International Journal for Parasitology 43 (2013) 1–10

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Invited Review

New approaches to studying *Plasmodium falciparum* merozoite invasion and insights into invasion biology

Michelle J. Boyle [a], Danny W. Wilson [b], James G. Beeson [a,c,*]

[a] The Burnet Institute for Medical Research and Public Health, Melbourne, Victoria 3004, Australia
[b] Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia
[c] Department of Microbiology, Monash University, Victoria, Australia

ARTICLE INFO

Article history:
Received 10 September 2012
Received in revised form 30 October 2012
Accepted 3 November 2012
Available online 5 December 2012

Keywords:
Malaria
Plasmodium falciparum
Merozoite
Invasion
Inhibitors
Vaccines

ABSTRACT

Merozoite invasion of human red blood cells by *Plasmodium falciparum* is essential for blood stage asexual replication and the development of malaria disease. Despite this, many of the processes involved in invasion are poorly understood. Recent advances have been made in methods to isolate viable merozoites for studies of invasion. The application of these approaches is providing new insights into the kinetics of invasion and merozoite survival, as well as proteins and interactions involved in invasion, and will facilitate the development and testing of anti-merozoite vaccines and the identification of invasion-inhibitory compounds with potential for drug development. This review discusses these recent advances and considers potential avenues for future research.

1. Introduction

Plasmodium falciparum causes the majority of malaria morbidity and mortality worldwide, with an estimated 781,000 deaths and 225 million cases of malaria annually (World Health Organization Global Malaria Programme, 2010). Malaria disease occurs during the asexual blood stage of infection, when the merozoite form of the parasites invades host red blood cells (RBCs) and establishes an intracellular trophozoite. The intraerythrocytic parasite grows and divides over approximately 48 h into a schizont form containing up to 30 daughter merozoites that are released for subsequent invasion and replication. Currently there is no vaccine against malaria and with the emergence of resistance to the artemisinin class of antimalarial drugs (Dondorp et al., 2009), new approaches to tackling disease are desperately needed. Since the merozoite form of *Plasmodium* spp. is extracellular and is exposed to antibodies and immune cells in the blood stream, merozoite antigens have long been regarded as attractive targets for vaccine development. There is also growing interest in the identification and development of novel drug therapies that target merozoite invasion as many of the proteins and processes required for invasion are essential and are unique to the parasite. Despite the importance of invasion to the *P. falciparum* life-cycle and malaria disease, our current understanding of the processes required for merozoite invasion is far from complete, impeding development and evaluation of vaccines and novel therapeutic compounds. This review will discuss recent advances in approaches to study merozoite invasion along with the impact of these methods on our understanding of merozoite invasion biology, invasion mechanisms and the potential to apply these methods in the development of vaccines and new therapeutic compounds.

2. Limitations to studying merozoite invasion

Much of our understanding of the basic biology of merozoite invasion comes from studies completed 20–30 years ago using live video microscopy (Dvorak et al., 1975) and electron microscopy (EM) to characterise invasion of merozoites of *Plasmodium knowlesi* (Bannister et al., 1975; Aikawa et al., 1978, 1981) and other non-human *Plasmodium* spp. (Ladda et al., 1969). From this work it is clear that merozoite invasion of the RBC progresses through a series of coordinated events in a step-wise-like manner. This consists of initial contact between the merozoite and RBC, reorientation of the merozoite so that the apical end binds irreversibly to the RBC surface, formation of tight junction and invasion (reviewed in Bannister and Dluzewski, 1990) (Fig. 1). Each step in the invasion process involves a range of receptor–ligand interactions, protein processing events and signalling cascades. Many merozoite

* Corresponding author at: The Burnet Institute for Medical Research and Public Health, 85 Commercial Road, Melbourne, Victoria 3004, Australia. Tel.: +61 3 9282 2111; fax: +61 3 9282 2126.
E-mail address: beeson@burnet.edu.au (J.G. Beeson).

0020-7519
http://dx.doi.org/10.1016/j.ijpara.2012.11.002

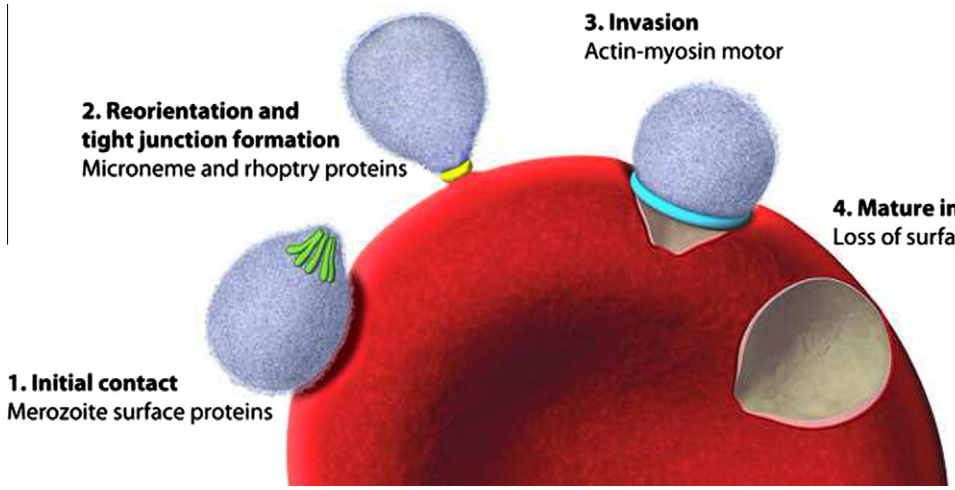

Fig. 1. Merozoite invasion of red blood cells. Merozoite invasion appears to occur in a step-wise process. (1) Initial contact occurs between any surface of the free merozoite and the human red blood cell, with interactions thought to be mediated by merozoite surface proteins; the apical organelles (micronemes and rhoptries) are coloured green. (2) The merozoite then re-orientates such that the apical end contacts the human red blood cell surface and secondary interactions occur, associated with the release of proteins from the rhoptries and micronemes, leading to tight junction formation (yellow) between the parasite and the human red blood cell. Re-orientation and tight junction formation are thought to be mediated by proteins of the apical organelles (micronemes and rhoptries), such as apical membrane antigen 1, erythrocyte binding antigens and *P. falciparum* reticulocyte-binding homologues. The release of apical organelles is thought to commence at or immediately following egress, and is completed after the commencement of invasion. (3) Following tight junction formation the merozoite invades using a unique actin–myosin motor (the moving junction is coloured blue). (4) Mature invasion is thought to result in the loss of specific surface proteins and leads to the intraerythrocytic development cycle. Figure adapted from Beeson and Crabb, (2007).

antigens with potential roles in invasion have been identified and localised to the merozoite surface or apical organelles known as the micronemes, rhoptries and dense granules. However, for most merozoite proteins a specific role during invasion is unknown or poorly defined. Part of this restricted knowledge of invasion events is due to technical challenges in studying merozoites. Until recently, no well-established or routinely used methods to isolate *P. falciparum* merozoites that retained their invasive capacity had been developed. Consequently, most studies of invasion have relied on indirect observations of invasion and subsequent blood-stage development. Alternatively, investigators have used merozoites of other *Plasmodium* spp. such as *P. knowlesi* as these have proven to be more suitable for isolation in vitro. Such studies have shed light on invasion kinetics (Dennis et al., 1975; Johnson et al., 1980), specific protein interactions (Mitchell et al., 2004) and invasion inhibitors (Hadley et al., 1983). However, while *P. knowlesi* merozoites are known to have similar invasion kinetics as *P. falciparum* (Dvorak et al., 1975; Gilson and Crabb, 2009), there remains significant differences between the two species in regards to mechanisms of invasion, including receptor–ligand use (reviewed in Gaur et al., 2004).

In *P. falciparum*, characterization of the role of specific proteins during merozoite invasion has been facilitated by the development of stable genetic transformation technologies, enabling the generation of modified parasites with specific deletions, truncations or other alterations in key proteins (for example loss or gain-of-function and tagging (Crabb and Cowman, 1996; Cowman et al., 2000) and targeting proteins for degradation in conditional knock-out phenotypes (Meissner et al., 2005; Dvorin et al., 2010)). Growth defects are typically measured in standard growth inhibitory assays (GIAs), which typically measure total parasite replication over 48 h (Persson et al., 2006); the assumption is that these assays mostly assess inhibition or alteration of merozoite invasion. Differential inhibition of invasion in the presence of vaccine-induced antibodies and enzyme-treated RBCs with genetically modified parasites has helped define a number of host cell receptor–ligand interactions (for recent reviews on invasion mechanisms see Farrow et al., 2011; Harvey et al., 2012). However, testing antibodies or compounds for activity in standard in vitro GIAs cannot clearly differentiate merozoite invasion inhibition from potential inhibitory effects on schizont rupture or merozoite egress and intra-erythrocytic parasite development.

