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Erythrocyte Invasion Receptors for *Plasmodium falciparum*: New and Old

Running Title: Erythrocyte Invasion Receptors for *P.falciparum*

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Abstract

Understanding the complex process by which the invasive form of the P*lasmodium falciparum* parasite, the merozoite, attaches to and invades erythrocytes as part of its blood stage life cycle represents a key area of research in the battle to combat malaria. Central to this are efforts to determine the identity of receptors on the host cell surface, their corresponding merozoite binding proteins and the functional relevance of these binding events as part of the invasion process. This review will provide an updated summary of studies identifying receptor interactions essential for or implicated in *P. falciparum* merozoite invasion of human erythrocytes, highlighting the recent identification of new receptors using groundbreaking high throughput approaches and with particular focus on the properties and putative involvement of the erythrocyte proteins targeted by these invasion pathways.

Introduction

Despite significant progress over the last decade, annual cases of malaria continue to number in the hundreds of millions resulting in up to half a million deaths every year (WHO, 2014). Malaria is caused by infection by parasites of the genus *Plasmodium* within the phylum apicomplexa. Five species of *Plasmodium* are capable of infecting humans, however despite justified concerns regarding increased incidence of *Plasmodium vivax* infection (Galinski and Barnwell, 2008) and the emergence of *Plasmodium knowlesi (Singh et al., 2004)*, P*lasmodium falciparum* remains the major source of morbidity associated with this parasite (Snow et al., 2005).

P. falciparum exhibits a complex life cycle which includes stages within the *Anopheles* mosquito vector and the hepatocytes of the human liver. The manifestation of symptoms and the burden of morbidity associated with malaria however occur almost entirely as a result of the asexual replication cycle that takes place within circulating erythrocytes (red blood cells). This rapid replication cycle, which follows initial invasion of an erythrocyte host cell takes approximately 42-48 hours and is characterised by multiplication and maturation of the parasite through so called ring stages, trophozoites and schizonts and culminates in eventual rupture of the erythrocyte releasing up to 32 new merozoites each of which is capable of rapidly infecting a new red blood cell. As the only extracellular stage of the parasite life cycle within the human host and with a view to development of an effective therapeutic vaccine, the process by which merozoites attach to and invade erythrocytes has provided a focus of malaria research for many years.

This review will summarise past and recent successes in determining the identity of receptors on the red blood cell surface that are essential for or implicated in *P. falciparum* merozoite invasion of human erythrocytes; highlight the structural and functional properties of these host proteins and provide speculation regarding their relevance as part of the merozoite invasion process.

The process of merozoite invasion

Blood stage invasion by the malarial parasite begins with the interaction between a merozoite (the invasive form of the parasite) and the erythrocyte surface. The interaction can occur irrespective of the orientation of the merozoite and most commonly begins as a 'side on' association. This initial low affinity and reversible interaction elicits waves of deformation of the erythrocyte membrane (Weiss et al., 2015) and is swiftly followed by reorientation of the merozoite such that the apical tip is in direct contact with the host membrane. Direct high affinity interactions established between membrane proteins expressed on the erythrocyte surface and merozoite adhesins such as the EBL (Erythrocyte Binding Ligand) and PfRh (*P. falciparum* Reticulocyte binding Homolog) proteins commit the parasite to active invasion which proceeds through an induced depression in the erythrocyte surface and is mediated by the formation of a 'tight junction' defined by merozoite proteins of the AMA1-RON complex inserted into the erythrocyte membrane. Penetrative force provided by the parasite actin myosin motor drives the merozoite across the host membrane into a parasitophorous vacuole compartment believed to be created by secretion of the rhoptries into the host cell and the erythrocyte membrane is resealed completing the process. This entire sequence of events is completed within approximately 30 seconds and the current model is summarised in Figure 1.

Alternate Invasion Pathways

Merozoites can use different pathways, defined by receptor–ligand interactions, for invasion of erythrocytes. Traditionally within the malaria field invasion by *P. falciparum* has been classified as occurring via two main pathways – sialic acid (SA) dependent and SA independent invasion, characterised by the capacity to invade erythrocytes in which sialic acid has been cleaved using the protease neuraminidase. Sialic acid dependent invasion involves the erythrocyte glycophorin binding proteins, EBA175, EBL-1 and EBA140 discussed in greater detail below as well EBA181 and PfRh1 which have been demonstrated to bind SA but of as yet unidentified receptor. Sialic acid independent invasion is predominantly believed to be reliant on the neuraminidase insensitive binding of PfRh4.

Different strains of *P. falciparum*, both clinical isolates and defined lab adapted clones vary in their dependence on sialated erythrocyte receptors for invasion. Functional redundancy within the repertoire of merozoite erythrocyte binding proteins such as the SA binding EBA family allied to variation in the expression or use of such proteins provide the parasite with powerful mechanisms by which to evade the development and efficacy of inhibitory antibodies by the immune system. However perhaps the most striking demonstration of the adaptability of *plasmodium* comes from the elegant work of Stubbs et al who demonstrated the capacity for sialic acid dependent parasites to reversibly switch to a SA independent invasion pathway via activation of PfRh4 expression in response to the removal of target erythrocytes sialic acid (by neuraminidase) or by genetic ablation of EBA175 (Stubbs et al., 2005). This illustration of the "plasticity of *P. falciparum* in the face of selective pressures such as altered erythrocyte receptors" (Stubbs et al., 2005) highlights the challenges faced by efforts to develop an effective vaccine to combat malaria erythrocyte invasion.

