P. falciparum Invasion and Erythrocyte Aging

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Plasmodium parasites need to find red blood cells (RBCs) that, on the one hand, expose receptors for the pathogen ligands and, on the other hand, maintain the right geometry to facilitate merozoite attachment and entry into the red blood cell. Both characteristics change with the maturation of erythrocytes.

Plasmodium erythrocyte senescence deformability invasion receptors cytoadherence

1. Malarial Erythrocyte Receptors and *Plasmodium* Ligands

The identification of crucial ligand–receptor interactions involved in parasite erythrocyte entry is a venue that has been explored to tackle the disease, as their blockage may lead to the development of multivalent and more effective vaccines [1][2].

In studies focused on determining the proteins involved in the invasion process of *P. falciparum*, it was made clear that the physical interaction of the merozoite (the invading stage) with its proper receptor in the red blood cell (RBC) is a well-orchestrated process and that the integrity of the RBC membrane with its scaffold of proteins is crucial for this interaction to be successful. Invasion mechanisms used by the merozoite involve several steps that have been well described throughout the years [3].

As far as it is known, the first merozoite–host interaction is reversible, but it becomes more stable as the merozoite starts expressing some proteins on its surface. Microneme proteins form strong linkages between the parasite and the erythrocyte, helping rhoptry proteins in the final entry of the merozoite into the host cell. These initial steps in *Plasmodium* invasion are followed by the deformation of the erythrocyte membrane to form a parasitophorous vacuole membrane, in which the merozoite encapsulates itself after the shedding of surface proteins into the extracellular environment [4][5].

Based on the host molecules used by the parasite and their response to enzymatic treatment, two main invasion pathways for *P. falciparum* have been defined. One is based on the interaction of the parasite with sialic acid (SA) residues and the other pathway works in a manner that is independent of these molecules [6].

The known receptors for the SA-dependent pathways are Glycophorin (Gly) A [7][8][9][10]. Glycophorins are heavily glycosylated transmembrane sialo glycoproteins, which partly explains why these multi-abundant proteins in the

erythrocyte membrane are responsible for the SA-dependent invasion by *P. falciparum*. These molecules have been characterized as carrying the antigens for several human blood groups: Gly A and Gly B carry the MN and SS blood groups, and Gly C carries the Gerbich blood group system [11][12]. Given the abundance of Glys on the RBC cell surface, it is likely that they also serve as substrates for glycosylation, which provides the RBC with a negatively charged complex glycan “coat” that allows for their circulation without adherence to other cells or walls of blood vessels [13].

As expected, the SA-dependent pathway efficiency is reduced upon enzymatic treatment of the erythrocyte with neuraminidase and the subsequent removal of sialic acid residues [14]. For this pathway, there is a redundancy in the parasite, comprising mainly several ligands of *P. falciparum* belonging to the Erythrocyte Binding Ligand (EBL) proteins and Erythrocyte Binding Antigens (EBAs).

Four main *P. falciparum* ligands have been identified for the SA-independent invasion: erythrocyte-binding antigen-175 (EBA-175), erythrocyte-binding antigen-181 (EBA-181), erythrocyte-binding ligand-1 (EBL-1), and erythrocyte-binding antigen-140 (EBA-140) [15]. It is generally accepted that Gly A is the receptor for the EBA-175 ligand, but *P. falciparum* can also invade erythrocytes using Gly B through EBL-1 [16] and through *P. falciparum* EBA-140 using Gly C as a receptor [17]. Regarding these receptors, Dankwa et al. reported that GPA and GPB are the key ones involved in the *P. falciparum* invasion route into human erythrocytes [18], probably due in part to their abundance on the surface of the erythrocyte.

The SA-independent parasite invasion ligands are dominated by the reticulocyte binding homologs family (PfRBL or PfRh) [10][19]. The family of sialic acid-independent or neuraminidase-resistant receptors [20][21][22] includes Receptor Z [19], Complement Receptor 1 (CR1) [23][24], Basigin (BSG) [25][26][27], and CD55 [28].

Receptor Z is used by the *Plasmodium falciparum* W2-mef and 3D7 strains. In related studies, the *P. falciparum* reticulocyte binding protein homolog 2b (PfRH2b) bound to RBC via this putative receptor, which was resistant to trypsin and neuraminidase treatment but sensitive to chymotrypsin [3]. Another ligand proposed for receptor Z is the Erythrocyte binding antigen-181 (EBA-181), for which no receptor is known [29].

The Complement Receptor 1 (CR1), also known as CD35 (cluster of differentiation 35), is an important polymorphic glycoprotein on the membrane surface of erythrocytes and many other nucleated cells. It is highly sensitive to treatment with trypsin. Along with other proteins, CR1 is also a regulator of the complement system, where it helps the RBC to avoid autologous complement attack [30].

The activation of a complement on the RBC membrane may occur due to the deposition of naturally occurring IgG autoantibodies, leading to the accumulation of C3b/C4b on the cell surface. In this scenario, C3b molecules bound to CR1 are deactivated by Complement Factor I, preventing erythrocytes against complement or phagocytosis-mediated destruction [30][31].

