CHAPTER 1

Introduction

1.1 Summary and Aims

The first aim of the work described in this PhD thesis was to investigate the potential of using monoclonal antibodies targeting the RH5-Basigin interaction, as novel anti-malarial therapeutics. To this end, I developed a series of humanised or chimeric monoclonal anti-Basigin and anti-RH5 antibodies, and characterised their ability to interfere with the RH5-Basigin interaction and *P. falciparum* erythrocyte invasion. The second goal was the identification of novel receptor-ligand pairs involved in erythrocyte invasion by *Plasmodium falciparum*. The current chapter provides background information concerning both these projects.

To mirror the twin experimental goals of the PhD thesis, this introductory chapter is split into two sections. In the first section, I discuss the general structure and functions of antibodies, as well as their value in the clinic for the treatment of various diseases. In the second part I describe malaria, focusing particularly on erythrocyte invasion, and on how this critical stage in parasite's lifecycle can be exploited for the development of anti-malarial intervention measures.

1.2 General structure and properties of antibodies

In the early 1890s, Emil von Behring together with Kitasato Shibasaburo, with their work in diphtheria and tetanus, were the first to propose that mediators in serum can react with foreign substances (A G N, 1931). These mediators were later termed antibodies (antikörper in German*)* by Paul Ehlirch in 1891, who reported that if two substances give rise to two different "antikörper", then they themselves must be different (Lindenmann, 1984). In 1920s, Michael Heidelberger and Oswald Avery found that antibodies "were made of proteins"(Van Epps, 2006).

Later studies demonstrated that antibodies are large Y-shaped proteins secreted by B-cells, and are responsible for organism's humoral immunity (Abbas *et al.*, 2011). An antibody is a tetrameric molecule consisting of four polypeptide chains; two identical heavy chains (α, δ, ε, γ, μ) pair with each other, as well as with two identical light chains (κ,λ), via disulfide bonds (Fig. 1.1A). The type of heavy chain present, defines the class of antibody (see below). Both heavy and light chains have a globular structure consisting of tandem immunoglobulin (Ig) domains, and are subdivided into constant (C) and variable (V) region. The V region is responsible for antibody specificity and binding to the antigen: within the variable regions of both

heavy (VH) and light (VL) chains, three polypeptide segments show exceptional variability between different antibodies and are known as hypervariable regions. These diverse stretches from both VH and VL chain are brought together to form an antigen-binding surface (Abbas *et al.*, 2011).

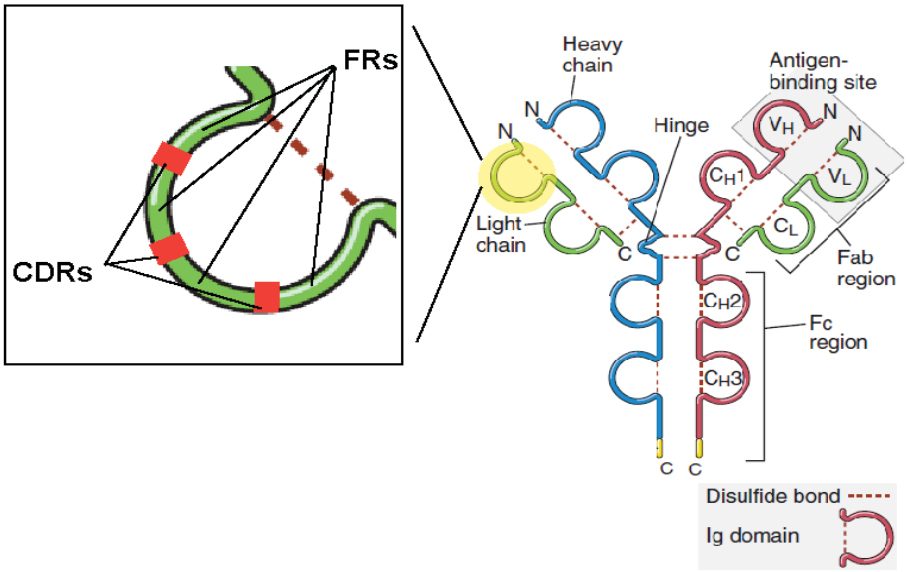
Hypervariable regions form a surface that is complementary to the structure of the bound antigen, and hence hypervariable regions are also called complementarity determining regions (CDRs) (Male *et al.*, 2006; Abbas *et al.*, 2011) (Fig. 1.1A). Amino acid residues within the CDRs form multiple contacts with the target antigen. The most extensive contact is with the third hypervariable region of the heavy chain (CDRH3). The latter CDR is also the most variable amongst the CDRs of both VH and VL because it is encoded by a stretch in the genome which is derived from an "error prone" recombination event between three different segments (V,D,J) in the genome (Male *et al.*, 2006; Abbas *et al.*, 2011).

CDRs are surrounded by four less variable sequences, known as framework regions (FRs) which are thought to play supporting role for the CDRs during binding to the antigen (Fig. 1.1A). Some residues within the FRs may directly contact the antigen, but the primary role of FRs is to sterically maintain the CDRs at a position that maximizes interaction with antigen binding sites (Male *et al.*, 2006; Abbas *et al.*, 2011).

1.3 Antibody classes

The C region Ig domains are separate from the antigen-binding site and do not participate in antigen recognition. The heavy chain C region interacts with other effector molecules and cells of the immune system and therefore, mediates most of the biological functions of antibodies (section 1.4). Antibodies are divided into five dinstict classes (isotypes) named IgA, IgD, IgE, IgG, and IgM, depending on which heavy chain (α,γ,δ,ε,μ) is present (Fig 1.1B). In humans, IgA and IgG isotypes can be further divided into subclasses called IgA1, IgA2 and IgG1, IgG2, IgG3, IgG4, respectively (Abbas *et al.*, 2011).

A.



B.

Isotope of Antibody	Subtypes (H Chain)	Serum Concentration (mg/mL)	Serum Half-life (days)	Secreted Form
IgA	IgA1,2 (α1 or α2)	3.5	6	IgA (dimer) Monomer, dimer, trimer
IgD	None (δ)	Trace	3	None
IgE	None (ε)	0.05	2	IgE Monomer
IgG	IgG1-4 (γ1, γ2, γ3, or γ4)	13.5	23	IgG1 Monomer
IgM	None (μ)	1.5	5	IgM Pentamer

Figure 1.1 Structure and properties of antibodies.

A. The structure of an IgG antibody is shown as representative of all antibody classes. IgG consists of two heavy and two light chains, each of which has a constant (CH1-3 for heavy chain, and CL for light chain) and a variable region (VH for heavy chain, and VL for light chain). The antibody specificity is due to the three Complementarity Determining Regions (CDRs) found in the variable regions of both heavy and light chains. The three CDRs of each heavy and light chain are surrounded by four Framework Regions (FRs). The antibody molecule is subdivided into Fab and Fc region, based on the fragments obtained after proteolytic processing by specific enzymes. Abbreviations: CH1-3, Constant Heavy Immunoglobulin Domains 1-3; CL, Constant Light; VH, Variable Heavy; VL, Variable Light.

B. Antibodies are divided into five different classes (IgG, IgE, IgA, IgM and IgD), depending on which heavy chain (α, γ, δ, ε, μ) is present. Subtypes of γ (γ1-4) and α (α1-2) chains further devide IgGs and IgAs in IgG1-4 and IgA1-2 subclasses, respectively. Heavy and light chains are indicated with magenta and green colour, respectively. Pictures adapted and modified from Abbas *et al.*, 2011.

IgG (Fig. 1.1) is the predominant form of antibodies in blood and extracellular fluids. IgGs account for the 70-75% of the total serum immunoglobulin pool, and provide the majority of antibody-based immunity (Male *et al.*, 2006; Abbas *et al.*, 2011). It is the most well studied class of antibodies and due to their high abundance, extended serum half-life and versatility in triggering antibody effector functions (sections 1.4 and 1.5), they have attracted most of the interest towards the development of antibody-based therapeutics for various diseases (section 1.6). Therefore, for the rest of this PhD thesis, I am focusing mainly on the properties and functions of IgGs.

1.4 How do antibodies function?

1.4.1 Direct neutralisation of microbes and microbial toxins

Antibodies against microbes inhibit the interaction of microbes with cellular components. Many microbes (e.g influenza virus, HIV, *P. falciparum* merozoites) use molecules exposed on their surface to recognise and infect host cells. Antibodies that bind to these microbial structures interfere with their ability to recognise and bind to host cell surface receptors. In this way, antibodies neutralise pathogens and prevent the initiation or continuation of infection (Abbas *et al.*, 2011).

Similarly, many microbial toxins mediate their pathologic effect by binding to host cell surface receptors. For example, diphtheria toxin binds to HB-EGF (Naglich *et al.*, 1992) to enter host cells where it inhibits protein synthesis, and tetanus toxin (tetanospasmin) binds to a cell surface receptor to infect inhibitory neurons and thereby inhibiting the release of neurotransmitters. Antibodies against such toxins hinder the interaction with host cells and therefore, prevent the onset of injury or disease (Abbas *et al.*, 2011).

1.4.2 Antibody effector functions

1.4.2.1 Antibody effector functions mediated by Fc receptors

Besides antibody direct neutralisation of pathogens and pathogenic toxins, more sophisticated effector systems have co-evolved with antibodies to maximize the protection against microbes: Antibody Dependent Cellular Phagocytosis (ADCP), Antibody Dependent Cellular Cytotoxicity (ADCC), and Complement Dependent Cytotoxicity (CDC) (Hogarth and Pietersz, 2012). ADCP and ADCC (see below) are mediated by a class of cell surface receptors - Fc receptors (FcRs) - which bind

specifically to the Fc portion of antibodies (Fig. 1.1). The engagement of Fc receptors to antibody Fc region initiates intracellular signalling cascades which activate, regulate and modulate immunity (Hogarth and Pietersz, 2012).