The role of specific proteins during invasion can further be assessed using standard or advanced microscopy techniques to localise proteins at different stages of the blood stage cycle (Tonkin et al., 2004; Hanssen et al., 2010a,b; Cho et al., 2012). However, localisation of proteins during invasion has been hampered by the inability to isolate sufficient viable merozoites for images of invasion to be consistently captured (for examples of invading merozoites captured serendipitously see Langreth et al., 1978; Baum et al., 2006; Khattab et al., 2008). A more direct way of studying merozoite invasion is the use of live video microscopy of merozoite egress and reinvasion (Glushakova et al., 2005, 2010; Gilson and Crabb, 2009; Treeck et al., 2009; Boyle et al., 2010a; Richard et al., 2010). However, this technique is labour intensive, typically infers phenotypes or inhibitory activity from low numbers of events and is not free from the confounding problem of potential inhibitory effects on the late stage parasite. Limitations on the isolation of viable *P. falciparum* merozoites has also made studies of complex and/or low affinity protein interactions, such as signalling cascades, difficult. As a result, researchers have frequently turned to studying similar processes in other *Apicomplexa* spp. that are more amenable to these studies (for recent review see Gaur and Chitnis, 2011). Recently significant advances have been made in developing methods to isolate viable *P. falciparum* merozoites (Boyle et al., 2010b; Singh et al., 2010). These approaches have led to new insights into merozoite invasion biology and the role of specific proteins during invasion as well as opening up new avenues of research.

3. New methods to isolate viable merozoites of *P. falciparum*

Our method to isolate viable merozoites involves the generation of highly synchronous cultures of mature schizonts at high purity, followed by purification of merozoites using membrane filtration (for a flow diagram of isolation methods see Fig. 2, detailed meth-

ods have been described elsewhere (Boyle et al., 2010b)). An important aspect of this approach was to develop methods to obtain very tight synchronisation of the developmental stages of *P. falciparum* in vitro. This was achieved by firstly taking advantage of the specific invasion-inhibitory activity of heparin (Boyle et al., 2010a), which can be used to treat cultures for defined periods to block invasion events and increase the synchronicity of parasite stages. In addition, we took advantage of the schizont rupture inhibition activity of the cysteine protease inhibitor trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64) which prevents rupture of the schizont, but does not disrupt merozoite maturation or inhibit merozoite invasion (Boyle et al., 2010b). Mature schizonts can then be disrupted by membrane filtration to release and isolate merozoites. The invasive potential of merozoites is thereby increased compared with other methods that isolate merozoites from naturally ruptured schizonts over long time periods during which time many merozoites lose their invasion potential prior to being harvested from cultures. Membrane filtration allows for the isolation of merozoites largely free from intact schizonts, other parasites stages or uninfected RBCs. Following filtration, merozoites can be incubated with uninfected RBCs allowing for invasion to proceed. Invasion rates can then be measured immediately by

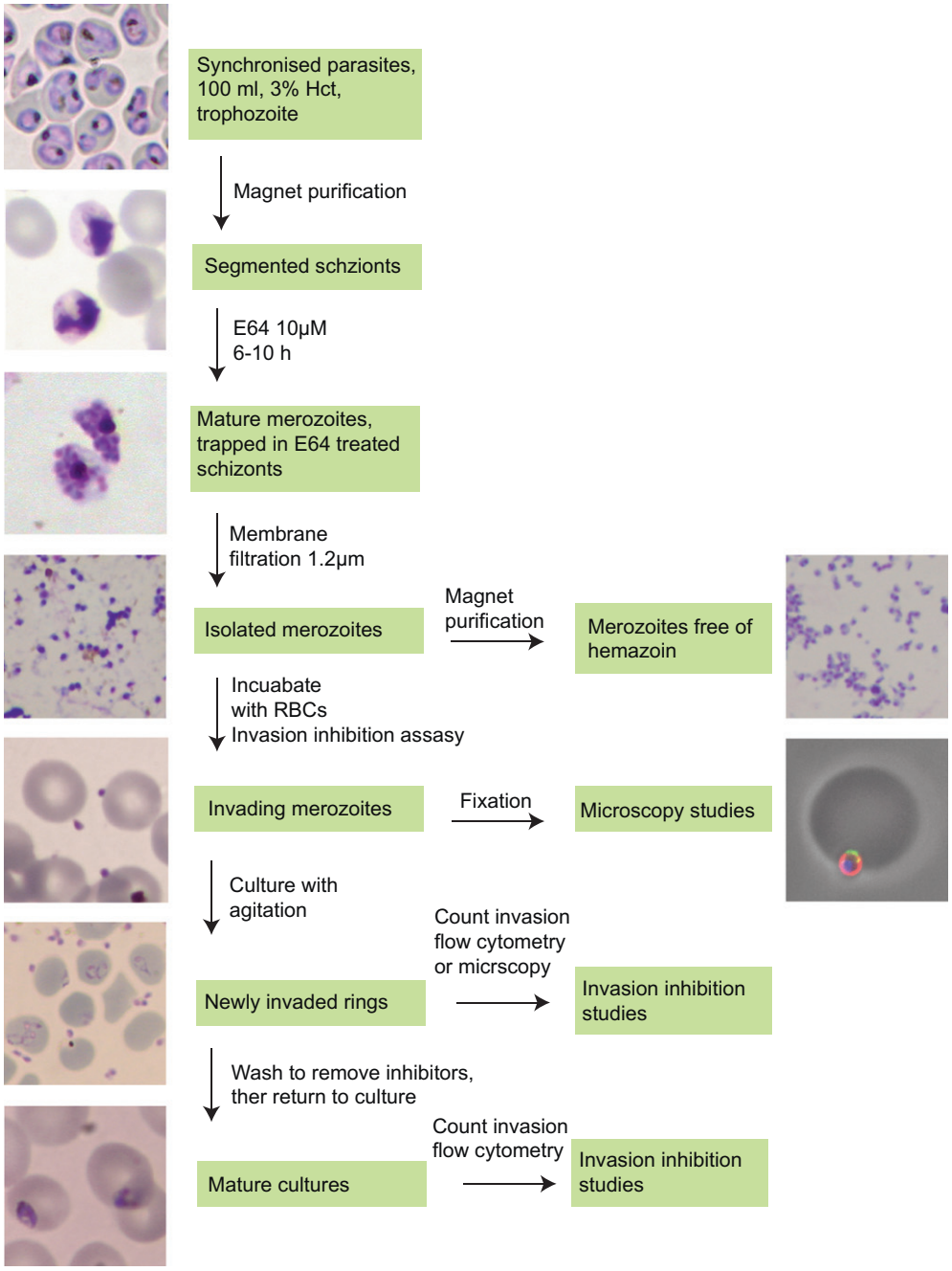

Fig. 2. Methods to isolate *Plasmodium falciparum* merozoites by membrane filtration. Highly synchronised trophozoites are magnet purified to remove uninfected red blood cells. When developed to segmented schizonts, parasites are treated with trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64) for 6–10 h until mature merozoites have formed. Merozoites are released by membrane filtration through a 1.2 μm filter. Isolated merozoites can be magnet purified to remove hemozoin for cellular applications or incubated with uninfected human red blood cells for invasion inhibition assays. Invading merozoites can be fixed for microscopy studies. For invasion inhibition assays, merozoites and human red blood cells are incubated in agitated cultures and then either analysed via flow cytometry or microscopy immediately, or following culture to mature parasites. Hct, haematocrit.

flow cytometry using GFP fluorescent parasites (Boyle et al., 2010b; Wilson et al., 2010, unpublished data), microscopy of ring-stages or by allowing parasites to develop over 24–48 h in standard culture conditions and then analysed by flow cytometry (Persson et al., 2006; Boyle et al., 2010b; Wilson et al., 2010). Potentially, an alternative methodology for the staining of ring stage parasites could also be applied, although the ability to distinguish between true intraerythrocytic early ring-stage parasites and merozoites bound to the outside of the RBC requires exploration (Jouin et al., 2004; Boyle et al., 2010b; Singh et al., 2010; Grimberg, 2011). Invasion rates of merozoites isolated by this method were estimated at approximately 15%, which is comparable with standard culture conditions of 20–40% for the same parasite strain (Boyle et al., 2010b). Importantly, this invasion efficiency was sufficient for the development of downstream applications such as specific merozoite invasion inhibition assays (mero-IIAs) and immunofluorescence and EM of invading merozoites (discussed below). Losses of merozoites occur during the purification process and are likely to result from schizonts being lost during magnet purification, loss of merozoites due to the large volume (300–400 μl) of filtrate trapped on the filter membrane, and losses due to schizonts not ruptured during membrane filtration. Further optimisation may increase isolation efficiency, however this isolation method has proven a robust and efficient approach for a number of different applications to date.

