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Rodent *Plasmodium*-infected red blood cells: Imaging their fates and interactions within their hosts

Carla Claser [a], Benoit Malleret [a], Kaitian Peng [a], Nadja Bakocevic [a], Sin Yee Gun [a,b], Bruce Russell [b], Lai Guan Ng [a], Laurent Rénia [a,b,*]

[a] Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Biopolis, 136648 Singapore
[b] Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

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ABSTRACT

Malaria, a disease caused by the *Plasmodium* parasite, remains one of the most deadly infectious diseases known to mankind. The parasite has a complex life cycle, of which only the erythrocytic stage is responsible for the diverse pathologies induced during infection. To date, the disease mechanisms that underlie these pathologies are still poorly understood. In the case of infections caused by *Plasmodium falciparum*, the species responsible for most malaria related deaths, pathogenesis is thought to be due to the sequestration of infected red blood cells (IRBCs) in deep tissues. Other human and rodent malaria parasite species are also known to exhibit sequestration. Here, we review the different techniques that allow researchers to study how rodent malaria parasites modify their host cells, the distribution of IRBCs *in vivo* as well as the interactions between IRBCs and host tissues.

1. Introduction

Malaria, a mosquito-borne infectious disease caused by a protozoan parasite of the *Plasmodium* genus, occurs primarily in the intertropical regions of the world. The *Plasmodium* life cycle begins when an infected female *Anopheles* mosquito injects infectious sporozoites into the skin of the host that subsequently migrate to the liver. After intra-hepatic multiplication, mature merozoites are released into the blood stream where they go on to infect red blood cells (RBCs). This marks the start of the erythrocytic stage of the disease that is responsible for clinical symptoms such as high fever, muscle aches and chills. In most cases, the administration of appropriate medical treatment clears the infection, but in some patients, severe malaria may develop. This is characterized by metabolic alterations, renal failure, liver and lung dysfunction, anemia and human cerebral malaria (HCM), which itself encompasses a whole range of neurological complications such as seizures, impaired consciousness and coma [1].

Due to ethical constraints, the study of *Plasmodium* biology as well as its associated pathology and immunity in humans has traditionally been restricted to analysis of the peripheral blood and post-mortem autopsy samples. This does not allow researchers to gain a full understanding of the parasite's dynamic distribution *in vivo* nor their potential interactions with immune and other host cells. Due to these limitations, rodent parasite *Plasmodium* species such as *P. berghei*, *P. yoelii*, *P. chabaudi and P. vinckei*, have been widely used in mouse models of infection to study different aspects of parasite biology, immunity and pathology [2,3]. In particular, studies that utilize the ANKA strain of *P. berghei* in an experimental model of cerebral malaria [1,4] have provided new insights into *in vivo* parasite distribution and sequestration in relation to disease severity [5–8].

One important feature of *P. falciparum* infection in humans is the absence of mature infected red blood cells (IRBCs) in the circulation [9,10] due to a phenomenon termed sequestration that results from the cytoadherence of *P. falciparum* IRBCs to endothelial cells [11]. In addition, *P. falciparum* IRBCs form rosettes by adhering to non-infected RBCs [3,12,13] and agglutinates by adhering to platelets [14]. Complete sequestration of mature IRBCs has generally not been observed for other human malaria parasite species but has been reported to occur to a certain degree in *P. vivax* [15]. This is probably due to the capacity of a fraction of *P. vivax* IRBCs to cytoadhere to endothelial cells [16,17]. Rodent malaria parasites such as *P. berghei* [18,19] and *P. chabaudi* [20,21] also exhibit sequestration *in vivo*. However, it has not been determined if rodent malaria parasites can cytoadhere *in vivo* although RBCs infected with *P. chabaudi* and *P. yoelii* have been demonstrated to cytoadhere to different sources of endothelial or fibroblast cells *in vitro* [22,23]. *P. chabaudi* IRBCs have also been shown to be capable of forming rosettes with naive RBCs [24].

