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Molecular Mechanisms of Malarial Parasite Invasion

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CONTENTS

ABBREVIATIONS

AMA-1: Apical membrane antigen**; BAEBL:** Binding antigen of erythrocyte binding–like family; **CSP:** Circumsporozoite protein; **CD81:** Hepatocyte surface protein of tetraspanin superfamily; **DBL:** Duffy binding-like family protein; **EBA:** Erythrocyte binding antigen; **EEF:** Exo-erythrocytic form; **HS:** Heparin sulphate; **HSPGs:** Heparin sulfate proteoglycans; **MACPF:** Membrane attack complex and perforin; **MAEBL:** Membrane protein of apical organelle proteins of erythrocyte binding-like family**; MAOP:** Membrane attack ookinete microneme protein; **RBL:** Reticulocyte binding protein like family; **RHOPH:** High molecular weight rhoptry; **SPECT2:** Sporozoites microneme protein essential for cell traversal; **SSP2:** Sporozoite surface protein; **TRAP:** Thrombospondin-related adhesive protein; **TEMs:** Tetraspanin enriched microdomains; **TSR-domain:** Thrombospondine type-1 repeat.

INTRODUCTION

Malaria is an ancient disease that continues to cause enormous human morbidity and mortality. It is still the most important parasitic disease in the world; responsible for 300 million to 500 million cases of illness and 1.5 million to 2.7 million deaths annually[1]. The life cycle of the causative parasite involves multiple tissues in two distinct hosts (mosquitoes and human). *Plasmodium* infection is initiated by the inoculation of sporozoites in the host by a female mosquito. Out of more than 20 well documented species of *Plasmodium* that cause malaria in various vertebrates, four species infect humans; *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*[2].

Plasmodium and other apicomplexan parasites are obligate intracellular pathogens that need to efficiently enter and exit their respective host cells in order to propagate and progress along the life cycle. Understanding the basic mechanisms that govern parasite invasion, remodeling, growth, reinvasion and other complex events may yield new diagnostics, treatment and vaccines[3]. Signaling processes and specific interaction of the malaria sporozoites with their target cells have been shown to play important roles in the invasion of both cell

types in the mosquito vector and vertebrate host during the course of the parasite's life cycle. Invasion process is mediated by different types of protein families which play a critical role in sporozoite traversal, cell passage activity and rupture of the cell membrane[4].

The defining feature of this phylum is the presence of an apical complex composed of secretory organelles termed micronemes, rhoptries and dense granules. The process of host cell invasion involves a series of steps. After initial contact of the parasite with a target cell, reorientation occurs, resulting in direct juxtaposition of its apical end with the host cell membrane, leading to formation of tight junction. This is an irreversible zone of contact where the host and parasite plasma membrane are brought together through interaction of parasite ligands with specific host cell receptors[5,6].

Within minutes of biting, the motile sporozoites reach the liver and infect hepatocytes. In the hepatocytes, they further differentiate into a replicative exo-erythrocytic form (EEF) that will ultimately give rise to thousands of merozoites that initiate the erythrocytic cycle[7]. After ingestion of infected blood by a mosquito, malarial parasites are fertilized in the mosquito midgut and develop into motile zygotes, called ookinetes. Ookinetes invade the midgut epithelial cell from the luminal surface by rupturing the cell membrane and move through the cytoplasm to the side of the basal lamina, sometimes penetrating additional epithelial cells laterally. In the basal lamina, ookinetes transform to oocysts and finally develop to sporozoites, the mosquito salivary gland-invasive stage. During this midgut invasion, many ookinetes are killed by the insect's defense system, and the number of malarial parasites is greatly reduced[8].

Maintenance of host specificity generally involves a complex interplay between pathogen and host factors, which are influenced by various evolutionary and genetic determinants. Invasion into the vertebrate and mosquito hosts is mediated by specific molecular interactions through which multiple parasite ligands are released from host cell internal organelles, and a series of protein families are also expressed on the cell surface of the target (hepatocytes-erythrocytes-midgut epithelium)[9].

This review discusses the process of host cell invasion by the malaria parasite, and the identification and functional analysis of the specific receptor-ligand interactions between host and parasite adhesive proteins. Understanding of the molecular mechanism by which these proteins mediate invasion facilitates their use in the design of vaccine and drug strategies.

Keywords: Malaria, Sporozoite, Molecular Invasion, Hepatocytes, Erythrocytes, Midgut, Salivary Glands, Ligands, Domain, Micronemes.

