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## Erythrocyte polymorphisms and malaria parasite invasion in Papua New Guinea

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### Abstract

*Plasmodium falciparum* merozoites engage the erythrocyte surface through several receptor (host)–ligand (parasite) interactions during a brief exchange that results in parasite invasion of the red blood cell. Tens of thousands of these events occur during the initial cycle of blood-stage infections but advance towards billions as the parasite becomes visible to microscopists attempting to diagnose the underlying cause of illness in febrile patients. Advancing blood-stage infection leads to massive proportions of erythrocytes that rupture during repetitive cycles of asexual reproduction. As the infection leads to illness, non-immune or semi-immune individuals can suffer from life-threatening consequences of severe malarial anemia that play a leading role in pathogenesis. Through natural selection, some erythrocyte membrane polymorphisms are likely to have reduced the invasion success of the *P. falciparum* merozoite and increased the fitness of the human host population.

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In the mid-1970s, initial insights into the molecular nature of the interactions between erythrocyte surface proteins and malaria parasites were gained using *Plasmodium knowlesi*, a simian parasite amenable to *in vitro* culture [1]. These studies ultimately led to the discovery that the erythrocyte membrane protein carrying the Duffy blood group was a crucial invasion receptor for *Plasmodium knowlesi* and the related human parasite *Plasmodium vivax* [2,3]. These seminal experiments performed by Louis Miller and his colleagues paved the way to the exploration of two key concepts that underlie susceptibility of the red blood cell to malaria infection [4]. First, specific proteins on the erythrocyte surface are co-opted by the merozoite to activate and promote parasite invasion [5,6]. This hypothesis implies that parasite proteins somehow bind erythrocyte receptors and work in partnership as the merozoite pulls itself into the cell and forms the parasitophorous vacuole. Second, polymorphisms that modify the structure or level of expression of erythrocyte membrane proteins alter the efficiency of invasion and, ultimately, susceptibility to malaria. As malaria kills millions of children each year in endemic areas, mutations that diminish the efficiency of the invasion process would confer a selective advantage to the host and might be expected to increase in frequency over time through natural selection. Given the relatively short generation time of malaria compared with humans (asexual stages of 2–4

days and life cycle from 1–4 months for *Plasmodium* spp. versus 20 years for humans) and an appreciation of the genetic diversity of malaria species, it is reasonable to consider that the parasite has adapted in response to the polymorphic erythrocyte landscape in which it lives. The blueprint for recent investigations of erythrocyte membrane polymorphisms in Papua New Guinea (PNG) is based upon these concepts. Results from these studies provide evidence that the Gerbich-negative ( $Ge^-$ ) blood group phenotype, highly prevalent in malarious regions of PNG, has arisen to confer protection from *P. falciparum* merozoite invasion [7,8].

## Gerbich-negativity and field studies in PNG

The  $Ge^-$  phenotype is caused by the deletion of exon 3 in the glycophorin C gene (*GYPC* $\Delta$ *ex3*; human chromosome 2q14-q21) and is found in Hardy–Weinberg equilibrium in malaria holoendemic regions of PNG [7–13]. Glycophorin C (128 amino acid residues, apparent 35 kDa, integral membrane sialoglycoprotein) [10] is a physiologically important monomer that interacts with the peripheral membrane protein 4.1 to mediate attachment of the sub-membranous cytoskeleton to the erythrocyte membrane. Our recent study conducted in the Wosera region of East Sepik Province revealed an association between *GYPC* genotypes and ovalocytic erythrocyte morphology [8]. An increased proportion of ovalocytic erythrocytes were observed in blood smears from individuals who were heterozygous or homozygous for *GYPC* $\Delta$ *ex3* compared with individuals who were homozygous *GYPC* wild-type. It is important to note that levels of ovalocytes were higher in Papua New Guineans than North American control individuals regardless of *GYPC* genotype. Furthermore, this association was independent of the 27 base-pair deletion in the band 3 gene (*SLC4A1* $\Delta$ 27) that deletes nine amino acids from the intracellular  $NH_2$ -terminal region of this abundant integral membrane protein [14]. Mgone *et al.* have previously described a strong correlation between *SLC4A1* $\Delta$ 27 heterozygosity and Southeast Asian ovalocytosis [15]. As no individuals who are homozygous for *SLC4A1* $\Delta$ 27 have yet been identified, it is presumed that the mutation in both copies of the gene is lethal during fetal development [16]. Overall, these findings suggest that multiple erythroid polymorphisms and as-yet unidentified environmental factors influence erythrocyte morphology.

