Blood-Stage Malaria Parasite Antigens: Structure, Function, and Vaccine Potential

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Abstract

Plasmodium parasites are the causative agent of malaria, a disease that kills approximately 450,000 individuals annually, with the majority of deaths occurring in children under the age of 5 years and the development of a malaria vaccine is a global health priority. *Plasmodium* parasites undergo a complex life cycle requiring numerous diverse protein families. The blood stage of parasite development results in the clinical manifestation of disease. A vaccine that disrupts the blood stage is highly desired and will aid in the control of malaria. The blood stage comprises multiple steps: invasion of, asexual growth within, and egress from red blood cells. This review focuses on blood-stage antigens with emphasis on antigen structure, antigen function, neutralizing antibodies, and vaccine potential.

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Introduction

Malaria is a global health problem with 216 million new cases with approximately 450,000 deaths annually. Tragically, the majority of these deaths occur in children under the age of 5 years. Although human malaria is caused by five species of *Plasmodium* parasites, *Plasmodium falciparum* and *Plasmodium vivax* cause the majority of the mortality and morbidity associated with the disease [1]. *P. falciparum* malaria is distributed throughout the tropics, while *P. vivax* malaria is predominant in Southeast Asia and South America [1]. *P. falciparum* and *P. vivax* share similar lifecycles but use distinct proteins to achieve host cell invasion, thus requiring the development of independent treatments for each species.

A long-term goal of malaria research has been the development of a viable vaccine. Several lines of evidence indicate that a vaccine is feasible. For example, individuals in malaria endemic areas can acquire immunity to *Plasmodium* infection [2]. Antibody levels to *Plasmodium* proteins accumulate with age, and these levels correlate with protection and a decrease in the severity of malaria [3–7]. The passive transfer of antibodies from protected individuals to naïve recipients results in marked reduction of parasitemia [8]. In addition, naïve individuals injected with irradiated parasites develop antibodies to the parasite, and this response leads to protection from *Plasmodium* infection [9]. Cumulatively, these studies suggest that antibodies could play a key role in the development of the desired protective immune response induced by a malaria vaccine, although any vaccine would need to be able to elicit high titers of antigen-specific neutralizing antibodies. RTS,S/AS01 is the most advanced malaria vaccine developed and has a demonstrated efficacy of approximately 30% that is short lived [10–18]. These developments suggest that a vaccine for malaria is achievable although improvements are necessary to achieve the long-lived high efficacy desired.

Unlike viruses that have a limited number of proteins and have proven the benefits of vaccination, *Plasmodium* parasites are eukaryotic pathogens that utilize a wide array of proteins and protein families to ensure survival during the parasite's life cycle. The life cycle involves two asexual life stages within a human host, and a sexual stage within the mosquito vector [19–21]. The life cycle within the

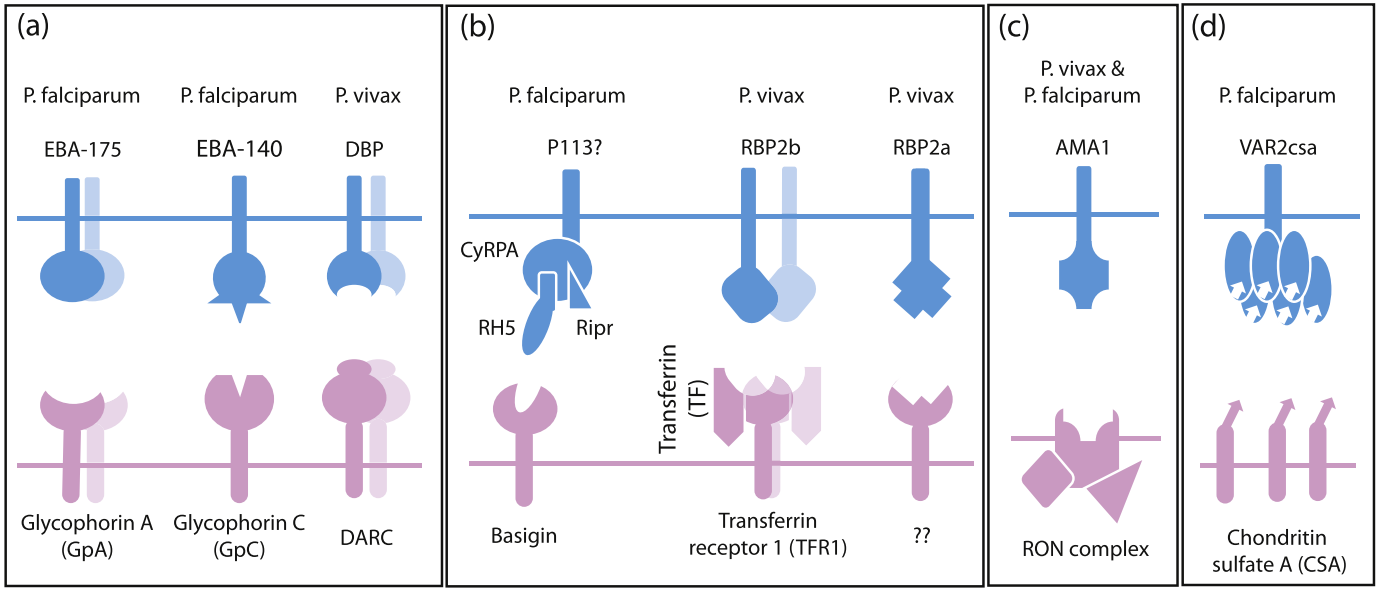

Fig. 1. Interaction between malaria parasite ligands and red cell receptors. Tight-junction formation relies on the (a) EBL and (b) RBL families of parasite proteins. These families are defined by conserved domains and contain members from both *P. falciparum* and *P. vivax*. Each family of proteins provides redundant pathways through which the parasite can form a tight junction utilizing different parasite ligand/RBC receptors pairs. (c) The moving junction is key to active invasion and involves the parasite AMA1/RON2 complex. The AMA1/RON2 complex is unique in that it is composed completely of parasite proteins, some of which are inserted into the RBC membrane to serve as RBC anchors. (d) Host–parasite receptor–ligand interactions are also critical for parasite sequestration into organs and tissues that prevents parasite clearance by the spleen. VAR2CSA is a PfEMP-1 family protein that is responsible for sequestration into the placenta during pregnancy. This sequestration has drastic effects on fetal viability and pregnancy outcomes and VAR2CSA is a prime vaccine candidate to prevent PM.

human begins when an infected *Anopheles* mosquito bites a human and injects sporozoite stage parasites into the host during the process of acquiring a blood meal [19]. The motile sporozoites must then traverse the epithelium to enter the circulatory system with the ultimate goal of reaching the liver, the site where infection is first established. Upon establishing infection in the liver, the parasite undergoes multiple development stages which culminate in the formation of thousands of merozoites. These merozoites are released from the infected hepatocyte to initiate the blood stage of the life cycle.

Merozoites attach to and actively invade red blood cells (RBCs) [20]. *P. vivax* shows a tropism for reticulocytes, while *P. falciparum* prefers to invade erythrocytes [22]. Invasion into erythrocytes is a multi-step process that includes (1) low-affinity interactions between the parasite and erythrocyte, (2) apical reorientation of the parasite relative to the erythrocyte, (3) formation of an electron dense contact between the two cells termed the tight junction, (4) active invasion via a moving junction coupled with an actin–myosin motor, and (5) shedding of the surface protein coat of the parasite and formation of the parasitophorous vacuole. Post-invasion, the parasite replicates asexually within a parasitophorous vacuole to produce daughter merozoites. Subsequent rupture of the cell releases the daughter merozoites that can go on to invade other RBCs perpetuating the blood stage. This constant cycle of invasion, replication, and lysis of RBCs leads to the clinical manifestation of malaria that includes anemia, fatigue, and fever as reviewed in Ref. [1]. In furtherance of this cycle, parasites within RBCs have the unique ability to sequester into tissues and organs maintaining infection and preventing clearance by the spleen [23].

During the blood stage, a small percentage of parasites undergo gametocytogenesis and are taken up during a blood meal by a second feeding *Anopheles* mosquito [21]. Thus, begins the sexual or sporogonic stage of the life cycle. The gametocytes develop into macro- and micro-gametes and the micro-gamete fertilizes the macro-gamete to form a zygote in the midgut of the mosquito [21]. The zygote then develops into an ookinete which traverses from the midgut to the basal lamina of the migut epithelium [21]. It is there that the ookinete develops into an oocyst where the parasite forms mature sporozoites [24]. The mature sporozoites egress from the oocyst and travel to the mosquito salivary gland where the parasites are poised to infect a second human host upon the next mosquito blood meal [24].