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1. Hepatic Cell Invasion by *Plasmodium* Sporozoite

Malaria sporozoites must cross at least two cell barriers to reach their initial site of replication in the mammalian host including hepatocytic and erythrocytic barriers.

1.1. Hepatic sinusoidal cell layer

1.1.1. Molecular interactions involved in the invasion of hepatocytes

Sporozoites invasion of hepatocytes occurs within minutes, after parasite injection during a blood meal by an infected mosquito. They migrate towards small dermal capillaries, traverse the vascular endothelial layer, and rapidly home to the liver. To infect hepatocytes, the parasite must cross the sinusoidal cell layer. The exact route *Plasmodium* sporozoites take to hepatocytes has been subjected to cell biology. Although hepatocytes lie beneath an endothelial cell lining, the liver is unique in that its endothelial cells have open fenestrations allowing for direct contact between the circulatory system and hepatocytes. Estimates, however, indicate that the diameter of these fenestrations is 0.1 μm, about ten times smaller than the diameter of a sporozoite[10].

It is possible that sporozoites may be arrested in the liver by sequential interactions with endothelial cell receptors similar to the way in which leukocytes roll, arrest, and extravasate at sites of inflammation[11]. This is an attractive hypothesis because the sporozoite surface protein, thrombospondin-related adhesive protein (TRAP), contains an adhesive domain called the A domain, which is also present in the leukocyte adhesion molecules. It has been shown that the binding of the A domains of the leukocyte to the endothelial cell receptors mediates leukocyte arrest at sites of inflammation[12]. However, further studies discovered that these molecules are not important for sporozoites infectivity by injecting *P. yoelii* sporozoites into knockout mice. When the investigators assessed infection of hepatocytes by sporozoites using quantitative PCR, they found no difference between infected and control mice suggesting that these receptors are not involved in sporozoites sequestration in the liver. So, trans-endothelial passage of sporozoites is the attractive hypothesis supported by increasing knowledge of organ-specific endothelial cell markers[13].

Biochemical and physiological studies suggested that sporozoites enter the liver by passing through Kupffer cell which is considered as a gate for sporozoites to the liver[14]. Different approaches have been used to confirm the role of Kupffer cells through saturation of the phagocytic capacity with silica or removal from the liver by intoxication with clodronate, prior to infection, leading to a decrease in the number of liver stages of *P. berghei*. Kupffer cells represent an integral component of the lining of the liver capillaries, they leave behind small gaps in the normally continuous sinusoidal cell layer. These gaps are large enough to allow sporozoites passage, and direct access to hepatocytes[15]. An argument against malaria sporozoites passage through kupffer cells is that parasites can cross endothelial cell barriers, since they are already able to penetrate the vascular capillary wall in the skin, however the conditions for sporozoites passage across the dermal capillary wall and the sinusoidal barrier differ markedly[16].

Vascular endothelial cells secrete heparin sulfate proteoglycans and sporozoites recognize proteoglycans in various tissues. So, the route the parasite takes across this barrier, whether trans-or-para endothelial remains to be established[17].

Sporozoites also enter dermal lymphatic vessels to the lymph node; and both vascular and lymphatic endothelia exhibit distinct molecular surface markers. Sporozoites enter lymphatic vessels passively through endothelial inlet valves and the observed lateral drifting of sporozoites along lymph vessels supports this interpretation[18].

Therefore, malaria sporozoites have to overcome each of the cell barriers during their journey to hepatocytes. These have their own characteristics, composition and orientation and must therefore be viewed individually with regard to the passage across the sinusoidal barrier. Available evidence points to kupffer cells being the gate to the liver. *Plasmodium* sporozoites migrate through several hepatocytes before finally settling down in one for development to a liver stage. It was concluded that sporozoites are able to enter cells in at least two different ways: transmigration and membrane invagination[19].

1.1.2. Families of plasmodial ligands involved in hepatocytes invasion

It was reported that a membrane attack complex and perforin (MACPF-related protein) is essential for cell passage activity of the sporozoite. This protein molecule participates in rupture of the cell membrane, and sporozoites disrupted in this gene completely lost cell wounding activity. Sporozoites protein with a MACPF or sporozoite protein essential for cell traversal (SPECT2) is very essential for cell traversal ability of sporozoites. SPECT2 is specifically produced in the liver-infective-sporozoite and localized to micronemes. The MACPF domain of SPECT2 is highly conserved with the mammalian MACPF - family proteins. It has been suggested to be important for integration of the cell membrane because mammalian MACPF family proteins act by forming pores in the target cell membrane [20].

