Plasmodium falciparum protein export and erythrocyte remodeling in blood-stage malaria infections

Tanner Peltier

Iowa State University

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Abstract

Malaria is a vector-borne disease caused by several unicellular *Plasmodium* species. *Plasmodium* are parasitic eukaryotic protozoans within the apicomplexan phylum named for an apical complex of secretory organelles and cytoskeletal components critical for host cell invasion. Infections are deadliest in children under five years of age and pregnant women. Here, we focus on the life cycle of *Plasmodium falciparum* and the molecular remodeling capabilities implemented against human erythrocytes. These changes can complicate Malaria infections and reach the microvasculature of the brain leading to death in the human host. Millions of Malaria infections occur each year and researching *Plasmodium falciparum* erythrocyte remodeling may uncover new mitigation efforts. This paper will review research topics covering *P. falciparum* and the mechanisms used to adapt the erythrocyte to its own needs.

Introduction

Overview of Malaria

Understanding Malaria as a disease is important in discussing the protozoan *Plasmodium falciparum*. Malaria remains endemic in tropical and subtropical regions of Africa, Asia, and South America. Disease transmission is carried out by infected female *Anopheles* mosquitoes obtaining their blood meal from humans. *P. falciparum* sporozoites migrate from the mosquito salivary glands into the bite-site created by the feeding mosquito thereby infecting the human host (Ashley et al., 2018). Sporozoites then migrate to the hepatic cells of the liver where they undergo a transformation into asexual merozoites that then invade erythrocytes (Jones & Rayner, 2009). The erythrocytic phase of *P. falciparum* causes all the symptoms of Malaria, including fever, tremors, and chills.

P. falciparum contributed to over 229 million new Malaria infections in 2019. Malaria is counted as one of the world's deadliest diseases contributing to 409,000 recorded deaths in 2019 (World Health Organization, 2020). Diagnosis of Malaria cases is an important management tool in assessing the global impact of Malaria as well as community spread. Typically, laboratory tests involving thin and thick peripheral blood smear staining, PCR, and rapid diagnostic tests are used with each method's strengths and weakness considered situationally (Tangpukdee et al., 2009). Limitations of peripheral blood smears for example, are that distribution of infected red blood cells (RBCs) is not uniform throughout the host resulting in potential misdiagnosis and highlighting the need for further development of effective and accessible testing options (Tran et al., 2012). Clinical diagnosis of Malaria can also be an important method to diagnose cases. What signs and symptoms these include are found on physical examinations and can range from fever, headache, weakness, myalgia, chills, etc. The challenge here is due to the wide-range of

symptoms which are common but not specific to Malaria and present in other diseases as well, but physical examinations remain an inexpensive and widespread method of diagnosis (Tangpukdee et al., 2009).

Malaria pathologies can range from uncomplicated fevers to life-threatening conditions like anemia and RBC adhesion to brain venule endothelial cells of younger individuals that results in death (Rowe et al., 2009; Tilley et al., 2011). Interventions aim at prevention of these life-threatening infections has led to several treatment options to combat infection. There are also therapeutic treatments that eliminate merozoites during the erythrocytic phase (World Health Organization, 2020). Insecticides against *Anopheles* spp. are also used to limit vector transmission, but widespread use has also resulted in mosquito resistance (Tran et al., 2012). The limited effect of these combined measures emphasizes the need to develop an effective long-term malaria vaccine.

Another key aspect of *P. falciparum's* evolutionary success as a microbe is its ability to avoid the human host's immune response. Upon invasion of RBCs, *P. falciparum* is able to place adhesins, known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), on the surface of the RBC which allows the infected RBC to avoid clearance by the spleen. Avoidance of the spleen is due to the fact that PfEMP1 adhesins are presented on *P. falciparum* induced knobs on the erythrocyte surface which facilitate adherence to the walls of blood vessels (Tilley et al., 2011). The ability to avoid clearance is unique to *P. falciparum* and allows infected RBCs to remain unchecked by the host's innate immune system. This means *P. falciparum* can continue modification of the RBC for nutrient acquisition and asexual reproduction without being phagocytized. However, this does not protect *P. falciparum* from other forms of adaptive immunity that hold promise for future vaccine development.