To assess the effect of *GYPC* genotype on susceptibility to blood-stage infection, we examined the malaria infection status of a population in the Wosera at monthly intervals over a seven-month period. The frequency and intensity of blood-stage *P. falciparum* or *P. vivax* infection were not associated with *GYPC* genotype [8]. This lack of, or equivocal, correlations between erythroid polymorphisms and malaria infection status have been reported for other erythroid polymorphisms. In a previous study in PNG, Allen *et al.* found that  $\alpha$ -globin polymorphisms associated with  $\alpha^+$ -thalassemia that have reached genetic fixation in malaria endemic regions of PNG, were protective against severe malaria, but were not associated with reduced parasitemia [17]. In regard to the balanced *SLC4A1* $\Delta$ 27 polymorphism, although two independent studies have observed significant association between this polymorphism and protection from severe malaria [18,19], the polymorphism was not consistently associated with reduced infection prevalence or parasitemia [18,19]. These findings are not unusual to PNG as numerous studies from Africa on the  $\beta$ -globin hemoglobin S (HbS) (sickle cell) [20] and HbC polymorphisms [21,22], human leukocyte antigen (HLA) [20] and glucose-6-phosphate dehydrogenase deficiency variants [23] have reported protection against severe malaria morbidity with equivocal reduction in susceptibility to infection.

## Erythrocyte receptors and parasite-binding ligand interactions

Early results identifying the Duffy blood group antigen as the invasion receptor for *P. knowlesi* and *P. vivax* [2,3] implicated the blood group proteins as merozoite targets of human *Plasmodium* parasite species. Strategies using protease and neuraminidase treatment to cleave and alter polypeptide and sialic acid side-chains, respectively, have identified at least four major erythrocyte receptor pathways leading to *P. falciparum* invasion [6,24]. These include pathways that use glycophorin A (GYPA; trypsin sensitive, neuraminidase sensitive), glycophorin B (GYPB) and receptor Y (trypsin resistant, neuraminidase sensitive), and receptor X (trypsin sensitive, neuraminidase resistant). Identification of *Plasmodium* erythrocyte-binding ligands (EBL) or antigens (EBA) is gaining momentum as these proteins exhibit genomic and structural similarities (e.g. single-copy genes, conserved multi-exon gene organization, intron–exon boundaries, and amino- and C-terminal cysteine-rich domains). A recent review by Adams *et al.* provides an update of this growing list of related molecules [25]. These findings are summarized in Fig. 1.

Parasite ligands known to interact with specific host receptors include the *P. knowlesi* and *P. vivax* Duffy-binding proteins (Pk- and Pv-DBP) and the Duffy blood group antigen, *P. falciparum* EBA175 and GYPA. Recently, data mining of the *P. falciparum* chromosome 13 DNA sequence by three groups identified a new 140 kDa EBL, termed EBA140 (alternatively, BAEBL or EBP-2) [26–28]. Evidence suggesting that the erythrocyte receptor for EBA140 is human GYPC has included traditional strategies using erythrocytes from donors with blood group deficiencies and enzyme modifications of erythrocyte cell surfaces [24].

In our more recent study [29], we have provided additional proof that GYPC is the receptor for EBA140. By overlaying parasite proteins shed into culture supernatant onto western blots of erythrocyte membrane proteins, we showed that EBA140 bound to the wild-type GYPC protein, but not to GYPC of Papua New Guineans homozygous for *GYPC* $\Delta$ *ex3*. Evidence that the EBA140–GYPC interaction is physiologically important was revealed by experiments that used *P. falciparum* strains 3D7 and W2mef, the respective isogenic EBA140 knockout progeny, Ge<sup>+</sup> and Ge<sup>-</sup> negative erythrocytes, antibodies against the EBA140 F2 domain (predicted GYPC-binding region [30]) and chymotrypsin treatment (to limit non-GYPC invasion pathways). Overall, we found that antibodies to EBA140 inhibited merozoite invasion most effectively when invasion of wild-type parasites was restricted to the GYPC pathway, suggesting that, when available, the EBA140–GYPC interaction is important during the process of parasite invasion. That relative invasion efficiencies were not influenced by EBA140 antibody in experiments using Ge<sup>-</sup> erythrocytes or EBA140-knockout parasites suggests that *P. falciparum* invades erythrocytes by alternative pathways when GYPC is not available as a receptor.

