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Malaria's Deadly Grip: Cytoadhesion of *Plasmodium falciparum* Infected Erythrocytes

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Summary

Cytoadhesion of *Plasmodium falciparum* infected erythrocytes to host microvasculature is a key virulence determinant. Parasite binding is mediated by a large family of clonally variant adhesion proteins, termed *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by *var* genes and expressed at the infected-erythrocyte surface. Although PfEMP1 proteins have extensively diverged under opposing selection pressure to maintain ligand binding while avoiding antibody-mediated detection, recent work has revealed they can be classified into different groups based on chromosome location and domain composition. This grouping reflects functional specialization of PfEMP1 proteins for different human host and microvascular binding niches and appears to be maintained by gene recombination hierarchies. In one extreme, a specific PfEMP1 variant is associated with placental binding and malaria during pregnancy, while other PfEMP1 subtypes appear to be specialized for infection of malaria naïve hosts. Here, we discuss recent findings on the origins and evolution of the *var* gene family, the structure-function of PfEMP1 proteins, and a distinct subset of PfEMP1 variants that have been associated with severe childhood malaria.

Introduction

Clonal antigenic variation is a strategy employed by many microbial pathogens to vary surface proteins to evade immunity. The human malaria parasite, *Plasmodium falciparum*, varies a family of adhesion proteins at the infected erythrocyte (IE) surface that it uses to bind to the endothelial lining of blood microvessels (Miller *et al.*, 2002). During blood stage infection, *P. falciparum* merozoites sequentially invade and egress from red blood cells every 48 hours. As the parasite matures, IEs exhibit reduced deformability and sequester from blood circulation. This allows the parasite to avoid splenic clearance mechanisms, but comes at a cost to the host. Sequestered IEs disrupt microvascular blood flow (Dondorp *et*

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al., 2008) and cause localized endothelial dysfunction by damaging endothelial barrier integrity and inducing pro-inflammatory, pro-adhesive, and coagulation pathways (Francischetti *et al.*, 2008; Miller *et al.*, 2013; Moxon *et al.*, 2009). This adhesive phenotype is also associated with organ-specific disease complications from IE adherence in brain (cerebral malaria) and placenta (placental malaria) microvasculature (Miller *et al.*, 2002).

Cytoadhesion is mediated by specific interactions between members of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family encoded by *var* genes and receptors on the surfaces of endothelial cells (Rowe *et al.*, 2010). Each parasite genotype encodes ~60 different *var* genes, which are expressed in a mutually exclusive fashion (Guizetti and Scherf, 2013). Switching between *var* genes facilitates parasite immune evasion and modifies IE binding specificity (Roberts *et al.*, 1993). A specific PfEMP1 variant, VAR2CSA, interacts with chondroitin sulfate A, which is abundant within the placental intervillous space (Fried and Duffy, 1996; Salanti *et al.*, 2004). In contrast, cerebral binding and severe childhood malaria is associated with specific PfEMP1 variants containing a combination of adhesion domains, termed domain cassettes (DC) 8 and 13 (Avril *et al.*, 2012; Claessens *et al.*, 2012; Lavstsen *et al.*, 2012). The vast majority of *P. falciparum* infections do not lead to severe malaria, suggesting that IE sequestration is relatively well-adapted to limit host death and favor parasite transmission to mosquitoes. It is therefore interesting that potentially lethal adhesion traits persist in the parasite population. This review covers recent advances in the molecular mechanisms of PfEMP1 binding, integrating findings on protein structure-function, *var* gene evolution, and adhesion phenotypes associated with severe malaria.

Major adhesion traits of *P. falciparum* infected erythrocytes

P. falciparum IEs have evolved to bind a diverse array of receptors on different human cell types. Two major adhesion traits that may contribute to severe childhood malaria are endothelial binding and rosetting with uninfected erythrocytes. Adhesion also occurs to platelets, dendritic cells, B cells, monocytes and macrophages to modulate host immune functions, a topic reviewed elsewhere (Chua *et al.*, 2013).