In contrast, the methods described by Singh et al. (2010) isolate merozoites following their natural egress from mature schizonts by differential centrifugation and synchronisation of parasite cultures was achieved using sorbitol. Intact schizonts are then separated from released merozoites by centrifugation at 500*g*, and merozoites are then harvested from supernatants by centrifugation at 3,300*g*. Isolated merozoites were reported to be approximately 25% viable, comparable with naturally released merozoites that were allowed to invade under standard culture conditions (Singh et al., 2010). Previously, it has been reported that naturally-released merozoites that are subsequently isolated by differential centrifugation are largely non-viable (Blackman, 1994). The success of the Singh et al. (2010) method maybe due to a greater level of synchronisation of parasite cultures that enables schizont rupture and merozoite release in cultures to occur over a shorter period that leads to better retention of viability; this is also important for maximising the viability of merozoites obtained using our membrane filtration approaches (Boyle et al., 2010b). While the reported rate of merozoite viability appears comparable between the two assays, to the best of our knowledge, no direct comparisons between the two methods of isolation have been performed. As our group has no experience in the Singh et al. (2010) method, this review will focus predominately on insights based on the isolation of merozoites via membrane filtration methods as described (Boyle et al., 2010b).

4. Insights into invasion biology

4.1. Merozoite survival

Previously, it has been widely accepted that *P. falciparum* merozoites have an intrinsically very short viability (minutes or less) in terms of the ability to invade a new RBC following egress from the schizont. The invasion process is indeed rapid and can occur within 1 min of initial contact with the RBC (Gilson and Crabb, 2009). However, until recently, direct evidence for survival of free merozoites was limited to studies of *P. knowlesi* merozoites that have an invasive half-life of approximately 5 min at 37 °C (Dennis et al., 1975; Johnson et al., 1980). It was speculated that the previous lack of success in isolating viable *P. falciparum* merozoites was a consequence of the half-life of *P. falciparum* being shorter than *P. knowlesi* spp. This concept has influenced other ideas surrounding invasion and immunity. For example, it was proposed that invasion inhibitory antibodies would require high concentrations to effectively block invasion due to rapid invasion kinetics (Saul, 1987) and the potential impact of cell-mediated immune mechanisms such as phagocytosis may be limited due to the small opportunity for phagocytosis to occur before a merozoite invades or becomes non-viable.

Using merozoites isolated via membrane filtration, the half-life of merozoites following release from schizonts was defined (Boyle et al., 2010b). Measurements of the time until invasion occurs after mixing merozoites with RBCs and the loss of invasive potential of merozoites when they are incubated for defined periods in the absence of RBCs suggest that the invasive half-life was approximately 8 min at 37 °C. This is substantially longer than previously proposed. Furthermore, it should be noted that a proportion of merozoites remained invasive for much longer periods of time, with 20% of invasion events occurring more than 10 min after contact with RBCs and invasion rates of up to 20% observed when merozoites were incubated for 10 min in the absence of RBCs prior to testing for invasive potential. Investigating the effect of temperature on the invasive half-life of merozoites resulted in a pattern of viability very similar to that previously reported for isolated *P. knowlesi* merozoites, with similar survival when merozoites were incubated on ice or at 37 °C and increased survival, with a half-life of approximately 20 min, when incubated at room temperature (Johnson et al., 1980; Boyle et al., 2010b).

It is hypothesised that the loss of invasiveness of merozoites may be due to aberrant triggering of invasion events such as the spontaneous cleavage and/or shedding of surface proteins, such as merozoite surface protein 1 (MSP1), which can occur in the absence of invasion (Langreth et al., 1978; Johnson et al., 1981; Blackman et al., 1991); premature release of rhoptry and microneme organelle proteins so that merozoites can no longer attach to RBCs (Johnson et al., 1980); or loss of metabolic activity. Our finding that a significant proportion of merozoites remain invasive for longer time periods has a number of implications for our understanding of merozoite invasion biology. Firstly, these data suggest that merozoites are not intrinsically short lived and may not be programmed to 'invade' immediately after egress from the schizont and instead need an additional signal to trigger invasion. This concept is supported by the observation that a proportion of merozoites maintain viability for long time periods in the absence of RBCs (>10 min). The ability of merozoites to maintain viability while inside E64-treated schizonts and then lose viability after membrane filtration supports the hypothesis that the trigger for invasion is an environmental change, such as the release of microneme proteins triggered by low potassium concentrations (Singh et al., 2010). However, it is also possible that microneme release is not directly linked to subsequent cascades of invasion steps, which may require other triggers such as contact with the RBC to commit to invasion (Riglar et al., 2011). Indeed, if the change in potassium levels was triggering the loss of viability due to microneme release, one would expect that this process would be uniform in all merozoites in the preparations used for these studies. In contrast, there is clearly a proportion of merozoites surviving for extended periods of time, suggesting that multiple mechanisms are at play in loss of viability.

The increased invasive half-life when kept at room temperature compared with 37 °C suggests that mechanisms such as metabolism or enzyme function resulting in cleavage and/or shedding of merozoite antigens involved in the invasion process may contribute to loss of viability. However, in preliminary experiments there is no evidence for the spontaneous shedding and loss of surface proteins over time, with similar proportions of isolated merozoites

labelled with MSP1-block two antibodies at 1–2 h post isolation, compared with immediately following filtration (M. Boyle, D. Wilson, J. Beeson, unpublished data). Interestingly, when merozoites were incubated at 40 °C prior to addition of RBCs to mimic febrile illness, a dramatic loss of invasiveness was seen compared with 37 °C (Boyle et al., 2010b). Febrile temperatures are known to inhibit intra-erythrocytic development of late stage parasites (Kwiatkowski, 1989; Long et al., 2001), however, the effect of fever on merozoite invasion per se is unknown. The loss of viability of merozoites when incubated at 40 °C may indicate that fever could be beneficial for malaria patients by reducing merozoite invasive capacity. The benefit of fever in malaria illness has been suggested previously, with increased parasite clearance times observed for patients treated with antipyretics (Brandts et al., 1997).

4.2. Implications of merozoite survival times on immune mechanisms and inhibition of invasion

It is difficult to extrapolate the estimated half-life of *P. falciparum* merozoites in vitro to the situation in vivo due to the challenges of reproducing normal physiological conditions in the laboratory, and the limitations in our knowledge of human infection. Indeed, currently there is no experimental evidence to inform our understanding of how long merozoites are extracellular and viable within the circulation or knowledge of the time over which invasion occurs in vivo. Late stage parasites are able to sequester in various microvascular beds and this process may result in sequestered schizonts undergoing rupture and release of merozoites in areas where blood flow is reduced (Dondorp, 2008), and where there are few RBCs for invasion by merozoites. It is interesting to speculate that under these conditions a longer invasive half-life may be advantageous to enable the merozoite to migrate to a capillary or small blood vessel containing uninfected RBCs receptive to invasion. Equally, it can also be hypothesised that when schizont rupture occurs in the blood-stream in vivo, merozoites might have ready access to a large number of uninfected RBCs. This could result in rapid invasion and there would be no need for merozoites to have extended viability. Further studies to understand these possibilities and the relevance of merozoite viability over time are needed.

From an immunological perspective, the longer than expected survival of merozoites may have important implications for our understanding of immunity to malaria. Approximately 5% of the blood volume circulates through the spleen every minute, and consequently it is likely that viable and intact merozoites would be captured by the spleen, which is thought to be able to trap particles greater than 200 nm in size (Moghimi et al., 1993; Champion et al., 2007). Correctly presented merozoite antigens would thus be exposed to splenic immune cells for phagocytosis, antigen presentation and activation of the immune system. Furthermore, if invasion occurs over several minutes, antibodies would have a much longer time to coat merozoites and block invasion than was previously estimated (Saul, 1987), and cellular immune responses such as phagocytic uptake of merozoites may play an important role, as previously proposed (Bouharoun-Tayoun et al., 1990). Based on the model by Saul (1987), if merozoites come into contact with RBCs and are able to invade within 1–2 min post egress, then higher concentrations of antibodies would be required to inhibit invasion (estimated at $\geqslant$10 μg/ml). While antibodies with invasion inhibitory activity against important invasion ligands, such as apical membrane antigen 1 (AMA1), have been reported at high enough concentrations to meet this theoretical level for vaccinated unexposed individuals (Duncan et al., 2011), naturally exposed children (Thera et al., 2011) and adults (Hodder et al., 2001), it is possible that lower concentrations of antibody are also inhibitory. Future studies are needed to better understand the in vivo kinetics of merozoite-RBC interactions and the implications of these kinetics on both vaccine-induced and naturally-acquired immune responses.