The complicated life cycle of the *Plasmodium* parasite offers many challenges and opportunities for the development of malaria vaccines. This is driven home by the diverse array of proteins and protein families that show stage-specific expression within the parasite. As such, research has focused on the formation of individual infection-blocking, blood-stage, or sexual stage (transmission-blocking) vaccines. Recently, a surge in the structure–function analysis of malaria vaccine candidates, and their effect on the immune response, has opened new avenues for structural vaccinology for malaria. This review focuses on a parasite proteins involved in the individual steps of RBC invasion and in sequestration (Fig. 1, Table 1). While there have been advances in understanding the diverse life cycle stages, here we have focused on the structural and immune response data available for a number of promising vaccine candidates of the blood stage (Fig. 1, Table 1), which is responsible for the clinical manifestation of malaria. A blood-stage vaccine would provide protection from clinical malaria, reduce parasite burden in the blood, and likely reduce the incidence of transmission.

EBA-175

P. falciparum erythrocyte binding antigen 175 (EBA-175) is a member of the erythrocyte binding-like (EBL) family of proteins that has members in both *P. falciparum* and *P. vivax* [60]. EBA-175 is involved in tight-junction formation [25,61–71], mediates cell signaling to facilitate invasion [72–74], alters RBC rigidity [72–74], and clusters RBCs to benefit the parasite [75]. While typically known for anchoring the parasite to an RBC during tight-junction formation [25,61–71], EBA-175 is also phosphorylated upon receptor binding to facilitate signaling within the cytoplasm of the merozoite [74]. RBC binding by EBA-175 also induces a phosphorylation cascade within the RBC that alters the rigidity of the RBC and influences invasion [72,73]. EBA-175 is proteolytically shed post-invasion [75–77] and facilitates RBC clustering that enhances parasite growth and immune evasion [75].

EBL family proteins share an overall domain structure which includes an N-terminal signal peptide, a short region I, a region II that contains either a single or double Duffy binding-like (DBL) domain required for receptor-binding, an unstructured predicted coil/coil domain consisting of regions III-V, a conserved cysteine-rich domain forming region VI, a transmembrane domain, and cytoplasmic domains [60] (Fig. 2a). EBA-175 binds to glycophorin A (GpA) on the RBC to facilitate its diverse functions [25,61–70] (Fig 1b). GpA is a glycosylated membrane receptor and is the second most abundant protein on the RBC [78,79]. Recognition of GpA by EBA-175 is dependent on specific terminal sialic acid residues on O-linked glycans in exon 3 of GpA and enzymatic removal or mutation of the glycosylated residues ablates binding *in vitro* and in parasite

Table 1. Structures of blood stage antigens

Antigen	PlasmoDB	PDB	Description	Ref.
EBA-175	PF3D7_0731500	1ZRL; 1ZRO	Region II; with sialyllactose	[25]
	PF3D7_0731500	2RJI	Region VI	[26]
	PF3D7_0731500	4K2U; 4QEX	Region II F1 with Fab 218; with Fab 217	[27]
EBA-140	PF3D7_1301600	4GF2	Region II	[28]
	PF3D7_1301600	4JNO	Region II with sialyllactose	[29]
PvDBP	PVX_110810	3RRC	Region II	[30]
	PVX_110810	4NUU; 4NUV	Region II dimer with one DARC; with two DARC	[31]
	PVX_110810	4YFS	DEKnull mutant	[32]
	PVX_110810	5F3J	Region II with scFv 2D10	[33]
	PVX_110810	6OAN	Region II with scFv 053054	[34]
	PVX_110810	6OAO	Region II with scFv 092096	[34]
	PVX_110810	6R2S	Region II with Fab DB9	[200]
PkDBP	PKNH_0623500	5X6N	Region II	[35]
RH5	PF7G8_040028400	4U0R; 4U1G; 4U0Q	RH5 with Fab 9AD4; with Fab QA1; with basigin	[36]
	PF7G8_040028400	5MI0	RH5	[37]
	PF3D7_0424100	4WAT	RH5	[38]
	PF3D7_0423800 PF7G8_040028400	6MPV	CyRPA + RH5 + Ripr	[39]
CyRPA	PF3D7_0423800	5EZN; 5EZO; 5EZI	CyRPA; with Fab c12; with Fab c1	[40]
	PF3D7_0423800	5TIK; 5TIH	CyRPA; with Fab 8A7	[41]
PvRBP2a	PVX_121920	4Z8N	Receptor binding domain	[42]
PvRBP2b	PVX_094255	5W53	Receptor binding domain	[43]
	PVX_094255	6BPA; 6BPC; 6BPE; 6D04; 6D05	Receptor binding domain with Fab 3E9; with Fab 4F7; with Fab 6H1; with TF + TFR; with TF + TFR	[44]
PfAMA1	PF3D7_1133400; PF3D7_1133400; PF3D7_1133400; PfIT_110038000	4R19; 4R1B; 4R1C; 4R1A	Domain I + II of 3D7 strain; of 3D7 strain; of 3D7 strain; of FVO strain;	[45]
	PF3D7_1133400	1Z40	3D7 strain	[46]
	PF3D7_1133400	2Z8V; 2Z8W	Domain I + II with V_{NAR} 14I-1; with V_{NAR} 14I1-M15	[47]
	PF3D7_1133400	2Q8A, 2Q8B	Domain I + II with Fab 1F9	[48]
	PfIT_11003800	2J5L	Domain III fragment with Fab F8.12.19	[49]
	PfIT_11003800	5NQF	Domain I + II with PvRON2	[50]
	PfIT_11003800	4Z09; 4Z0D; 4Z0E; 4Z0F	Domain I + II with RON2	[51]
	PfIT_11003800	3SRI; 3ZWZ; 3SRJ	Domain I + II with RON2	[52]
PvAMA1	PVX_092275	1W81; 1W8K	Domain I + II	[53]
	PVX_092275	2J4W	Domain II peptide with Fab F8.12.19	[49]
	PVX_092275	5NQG	Domain I + II with RON2	[50]
PkAMA1	PKNH_0931500	4UV6; 4UAO	Domain I + II; with Fab R31C2	[54]
Var2csa	PF3D7_1200600	2WAU	DBL6ϵ	[55]
	PfIT_120006100	2Y8D	DBL6ϵ	[56]
	PfIT_120006100	3BQI; 3BQL; 3BQK	DBL3x	[57]
	PfIT_120006100	3CML; 3CPZ	DBL3x	[58]
	PfIT_120006100	4P1T	DBL3x + DBL4ϵ	[59]

cultures [62,70,80]. EBA-175 binding to GpA occurs through the region II (RII-175) of EBA-175 that comprises two DBL domains named F1 and F2 [25,64,69] (Fig. 2b). The crystal structure of RII alone and co-crystallized with sialyllactose revealed that RII-175 forms a handshake dimer [25]. The glycan binding sites reside at the dimer interface and a majority of the glycan contact residues are from the F2 domain [25]. The other regions of EBA-175 also play a role in binding as the full-length ecto-domain of EBA-175 has a higher affinity for GpA when compared to the RII domain alone [68].

In addition to the structure of the RII domain, the structure of the RVI-175 domain was solved (Fig. 2b) [26]. This domain is proposed to be involved in trafficking of EBA-175 to micronemes, a *Plasmodium* secretory organelle priming EBA-175 for release during invasion [81]. This domain, which is highly conserved within EBL proteins, has a structure composed of 5 α-helices in a tight bundle resembling the KIX domain from eukaryotic transcription factor CREB-binding protein (CBP) [26]. The CBP KIX domain is involved in protein:protein interactions and may explain RVI's role in trafficking by potentially binding to other *Plasmodium* proteins necessary for proper protein trafficking within the parasite [26]. RVI-175 may also form a dimer as the crystal packing identified a viable dimeric interface