Cytoadhesion to microvascular endothelial cells

A common adhesion property of *P. falciparum* field isolates is CD36 binding (Newbold *et al.*, 1997). CD36 is widely expressed on microvascular endothelia, as well as monocytes, macrophages, dendritic cells and platelets (Silverstein and Febbraio, 2009). CD36-binding not only mediates sequestration of IEs, but also plays a role in non-opsonic phagocytosis of IEs (McGilvray *et al.*, 2000) and modulation of dendritic cell function (Urban *et al.*, 1999). Binding to CD36 is associated with uncomplicated malaria (reviewed in (Rowe *et al.*, 2010)), possibly by targeting sequestered IEs to non-pathogenic sites such as adipose tissue and skeletal muscles, rather than vital organs such as the brain. Indeed, human CD36 deficiency polymorphisms that reduce *P. falciparum*-binding do not protect against severe malaria (Fry *et al.*, 2009).

A key endothelial cell cytoadherence receptor that has recently been discovered is the Endothelial Protein C receptor (EPCR) (Turner *et al.*, 2013). EPCR is widely expressed on endothelial cells and leukocytes. The protein C-EPCR signalling pathway has anti-inflammatory activities on leukocytes and has anti-thrombotic and anti-inflammatory effects that protect endothelial cells and help maintain vascular integrity (Mosnier *et al.*, 2007). Binding to EPCR is associated with severe malaria (Turner *et al.*, 2013) and modifications in EPCR expression in cerebral malaria have been reported (Moxon *et al.*, 2013). Parasites selected for binding to human brain microvascular endothelial cells express PfEMP1 variants (Avril *et al.*, 2012; Claessens *et al.*, 2012) that bind to EPCR (Turner *et al.*, 2013).

Numerous other endothelial receptors have been described (reviewed in (Rowe *et al.*, 2010)), but many are poorly understood and their role in malaria disease is unclear. Cytoadhesion studies under flow conditions show that while some interactions, such as CD36-binding, cause stationary adhesion, others, such as ICAM1-binding, promote rolling of IEs (Cooke *et al.*, 1994). Interactions between IE and multiple receptors may co-operate *in vivo* to maximise adhesion and sequestration (Yipp *et al.*, 2007).

Rosetting with uninfected erythrocytes

Binding of IEs to uninfected erythrocytes (rosetting), is an adhesion phenotype that varies between clinical isolates, with high levels of rosetting associated with severe malaria in African children (reviewed in (Rowe *et al.*, 2010). Rosetting may contribute to malaria pathology by causing greater obstruction to microvascular blood flow than cytoadherence alone (Kaul *et al.*, 1991). Rosetting is mediated by specific PfEMP1 variants binding to receptors on uninfected erythrocytes, including Complement Receptor 1 (CR1) (Rowe *et al.*, 1997) and A and B blood group tri-saccharides (Carlson and Wahlgren, 1992). Some rosetting variants also bind the Fc region of human IgM (Ghumra *et al.*, 2008), a process that strengthens rosetting interactions (Scholander *et al.*, 1996). Naturally occurring polymorphisms in human erythrocyte rosetting receptors, such as CR1 deficiency and blood group O, impair rosette formation, and confer significant protection against severe malaria, confirming the importance of rosetting as a virulence factor in malaria (Cockburn *et al.*, 2004; Rowe *et al.*, 2007).

Molecular mechanisms of IE binding

PfEMP1 proteins contain a polymorphic ectodomain, linked through a single transmembrane helix to a relatively conserved cytoplasmic region (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995). The cytoplasmic tail anchors PfEMP1 proteins at parasite-induced knob-like protrusions at the erythrocyte surface (Maier *et al.*, 2009). The PfEMP1 ectodomain is built from a combination of Duffy binding-like (DBL) and cysteine-rich interdomain region (CIDR) adhesion domains (Smith *et al.*, 2000). These binding modules are only found in *Plasmodium*. The DBL superfamily bridges two important adhesive properties of *P. falciparum*, having a key role in erythrocyte invasion (DBL-containing erythrocyte binding proteins, DBL-EBPs) and IE cytoadhesion interactions (DBL-PfEMP1) (Miller *et al.*, 2002).