4.3. The role of serum components and complement in invasion

Serum components, such as complement, are important contributors to innate immunity and have roles in inhibiting pathogen replication. However, many infectious organisms co-opt serum components to facilitate infection. Using conventional growth assays, it has been previously shown that parasite replication in vitro does not require human serum components (many groups routinely culture parasites using AlbumaxII supplemented medium and not human serum). However, these assays can only measure serum at 10–20% concentrations, well below physiological concentrations, and have not specifically assessed the involvement of serum components for invasion. Utilising isolated merozoites in mero-IIAs, it was observed that merozoite invasion occurred efficiently in the presence or absence of serum; at high serum concentrations invasion occurred efficiently, but was slightly reduced compared with serum-free conditions (Boyle et al., 2010b). Subsequent experiments have shown that complement does not appear to inhibit or enhance merozoite invasion, with comparable levels of invasion observed in heat-inactivated and normal human serum (at concentrations up to 80%) (M. Boyle, J. Beeson, unpublished data). Together these findings suggest that host serum components are not important in merozoite invasion. Of note, an interaction between the merozoite protein reticulocyte binding homologue 4 (Rh4) and human complement receptor 1 (CR1) on the RBC surface has been implicated in invasion via the sialic-acid independent pathway (Spadafora et al., 2010; Tham et al., 2010). Additionally, a complex between CR1, Rh4 and the serum complement component C4b has recently been described (Tham et al., 2011). It has been proposed that this tertiary structure may be beneficial to the parasite by enhancing the affinity of the Rh4–CR1 interaction (Tham et al., 2011) presumably resulting in higher invasion efficiency. If CR1/Rh4 pathway was enhanced by binding C4b, it would be expected that higher invasion would be observed in normal serum compared with heat-inactivated serum, which lacks active complement. Previously published data showed that the addition of C4b or C3b to standard GIA did not enhance or disrupt invasion (Tham et al., 2011). This suggests that complement components are not involved in enhancing merozoite invasion.

4.4. Insights into mechanisms of invasion with microscopy studies

Using isolated invasive merozoites, methods were developed to fix merozoites during invasion for microscopy studies. Previously, EM imaging of merozoite invasion has been limited to other *Plasmodium* spp. such as *P. knowlesi*. To the best of our knowledge, there had only been one previously reported EM of *P. falciparum* invading merozoites that was captured serendipitously (Langreth et al., 1978). With isolated viable merozoites, fixation of invading merozoites can be consistently achieved, allowing for in-depth investigation and localisation of proteins in both immunofluorescence and EM studies (Fig. 3). Although these imaging techniques only capture merozoites invading at single snap-shots in time (as opposed to single cell time-lapse microscopy), and therefore may not entirely reflect the true sequence of events, this technique has none-the-less led to a number of important insights into specific mechanisms of invasion; the confirmation that commitment to invasion occurs after attachment of the merozoite to RBCs and formation of the tight junction, triggering subsequent events of rhoptry secretion, surface-protein shedding and actin–myosin motor activation (Riglar et al., 2011). The proposed shedding of the major surface protein MSP1 at the tight junction between the

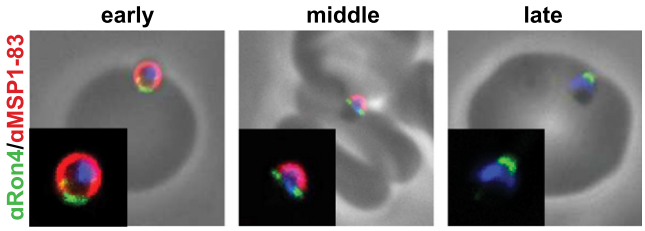

Fig. 3. Imaging of merozoites during invasion. Isolated merozoites are able to be fixed during invasion for microscopy studies. Here, merozoites were fixed during invasion and labelled with antibodies to *Plasmodium falciparum* rhoptry neck protein (PfRON)4 (green) and merozoite surface protein (MSP)1-83 kDa fragment (red). PfRON4 effectively marked the point of invasion between the merozoite and red blood cell allowing for analysis of surface protein shedding. MSP1-83 was shed at the point of tight junction between the merozoite and human red blood cell. Preparation and staining of cells was performed as described (Boyle et al., 2010b). Antibodies for labelling were kindly provided by D. Conway and K. Tetteh (MSP1-83) and A. Cowman (RON4).

merozoite and RBC has also been confirmed (Boyle et al., 2010b; Riglar et al., 2011), as shown previously with *P. knowlesi* (Blackman et al., 1996). The mechanisms mediating MSP1 shedding now require further study; shedding is known to occur with the cleavage of MSP1-42 to MSP1-33 and MSP1-19, mediated by the by subtilisin protease, PfSUB2 (Harris et al., 2005). However, the localization of PfSUB2 during invasion appears different from the point of shedding with PfSUB2 tracking to the posterior of the invading merozoite before MSP1 shedding occurs (Riglar et al., 2011). Furthermore, it has been proposed by the earliest EM images of invading *Plasmodium* merozoites that all merozoite surface proteins are shed during invasion, with a complete loss of surface coat on the merozoite after the point of tight junction with the RBC (Bannister et al., 1975; Aikawa et al., 1978, 1981). However, while this shedding has been clearly shown for MSP1, direct evidence for the loss of other surface proteins during invasion is currently lacking. Our ongoing studies suggest that cleavage and shedding of surface proteins is not universal, but instead is selective to specific proteins (M. Boyle, J. Beeson, unpublished data). While it is thought that surface proteins are involved in mediating initial contact steps between the merozoite and the RBC, there are limited data to support the role of surface proteins directly in initial contact. To the best of our knowledge only one RBC receptor has been reported for a merozoite surface protein, with MSP1-19 reported to bind Band3 (Goel et al., 2003). Other studies suggest that MSP1-42, but not the full-length unprocessed form of MSP1, may bind heparin-like molecules on the RBC (Boyle et al., 2010a). The characterization of surface protein localisation during invasion may shed light on to the potential multiple functions of surface proteins during invasion and downstream of invasion.

Further application of these methods in merozoite isolation and microscopy have enabled the localisation of a number of merozoite proteins involved in invasion, including rhoptry and motor complex associated proteins (Chen et al., 2011; Triglia et al., 2011; Wong et al., 2011; Angrisano et al., 2012). In other studies, isolated merozoites have been used for the investigation of cellular immune responses (Costa et al., 2011), with the ability to remove contaminating hemozoin crystals from filtrates allowing the application of isolated merozoites to other cellular assays (Boyle et al., 2010b). In addition, isolation of viable merozoites using differential sedimentation techniques has shed light on the triggers and signalling pathways involved in invasion (Singh et al., 2010). The development of methods to isolate viable merozoites has therefore opened new avenues to study a range of aspects of merozoite invasion biology and immune mechanisms targeting merozoite stages.

5. Drug and vaccine development

5.1. Development of specific invasion inhibition assays

Prior to the development of a robust method for purification of invasive *P. falciparum* merozoites, in vitro studies looking at potential invasion inhibitory activity of antibodies and compounds for vaccine and drug development have relied on measuring inhibition of total blood-stage replication of parasites, rather than inhibition of invasion. This approach has the obvious disadvantage that any inhibitory activity measured could also result from inhibition of late stage parasite development, schizont rupture and/or growth of the newly invaded ring-stage parasite. The development of reliable methods to isolate viable *P. falciparum* merozoites with high yield has now allowed for the study of invasion inhibitors specifically (Boyle et al., 2010b).

As a proof of principle during the initial assessment of mero-IIAs, the activity of known invasion inhibitors was confirmed; this included heparin (inhibits initial attachment of the merozoite to RBC), AMA1 targeting peptide R1 and monoclonal antibody 1F9 (which inhibit the formation of the tight junction), and actin inhibitor cytochalasin D (inhibits gliding motility and merozoite penetration of the RBC). Comparison of the inhibitory activity of the cysteine protease inhibitor E64 and the serine/cysteine protease inhibitor N-α-Tosyl-L-lysine chloromethyl ketone (TLCK) also highlighted the value of mero-IIAs; while E64 is inhibitory to schizont rupture, it has no inhibitory activity against merozoite invasion, suggesting that cysteine proteases may have a limited role in merozoite invasion. In contrast, the serine/cysteine protease inhibitor TLCK was inhibitory to both invasion and rupture (Boyle et al., 2010b), as has been shown for *P. knowlesi* (Hadley et al., 1983), suggesting that serine proteases may play an important role in invasion.

In the case of the AMA1 inhibitors R1 and 1F9 (Boyle et al., 2010b), as well as heparin (M. Boyle, D. Wilson, J. Beeson, unpublished data), mero-IIAs were more sensitive than GIAs for measuring inhibitory activity. While the reasons for greater sensitivity in mero-IIA were not explored, it is possible that degradation of inhibitory compounds occurs in GIAs (typically conducted over 48–72 h incubation) compared with mero-IIAs where the compound is added immediately prior to invasion. It is also possible that differences in haematocrit and merozoite: RBC ratios between the two assays may affect inhibition activity, a phenomenon which has been suggested in previous reports on inhibitory antibodies (Saul et al., 1982); however, these factors have not been found to have a major effect in other studies by our group (Persson et al., 2006; Wilson et al., 2010). Although mero-IIAs were more sensitive and specific than GIAs, at present it is not known which assay is likely to be more relevant to the activity of compounds or antibodies in vivo. Indeed, compounds that function on inhibiting intra-erythrocytic development, or targeting processes upstream of invasion such as merozoite protein maturation, are more appropriately tested in GIA.

