Erythrocyte invasion by *Plasmodium* merozoites is mediated by molecular interactions between parasite proteins and host receptors. We describe molecular, genetic, biochemical and structural studies to understand the functional roles played by a family of erythrocyte binding proteins (EBPs) from malaria parasites in the process of red cell invasion. These together with field-based immuno-epidemiology studies have been used to build the rationale for the use of EBPs as malaria vaccine candidates. Parasite proteins such as EBPs that mediate interactions with host receptors are commonly localized in apical organelles. Successful invasion requires timely secretion of these parasite proteins during invasion. We describe here efforts to understand signalling mechanisms that trigger timely secretion of EBPs and other parasite ligands from apical organelles during invasion. Such signalling pathways may provide novel drug targets for inhibition of invasion and parasite growth.

**Keywords:** Erythrocyte binding proteins, host–parasite interactions, malaria vaccines, *Plasmodium* merozoites.

**Introduction**

Malaria continues to be a major public health problem in many parts of the tropical world including India. *Plasmodium falciparum* is responsible for ~500 million cases of malaria worldwide each year, resulting in ~1 million deaths primarily in children and pregnant women residing in malaria endemic regions of sub-Saharan Africa. In addition, *Plasmodium vivax* accounts for ~90 million cases of malaria distributed across South and South East Asia, Papua New Guinea, Pacific islands and Latin America. Originally described as ‘benign malaria’, recent studies from different geographic locations have shown that *P. vivax* infections can also progress to severe complications and cause deaths. According to estimates of the World Health Organization, there are ~60 million cases of malaria each year in India that lead to ~20,000 deaths. *P. falciparum* and *P. vivax* contribute equally to malaria cases in India. Regions of India that are most affected by malaria include states of Central India such as Odisha, Chhattisgarh, Jharkhand, Madhya Pradesh and Bihar, as well as the northeastern states bordering Myanmar and Bangladesh. Epidemiology studies conducted in some of the worst affected parts of India such as the forest regions of Odisha are as high as malaria-endemic regions in Africa. *P. falciparum* accounts for more than 80% of malaria in such forest villages of Odisha. Infants and children residing in these high-transmission regions suffer multiple episodes of malaria each year, whereas older children and adults develop partial immunity with lower malaria incidence rates and parasite densities. Repeated infection in infants and young children residing in such high malaria-transmission areas commonly leads to moderate and severe anaemia, which can have serious detrimental effects on child development. There is an urgent need to develop vaccines to protect children from malaria in such endemic regions.

All the clinical symptoms of malaria are attributed to the blood stage of the malaria parasite life cycle. During the blood stage, *Plasmodium* merozoites invade and multiply within host erythrocytes. Inhibition of erythrocyte invasion by antibodies directed against merozoite proteins that mediate invasion should limit parasite growth and provide protection against malaria. Similarly, small-molecule inhibitors that inhibit molecular mechanisms that mediate invasion should block parasite growth and clear blood-stage infections. A clear understanding of the molecular events and mechanisms that mediate erythrocyte invasion by *Plasmodium merozoites* is critical for the development of such novel prophylactic and therapeutic strategies to block invasion and protect against malaria.

Our laboratory has focused on understanding molecular interactions and mechanisms that mediate erythrocyte invasion by *Plasmodium* merozoites. We have used molecular, genetic, biochemical and structural approaches to study the functional roles of key parasite proteins that mediate erythrocyte invasion by *Plasmodium merozoites*. We have combined these laboratory-based functional studies with field-based immuno-epidemiology studies to build the rationale for the development of blood-stage malaria vaccines that target key parasite proteins involved in erythrocyte invasion. The first-generation, novel, recombinant, blood-stage malaria vaccines developed at our Centre (ICGEB) are now being tested in human clinical trials. We have also uncovered key signalling mechanisms that regulate key steps in the invasion
process. This has opened up new strategies to block signalling pathways to inhibit erythrocyte invasion and blood-stage parasite growth.