Evolutionary origins of var genes

Of the five *Plasmodium* spp. that infect humans, *P. falciparum* is responsible for nearly all deaths and is the only one that undergoes massive IE sequestration in host microvasculature (Smith and Deitsch, 2012). As homologs of DBL-EBP erythrocyte invasion ligands are found in human, rodent, and primate malaria species (Duraisingh *et al.*, 2012), it is likely that this unique cytoadhesion trait evolved from parasite invasion ligands. Although it was originally postulated that *P. falciparum* was more virulent because it had recently jumped from avian malaria species into humans and was less well adapted (Waters *et al.*, 1991), more recent phylogenetic and genome analysis does not support this interpretation and instead suggests that *var* genes have co-evolved for a long time with primates. At least six distinct *falciparum*-like species infect African Great Apes (chimpanzees and gorillas) (Prugnolle *et al.*, 2011) and the *var* gene family is restricted to this subgenus of *Plasmodium*. One of the closest known relatives of *P. falciparum* is the chimpanzee malaria *P. reichenowi*. Both encode *var* genes (Rask *et al.*, 2010) and are estimated to have diverged 6–10 million years ago (Escalante and Ayala, 1995). Little is known about the role of PfEMP1 proteins in non-human primate infections or pathogenesis, but these findings have important implications for our understanding of malaria pathogenesis. It indicates that the cytoadhesion trait is not a recent adaptation and it will be interesting to learn if there are

ancestral PfEMP1 binding properties that may confer selective advantages for parasite growth and transmission.

Genomic organization of var genes

Members of the *var* gene family are concentrated in sub-telomeric regions of all chromosomes and internal regions on chromosomes 4, 7, 8, and 12 (Gardner *et al.*, 2002). Comparison of the 3D7 genome reference isolate and six partial *P. falciparum* genomes highlights many commonalities between *var* repertoires (Rask *et al.*, 2010). In particular, the majority of *var* genes can be classified into three main groups (A, B, and C) on the basis of upstream sequence (Ups), chromosome location, and direction of transcription (Fig. 1). Surprisingly, the *var* gene repertoire also contains three strain-transcendent variants (*var1*, *var2csa*, and *type3 var*) found in most or all parasite genotypes. It is thought that this genome organization contributes to gene recombination hierarchies and functional specialization of PfEMP1 proteins (Kraemer and Smith, 2006). Intriguingly, UpsA, UpsB, and UpsC *var* genes and partial *var1* and *var2csa* homologs are present in the highly fragmentary *P. reichenowi* genome sequence (Rask *et al.*, 2010). This reinforces the ancient origins of the *var* genome organization and suggests the possibility that functional specialization of proteins may have occurred early.

Structure-Function of PfEMP1 proteins

The modular architecture of PfEMP1s and arrangement of ligand binding domains

Although PfEMP1 differ in size and number of extracellular domains, nearly all proteins have a tandem DBL-CIDR domain at the N-terminus, called the semi-conserved head structure. In small proteins, this is followed by a second DBL-CIDR tandem forming a 4 domain extracellular unit, while large proteins have additional types of DBL domains (Fig. 1). By sequence similarity, DBL and CIDR domains are classified into major types (α, β, γ, δ, etc.) and sub-types (e.g. CIDRα1.1) (Rask *et al.*, 2010; Smith *et al.*, 2000). Sequence classification likely reflects structural and functional specialization of domains. For instance, the PfEMP1 head structure is nearly always comprised of a DBLα-CIDRα tandem and other DBL and CIDR sequence types (β, γ, δ, etc.) are located C-terminal to these (Fig. 1) (Rask *et al.*, 2010). Furthermore, group A head structures have diverged in sequence and binding properties from group B and C (Robinson *et al.*, 2003) (Fig. 2).