Sialic Acid Dependent Invasion Receptors

Glycophorin A (EBA175)

Glycophorin A is a 131 amino acid type I transmembrane glycoprotein, expression of which is restricted to the erythroid lineage of cells. Together with the chloride bicarbonate transporter band 3 it represents the most abundant protein within the erythrocyte membrane, present at approximately 1×10^6 copies per cell (Reid ME, 2012). It is the major erythrocyte sialoglycoprotein in the erythrocyte membrane, existing as a heavily glycosylated transmembrane dimer with one N glycan and at least 15 O glycans per monomer. Although it exists predominantly as homodimers, GPA is also capable of forming heterodimers with glycophorin B and interacts directly with the eighth transmembrane helix of band 3 (forming the dual protein blood group antigen Wrb) (Telen and Chasis, 1990) and via its C-terminus. Glycophorin A was the first erythrocyte receptor for which a corresponding *P. falciparum* ligand was identified and is bound by the micronemal erythrocyte binding protein EBA175 (Orlandi et al., 1992). Interaction between these two proteins has now been extensively characterised, with the binding site on GPA mapped to sialylated glycan epitopes within the region of the N terminal domain covered by amino acids 55-72, which is bound by the RII domain of EBA175(Tolia et al., 2005). Structural analysis suggests that the RII domain of EBA175 assembles as a dimer around the dimeric GPA extracellular domain in response to binding of the parasite protein to its erythrocyte receptor during invasion (Tolia et al., 2005). Dimerisation of EBA175 in response to GPA binding may act as a signal to induce signalling within the merozoite. Binding of antibody fragments to GPA has also been shown to alter erythrocyte membrane deformability

(Chasis et al., 1985), in a process which may result from conformational changes within the cytoplasmic domain induced by binding to the extracellular region of GPA, raising the possibility that EBA175-GPA binding induces downstream alterations in both host and parasite which may contribute to the progress of the invasion process.

Glycophorin B (EBL-1)

Glycophorin B is structurally similar to glycophorin A with the exception of the cytoplasmic tail which is much shorter in GPB; it is encoded by a separate gene to GPA which probably arose by gene duplication of the original GYPA gene. It has a copy number of 170-200,000 copies per cell (Reid ME, 2012), 5-6 fold less than that of glycophorin A and exists both as homodimers and as part of heterodimers with GPA. Glycophorin B negative cells show moderate resistance (40-79% depending on the parasite strain studied) (Rayner et al., 2001, Gaur et al., 2003)) to malarial invasion but almost complete resistance following trypsin removal of glycophorin A which likely indicates a degree of functional redundancy in the roles of the parasite proteins involved in binding these similar glycophorins during merozoite attachment and invasion. Interestingly it has been hypothesised that glycophorin B may have arisen as a result of selective pressure to lose the EBA175 binding site on GPA (Salinas et al., 2014).

In 2009, Mayer et al identified GPB as the receptor for the parasite protein erythrocyte binding ligand 1 (EBL-1) by demonstrating the inability of GPB null erythrocytes to adsorb this protein from *P. falciparum* culture supernatants (Mayer et al., 2009). These data were later corroborated through the screening of a *P. falciparum* cDNA phage display library using purified glycophorins, which also identified the binding site for GPB on EBL-1 (Li et al., 2012). As in the case of EBA175 binding to GPA, EBL-1 binding to erythrocytes is neuraminidase sensitive indicating that the binding site on GPB resides within a sialylated glycan epitope.

Glycophorin C (EBA140)

Glycophorin C represents the third member of the erythrocyte glycophorin family, it comprises 128 amino acids, 12 O linked glycans and a single N linked glycan, and is present at $135-143 \times 10^3$ copies per erythrocyte (Reid ME, 2012). It resides within the junctional complex of proteins where it associates with the spectrin cytoskeleton via interactions with protein 4.1 and p55 (Alloisio et al., 1993). GPC represents the erythrocyte receptor for the *P. falciparum* ligand EBA 140 (Lobo et al., 2003, Maier et al., 2003), a homologue of EBA175 which binds to erythrocytes in a neuraminidase and trypsin sensitive but chymotrypsin insensitive manner. The precise nature of the binding site for EBA140 on GPC has proved difficult to determine unequivocally. Neuraminidase sensitivity indicates a dependence upon sialic acid for binding however this observation appears to be confounded by the inability of EBA140 to bind erythrocytes expressing the poorly glycosylated natural deletion variant of GPC termed Gerbich (prevalent within the population of the malaria endemic Melanesia region of Papua New Guinea (Patel et al., 2001)). Despite this, recent evidence has confirmed the importance of oligosaccharides and particularly the N glycan for EBA140 binding of GPC (Mayer et al., 2006, Rydzak et al., 2015). Sialylated residues attached to this glycosidic chain (resident on Asn8) and proposed to be modified in Gerbich erythrocytes represent the most likely binding site for the parasite ligand. As yet no alteration of erythrocyte membrane properties has been attributed to GPC binding that could indicate a role beyond surface attachment however antibody ligation of wild type

but not Gerbich erythrocytes was reported to induce phosphatidylserine exposure (Head et al., 2005), a phenomenon also observed in *Plasmodium* infected erythrocytes (Eda and Sherman, 2002).