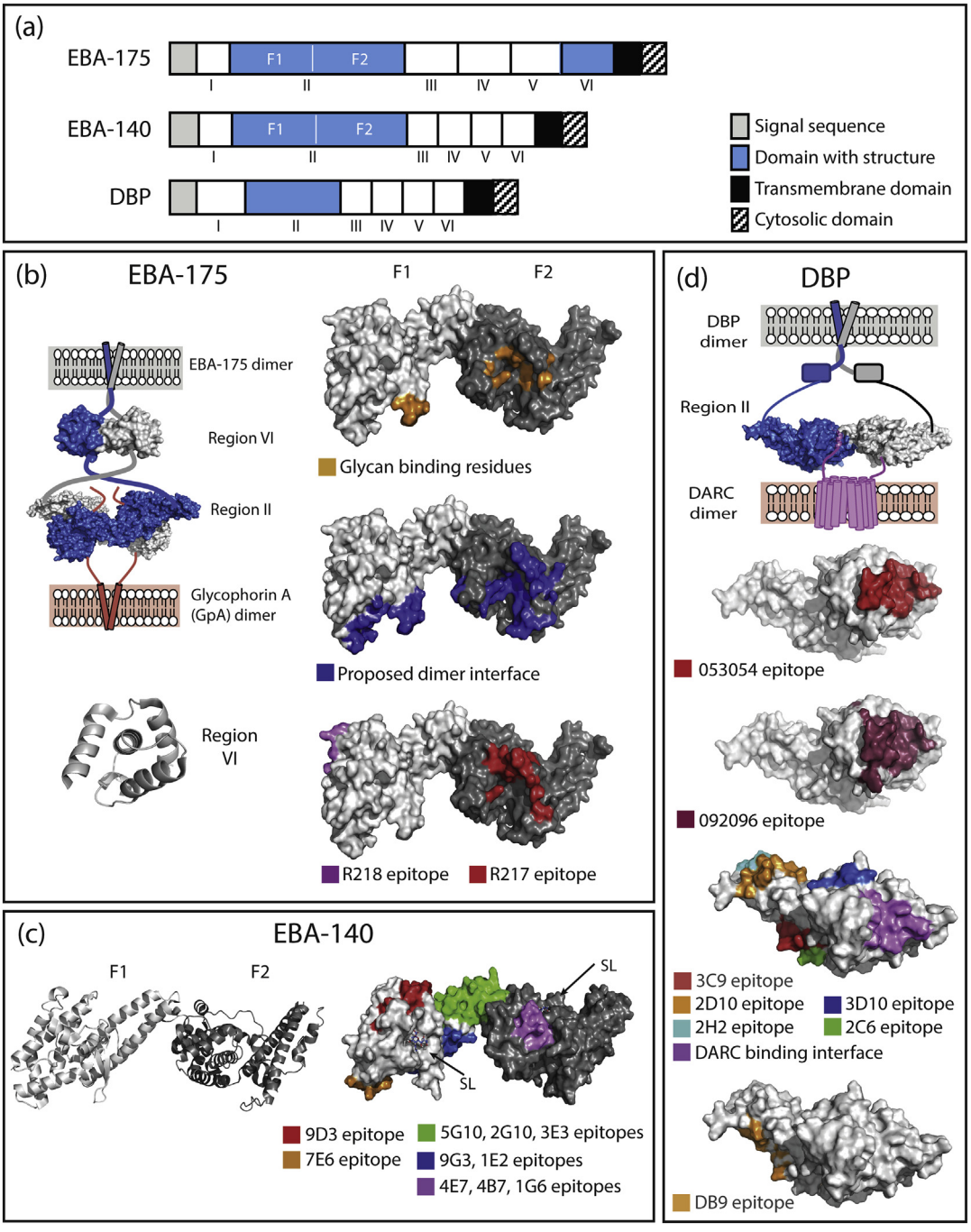

Fig. 2. Structure of EBL family antigens. (a) Domain organization of EBL family antigens. (b) Molecular interaction between a dimer of EBA-175 (represented in gray and blue; PDB: 1ZRL, 2RJI) and the RBC receptor GpA (PDB: 1ZRO). The two DBL domains F1 and F2 of region II are colored in light and deep gray, respectively. The binding interfaces of the two inhibitory antibodies R217 (potent; PDB: 4QEX) and R218 (mild; PDB: 4K2U) are highlighted on the surface representations of region II. (c) Structure of region II of EBA-140 is shown in ribbon (PDB: 4GF2). Surface representation of HDX-MS epitopes are highlighted and the binding sites for sialyllactose (SL) (showed in sticks) are pointed with arrows (PDB: 4JNO). (d) Molecular interaction between a dimer of DBP (represented in blue and gray surfaces) and the RBC receptor DARC (PDB: 4NUV). Monomer of DBP is shown in surface representation. Epitopes of naturally acquired neutralizing antibodies 053054 (PDB: 6OAN) and 092096 (PDB: 6OAO); non-inhibitory antibody 3D10; inhibitory antibodies 2D10 (PDB: 5F3J), 2H2, 2C6 and 3C9; and vaccine-induced inhibitory antibody DB9 (PDB: 6R2S) are highlighted on the surface representations of DBP monomer.

and dimerization of RVI-175 could be induced *in vitro* [26].

Antibodies to various domains of EBA-175, including RII-175, are observed in individuals in endemic areas. Antibody levels to EBA-175 are correlated with age and with a decrease in the severity of disease [82–85]. This suggests that EBA-175 is an attractive vaccine candidate as it is likely a target of protective immunity. Immunization of mice with the complete RII-175 domain resulted in the production of two monoclonal antibodies (mAbs): R217 and R218 [86]. Both antibodies had high-affinity binding to RII-175. However, R217 showed a 100-fold lower IC_{50} than R218 against parasites in culture [86]. Structure determination of the Ab:Antigen complex revealed that R217's conformational epitope comprised amino acid residues 475–485 and 561–567 in the F2 domain [27] (Fig. 2b). This epitope overlapped with both the dimer interface and the glycan binding residues of F2-175, indicating that R217 is neutralizing as it prevents dimerization and receptor binding by EBA-175 [27,87]. The structure of the Ab:antigen complex for R218, which is only mildly inhibitory, revealed that the epitope comprised amino acids 293–313 of the F1 domain [27]. This epitope is located on the outer face of the F1 domain distant from any known receptor binding residues of EBA-175 and explains the difference in parasite IC_{50} of the two mAbs. These studies emphasize that both inhibitory and non-inhibitory epitopes can be found in parasite proteins. Similarly, antibodies against RIII-RV domains of EBA-175 can be found in the population in endemic areas and these antibodies also correlate with protection against the clinical symptoms of malaria [83,84].

The ability of EBA-175 antibodies to neutralize parasites *in vitro*, coupled with the correlation to immunity and protection in endemic populations indicate EBA-175, has a high potential as a vaccine candidate. This is in contrast to knockout studies that demonstrate that EBA-175 is dispensable for *in vitro* RBC culture of *P. falciparum* [67]. Nevertheless, phase 1 clinical trials of multiple forms of EBA-175 have been performed to examine the immunogenicity and safety of RII-175 [88,89]. Vaccination of both malaria-naïve and malaria-exposed individuals resulted in sera with a limited 20% neutralization of parasites *in vitro* [88,89]. A third study used a combined administration of F2-175 and a second blood-stage antigen, MSP1-19, and demonstrated a 49% neutralization of homologous parasites and 21% neutralization of heterologous strains of *P. falciparum* [90]. The combined vaccine elicited a minimal response to MSP1-19 and responses to F2-175 were 5- to 22-fold higher, suggesting that the majority of parasite neutralization was through F2-specific antibodies [90]. Immunization with F2-175 would have eliminated the non-inhibitory epitope found in F1-175 and may explain why the vaccine containing F2-175 had a greater ability to inhibit parasite growth *in vitro* compared to the complete RII-175.

Recently, EBA-175 has been shown to play a role outside of tight-junction formation [75]. EBA-175 is shed post-invasion and the shed protein clusters uninfected red cells around an infected cell. This clustering leads to an enhancement of parasite growth as daughter merozoites can rapidly invade a neighboring cell, and also protects from antibodies that target other components of the invasion machinery [75]. This new role for EBA-175 might explain the limited protection observed upon vaccination with any single blood-stage antigen. A potent blood-stage vaccine will likely have to prevent both clustering and tight-junction formation and this will require targeting multiple antigens simultaneously.

EBA-140

P. falciparum erythrocyte binding antigen 140 (EBA-140) is another member of the EBL family of proteins [28,29,91–93]. EBA-140, like EBA-175, is involved in tight-junction formation during invasion and shares a similar domain architecture with EBA-175 and DBP [28,29,91–93]. Region II of EBA-140 (RII-140) contains two DBL domains called F1 and F2 respectively [28,29] (Fig. 2a). EBA-140 utilizes RII-140 to bind glycophorin C on the surface of RBCs during tight-junction formation [91,92,94,95]. This binding, like EBA-175 binding to GpA, is sialic acid-dependent as neuraminidase treatment of RBCs abolishes binding [91,92,94].

The structures of RII-140 alone and in complex with sialyllactose have been solved [28,29]. RII-140, in contrast to RII-175, is a monomer with two receptor-binding pockets located individually in each DBL domain [28,29] (Fig. 2c). Each receptor-binding pocket is embedded within each DBL domain in contrast to the receptor-binding pockets of EBA-175 and DBP, which are located near or at their respective dimer interfaces [29]. Structure-guided studies revealed that the binding pocket of the F1 domain contributed the majority of the receptor binding residues and mutation of the F2 domain binding pocket resulted in a minor reduction in receptor binding [29].