Another hypothesis is that sporozoites may bind to a receptor and invade hepatocytes directly. Circumsporozoite (CSP), a sporozoite cell surface protein, plays an important role in cell invasion, and arrest in liver sinusoid[21]. *Plasmodium* sporozoites express CSP on the surface of all species. It is an important molecule for the parasite because: (1) It is involved in the development of infectious sporozoites in mosquitoes, (2) It plays a role in invasion of the salivary gland, (3) It is essential to the binding and invasion of liver cells in the vertebrate host, and (4) It is also a malaria vaccine candidate. In addition, CSP contains a known cell-adhesive motif that is highly conserved in all species of plasmodia. This motif is called region IL-plus (about 20 amino acids in length), and CSP lacking this region does not have binding activity. Studies performed to define the structural properties of region IL-plus required for binding to heparin sulfate proteoglycans (HSPGs), demonstrated that the downstream positively-charged residues and interspersed hydrophobic amino acids were required for binding activity[22].

Studies have shown that many of the proteins containing this motif bind to sulfated glycoconjugates. CSP binds to the glycosaminoglycan chains of HSPGs. It was thought that HSPGs on the surface of hepatocytes extend through fenestrae into the circulation, so the sporozoites may be captured in the liver by protruding HSPGs through the open fenestrae of the endothelial cells[15,23].

On the hepatocyte side, the only surface protein known to play a key role in the infection by several *Plasmodium* species is the tetraspanin (CD81). It belongs to a family of proteins which have been implicated in various biological processes such as cell adhesion, migration, cell fusion, co-stimulation, signal transduction and differentiation. It is also essential for the infection of hepatocytic cells by the hepatitis C-virus (HCV). Biochemtcal studies have shown that tetraspanins associate with many other surface molecules and they have been suggested to function as molecular organizers of membrane multi-molecular complexes. In addition, tetraspanins form primary complexes with a limited number of proteins called tetraspanin partners; they are highly specific for host cell invasion by sporozoites[24,25]. Invasion of hepatocytes by *P. falciparum* and *P. yoelii* depends on CD81 and cholesterol-dependent tetraspanin-enriched microdomains (TEMs) on the hepatocytes surface[26].

2. Erythrocyte Invasion by Merozoite

2.1. Plasmodial ligands involved in erythrocyte invasion

Invasion of erythrocytes by malarial parasite is an obligatory step during blood stage. It involves a cascade of molecular events between the merozoites and host erythrocytes, and it is a tightly controlled process that involves specific receptor-ligand interactions between host RBCS and parasite molecules[27,28]. Proteins spread over the entire surface of the merozoite are involved in the initial attachment of the parasite to host erythrocytes. Erythrocyte invasion is a multistep process where interaction between the merozoites and erythrocytes is followed by reorientation of the merozoites. This results in the formation of a tight junction between merozoite and apical end of the erythrocyte membrane[3].

The malarial parasite senses the environment to modulate its own cycle so erythrocyte invasion is critical to the pathogenesis and survival of the malarial parasite. This process is partly mediated by proteins that belong to the Duffy binding-like family (DBL), which are expressed on the merozoite surface[7]. *Plasmodium* sporozoite surface proteins (SPECT2) are restricted to invading erythrocytic cells in the blood stream in contrast to other members of Apicomplexa, such as *Toxoplasma gondii,* that invade a wide range of cell types from many different species[29]. The sequence of events leading to erythrocyte invasion is similar for all *Plasmodium spp.*[30].

2.2. Thrombospondin related adhesive protein (TRAP)

This protein is essential for sporozoite gliding, cell invasion and *in vivo* infectivity. It is a protein expressed in sporozoites and conserved in all *Plasmodium* species. It is localized to the parasite micronemes and becomes surface exposed at the sporozoite's anterior tip, particularly upon contact with the host[31]. Initial contact between the merozoites and new red cells occur at any point on the surface of merozoites. Then, the parasite re-orientates to juxtapose its apex with the erythrocyte surface[32,33].

A high affinity irreversible contact occurs with the formation of a close membrane to membrane contact known as "tight junction". The highly specialized conoid apical organelle known as rhoptries and smaller organelle known as micronemes, contain proteins required for invasion process. The contents of the micronemes and rhoptries are discharged and a nascent vacuole forms within the erythrocyte. Microneme discharge is regulated by free Ca^{2+} level within the cytoplasm of the parasite[19]. The merozoites enter the red cell with the tight junction bridging and the erythrocyte moving from anterior to posterior. The invasion process is complete when the newly formed parasitopharous vacuole closes[34].