Overview of *P. falciparum*

As mentioned, *P. falciparum* has a complex lifecycle, so I will be presenting a simplified version of the initial infection process. The vertebrate host portion of *P. falciparum's* cycle starts at the sporozoite stage located in the salivary glands of an infected female *Anopheles* mosquito. The sporozoites migrate through the proboscis and into the bite-site. It's at this point that the highly motile *P. falciparum* find their way to the liver and invade hepatocytes which develop into merozoites. This process is termed the exoerythrocytic stage as the sporozoites infect and develop within hepatocytes and not erythrocytes. Initiation of the blood stage also known as the erythrocytic stage, occurs when merozoites exit the hepatocytes and enter the blood where they invade erythrocytes (Spillman et al., 2015). Host cell invasion depends on an interaction known as a Tight Junction (TJ) formed between the surface of the merozoite plasma membrane and the erythrocyte plasma membrane. The apical prominence of the merozoite is oriented directly toward erythrocyte and a series of molecular motors drives the parasite into the RBC (Jones & Rayner, 2009). Though each stage of *P. falciparum's* life cycle is distinct and specialized there are shared mechanisms that remain similar and much of the invasion machinery described above is also used by sporozoites to invade hepatocytes. Another protein involved with *Plasmodium* invasion and survival are calcium-dependent protein kinases (CDPKs). At the sporozoite stage, knockout of PfCDPK1 genes result in less gliding motility essential for proper orientation (Green et al., 2008). Rhoptries are another shared feature for invasion. These secretory organelles release proteins during invasion and are located in the apical complex of *P. falciparum* merozoites and sporozoites alike (Baum et al., 2008). The resulting TJ formation represents an

irreversible attachment with the erythrocyte plasma membrane, committing the parasite to invasion.

Entering the erythrocyte by sporozoites is a complex process that starts with attachment and ends with the creation of a parasitophorous vacuolar membrane (PVM). Motility of *P. falciparum* just prior to invasion is achieved by thrombospondin related anonymous protein (TRAP), a transmembrane adhesin which works with myosin motors to drive the parasite forward (Baum et al., 2008; Jones & Rayner, 2009; Wright & Rayner, 2014). It is also important to note that at this stage many protein-protein interactions are necessary to invade the erythrocyte. One study highlighted that without the PfRipr/PfRh5/CyRPA complex formed at the apical end of *P. falciparum*, erythrocyte invasion is not possible (Volz et al., 2016). The TJ formation occurs just after this where PfAMA1, which is secreted by the micronemes into the parasite membrane, binds to RON2, which is released from the rhoptries into the host membrane, creating the TJ and fully committing the parasite towards invasion (Volz et al., 2016). PfAMA1 is a transmembrane protein and binds to an exposed loop of RON2. The TJ formed by *P. falciparum* is powered by the parasites actin-myosin motor and creates an invagination within the erythrocyte that eventually forms the PVM (Weiss et al., 2015). The process of invasion is complex, rapid, and conserved across *Plasmodium* spp. as an essential survival mechanism at the merozoite and sporozoite stages.

The PVM represents an additional barrier within infected RBCs that parasite protein transport must overcome (Hanssen et al., 2010). After invasion, initiation of intraerythrocytic development occurs with parasite morphology proceeding through ring, trophozoite, and schizont stages. After this cycle of replication daughter merozoites are released and invade new erythrocytes to continue the process. A variety of proteins exposed on the

merozoite's and infected RBC surface helps *P. falciparum* to avoid the immune response (Cowman et al., 2012; Wright & Rayner, 2014). The hypervariability studied in each of these mechanisms means that they make for difficult targets. Parasite gametes known as gametocytes are also produced during erythrocyte infection, and the specific mechanisms that control gametocytogenesis remains a topic of study in current literature (Talman et al., 2004). Mature gametocytes enter the peripheral circulation to be taken up by *Anopheles* mosquitoes. Mating then occurs in the mosquito midgut where male and female gametocytes exit the RBC and fuse to form a zygote. The zygote develops into an oocyst and eventually forms new sporozoites which migrate to the salivary glands and start the cycle over (Drakeley et al., 2006; Spillman et al., 2015).