Population studies in endemic areas globally have shown that antibodies to EBA-140 are correlated with acquired immunity and protection in individuals. High antibody titers to EBA-140, particularly to the RII and RIII-V domains, have been linked with greater protection against infection [82,96–99]. Similar to EBA-175, EBA-140 appears dispensable for *in vitro* RBC culture of *P. falciparum* [95] despite the strong acquired immunity and antibody data. Immunization of rabbits with the F2 domain resulted in minimal neutralization of parasites in culture

(17.6%) that was strain-transcending as the F2 domain does not contain any polymorphisms [100]. In contrast, immunization of rabbits with the highly polymorphic RIII-V resulted in a strain-specific 50%–80% neutralization of parasites in culture. These studies focused on the polyclonal antibody response to both immunogens [101]. A more recent study mapped the monoclonal antibody response from mice immunized with either RII-140 or F1-140 [102]. Surprisingly, immunization with RII-140 resulted in four mAbs (4E7, 4B7, 1G6, and 1B10) that were mildly inhibitory with 10-15% neutralization of parasites in culture [102]. In contrast, immunization of mice with F1-140 resulted in seven F1-140-specific mAbs (1E2, 9G3, 7E6, 9D3, 5G10, 2G10, and 3E3) with a 2-fold increase in parasite neutralization compared to the mAbs isolated from the RII-140 immunization [102].

RII-140 immunization leads to a similar neutralizing response as immunizing with F2-140 [100,102]. This is likely due to production of F2-140-specific mAbs by immunization with RII-140 [102]. F2-140 appears dispensable for binding [29], and this may explain the limited parasite neutralization observed. The seven F1-140 mAbs derived from immunization with F1-140 all mapped to epitopes distant from the F1-140 receptor-binding pocket. Together, the data suggest that the functionally important F1-140 receptor binding pocket is an immunologically privileged site. The parasite neutralization observed with F1-140-specific mAbs is less than that of the polyclonal antibodies from immunization with RIII-V-140 [101,102]. However, the F1-140-specific mAbs exhibited strain transcending neutralization in contrast to the polyclonal antibodies to RIII-V [101,102]. Further refinement of both F1-140 and RIII-V-140 as immunogens is warranted to raise the potential of EBA-140 as a vaccine candidate.

DBP

P. vivax Duffy binding protein (DBP) binds the RBC receptor Duffy antigen for chemokines (DARC) during tight-junction formation [60,103–106] (Fig. 2d). DBP is the sole EBL family member expressed in *P. vivax*, is believed to be essential for invasion, and shares an overall domain architecture with EBA-175 [60,103–106] including a structurally conserved DBL domain in region II [25,30,31,35] (Fig. 2a). Binding to DARC is facilitated through region II (RII-DBP) [30,104], and RII-DBP, in contrast to RII-175, is composed of a single DBL domain that can form a dimer [30] (Fig. 2d). RII-DBP binds to DARC in a two-step process where two DBP molecules bind one DARC to form a heterotrimer followed by binding of a second DARC to form a 2:2 heterotetramer [31]. The structures of the heterotrimer and heterotetramer show that the DARC binding pockets of RII-DBP are located at the RII-DBP dimer interface in sub-domain 2 (SD2) of RII-DBP [31].

Antibodies to RII-DBP are found in individuals living in endemic regions and correlate with protection from symptomatic malaria [107–109]. Linear epitope mapping of the immune response of individuals from Papua New Guinea, where *P. vivax* is endemic, identified three epitopes highly correlated with immunity, three-moderately correlated, and four epitopes with low correlation [110]. The epitopes with the highest correlation with protection are located in the dimer interface and the DARC binding sites of RII-DBP [110] (Fig. 2d). Comparison of these epitopes from diverse strains showed that seven of the ten epitopes contained polymorphisms [111].

Recently, naturally acquired neutralizing mAbs to RII-DBP have been isolated from individuals living in malaria endemic areas [34,112]. A majority of the mAbs recognized an overlapping epitope [112], and this epitope was recognized by diverse populations worldwide suggesting that these epitopes are key components of the naturally acquired immune response to *P. vivax* [34,112]. Crystal structures and hydrogen–deuterium exchange mass spectrometry analysis of two mAbs, 053054 and 092096, in complex with RII-DBP revealed overlapping but distinct epitopes on RII-DBP [34] (Fig. 2d). The epitopes are contained in subdomain 2 and comprise the DARC-binding site and dimer interface of RII-DBP [34]. These mAbs prevent RII-DBP binding to RBCs, and 092096 neutralized *P. vivax* invasion into reticulocytes [34]. Together, these results demonstrate that naturally acquired human antibodies that target the DARC-binding site and dimer interface of RII-DBP can neutralize *P. vivax* [34]. 092096 could compete with polysera of individuals with naturally acquired immunity from a wide geographical distribution showing that antibody recognition of this epitope is conserved in worldwide populations [34].

In addition to investigating naturally acquired immunity, animal immunizations utilizing various forms of RII-DBP have been conducted. A panel of murine mAbs, 2D10, 2H2, 2C6, 3C9, and 3D10, was generated against RII-DBP with varying inhibition of RII-DBP binding to RBCs [113]. Epitopes were identified for the inhibitory mAbs 2D10, 2H2, 2C6, and the non-inhibitory mAb 3D10 [33]. The crystal structure of the 2D10:DBP complex revealed that the 2D10 epitope is located in sub-domain 3 of RII-DBP [33]. Combined analyses using small angle x-ray scattering and hydrogen–deuterium exchange mass spectrometry revealed 2H2 shares an overlapping epitope with 2D10, while the 2C6 epitope was to a distinct portion of sub-domain 3, and the non-inhibitory 3D10 epitope mapped to sub-domain 1 (Fig. 2d) [33]. The inhibitory mAb epitopes are well

conserved among all the polymorphic variants of DBP with >92% invariance in global sequence of DBP examined [33]. The epitope for murine monoclonal antibody 3C9 was mapped by phage display and is adjacent to and overlaps with 2C6 [201]. The location of inhibitory epitopes outside sub-domain 2 the receptor binding site and dimer interface suggest that these antibodies function outside of receptor-blockade and may sterically disrupt DBP's ability to engage DARC. The naturally acquired human antibodies demonstrated greater potential to inhibit RII-DBP binding to RBCs than the murine antibodies [112] consistent with different mechanisms of action: direct receptor-blockade by the human antibodies and steric hinderance by the murine mAbs.

DBP is highly polymorphic, and these variations may limit a strain-transcendent immune response. One region of DBP is particularly polymorphic and is termed the DEK sequence [114–117]. This region is far removed from the receptor-binding and dimer-interface residues of DBP. Mutation of the DEK segment resulted in the immunogen DEKnull that proved just as immunogenic as the wild-type Sal-1 RII-DBP and was able to elicit inhibitory antibodies [114–117]. Structural analysis of DEKnull revealed no significant difference between Sal-1, the reference strain of *P. vivax*, RII-DBP and DEKnull outside of the region mutated [32].

Based on the promising results seen in population and animal studies for DBP, two phase 1 clinical trials have been conducted in malaria-naïve individuals [118,119]. Both studies showed that immunization with RII-DBP produced strain transcending antibodies that were able to inhibit RII-DBP binding to DARC *in vitro* for a limited subset of DBP variants [118,119]. Vaccine-induced human monoclonal antibodies were isolated from vaccinees from one of the clinical trials [200]. Epitope binning identified four distinct epitope groups with the major epitope group represented by antibody DB9 that is capable of parasite neturalization [200]. X-ray crystallography revealed DB9 tragets an epitope located in subdomain 3 of DBP [200]. This epitope overlaps with the epitope of murine monoclonal antibody 3C9 [201]. It is interesting to note that the epitopes targeted by naturally acquired immunity in subdomain 2 [34] are entirely distinct from the epitopes identified upon immunization of mice [33] or humans [200]. These results indicate that new immunogens can be developed based on the structural information now available for receptor engagement and antibody inhibition.

RH5

P. falciparum parasites contain a second family of proteins, the reticulocyte-binding homolog (RH) proteins, part of the larger RBL family of *Plasmodium* proteins, that are involved in RBC invasion and tight-junction formation [120,121]. RH proteins are characterized by the presence of an RH family domain at the N-terminus of the proteins [120,121]. RH5 is unique among the RH proteins as it is much smaller than the others and lacks the transmembrane domain present in the other family members [120,121]. RH5 is a 63-kDa protein composed of a signal peptide, an unstructured N-terminal domain, and a RH family domain [120,121] (Fig. 3a). The full-length 63-kDa protein can be observed within the parasite secretory organelles prior to invasion, while post-secretion, RH5 predominantly exists as two shorter processed forms of 45 kDa and approximately 20kDa [120,121]. The processing occurs upstream of the RH family domain [120,121].