The modular nature of PfEMP1s naturally led to the suggestion that they operate as strings of distinct ligand binding modules and that DBL-CIDR domains may form a structure-function unit. This prediction is supported in that many binding properties have been ascribed to single domains, with for example, ICAM1 binding to DBLβ domains (Howell *et al.*, 2008) and CD36 to CIDRα in the PfEMP1 head structure (Baruch *et al.*, 1997). However, specific adhesion traits, such as CSA binding, appear to involve a combination of domains (Clausen *et al.*, 2012; Srivastava *et al.*, 2011). Recent computational analysis identified 21 tandem domain arrangements of two or more domains (e.g. DC8 or DC13) (Fig. 1), which are highly conserved across parasite genotypes (Rask *et al.*, 2010). This conservation suggests that DCs may be maintained as larger structure-function units, encoding one or more adhesion properties.

Several lines of evidence suggest PfEMP1 ectodomains have higher order organization. For instance, the structure of a DBL1α-CIDR1γ tandem domain reveals close packing between the DBL and CIDR domains to generate a rigid structural unit (Vigan-Womas *et al.*, 2012). In addition, two small angle x-ray scattering (SAXS) studies show that full-length PfEMP1 ectodomains can adopt different shapes. Whereas full-length VAR2CSA ectodomain folds back on itself into a compact structure (Clausen *et al.*, 2012; Srivastava *et al.*, 2010), a CD36 and ICAM-1 binding PfEMP1 variant has an extended structure (Brown *et al.*, 2013). These

different arrangements may facilitate specific PfEMP1-receptor interactions and align pRBCs with cell surface receptors. Despite the availability of isolated DBL and CIDR structures, a major unanswered question is how PfEMP1 proteins recognize different protein or carbohydrate substrates. Additional sequencing and analysis of *var* genes paired with studies of PfEMP1 domain and ectodomain structures in complex with host receptors will be needed to fully understand which surface determinants are needed for PfEMP1 binding specificity and how PfEMP1 ectodomains are organized to present these binding surfaces to host receptors.

Binding properties of the PfEMP1 head structure and recombination hotspots

The PfEMP1 head structure plays a key role in binding and has diverged into three major adhesion properties. Whereas most group B and C PfEMP1 proteins appear to be under selection to bind CD36 (Robinson *et al.*, 2003), many group A variants were recently shown to bind EPCR (Turner *et al.*, 2013) or to form rosettes with uninfected erythrocytes (Fig. 2). Rosetting variants tend to encode CIDR sub-types (β, γ, δ) (Ghumra *et al.*, 2012), which are more typically found in the membrane proximal DBL-CIDR tandem (Fig. 1).

Sequence analysis suggests there is a recombination hotspot between DBL subdomains 2 and 3 (Rask *et al.*, 2010). From genome comparisons, recombination appears to be extremely rare between group A and non-group A genes (Kraemer *et al.*, 2007; Rask *et al.*, 2010). The reduced recombination between UpsA and UpsB genes is not unexpected given their inverted gene orientation would lead to a lethal event for a single exchange. However, DC8 is an unusual chimeric gene between an UpsB and group A *var* gene with the breakpoint between subdomain 2 and subdomain 3 in the DBLα domain (Lavstsen *et al.*, 2012). By limiting recombination between *var* groups, the parasite is able to evolve specialized PfEMP1 subsets for different microvascular niches, which differ in CD36 and EPCR expression levels. For instance, CD36 is weakly expressed in brain and placental microvasculature. Platelets express CD36 and it has been speculated that these may bridge *P. falciparum* IE binding to CD36 negative endothelium (Wassmer *et al.*, 2004). However, in the case of cerebral binding, the selective advantage for the parasite of this tissue location is puzzling because this adhesion trait may kill the host. This paradox is starting to be explained by recent findings on severe malaria variants.