5.2. Inhibitors of invasion as novel therapeutic agents

Currently, all licensed malaria therapeutic agents target the intra-erythrocytic development of the parasite. Merozoite invasion, as a key point in parasite development, may also have potential for intervention with targeting of proteases or essential proteins, as discussed previously (O'Donnell and Blackman, 2005; Wegscheid-Gerlach et al., 2010; Macraild et al., 2011). There may be potential for the use of merozoite invasion inhibitory drugs in combination with drugs targeting the intra-erythrocytic development. Combination treatments targeting different stages may

increase treatment efficacy or the rate of parasite clearance, and decrease the emergence of drug resistance. Our ongoing studies of antimalarial drug activity suggest that none of the antimalarial agents in widespread use have activity against merozoite invasion (D. Wilson, J. Beeson, unpublished data).

Polysulphated heparin-like-molecules (HLMs) are inhibitory to invasion (Boyle et al., 2010a) and may have potential as antimalarial agents (Havlik et al.,1994, 2005; Clark et al., 1997; Evans et al., 1998; Adams et al., 2006; Boyle et al., 2010a). From testing a number of modified HLMs and semi-synthetic carbohydrates based on K5 polysaccharides that have different compositions, it has been previously shown that the invasion-inhibitory activity of these compounds is specific and dependent on the level and degree of sulphation, as well as backbone structure and chain length (Boyle et al., 2010a). The activity of these compounds appears to result from their ability to bind to MSP1-42 (Boyle et al., 2010a), but they may bind other proteins involved in invasion (Baum et al., 2009; Kobayashi et al., 2010). In human malaria, heparin has previously been used to treat disseminated intravascular coagulation (Smitskamp and Wolthuis, 1971; Mitchell et al., 1974; Munir et al., 1980; Rampengan, 1991). However, due to the high risk of bleeding resulting from anticoagulant activity, heparin treatment is no longer recommended (World Health Organization, 2010). Our studies and others suggest it is possible to reduce or remove the anticoagulant activity of HLMs while maintaining inhibitory activity against *P. falciparum* (Vogt et al., 2006; Boyle et al., 2010a). Indeed, periodate-treated HLMs have been identified that have no anticoagulation activity nor toxicity in heart, liver, kidney or lung tissue (Yu et al., 2010) and shown to have activity in a murine model (Vogt et al., 2006). Furthermore, the polysaccharide curdlan sulphate has been tested in a small Phase IIB human trial which suggested that treatment reduced malaria disease severity (Havlik et al., 2005). Curdlan sulphate inhibits invasion, but also has broader biological activities that may contribute to its activity. It is possible that in the future oligosaccharide HLM compounds with increased bioavailability/kinetics/safety and improved inhibitory activity may be developed.

The potential for the development of antimalarial drugs targeting merozoite invasion is not limited to HLMs. A number of potential drug targets have been identified such as proteases and kinases that have essential roles in processes such as protein cleavage, rhoptry and microneme release, signalling and activation of the invasion motor. Further, new libraries of drugs with antimalarial properties have recently been provided to the research community for further drug research development (Gamo et al., 2010; Guiguemde et al., 2010), some of which may have inhibitory activity against merozoite invasion.

5.3. Vaccines and immunity

The importance of humoral responses against blood stages has been well demonstrated, with studies showing that immunoglobulin from immune adults can be transferred to infected children, resulting in recovery and parasite clearance (McGregor, 1964; Sabchareon et al., 1991). However, the mechanisms mediating protective immunity are unclear as correlates of protection remain poorly characterised with respect to both the mechanisms and targets of immunity. Antibodies targeting merozoites surface antigens and invasion ligands are thought to contribute to immunity (Richards and Beeson, 2009; Fowkes et al., 2010). Assays that are currently used to measure functional antibodies to merozoite antigens include GIAs, antibody dependent cellular inhibition assays (ADCI), and neutrophil respiratory burst (Bouharoun-Tayoun and Druilhe, 1992; Persson et al., 2006; Joos et al., 2010). GIAs typically measure the ability of total or dialysed serum, or purified serum IgG, to inhibit growth of parasites over one or two cycles of replication in in vitro, with parasitemia measured by microscopy, flow cytometry or other methods (O'Donnell et al., 2001; Kennedy et al., 2002; Bergmann-Leitner et al., 2006; Persson et al., 2006, 2008; McCallum et al., 2008; Wilson et al., 2010, 2011). These assays are often reported to be a measure of 'invasion inhibitory' activity; however, as discussed earlier, the nature of the assay means that inhibitory effects measured may be due to activity distinct from direct merozoite invasion inhibition, such as inhibition of intra-erythrocytic growth, schizont rupture and/or merozoite dispersal.

Significant efforts by multiple laboratories has been undertaken to increase the validity and throughput of GIAs by increasing accuracy of parasitemia measurements using flow cytometry (compared with microscopy), using dialyzed serum samples to remove inhibitory drugs, comparing multiple GIA techniques, testing serum against drug-resistant lines or by the use of purified IgG (Kennedy et al., 2002; Persson et al., 2006; Bergmann-Leitner et al., 2008; Dent et al., 2008; Wilson et al., 2010). However, despite the best efforts, the validity of GIA as a measure of protective immune function remains unclear. Investigators have reported either no associations between GIA and protection from malaria (Marsh et al., 1989; McCallum et al., 2008) or only weak associations (John et al., 2004; Dent et al., 2008; Crompton et al., 2010). Furthermore, some investigators have reported growth enhancement by some purified IgG samples (Shi et al., 1999). This does not necessarily suggest that growth inhibition of *P. falciparum* is not important in protective immunity, but may indeed reflect the limitations of GIAs to detect activity due to assay limitation or that GIA is only one component mediating protection. In the testing of antibodies in serum, GIAs are limited to 1:10 or 1:5 serum dilutions as high serum concentrations in vitro causes non-specific inhibition. Consequently, concentrations of antibodies in GIA may be below the threshold for the detection of growth inhibition activity (Wilson et al., 2011). While others have used purified IgG at higher concentrations, GIAs are further limited as they do not account for the possibility of antibodies interacting with complement factors as most studies have tested serum after heat inactivation (which disrupts complement function) or have used purified IgG. The development of a specific mero-IIA with isolated viable merozoites may overcome some of these limitations of standard GIA. Our laboratory is now applying mero-IIA to the study of mechanism of antibody targeting merozoite invasion specifically. In ongoing studies, we have established that some individuals in malaria-endemic areas have antibodies that can directly inhibit invasion, but the relative importance of these antibodies in protective immunity remains to be established (M. Boyle, J. Beeson, unpublished data).

6. Future directions

The continuing high burden of malaria globally is a sobering reminder of the urgent need for vaccines and new therapeutic agents for malaria prevention and treatment. Merozoite invasion of RBCs is an attractive target for both vaccine and drug development, highlighting the importance of further studies to understand the mechanisms and interactions involved in invasion, and to identify and evaluate candidate anti-merozoite vaccines and invasion-inhibitory compounds. New methods to isolate viable merozoites provide an important tool for studies of invasion and invasion inhibitors, as described in this review. These methods have many potential further applications, such as proteomics, metabolomics, and transcriptional analyses. Furthermore, it may be possible to use isolated merozoites for transfection to obtain higher transfection efficiencies, as is done with *Plasmodium berghei* (van Dijk et al., 1995). Further advances to the methods described recently

(Boyle et al., 2010b; Singh et al., 2010) could lead to major new insights that enhance our understanding of the process of *P. falciparum* merozoite invasion, the most fleeting stage of the intracellular parasite lifecycle.

Acknowledgements

The authors were supported by funding from the Australian Research Council (Future Fellowship to J.G.B.), National Health and Medical Research Council of Australia (fellowship to D.W.W. and Infrastructure for Research Institutes Support Scheme), the Australian Government (Australian postgraduate award to M.J.B), the University of Melbourne, Australia, Faculty of Medicine, Dentistry and Health Sciences (Postgraduate Top-Up Award M.J.B) and the Victorian State Government Operational Infrastructure Support, Australia.

References

Adams, Y., Freeman, C., Schwartz-Albiez, R., Ferro, V., Parish, C.R., Andrews, K.T., 2006. Inhibition of *Plasmodium falciparum* growth in vitro and adhesion to chondroitin-4-sulfate by the heparan sulfate mimetic PI-88 and other sulfated oligosaccharides. Antimicrob. Agents Chemother. 50, 2850–2852.

Aikawa, M., Miller, L.H., Johnson, J., Rabbege, J., 1978. Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. J. Cell Biol. 77, 72–82.

Aikawa, M., Miller, L.H., Rabbege, J.R., Epstein, N., 1981. Freeze-fracture study on the erythrocyte membrane during malarial parasite invasion. J. Cell Biol. 91, 55–62.

Angrisano, F., Riglar, D.T., Sturm, A., Volz, J.C., Delves, M.J., Zuccala, E.S., Turnbull, L., Dekiwadia, C., Olshina, M.A., Marapana, D.S., Wong, W., Mollard, V., Bradin, C.H., Tonkin, C.J., Gunning, P.W., Ralph, S.A., Whitchurch, C.B., Sinden, R.E., Cowman, A.F., McFadden, G.I., Baum, J., 2012. Spatial localisation of actin filaments across developmental stages of the malaria parasite. PLoS One 7, e32188.

