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Invited Review

The reciprocal influence of the liver and blood stages of the malaria parasite's life cycle

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ABSTRACT

While the liver and blood stages of the *Plasmodium* life cycle are commonly regarded as two separate fields of malaria research, several studies have pointed towards the existence of a bidirectional cross-talk, where one stage of mammalian infection may impact the establishment and progression of the other. Despite the constraints in experimentally addressing concurrent liver and blood stage *Plasmodium* infections, animal models and clinical studies have unveiled a plethora of molecular interactions between the two. Here, we review the current knowledge on the reciprocal influence of hepatic and erythrocytic infection by malaria parasites, and discuss its impacts on immunity, pathology and vaccination against this deadly disease.

1. Introduction

Malaria, a disease caused by *Plasmodium* parasites transmitted through the bites of infected *Anopheles* mosquitoes, has been a major threat to global health throughout history, and remains a leading cause of death and disease across many tropical and sub-tropical regions. According to the latest WHO World Malaria Report, in 2020 malaria led to an estimated 241 million cases in 87 endemic countries, resulting in over 627,000 deaths globally (WHO, 2020). Although efforts towards malaria control have significantly reduced disease prevalence over the last two decades, in recent years progress appears to have stalled or even reversed. The recent coronavirus disease 2019 (COVID-19) pandemic constitutes a further blow to the prospect of attaining the goals of the WHO's global technical strategy for malaria, which include a reduction of at least 90% in malaria mortality and case incidence rates globally by 2030 compared with 2015 (WHO, 2015). While the exact impact of COVID-19 on malaria control efforts is hard to predict, it has been convincingly argued that indirect effects of the pandemic have the potential to seriously undermine the health system in sub-Saharan Africa, resulting in increased incidence of malaria, tuberculosis and HIV infections (Velavan et al., 2021). This grim scenario increases the urgency to develop new tools to combat malaria, an effort that demands an increased understanding of both the disease and the biology of human infection by *Plasmodium* parasites.

Of the six species of *Plasmodium* that infect humans, *Plasmodium falciparum* and *Plasmodium vivax* present the most significant health threats for the populations of malaria-endemic regions. Malaria parasites have been associated with their hosts for millions of years, and it has been argued that *P. falciparum* might have exerted greater selective pressure on human evolution than any other pathogen (Cowman et al., 2016). Throughout history, both parasites and hosts have co-evolved complex host-parasite molecular interactions that have shaped the host's immune responses to infection and the parasite's mechanisms to evade them, the development of sophisticated cell invasion mechanisms, and the ability of intracellular parasites to access nutrients (Su et al., 2020). A complete understanding of the multifaceted nature of these interactions remains elusive, and is rendered even more challenging by the complexity of the *Plasmodium* parasite's life cycle. In this review, we will discuss the complex relations between the two stages of the mammalian infection by malaria parasites, as well as their impact on each other, on pathology, and on vaccination against this devastating disease.

2. The *Plasmodium* life cycle

Plasmodium parasites alternate between female *Anopheles* mosquitoes, where they undergo both sexual and asexual replication phases, and vertebrate hosts, where they go through two consecutive stages of asexual development that occur inside two very different types of host cells. Briefly, the sexual forms of the parasite ingested by a mosquito during a blood meal undergo a process of sexual fusion followed by asexual replication, which culminates in the generation of thousands of sporozoites that migrate to the