A recent study by Lobo *et al.* [31] adds further support to the prediction that the GYPC (receptor)–EBA140 (ligand) interaction is an important invasion pathway for *P. falciparum*. However, their observations suggesting that the binding region for EBA140 resides in a nine-amino-acid region encoded by exon 2 of the *GYPC* gene appear to conflict with our studies using erythrocytes from *GYPC* $\Delta$ *ex3* homozygous (Ge<sup>-</sup>) donors from PNG. Potential explanations for these differences include the possibility that deletions of either *GYPC* exon 2 or exon 3 would either remove sialic acid epitope(s) necessary for EBA140 binding or alter the presentation of sialic acid epitope(s) in a neighboring region of the GYPC protein. Additional experiments using erythrocytes characterized for various Gerbich phenotypes and underlying *GYPC* gene polymorphisms, along with additional EBL control interactions, will be helpful in sorting out these differences.

## Concluding remarks

Ongoing studies continue to investigate how malaria parasites in PNG interact with the erythrocyte membrane polymorphic proteins that have already been identified. Evidence for EBA140 polymorphism and differences in GYPC binding have already been observed [29–32]. Given the number of erythrocyte membrane polymorphisms observed in PNG so far, the availability of a more global proteomic analysis [33] could provide further insight into how malaria is involved in selection of erythrocyte membrane protein polymorphisms. Finally, given the impact of the *SLC4A1Δ27* mutation on protection from severe malaria in PNG, it is clearly important to investigate how *GYPCΔex3* influences malaria pathogenesis through case control studies.

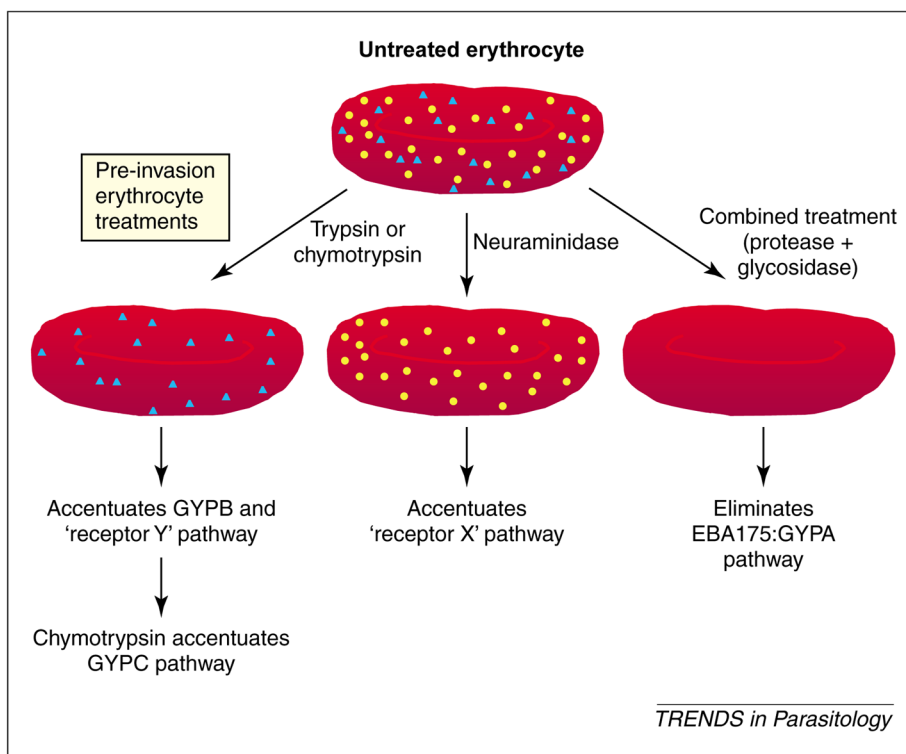
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**Fig. 1.** Pathways of erythrocyte invasion. *Plasmodium falciparum* uses protein and polysaccharide epitopes to engage erythrocytes using a multi-protein invasion complex (reviewed in Ref. [34]). Enzyme treatments modify erythrocytes to accentuate specific generalized pathways by limiting or eliminating access to epitopes that are sensitive to treatment. The pathway denoted by receptor 'x' represents erythrocyte invasion that is accentuated by neuraminidase pre-treatment of erythrocytes. The pathway denoted by GYPB and receptor y is accentuated by trypsin or chymotrypsin pre-treatment of erythrocytes. Yellow circles and blue triangles indicate polypeptide epitopes and polysaccharide epitopes, respectively. Abbreviations: EBA, erythrocyte-binding antigen; GYP, glycophorin.