The Fc receptors that bind IgGs are called Fcγ receptors (FcγR). FcγRs comprise a diverse family of cell surface receptors consisting of three classes of receptors (FcγRI-III) encoded by six genes: *FCGR1A* (which encodes FcγRI; also known as CD64), *FCGR2A* (which encodes FcγRIIA; also known as CD32A), *FCGR2B* (which encodes FcγRIIB; also known as CD32B), *FCGR2C* (which encodes FcγRIIC; also known as CD32C), *FCGR3A* (which encodes FcγRIIIA; also known as CD16A) and *FCGR3B* (which encodes FcγRIIIB; also known as CD16B) (Hogarth and Pietersz, 2012). FcγRs cellular distribution is shown in Fig. 1.2A.

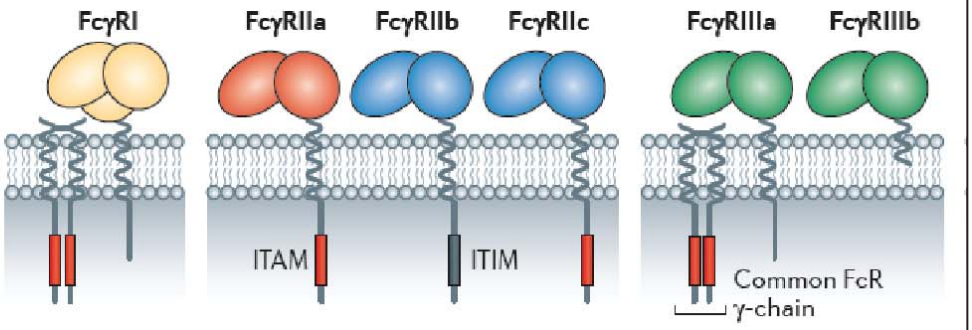
Within the three classes of FcγRs, several polymorphisms with functional relevance have been described. Position 158, in the amino acid sequence of FcγRIIIA, has been shown to be polymorphic, and either valine or phenylalanine are found in this position. The V_{158} allotype has been shown to be associated with more efficient natural killer (NK) cell activity (Male *et al.*, 2006). Similarly, high- or low-responder forms of FcγRIIA are defined by either Arginine (R_{131}) or Histidine (H_{131}) residues at position 131, respectively (Hogarth and Pietersz, 2012). Finally, polymorphisms in FcγRIIIB result in three allotypes, FcγRIIIB-NA1 (R_{36}, N_{65}, D_{82} , V_{106}), FcγRIIIB-NA2 (S_{36}, S_{65}, N_{82}, I_{106}) and FcγRIIIB-SH ($S_{36,}$ S_{65}, D_{78}, $N_{82,}$ I_{106}), which differ in their glycosylation pattern (Bruhns, 2012).

Structurally, each FcγR has a unique Fc-binding α-chain of which the extracellular region consists of two immunoglobulin-like domains (Fig. 1.2B). FcγRI is an exception, since it has three immunoglobulin-like domains (Fig. 1.2B). Of note, is that FcγRI displays the highest affinity for antibody Fc regions, in comparison to the rest FcγRs (Fig. 1.2C) (Woof and Burton, 2004; Hogarth and Pietersz, 2012). The α-chain of FcγRI and FcγRIIIA is incapable of intracellular signal transduction upon receptor engagement. Hence, it is complexed through its transmembrane region with a dimer of the common FcR γ-chain which contains an immunoreceptor tyrosine based activation motif (ITAM), responsible for the initiation of stimulating signals (Fig. 1.2B)

A.

	FcγRI	FcγRIIA	FcγRIIB	FcγRIIC	FcγRIIIA	FcγRIIIB
B cell	-	-	+	-	-	-
T cell	-	-	-	-	-	-
NK cell	-	-	-	+	+	-
Monocyte/Macrophage	+	+	+/-	+	+	-
Neutrophil	(+)	+	+/-	+	-	+
Dendritic cell	+	+	+	-	-	-
Basophil	-	+	+	-	-	+/-
Mast cell	(+)	+	-	-	-	-
Eosinophil	-	+	-	-	-	-

B.



C.

	FcγRI	FcγRIIA		FcγRIIB	FcγRIIC	FcγRIIIA		FcγRIIIB
		H_{131}	R_{131}			V_{158}	F_{158}	NA1,NA2,SH
IgG1	**650**	52	35	1.2	1.2	20	11.7	2.0
IgG2	-	4.5	1.0	0.2	0.2	0.7	0.3	-
IgG3	**610**	8.9	9.1	1.7	1.7	**98**	77	10
IgG4	**340**	1.7	2.1	2.0	2.0	2.5	2.0	-

Figure 1.2 The Fcγ family of receptors.

A. Cellular distribution of human FcγRs. ***B.*** Diagrammatic representation of the human FcγR general protein structure. Of note, is that the extracellular region of FcγRI has an additional immunoglobulin-like domain in comparison to the other FcγRs. ***C.*** A table showing the affinity constants (K_A) of human FcγRs for human IgG subclasses (x10^5 M^{-1}). For details see text. Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; +, constitutive expression; (+), inducible expression; +/-, expression in some cell subsets only. Pictures adapted and modified from Bruhns, 2012 and Hogarth & Pietersz, 2012.

(Woof and Burton, 2004). FcγRIIIA can associate with other signalling molecules as well, such as homodimers of the T cell receptor (TCR) ζ-chain or heterodimers composed of γ and ζ chains in NK cells. FcγRIIA, FcγRIIB and FcγRIIC do not associate with other molecules for signal transduction but they carry their signalling motifs in the cytoplasmic tail of the α-chain. FcγRIIB, instead of an ITAM, contains an immunoreceptor tyrosine based inhibitory motif (ITIM) in its α-chain cytoplasmic region, through which it transduces suppressing signals (Nimmerjahn and Ravetch, 2008). The simultaneous expression of activating and inhibitory molecules on the same cell is the key for the generation of a balanced immune response (Nimmerjahn and Ravetch, 2006).

1.4.2.1.1 Antibody Dependent Cellular Phagocytosis (ADCP)

An important biological function of antibodies is the Antibody Dependent Cellular Phagocytosis (ADCP). Phagocytosis of IgG-coated microbes is mediated by the engagement of the Fc portions of antibodies to FcγRs expressed on a phagocyte's cell surface (Abbas *et al.*, 2011). Phagocytes express a combination of FcγRs including FcγRI and FcγRIIA (Desjarlais and Lazar, 2011). Following the binding to the antibody Fc portion, the target foreign particle is internalized by the phagocytes, and is finally destroyed in phagolysosomes. Besides the phagocytosis itself, the binding of IgGs to FcγRs on phagocytes triggers the secretion of lytic enzymes and reactive oxygen species which are directed against microbes that are too large to be phagocytosed (Abbas *et al.*, 2011).

1.4.2.1.2 Antibody Dependent Cell mediated Cytotoxicity (ADCC)

In a process called antibody dependent cell mediated cytotoxicity (ADCC), NK-cells recognise and destroy antibody-coated target cells. This process is primarily mediated by the low affinity, NK-cell surface exposed, FcγRIIIA, which binds to the Fc region of antibodies aggregated on target cells. The engagement of FcγRIIIA, activates NK-cell and triggers the discharge of perforins and granzymes which form pores and lyse the foreign cells (Male *et al.*, 2006; Abbas *et al.*, 2011).

1.4.2.2 Complement dependent cytotoxicity

Antibodies bound to the surface of target cells can activate the classical pathway of the complement cascade. IgG subtypes have different ability in triggering the classical pathway cascade with IgG3>IgG1>>IgG2>>IgG4 (Woof and Burton,

2004). The first component of this pathway, C1, is a complex between a hexameric recognition unit C1q, and two molecules of each of the proteases C1r and C1s. Once C1q in the C1 complex binds to the Fc region of immobilized antibodies – at least two out of six arms of C1q must be bound - it undergoes conformational changes which trigger autocatalytic activation of C1r. C1r subsequently cleaves and activates C1s. C1s then cleaves C4 into 2 fragments C4a and C4b, of which the latter stays associated with C1 to ensure that the reaction proceeds on the target cell surface (Male *et al.*, 2006).

The pathway continues with a series of enzymatic events and finally results in the formation of the so called membrane attack complex (MAC) which is a pore on the target cell membrane, formed by multiple copies of C9 protein, arranged like barrel staves around a central cavity. The MAC complex allows free flow of solutes and water across the target membrane. The entry of water results in osmotic swelling and rupture of the cells upon whose surface the MAC is deposited (Male *et al.*, 2006).

1.5 The interactions between IgG Fc portion and C1q or Fcγ receptors have been characterised

The interactions between IgG Fc region and C1q or FcγRs have been studied extensively, and a number of amino acids within antibody Fc portion have been suggested to participate in the binding to C1q and FcγRs. The latter amino acids are of great therapeutic interest, and provide a means of modulating antibody effector functions *in vivo* when antibodies are used for treatment of various diseases.