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mosquito's salivary glands. Upon a subsequent blood meal, mosquitoes inject between a few dozen and a few hundred salivary gland-resident, mammalian-infective sporozoites into the skin of the vertebrate host. Injected sporozoites use gliding motility to reach a blood vessel, and travel in the bloodstream until they reach the liver sinusoids. Here, sporozoites traverse the endothelium to gain access to the liver, where they cross Kupffer cells and traverse several hepatocytes before productively invading one, with formation of a parasitophorous vacuole. These events mark the beginning of the liver or hepatic stage of mammalian infection, an obligatory yet clinically silent phase of the *Plasmodium* life cycle that lasts between 2 and 3 days for rodent *Plasmodium* parasites, and between 7 and 14 days depending on the human-infective malaria parasite species. During this phase, the parasite undergoes a process of extensive asexual replication that culminates in the formation of tens of thousands of merozoites, which are eventually released into the bloodstream (Prudêncio et al., 2006). Merozoite release defines the initiation of the blood or erythrocytic stage of infection, during which the *Plasmodium* parasites cyclically invade, asexually replicate and burst red blood cells (RBCs), causing the symptoms of disease. Sexual parasite forms that develop during the blood stage of infection can be ingested by a feeding mosquito, thus completing the cycle (Cowman et al., 2016). The study of the intricate relationships between malaria parasites and their human hosts presents obvious ethical and technical constraints. As such, in vitro culture systems and, most importantly, rodent models of infection have been used extensively to elucidate not only the fundamentals of the biology of *Plasmodium* parasites, but also key aspects of the pathophysiology of infection and of the host's immunity against malaria parasites (Prudêncio et al., 2011).

3. Challenges to the investigation of the liver and blood stages of *Plasmodium* infection

Over decades, the liver and blood stages of *Plasmodium* infection have been viewed as somewhat self-enclosed entities, and the investigation of one is more often than not carried out in the absence of the other. While this can be partly explained by a human tendency to compartmentalise knowledge (Leary and Tangney, 2012), there are also historical and practical reasons why that distinction is so often present in the laboratory. Used as we are to viewing the life cycle of *Plasmodium* parasites in its entirety, we may be forgiven for overlooking the fact that the different pieces that make up this complex puzzle became known at very different times in history. Indeed, while the blood and mosquito forms of *Plasmodium* were first identified in the late nineteenth century, it was not until 1948 that malaria parasites were first detected in the liver of a mammalian host (Arrow et al., 2004). On the other hand, the tools available to experimentally address the erythrocytic stages of malaria parasites differ significantly from those accessible to study hepatic infection by *Plasmodium*. In fact, while viable *Plasmodium*-infected red blood cells can be preserved at low temperatures, and continuous cultures of human blood stage malaria parasites have been possible since 1976 (Trager and Jensen, 1976), cryopreservation of *Plasmodium* sporozoites remains challenging. In practice, this means that whereas blood stages of malaria parasites for research purposes are relatively easy to obtain, access to the sporozoites required to investigate the liver stage of infection is largely restricted to laboratories that host an *Anopheles* insectary and appropriate conditions for experimental mosquito infections. Besides, the study of liver stage *Plasmodium* infection is further challenged by its extremely low infection rate compared with blood stage infection. Finally, while the symptomatic nature of the erythrocytic infection enables the collection of blood samples from infected patients, the study of liver infection depends almost exclusively on in vitro cultures of suitable hepatic cells and on appropriate in vivo animal models (Prudêncio et al., 2011; De Niz and Heussler, 2018). Thus, it is not surprising that the two stages largely continue to be seen as if they represent two distinct infections. However, an increasing amount of evidence suggests that they may influence each other, and that the outcome of infection may depend on the interactions taking place between them.

4. Concomitant infections are the rule, not the exception

Although the two stages of *Plasmodium* development within the mammalian host are sequential, with the infection of red blood cells starting after the completion of the parasite's developmental phase in the liver, in endemic regions the chances of a host concomitantly harbouring both the liver and the blood stages of infection are substantial. In fact, in high-transmission settings, concurrent infections are likely the rule rather than the exception. Indeed, while the intensity of malaria transmission can range from unstable epidemic transmission to high perennial transmission – and can be characterised by 100-fold differences in entomological measures of transmission (or entomological inoculation rates, EIRs) – in regions of high malaria transmission, individuals can be exposed to several hundred infected mosquito bites per year (Robert et al., 2003). As such, mosquitoes repeatedly transmit *Plasmodium* sporozoites to individuals who already have ongoing infections. Interestingly, an important but unexplained feature of epidemiological studies carried out in highly endemic areas is that the simultaneous carriage of various parasite genotypes at low asymptomatic parasitemias is frequently seen in older children but rarely seen in infants (Molineaux and Gramiccia, 1980; Owusu-Agyei et al., 2002; Mayor et al., 2003; Sama et al., 2006). Moreover, the incidence of infection in young children initially increases with age, before it declines as a result of acquired immunity (Molineaux and Gramiccia, 1980; Sama et al., 2006). That being the case, why are mixed infections not frequently observed in younger, less immune children, who ought to be prone to sequential infections?