**Functional analysis of erythrocyte binding proteins and their role in erythrocyte invasion by malaria parasites**

The invasion of erythrocytes by *Plasmodium* merozoites is a complex, multi-step process. Live-cell video microscopy and electron microscopy studies of the invasion process have shown that following initial attachment, the merozoite reorients so that its apical end, which is marked by the presence of membrane-bound organelles called micronemes and rhoptries, faces the erythrocyte membrane8–11. Following apical reorientation, a junction, which is visible at the point of contact with the erythrocytes, develops between the apical end of the merozoite and target erythrocyte. As invasion proceeds, the junction moves around the merozoite and the parasite finally resides within a vacuole surrounded by a vacuolar membrane. This multi-step invasion process is mediated by multiple specific molecular interactions between erythrocyte receptors and parasite ligands.

*P. vivax* and *P. knowlesi* merozoites are completely dependent on interaction with the Duffy antigen receptor for chemokines (DARC) on human erythrocytes for invasion12,13. However, *P. knowlesi* can invade rhesus erythrocytes by multiple pathways using DARC as well as other receptors for invasion14. *P. falciparum* commonly uses sialic residues on glycoporphin A as invasion receptors15,16. However, like *P. knowlesi, P. falciparum* also uses alternative receptors to invade human erythrocytes by multiple pathways15,16. Parasite proteins that mediate interactions with these host receptors during invasion belong to a family of erythrocyte binding proteins (EBPs; Figure 1). Members of the EBP family include *P. vivax* and *P. knowlesi* Duffy binding proteins (PvDBP and PkDBP), *P. knowlesi* β and γ proteins, which bind alternative receptors on rhesus erythrocytes, the 175 kD *P. falciparum* erythrocyte binding antigen (EBA175), which binds glycoporphin A, and EBA175 homologues, EBA140, EBA181 and EBL1, which bind alternative receptors17.

**Molecular genetic approaches to study functional roles of EBPs**

In an effort to understand the functional roles of EBPs in the invasion process, we used molecular genetic methods to knockout the *P. knowlesi* α gene that encodes PkDBP. *P. knowlesi* α gene knockout parasites (PkαKO) lack PkDBP and lose the ability to invade human erythrocytes, confirming the absolute dependence of *P. knowlesi* interaction with DARC for invasion18. However, PkαKO parasites invade rhesus erythrocytes normally due to the presence of *P. knowlesi* β and γ proteins, which mediate invasion of rhesus erythrocytes by binding alternative invasion receptors on rhesus erythrocytes. Analysis by electron microscopy revealed that while PkαKO parasites attach to human erythrocytes and undergo apical reorientation, a junction does not develop and invasion is aborted at this step18. In contrast, PkαKO parasites form a normal junction with rhesus erythrocytes and invade normally due to the presence of *P. knowlesi* β and γ proteins. These studies highlight the essential role of the interaction of Duffy binding protein with DARC for junction formation during erythrocyte invasion and validate EBPs as targets for intervention strategies to block invasion18.

**Structure–function studies of EBPs**

In addition to molecular genetic approaches to decipher the functional roles of EBPs, we have used biochemical, molecular and structural studies to understand how EBPs interact with their receptors. We have demonstrated that the receptor-binding domains of EBPs map to their N-terminal, conserved, cysteine-rich regions (region II), that are also referred to as Duffy-binding-like (DBL) domains after the first binding domains identified from PvDBP and PkDBP19,20 (Figure 1). DBL domains contain 12 to 14 conserved cysteines that form structurally important disulphide linkages. In addition, DBL domains contain conserved motifs that include hydrophobic amino acid residues. Using a combination of molecular and biochemical approaches, we have demonstrated that receptor-
binding residues lie in the central regions of the DBL domains\textsuperscript{21–23}. Alanine replacement mutagenesis of potential binding residues within region II of PvDBP (PvDBPII) identified a number of positively charged and hydrophobic residues between cysteines 4 and 7 that are likely to make contact with DARC\textsuperscript{24}. The binding residues identified within PvDBPII are also present in region II of PkDBP (PkDBPII)\textsuperscript{25}. We have also mapped the receptor-binding site for PvDBPII and PkDBPII to a 35 amino acid extracellular region at the N-terminus of DARC\textsuperscript{26}. A sulphated tyrosine (Y41) within this extracellular sequence plays a key role in receptor recognition by PvDBPII and PkDBPII\textsuperscript{24,26}. Replacement of Y41 with F41 on DARC completely abrogates binding with PvDBPII and PkDBPII.