Sialic Acid Independent Invasion Receptors

Complement Receptor 1 (PfRh4)

Essential to the sialic acid independent pathway of *P. falciparum* invasion is the initially rhoptry localised protein PfRh4. Disruption of the gene encoding PfRh4 in a strain of normally sialic acid dependent parasites W2Mef abolishes the ability of this strain to switch invasion pathways to enable invasion of neuraminidase treated erythrocytes in which sialic acid has been enzymatically removed (Duraisingh et al., 2003a). In 2010, the erythrocyte receptor for PfRh4 was identified as complement receptor 1 (CR1) (Tham et al., 2010). CR1 is a widely expressed complement regulatory protein that binds C3b and C4b and mediates the clearance of immune complexes by splenic macrophages. It is a very large protein (220kDa in its most common variant) and is composed of a string of 30 short complement control protein (CCP) repeats which project 85nm from the surface of the erythrocyte plasma membrane (Moulds, 2010). The PfRh4 binding site has been mapped to the C3b/C4b binding domain located within the site encompassing CCP repeats 1-3 at the extreme N terminus of the protein (Tham et al., 2010).

CR1 is expressed on a variety of blood cell types and mediates a range of immune functions. In erythrocytes, expression of CR1, which is low relative to other that observed on other blood cell types is variable, with up to 10 fold differences between individuals, ranging from as low as 1-200 up to 1200 copies per erythrocyte (Moulds, 2010). Erythrocytes from individuals with higher expression of CR1 exhibit increased merozoite, PfRh4 binding and sialic acid independent invasion rates respectively. Several studies have also correlated homozygosity (and even heterozygosity) for the CR1-L (CR1 low allele) with protection against severe malaria (Panda et al., 2012, Cockburn et al., 2004, Fowkes et al., 2008). In interpretation of these data it is important to consider that CR1 also provides a binding site for the parasite rosetting ligand *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Rowe et al., 1997) which is expressed on the surface of infected erythrocytes and thus the influence of CR1 levels on severity of clinical episodes of malaria is likely to be multifactorial.

The precise role that PfRh4 binding to CR1 plays in facilitating the invasion process, as for the majority of the merozoite erythrocyte receptor interactions is unknown. Whilst there appears to be functional redundancy between the interaction of these two proteins and that between the EBA proteins and the sialic acid invasion pathway glycophorin receptors, the structural properties of CR1 and GPA, B and C are very different. The glycophorins, each present at a copy number several orders of magnitude greater than CR1 are localised within multiprotein complexes with indirect linkage to the erythrocyte spectrin cytoskeleton. CR1 in contrast has not been identified to associate with the cytoskeleton or to exist as part of a larger complex. Nevertheless ligation of CR1 on uninfected erythrocytes by either antibodies or complement opsonised particles results in intracellular Ca2+ flux and a corresponding increase in membrane deformability associated with phosphorylation of β spectrin (Glodek et al., 2010) which speculatively could be exploited by the parasite as part of the invasion process.

<u>Basigin (PfRh5)</u>

The degree of functional redundancy between EBA and PfRh proteins is highlighted by the fact that the genes encoding all of these proteins can be genetically disrupted (reviewed in (Tham et al., 2012). PfRh5 provides the sole exception to this rule, apparently providing an essential non redundant function at some stage of the blood stage parasite life cycle. Early work showed PfRh5 binding to erythrocytes via an unknown glycosylated receptor resistant to chymotrypsin, trypsin and neuraminidase however the specific identity of the receptor remained uncovered (Baum et al., 2009). In 2011 using a novel systematic screening approach optimised for the detection of low affinity interactions (AVEXIS - AVidity-based EXtracellular Interaction Screen) Crosnier et al identified the Ok blood group antigen basigin (CD147) as the much sought erythrocyte membrane protein receptor responsible for PfRh5 binding (Crosnier et al., 2011).

Basigin (CD147) is a broadly expressed, highly glycosylated type I integral membrane protein that is a member of the immunoglobulin superfamily of proteins. Within the erythrocyte membrane it is present at approximately 3000 copies per cell (Reid ME, 2012) and exists as a homodimer in complex with two monomers of the monocarboxylate transporter 1 (MCT1) lactate transporter (Wilson et al., 2002). As part of the biogenesis of the erythrocyte membrane during erythropoiesis, basigin is believed to act as a chaperone for MCT1 facilitating its delivery to the plasma membrane (Kirk et al., 2000) . Basigin is not associated with the spectrin cytoskeleton and the majority of this protein expressed within the plasma membrane of orthochromatic erythroblasts is lost during the process of erythroblast enucleation (Griffiths et al., 2012). Whether the population that remains in complex with MCT1 in mature erythrocytes is vestigial at this stage or performs an additional unidentified function is unknown. It will be intriguing to see within the coming years whether the increased scrutiny of this protein uncovers features relating to its structure, properties or function under both basal and invasion conditions that account for its specific involvement in the merozoite invasion process.