Several studies reported that the so called lower hinge region (Fig. 1.1A), is of key importance for the binding of C1q and FcγRs to antibody Fc region. For example Morgan and colleagues demonstrated that changing the leucine 235 to glutamic acid (Eu numbering) abolished FcγRI binding (Morgan *et al.*, 1995). The same group also reported that the mutation of glycine to alanine at position 237 abolished FcγRI binding and reduced complement lysis and FcγRIII-mediated function (Morgan *et al.*, 1995). Chappel and colleagues proposed that the entire sequence spanning residues 234-237 of hIgG1, is required for binding to FcγRI (Chappel *et al.*, 1991). Similarly, Shields and colleagues exchanged the amino acid residues 233-236 of hIgG1 with those found in hIgG2 at the same positions. They showed that the

E233P/L234V/L235A/G236Δ hIgG1 variant binds to all Fcγ receptors with reduced affinity (Shields *et al.*, 2001)

Another area in antibody Fc region that has attracted interest and has been proposed to play important role in driving antibody effector mechanisms, is the area spanning the residues 327–331 from a bend, joining two β-strands close in the tertiary structure to the lower hinge region (Armour *et al.*, 1999). Alanine substitution at positions P329, and P331 significantly reduced the ability of C1q binding to hIgG1 (Idusogie *et al.*, 2000). Moreover, the introduction of P331S mutation in IgG3, as found in IgG4, reduced affinity for FcγRI by a factor of 10 (Canfield and Morrison, 1991). Lysine at position 322, which is located N-terminally to the 327–331 region, has also been implicated in the stimulation of antibody effector functions. Armour and colleagues in two elegant studies combined mutations in the two Fc regions mentioned above and demonstrated that the E233P/L234V/L235A/G236Δ/A327G/A330S/P331S mutant has impaired binding to FcγRI, FcγRIIA$_{H131}$, FcγRIIA$_{R131}$ and FcγRIIB (Armour *et al.*, 1999, 2003). A number of other amino acid residues in the Fc region have also been implicated in the modulation of antibody effector functions (Idusogie *et al.*, 2000; Hezareh *et al.*, 2001; Shields *et al.*, 2001).

Finally, the presence of oligosaccharides attached to the asparagine at position 297, has been shown to be important for most antibody effector functions (Idusogie *et al.*, 2000). Previous studies provided evidence that that interaction sites on IgG Fc for FcγRI, FcγRII, FcγRIII and C1q are comprised principally of only the protein moiety (Jefferis, 2009). Nevertheless, the generation of the interaction sites for these ligands appears to be dependent on IgG Fc protein – carbohydrate interactions. Indeed, it has been shown that effector mechanisms mediated through FcγRI, FcγRII, FcγRIII and C1q are severely affected for aglycosylated forms of IgG (Jefferis, 2009).

1.6 The discovery of hybridoma technology and the emergence of antibody therapeutics

In 1975 Kohler and Milstein were the first to introduce the hybridoma technology (Köhler and Milstein, 1975). They demonstrated that cell lines (hybridomas) derived from the fusion of myeloma and mouse spleen cells from an immunised donor can grow indefinitely in culture, and have the ability to secrete

antibodies. Each hybridoma clone produces antibodies of a single specificity (monoclonal antibodies) and therefore, if the spleen cell donor is immunised with an antigen of interest, it is possible to isolate clones which secrete antibodies of the desired specificity. Hybridoma technology provided an unlimited source of monoclonal antibodies, and enabled of the possibility that monoclonal antibodies could be used as putative therapeutics.

Monoclonal antibodies have rapidly emerged as a clinically important class of biological drugs. Their fine specificity, long serum half-lives (Fig. 1.1B) and the ability they offer to modulate antibody effector functions simply by mutating amino acids within antibody Fc region (section 1.5), have established antibody based therapies as one of the most successful therapeutic strategies (Beck *et al.*, 2010). The first antibody that was approved for clinical therapy in 1986 was OKT3, a mouse anti-CD3 monoclonal, used for the treatment of acute allograft rejection in renal transplantation (Smith, 1996; Chan and Carter, 2010). Since then, more than 30 antibodies have been approved by FDA for human therapy (Beck *et al.*, 2010) and more than 240 antibodies are currently under clinical trials for a variety of diseases, ranging from cancer and organ transplantation, to autoimmunity and infectious diseases (Chan and Carter, 2010; Reichert, 2010).

Alemtuzumab (marketed as Campath) was amongst the first antibodies to be used in clinic (Riechmann *et al.*, 1988; Gorman and Clark, 1990). It is an anti-CD52 humanised antibody (for the humanisation technology see Chapter 4), and it was originally intended for the treatment of leukemias (Gorman and Clark, 1990; Magliocca and Knechtle, 2006; Coles, 2013). Because of its ability to transiently deplete peripheral lymphocytes, it is now also being considered for the treatment of multiple sclerosis (Coles, 2013). Alemtuzumab has also been used as an induction agent during organ transplantations (Magliocca and Knechtle, 2006).

Trastuzumab (Herceptin) is another well-known therapeutic antibody. It is directed against Human Epidermal growth factor Receptor 2 (HER2) and has been used to treat HER2 positive metastatic breast cancer, in patients who had received one or more chemotherapy regimens (Reichert, 2010). In 2012, FDA approved trastuzumab to be used in combination with pertuzumab – another anti-HER2 antibody which inhibits HER2 dimerization- and docetaxel, for the treatment of patients with HER2-positive metastatic breast cancer who have not previously

received anti-HER2 therapy or chemotherapy for metastatic disease (Baselga *et al.*, 2012; Blumenthal *et al.*, 2013).

1.7 Malaria is a significant global health problem

The parasitic protozoon of the genus *Plasmodium* is the causative agent of malaria, a serious infection which accounted for 219 million clinical cases and 1.2 million deaths in 2010 alone (Murray *et al.*, 2012; World Health Organization, 2012). Malaria is transmitted to humans through the bites of female *Anopheline* mosquitoes (section 1.9), and is strongly associated with poverty as mortality rates are highest in developing countries with lower gross national income (GNI) per capita (World Health Organization, 2012). The vast majority of malaria fatalities (85–90%) occur in sub-Saharan Africa, mainly in the vulnerable populations of children under the age of five and pregnant women (Geels *et al.*, 2011; World Health Organization, 2012). Globally, an estimated 3.3 billion people (nearly half the world's population) were at risk of malaria in 2011, and today there is on-going malaria transmission in 99 countries (World Health Organization, 2012).

1.8 Five species of *Plasmodium* are infective to humans

Historically, it was thought that four species *of Plasmodium* naturally infect humans: *P. falciparum, P. vivax, P. ovale* (recently subdivided into two closely related species, *P. ovale curtisi* and *P. ovale wallikeri*; Sutherland *et al.*, 2010), and *P. malariae.* The number of *Plasmodium* species that infect humans has recently been increased by the observation that *P. knowlesi,* a simian malaria parasite which primarily infects macaques, causes a substantial number of naturally-acquired infections in human populations in South East Asia (Singh *et al.*, 2004; Antinori *et al.*, 2013). It has yet to be established whether *P. knowlesi* has switched hosts and transmission is between humans or whether all infections are zoonotic (Singh *et al.*, 2004; Antinori *et al.*, 2013).

The work described in this thesis focuses primarily on *P. falciparum*, the most virulent amongst human *Plasmodium* spp., and a major cause of mortality in children below five years of age, globally (Elliott and Beeson, 2008; Richards and Beeson, 2009).

1.9 *P. falciparum* has a complex lifecycle

The lifecycle of *P. falciparum* is complex, and the parasite alternates between the human host and a mosquito vector (Fig. 1.3) (Bannister and Sherman, 2009). Malaria infection is initiated when the transmissive form of *P. falciparum*, the sporozoites, enter the skin of the human host through the bite of an infected female *Anopheline* mosquito (Prudêncio *et al.*, 2006; Bannister and Sherman, 2009). Sporozoites migrate to the liver where they invade hepatocytes, and subsequently replicate and differentiate to form thousands of invasive merozoites (the exo-erythrocytic form of the parasite). After about nine days, merozoites enter the blood stream and invade erythrocytes initiating an asexual replication cycle that has a duration of 48 hours. This cycle ends with the release of new merozoites from the mature infected erythrocyte (schizont), which can, in turn, infect new erythrocytes (Bannister and Sherman, 2009). In the blood, some intra-erythrocytic stages develop into the sexual parasite stages, the male and female gametocytes, which, during transmission, are ingested by mosquitoes as part of their blood meal (Cowman and Crabb, 2006; Prudêncio et al., 2006). Once within the mosquito gut, the gametocytes develop into mature gametes, and undergo fertilization to form a zygote. The zygote then develops into a motile ookinete which penetrates the mosquito midgut wall, and transforms into an oocyst. The parasite replicates within the oocyst to form mature sporozoites which are finally released and migrate to mosquito salivary glands, ready to be transmitted to human again.

The asexual erythrocytic stages of the parasite are responsible for the clinical manifestations of the disease (Miller *et al.*, 2002a). The synchronised release of merozoites into the blood stream due to erythrocyte rupture at the end of each 48-hour asexual blood cycle, is accompanied by recurrent attacks of chills and fever (Chen *et al.*, 2000). Moreover, sequestration of infected erythrocytes injures endothelial cells and disrupts blood flow, causing tissue hypoxia and lactic acidosis. When sequestration occurs in the brain or placenta, it can lead to life threatening conditions known as cerebral malaria and placental malaria, respectively (Miller *et al.*, 2013).