This feature also takes place in *P. falciparum*-infected erythrocytes. In ring-stage infections, phagocytosis is almost entirely dependent on the intervention of CR1 complement activation. This role of CR1 is reduced, however, when more mature forms of the parasite become present.

CR1 levels have been associated with malarial susceptibility and/or severity of the disease in different population groups [32]. Spadafora et al. observed that the amount of these molecules per erythrocyte varies depending on the donor and that levels of CR1 decrease in older erythrocytes when compared with younger ones [23]. The *P. falciparum* protein reticulocyte homology 4 (PfRh4) was reported as the CR1 ligand on the parasite [33].

2. Effect of *Plasmodium* spp. Invasion on Mechanical and Molecular Erythrocyte Properties

When erythrocytes are infected with *P. falciparum*, their natural aging process is accelerated and many of the RBC-aging features appear in infected RBCs, even though they are young [34]. The intra-erythrocytic phase of *Plasmodium* infection is initiated by erythrocyte invasion by merozoites, followed by the asexual replication cycle, which progresses through the ring, trophozoite, and schizont stages, until the new release of merozoites, a step which is associated with the clinical symptoms of malaria [35].

It has been well documented that erythrocytes that have been infected with the *Plasmodium* parasite undergo changes in their membrane composition, particularly in components such as phospholipids and cholesterol, and how these are organized [36][37]. In addition, *P. falciparum* also elicits the formation of hemichromes and the aggregation of Band 3 molecules for further opsonization and phagocytosis of the infected RBCs [38][39][40].

There is also an effect seen on the osmotic, antigenic, transportation, and deformation properties of *P. falciparum*-infected erythrocytes [41][42]. This is also true for uninfected red blood cells when malaria infection takes place. In fact, infected red blood cells (iRBCs) cause a bystander effect, wherein RBCs hosting the parasite provoke changes in the physical properties of the surrounding non-hosting RBCs [43][44]. These rigidified, uninfected red blood cells are mainly removed by splenic macrophages [45].

Changes in the membrane of the erythrocyte that are induced by malaria infection also affect their deformability. As previously explained, this property is crucial for their intrinsic ability to pass through capillaries and other narrow passages of the vascular system.

As the parasite grows inside the RBC, the latter becomes rounder and wrinkled. During asexual stages, the membrane of iRBCs presents knobs, or protrusions, elicited by parasitic proteins known as Knob Associated Histidine–Rich proteins [46][47][48]. Knobs act as a scaffold for the presentation of PfEMP1, which is a protein known as the main actor for the adhesion of the infected RBCs to the endothelium [49].

Red blood cells containing mature parasites exhibit unusual rigidity, and the primary factor responsible for their absence in the bloodstream is not their splenic uptake but an increase in their adherence to endothelial cells. The

increased cytoadherence of the iRBCs to the endothelial lining of capillaries and deep tissues through Intercellular Adhesion Molecule-1 (ICAM-1), vascular cellular adhesion molecule (VCAM), Thrombospondin (TSP), P-selectin (CD36), E-selectin, and other molecules [50] is facilitated by specific parasite proteins exposed on the surface of the iRBCs (e.g., PfEMP1) [51]. Their increased adherence is responsible for the clinical occlusion of deep blood vessels in the brain when infected erythrocyte rosettes are formed, leading to the dreaded condition of cerebral malaria, which is a major complication in the malarial pathology [52][53].

Most of the RBC deformability studies were performed on erythrocytes infected with the asexual forms of the parasites [54][55][56]. However, other lines of investigation on deformability were based on the effects that the sexual forms of the parasite exert on the erythrocyte [47][48]. It is worth noting that throughout their development, *P. falciparum* gametocytes alter the structural and mechanical characteristics of their host erythrocyte membrane.

The gametocyte has at least five (I–V) morphologically distinct stages in which it transforms; each one of them has different characteristics and effects on the host cell [57]. Through mathematical modeling, 3D imaging, and the use of transgenic parasites, Aingaran et al. demonstrated that early gametocytes increase the rigidity of the erythrocyte stages I to IV [47] and that immature sexual stages are enriched in proximity to erythroblastic islands [58]. Thus, the increased stiffness of immature gametocyte-infected erythrocytes could play a role in their entrapment within the bone marrow due to mechanical retention, favoring their maturation in the hematopoietic system. Mature gametocytes (stage V) exit this microenvironment possibly due to the restoration of their deformability [48], regaining their capacity to pass through narrow openings and be released into the bloodstream. This strategy can enable them to be available for ingestion by mosquitoes only once they have matured [49][59][60].

Many studies provided evidence about the role of several proteins expressed during the sexual stages of the parasite, such as Knob-associated histidine-rich protein, PfEMP3 [61], and *P. falciparum* Ring infected Erythrocyte Surface Antigen (RESA) [62] in the reduction of the erythrocyte deformability [49] (see a detailed list in Neveu et al., 2019). Furthermore, the expression of another family of proteins called STEVOR (Subtelomeric Variable Open Reading Frame) was studied in both the sexual and asexual stages of the parasite regarding this issue [63][64][65][66].