Confronted by this question, we previously investigated the impact of an ongoing blood stage infection on a concurrent liver infection. By using rodent models of *Plasmodium* infection, we showed that ongoing blood stage infections impair the growth of concurrently inoculated *Plasmodium berghei* sporozoites such that they become growth-arrested in liver hepatocytes and fail to develop into blood stage parasites (Portugal et al., 2011). This impairment is transient in nature and independent of the *Plasmodium* species, but only occurs above a certain threshold of blood parasite density. This phenomenon has some resemblance with those described for quorum-sensing in bacteria (Mukherjee and Bassler, 2019). In the case of *Plasmodium* infections, the blood stage is able to protect its niche from the threat of new infections or superinfection (Portugal et al., 2011). Notably, mathematical modelling showed that this effect is dependent on the transmission intensity and is most prominent under moderate-to-high transmission settings, as observed in epidemiological studies (Molineaux and Gramiccia, 1980; Owusu-Agyei et al., 2002; Mayor et al., 2003; Sama et al., 2006). This strongly suggests that the probability of a new *Plasmodium* infection occurring in humans is also dependent on the level of the peripheral blood parasitaemia. As such, we proposed that blood stage density-dependent inhibition of new liver stage infections alone can explain the changes in infection risk and complexity of infections in young individuals observed in the field (Portugal et al., 2011).

Prevention of superinfection by ongoing blood stage infections might have direct implications in protection of the host. The *Plasmodium* parasites that initially infect the blood protect their niche,

possibly attempting to ensure transmission on a first-come-first-served basis. Thus, the host's immune system would only have to fight infections by parasites with lower genetic diversity, raising the chances of eliminating the circulating infected RBCs and clearing infection, and thereby increasing the host's chances of survival. This is particularly relevant for non-immune individuals, typically the younger ones in endemic regions. In older, semi-immune children, the risk of severe disease decreases and, as circulating parasitaemia is also lower, the complexity of blood stage infections increases. Indeed, it has also been reported that superinfections are much more frequently observed among asymptomatic carriers - who have lower peripheral blood parasitaemias and thus are probably below our proposed threshold of protection – than in clinical cases (Al-Yaman et al., 1997). *Plasmodium* has co-evolved with mammals for millions of years, and mechanisms such as protection from superinfection seem to have been selected to benefit both the host and the incumbent pathogen by maintaining parasite density at levels that are not life threatening before acquired immunity takes up its role.

5. The impact of the blood stage of *Plasmodium* infection on pre-erythrocytic malaria vaccination

The obligatory but asymptomatic nature of the liver stage of *Plasmodium* infection makes it an ideal target for vaccination against malaria, including the subunit vaccine RTS,S, based on the *P. falciparum* circumsporozoite protein (CSP), recently endorsed by the Word Health Organisation (WHO, 2015, Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: Final results of a phase 3, individually randomised, controlled trial, Doi: 10.1016/S0140-6736(15)60721-8). A promising alternative to subunit vaccines can be found in whole-sporozoite (Wsp) immunisation, which relies on the generation of immune protection against pre-erythrocytic parasite stages following immunisation with *Plasmodium* sporozoites under conditions that prevent the appearance of clinical symptoms. This includes radiation-attenuated sporozoites (RAS) (Seder et al., 2013), genetically attenuated parasites (GAP) (Roestenberg et al., 2020), immunisation with infective sporozoites in combination with chemoprophylaxis (CPS) (Roestenberg et al., 2009), and genetically modified rodent *P. berghei* sporozoites expressing human *Plasmodium* antigens (Reuling et al., 2020).