X-ray crystallography has been used to determine the three-dimensional structure of PkDBPII (Figure 2)\textsuperscript{27}. DBL domains have a conserved structure and are composed of three sub-domains. Sub-domain 1 contains a random coil with two disulphide bonds. Sub-domains 2 and 3 are composed of six $\alpha$-helices with one and three intra-domain disulphide bonds respectively. The three sub-domains are held together by hydrophobic interactions. The binding residues for DARC identified within PkDBP are localized to sub-domain 2. The three-dimensional structure predicts that the binding residues for DARC identified within sub-domain 2 come together on the surface of PvDBPII to form the receptor recognition site (Figure 2)\textsuperscript{27}. The DARC binding site on PvDBPII is fully exposed and accessible to antibodies. Analysis of PvDBP sequences from diverse field isolates reveals that the binding residues predicted to make contact with DARC are highly conserved\textsuperscript{27}. While PvDBPII contains regions that are polymorphic, these appear to be distant to the binding site\textsuperscript{27}. These observations suggest that it should be possible to elicit cross-reactive binding inhibitory antibodies against PvDBPII that inhibit binding of PvDBPII from diverse $P.\ \text{vivax}$ isolates to DARC.

### Immuno-epidemiology studies on PvDBP following natural exposure to $P.\ \text{vivax}$ in endemic areas

Following repeated exposure to $P.\ \text{vivax}$ infections, children residing in malaria endemic regions develop immunity to $P.\ \text{vivax}$ malaria\textsuperscript{28}. Malarialometric indices such as incidence of $P.\ \text{vivax}$ malaria, frequency of $P.\ \text{vivax}$ infection and parasite densities decline with age, suggesting that protective immune responses are acquired with age\textsuperscript{28}. We analysed sera from 5 to 15-year-old children residing in a malaria endemic region of Papua New Guinea, to study the presence and role of antibody responses against PvDBPII in naturally acquired immunity to $P.\ \text{vivax}$ infection\textsuperscript{29}. The study demonstrated that following repeated exposure, children residing in $P.\ \text{vivax}$ endemic regions develop binding inhibitory antibodies against PvDBPII. Importantly, the study also demonstrated that the development of high titre binding inhibitory antibodies directed against PvDBPII is associated with reduced risk of $P.\ \text{vivax}$ infection in these children\textsuperscript{29}.

The presence of high titre anti-PvDBPII binding inhibitory antibodies is also associated with reduced $P.\ \text{vivax}$ densities, indicating that these antibodies play a role in limiting parasite growth in vivo\textsuperscript{29}. The association of the presence of high titre anti-PvDBPII binding inhibitory antibodies with protection against $P.\ \text{vivax}$ infection provides support for the development of a vaccine based on this functional receptor-binding domain. Importantly, as predicted by the structural studies, sera from individuals with high titre binding inhibitory antibodies against PvDBPII inhibit binding of polymorphic PvDBPII domains from diverse $P.\ \text{vivax}$ strains\textsuperscript{29}. A vaccine based on PvDBPII that elicits binding inhibitory antibodies should thus be effective against diverse $P.\ \text{vivax}$ strains.

### Development of blood stage malaria vaccines based on invasion-related proteins

Global efforts to develop malaria vaccines have primarily focused on development of a $P.\ \text{falciparum}$ vaccine due to the higher risk of mortality associated with $P.\ \text{falciparum}$ malaria. While this strategy is appropriate for Africa, parts of the world such as India, South East Asia and Latin America will need vaccines for both $P.\ \text{falciparum}$ and $P.\ \text{vivax}$ malaria. The ICGEB malaria vaccine programme thus includes vaccines for both $P.\ \text{vivax}$ and $P.\ \text{falciparum}$. Given that all clinical symptoms of malaria are attributed to the blood stage of the parasite infection, we have focused on developing blood stage vaccines that target invasion-related proteins to inhibit parasite growth and provide protection against $P.\ \text{vivax}$ and $P.\ \text{falciparum}$ malaria.