Group A *var* genes and severe childhood malaria

African children with severe malaria suffer from four main complications — coma (cerebral malaria), respiratory distress, hypoglycaemia and severe anaemia. There is immunoepidemiological data that group A PfEMP1 variants are associated with early childhood infections (Cham *et al.*, 2009; Jensen *et al.*, 2004; Lavstsen *et al.*, 2012) and that severe childhood malaria infections are linked to the expression of a restricted subset of PfEMP1 (Bull *et al.*, 2000; Nielsen *et al.*, 2002). The continuous release of *var* sequence data has enabled increasingly more sophisticated molecular tools to solve the challenge of detecting and typing transcript expression of this diverse gene family. Although different approaches are used for *var* profiling making it difficult to compare between studies, a consensus is emerging that group A-like *var* genes are increased in severe malaria infections. Within the group A, rosetting parasites were more strongly linked to respiratory distress, and non-rosetting, group A-like genes with cerebral malaria in Kenya (Warimwe *et al.*, 2012). Furthermore, using new primers sets that are specific to different PfEMP1 domain types, DC8 and DC13 *var* genes (Lavstsen *et al.*, 2012) and EPCR binding (Turner *et al.*, 2013) are strongly linked to cerebral malaria and other severe malaria complications. Taken together, these findings suggest that PfEMP1 proteins have diverged into CD36 binding and non-CD36 binding subsets (Fig. 2). The non-CD36 binding group A subset may confer a growth and transmission advantage in malaria naïve hosts, although this is balanced by a

greater risk of severe malaria outcomes. An important question is whether all EPCR-binding or rosetting variants carry the same risk of severe outcomes, or if the combination of adhesion traits associated with specific EPCR-binding or rosetting PfEMP1 variants influences the extent of cerebral sequestration or disease severity. Currently, most studies have used broadly cross-reactive DBLα primers for *var* profiling and the new PfEMP1 domain type specific primers (e.g. DC8) have been only been evaluated on patient isolates from Tanzania (Lavstsen *et al.*, 2012). The two approaches amplify different regions of *var* genes and are subject to different limitations in detecting different *var* types (Lavstsen *et al.*, 2012). In the future, further application of these combined molecular tools for profiling *var* gene expression in combination with EPCR binding and rosetting assays are needed to fully resolve the question of which PfEMP1 subtypes are most likely to cause severe disease in patients from different regions of the world.

Conclusions

Despite the extensive diversification of *var* genes, recent work suggests that different *var* gene repertoires encode similar compositions of proteins, which are under selection for different host or microvascular niches. These protein groups can be classified by chromosome location and domain composition and appear to be maintained by ancient gene recombination hierarchies. Analysis of the PfEMP1 head structure indicates it has diverged into CD36-binding, EPCR-binding, and rosetting variants, which have different roles in mild versus severe malaria. Intriguingly, many of the known parasite cytoadhesion receptors are shared between endothelial cells and leukocytes, and thus it will be important to understand if there is selection on PfEMP1s between strong endothelial adhesion and host inflammatory properties. The finding that DC8 and DC13 variants encode EPCR binding activity has important implications for malaria pathogenesis. The ability of DC8 and DC13 variants to bind avidly to diverse endothelial cell types (Avril *et al.*, 2013) may benefit parasites by conferring a growth and transmission advantage in malaria naïve hosts. However, this adhesion trait may also interfere with protein C-EPCR signaling pathways (Moxon *et al.*, 2013; Turner *et al.*, 2013), resulting in deregulated inflammation and coagulation pathways and contribute to severe malaria complications. To design disease interventions, it will be important to better understand how IE-host receptor binding interactions may be manipulating the host environment to the parasite benefit.