In addition to identifying basigin as the receptor for PfRh5 binding, employing a high throughput approach in which a pentamerised recombinant PfRh5 prey was used to screen a library comprised of 40 major erythrocyte membrane protein ectodomain, Crosnier and colleagues were able to demonstrate that both soluble basigin and basigin antibodies potently inhibit invasion. The essentiality of basigin as a *P. falciparum* invasion receptor was further confirmed using an exciting approach made possible through advances within the erythropoiesis field that have enabled the *in vitro* culture of invasion susceptible red blood cells differentiated from haematopoietic stem cells. By transducing immature nucleated erythroid precursors with lentiviruses containing shRNA targeting BSG prior to subsequent differentiation, the authors were able to generate *in vitro* derived red blood cells exhibiting a 50-60% reduction in cell surface expression of basigin. In so doing they were able to monitor invasion of a novel red blood cell phenotype and confirm the reduced susceptibility of these cells to invasion by *P. falciparum* and hence the importance of the PfRh5-basigin interaction for this process. Additional work was able to establish the importance of a region of basigin that covers a naturally occurring variant of the protein harbouring the E92K Oka- mutation with erythrocytes from these rare donors demonstrating reduced susceptibility to invasion by *P. falciparum.* The crystal structure of PfRh5 in complex with basigin has since been reported providing detailed structural insight into the interaction between these two proteins (Wright et al., 2014).

Most excitingly, unlike in the case of other merozoite-erythrocyte receptor interactions which display varying strain dependent sensitivities to blocking, all detectable erythrocyte invasion by every *P. falciparum* strain tested was prevented by anti-basigin antibodies. The interaction between basigin and PfRh5 appears therefore to define a fundamentally distinct function from that of all previously identified EBA and PfRh protein interactions. This apparent cross strain dependence upon the binding of a single receptor-ligand pair has elevated the disruption of this interaction to the top of worldwide efforts for malaria therapeutic intervention and PfRh5 now represents the highest priority vaccination target.

Semaphorin 7a (MTRAP)

The TRAP family describes a group of proteins that facilitate linkage between target host cells and the actomyosin based molecular motors required to drive invasion of the various motile forms of malaria parasites including sporozoites, ookinetes and merozoites (Morahan et al., 2009). Each of these forms can be distinguished by expression of distinct TRAP family proteins. Whilst the importance of MTRAP, which is expressed on the surface of the blood stage invasive merozoite for successful erythrocyte invasion or growth, was illustrated by the inability to successfully disrupt the gene encoding this protein (Baum et al., 2006), the identity of the erythrocyte receptor for this parasite protein remained elusive for many years. In 2012 Bartholdson and colleagues, again employing the AVEXIS assay, demonstrated the identity of the human erythrocyte receptor for MTRAP as Semaphorin 7a (Bartholdson et al., 2012), an 80kDa GPI anchored glycosylated membrane protein also known as the John Milton Hagen (JMH) blood group antigen. Stoichiometric analysis indicates that MTRAP associates weakly with the dimeric Sema7a in a 1:1 ratio with two MTRAP monomers interacting with each Sema7a homodimer via the Sema domains. Naturally occurring polymorphisms within the extracellular domain of Sema7a did not affect the affinity of MTRAP binding and interestingly, the addition of antibodies to Sema7a did not inhibit merozoite invasion in *in vitro* invasion assays (Bartholdson et al., 2012). Although, Sema7a (CD108) has been shown to play diverse roles in neuronal and immune cells where it associates with integrins, neither the copy number nor function of Semaphorin 7a on erythrocytes is currently known hindering speculation regarding the relevance of the properties of this host cell protein to the function of this interaction. Studies of invasion phenotypes using erythrocytes from rare JMH negative individuals or from the complement sensitive erythrocytes of patients with paroxysomal nocturnal haemoglobinuria deficient in GPI anchored proteins may provide further insight into the timing and importance of this interaction for successful merozoite invasion.

Band 3/GPA (MSP-1)

Band 3, together with glycophorin A, represents the most abundantly expressed protein within the erythrocyte plasma membrane, present at approximately $1x10^6$ copies per cell (Reid ME, 2012). As an ankyrin-binding mediated tetramer it forms the core of a major multiprotein complex which also contains glycophorin A, protein 4.2 and components of the Rh (Rhesus protein) subcomplex, RhCe/D, RhAG, CD47, GPB and ICAM4 (Bruce et al., 2003) and provides the major site of vertical connectivity between plasma membrane and spectrin cytoskeleton required for maintenance of the erythrocyte structural integrity. In its dimeric state it resides within the junctional complex of proteins providing a supplementary site of cytoskeletal anchorage via its interaction with adducin

(Anong et al., 2009) and exists also as an additional population of more freely mobile dimers probably in association with GPA.