RH5 is part of a larger complex that facilitates binding to the receptor basigin (BSG) on RBCs [122,123] (Fig. 3b). The parasite interaction with BSG appears essential for merozoite entry into the erythrocyte [122], in contrast to the dispensability of other EBL and RH family interactions. RH5 forms a complex with *P. falciparum* RH5-interacting protein (RIPR) [39,124,125], and cystein-rich protective antigen (CyRPA) [39–41,126] (Fig. 3c). This complex plays a critical role in invasion as conditional knockdown of either RIPR or CyRPA greatly reduces invasion of the parasite into RBCs [127]. RIPR and CyRPA form a complex with the RH family domain of RH5 and may be involved in BSG binding although direct binding of the proteins to RBCs has not been shown. This complex is also linked to Ca^{2+} release from the merozoite to the RBC and is dependent on RH5 binding to BSG [74,127]. In addition, RH5 can form a complex with another parasite protein P113 through the N-terminal unstructured domain of RH5 [128]. Binding to P113 has been proposed to secure the RH5 complex to the parasite membrane as P113 contains a transmembrane domain [128]. There have also been conflicting reports regarding the presence of a GPI anchor on CyRPA which may also facilitate anchoring of RH5 to the parasite membrane [40,41,126].

The structures of RH5 alone [36,38] and in complex with BSG [36], CyRPA alone [40,41], and RH5 in complex with CyRPA and RIPR [39] have been solved. The RH family domain of RH5 forms a flat "kite-like" structure composed predominantly of α-helices and includes two disulfide bonds [36,38] (Fig. 3b). The structure of RH5 and BSG shows that two immunoglobulin domains of BSG bind to the loops at one pole of RH5 [36]. BSG is expressed on a variety of cell types and has four isoforms all of which are glycosylated [129]. The most common isoform is BSG-S, which contains two immunoglobulin domains [129]. The crystal structures of RH5 and BSG emphasize that the glycans on BSG are not important for binding as most of the RH5/BSG contacts are through the backbone of BSG, which helps explain why binding to a glycosylated protein is

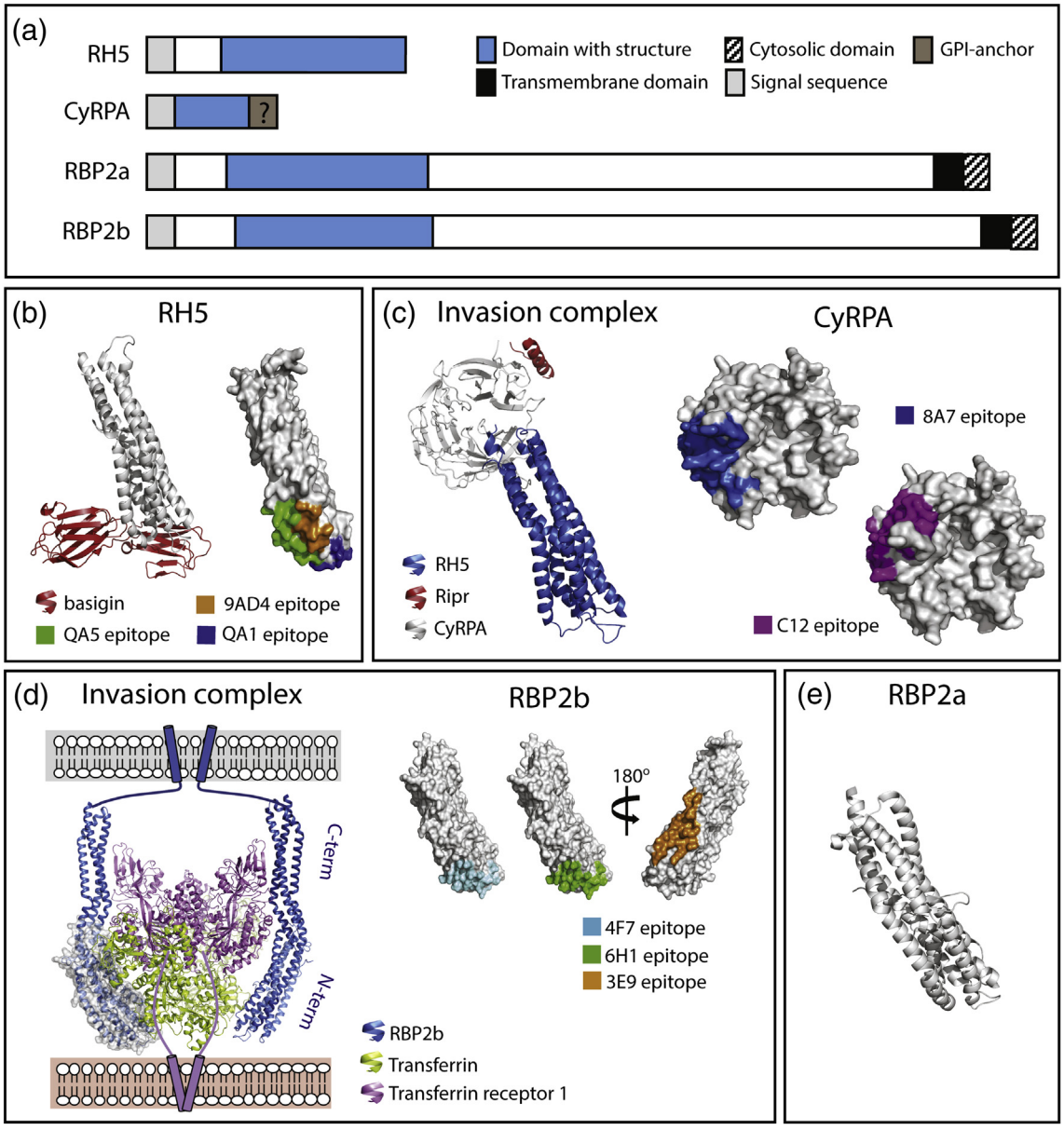

Fig. 3. Structures of the RH family. (a) Domain organization of RH and RBP families. (b) C-terminal RH domain of RH5 in complex with basigin is shown in gray and red ribbons, respectively (PDB: 4U0Q). Epitopes of inhibitory mouse antibodies are highlighted on the surface representation (PDB: 4U0R, 4U1G). (c) The invasion complex of CyRPA, RH5 and RIPR (PDB: 6MPV). Right panels show the surface representation of CyRPA oriented the same as in the complex. The epitopes of inhibitory antibodies 8A7 (PDB: 5TIH) and C12 (PDB: 5EZO) are highlighted. (d) Left panel is the hetero-hexamer invasion complex consisting of a dimer containing the receptor-binding domain of RBP2b, transferrin (TF), and transferrin receptor 1 (TFR1) (PDB: 6D04). The receptor-binding domain of RBP2b is folded into two subdomains, N-terminal and C-terminal. The binding epitopes of various inhibitory mouse antibodies (PDB: 6BPA, 6BPC, 6BPE) are all found in the N-terminal subdomain of RBP2b highlighted in the surface representations on the right panel. (e) Structure of RBP2a (PDB: 4Z8N) highly resembles the N-terminal subdomain of RBP2b. The molecule is in the same orientation as the ribbon RBP2b molecule on the left.

important for one of the sialic acid independent invasion pathways in *P. falciparum* [36]. Two structures of CyRPA alone have been solved and reveal that CyRPA forms a monomeric 6-bladed β-propeller structure with four intra-sheet disulfide bonds and one inter-sheet disulfide bond [40,41] (Fig. 3c). The ternary complex of Rh5–CyRPA–Ripr was recently solved by cryo-electron microscopy to ~7 Å and showed that RH5 binds to CyRPA at blades 4 and 5 of CyRPA, while RIPR binds to blade

6 of CyRPA sandwiching CyRPA between the two proteins [39] (Fig. 3c). The CyRPA binding site is at the opposite pole of RH5 from the BSG binding site [39]. Interaction studies showed that the ternary complex was unable to bind soluble BSG but could interact with full-length BSG in a lipid environment and on the surface of cells [39]. Lipid interaction studies suggest that the ternary complex, upon binding to BSG, dissociates with RH5 and RIPR inserting into the membrane and CyRPA being released [39].

The RH5 complex is of interest to the vaccinology community as antibodies to RH5 in human population studies have been correlated with age and protection [130]. In addition, antibodies to RH5, RIPR, and CyRPA neutralize parasite growth and invasion *in vitro* and *in vivo*. Murine mAbs have been purified for both RH5 and CyRPA, and the structures determined in complex with their respective antigens [36,40,41]. The structure of RH5 with inhibitory mAbs 9AD4 and QA1 revealed that 9AD4 binds adjacent to the BSG binding site on RH5, and explains why this antibody is unable to block RH5/BSG binding, but currently offers no definitive explanation for its mechanism of parasite neutralization [36]. The mechanism of inhibition by QA1 is clear as the binding site for QA1 overlaps with the BSG binding surface of RH5 and directly blocks receptor binding [36]. The epitope of another inhibitory mAb QA5 was also determined by SAXS and also overlaps with the BSG-binding surface of RH5 [36]. The structure of CyRPA in complex with inhibitory mAbs from two different studies provides insight into RH5 binding to CyRPA. mAb 8A7 binds to the first and second β-sheets of CyRPA perpendicular to the propeller axis [41]. mAb C12 also binds to this face of CyRPA, although in contrast to 8A7, it binds to the second and third β-sheets [40]. Both mAbs block RH5 binding to CyRPA, yet the epitopes do not appear to overlap with the RH5-CyRPA binding site [39–41]. This suggests that the mechanism of inhibition may be through steric clashes from mAb binding to CyRPA.

