensure re-infection and systematically deactivate, rather than boost, vaccine-induced immune responses to skin-derived antigens¹⁴⁸.

The ultimate question is whether a PE vaccine could be deployed on a large scale. LAP approaches are efficient, but their implementation still requires major technical and logistical hurdles to be overcome¹⁴⁹ (BOX 3). Regardless, LAP-based studies remain valuable and might lead to the identification of specific sets of protective antigens. In the meantime, efforts to develop subunit vaccines, primarily virus vectored, have yielded promising results¹⁵⁰. For example, immunization against epitopes from two sporozoite proteins using prime–boost strategies based on adenovirus and modified vaccinia Ankara (MVA) virus confers potent protection and elicits

a prolonged effector memory response in the liver¹⁵¹. Until now, subunit vaccinology has focused on just a few antigens, and mainly CSP. Although CSP is clearly a major protective antigen following RAS vaccination¹⁵², it is also obvious that CD8⁺ T cell-mediated protection can be mounted independently of CSP¹⁵³. An important priority for future research is therefore to identify new protective T cell antigens that possibly elicit greater protection than CSP. The recent technical breakthroughs in the malaria research field, from genome sequencing to cellular imaging, now make it possible to envisage systematic approaches towards achieving this goal¹⁵⁴.

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Competing interests statement

The authors declare no competing financial interests.

CORRIGENDUM

Looking under the skin: the first steps in malarial infection and immunity

Robert Ménard, Joana Tavares, Ian Cockburn, Miles Markus, Fidel Zavala and Rogerio Amino

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In Box 3 of the above article (page 709) the sentence “Recently, it was found that five intravenous injections of $>10^5$ cryopreserved RAS protected all six tested patients against a challenge by bites of five mosquitoes 3 weeks after the last immunization dose, whereas four intravenous immunizations protected only three of nine patients¹³⁹” should have read “Recently, it was found that five intravenous injections of $>10^5$ cryopreserved RAS protected all six tested patients against a challenge by bites of five mosquitoes 3 weeks after the last immunization dose, whereas four intravenous immunizations protected only six of nine patients¹³⁹.” The authors apologize to readers for any misunderstanding caused. This has now been corrected online.