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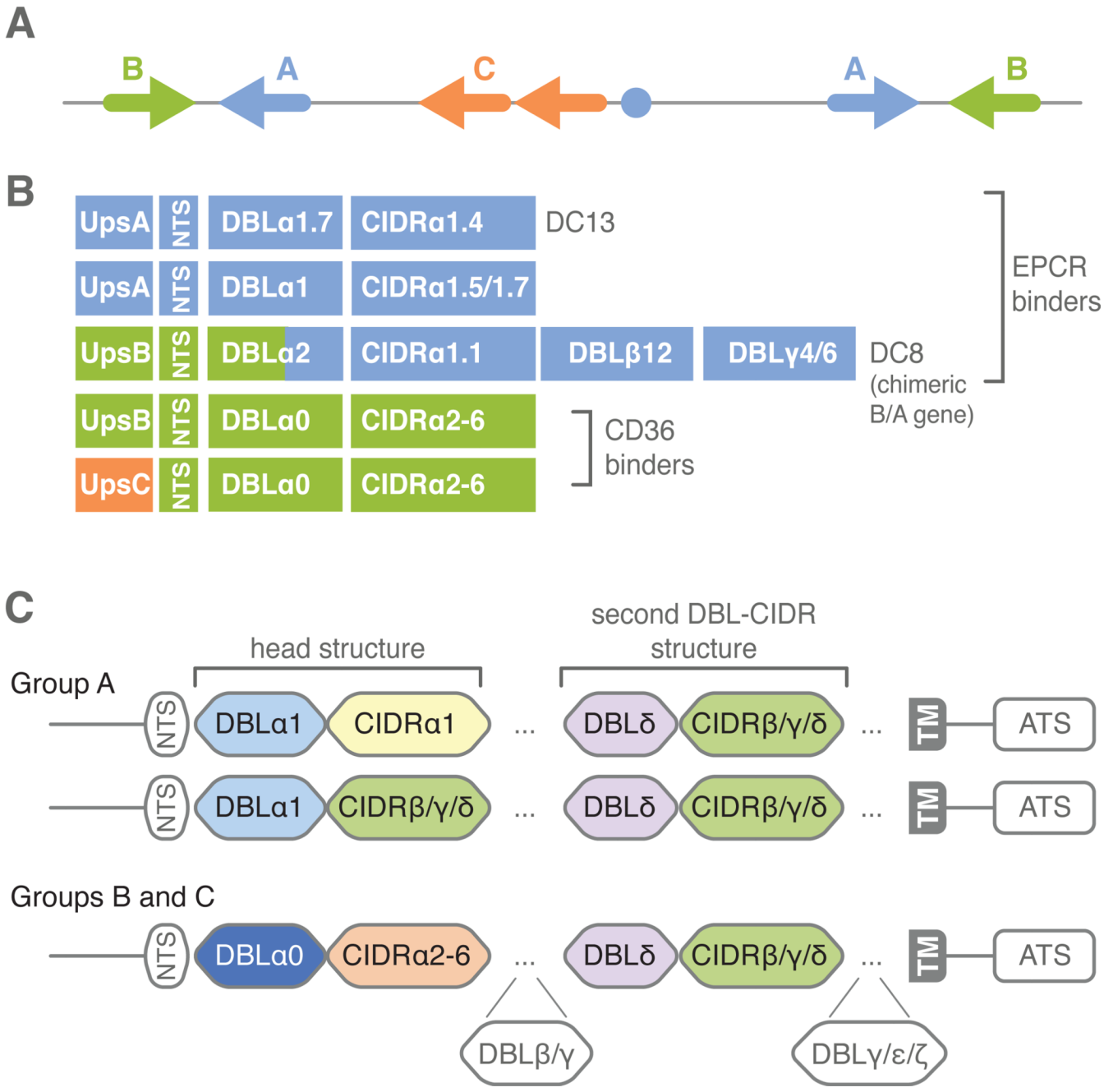

Fig. 1.

Var gene organization and protein domain architectures. A. Chromosomal organization of *var* genes. Group B and A *var* genes are located in the subtelomeric regions and transcribed in opposite orientations. Group C are typically found on central chromosome clusters. B. There is a recombination hot spot between subdomains 2 and 3 in the DBL domain of the PfEMP1 head structure. DC8 is an unusual chimeric gene between a group B and group A *var* gene. C. Different types of DBL and CIDR domains are located at the N- and C-terminus of proteins. Small PfEMP1 have four extracellular domains, large PfEMP1 have additional DBL domains after the head structure or the second DBL-CIDR structure.