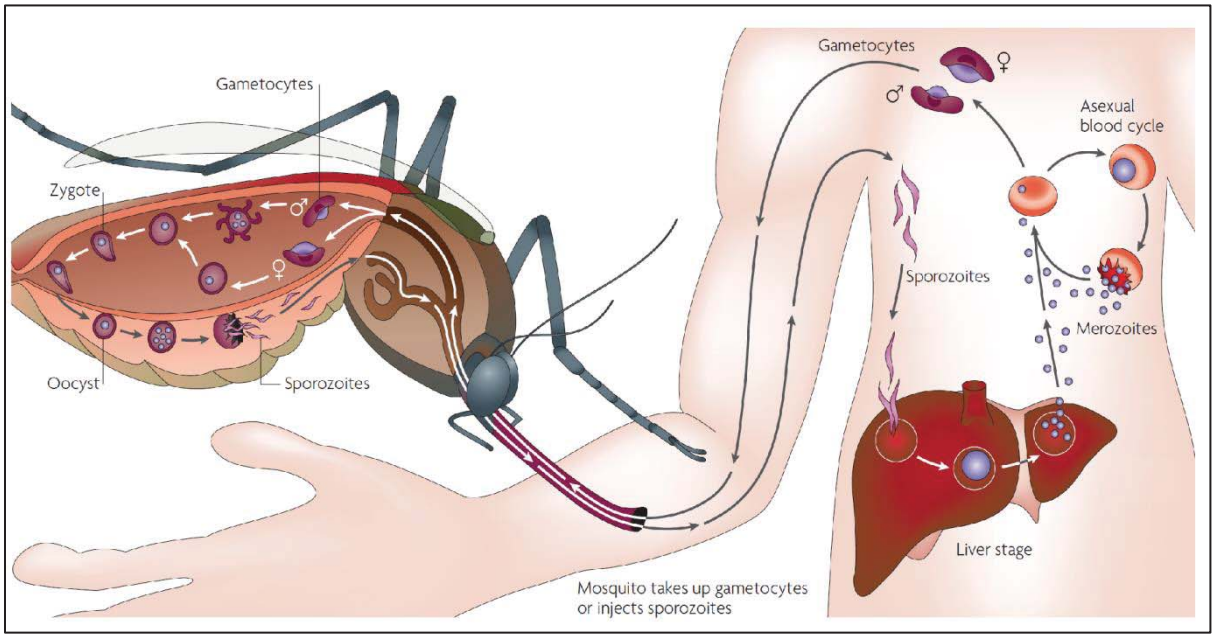


Figure 1.3 The lifecycle of *Plasmodium falciparum*. The lifecycle begins when sporozoites enter human host body, after a bite from a *P. falciparum* infected female *Anopheline* mosquito. Sporozoites migrate to the liver and invade hepatocytes, where they grow and replicate. After about nine days, thousands of merozoites are released into the bloodstream (Bannister and Sherman, 2009), where they rapidly invade host erythrocytes. Erythrocyte invasion marks the initiation of a new proliferation cycle which ends up with the release of new merozoites from matured infected erythrocytes (schizonts). The free merozoites are then able to invade new erythrocytes to continue the asexual blood-stage lifecycle. During blood stages, a number of parasites develop into male or female gametocytes which can then be ingested by a mosquito during a blood meal. Gametocytes mature in mosquito gut, and fuse to form a zygote. The zygote develops into a motile ookinete which transverses the midgut epithelium and transforms into an oocyst. The parasite replicates within the oocyst to form mature sporozoites, which are finally released and migrate to mosquito salivary glands, ready to transmitted to be human again (Cowman and Crabb, 2006; Prudêncio et al., 2006). Picture adapted from Su *et al.*, 2007.

1.10 Erythrocyte recognition and invasion are critical steps in the *P. falciparum* lifecycle

Erythrocyte host entry is an obligatory step in the *P. falciparum* lifecycle (Cowman and Crabb, 2006). On lysis of infected erythrocytes, 16-32 daughter merozoites – the only extracellular form of the parasite during blood stages - are released into the bloodstream, and are capable of rapidly invading (within 30 to 40 seconds) new erythrocytes (Dvorak *et al.*, 1975; Gilson and Crabb, 2009). Merozoites have evolved to carry a full complement of organelles (see below) needed to invade erythrocytes in a rapid and efficient manner (Garcia *et al.*, 2008). Nevertheless, the brief extracellular exposure of merozoites outside their intra-erythrocytic niche, render them vulnerable to host immune defences. Indeed, a significant arm of naturally-acquired immunity to malaria is directed against merozoite antigens (Cohen *et al.*, 1969). Therefore, erythrocyte invasion has long been considered to be an exploitable target for therapeutic intevention; consequently, a deep understanding of the molecular processes that underpin erythrocyte invasion may aid the efforts to control malaria globally.

Below, I describe what is known about the structure of merozoite and the molecular events that drive erythrocyte invasion. Particular emphasis is given to the structure and function of proteins exposed on merozoite cell surface. Such proteins are directly accessible to human host humoral system and thus, they are of fundamental importance for the development of anti-malarial therapeutics.

1.10.1 Merozoite structure

P. falciparum merozoites are ovoid-shaped cells, ~1.2μm in length, and have a distinct structural organization of organelles within their apical protuberance (Bannister and Mitchell, 2003) (Fig. 1.4). A nucleus, a mitochondrion, and a plastid called apicoplast, are responsible for genetic and metabolic processes, and are located basally. Trafficking organelles such as the rough endoplasmic reticulum and Golgi complex are absent or residual in the mature merozoite (Garcia *et al.*, 2008). Underlying the plasma membrane, two additional membranes form a closed flat cisterna called the inner membrane complex (IMC) (Cowman *et al.*, 2012). The IMC together with the plasma membrane form the merozoite pellicle that lines the whole cell apart from the apical end (Bannister and Mitchell, 2003; Garcia *et al.*, 2008).

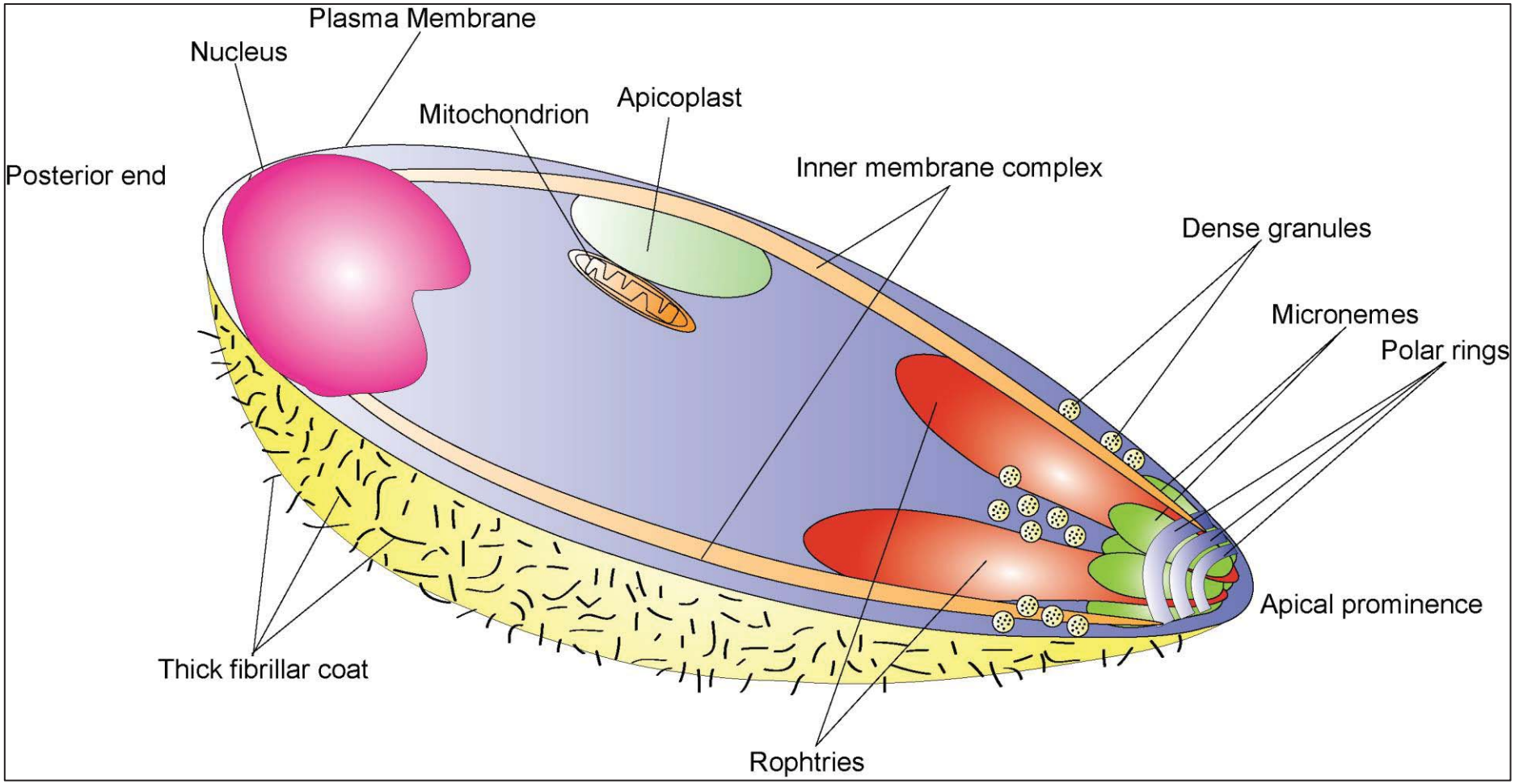


Figure 1.4 Merozoite structure. Merozoite has a polarised morphology and is covered by a proteinaceous fibrillar coat (~15nm thick) (Garcia *et al.*, 2008). Merozoite carries a nucleus, an apicoplast and a single mitochondrion. The secretory organelles (rhoptries, micronemes and dense granules) are located apically and play key role in erythrocyte invasion. The three polar rings are located at the apical end of merozoite. The inner membrane complex is necessary for anchoring the actin-myosin motor, which generates the force required during invasion.

The IMC appears to provide anchorage to several accessory proteins (e.g *Pf*GAP45, *Pf*GAP50) which support the actin-myosin motor within the merozoite, during erythrocyte invasion (Farrow *et al.*, 2011)

Two (or sometimes three) subpellicular microtubules (omitted for clarity in Fig. 1.4) line up in parallel along the side of the merozoite (Garcia *et al.*, 2008). This structure is called the *P. falciparum* merozoite assemblage of subpellicular microtubules (*f*-MAST) (Pinder *et al.*, 2000), and has been proposed to provide mechanical support for the merozoite (Garcia *et al.*, 2008). A possible role of *f*-MAST in invasion was proposed, due to the inhibitory effects of microtubule disrupting drugs in erythrocyte invasion (Pinder *et al.*, 2000). Consistent with this, evidence from electron microscopy also implicates microtubules in microneme targeting to the merozoite apex (see below) (Bannister and Mitchell, 2009). Another cytoskeletal structure is the three polar rings which are located at the apical end of merozoite, and define the site for rhoptry and microneme secretion during invasion (see below; Garcia *et al.*, 2008).