Given its abundance, importance and interactions with other erythrocyte sites of malaria attachment, a role for band 3 in merozoite attachment and invasion has long been assumed to exist. It is only recently however that the potential nature of this role, during attachment at least, has begun to fully emerge. In 2003, Goel et al reported the identification of a sialic acid independent interaction of host and parasite proteins *in vitro* between two non-glycosylated extracellular regions of band 3 (within the regions covered by amino acids 720-761, and by 807-826) and fragments of the Major Merozoite Surface Protein (MSP1), $MSP1_{42}$ and $MSP1_{19}$ (Goel et al., 2003). MSP1 is the most abundant protein on the surface of the merozoite (Holder and Freeman, 1984); it undergoes extensive proteolytic processing around the time of merozoite release following schizont rupture resulting in the generation of four proteolytic fragments of 83kDa, 30kDa, 38kDa and 42kDa which remain as a multisubunit complex on the surface of the merozoite (McBride and Heidrich, 1987) . Secondary processing of the 42kDa fragment results in the generation of a 33kDa and 19kDa fragment and whilst the majority of these fragments are shed from the merozoite surface, $MSP1_{19}$ remains attached during the penetrative process of invasion(Blackman et al., 1991). Recently, a yeast two hybrid study in which the 720-761 so called 5ABC peptide of band 3 was used as bait, identified an association between this extracellular region of band 3 and the C terminus of RhopH3 (Baldwin et al., 2014), a *P. falciparum* protein which was in turn was shown to bind directly to $MSP1_{19}$. There is thus accumulating evidence for a role for band 3 as a binding site for the major multi component MSP complex to which RhopH3 has now been assigned.

Band 3 associates directly with the equally abundant single transmembrane spanning EBA175 receptor glycophorin A via its eighth transmembrane helix. The association of these two proteins at this location is marked by the presence of a well characterised blood group antigen Wrb. Earlier this year using phage display screening technology, Baldwin et al identified a region of the 83kDa fragment of MSP1 (a stably expressing 12kDa recombinant fragment) that bound to immobilised glycophorin A as well as native and neuraminidase treated erythrocytes (Baldwin et al., 2015). Interestingly this fragment also binds trypsin treated erythrocytes indicating binding to the trypsin insensitive 40 amino acid region of GPA close to the base of the extracellular domain of the protein. *P. falciparum* invasion is inhibited up to 40% in the presence of high concentrations of either $MSP1_{83}$ or $MSP1_{12}$.

The abundance within and specificity for the erythrocyte membrane of the band 3/GPA complex make it an ideal target for binding of merozoite proteins involved in initial pre-invasion adhesion, potentially enabling the parasite to attach at any site on the erythrocyte. It is easy to envision a scenario in which the merozoite has evolved in order to exploit association between its most abundantly expressed surface protein and that of the corresponding host cell equivalent in order to increase the chances of adhesion, which can take place regardless of the orientation of either cell. Band 3 exists as both tetramers and dimers, within at least two major multiprotein complexes and as populations both attached to the spectrin cytoskeleton (via ankyrin or adducin respectively) and mobile within the corrals imposed by the erythrocyte membrane cytoskeletal architecture. To what degree these different populations of band 3 (and associated GPA) represent the target for MSP1 binding, the relevance of cytoskeletal association at these sites and the interplay between MSP1 band 3/GPA interactions and subsequent binding of overlapping (in the case of EBA175-GPA) or

sterically adjacent erythrocyte receptors during the rapid progression of committed invasion will all provide exciting avenues of future research.

<u>Kx (AMA1)</u>

Invasion of the erythrocyte membrane by the apically orientated merozoite occurs via a tight junction which forms at the apposition of host and parasite membranes. Formation of this tight junction is mediated by the insertion of the merozoite RON (Rhoptry Neck Protein) complex protein RON2 into the erythrocyte membrane (Lamarque et al., 2011). AMA1 (Apical Membrane Antigen 1), a microneme protein which is secreted onto the surface of the merozoite binds to RON2 (Srinivasan et al., 2011) providing a tight and direct association between the invading parasite and host membrane. This remarkable mechanism enables the parasite to actually encode both ligand and receptor and with it provides an ingenious means by which to avoid the effects of evolutionary adaptation of host encoded proteins. Competitive inhibition of the RON2-AMA1 interaction by synthetic peptides does not prevent merozoite attachment to erythrocytes but potently inhibits invasion by preventing tight junction formation and internalisation (Richard et al., 2010, Weiss et al., 2015).

The site of tight junction formation within the erythrocyte membrane is an understandable source of interest within the malaria invasion research community. Although it is possible and perhaps even likely that RON complex components translocated to the cytoplasmic side of the erythrocyte membrane (Riglar et al., 2011) associate with host cell proteins to provide anchorage, no such associations have as yet been identified. A potential clue to the localisation of the tight junction was provided by the study of Kato et al (Kato et al., 2005). Using recombinantly expressed domains of AMA1 they showed that Domain III binds to trypsin treated wild type erythrocytes but not to erythrocytes null for the Kx protein suggesting that AMA1 binds to Kx as part of a process that also involves enzymatic exposure or modification of the erythrocyte surface protein. The authors also report reduced *P. falciparum* invasion of Kx null erythrocytes of the McLeod phenotype. It should be noted however that erythrocytes from these patients are severely acanthocytic (Galey et al., 1978) and possess in addition a myriad of additional membrane protein modifications (Tang et al., 1981) and altered properties (Ballas et al., 1990) that complicate interpretation of this effect. Whether this putative interaction plays an active role in tight junction formation/internalisation or occurs incidentally, the identification of a candidate erythrocyte receptor for a component of the tight junction, localised within the major junctional complex provides a useful starting point for efforts to characterise host-parasite interactions at this crucial structure.