Bannister, L.H., Butcher, G.A., Dennis, E.D., Mitchell, G.H., 1975. Structure and invasive behaviour of *Plasmodium knowlesi* merozoites in vitro. Parasitology 71, 483–491.

Bannister, L.H., Dluzewski, A.R., 1990. The ultrastructure of red cell invasion in malaria infections: a review. Blood Cells 16, 257–292.

Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T.-W., Green, J.L., Holder, A.A., Cowman, A.F., 2006. A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. J. Biol. Chem. 281, 5197–5208.

Baum, J., Chen, L., Healer, J., Lopaticki, S., Boyle, M., Triglia, T., Ehlgen, F., Ralph, S.A., Beeson, J.G., Cowman, A.F., 2009. Reticulocyte-binding protein homologue 5 – an essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. Int. J. Parasitol. 39, 371–380.

Beeson, J.G., Crabb, B.S., 2007. Towards a vaccine against *Plasmodium vivax* malaria. PLoS Med. 4, e350.

Bergmann-Leitner, E.S., Duncan, E.H., Mullen, G.E., Burge, J.R., Khan, F., Long, C.A., Angov, E., Lyon, J.A., 2006. Critical evaluation of different methods for measuring the functional activity of antibodies against malaria blood stage antigens. Am. J. Trop. Med. Hyg. 75, 437–442.

Bergmann-Leitner, E.S., Duncan, E.H., Burge, J.R., Spring, M., Angov, E., 2008. Miniaturization of a high-throughput pLDH-based *Plasmodium falciparum* growth inhibition assay for small volume samples from preclinical and clinical vaccine trials. Am. J. Trop. Med. Hyg. 78, 468–471.

Blackman, M.J., Ling, I.T., Nicholls, S.C., Holder, A.A., 1991. Proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains. Mol. Biochem. Parasitol. 49, 29–33.

Blackman, M.J., 1994. Purification of *Plasmodium falciparum* merozoites for analysis of the processing of merozoite surface protein-1. Methods Cell Biol. 45, 213–220.

Blackman, M.J., Dennis, E.D., Hirst, E.M., Kocken, C.H., Scott-Finnigan, T.J., Thomas, A.W., 1996. *Plasmodium knowlesi*: secondary processing of the malaria merozoite surface protein-1. Exp. Parasitol. 83, 229–239.

Bouharoun-Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T., Druilhe, P., 1990. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J. Exp. Med. 172, 1633–1641.

Bouharoun-Tayoun, H., Druilhe, P., 1992. *Plasmodium falciparum* malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. Infect. Immun. 60, 1473–1481.

Boyle, M.J., Richards, J.S., Gilson, P.R., Chai, W., Beeson, J.G., 2010a. Interactions with heparin-like molecules during erythrocyte invasion by *Plasmodium falciparum* merozoites. Blood 115, 4559–4568.

Boyle, M.J., Wilson, D.W., Richards, J.S., Riglar, D.T., Tetteh, K.K.A., Conway, D.J., Ralph, S.A., Baum, J., Beeson, J.G., 2010b. Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. Proc. Nat. Acad. Sci. U.S.A. 107, 14378–14383.

Brandts, C.H., Ndjavé, M., Graninger, W., Kremsner, P.G., 1997. Effect of paracetamol on parasite clearance time in *Plasmodium falciparum* malaria. Lancet 350, 704–709.

Champion, J.A., Katare, Y.K., Mitragotri, S., 2007. Particle shape: a new design parameter for micro- and nanoscale drug delivery carriers. J. Control. Release 121, 3–9.

Chen, L., Lopaticki, S., Riglar, D.T., Dekiwadia, C., Uboldi, A.D., Tham, W.-H., O'Neill, M.T., Richard, D., Baum, J., Ralph, S.A., Cowman, A.F., 2011. An EGF-like protein forms a complex with PfRh5 and is required for invasion of human erythrocytes by *Plasmodium falciparum*. PLoS Pathog. 7, e1002199.

Cho, S., Kim, S., Kim, Y., Park, Y., 2012. Optical imaging techniques for the study of malaria. Trends Biotechnol. 30, 71–79.

Clark, D.L., Su, S., Davidson, E.A., 1997. Saccharide anions as inhibitors of the malaria parasite. Glycoconjugate J. 14, 473–479.

Costa, G., Loizon, S., Guenot, M., Mocan, I., Halary, F., de Saint-Basile, G., Pitard, V., Déchanet-Merville, J., Moreau, J.-F., Troye-Blomberg, M., Mercereau-Puijalon, O., Behr, C., 2011. Control of *Plasmodium falciparum* erythrocytic cycle: γδ T cells target the red blood cell-invasive merozoites. Blood 118, 6952–6962.

Cowman, A.F., Baldi, D.L., Healer, J., Mills, K.E., O'Donnell, R.A., Reed, M.B., Triglia, T., Wickham, M.E., Crabb, B.S., 2000. Functional analysis of proteins involved in *Plasmodium falciparum* merozoite invasion of red blood cells. FEBS Lett. 476, 84–88.

Crabb, B.S., Cowman, A.F., 1996. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. Proc. Nat. Acad. Sci. U.S.A. 93, 7289–7294.

Crompton, P.D., Miura, K., Traore, B., Kayentao, K., Ongoiba, A., Weiss, G., Doumbo, S., Doumtabe, D., Kone, Y., Huang, C.-Y., Doumbo, O.K., Miller, L.H., Long, C.A., Pierce, S.K., 2010. In vitro growth-inhibitory activity and malaria risk in a cohort study in mali. Infect. Immun. 78, 737–745.

Dennis, E.D., Mitchell, G.H., Butcher, G.A., Cohen, S., 1975. In vitro isolation of *Plasmodium knowlesi* merozoites using polycarbonate sieves. Parasitology 71, 475–481.

Dent, A.E., Bergmann-Leitner, E.S., Wilson, D.W., Tisch, D.J., Kimmel, R., Vulule, J., Sumba, P.O., Beeson, J.G., Angov, E., Moormann, A.M., Kazura, J.W., 2008. Antibody-mediated growth inhibition of *Plasmodium falciparum*: relationship to age and protection from parasitemia in Kenyan children and adults. PLoS One 3, e3557.

Dondorp, A.M., 2008. Clinical significance of sequestration in adults with severe malaria. Transfus. Clin. Biol. 15, 56–57.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W., Lee, S.J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S.S., Yeung, S., Singhasivanon, P., Day, N.P.J., Lindegardh, N., Socheat, D., White, N.J., 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. New Engl. J. Med. 361, 455–467.

Duncan, C.J.A., Sheehy, S.H., Ewer, K.J., Douglas, A.D., Collins, K.A., Halstead, F.D., Elias, S.C., Lillie, P.J., Rausch, K., Aebig, J., Miura, K., Edwards, N.J., Poulton, I.D., Hunt-Cooke, A., Porter, D.W., Thompson, F.M., Rowland, R., Draper, S.J., Gilbert, S.C., Fay, M.P., Long, C.A., Zhu, D., Wu, Y., Martin, L.B., Anderson, C.F., Lawrie, A.M., Hill, A.V.S., Ellis, R.D., 2011. Impact on malaria parasite multiplication rates in infected volunteers of the protein-in-adjuvant vaccine AMA1-C1/alhydrogel+CPG 7909. PLoS One 6, e22271.

Dvorak, J.A., Miller, L.H., Whitehouse, W.C., Shiroishi, T., 1975. Invasion of erythrocytes by malaria merozoites. Science 187, 748–750.

Dvorin, J.D., Martyn, D.C., Patel, S.D., Grimley, J.S., Collins, C.R., Hopp, C.S., Bright, A.T., Westenberger, S., Winzeler, E., Blackman, M.J., Baker, D.A., Wandless, T.J., Duraisingh, M.T., 2010. A plant-like kinase in *Plasmodium falciparum* regulates parasite egress from erythrocytes. Science 328, 910–912.

Evans, S.G., Morrison, D., Kaneko, Y., Havlik, I., 1998. The effect of curdlan sulphate on development in vitro of *Plasmodium falciparum*. Tran. R. Soc. Trop. Med. Hyg. 92, 87–89.

Farrow, R.E., Green, J., Katsimitsoulia, Z., Taylor, W.R., Holder, A.A., Molloy, J.E., 2011. The mechanism of erythrocyte invasion by the malarial parasite, *Plasmodium falciparum*. Sem. Cell. Dev. Biol., 1–8.

Fowkes, F.J.I., Richards, J.S., Simpson, J.A., Beeson, J.G., 2010. The relationship between anti-merozoite antibodies and incidence of *Plasmodium falciparum* malaria: a systematic review and meta-analysis. PLoS Med. 7, e1000218.

Gamo, F.J., Sanz, L.M., Vidal, J., de Cozar, C., Alvarez, E., Lavandera, J.L., Vanderwall, D.E., Green, D.V., Kumar, V., Hasan, S., Brown, J.R., Peishoff, C.E., Cardon, L.R., Garcia-Bustos, J.F., 2010. Thousands of chemical starting points for antimalarial lead identification. Nature 465, 305–310.