The merozoite is exquisitely adapted for invading host erythrocytes, and contains a set of secretory organelles (micronemes, rhoptries, dense granules, mononeme, exoneme) within which the molecules necessary for erythrocyte invasion are stored. Micronemes are elongated, densely staining vesicles that are clustered apically (Garcia *et al.*, 2008) (Fig. 1.4). It is likely that more than one subpopulation of micronemes exist, allowing sequential secretion of different micronemal contents in different phases of invasion (Singh *et al.*, 2007; Cowman *et al.*, 2012). Rhoptries are two pear-shaped organelles, much larger than micronemes, with their apical ends converging on the centre of the merozoite prominence (Kats *et al.*, 2006). Rhoptries can be subdivided into two functional domains, the bulb and the neck, which are not physically separated; however, their contents are distinct, and are thought to be released at different time points during erythrocyte invasion (see below; Kats *et al.*, 2006; Cowman *et al.*, 2012; Zuccala *et al.*, 2012). Rhoptry neck proteins are thought to be released on the merozoite cell surface before their counterparts located in rhoptry bulb (Kats *et al.*, 2006). Rhoptry secretion is thought to follow microneme, though the exact sequence of the molecular events that drive erythrocyte invasion, is not yet fully understood (Harvey *et al.*, 2012).

Dense granules are small, spheroidal vesicles scattered in the apical half of the merozoite (Garcia *et al.*, 2008) (Fig. 1.4). In *P. falciparum* is thought that the contents of dense granules are discharged by exocytosis onto the merozoite surface after invasion, and these proteins decorate the parasitophorus vacuolar membrane (PVM) to enable exchange of molecules between the parasite and the host erythrocyte (Kats *et al.*, 2006). Mononemes and a type of vesicle called exonemes are two secretory organelles that have only recently been described, and both contain proteases with pivotal roles in merozoite invasion(Singh *et al.*, 2007; Yeoh *et al.*, 2007).

1.10.2 Erythrocyte invasion is a multistep process

The cellular steps of erythrocyte invasion have been well studied by microscopy (Dvorak *et al.*, 1975; Gilson and Crabb, 2009). Mature merozoites are released from the bursting schizont, and disperse among surrounding red blood cells (Harvey *et al.*, 2012). Merozoites then contact, probably at random, uninfected erythrocytes and loosely adhere on their cell surface (Cowman and Crabb, 2006) (Fig 1.5A). Primary merozoite contact is associated with an obvious warping of erythrocyte cell membrane during which, the merozoite reorients itself to orient its apical end in direct apposition with the erythrocyte cell membrane (Gilson and Crabb, 2009). Merozoite reorientation is followed by the establishment of additional protein-protein contacts between the two juxtaposed cell membranes, probably committing the merozoite to invasion (Riglar *et al.*, 2011; Cowman *et al.*, 2012; Harvey *et al.*, 2012). These contacts are followed by the formation of a so called tight or moving junction (section 1.10.5) which is finally transformed into to a migrating ring that moves towards the basal end of merozoite, encircling merozoite within the erythrocyte (Aikawa *et al.*, 1978) (Fig 1.5).

1.10.3 The initial attachment to erythrocytes is primarily mediated by proteins displayed on the merozoite cell surface

Once released from the erythrocyte, the merozoite surface is observed to be covered by a coat of proteins (Fig 1.4). These proteins are synthesised and transported to the merozoite cell surface during merozoite maturation within the

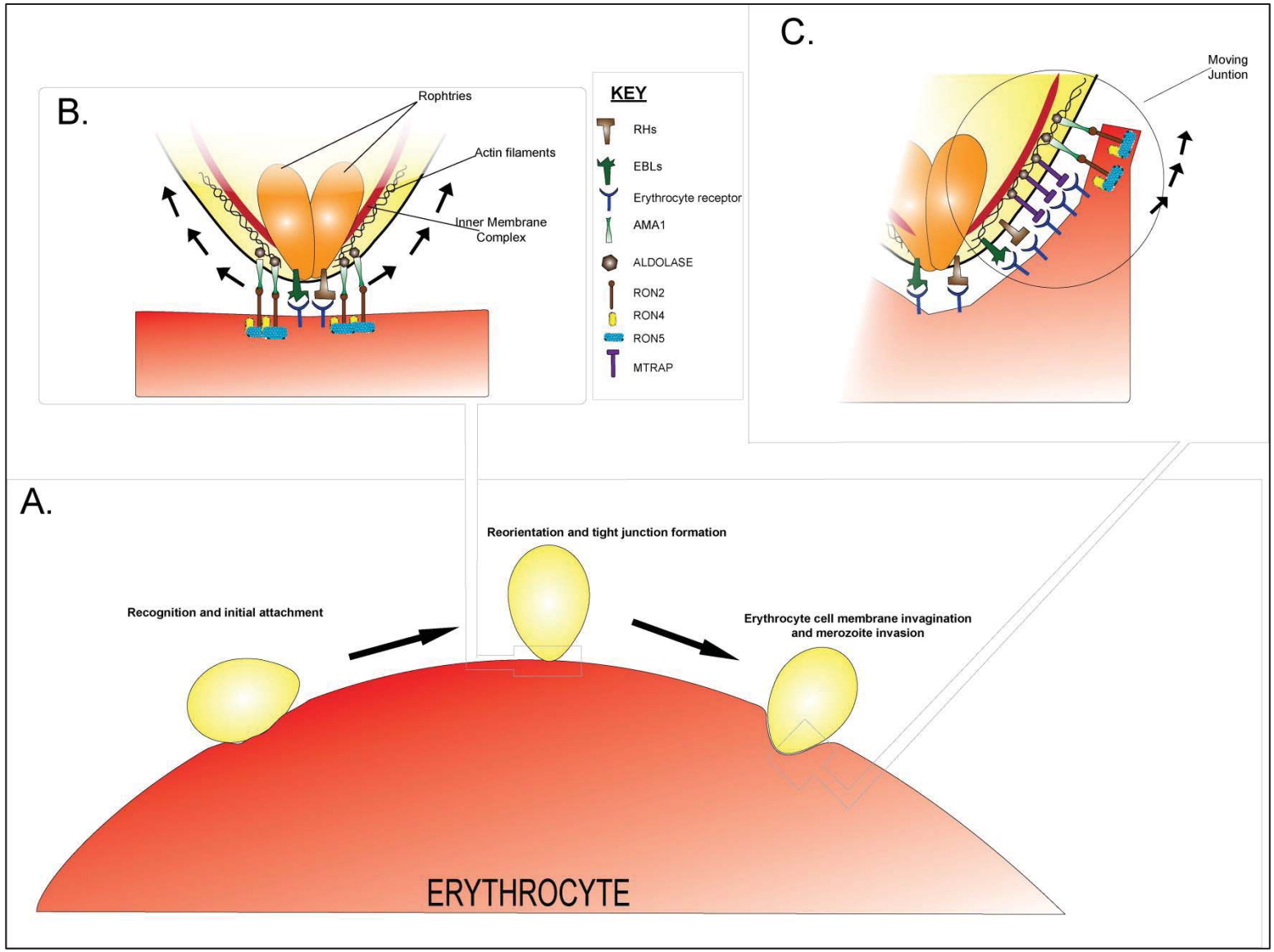

B.
Rophtries
Actin filaments
Inner Membrane Complex
KEY
RHs
EBLs
Erythrocyte receptor
AMA1
ALDOLASE
RON2
RON4
RON5
MTRAP
C.
Moving Juntion
A.
Reorientation and tight junction formation
Recognition and initial attachment
Erythrocyte cell membrane invagination and merozoite invasion
ERYTHROCYTE

Figure 1.5 Erythrocyte invasion is a multistep process.

A. **Sequence of events leading to erythrocyte invasion**. First, merozoite recognises and loosely adheres to erythrocyte cell surface. Then, it reorients itself, putting its apical end facing the erythrocyte cell membrane. At this point a so called tight or moving junction is formed. During erythrocyte invasion, the tight junction starts moving towards the basal end of the merozoite, encircling merozoite within the erythrocyte.

B. **The formation of the tight junction**. The RON complex (RON2, RON4 and RON5) is embedded into erythrocyte's plasma membrane, marking the initiation of an irreversible series of events which end by the completion of erythrocyte invasion. Amongst RON complex proteins, RON2 is thought to directly interact with the microneme protein AMA-1 which is secreted on parasite's cell surface (Besteiro *et al.*, 2011). There is some evidence that AMA-1 cytoplasmic region is bound to aldolase which in turn is associated with parasite's actin myosin-motor (see text). Around the time of the tight junction formation, members of the reticulocyte binding protein homologues (RHs) and erythrocyte binding ligands (EBLs) families of proteins are secreted on parasite's cell surface, and bind to specific erythrocyte receptors.

C. **Moving junction migration**. During the invasion process, the moving junction migrates from the apical to the basal end of the merozoite (direction of arrows; also see *B.*). MTRAP is thought to bridge the gap between parasite actin-myosin motor and host erythrocyte plasma membrane: its cytoplasmic tail binds to aldolase which is associated with parasite's actin-myosin motor, and MTRAP ectodomain binds to receptors on erythrocyte cell surface.

schizont and are thought to mediate the initial attachment of the merozoites to erythrocytes (Garcia *et al.*, 2008). Studies have demonstrated that more than 20 proteins are associated with the merozoite cell surface prior to egress (Sanders et al., 2005; Cowman and Crabb, 2006; Cowman et al., 2012). These are divided into proteins that are directly anchored to the merozoite plasma membrane (usually through a Glycophosphatidylinositol (GPI anchor)) and peripheral proteins which are associated by interactions with surface-tethered proteins (Sanders *et al.*, 2005; Cowman *et al.*, 2012).