The promising animal studies have led to phase 1 clinical trials of an adenovirus vectored RH5 vaccine [131]. All naïve vaccinees produced both RH5-specific T-cell responses and RH5-specific antibodies at the end of the trial [131]. Antibodies from these individuals showed growth inhibition of parasites *in vitro* [131]. In addition to the adenovirus vectored RH5 vaccine, the production of stabilized variants of RH5 has been investigated in insect cells [132,133] and *Escherichia coli* [37]. It should be noted that some animal studies showed that full-length RH5 was required to generate neutralizing antibodies in animals [36,134–138] and the RH family domain alone was not sufficient [120,134]. It appears that the N-terminal unstructured domain of RH5 alone can elicit neutralizing antibodies [128]. mAbs to the N-terminal unstructured region show a wide range of neutralization with mAb 5A08 exhibiting the highest level with >95% neutralization of parasite cultures at a concentration of 0.1 mg/mL [138]. The epitope for 5A08 contains a segment AIKK within the N-terminal unstructured domain [138]. Immunization with virus-like particles that retain the sequence encompassing the 5A08 epitope resulted in >90% growth inhibition of parasites at a polysera concentration of 1 mg/mL [138]. These studies emphasize that the design of an RH5-based immunogen may utilize multiple segments of RH5.

RBP2b

DBP has long been thought to be the sole invasion ligand of *P. vivax*, but recent work has identified the 11-member reticulocyte binding protein (RBP) family, part of the larger RBL family of *Plasmodium* proteins, as important for invasion of reticulocytes [43,44,139–141]. RBP2b is a member of the RBP family and binds to both transferrin receptor 1 (TFR1), a reticulocyte type II transmembrane protein, and transferrin (TF) to form a heterohexamer to facilitate invasion [43,44]. The hexamer is composed of the homodimer of TFR1 bound to two TF's sandwiched between two RBP2b molecules [44] (Fig. 3d). RBP2b is a large 326-kDa protein composed of a signal peptide, an N-terminal disordered domain, two consecutive domains involved in binding to TFR1 and TF, an uncharacterized C-terminal region and a transmembrane domain [43,44,140,141]. Of the two receptor binding domains, the N-terminal domain composed of amino acid residues 168–460 is predominantly positively charged and binds to both TFR1 and TF [43,44]. The second receptor-binding domain composed of amino acid residues 461–633 is predominantly negatively charged and binds to TFR1 [44]. The N-terminal receptor binding domain resembles the structure of RH5 and the related erythrocyte binding domain of RBP2a and is primarily α-helical in nature [36,38,42–44] (Fig. 3e). In comparison, the more C-terminal domain is an elongated α-helical domain [44].

Immunization of mice with a long construct of RBP2b, amino acid residues 161–1454, resulted in the isolation of four murine mAbs that neutralize parasite invasion and erythrocyte binding, in addition to one non-neutralizing mAb [43,44]. Crystal structures of three inhibitory mAbs and a SAXS structure for one inhibitory mAb in complex with RBP2b show that all four bind to the N-terminal receptor binding domain [44]. The mechanism of inhibition for three of the four mAbs (10B12, 4F7, and 6H1) is through steric hindrance of RBP2b with antibody binding preventing access to the reticulocyte membrane [44]. The fourth mAb, 3E9, is neutralizing as the epitope in the N-terminal domain of RBP2b overlaps with the TFR1 binding site [44]. Furthermore,

naturally acquired antibodies against RBP2b have been shown to correlate with protection from *P. vivax* malaria [139] making RBP2b an exciting new vaccine candidate for the field in light of the new structural data now available.

AMA1/RON2

Apical membrane antigen 1 (AMA1), which is conserved across *Plasmodium* species, is both a blood-stage and a liver-stage antigen although the majority of the research conducted has focused on its functions during the blood stage [142–145]. AMA1 is involved in the invasion of hepatocytes during the liver stage and RBCs during the blood stage [143–145]. AMA1 is unique in that its receptor is a protein complex composed of RON2, RON4, and RON5 that is secreted by the parasite and injected into the host cell membrane prior to invasion [52,146,147] (Fig. 4b). AMA1 binds to a short segment of RON2 composed of a long β-hairpin loop in a hydrophobic groove along one side of the AMA1 receptor-binding domain [52,146,147]. This interaction has been shown to be critical for invasion of both hepatocytes and RBCs [143–145]. AMA-1 is thought to be essential for parasite viability as knockout of AMA1 in *Plasmodium* has proven difficult, and knockdown of AMA1 in the related parasite *Toxoplasma gondii* causes severe reduction of invasion events by this parasite [148].

AMA1 is processed during the invasion process. Within the parasite, AMA1 exists as an 83kDa protein consisting of a signal peptide, a short N-terminal segment, two tandem PAN/apple domains (D1 and D2), a third domain (D3), a transmembrane domain, and a short cytoplasmic domain [45,46,149] (Fig. 4a). Upon export to the parasite surface, AMA1 is processed to remove the signal peptide and the N-terminal segment resulting in a 66-kDa protein with an ectodomain composed of domains 1–3 [149]. At the end of invasion, AMA1 is cleaved into either a 48- or 44-kDa protein that removes the majority of the ectodomain from the surface of the parasite [149].

The structures of AMA1 alone and in complex with RON2 have been solved from both *P. falciparum* and *P. vivax* [45,46,50–53] (Fig. 4). There are eight disulfide bonds in the ectodomain: three in D1, two in D2, and three in D3 [45,46,150]. The structures also revealed that D1 and D2 are highly modified PAN domains possessing a PAN domain core with multiple long loops incorporated into each domain

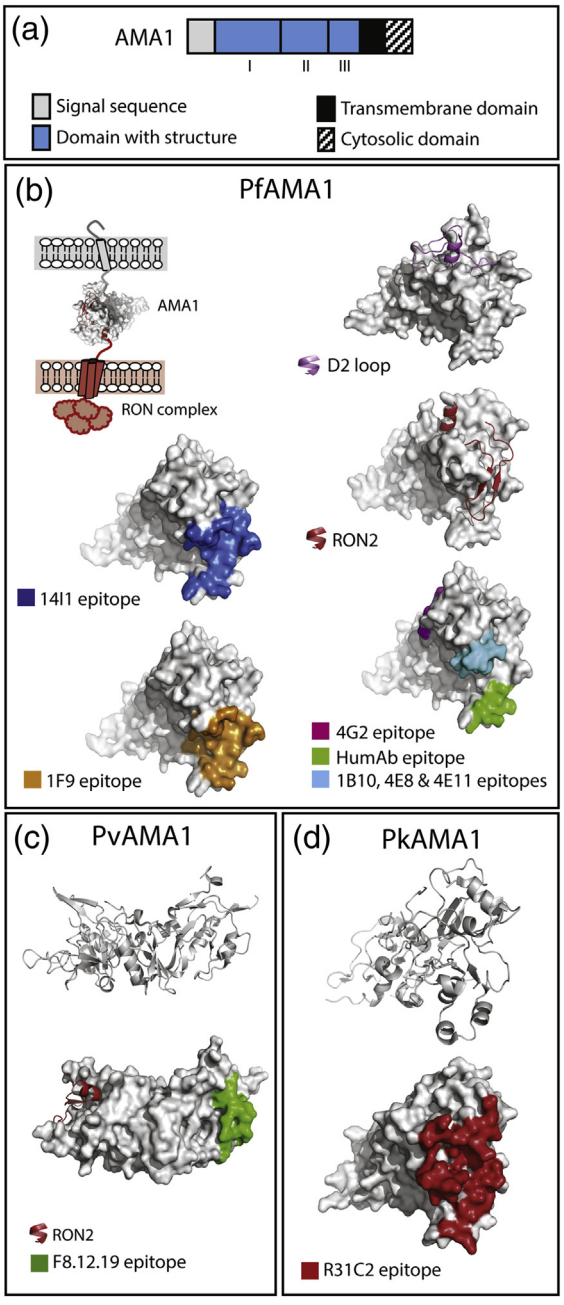

Fig. 4. Structures of AMA1. (a) Domain organization of AMA1. (b) Molecular interaction between the RON complex and AMA1. Structure of PfAMA1 D1–D2 is shown in gray. The D1 loop of PfAMA1 is shown in magenta ribbon (PDB: 2Z8V). Structure of PfAMA1 in complex with RON2 peptide is shown in gray surface and red ribbon, respectively (PDB: 5NQF). The complex structure showed the helix of RON2 displaced the D2 loop of AMA1 which becomes disordered. Epitopes of neutralizing mouse antibodies 14I (PDB: 2Z8V), 1F9 (PDB: 2Q8A), 4G2, 1B10, 4E8, and 4E11 and human antibody HumAb, and non-inhibitory mouse antibody 5G8 are highlighted on the surface representation of PfAMA1. (c) Structure of PvAMA1 D1–D3 domain is shown in gray. The binding of RON2 peptide (red ribbon; PDB: 5NQG) and the epitope of antibody F8.12.19 (PDB: 2J4W) is shown on surface representation of PvAMA1. (d) Structure of PkAMA1 D1–D2 domain is shown in the same orientation as PfAMA1 (PDB: 4UAO).