Blood stage protein export

Current *P. falciparum* research seeks to identify additional targets to treat infections effectively. *P. falciparum* infects RBCs which lack a nucleus, secretory pathway, protein synthesis, and other trafficking processes. This means, *P. falciparum* must carry out major modifications of the host erythrocyte in order to acquire essential nutrients to fulfill the replication process. Some of these changes include modifications of the permeability, rigidity, and cytoadherence properties of the erythrocyte (Spillman et al., 2015). An early example of the mechanical changes made to RBCs tested membrane stiffening in infected cells. By striking parasitized cells with a pipette tip this work demonstrated noticeable impairment of deformability which is known to contribute to trapping of RBCs in the microcirculatory system (Nash et al., 1989). Research then needed to focus on how protein action and transport contributed to these changes.

When studying distribution of proteins that remodel the erythrocyte it is essential to understand the barriers that *P. falciparum* overcomes in order to facilitate these changes as well as nutrient acquisition and immune system avoidance. Like other secreted proteins, proteins exported into the RBC initially enter the parasite endoplasmic reticulum (ER). Most of these proteins contain a *Plasmodium* export element (PEXEL) motif, a pentameric sequence that marks proteins for transport to the host cell (Elsworth et al., 2014)(Florentin et al., 2020). The PEXEL motif is processed by an aspartic protease called Plasmepsin 5 (PMV) in the ER and this is critical to export of these proteins (Boddey et al., 2013, 2016; Russo et al., 2010; Sleebs et al., 2014). In the vacuole, protein unfolding is then required for proper transport through the PVM (Gehde et al., 2009). In addition to a protein translocon, the PVM also contains a nutrient-pore that allows *P. falciparum* to obtain nutrients as well as export waste products across this membrane (Hanssen et al., 2010; Spillman et al., 2015).

Secretion of proteins through the parasite secretory pathway, across the parasite plasma membrane (PPM), through the vacuolar lumen, past the PVM, and finally through the erythrocyte cytosol to the host cell membrane is a complicated route all on its own. Yet, there still needs to be proper folding of the protein as well as signals to indicate entry through the PVM translocon. The PVM translocon's essential function then is to be a passive hydrophilic pathway for proteins (Goldberg & Zimmerberg, 2020). This has been described as a two-step process whereby a protein is folded, marked with the proper signal, and secreted through the PPM into the vacuolar lumen being unfolded and passed through the vacuole membrane translocon at the PVM to enter the erythrocyte cytosol (Charpian & Przyborski, 2008; Mesén-Ramírez et al., 2016). Hundreds of parasite proteins are exported in this way, including PfEMP1 adhesin described above.

Studies show that PfEMP1 is essential to cytoadherence to avoid destruction in the spleen and requires translocation across the PVM via the *Plasmodium* translocon of exported proteins (PTEX), as shown by conditional knockdown of PTEX components, resulting in PfEMP1 being unable to be exported beyond the PVM (Batinovic et al., 2017; Goldberg & Zimmerberg, 2020). PTEX is a membrane protein complex formed from of a hexameric HSP101 protein-unfolding motor bound to seven copies of the protein EXP2 that span the PVM, forming a pore. Seven copies of a protein known as PTEX150 connect HSP101 to EXP2 (C. M. Ho et al., 2018). This organization allows PTEX to function by making conformational changes to facilitate protein transport. In order for cargo to pass through PTEX, proteins are unfolded and passed through the HSP101/PTEX150/EXP2 assembly, allowing transport across the PVM and into the RBC (C. M. Ho et al., 2018). PTEX is essential to the transportation of proteins as shown in research involving knockdown mutants where PTEX inhibition resulted in decreased *P. falciparum* capacity to remodel the RBC and parasite death (Charnaud et al., 2018; Elsworth et al., 2014). PTEX has an essential role in *P. falciparum's* lifecycle and studies are attempting to uncover how proteins are recognized by PTEX for transport.

While most exported proteins are marked with PEXELs (Maier et al., 2008), there are also exported proteins that do not contain this feature and they are known as PEXEL/HT negative exported protein (PNEPs). Though PNEP's have been far less studied they play an essential role in erythrocyte remodeling. Many exported proteins travel through Maurer's clefts, which are membrane cisternae analogous to the Golgi apparatus created by the parasite in the RBC. Maurer's clefts direct exported proteins to the erythrocyte membrane (Spillman et al., 2015). The PNEP REX2 is one component of Maurer's clefts which have an important role in RBC remodeling. Most PNEPs have a transmembrane domain (TM) although this is not the case

for all PNEPs as a general understanding of their features has been better documented in recent years (Heiber et al., 2013). Expanding research into knobby adhesive protrusions which are a key phenotype of the infected RBC membrane has played an essential role in discovery. It is known that these knobs are made up of knob-associated His-rich protein (KAHRP) which act as the platform for exported PfEMP1(Maier et al., 2009). This key function of PNEPs has shown how crucial research into discovery of other lesser known proteins might explain more about *P. falciparum's* complex protein export.