<u>Receptors with Unknown Merozoite Binding Partners</u>

<u>CD55</u>

CD55 (Decay Accelerating factor or DAF) is a highly glycosylated 319 amino acid glycoprotein, it is comprised of a large extracellular domain containing four CCP repeats and a heavily O glycosylated stalk and is anchored to cholesterol rich domains or 'lipid rafts' within the extracellular face of the erythrocyte lipid bilayer by means of a GPI anchor (Lublin, 2005). CD55 is widely expressed on cells throughout the body but in erythrocytes is present at about 20,000 copies per cell (Reid ME, 2012). It serves as an inhibitor of autologous complement activation by preventing the assembly and accelerating the decay of C3 convertase and together with CD59 (Membrane Inhibitor of Reactive Lysis or MIRL), protects erythrocytes from complement mediated lysis. Absence of CD55 (and other

GPI linked proteins) on erythrocytes represents a characteristic phenotype used for the diagnosis of Paroxysomal Nocturnal Haemoglobinuria (PNH), a rare clonal disorder of haematopoietic stem cells that results from mutations in phosphatidylinositol glycan A (PIG-A), one of several enzymes required for the synthesis of GPI anchors. The absence of haematological phenotype in patients with a rare naturally occurring complete deficiency of CD55 alone (Inab phenotype) indicates that increased susceptibility of PNH erythrocytes to complement mediated lysis is not a direct result of the loss of CD55 expression on these cells.

Earlier this year, a seminal study employed a high throughput forward genetic screening approach to the identification of erythrocyte membrane proteins involved in merozoite invasion. Using cultured red blood cells derived from haematopoietic stem cells transduced with a library of shRNAs to known blood group proteins they demonstrated that knock down of CD55 reduced susceptibility of host cells to *P. falciparum* invasion by up to 30%. Invasion of mature erythrocytes from the haematologically benign but rare naturally occurring CD55 null Inab phenotype by a variety of lab adapted *P. falciparum* strains and clinical isolates was almost completely abolished (Egan et al., 2015). Puzzlingly, the identification of CD55 as a strain transcendent mediator of parasite invasion is at odds with an earlier study that reported increased parasitemia within the CD55 negative population compared to CD55 positive erythrocytes within the same PNH patient (Pattanapanyasat et al., 2003). This is contrary to the observations of Egan et al who reported complete absence of invasion in erythrocytes from the rare CD55 null Inab individuals (Egan et al., 2015). Further investigation of this apparently contradictory increase in invasive susceptibility in the absence of CD55 in these patients may illuminate the role of additional codeficient GPI linked erythrocyte membrane proteins during the invasion process.

Interestingly, Egan et al reported similar attachment of *P. falciparum* merozoites to CD55 null and wild type cells with subsequent detachment accounting for the defective invasion leading them to propose that CD55 may in fact be critical for committed tight junction formation rather than initial attachment. CD55 has also been reported to localise to the PVM together with other lipid raft associated proteins during invasion (Murphy et al., 2004). Binding of C3b to glycophorin A has been shown to result in the lateral confinement of CD55 via the formation of a cytoskeletally immobilised DAF-C3b-GPA-band 3 complex (Karnchanaphanurach et al., 2009). One interesting possibility therefore is that CD55 is recruited to membrane protein complexes at the site of invasion in response to binding of glycophorin A, for example by EBA175, positioning it for the establishment of an interaction with an as yet unidentified merozoite protein essential for committed invasion. Exploration of this hypothesis in concert with efforts to identify the merozoite binding partner for CD55 should represent an area of particular focus.

CD44

In addition to the identification of CD55 as an erythrocyte receptor essential for *P. falciparum* invasion, Egan et al also demonstrated a 30% drop in parasitemia of *in vitro* cultured red blood cells with severe knockdown of CD44 (Egan et al., 2015). CD44 (H-CAM) is a widely expressed 85kDa glycoprotein that exists as multiple alternatively spliced variants. It provides a receptor for hyaluronic acid (HA), is present at approximately 6000-10,000 copies per cell (Anstee et al., 1991) and forms the Indian blood group in erythrocytes. CD44 has also been shown to bind ankyrin (Kalomiris and Bourguignon, 1988, Nunomura et al., 1997) and protein 4.1 (Nunomura et al., 1997),

with binding of the latter inhibiting binding to ankyrin, and also subject to regulation by calmodulin and Ca^{2+} in *in vitro* binding studies (Nunomura et al., 1997). Influx of calcium into the erythrocyte, known to be associated with merozoite invasion could alter the affinity of CD44 for protein 4.1 leading to a reconfiguration of its cytoplasmic domain interactions and potential conformational changes in the extracellular domain that could facilitate interactions with merozoite proteins. As in the case of CD55, identification of the merozoite binding partner as well as determination of the timing and nature of the disruption to the invasion process induced by the absence of CD44 will hopefully inform further work.