2.3. Merozoite adhesive proteins

Proteins spread over the entire surface of the merozoites are involved in the initial, reversible attachment of the parasite to host erythrocytes. Apical orientation precedes microneme and rhoptry discharge. Specific proteins sense the apical orientation of the merozoites with the erythrocyte surface such as apical membrane antigen-1 (AMA-1) and the components of the high molecular weight rhoptry (RHOPH) complex[17].

2.4. Apical membrane antigen-1 (AMA-1)

It is a transmembrane protein present on the surface of merozoites that is thought to be involved in the process of parasite invasion of erythrocytes and hepatocytes. Although conventional antibodies to AMA-1 can prevent such invasion, extensive polymorphorphisms within surface exposed loops may limit the ability of these induced antibodies to protect against all parasite genotypes[35]. Although AMA-1 is the target of a natural immune response that can inhibit invasion, little is known about the molecular mechanisms by which AMA-1 facilities the invasion process. Many studies were done to identify peptides that specifically interact with and block the function of AMA-1. Results indicated that they bind to a similar region on the surface and so, block interaction between AMA-1 and ligand on the erythrocyte surface which is a critical step in malarial invasion[36,37].

Another family of adhesive ligands is the DBL[28,38]. It is composed of adhesion molecules that are critical for junction formation between the apical end of the merozoites and the erythrocyte surface. Proteins in this family including erythrocyte-binding antigen-175 (EBA-175) are homologous to *P. vivax* DBL. These proteins contain one or more DBL domains, which are composed of cysteine residues associated with erythrocyte binding. EBA-175 is bound to glycophorin A (RBC receptor) on the erythrocyte surface in a silaic acid-dependent invasion pathway[39,40].

Additionally, binding antigen of erythrocyte binding-like family (BAEBL) is a paralog of erythrocyte binding like proteins, and it is also a membrane protein of apical organelle proteins essential for both RBCs invasion by merozoites and mosquito salivary glands by sporozoites. It binds to erythrocytes in a heparin sulfate (HS)-dependent manner which plays a role in merozoites invasion. Moreover, heparin was demonstrated to inhibit both theses binding pathways. Further research on the mechanisms of inhibition by heparin will facilitate the development of new anti-malarial drugs that inhibit invasion[23]. *Plasmodium falciparum* invasion is mediated also by merozoite surface protein EBA-140 that is thought to bind to glycophorin C in a silaic acid dependent manner and also through HS[41,42].

2.5. Alternative invasion pathways into human erythrocytes

Plasmodial parasites use alternate erythrocyte receptors for invasion. The specific recognition of receptors on the erythrocyte is a critical step in the invasion process. *P. vivax* uses the Duffy receptor for invasion into human erythrocytes and this parasite is unable to invade Duffy negative erythrocytes. This phenomenon is thought to account for the lack of *P. vivax* in West Africa, where the prevalence of Duffy negativity approaches 95%[43]. In contrast, *P. falciparum* invades human erythrocytes using multiple and alternate receptor-ligand interactions. The variability of different lines on alternative ligand receptor interactions are considered a feature of *P. falciparum*[5,44]. For instance, some strains of *P. falciparum* such as Dd2 are unable to invade neuraminidase-treated erythrocytes, which are bereft of sialic acid, and are therefore dependent on sialic acid containing receptors for invasion. However, other strains such as 3D7 are able to invade neuraminidase-treated erythrocytes with high efficiency and are therefore sialic acid independent. Similarly, different parasites are variantly dependent on trypsin-sensitive and/or chymotrypsin-sensitive receptors for invasion[45].

3. Molecular Interactions in Mosquito

3.1. Mid-gut invasion

3.1.1. Ookinetes related proteins

After ingestion of infected blood by a mosquito, malarial parasites are fertilized in the mosquito midgut and develop into motile ookinetes. These ookinetes invade epithelial cells by rupturing the cell membrane, and migrate through the cytoplasm toward the basal lamina, on which they develop to oocysts. A protein with a MACPF–related domain, is produced, and is named membrane arrack ookinete protein (MAOP). It is produced in the ookinete stage and plays an essential role in midgut invasion by the ookinete[46]. In 2012, the investigators showed that ookinetes with inactivated MAOP gene could not invade the midgut epithelium. Moreover, analysis by electron microscopy revealed that they attached to the epithelial cell surface, but could not proceed into the cytoplasm confirming that this molecule is essential for rupturing the epithelial membrane before midgut invasion. This finding indicates that conserved mechanisms for membrane rupture are used by the ookinete and the sporozoite for host cell passage[6].