Several merozoite cell surface proteins contain domains that have been implicated in mediating protein–protein interactions. These include Duffy binding–like (DBL) or erythrocyte binding–like (EBL) domains (carried by MSPDBL-1 and MSPDBL-2; Wickramarachchi *et al.*, 2009; Hodder *et al.*, 2012; Sakamoto *et al.*, 2012) that are specific to *Plasmodium* spp. and are present in many proteins with diverse functions, ranging from erythrocyte invasion (section 1.10.4) to erythrocyte remodelling and cytoadherence (e.g. *Pf*EMP-1; Cowman *et al.*, 2012). Other proteins which carry six-cysteine (6-cys) domains (e.g. Pf12, Pf38, Pf41) are again likely to be involved in protein–protein interactions (Cowman *et al.*, 2012).

Structural studies have demonstrated that the structure of the 6-cys proteins is similar to that of the surface antigen (SAG) related sequence (SRS) superfamily found in *Toxoplasma gondii*, a parasite which, like *Plasmodium,* belongs to the Apicomplexa phylum (Gerloff *et al.*, 2005). The cysteine rich domains in the prototype protein *Tg*SAG1, dimerise to form a receptor binding site at the tip of the molecule (Cowman *et al.*, 2012). Initial experiments suggested that the structural similarity between the *P. falciparum* 6-cys proteins and the *Tg*SAG1 is very unlikely to extend to their molecular functions (Gerloff *et al.*, 2005). More recent studies demonstrated that at least two members of *P. falciparum* 6-cys family of proteins, P12 and P41, are able to form a heterodimer on the infective merozoite surface, but the complex ,however, does not appear to have a major role in erythrocyte invasion (Taechalertpaisarn *et al.*, 2012). Another protein motif that is carried by proteins associated with merozoite cell surface is the EGF-like domain, which has also been implicated in protein-protein binding (Cowman and Crabb, 2006).

1.10.3.1 Merozoite Surface Proteins (MSPs) are important components of merozoite cell surface

Proteins belonging to the Merozoite Surface Protein (MSP) family (e.g. MSP-1, MSP-2, MSP-3, MSP-6, MSP-7, MSP-9, MSP-10) have been shown to be components of the protein coat that covers the mature merozoite (Sanders *et al.*, 2005). Among all the merozoite cell surface proteins, MSP-1 is most likely to be the most abundant (Cowman *et al.*, 2012). The *msp-1* gene is refractory to genetic deletion suggesting that it is essential for blood stage growth. Structurally, MSP-1 is a GPI-anchored, 195 kDa protein which translocates to the merozoite cell surface during schizogony (Koussis *et al.*, 2009; Kadekoppala and Holder, 2010). Just prior to erythrocyte egress, a serine protease, *Pf*SUB1, is discharged from the exonemes (Yeoh *et al.*, 2007) (section 1.10.1) into the parasitophorous vacuole (PV) and processes the 195kDa MSP-1 precursor into four fragments: an N-terminus MSP-1_{83}, two central fragments MSP-1_{30} and MSP-1_{38}, and a C-terminal MSP-1_{42} , all remain associated with each other (Koussis *et al.*, 2009; Kadekoppala and Holder, 2010). Two polypeptides (MSP-6_{36} and MSP-7_{22}), derived from proteolytic processing, by *Pf*SUB1, of another two merozoite surface proteins, MSP-6 and MSP-7, are non-covalently associated with the four MSP-1 fragments to form the MSP1/6/7 complex (Koussis *et al.*, 2009). The latter protein complex is attached to the merozoite cell surface via the GPI-anchor of MSP-1_{42}, and is likely to be implicated in the primary attachment of the merozoite to the erythrocyte cell surface (Perkins and Rocco, 1988; Nikodem and Davidson, 2000; Kadekoppala *et al.*, 2008; Koussis *et al.*, 2009; Boyle *et al.*, 2010; Kadekoppala and Holder, 2010). Interestingly, Band 3 has been proposed to be the host erythrocyte receptor for MSP-1 (Goel *et al.*, 2003); however, this remains controversial and antibodies against Rhesus Band 3 did not inhibit *P. knowlesi* merozoite initial binding to erythrocytes, but they did abolish erythrocyte invasion (Miller *et al.*, 1983).

During the invasion process, and while the moving junction migrates towards the basal end of the merozoite (section 10.1.5), the proteinaceous coat that covers the merozoite cell surface is shed into the supernatant by the activity of proteases (Harvey *et al.*, 2012). As part of the merozoite protein coat, the MSP1/6/7 complex is also shed by *Pf*SUB2 cleavage (Harris *et al.*, 2005). *Pf*SUB2 cleaves MSP-1_{42} into two fragments: an N-terminal 33kDa (MSP-1_{33}) fragment, which is released into the supernatant together with the other components of the MSP1/6/7 complex, and a 19kDa peptide (MSP-1_{19}) that remains bound to the merozoite cell surface

(Kadekoppala and Holder, 2010). MSP-1_{19} contains two epidermal growth factor (EGF)-like domains and following invasion is transferred to the developing food vacuole, where it remains until the end of the next intracellular cycle. MSP-1_{19} is the first marker of the biogenesis of the food vacuole and it is likely that the protein has an important role in this location (Blackman *et al.*, 1994; Kadekoppala and Holder, 2010). Antibodies that inhibit the processing of MSP-1_{42} by *Pf*SUB2 have been shown to prevent erythrocyte invasion (Blackman *et al.*, 1994).

1.10.3.2 Members of the Serine Rich Antigen (SERA) family of proteins are exposed on the merozoite cell surface

Besides MSPs, members of the SERA family of proteins are also components of merozoite cell surface. SERA is a protein family which consists of nine members (SERA1-9; Blackman, 2008). All SERAs were found to carry a centrally conserved papain-like domain (Miller *et al.*, 2002b). Interestingly, SERA1–5 and 9 have a cysteine to serine substitution within the active site (Miller *et al.*, 2002b). Among SERA family members, SERA5 and SERA6 are the most abundant during parasite blood stages and the genes encoding for both these proteins cannot be disrupted, suggesting that they are essential in this lifecycle stage (Miller *et al.*, 2002b; McCoubrie *et al.*, 2007). SERA5 is translocated in the PV as a 126kDa precursor, and similar to MSP-1, is processed by *Pf*SUB1, into an N-terminal 47kDa fragment (P47), a central 56kDa polypeptide which carries the papain-like domain (P56), and a small 18kDa C-terminal fragment (P18) (Yeoh *et al.*, 2007; Blackman, 2008). P56 is further processed by a C-terminal truncation to result a P50 peptide which eventually accumulates in culture supernatants. On the contrary, the N- and C-terminal fragments, P47 and P18, remain associated in via disulfide bonds and appear to bind to the surface of the released merozoites. Intriguingly, antibodies against P47 but not against P50, block erythrocyte invasion (Blackman, 2008).

SERA6 is also released in PV and is processed by *Pf*SUB1 (Yeoh *et al.*, 2007). Cleavage of SERA6 by *Pf*SUB1 converts it to an active cysteine protease (Ruecker *et al.*, 2012). Intriguingly, pharmacological blockade of *Pf*SUB1, inhibits egress and ablates the invasive capacity of released merozoites (Yeoh *et al.*, 2007). Moreover, mutations that replace the predicted catalytic cysteine of SERA6 could not be stably introduced into the parasite SERA6 encoding gene, indicating that SERA6 is an essential enzyme (Ruecker *et al.*, 2012).

1.10.4 EBL and RH are two protein families with major roles in erythrocyte invasion

Erythrocyte Binding Ligands (EBLs) and Reticulocyte binding-protein Homologues (RHs) are two protein families which play a pivotal role in erythrocyte invasion (Iyer *et al.*, 2007) (Fig. 1.6). The *Pf*EBL family is comprised of five proteins, EBA-175, EBA-140 (BAEBL), EBA-181 (JESEBL), EBL1 and EBA-165 (PAEBL) (Iyer *et al.*, 2007). However, only three *Pf*EBL proteins are likely expressed in most *P. falciparum* strains. In the 3D7 reference genome *eba-165* is a pseudogene, containing two frameshift mutations which result in premature termination of protein translation (Triglia *et al.*, 2001; Rayner *et al.*, 2004; Stubbs *et al.*, 2005). Similarly, *ebl-1* is likely to be a pseudogene in a number of *P. falciparum* strains (Drummond and Peterson, 2005; Githui *et al.*, 2010), although an erythrocyte receptor for a truncated form of EBL-1 has been identified (see below; Mayer *et al.*, 2009). EBLs are stored in the micronemes from where they are then released at the apical merozoite cell surface. Structurally, EBLs are type I transmembrane proteins (Fig.1.6), divided into six regions. Region II (RII) carries two tandem cysteine rich DBL domains (F1 and F2; Fig. 1.6), which are responsible for receptor binding. Region III-V links RII to RVI, which also consists of a small cysteine-rich domain (Tham *et al.*, 2012).

The *Pf*RH family consists of six proteins, RH1, RH2a, RH2b, RH3, RH4 and RH5 (Fig 1.6) (Tham *et al.*, 2012). RH2a and RH2b are identical over 80% of the protein sequence, differing only at the C-terminus (Tham *et al.*, 2012). With the exception of RH5 (section 1.10.6.1) which lacks a transmembrane domain, all *Pf*RH members are predicted to be type I transmembrane proteins, localised in the rhoptry neck from where they are secreted at the merozoite apex, during invasion (Tham *et al.*, 2012). Similar to *ebl-1* and *eba-165, rh3* is transcribed but it appears not to be translated, probably due to two reading frameshifts at the 5' end of the gene (Taylor *et al.*, 2001).