Apart from cytoadherence, other factors can play a role in parasite sequestration *in vivo*. During the development of *P. falciparum*, for example, the parasite progressively occupies most of the available cytoplasmic space of the infected red blood cell (IRBC) [25,26] and induces remodeling of the cell surface [27,28]. This leads to changes in the

* Corresponding author at: Singapore Immunology Network, A*STAR, Immunos, #3-15, 8A Biomedical Grove, Biopolis, 138648 Singapore. Tel.: +65 6407 0005; fax: +65 6464 2056.
E-mail address: renia_laurent@immunol.a-star.edu.sg (L. Rénia).

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deformability and rheological properties of the IRBCs [29–32], with less deformable and denser IRBCs likely to be pushed to the side of the blood stream closer to the blood vessel walls [33]. Under physiological non-disease conditions, blood flow varies between and even within organs [34,35]. These differences in blood flow are further acerbated under inflammatory conditions when vessel constriction may lead to localized obstruction [36] and further sequestration of IRBCs. In addition, production of pro-inflammatory cytokines during systemic and local inflammation may increase expression of adhesion molecules on endothelial cells of blood capillaries [37], thus further enhancing interactions between IRBCs and endothelial cells [38]. All these varied factors are likely to contribute significantly toward the pathogenesis of severe malaria.

In recent years, many studies have attempted to understand the cellular and molecular mechanisms that control IRBC distribution *in vivo* and their interactions with endothelial as well as immune cells. Here, we have reviewed how various imaging techniques can be utilized to provide further insight into parasite biology as well as potential applications in disease diagnosis and intervention.

2. Static imaging

2.1. Transmission electron microscopy

Transmission electron microscopy (TEM) was used extensively in the 1950s to 1970s to describe the surface and submicroscopic structures of a range of human, avian, simian and rodent malaria parasite species [49,50]. TEM studies have revealed that the membrane of the RBC is altered during infection by human *Plasmodium* species [51]. For instance, *P. falciparum* and *P. malariae* IRBCs display electron-dense protrusions called knobs [52,53] or spiky excrescences [54,55] respectively while *P. vivax* IRBCs display caveolea-like structures [56]. TEM has also been instrumental in dissecting the interactions between IRBCs and other host cells like macrophages [57]. It was TEM studies that first revealed that cytoadherence of *P. falciparum* IRBCs results from the intimate association of knobs present on the surface of IRBCs with endothelial cells [52]. In fact, this technique remains the gold standard for the visualization of IRBC cytoadherence to endothelial and other cells both *in vitro* and *in vivo*. In contrast to human malaria, rodent malaria IRBCs do not express knob-like structures [20]. However, it has been shown that *P. yoelii* 17X clone YM, PbA and *P. chabaudi* clone AS IRBCs may interact with endothelial [23,48,58] or fibrocytic cells [22].

2.2. Scanning electron microscopy

Scanning electron microscopy (SEM) has been used extensively for visualization of the surfaces of various cells or tissues. This technology is based upon the detection of diffracted electron beams and requires fixed biological samples to be coated with a thin layer of conductive material such as gold, carbon or palladium [59]. The level of magnification that can be achieved with electron microcopy (EM) is much higher compared to that of optical microscopy, since EM utilizes much shorter electron wavelengths compared to the photon wavelengths traditionally used in optical microscopy. In the malaria field, SEM has been applied mainly towards description of the surface of normal [60,53] as well as IRBCs [61]. In 1976, the first SEM image of *P. berghei* IRBCs were reported by Gorenflot and colleagues [60], where it was demonstrated that these infected RBCs were devoid of any knob-like structures. Instead, small characteristic anfractuosities at the surface of the IRBCs were observed [53]. SEM has also been used to study the interactions between IRBCs and macrophages [62] as well as having been applied towards the visualization of brain capillaries and their cellular contents during ECM (experimental cerebral malaria) [63]. In future, high resolution imaging of the brain vessels associated with backscatter electron imaging of gold conjugated antibodies [64] will allow for the precise identification and localization of IRBCs and immune cells in capillaries during infection. In addition, the advent of a new technique based upon coupling of focused ion beams with SEM will allow for 3D reconstruction of the *Plasmodium* IRBC surface on a nanoscale level [65].