[45,46]. This results in the loss of the classical PAN disulfide bond patterns [45,46]. Of these loops, the D2 loop extends up to contact a hydrophobic channel on one face of D1 and blocks the RON2 receptor binding groove [45,46]. During receptor binding, as revealed by the AMA1/RON2 complex structure, a long β-hairpin loop from RON2 sits in this channel and displaces the D2 loop, exposing a new surface to the surrounding environment (Fig. 4b) [52]. The AMA1 ectodomain has a polymorphic face that contains the majority of the polymorphisms found in all three domains and a conserved face [45,46]. The hydrophobic channel is located on the polymorphic face of AMA1 and one end of the channel comprises several of the most polymorphic residues in D1 [45,46]. The opening and closing of this channel for receptor binding may be important to evade recognition by the host immune system.

Antibodies to AMA1 in populations from endemic areas have been correlated with protection [151]. In particular, IgG_3 antibodies have been shown to more strongly correlate with protection compared to IgG_1 antibodies [151]. Immunizations of animals with AMA1 have also been shown to be protective [152,153]. These studies have reinforced AMA1 as a vaccine candidate in addition to the fact that AMA1 appears essential for parasite viability. Multiple groups have produced panels of murine mAbs and mapped their epitopes to further understand AMA1 immune responses. The two most well-studied mAbs are 1F9 and 4G2 [48,154–157], both of which are neutralizing antibodies that function through blocking various aspects of receptor binding by AMA1 (Fig. 4b) [48,154–157]. The crystal structure of 1F9 in complex with AMA1 revealed 1F9 binds to the polymorphic end of the RON2 binding site [48]. Although the structure of the complex has not been solved, the epitope of 4G2 has been mapped to the D2 loop that binds to the RON2 binding site in the absence of RON2 [156]. It has been proposed that 4G2 binding to AMA1 blocks the ability of the D2 loop to be displaced for RON2 binding. Studies of naturally occurring human antibodies have shown that these antibodies can recognize both the 1F9 and 4G2 epitopes and that those that target 1F9 are correlated with protection from malaria [48,154].

A number of other neutralizing mAbs have been shown to block RON2 binding including 1B10 [158], 4E8 [158], 4E11 [158], R31C2 [54], 14I-1 [47], 14I-M15 [47], and HumAbAMA1 [159]. R31C2 is a mouse mAb raised against *Plasmodium knowlesi* AMA1 and binds to the *P. knowlesi* RON2 binding site similar to that seen in anti-*P. falciparum* AMA1 antibodies [54]. 14I-1 and 14I-M15 are shark immunoglobin new antigen receptors that have been engineered to be strain transcending [47]. Structures of 14I-1 and 14I-M15 in complex with AMA1 show that the CDR3 loops extend into the RON2 binding site almost in a mimic of the RON2 binding [47]. These studies identify promising avenues for exploitation of engineered antibodies to combat malaria. HumAbAMA1 is a human monoclonal antibody isolated from a Ghanaian donor that engages an epitope mapped to the RON2 binding site within AMA1 and overlaps with the 1F9 epitope [159].

An additional class of antibodies against AMA1 are mildly neutralizing *in vitro* map to the D3 domain and have a completely different mechanism of neutralization from the D1 antibodies [158]. These antibodies map to the polymorphic face of the D3 domain and block cleavage of AMA1 from the surface of the parasite resulting in incomplete invasion by the parasite [158]. One such antibody is 1E10 which on its own is only mildly neutralizing [158]. When tested against parasite growth *in vitro* in combination with 4G2, a D1 receptor blocking antibody, the combination showed synergistic inhibition of parasite growth [158]. This was not seen when a combination of two D1 receptor-blocking antibodies was tested [158]. These results suggest that future designs of AMA1 based vaccines may be improved by targeting multiple functional segments of AMA1. In addition to the inhibitory D3 mAbs, the structure of one non-inhibitory cross-reactive mAb F8.12.19 in complex with its cognate antigen PvAMA1 has been solved [49].

A number of phase 1 and 2 clinical trials have been conducted for AMA1 vaccines [160–169]. These vaccines have consisted of viral-vectored, DNA vectored, recombinant protein, and synthetic engineered antigen based on AMA1. Many of the vaccines have included multiple alleles of AMA1 to overcome strain-specific responses, and some have included other vaccine candidates in combination with AMA1 [160–169]. All of these vaccines have been proven safe in phase 1 trials with little to no adverse events related to immunization [160–169]. All trials have produced AMA1-specific antibodies and AMA1-specific T-cell responses [160–169]. Although all of these studies showed a >90% seroconversion of participants, these trials showed 20% efficacy at best and no protection at worst with significantly diminishing protection by the second malaria season [160–169]. This is in stark contrast to what has been observed in animal studies where sterile protection upon parasite challenge can be observed [153,158].

The dichotomy between high seroconversion and low protection may be explained by the polymorphic nature of AMA1. AMA1 is highly polymorphic particularly around the receptor binding site in D1 [170]. Analysis of 355 *P. falciparum* AMA1 sequences identified 64 polymorphisms indicating that over 10% of AMA1 is variable across strains [170]. D1 alone contains 32 polymorphisms and 20 more are distributed between D2 and D3 [170]. Many

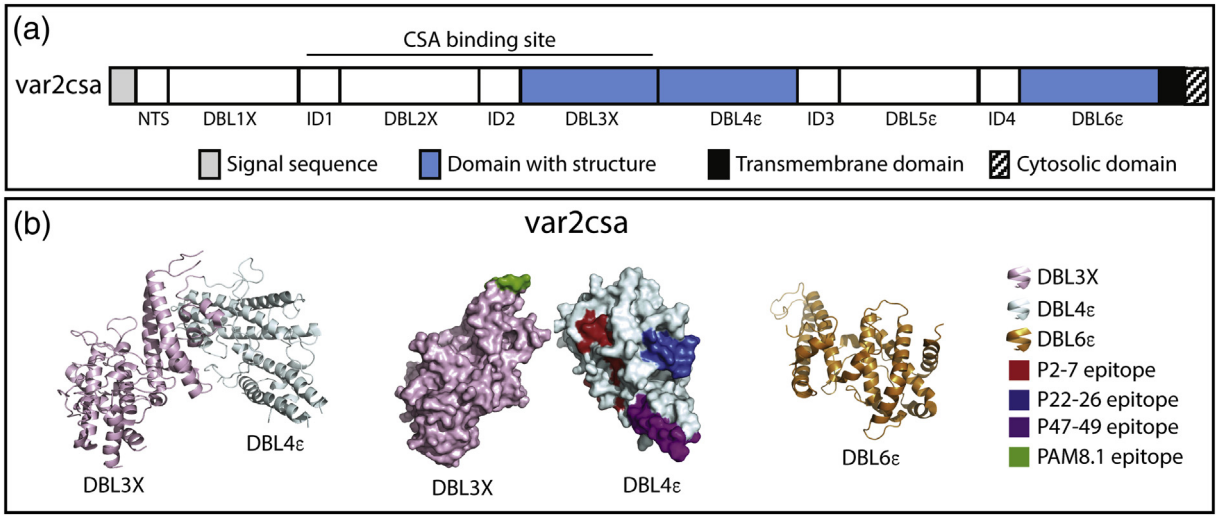

Fig. 5. Structure of VAR2CSA. (a) Domain organization of var2csa. (b) Structures of DBL3X, DBL4ϵ (PDB: 4P1T), and DBL6ϵ (PDB: 2Y8D) domain of VAR2CSA are shown in ribbons. Binding epitopes of PAM8.1 and various linear peptides are all highlighted on corresponding surface representations.

mAbs with epitopes that map to the RON2 binding site are affected by these polymorphisms.

Both animal immunization and population studies have resulted in strain-specific antibodies based on the antigen used or the geographic area studied [157,158,171,172]. Most of the current vaccine trials have utilized only one or two alleles of AMA1 and saw minimal parasite neutralization *in vitro*, and this minimal protection was strain-specific. The AMA1-DiCo vaccine has attempted to correct this flaw by designing three recombinant proteins that encompass the majority of the polymorphisms observed in AMA1 alleles [170]. Early testing in animals produced strain-transcending antibodies in rabbits that neutralized parasite growth *in vitro* across diverse strains [170]. The AMA1-DiCo vaccine has been proven safe in phase 1 clinical trials [168], and it will be informative to see if this vaccine has better efficacy than previous designs.