*Pf*SPZ-CVac is a form of CPS vaccination in which replication-intact, cryopreserved, *P. falciparum* sporozoites manufactured by Sanaria, Inc. (USA) are administered with concurrent administration of antimalarial chemoprophylaxis (Bastiaens et al., 2016). Vaccination with *Pf*SPZ-CVac was shown to be highly protective against homologous (Mordmüller et al., 2017) and heterologous (Sulyok et al., 2021) controlled human malaria infection (CHMI) in malaria-naïve volunteers, but was somewhat less effective against homologous CHMI in African vaccinees (Jongo et al., 2021). Whether and to what exact extent the poorer performance of Wsp vaccines in malaria-endemic regions compared with that observed in healthy volunteers in Europe and in the USA can be explained by an interaction between blood stage infection and vaccination remains to be fully understood. Nevertheless, it has been noted that parasitemia prior to immunisation does appear to negatively impact Wsp vaccine responsiveness (Mo et al., 2020), although it has also been argued that the explanation may reside in the skewing of the hosts' immune status in malaria-endemic regions due to chronic immune stimulation and immune exhaustion (Mo et al., 2020). Crucially, the efficacy of *Pf*SPZ-CVac immunisation against malaria was recently shown to decrease from 75% to 0% when the vaccine was administered in the presence of erythrocytic stage parasites (Murphy et al., 2021). The authors conclude that the blood stage of infection has a negative impact on the formation of pre-erythrocytic immunity, and hypothesise that the primary effector mechanism might reside in liver-resident $CD8^+$ T cells (Murphy et al., 2021). Of note, it has been shown in rodent models that *Plasmodium* blood stage infection affects the maturation of dendritic cells (DCs) and suppresses $CD8^+$ T cell immune responses, leading to the inability of mice infected with blood stage *Plasmodium yoelii* to initiate a $CD8^+$ T cell response when immunised with irradiated *P. yoelii* sporozoites (Ocaña-Morgner et al., 2003). The authors attribute this to a functional dysregulation of dendritic cells (DCs) that occurs when they encounter *Plasmodium*-infected erythrocytes in the blood and spleen, and leads to the secretion of suppressive factors that inhibit the activation of circulating $CD8^+$ T cells (Ocaña-Morgner et al., 2003).

Similarly, the implementation of RTS,S vaccination programmes must consider the seasonality of malaria transmission. In fact, it is interesting to note that R21/MM, another CSP-based subunit pre-erythrocytic vaccine candidate, displayed a remarkable 77% efficacy in a recent clinical study in Burkina Faso when the primary vaccination series was administered before the malaria season (Datoo et al., 2021). However, as noted by Moorthy and Binka (2021), while this is higher than many of the published RTS,S studies, there is no direct comparison between vaccinations timed to occur with the beginning of each malaria season, leaving the question of superiority largely unanswered. This raises the possibility that RTS,S vaccine efficacy might be increased by this immunisation schedule in seasonal areas, an hypothesis that is currently under investigation (Chandramohan et al., 2020). This reciprocal influence may have several implications for the design of clinical trials and for interventions aimed at malaria prevention. As such, the immunisation schedules employed in future pre-erythrocytic vaccine trials in endemic areas should take into account the impact of blood stage *Plasmodium* infection on the candidate's protective efficacy (Murphy et al., 2021).

Additionally, non-immune effects exerted by the blood stage of infection on the development of pre-erythrocytic stage immunity may also be considered. In rodents, a metabolic imbalance due to an ongoing blood stage infection restrains parasite control by hindering germinal centre responses, which can be partially restored upon dietary supplementation with L-glutamine (Vijay et al., 2020). Reduced serum levels of this amino acid during the acute phase of *Plasmodium* erythrocytic infection in humans correlate with poorer infection outcomes (Cordy et al., 2019). It is, therefore, reasonable to speculate that alterations in the nutritional status of infected humans in malaria-endemic areas (Friedman et al., 2003; Alexandre et al., 2015) may hinder protective humoral responses not only against blood stage *Plasmodium* forms but also against hepatotropic parasites in an immunisation setting, and even during natural transmission. While the impact of blood stage parasites on pre-erythrocytic immunity may result from a complex mix of innate and adaptive immune factors (Murphy et al., 2021), the observations outlined above further support the notion that the erythrocytic phase of mammalian infection by *Plasmodium* exerts a marked influence on a concurrent liver infection by this parasite. This may be particularly relevant in moderate-to-high transmission settings, where a previously infected host is likely to be exposed to an infectious mosquito bite, as well as in the context of pre-erythrocytic vaccination of individuals in endemic regions, who may harbour blood stage parasites during the immunisation process.