2.3. Atomic force microscopy

Atomic force microscopy (AFM) is a very sensitive technique that allows one to collect topographical information and measure surface mechanical properties of any material, be it cells or organisms, at a nanometer scale resolution under conditions that are nearly impossible to achieve with standard electron microscopy techniques. In contrast to other systems that depend on the measuring of deflected or scattered light or electrons from the sample, AFM is based upon the detection of any variable movement of a flexible cantilever that possesses a sharp tip at its end for the purpose of sensing the sample surface. AFM allows the investigation of *Plasmodium* infected air-dried RBCs without the need for prior fixation and/or surface coating that may modify fragile nanostructures present on the surface of IRBCs and create artifacts [64,66–68]. In contrast to *P. falciparum* IRBCs, AFM scans of rodent malaria IRBCs have confirmed previous TEM and SEM findings that *P. yoelii* or PbA IRBCs do not express any knob-like structures (Russell and Renia, unpublished results). Thus, like *P. vivax* IRBCs, RBCs infected with other rodent malaria species are likely to adhere to endothelial cells *via* a different mechanism.

3. Dynamic *in vivo* imaging in the study of parasite sequestration

Diverse techniques have been previously used to determine if parasite accumulation/sequestration occur in infected mice. Giemsa-stained blood smears, the gold standard technique for malaria diagnosis [39–43], though routinely used to detect for and quantify parasites present in the peripheral circulation, has seldom been used to assess parasite sequestration in organs. Histopathological post-mortem studies on patients who died from HCM revealed substantial mature IRBC sequestration in the brains of a large proportion of them [44,87–89]. However, this massive IRBC sequestration in the brain microvasculature has not been observed in mice infected with *P. berghei* during ECM [4,45–48]. This discrepancy has led many researchers to dismiss the relevance of using PbA infected mice as a model for studying HCM [90]. However, it should be noted that since histopathological studies in human samples are performed post-mortem, one should take into account the possibility that the observed massive IRBC accumulation is a post-agonal event. The converse is true for histopathological studies performed on PbA-infected mice, where brain samples are taken from anesthetized mice that display neurological symptoms. Thus, histopathological studies in the two systems should not be directly compared. Quantitative polymerase chain reaction (qPCR) techniques [69–73,77,78] that allow for the detection of sub-patent infections missed by traditional microscopy techniques have also been used in the study of parasite load and distribution in tissues. They have demonstrated that IRBCs do accumulate in tissues of mice during infection with PbA and this is mainly in the brain [74–76]. Lastly, using flow cytometry to detect GFP-transgenic parasites in tissue extracts, it was shown that GFP-expressing IRBCs accumulate in the lungs [80–86]. Recently, using GFP-transgenic parasites that were concurrently stained with Hoechst, it was also demonstrated that RBCs infected with mature PbA parasites sequester in higher numbers compared to those infected with younger parasites in the brain of mice upon the onset of ECM signs. Interestingly, it was observed that brain sequestration of PbA IRBCs was controlled by a subset of dendritic cells (DC) that express the Clec9A marker [85]. The effect of Clec9A + DC on IRBC sequestration is likely to be indirect, since this DC subset induces $CD8^+$ T cells that control IRBC accumulation in the brains of PbA-infected mice during ECM [18,19].

In all, these methodologies have presented new evidence for the accumulation and possibly sequestration of PbA IRBCs in various organs *in vivo* during infection [74,75]. Recent advances in new imaging technologies as detailed below have also allowed for the *in vivo* visualization of

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