Varied functions in different parasite life cycle stages have been reported for the STEVORs and the Repetitive Interspersed Family (RIFIN) of proteins, such as rosetting, alteration of iRBC rigidity, and immune evasion [67][68].

3. Changes in *Plasmodium* Invasion Strategies during Erythrocyte Senescence

As stated before, as the erythrocyte ages, changes in its membrane become evident, making this cell susceptible to opsonization and eventual phagocytosis. At first sight, erythrocyte senescence could impair the ability of parasites to survive due to decreased receptor availability and loss of deformability.

Indeed, the loss of SA receptors would pose another obstacle for the malaria parasite to overcome since most strains of *P. falciparum* primarily invade through the SA-dependent pathway, using alternate ways of invasion only

when these glycoproteins are not available due to mutations, blockage with antibodies, enzymatic cleavage, or natural loss due to aging. However, Huang et al. showed that the RBC charge density is affected by the loss of NANA (SA) during erythrocyte aging [69]. The loss of this negative charge favors a stronger adhesion of RBCs to other cells and tissues.

The parasite, thus, maximizes the above scenario, adding the concomitant help of a reduced negative charge in the RBC to the endothelium-adhering action of its exported proteins, which provides an opportunity to better stick to capillaries and other vessels and avoid the spleen clearance.

To add to the remarkable resilience of this parasite, *P. falciparum* can infect red blood cells at different stages of the erythrocyte, including earlier maturation stages [70][71]. This observation could be added to the many resources it has, which might explain its high virulence and set it apart from other *Plasmodium* spp. that invade red blood cells at specific differentiation stages, such as *P. knowlesi*, *P. vivax*, and *P. ovale*. The entry of the latter into the cell is almost restricted to the very youngest circulating class of RBCs [72][73]. *P. malariae*, in contrast, prefers to invade older erythrocytes [74]. Receptors expressed in younger or older erythrocytes, for which each species probably expresses more ligands, might help to determine their invasion preference.

Just as *P. falciparum* relies on the presence of erythrocyte receptors, *Plasmodium vivax* [75][76] and *Plasmodium knowlesi* [14] also depend on the presence of the Duffy antigen (Fy) in its different polymorphic expressions. Fy is an almost obligatory receptor for the invasion of these parasites into host reticulocytes, wherein it is expressed at higher levels than in older RBCs [77][78]. This could be one of the reasons why *P. vivax* and *P. knowlesi* show a marked preference for the invasion of reticulocytes rather than the invasion of the more mature erythrocytes. This is not the case for *P. falciparum*, which is sufficiently resilient to survive a myriad of challenges, including that of erythrocyte aging.

Regarding the molecular changes experienced by senescent erythrocytes, which involve the loss of parasite receptors, either through aggregation and subsequent loss of function (e.g., Band 3), vesicle release, or downregulation, the parasite exhibits enough flexibility to compensate for their disappearance by adopting alternative entry pathways. While the diversity of receptors is reduced due to the aging process, the molecular availability of ligands remains high enough to promote infection, thereby conferring the characteristic virulence of *P. falciparum*. The extensive range of molecular options that enable the parasite to invade red blood cells complicates the development of effective blocking vaccines.

In terms of mechanical changes, the loss of elasticity cannot, by itself, be considered a marker of erythrocyte maturity, for this loss is also observed in young red blood cells infected with *Plasmodium* or even uninfected red blood cells subjected to bystander effects during infection [46]. Under normal circumstances, erythrocytes that have lost their deformability are captured by splenic macrophages, leading to their rapid removal. However, during infection, the parasite evades this process by expressing surface proteins on erythrocytes, allowing them to bind to the molecules present on endothelial cells. This enables the parasite to remain in vascular niches of organs, such

as the heart, bone marrow, gastric mucosa, and even the brain, until reaching maturity [79], reducing its circulation in the bloodstream.

Under these circumstances, senescent red blood cells, or young red blood cells subjected to bystander effects when there is infection with *Plasmodium*, or even infected red blood cells that have not reached enough stiffness and remain in circulation, may act as decoys to partially saturate the phagocytic capacity of splenic macrophages, favoring the progression of the infectious process.

Some questions linger regarding the strategies of *P. falciparum* and other Plasmodia to select their receptors: Do they depend solely on their availability? How much of this preference could be attributed to changes in the geometry of the RBC due to the aging process? Do their preferred invasion receptors change over the lifespan of the RBC? Could other factors influence their invasion receptor selection?

It is also known that choline, which is a precursor of the phospholipid composition of cells, is avidly taken in by *P. knowlesi*-infected simian red blood cells. The permeability of simian erythrocytes to choline was found to be considerably increased after infection by the malaria parasite [80][81]. In addition, it was reported that young malaria-infected erythrocytes showed an increase in choline permeability [82].

The increased expression of the key *P. falciparum* receptor, namely, Basigin, on early reticulocytes [83][84][85] could augment *P. falciparum* merozoite binding and invasion into reticulocytes, although this effect has not been directly demonstrated [70].

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