ICAM4

Members of the Intercellular adhesion molecules (ICAMs) which (like the PfRh5 receptor basigin) form part of the immunoglobulin superfamily, are expressed on an array of cell types mediating or participating in a variety of cell-cell interactions. ICAM-4 was originally identified as the protein responsible for the erythrocyte LW (Landsteiner Wiener) blood group and later assigned to the ICAM family based on homology with other members. Its expression is restricted to the erythroid lineage residing within the Rh protein subcomplex (Rh, RhAG, GPB, CD47) within the larger tetrameric band 3/ankyrin based multiprotein complex in mature erythrocytes.

Earlier this year, Bhalla et al reported binding of recombinant ICAM4 to merozoites accompanied by an 80% drop in invasion upon addition to *in vitro* invasion assays (Bhalla et al., 2015). Invasion assays performed in the presence of a synthetic protein (M5) that binds ICAM4 in erythrocytes resulted in dose dependent cross strain invasion inhibition of both untreated and neuraminidase treated erythrocytes, with microscopic analysis reportedly unaffected leading the authors to conclude that ICAM4 is important at a later stage of invasion post attachment. Erythrocytes completely deficient in ICAM4 have been reported – both individually (LWnull) and as part of the Rhnull phenotype. Interestingly, although relative invasion efficiency was not explicitly reported for untreated Rh null erythrocytes compared to wild type, Chung et al demonstrated efficient and even increased invasion of Rh null erythrocytes treated with a panel of proteases (Chung et al., 2008). Further work is warranted to clarify the involvement of ICAM4 during merozoite invasion.

Merozoite Proteins with Unidentified Erythrocyte Receptors

Whilst major strides have been made in the identification of receptor ligand interactions associated with merozoite binding to the erythrocyte, additional interactants undoubtedly remain. Even amongst the highly characterised EBL and PfRh families, receptors for merozoite proteins with recognised capacity to bind the erythrocyte remain unidentified. Chief amongst these are the sialic acid dependent receptors termed W, Y and Z, binding partners for EBA181(Gilberger et al., 2003), PfRh1 (Rayner et al., 2001, Triglia et al., 2005) and PfRh2a/b (Duraisingh et al., 2003b) respectively and for which protease sensitivity profiles have long been characterised. Although the capacity for genetic disruption of each of these *P. falciparum* genes indicates non crucial or redundant functionality during the invasion process it will be fascinating to observe whether any of the erythrocyte proteins with demonstrated (Egan et al., 2015, Bhalla et al., 2015) or speculated (Storry et al., 2013) involvement in parasite binding represent targets for binding of these prominent parasite adhesins.

Sequence of merozoite-erythrocyte protein interactions during invasion

A detailed understanding of the sequence of specific protein interactions that occur between host and parasite proteins during attachment and invasion and how these correlate with the characteristic stages of the process outlined in Figure 1 is crucial to efforts to determine the molecular mechanism by which merozoite invasion of the red blood cell occurs from both a parasite and host perspective. Whilst significant insight has been derived from the localisation of specific proteins within compartments of the merozoite, the rapidity of the invasion process remains a major barrier to the detailed study of this process. Earlier this year Weiss et al published an extensive study in which they used live cell microscopy to perform a detailed visual characterisation of merozoite invasion (Weiss et al., 2015). By using a combination of inhibitors, protease treatments and parasite strains with defined invasion pathways they were able to develop a proposed model that describes the sequence of interactions that occur during *P. falciparum* invasion of an erythrocyte. With reference to the identities of erythrocyte receptors discussed in this review this can be summarised as the likely sequential interaction of 1) MSP1 with band 3/GPA (with accompanying weak deformation of the erythrocyte membrane), 2) the redundant interaction of EBA/PfRh ligands with glycophorins or CR1 associated with a strong merozoite motor dependent erythrocyte deformation, 3) PfRh5 binding to basigin resulting in formation of a pore connecting parasite and host and 4) establishment of an AMA1-RON2 interaction mediated tight junction within the erythrocyte membrane at unknown site with as yet speculative links to CD55 (Egan et al., 2015) or the junctional complex (Kato et al., 2005).

Perspectives

This review summarises a body of work that spans in excess of 40 years. During this time huge strides have been made in our understanding of the interactions that occur at the interface of malaria parasite and erythrocyte host. From early studies characterising protease sensitivity of erythrocytes to *P.falciparum* invasion in the 1970s and 80's to cutting edge screening approaches of recombinant protein libraries and the generation of haematopoietic stem cell derived novel red blood phenotypes as recently as this year, an array of complementary approaches have been employed over the years to identify the sites of malaria parasite attachment on the surface of the red blood cell (summarised in Figure 2). Identification of these receptors and of the corresponding *P.falciparum* proteins by which they are bound continues to inform our understanding of the mechanism of merozoite attachment and invasion of the erythrocyte membrane. Nevertheless many important questions still remain: Why are specific proteins on the surface of the erythrocyte targeted for attachment over others? Does this relate purely to structural characteristics of the host proteins? It is perhaps unsurprising that many of the erythrocyte proteins targeted are those that extend furthest from the surface of the plasma membrane. GPA,B,C, CR1, basigin, CD44 and ICAM4 are all single transmembrane helix proteins, Sema 7a and CD55 are attached to the plasma membrane via a GPI anchor – although these proteins vary considerably in size (contrast the 85nm CR1 with the 70 amino acid extracellular domain of GPA) (Bartholdson et al., 2013), all possess exposed flexible N terminal extracellular domains which likely facilitate interactions with the proteins expressed on the surface of the merozoite. At present, with the exception of the band 3-MSP1 association (itself seemingly dependent on a dual interaction with GPA) and possibly Kx-AMA1, no associations of merozoite proteins with extracellular domains of multi- transmembrane helix proteins have been reported. Whether this is indeed due to the reduced accessibility of