Both stages play central roles in malaria transmission, breaking through the cellular barrier of the new host and migrating to the site where they can develop into the next invasive forms. MACPF proteins may support this invasion by disrupting the host cell membrane. Primary structures of these malarial MACPF-related proteins show high homology with mammalian MACPF family proteins in the MACPF domain[13,47].

3.1.2. Mechanism of oocyst rupture

Ultrastructural study of disruptant ookinetes showed that membrane rupture is initiated by parasite attachment to the host cell membrane at the apical tip. The attachment seems tight and irreversible because most disruptants remained attached to the cell surface even when wild-type ookinetes had already arrived at the basal lamina. This apical attachment of the ookinetes is similar to that of a merozoite just about to enter the erythrocyte. Before cell entry, the merozoite makes apical junction with the erythrocyte membrane, which involves specific interactions between erythrocyte surface receptors and parasite attachment proteins. Therefore, it is possible that the ookinete attachment involves adhesive proteins interacting with receptors on the epithelial cell surface to initiate its commitment to midgut invasion[48].

It was reported that MAOP, a microneme protein paralogous to a sporozoite MACPF-related protein, is produced in the ookinete stage and also plays an essential role in midgut invasion by the ookinete[49]. On the other hand, it was reported that MAOP-distributed ookinetes cannot invade the midgut epithelium by rupturing the epithelial cell membrane. This finding suggests that conserved mechanisms for membrane rupture are used in different host invasive stages and play a key role in breaching biological barriers of host organs[50].

Sultan[51] reported that CSP, the major surface protein of both oocysts and salivary gland sporozoites, binds to mosquito salivary glands and not to other organs exposed to the hemolymph. Binding is strongest on the medial lobe and distal portion of the lateral lobes, the portions of the glands that are preferentially invaded by sporozoites. Another surface protein called TRAP also known as sporozoites surface protein 2 (SSP2), and thought to be expressed only in salivary gland sporozoites, is required for salivary gland infectivity although the mechanism involved is unknown[52].

3.2. Invasion of salivary glands of mosquitoes

Molecular interactions involved in the invasion of salivary glands by sporozoites remain largely unknown, sporozoites are found dispersed throughout the mosquito hemocoel, particularly in the thorax, suggesting that they are passively transported by the mosquito's open circulatory system[32]. Despite their dispersion throughout the hemocoel adhesion of sporozoites and their major surface protein is always greater in salivary glands, suggesting a specific recognition event. Some experiments strongly suggest that invasion by sporozoites is specific and receptor mediated[53]. In addition, oocyst sporozoites posses the surface protein (SSP2) required for sporozoite gliding motility. The invasive ability of salivary gland by sporozoites is directly correlated with their ability to glide and their ligand-binding properties[54].

3.2.1. Micronemal proteins

Molecular mechanisms underlying the interaction between malarial sporozoites and putative receptors on the salivary glands of *Anopheles* mosquito remain under research. Initially, the sporozoite interacts with the salivary gland basal lamina via both CSP and TRAP proteins which contain a region II plus sequence homalogus to that of CSP and have similar binding properties.

Another type of micronemal protein peptide like ligand named MAEBL, is expressed from salivary gland, and has a single transmembrane domain, about 200 kD. It is structurally related to members of *Plasmodium* DBL. Its role is based on recognition of the protein by a monoclonal antibody that caused more than 70% reduction in the average number of sporozoites per infected salivary gland[8].

Another interaction is that the extra cellular portion of TRAP contains two adhesive domains; A-domain and TSR-domain (thrombospondine type -1 repeat). These domains are involved in recognition and attachment to salivary gland receptor molecules. In addition, there is a certain structure of the domain which is a mimotope of the TRAP domain-A that interacts specifically with saglin on the surface of the salivary gland; and this interaction is essential for sporozoite invasion of the salivary gland[55].

Concluding Remarks

Plasmodium invasion is a complex process that requires specific recognition events that lead to arrest of the parasite. These events are in the form of a number of parasite proteins and their congnate receptors in both mammalian and vertebrate hosts. Multiple ligand-receptors interactions are also thought to provide a biological advantage, allowing parasite invasion through polymorphic receptors that help parasites to evade the immune system. So, immunological selection of surface proteins can be used as a mean of immune evasion. Therefore, different invasion pathways can be linked with protective immunity and/or virulence. A useful approach might be to block parasite antigens which are important in red cell invasion.

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