As mentioned in section 1.10.1, the exact sequence of the molecular events mediating erythrocyte invasion, is not yet completely clear. The EBLs and RHs are thought to bind to specific erythrocyte receptors at some point between the initial merozoite attachment and the formation of the tight junction (Harvey *et al.*, 2012). A model suggests that upon schizont rupture, merozoites are exposed to the low

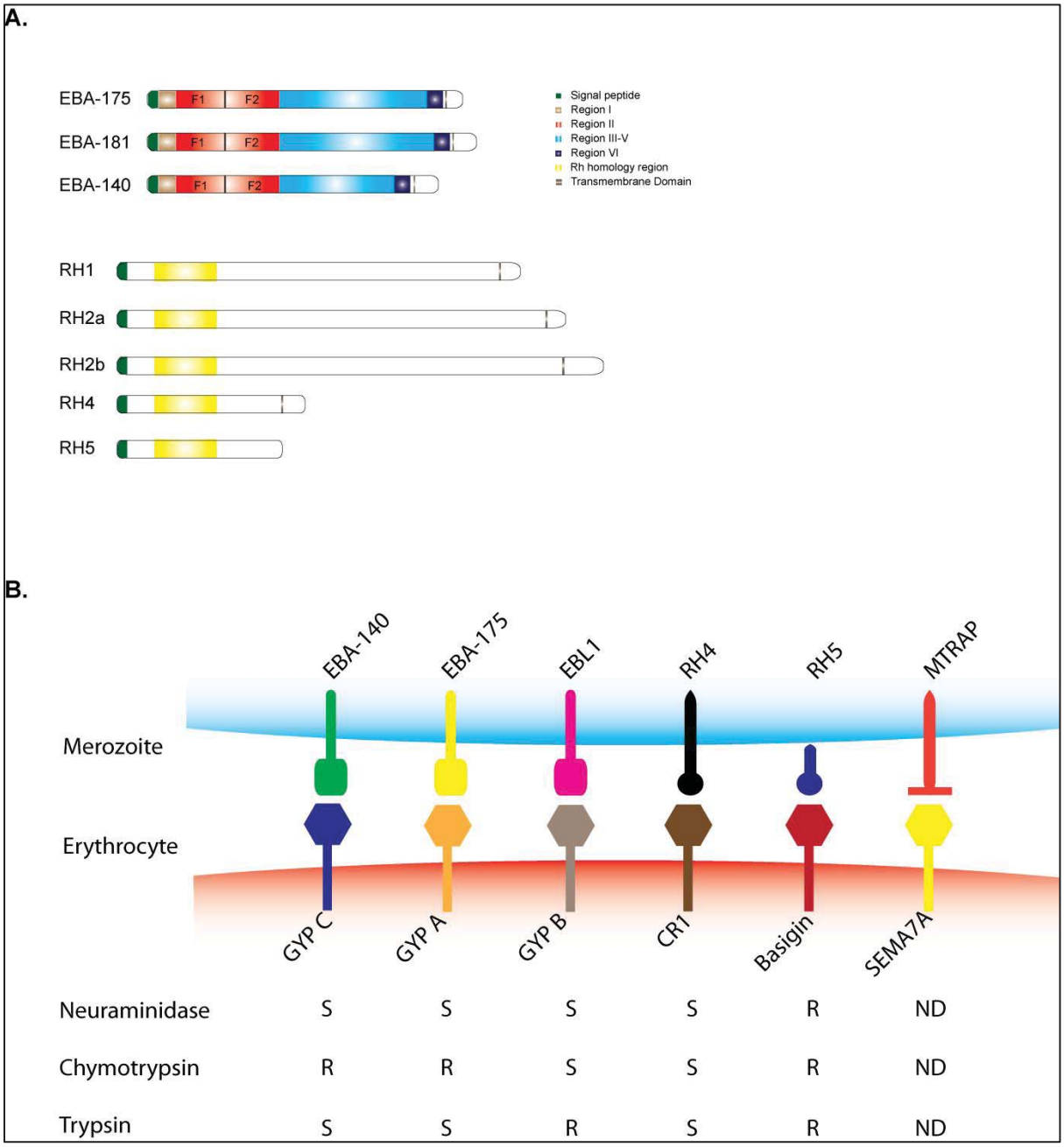


Figure 1.6 Cell surface Proteins involved in erythrocyte invasion.

A. A schematic representation of the structure of Erythrocyte Binding Ligands (EBLs) (top) and Reticulocyte binding Homologues (RHs) (bottom) families of invasion ligands. Apart from RH5 which is secreted, all the members of both protein families are type I integral membrane proteins. EBL Region II is cysteine rich, and contains 2 Duffy Binding Like domains (F1 and F2) which are known to mediate binding of EBLs to erythrocytes. Region VI is also cysteine rich and it was suggested to control the subcellular localisation of the protein prior to secretion (Culleton and Kaneko, 2010). The region of homology between RHs is indicated in yellow.

B. Known receptor-ligand pairs between merozoite and erythrocytes, during erythrocyte invasion. Abbreviations: GYPA, Glycophorin A; GYPB, Glycophorin B; GYPC, Glycophorin C; SEMA7A, Semaphorin7A; CR1, Complement Receptor 1; S, Sensitive; R, Resistant; ND, Not Determined.

potassium levels of the plasma, which triggers calcium release from the endoplasmic reticulum (Singh *et al.*, 2010). The rise in cytosolic calcium induces secretion of microneme contents onto the merozoite cell surface (Singh *et al.*, 2010). Following the primary contact of the merozoite with the erythrocyte, EBLs and RHs are engaged with their receptors, likely committing the merozoite to invasion by activating subsequent events which lead to erythrocyte entry (Cowman *et al.*, 2012; Harvey *et al.*, 2012) .

The erythrocyte receptors for EBA-175, EBL-1, EBA-140, RH4 and RH5 have been identified to be Glycophorin A (GYPA; Sim *et al.*, 1994), GYPB (Mayer *et al.*, 2009), GYPC (Lobo *et al.*, 2003; Maier *et al.*, 2003), Complement Receptor 1 (CR1;Tham *et al.*, 2010) and Basigin respectively (Crosnier *et al.*, 2011) (Fig. 1.6). EBA-181 binds to an unknown sialylated, trypsin-resistant red blood cell receptor which is cleaved after chymotrypsin treatment (Gilberger *et al.*, 2003). RH2a and RH2b appear to bind a yet unidentified trypsin-resistant, chymotrypsin- and neuraminidase-sensitive cell surface receptor (Triglia *et al.*, 2011). On the other hand, RH1 binds to a trypsin resistant receptor, in a sialic acid dependent manner (Rayner *et al.*, 2001).

1.10.4.1 EBLs and RHs mediate alternative invasion pathways

Several studies have highlighted that there is a remarkable functional redundancy between EBLs and RHs across different *P. falciparum* strains, but also within the same parasite strain (Tham *et al.*, 2012). EBLs and RHs have been shown to define a series of alternative invasion pathways where distinct ligand-receptor combinations can operate independently to mediate invasion with equal efficiency (Harvey *et al.*, 2012). For example, genetic disruption of *eba-175* in the W2mef strain - which primarily invades erythrocytes in a sialic acid dependent manner - results in up-regulation of the normally silenced *rh4*. As a result, the parasite switches to a sialic acid independent invasion pathway, concomitantly suggesting that the parasite is capable of accessing different invasion pathways by using different invasion ligands (Stubbs *et al.*, 2005).

Moreover, the targeted deletion of *rh2a* and *rh2b* in the 3D7 parasite line resulted in no detectable reduction in erythrocyte invasion efficiency (Duraisingh *et al.*, 2003). Nevertheless, 3D7 parasite lines lacking RH2b expression invaded both

neuraminidase- and trypsin-treated erythrocytes at significantly lower efficiency than the 3D7 parental line, suggesting a switch in invasion pathway. No alternation in the invasion phenotype was observed for 3D7Δ*rh2a* (Duraisingh *et al.*, 2003). Interestingly, the expression pattern of RH2b varies significantly among *P. falciparum* parasite strains with some strains completely lacking expression. The latter suggests that RH2b is of different functional significance among *P. falciparum* strains (Duraisingh *et al.*, 2003).

Similarly, experiments show that RH1 presents a differential expression pattern among different parasite strains (Triglia *et al.*, 2005). Gene deletion of *rh1* in Tak994 *P. falciparum* line, resulted in an unchanged growth rate (Triglia *et al.*, 2005). However, T994Δ*rh1* parasites demonstrated increased ability to invade both trypsin and neuraminidase-treated erythrocytes, reflecting a shift in these parasites towards the utilization of receptors that are more neuraminidase- and trypsin-resistant compared to the Tak994 parent line (Triglia *et al.*, 2005).

Other studies suggested that polymorphisms which result in amino acid changes in the protein sequence may represent another mechanism through which the parasite accesses alternative invasion pathways. Polymorphisms within the receptor-binding regions of EBA-140 and EBA-181 changed the erythrocyte binding profile of these proteins in a way that suggested an alteration in erythrocyte receptor binding specificity (Mayer *et al.*, 2002, 2004). However, later studies argued that these polymorphisms affected the affinity of EBA-140 and EBA-181 binding to receptors, but not the receptor specificities of these proteins (Maier *et al.*, 2009).

Individual EBLs and RHs appear to have overlapping functions which renders them individually dispensible for erythrocyte invasion. Other studies, however, have underscored the collective indispensability of EBLs and RHs, and highlight the requirement for a minimal complement of these proteins, necessary for erythrocyte invasion in each parasite strain (Harvey *et al.*, 2012; Tham *et al.*, 2012). For example, whereas *eba-181* and *rh4* can be deleted in the W2mef strain, genetic disruption of the same genes cannot be achieved in 3D7 suggesting that, at least in the 3D7 strain, these genes are essentially required for erythrocyte invasion (Gilberger *et al.*, 2003; Stubbs *et al.*, 2005). Furthermore, addition of anti-RH2b antibodies into parasite growth assays demonstrated a higher growth inhibitory effect in parasite lines in which different *ebl* genes were disrupted, suggesting that RH2b

complements the function of EBLs (Lopaticki *et al.*, 2011). In support of the notion of synergy between the EBLs and RHs, a combination of anti-EBA-175, RH2a/b, and RH4 antibodies were much more efficient in erythrocyte invasion, than individual antibodies alone (Lopaticki *et al.*, 2011).