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extracellular domains within these proteins or merely reflects the current practical difficulties associated with expression and characterisation of these proteins (Bartholdson et al., 2013), with additional important associations still to be identified remains to be determined. Alternatively, does the abundance and cell lineage restricted expression of specific proteins, for example band 3 and the glycophorins, alone account for their involvement in the early attachment events of the invasion process? Or are specific receptors involved at different stages of the invasion process based on more functional characteristics beneath the surface of the exposed extracellular domains?

Here, in particular there remains huge scope for further work. Consideration of these receptors, not as isolated sites for binding but in their native red blood cell membrane environment as part of multiprotein complex assemblies (Mankelow et al., 2012), cytoskeletally associated and with the capacity for dynamic rearrangement and modification is crucial to understanding the mechanisms that link merozoite attachment with active penetrative invasion. Many of the erythrocyte membrane proteins targeted for binding by merozoite proteins are involved either directly or indirectly in important red blood cell processes that range from regulation of membrane deformability to mediation of immune clearance. The degree to which these normal functions may be co-opted by the parasite to facilitate invasion is a fascinating area for future work. In this context, understanding the relevance of cytoskeletal linkage of specific receptors (and its absence for others), its establishment and disruption as part of the invasion process, is also key. As is an active appreciation of the effect that binding can have not just on adjacent proteins but at distant sites through modulation of cell signalling and through alteration of membrane biophysical properties. In recent years, the erythrocyte membrane biology field has been rejuvenated by developments in the *in vitro* culture and manipulation of erythroid cells. Undoubtedly significant challenges exist in the application of these technologies to more mechanistic studies of malaria invasion. However this author is hopeful that the substantial knowledge gained from identification of binding sites at the extracellular face of the erythrocyte and outlined in this review can be exploited to uncover the detailed and complex mechanism that underlies merozoite attachment and invasion of the erythrocyte membrane.

Disclosure of Conflict of Interest

The author declares no competing financial interests.

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Boxes

What is Known? *Plasmodium falciparum* merozoite invasion of red blood cells is mediated via specific binding to prominent erythrocyte membrane proteins. Different invasion pathways are characterised by functionally redundant binding to erythrocyte proteins that characterise sialic acid dependent and independent invasion pathways.

What is New? Exciting high throughput approaches employing novel screening approaches of recombinant protein libraries and invasion of in vitro derived red blood cells derived from genetic manipulation of haematopoietic stem cells have identified new erythrocyte protein receptors with essential roles in invasion.

What are the Main Questions for Future Work? Why are specific erythrocyte membrane proteins targets for merozoite binding? How are the intrinsic properties and erythrocyte functions of individual receptor proteins exploited or coopted by merozoite binding at different stages of the attachment and invasion process? How does binding to extracellular receptors facilitate the host cytoskeletal disruption required for merozoite penetration of the erythrocyte?

Figure Legends

Figure 1 Scheme depicting current model of merozoite invasion of the erythrocyte membrane. 1) Merozoites attach to the erythrocyte membrane in a random orientation most commonly 'side on' 2) Reorientation of the merozoite occurs such that the apical tip is in direct contact with the host membrane 3) A tight junction is established at the interface of merozoite and erythrocyte membrane likely facilitated by the association of EBL and PfRh proteins (secreted to the merozoite surface from their initial micronemal and rhoptry localisation) with their corresponding erythrocyte receptors. This process also involves insertion of the RON complex of parasite proteins into the host cell membrane 4) Penetrative force provided by the parasite actin myosin motor drives active invasion into a parasitophorous vacuole compartment believed to be created by secretion of the rhoptries into the host cell 5) The erythrocyte membrane is resealed completing the invasion process. Invasion process timings are taken from (Harvey et al., 2012, Weiss et al., 2015).

Figure 2 Schematic representation of human erythrocyte P*lasmodium falciparum* 'invasion receptors' labelled with corresponding merozoite binding proteins where known. Binding sites for *P. falciparum* proteins, where known, are indicated in red. Receptor dimensions are not to scale.

rhoptries
pfRH4
pfRh5
MTRAP

MSP-1

micronemes
EBA-175
EBA-140
EBL-1
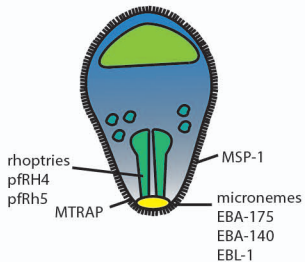
INITIAL ATTACHMENT

REORIENTATION

TIGHT JUNCTION FORMATION

INVASION

RESEALING

RED BLOOD CELL DEFORMATION (~ 6s)

APICAL RESTING (~5s)

INTERNALISATION (~17s)