### $P.\ \text{vivax}$ vaccine candidate PvDBPII

Our lead $P.\ \text{vivax}$ vaccine candidate is based on region II, the binding domain of PvDBP (PvDBPII; Figure 1). We have developed methods to produce recombinant PvDBPII in its correctly folded functional form\textsuperscript{30–32}. Recombinant PvDBPII is expressed in Escherichia coli, purified from inclusion bodies under denaturing conditions, refolded in vitro and purified to homogeneity by ion exchange chromatography. The immunogenicity of recombinant PvDBPII formulated with human compatible adjuvants, including alhydrogel, Montanide ISA720, MF59, QS21, AS02A, GLA-OF, R848-OF and GLA+R848-OF has been tested in animal models\textsuperscript{33–35}. Following initial studies in mice\textsuperscript{33}, PvDBPII formulated with alhydrogel, Montanide ISA720 and AS02A were selected for further immunogenicity studies in rhesus monkeys\textsuperscript{34}. 

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Recombinant PvDBPII formulated with alhydrogel, Montanide ISA720 and AS02A formulations was highly immunogenic in rhesus monkeys. ELISA recognition titres and binding inhibitory titres of Montanide ISA720 and AS02A formulations of PvDBPII were marginally higher than the alhydrogel formulation. More recently, we have tested the immunogenicity of recombinant PvDBPII formulated with toll-like receptor 4 (TLR4) agonist, glycolipid A (GLA-OF) and TLR7/8 agonist, R848-OF, in mice. The presence of a TLR4 agonist diversifies the repertoire of induced IgG leading to more efficient inhibition of binding of polymorphic PvDBPII domains derived from diverse P. vivax field isolates to DARC. These studies have identified AS02A and GLA-OF as potential adjuvants that can be used for clinical development of PvDBPII formulations. Recombinant PvDBPII formulated with such adjuvants will be tested in clinical trials to evaluate their safety, immunogenicity and efficacy.

**P. falciparum vaccine candidate JAIVAC-1**

Our lead *P. falciparum* vaccine candidate, JAIVAC-1, is composed of recombinant PfMSP119, the conserved, cysteine-rich C-terminal region of *P. falciparum* merozoite surface protein-1 (PfMSP1), and the receptor-binding domain, PfF2, of the 175 kD *P. falciparum* erythrocyte binding antigen (EBA175; Figure 1). Recombinant PfF2 specifically binds sialic acid residues on glycophorin A. Antibodies raised against PfF2 inhibit erythrocyte binding and invasion by diverse *P. falciparum* isolates in vitro. PfMSP1 is also thought to play a critical role in invasion. It undergoes a series of proteolytic processing events during invasion, as a result of which only a 19 kDa conserved, cysteine-rich C-terminal region, PfMSP119, remains on the merozoite surface during invasion. Antibodies targeting PfMSP1 inhibit the proteolytic processing of PfMSP1 and block erythrocyte invasion. Naturally acquired antibodies against both PfMSP119 and PfF2 have been shown to be associated with protection against *P. falciparum* malaria in endemic populations.

We have developed methods to produce recombinant PfMSP119 and PfF2 in *E. coli*. Pre-clinical studies in small animal models demonstrated that both antigens formulated with Montanide ISA720 are immunogenic and elicit invasion inhibitory antibodies against diverse *P. falciparum* isolates. The process for production of recombinant PfMSP119 and PfF2 was transferred to Bharat Biotech International Ltd (BBIL), Hyderabad. JAIVAC-1 was manufactured under cGMP at BBIL and is currently being tested in a phase-I clinical trial to evaluate safety and immunogenicity in healthy adults. The decision to proceed to phase II efficacy trials with JAIVAC-1 will be made based on the safety and immunogenicity data from the phase-I trial.
**Figure 3.** Levels of cytosolic Ca$^{2+}$ in *P. falciparum* merozoites. *P. falciparum* merozoites labelled with Ca$^{2+}$-sensitive fluorescent dye, Fluo-4AM indicate that Ca$^{2+}$ levels are high in free merozoites following release from ruptured schizonts. Merozoites are also stained with a fluorescent DNA dye, DAPI. a, *P. falciparum* merozoite in bright field. b, Fluo-4AM-labelled merozoite. c, DAPI-labelled merozoite showing nucleus. d, Overlay of b and c.