Maurer's clefts also signal the development stage of *P. falciparum* within RBCs. At infection, there is considerable growth of the ring stage and highly mobile Maurer's clefts. Upon transitioning to the trophozoite stage we can see settling of clefts in their final positions close to the RBC membrane (Grüring et al., 2011). It is important to note that Maurer's clefts function in adherence of the infected erythrocyte to the blood vessel walls mentioned earlier by transporting protein anchors in a complex trafficking pathway (Lanzer et al., 2006). Adherence can also form between other RBCs in what are called rosettes which play a major role in severe malaria infections (M. Ho et al., 1991). At the point of merozoite formation, Maurer's clefts were recorded collapsing. Speculation is that dynamic disassembly of clefts may aid in egress out of the erythrocyte (Grüring et al., 2011). This identified how control over Maurer's cleft formation can signal the stage of development within RBC. It was also discovered that cleft formation was initiated much earlier than previously thought (Lanzer et al., 2006). While much is now known, much is still unknown about factors involved in protein sorting for export in the ER of the parasite and researchers are looking into how it functions as the master regulator though our current understanding is limited (Florentin et al., 2020).

The step-wise fashion by which *P. falciparum* ruptures the erythrocyte is well documented and explains the cyclic nature of fevers experienced by infected individuals. As maturation of the merozoites begins, first the PVM and then the RBC cytoskeletion are degraded. Finally, the RBC plasma membrane is rapidly burst and releases mature merozoites (Glushakova et al., 2005). *P. falciparum* is able to alter the erythrocyte to devastating effect making modifications leading up to the final release of parasites which continue on to infect other RBCs.

Related topics

Some of the difficulties related to studying *P. falciparum* range from creating working models to study *in vivo* infections, incomplete knowledge of the exportome, and slow discovery of specific drugs to combat the parasites lifecycle. Restrictions involving host specificity of *P. falciparum* highlights the need for better model organisms to understand human infection. Research surrounding humanized liver mouse models aim to give better methods of studying the transition of *P. falciparum* from the hepatic stage to the blood stage and a means for preclinical drug tests (Soulard et al., 2015). Further studies geared towards exploring more of the parasite's export system is also necessary in understanding how export is achieved.

Drug discovery and resistance are presented by the parasite but avenues explored may be able target *in vivo* infections and functional activity within the erythrocyte. It's worth noting the chance at repurposing existing drugs to provide additional options to supplement current first-line artemisinin-based therapies (Teixeira et al., 2014). Though treating drug resistant *P. falciparum* remains problematic, further studies implementing models representative of the human infection and characterization of the entire export pathway could lead to potential treatment options that target the export system to prevent parasite survival. One current vaccine

development approach is focused on a specific adhesin, PfRH5, as anti-RH5 monoclonal antibodies have been shown to completely inhibit erythrocyte invasion (Douglas et al., 2015; Ord et al., 2014). Development of this vaccine is seen as one of the most important methods being developed to combat Malaria. Additional approaches to develop a vaccine may target proteins exported within infected RBCs, and this shows how applicable research into erythrocyte remodeling can open new opportunities for Malaria control.

Conclusion

Understanding *P. falciparum's* life cycle and how it establishes itself within an erythrocyte has been key to controlling Malaria infections globally. Learning the methods of infection through vector mediated life cycles have shown ways to mitigate this deadly disease. Establishing an understanding of the complex invasion of human erythrocytes and the establishment of protein export for function can be exploited through current technology. We've seen how proteins are transported across the various membranes created during infection and how *P. falciparum* evolved complex machinery to facilitate protein transport. Trafficking proteins is important in this case due to the unique environment of the RBC which is overcome by *P. falciparum*. Protein export facilitates avoidance of the host's immune system which is unique to *P. falciparum* and research discoveries are uncovering even more protein functions. Advancing the knowledge base surrounding the biology of *P. falciparum* has proven to be the way forward. Mitigating the global impact of Malaria associated infections and deaths is accomplished by studying this amazingly complex take down of the human erythrocyte.

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