Gaur, D., Mayer, D.C.G., Miller, L.H., 2004. Parasite ligand–host receptor interactions during invasion of erythrocytes by *Plasmodium* merozoites. Int. J. Parasitol. 34, 1413–1429.

Gaur, D., Chitnis, C.E., 2011. Molecular interactions and signaling mechanisms during erythrocyte invasion by malaria parasites. Curr. Opin. Microbiol. 14, 422–428.

Gilson, P.R., Crabb, B.S., 2009. Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. Int. J. Parasitol. 39, 91–96.

Glushakova, S., Yin, D., Li, T., Zimmerberg, J., 2005. Membrane transformation during malaria parasite release from human red blood cells. Curr. Biol. 15, 1645–1650.

Glushakova, S., Humphrey, G., Leikina, E., Balaban, A., Miller, J., Zimmerberg, J., 2010. New stages in the program of malaria parasite egress imaged in normal and sickle erythrocytes. Curr. Biol. 20, 1117–1121.

Goel, V.K., Li, X., Chen, H., Liu, S.-C., Chishti, A.H., Oh, S.S., 2003. Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. Proc. Nat. Acad. Sci. U.S.A. 100, 5164–5169.

Grimberg, B.T., 2011. Methodology and application of flow cytometry for investigation of human malaria parasites. J. Immunol. Methods 367, 1–16.

Guiguemde, W.A., Shelat, A.A., Bouck, D., Duffy, S., Crowther, G.J., Davis, P.H., Smithson, D.C., Connelly, M., Clark, J., Zhu, F., Jiménez-Díaz, M.B., Martinez, M.S., Wilson, E.B., Tripathi, A.K., Gut, J., Sharlow, E.R., Bathurst, I., El Mazouni, F., Fowble, J.W., Forquer, I., Mcginley, P.L., Castro, S., Angulo-Barturen, I., Ferrer, S., Rosenthal, P.J., Derisi, J.L., Sullivan, D.J., Lazo, J.S., Roos, D.S., Riscoe, M.K., Phillips, M.A., Rathod, P.K., van Voorhis, W.C., Avery, V.M., Guy, R.K., 2010. Chemical genetics of *Plasmodium falciparum*. Nature 465, 311–315.

Hadley, T., Aikawa, M., Miller, L.H., 1983. *Plasmodium knowlesi*: studies on invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. Exp. Parasitol. 55, 306–311.

Hanssen, E., Goldie, K.N., Tilley, L., 2010a. Ultrastructure of the asexual blood stages of *Plasmodium falciparum*. Methods Cell Biol. 96, 93–116.

Hanssen, E., McMillan, P.J., Tilley, L., 2010b. Cellular architecture of *Plasmodium falciparum*-infected erythrocytes. Int. J. Parasitol. 40, 1127–1135.

Harris, P.K., Yeoh, S., Dluzewski, A.R., O'Donnell, R.A., Withers-Martinez, C., Hackett, F., Bannister, L.H., Mitchell, G.H., Blackman, M.J., 2005. Molecular identification of a malaria merozoite surface sheddase. PLoS Pathog. 1, 241–251.

Harvey, K.L., Gilson, P.R., Crabb, B.S., 2012. A model for the progression of receptor–ligand interactions during erythrocyte invasion by *Plasmodium falciparum*. Int. J. Parasitol. 42, 567–573.

Havlik, I., Rovelli, S., Kaneko, Y., 1994. The effect of curdlan sulphate on in vitro growth of *Plasmodium falciparum*. Trans. R. Soc. Trop. Med. Hyg. 88, 686–687.

Havlik, I., Looareesuwan, S., Vannaphan, S., Wilairatana, P., Krudsood, S., Thuma, P.E., Kozbor, D., Watanabe, N., Kaneko, Y., 2005. Curdlan sulphate in human severe/cerebral *Plasmodium falciparum* malaria. Trans. R. Soc. Trop. Med. Hyg. 99, 333–340.

Hodder, A.N., Crewther, P.E., Anders, R.F., 2001. Specificity of the protective antibody response to apical membrane antigen 1. Infect. Immun. 69, 3286–3294.

John, C.C., O'Donnell, R.A., Sumba, P.O., Moormann, A.M., de Koning-Ward, T.F., King, C.L., Kazura, J.W., Crabb, B.S., 2004. Evidence that invasion-inhibitory antibodies specific for the 19-kDa fragment of merozoite surface protein-1 (MSP-1 19) can play a protective role against blood-stage *Plasmodium falciparum* infection in individuals in a malaria endemic area of Africa. J. Immunol. 173, 666–672 (Baltimore, Md: 1950).

Johnson, J.G., Epstein, N., Shiroishi, T., Miller, L.H., 1980. Factors affecting the ability of isolated *Plasmodium knowlesi* merozoites to attach to and invade erythrocytes. Parasitology 80, 539–550.

Johnson, J.G., Epstein, N., Shiroishi, T., Miller, L.H., 1981. Identification of surface proteins on viable *Plasmodium knowlesi* merozoites. J. Protozool. 28, 160–164.

Joos, C., Marrama, L., Polson, H.E.J., Corre, S., Diatta, A.-M., Diouf, B., Trape, J.-F., Tall, A., Longacre, S., Perraut, R., 2010. Clinical protection from falciparum malaria correlates with neutrophil respiratory bursts induced by merozoites opsonized with human serum antibodies. PLoS One 5, e9871.

Jouin, H., Daher, W., Khalife, J., Ricard, I., Puijalon, O.M., Capron, M., Dive, D., 2004. Double staining of *Plasmodium falciparum* nucleic acids with hydroethidine and thiazole orange for cell cycle stage analysis by flow cytometry. Cytometry Part A J. Int. Soc. Anal. Cytol. 57, 34–38.

Kennedy, M.C., Wang, J., Zhang, Y., Miles, A.P., Chitsaz, F., Saul, A., Long, C.A., Miller, L.H., Stowers, A.W., 2002. In vitro studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. Infect. Immun. 70, 6948–6960.

Khattab, A., Bonow, I., Schreiber, N., Petter, M., Schmetz, C., Klinkert, M.-Q., 2008. *Plasmodium falciparum* variant STEVOR antigens are expressed in merozoites and possibly associated with erythrocyte invasion. Malaria J. 7, 137.

Kobayashi, K., Kato, K., Sugi, T., Takemae, H., Pandey, K., Gong, H., Tohya, Y., Akashi, H., 2010. *Plasmodium falciparum* BAEBL binds to heparan sulfate proteoglycans on the human erythrocyte surface. J. Biol. Chem 285, 1716–1725.

Kwiatkowski, D., 1989. Febrile temperatures can synchronize the growth of *Plasmodium falciparum* in vitro. J. Exp. Med. 169, 357–361.

Ladda, R., Aikawa, M., Sprinz, H., 1969. Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. J. Parasitol. 55, 633–644.

Langreth, S.G., Nguyen-Dinh, P., Trager, W., 1978. *Plasmodium falciparum*: merozoite invasion in vitro in the presence of chloroquine. Exp. Parasitol. 46, 235–238.

Long, H.Y., Lell, B., Dietz, K., Kremsner, P.G., 2001. *Plasmodium falciparum*: in vitro growth inhibition by febrile temperatures. Parasitol. Res. 87, 553–555.

Macraild, C.A., Anders, R.F., Foley, M., Norton, R.S., 2011. Apical membrane antigen 1 as an anti-malarial drug target. Curr. Top. Med. Chem. 11, 2039–2047.

Marsh, K., Otoo, L., Hayes, R.J., Carson, D.C., Greenwood, B.M., 1989. Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. Trans. R. Soc. Trop. Med. Hyg. 83, 293–303.

McCallum, F.J., Persson, K.E.M., Mugyenyi, C.K., Fowkes, F.J.I., Simpson, J.A., Richards, J.S., Williams, T.N., Marsh, K., Beeson, J.G., 2008. Acquisition of growth-inhibitory antibodies against blood-stage *Plasmodium falciparum*. PLoS One 3, e3571.

McGregor, I.A., 1964. The passive transfer of human malarial immunity. Am. J. Trop. Med. Hyg. 13 (Suppl.), 237–239.

Meissner, M., Krejany, E., Gilson, P.R., de Koning-Ward, T.F., Soldati, D., Crabb, B.S., 2005. Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood stages using *Toxoplasma gondii* transactivators. Proc. Nat. Acad. Sci. U.S.A. 102, 2980–2985.

Mitchell, G.H., Butcher, G.A., Cohen, S., 1974. A merozoite vaccine effective against *Plasmodium knowlesi* malaria. Nature 252, 311–313.

Mitchell, G.H., Thomas, A.W., Margos, G., Dluzewski, A.R., Bannister, L.H., 2004. Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. Infect. Immun. 72, 154–158.