An alternate strategy for AMA1 immunization has shown promise in animal studies and holds potential for future vaccines. Mice immunized with the complex of AMA1 and the minimal binding domain of RON2 produced growth-neutralizing antibodies and were protected from malaria [172]. The protective effect is AMA1/RON2 complex dependent as immunization with AMA1 or RON2 alone or together but injected into separate sites did not protect mice from malaria [172]. A further study in *Aotus* monkeys with the AMA1/RON2 complex resulted in higher levels of protection against sporozoites challenge with longer times to blood-stage infection [173]. Furthermore, this immunization protocol elicited strain-transcending antibodies when tested *in vitro* and resulted in sterile immunity in a subset of the monkey vaccinees [173]. Immunization with the complex may result in the exposure of new epitopes to the immune system and together with the mAb epitope mapping may inform the next generation of AMA1 vaccines.

VAR2CSA

VAR2CSA is a member of the *var* family of genes found in *P. falciparum* that includes PfEMP1 [174–179]. The *var* genes are membrane proteins expressed during the trophozoite stage of the erythrocytic stage and are transported to the surface of the infected RBC (iRBC) to facilitate adhesion and sequestration of the iRBC to host tissues [174–179]. VAR2CSA is unique in that it is expressed specifically in parasites that are responsible for placental malaria (PM) [174–179]. VAR2CSA binds to chondroitin sulfate A (CSA) on Syndecan-1 (SDC1) expressed in placental tissues [180]. SDC1 presentation of CSA in placental tissue may be key to the placental tropism observed as SDC1 is expressed on other host cells, but these versions of SDC1 do not facilitate binding to iRBCs [180]. Binding of SDC1 by iRBC's allows for the sequestration of parasites in the placenta and leads to adverse outcomes during pregnancy [180]. A secondary effect of iRBCs binding to SDC1 is that this binding blocks SDC1 internal signaling [180]. How this may affect the parasite during the course of infection is not known.

VAR2CSA is a highly polymorphic ~350-kDa protein composed of multiple regions, Duffy-binding like domains and interdomain segments (ID). VAR2CSA contains an N-terminal segment, DBL1x, ID1, DBL2X, ID2, DBL3x, DBL4ϵ, DBL5ϵ, DBL6ε, a transmembrane domain, and a short

cytoplasmic domain [174–179] (Fig. 5a). Although VAR2CSA is highly polymorphic, it can be classified into four main clades based on the sequence of the DBL2X domain [181]. Clades 1 and 2 are the 3D7-like and FCR3-like clades, respectively [181]. Interestingly, like most *var* genes, the majority of VAR2CSA-containing strains have only one copy, but a small percentage have two copies of this gene, which can range from 87%–93% sequence similarity between copies [181]. What implications this has for vaccine design have yet to be determined.

Three of the domains in VAR2CSA have been implicated in CSA binding: DBL2X, DBL3X, and DBL6ϵ (Fig. 5a). The structures of DBL3X, DBL3X-DBL4ϵ, and DBL6ϵ have been solved and shed light on how DBL3X and DBL6ϵ bind to CSA [55–59] (Fig. 5b). All three DBL domains share an overall DBL fold with other DBL domains from EBA-175 and DBP [55–59]. The main differences arise from loop extensions and the conservation of certain disulfide bonds within the domains. DBL3X is much larger than other DBL domains crystallized in that it has multiple loop extensions including a loop insertion in sub-domain 2 that is involved in CSA binding [55,57–59]. DBL4ϵ also has an eleven amino acid residue insertion in helix 5 of subdomain 2 [59]. DBL6ϵ lacking loop insertions is more compact in comparison to DBL3X and DBL4ϵ, and it has fewer disulfide bonds than DBL3X [56,58,59]. The disulfide bond pattern in all three domains is only partially conserved when compared to EBA-175 and DBP [55–59].

DBL3X and DBL6ϵ also have distinct modes of binding to CSA [55]. DBL3X's CSA binding site is composed of a loop from subdomain 2 and residues toward one end of subdomain 3 [55]. In contrast, DBL6ϵ's CSA binding site is primarily on the opposite end of subdomain 2 from that of DBL3X's CSA binding site, although DBL6ϵ's binding site extends perpendicularly across subdomain 2 to subdomain 3 [55]. The identification of multiple distinct binding sites within DBL3X and DBL6ϵ is of importance, as both domains bind CSA with similar affinity and vaccine design may require targeting both sites simultaneously. Furthermore, the recombinant full-length VAR2CSA has stronger binding affinity to CSA than each domain in isolation. SAXS data suggest that the full-length VAR2CSA might adopt a compact shape and form a high-affinity binding site for CSA [182].

Antibodies to VAR2CSA are thought to be restricted to pregnancy. High levels of antibodies to all domains of VAR2CSA are observed in pregnant women from endemic areas, and high levels of antibodies, particularly high avidity antibodies, are correlated with protection from PM and reduced adverse pregnancy outcomes [183–189]. However, reactivity in men and children has been observed in Colombia [190], although it has been suggested that this is due to cross-reactivity to a different var protein [191]. VAR2CSA is unique as it is only expressed during PM and antibodies to this antigen are predominantly found in pregnant women [183–189,192,193]. VAR2CSA antibodies levels increase during the duration of the pregnancy and women who have had multiple pregnancies (multigravidae) have higher antibody levels [185–189,191]. The antibodies found in multigravidae are longer lasting and have higher avidity than those seen in primigravidae. These multigravidae antibodies inhibit CSA binding in recombinant protein assays and *in vitro*/*ex vivo* parasite binding assays [185,192–194], recognize all DBL domains of VAR2CSA and are more cross reactive than primigravidae antibodies [183,188,193], and induce opsonic phagocytosis *in vitro* [183].

Animal studies have also demonstrated the production of CSA-binding inhibitory antibodies, opsonic antibodies, and antibodies that map to all DBL domains [184,192,193,195,196]. Peptide arrays identified three regions in DBL4ϵ recognized by inhibitory polysera from immunized rats: peptides P2–7, P22–26, and P47–49 [195]. CSA-binding inhibitory multigravidae polysera also bound to a region within P22–P26, which covered P23–P25 [195]. This region is composed of a loop between two helices [195].

Human mAbs have been isolated from multigravidae donors, and both strain-specific antibodies (PAM4.7 and PAM8.1) and cross-reactive antibodies (PAM1.4, PAM2.8, PAM3.10, PAM 5.2, PAM6.1, PAM7.5) have been characterized [185]. These human mAbs were specific for either DBL3x (PAM2.8, PAM6.1, PAM8.1) or DBL5ϵ (PAM3.10, PAM4.7, PAM 5.2, PAM7.5) [185]. PAM8.1's epitope mapped to a 12-residue insertion seen in FCR3 that is missing in 3D7 and occurs between residues G1474 and Q1475 in 3D7 explaining the strain specificity exhibited by PAM8.1 [185]. PAM8.1 is inhibitory for CSA binding in both recombinant protein and parasite assays [194].

The studies of multigravidae antibodies and those raised in animals have led to two phase 1 clinical trials [197]. The first clinical trial is led by the PRIMALVAC project, and the vaccine consists of a recombinant protein produced in *E. coli* that corresponds to DBL1X–DBL2X of VAR2CSA [197]. The second clinical trial is led by the PAMCPH/PlacMalVac project and consists of ID1–DBL2X–ID2a of VAR2CSA produced in insect cells [197]. These highly important studies will provide critical insight to help establish a viable vaccine for PM.

Future Outlook

While most studies focus on immunization with single antigens, antigen combinations may provide enhanced protection [40,198]. However, polysera

from rabbits immunized with multiple antigens revealed that some antigen combinations were additive, others were synergistic, and certain combination was antagonistic in inhibiting blood-stage parasite growth in culture [198]. Determining the best combination of antigen will be essential for any future combination vaccines given diverse outcomes on parasite growth. Nevertheless, great progress has been made in the determination of antigen structures and antigen-specific epitopes for many of the leading blood-stage vaccine candidates. The new structural information and mapping of both inhibitory and non-inhibitory antibody epitopes provides a new foundation for malaria structural vaccinology. These studies and all those cited within in this review provide a foundation that is expected to lead to a new generation of more effective vaccines against blood-stage malaria.

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Abbreviations used:
RBCs, red blood cells; EBL, erythrocyte binding-like; DBL, Duffy binding-like; GpA, glycophorin A; mAbs, monoclonal antibodies; DBP, Duffy binding protein; DARC, Duffy antigen receptor for chemokines; RH, reticulocyte-binding homolog; BSG, basigin; RBP, reticulocyte binding protein; PM, placental malaria; CSA, chondroitin sulfate A.

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