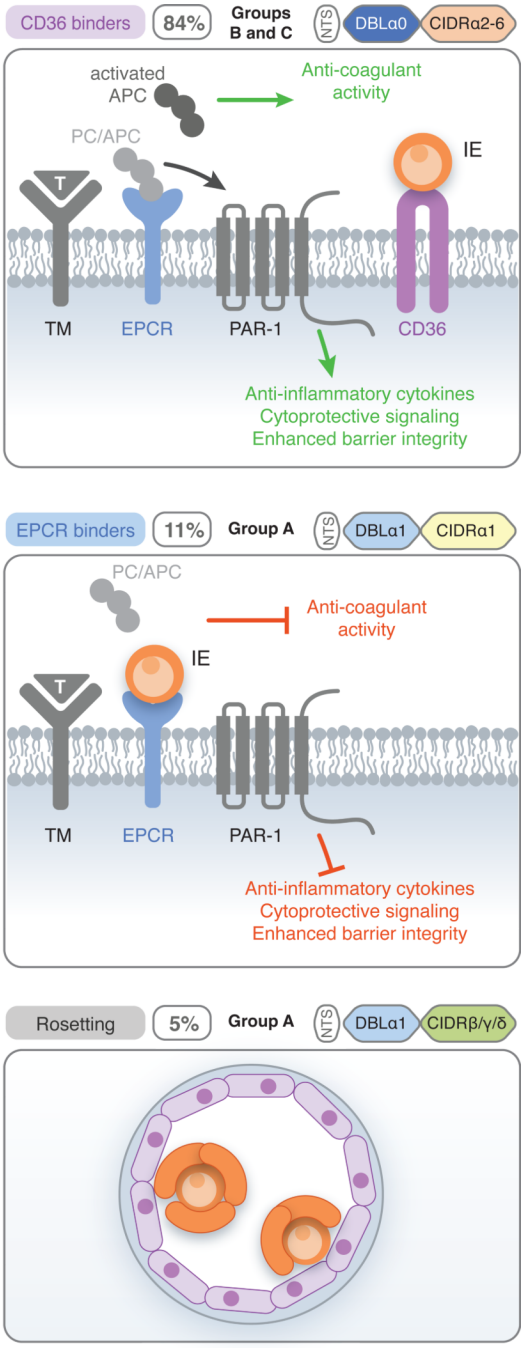

Fig. 2.

Model of PfEMP1 head structure divergence. The PfEMP1 head structure has diverged into three major binding groups under selection for EPCR-binding, CD36-binding, or to form rosettes with uninfected erythrocytes. The proportion of different head structure types in the 3D7 reference genome isolate and their predicted binding properties is shown. Note there may be some binding exceptions (e.g. only a subset of CIDRα1 domains bind EPCR, see Fig. 1). CD36 binding is the most common PfEMP1 adhesion trait and is associated with mild malaria. EPCR-binding and rosetting is linked to group A head structures. Group A PfEMP1 tend to be expressed in early childhood infections or malaria naïve, and are also associated with severe malaria. Under normal circumstances, protein C (PC) binds to EPCR

and is activated by the thrombin (T)/thrombomodulin (TM) complex. Activated protein C (APC) that is released into the plasma has anti-coagulant activity and the APC/EPCR complex activates the protease activated receptor 1 (PAR1) to mediate intracellular signaling. The protein C-EPCR signalling pathway has anti-inflammatory, anti-thrombotic and endothelial cytoprotective activities that help maintain vascular integrity (Mosnier *et al.*, 2007). The loss of EPCR at sites of *P. falciparum* IE sequestration (Moxon *et al.*, 2013) and EPCR-binding parasites (Turner *et al.*, 2013) may combine to interfere or subvert these pathways and contribute to disease pathogenesis.