Moghimi, S.M., Hedeman, H., Muir, I.S., Illum, L., Davis, S.S., 1993. An investigation of the filtration capacity and the fate of large filtered sterically-stabilized microspheres in rat spleen. Biochim. Biophys. Acta 1157, 233–240.

Munir, M., Tjandra, H., Rampengan, T.H., Mustadjab, I., Wulur, F.H., 1980. Heparin in the treatment of cerebral malaria. Paediatr. Indones. 20, 47–50.

O'Donnell, R.A., de Koning-Ward, T.F., Burt, R.A., Bockarie, M., Reeder, J.C., Cowman, A.F., Crabb, B.S., 2001. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J. Exp. Med. 193, 1403–1412.

O'Donnell, R.A., Blackman, M.J., 2005. The role of malaria merozoite proteases in red blood cell invasion. Curr. Opin. Microbiol. 8, 422–427.

Persson, K.E.M., Lee, C.T., Marsh, K., Beeson, J.G., 2006. Development and optimization of high-throughput methods to measure *Plasmodium falciparum*-specific growth inhibitory antibodies. J. Clin. Microbiol. 44, 1665–1673.

Persson, K.E.M., McCallum, F.J., Reiling, L., Lister, N.A., Stubbs, J., Cowman, A.F., Marsh, K., Beeson, J.G., 2008. Variation in use of erythrocyte invasion pathways by *Plasmodium falciparum* mediates evasion of human inhibitory antibodies. J. Clin. Investig. 118, 342–351.

Rampengan, T.H., 1991. Cerebral malaria in children. Comparative study between heparin, dexamethasone and placebo. Paediatr. Indones. 31, 59–66.

Richards, J.S., Beeson, J.G., 2009. The future for blood-stage vaccines against malaria. Immunol. Cell Biol. 87, 377–390.

Richard, D., Macraild, C.A., Riglar, D.T., Chan, J.-A., Foley, M., Baum, J., Ralph, S.A., Norton, R.S., Cowman, A.F., 2010. Interaction between *Plasmodium falciparum* apical membrane antigen 1 and the rhoptry neck protein complex defines a key step in the erythrocyte invasion process of malaria parasites. J. Biol. Chem. 285, 14815–14822.

Riglar, D.T., Richard, D., Wilson, D.W., Boyle, M.J., Dekiwadia, C., Turnbull, L., Angrisano, F., Marapana, D.S., Rogers, K.L., Whitchurch, C.B., Beeson, J.G., Cowman, A.F., Ralph, S.A., Baum, J., 2011. Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. Cell Host Microbe 9, 9–20.

Sabchareon, A., Burnouf, T., Ouattara, D., Attanath, P., Bouharoun-Tayoun, H., Chantavanich, P., Foucault, C., Chongsuphajaisiddhi, T., Druilhe, P., 1991. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am. J. Trop. Med. Hyg. 45, 297–308.

Saul, A., Myler, P., Mangan, T., Kidson, C., 1982. *Plasmodium falciparum*: automated assay of erythrocyte invasion using flow cytofluorometry. Exp. Parasitol. 54, 64–71.

Saul, A., 1987. Kinetic constraints on the development of a malaria vaccine. Parasite Immunol. 9, 1–9.

Shi, Y.P., Udhayakumar, V., Oloo, A.J., Nahlen, B.L., Lal, A.A., 1999. Differential effect and interaction of monocytes, hyperimmune sera, and immunoglobulin G on the growth of asexual stage *Plasmodium falciparum* parasites. Am. J. Trop. Med. Hyg. 60, 135–141.

Singh, S., Alam, M.M., Pal-Bhowmick, I., Brzostowski, J.A., Chitnis, C.E., 2010. Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. PLoS Pathog. 6, e1000746.

Smitskamp, H., Wolthuis, F.H., 1971. New concepts in treatment of malignant tertian malaria with cerebral involvement. Brit. Med. J 1, 714–716.

Spadafora, C., Awandare, G.A., Kopydlowski, K.M., Czege, J., Moch, J.K., Finberg, R.W., Tsokos, G.C., Stoute, J.A., 2010. Complement receptor 1 is a sialic acid-independent erythrocyte receptor of *Plasmodium falciparum*. PLoS Pathog. 6, e1000968.

Tham, W.-H., Wilson, D.W., Lopaticki, S., Schmidt, C.Q., Tetteh-Quarcoo, P.B., Barlow, P.N., Richard, D., Corbin, J.E., Beeson, J.G., Cowman, A.F., 2010. Complement receptor 1 is the host erythrocyte receptor for *Plasmodium falciparum* PfRh4 invasion ligand. Proc. Nat. Acad. Sci. U.S.A. 107, 17327–17332.

Tham, W.-H., Schmidt, C.Q., Hauhart, R.E., Guariento, M., Tetteh-Quarcoo, P.B., Lopaticki, S., Atkinson, J.P., Barlow, P.N., Cowman, A.F., 2011. *Plasmodium falciparum* uses a key functional site in complement receptor type-1 for invasion of human erythrocytes. Blood 118, 1923–1933.

Thera, M.A., Doumbo, O.K., Coulibaly, D., Laurens, M.B., Ouattara, A., Kone, A.K., Guindo, A.B., Traore, K., Traore, I., Kouriba, B., Diallo, D.A., Diarra, I., Daou, M., Dolo, A., Tolo, Y., Sissoko, M.S., Niangaly, A., Sissoko, M., Takala-Harrison, S., Lyke, K.E., Wu, Y., Blackwelder, W.C., Godeaux, O., Vekemans, J., Dubois, M.-C., Ballou, W.R., Cohen, J., Thompson, D., Dube, T., Soisson, L., Diggs, C.L., House, B., Lanar, D.E., Dutta, S., Heppner, D.G., Plowe, C.V., 2011. A field trial to assess a blood-stage malaria vaccine. New Engl. J. Med. 365, 1004–1013.

Tonkin, C.J., van Dooren, G.G., Spurck, T.P., Struck, N.S., Good, R.T., Handman, E., Cowman, A.F., McFadden, G.I., 2004. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol. Biochem. Parasitol. 137, 13–21.

Treeck, M., Zacherl, S., Herrmann, S., Cabrera, A., Kono, M., Struck, N.S., Engelberg, K., Haase, S., Frischknecht, F., Miura, K., Spielmann, T., Gilberger, T.W., 2009. Functional analysis of the leading malaria vaccine candidate AMA-1 reveals an essential role for the cytoplasmic domain in the invasion process. PLoS Pathog. 5, e1000322.

Triglia, T., Chen, L., Lopaticki, S., Dekiwadia, C., Riglar, D.T., Hodder, A.N., Ralph, S.A., Baum, J., Cowman, A.F., 2011. *Plasmodium falciparum* merozoite invasion is inhibited by antibodies that target the PfRh2a and b binding domains. PLoS Pathog. 7, e1002075.

van Dijk, M.R., Waters, A.P., Janse, C.J., 1995. Stable transfection of malaria parasite blood stages. Science 268, 1358–1362.

Vogt, A.M., Pettersson, F., Moll, K., Jonsson, C., Normark, J., Ribacke, U., Egwang, T.G., Ekre, H.-P., Spillmann, D., Chen, Q., Wahlgren, M., 2006. Release of sequestered malaria parasites upon injection of a glycosaminoglycan. PLoS Pathog. 2, e100.

Wegscheid-Gerlach, C., Gerber, H.-D., Diederich, W.E., 2010. Proteases of *Plasmodium falciparum* as potential drug targets and inhibitors thereof. Curr. Top. Med. Chem. 10, 346–367.

Wilson, D.W., Crabb, B.S., Beeson, J.G., 2010. Development of fluorescent *Plasmodium falciparum* for in vitro growth inhibition assays. Malaria J. 9, 152.

Wilson, D.W., Fowkes, F.J.I., Gilson, P.R., Elliott, S.R., Tavul, L., Michon, P., Dabod, E., Siba, P.M., Mueller, I., Crabb, B.S., Beeson, J.G., 2011. Quantifying the importance of MSP1-19 as a target of growth-inhibitory and protective antibodies against *Plasmodium falciparum* in humans. PLoS One 6, e27705.

Wong, W., Skau, C.T., Marapana, D.S., Hanssen, E., Taylor, N.L., Riglar, D.T., Zuccala, E.S., Angrisano, F., Lewis, H., Catimel, B., Clarke, O.B., Kershaw, N.J., Perugini, M.A., Kovar, D.R., JGulbis, M., Baum, J., 2011. Minimal requirements for actin filament disassembly revealed by structural analysis of malaria parasite actin-depolymerizing factor 1. Proc. Nat. Acad. Sci. U.S.A. 108, 9869–9874.

World Health Organization Global Malaria Programme, 2010. World Malaria Report 2010. WHO, Geneva.

World Health Organization, 2010. Guidelines for the Treatment of Malaria. WHO, Geneva.

Yu, L., Garg, H.G., Li, B., Linhardt, R.J., Hales, C.A., 2010. Antitumor effect of butanoylated heparin with low anticoagulant activity on lung cancer growth in mice and rats. Curr. Cancer Drug Targets 10, 229–241.