**Figure 4.** Signalling events involved in apical organelle release during erythrocyte invasion by *Plasmodium* merozoites. Exposure of merozoites to a low K$^+$ environment activates phospholipase C (PLC) through an unknown mechanism, which leads to production of inositol tri-phosphate (IP$_3$) and diacylglycerol (DAG). Activation of IP$_3$-receptor (IP$_3R$) gated channels by IP$_3$ leads to release of calcium (Ca$^{2+}$) from the endoplasmic reticulum (ER). Rise in cytosolic Ca$^{2+}$ levels results in the release of microneme (Mn) proteins such as EBA175. In the next step, binding of EBA175 to glycophorin A (Gly A) on the red blood cells (RBC) leads to the release of rhoptry (Rh) proteins such as PfRH2a/b through an unknown mechanism. Mt, Mitochondrion.

**Signalling events involved in erythrocyte invasion by *Plasmodium* merozoites**

Key parasite proteins that mediate interactions with host receptors during erythrocyte invasion by *Plasmodium* merozoites are commonly localized in apical organelles referred to as micronemes and rhoptries. For example, members of the EBP family are localized in micronemes, whereas members of the PIRH family of *P. falciparum* proteins such as PIRH1 and PIRH2a/b, which share homology with *P. vivax* reticulocyte binding proteins, are localized in rhoptries. The sequence, timing and mechanisms that trigger the coordinated release of such key microneme and rhoptry proteins during invasion are not completely understood. Live-cell imaging of *P. falciparum* merozoites labelled with calcium-sensitive fluorescent dye, Fluo-4AM demonstrated that cytosolic Ca$^{2+}$ levels are high in merozoites released from ruptured schizonts (Figure 3). Ca$^{2+}$ could thus play a role as a second messenger in signalling pathways related to invasion. These studies also demonstrated that following attachment of the merozoites to a target erythrocyte, basal cytosolic Ca$^{2+}$ levels are restored and invasion then proceeds to completion. Given the observed rise in cytosolic Ca$^{2+}$ levels in free merozoites, we studied whether cytosolic Ca$^{2+}$ can play a role as a second messenger to trigger apical organelle release during invasion. We demonstrated using flow cytometry that treatment of *P. falciparum* merozoites with calcium ionophore A23187 triggers increase in cytosolic Ca$^{2+}$ levels and release of microneme proteins such as EBA175 to the merozoite surface. Interestingly, we found that rise in cytosolic Ca$^{2+}$ levels does not trigger release of rhoptry proteins to the merozoite surface, suggesting that release of microneme and rhoptry proteins are likely triggered by different signals. Next, we demonstrated that exposure of merozoites to a low K$^+$ concentration as found in the blood plasma provides the external signal that triggers a rise in cytosolic Ca$^{2+}$ and release of microneme proteins. Following translocation to the merozoite surface, engagement of microneme proteins such as EBA175 with their cognate receptors such as glycophorin A on the erythrocytes...
triggers the release of rhoptry proteins such as PfRH2a/b. Interestingly, receptor-engagement by EBA175 also restores basal cytosolic Ca\(^{2+}\) levels, as observed by live-cell imaging following erythrocyte attachment prior to invasion. These studies have for the first time identified the sequence of events and external signals that trigger apical organelle release during invasion\(^{3,4}\).

Apical organelle release during invasion thus plays a vital role in the invasion and parasite development. These studies show a missing complex process that is mediated by multiple specific pathways and receptor interactions during invasion of the human erythrocyte.

### Summary

The invasion of erythrocytes by malaria parasites is a complex process that is mediated by multiple specific molecular interactions between parasite ligands and host receptors. We have used diverse molecular, genetic, biochemical, and structural approaches to understand the functional roles of members of the EBP family in erythrocyte invasion by *Plasmodium* merozoites. Together with field-based approaches, these studies have helped build the rationale for development of malaria vaccines based on EBPs. We have also studied the signalling mechanisms that regulate key processes such as secretion of apical organelle proteins during the process of erythrocyte invasion. Understanding the signalling pathways involved in erythrocyte invasion will allow the development of novel therapeutic strategies to block erythrocyte invasion and limit parasite growth.

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Duffy binding protein.}


ACKNOWLEDGEMENTS. Work in the author’s laboratory has been supported by the Department of Biotechnology (DBT), TATA Innovation Fellowship (DBT), Malaria Vaccine Initiative, PATH, European Vaccine Initiative, Bill and Melinda Gates Foundation, European Commission (EVIMalarR, MalSig and Pregvax grants), The Wellcome Trust, Howard Hughes Medical Institute and WHO-TDR. I thank Shailja Singh and Anuj Kumar for help with the figures.