6. Does the liver stage of *Plasmodium* infection impact the outcome of malaria?

The fact that the liver stage of *Plasmodium* infection is symptomatically silent and takes place in an immune-privileged organ (Kubes and Jenne, 2018) sustained for many years the perception that this phase of the parasite's life cycle, crucial for its successful

establishment under natural conditions, was also immunologically silent. However, we now know that this is not the case. Small inflammatory foci, mostly composed of myeloid cells of the macrophage and neutrophil lineages, have been observed surrounding infected hepatocytes in mice infected with rodent malaria parasites (Khan and Vanderberg, 1991; Van De Sand et al., 2005; Epiphanio et al., 2008). Importantly, exuberant accumulation of immunocompetent cells hamper productive liver stage infections in the absence of host anti-inflammatory factors, such as heme oxygenase-1 (Epiphanio et al., 2008). This protective response is orchestrated, at least in part, by the cytoplasmatic RNA sensor Mda5, the adaptor molecule Mavs and the transcription factors Irf3 and Irf7 (Liehl et al., 2014). The resulting production of type I interferons (IFNs) propagates the innate immune response in an autocrine and paracrine manner, resulting in the recruitment of natural killer (NK) and NK T cells (Liehl et al., 2014; Miller et al., 2014).

The host's ability to sense *Plasmodium* hepatic infection and mount an immune protective response to limit it opens the possibility that such a response may pre-condition the host's immunity to the ensuing blood stage of infection. In fact, both host and parasite factors have been identified that alter the course and outcome of experimental infections initiated by the natural route (i.e., administration of sporozoites) or transfusion of infected RBCs (Ribot et al., 2019; Sato et al., 2019), suggesting that such cross-talk between both stages of the parasite's development does indeed occur.

Furthermore, evidence suggests that the influence of the liver stage of infection on the ensuing erythrocytic stage of parasite development may also impact the immune response to the latter and, thereby, its associated pathology. Throughout the parasite's development within the hepatocyte, monocyte-derived dendritic cells infiltrate the infected liver and present antigens that directly stimulate *Plasmodium*-specific $CD8^+$ T cells (Kurup et al., 2019). The fact that hepatic and erythrocytic merozoites have highly comparable protein expression profiles (Shears et al., 2019) raises the possibility that priming and activation of effector $CD8^+$ and $CD4^+$ T lymphocytes against hepatic late-stage antigens may result in cross-stage immune adaptive responses (Lau et al., 2014; Müller et al., 2017; Fernandes et al., 2018), thus biasing the progression of the ensuing blood stage of infection. Indirect evidence supporting this notion is provided by the observation that immunisation with late liver stage-arresting parasites provides relevant protection following challenge with erythrocytic parasite forms (Butler et al., 2011; Vaughan et al., 2018). However, whether such mechanisms are at play during natural infections remains to be established.

7. Conclusions

While the liver and blood stages of mammalian infection by *Plasmodium* are commonly regarded as two separate fields of malaria research, the possibility of a bi-directional interaction where one stage may impact the establishment and progression of the other bears important implications in terms of infection, immunity and pathology. From our standpoint, this warrants the detailed investigation of other host-mediated processes that may dictate the reciprocal influence of the hepatic and erythrocytic stages of the *Plasmodium* life cycle within the same host. Such knowledge would potentially bear profound implications for not only our understanding of the epidemiology of malaria, but also for the development of novel strategies aimed at controlling infection and reducing disease burden.

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