Taken together, the functional redundancy between EBLs and RHs is believed to enable the parasite to switch between different invasion pathways, and enables different strains of *P. falciparum* to invade using different host receptors (Cowman and Crabb, 2006; Harvey et al., 2012). This phenotypic variation of invasion pathways has major implications for the parasite survival within the human host. It most likely guarantees that the parasite will gain access to erythrocytes even in the case where the availability of certain erythrocyte receptors is limited (Cowman and Crabb, 2006). For example, the expression of GYPA – the host receptor of EBA-175 - is significantly reduced in aged erythrocytes (Sparrow *et al.*, 2006). Moreover, this phenotypic variation provides the parasite with a mechanism to evade host humoral immune responses targeting a subset of ligands that would block invasion, and with the means to counteract the extremely polymorphic nature of the erythrocyte surface receptors in terms of their primary sequence and levels of expression (Tham *et al.*, 2012).

1.10.5 The formation of the tight junction and the role of AMA1, MTRAP and RON proteins in mediating erythrocyte invasion

Once the apex of the merozoite is apposed directly towards the erythrocyte membrane, the discharge of rhoptry neck contents is triggered (Richard *et al.*, 2010; Riglar *et al.*, 2011; Cowman *et al.*, 2012). Among the proteins secreted from the rhoptry are three rhoptry neck proteins (RONs), RON2, RON4 and RON5. These three proteins appear to form a complex that is embedded in the erythrocyte plasma membrane (Fig. 1.5) (Richard *et al.*, 2010; Srinivasan *et al.*, 2011; Harvey *et al.*, 2012). RON4 and RON5 are predicted to be peripheral proteins located entirely within the erythrocyte cytosol, whereas RON2 localises to the erythrocyte membrane such that an exposed C-terminal loop protrudes to the extracellular space (Srinivasan *et al.*, 2011; Harvey *et al.*, 2012). The extracellular part of RON2 directly interacts with the Apical Membrane Antigen 1 (AMA1), a micronemal protein which - together with other microneme proteins - is secreted on merozoite apical cell surface following merozoite egress (Srinivasan *et al.*, 2011). This remarkable process where

the parasite provides both the receptor and ligand leads to the formation of the tight junction (Fig. 1.5), that the nexus bridging both host and parasite cell membranes (Farrow *et al.*, 2011) and appears as an electron dense thickening (by electron microscopy) below the erythrocyte membrane (Aikawa *et al.*, 1978).

AMA1 is a key component of the tight junction and is essential for parasite invasion. It is synthesised during schizogony as an 83kDa precursor and is proteolytically cleaved within the micronemes to yield a 66kDa protein. It is a type I integral membrane protein with a large extracellular domain and a short cytoplasmic tail (Harvey *et al.*, 2012). Similar to MSP-1, AMA-1 is processed by *Pf*SUB2 during the late stages of erythrocyte invasion resulting into two alternative fragments, 44kDa and 48kDa, which are released into the supernatant (Howell *et al.*, 2001; Harris *et al.*, 2005).

The engagement of AMA-1 with RON2 appears to initiate downstream signalling cascades that eventually result in the release of the rhoptry bulb contents (Riglar *et al.*, 2011; Srinivasan *et al.*, 2011; Cowman *et al.*, 2012). The cytoplasmic domain of AMA1 is not required for correct trafficking and surface translocation, but is essential for AMA1 function (Treeck *et al.*, 2009). Indeed, the cytoplasmic tail *of P. falciparum* AMA1 is phosphorylated at serine 610. It was suggested that the enzyme responsible for serine 610 phosphorylation is the cAMP regulated protein kinase A (*Pf*PKA). Importantly, mutation of AMA1 serine 610 to alanine abrogates phosphorylation of AMA1 *in vivo* and impedes erythrocyte invasion (Leykauf *et al.*, 2010).

Following the binding of AMA1 to RON2, the tight junction is transformed into a circumferential ring of contacts between the erythrocyte and merozoite, which migrates towards the basal end of merozoite (Aikawa *et al.*, 1978) (Fig. 1.5). The migrating ring is called moving junction and is thought to be powered by an actin myosin motor. Studies show that the cytoplasmic tail domain (CTD) of AMA-1 binds to the actin binding protein aldolase *in vitro* (Fig. 1.5) and thus, likely links parasite surface ligands with the intracellular actin–myosin motor (Srinivasan *et al.*, 2011). Phosphorylation of serine 610 does not appear to be required for aldolase binding (Srinivasan *et al.*, 2011). Other studies, however, report that the localisation RON4, which is also part of the moving junction, does not overlap with the localisation of actin filaments, suggesting that other proteins – besides the already known

components of the moving junction - are involved in the engagement of the actin-myosin motor with the moving junction (Angrisano *et al.*, 2012).

Merozoite Thrombospondin-Related Anonymous Protein (MTRAP) is another micronemal protein which is thought to be an important constituent of the moving junction (Baum *et al.*, 2006). Similar to AMA-1, MTRAP has been proposed to be involved in bridging the gap between parasite ligands and the actin-myosin motor (Baum *et al.*, 2006; Morahan *et al.*, 2008). Indeed, the MTRAP CTD has been shown to bind to aldolase *in vitro* and this interaction was reduced when a conserved tryptophan residue was mutated to alanine (Baum *et al.*, 2006). A receptor for MTRAP has recently been recently identified to be Semaphorin7A (Bartholdson *et al.*, 2012) (Fig. 1.6). Despite MTRAP being essential for erythrocyte invasion, attempts to block the interaction between MTRAP and Semaphorin7A during *in vitro* invasion assays, using recombinant proteins and antibodies, showed no significant inhibitory effect and thus, the functional relevance of this interaction is still unclear (Bartholdson *et al.*, 2012).

1.10.6 The interaction between RH5 and Basigin is essential and universally required for erythrocyte invasion

RH5 is exceptional across EBLs and RHs, since it is indispensable for erythrocyte invasion in all *P. falciparum* strains tested to date (Douglas *et al.*, 2011; Bustamante *et al.*, 2013). Basigin has been identified as the erythrocyte receptor for RH5 (Crosnier *et al.*, 2011) and antibodies against either RH5 or Basigin can potently block erythrocyte invasion in *P. falciparum* parasite culture (Crosnier *et al.*, 2011; Douglas *et al.*, 2011; Bustamante *et al.*, 2013)

1.10.6.1 RH5 is a merozoite ligand, required for erythrocyte invasion

RH5 is structurally different from the other RH family members, as it is much smaller and lacks an obvious transmembrane and cytoplasmic domain and is therefore predicted to be secreted (Fig. 1.6). Unlike the redundancy displayed by the EBLs and RHs, *rh5* is broadly refractory to genetic deletion (Hayton *et al.*, 2008; Baum *et al.*, 2009), suggesting that is essential for parasite survival during the blood stages. RH5 is expressed as a 63kDa protein and in mature merozoites it localises to the rhoptry body (Rodriguez *et al.*, 2008; Baum *et al.*, 2009). During erythrocyte invasion, RH5 is located at the tight junction (Baum *et al.*, 2009). Interestingly,

polymorphisms in RH5 have been implicated in the determination of species-specific pathways of *P. falciparum* erythrocyte invasion (Hayton *et al.*, 2008).

A recent study identified a binding partner for RH5, the RH5-interacting protein (RIPR), which is localised in the micronemes of mature merozoites (Chen *et al.*, 2011). RIPR complexes with RH5 on the surface of the free merozoite, and the complex is located at the leading edge of the moving junction during erythrocyte invasion. Similar to *rh5*, *ripr* is refractory to genetic disruption and polyclonal antibodies raised against RIPR inhibit erythrocyte invasion consistent with an essential role in this process (Chen *et al.*, 2011). Nevertheless, no direct binding of RIPR to erythrocytes could be detected and thus, the precise functional role of the RH5/RIPR complex remains elusive (Chen *et al.*, 2011). Interestingly, RIPR also lacks a transmembrane domain (Chen *et al.*, 2011) suggesting that additional proteins are likely to be required if RH5 and RIPR function at the merozoite surface.

1.10.6.2 Basigin is a multifunctional transmembrane glycoprotein

Basigin, "*bas*ic *i*mmuno*g*lobul*in* superfamily" (BSG in human; Bsg in mouse), is a widely expressed transmembrane glycoprotein belonging to the immunoglobulin superfamily (Muramatsu and Miyauchi, 2003; Liao *et al.*, 2011a). Studies showed that Basigin is likely to become differentially glycosylated in different tissues (Kanekura *et al.*, 1991; Fadool and Linser, 1993). Four protein isoforms (Basigin-1, -2, -3, -4) derived from alternative splicing and the use of an alternative promoter, have been described (Fig 1.7). Basigin-2, which carries two immunoglobulin-like domains, is thought to be the major isoform expressed on erythrocytes and corresponds to the Ok^a blood group antigen (Crosnier *et al.*, 2011).

1.10.6.2.1 Physiological functions of Basigin

Basigin has been assigned with a number of physiological functions. *Bsg* null mouse embryos developed normally during pre-implantation stages but the majority of mutant embryos died around the time of implantation (Igakura *et al.*, 1998). Bsg is strongly expressed in both the embryo trophectoderm and the uterine endometrium, suggesting that Bsg is likely to be involved in intercellular recognition processes required for implantation (Igakura *et al.*, 1998; Muramatsu and Miyauchi, 2003). Male *bsg* deficient mice are sterile due to an arrest of spermatogenesis at the metaphase of the first meiosis. Female mice lacking Bsg expression are also sterile, probably

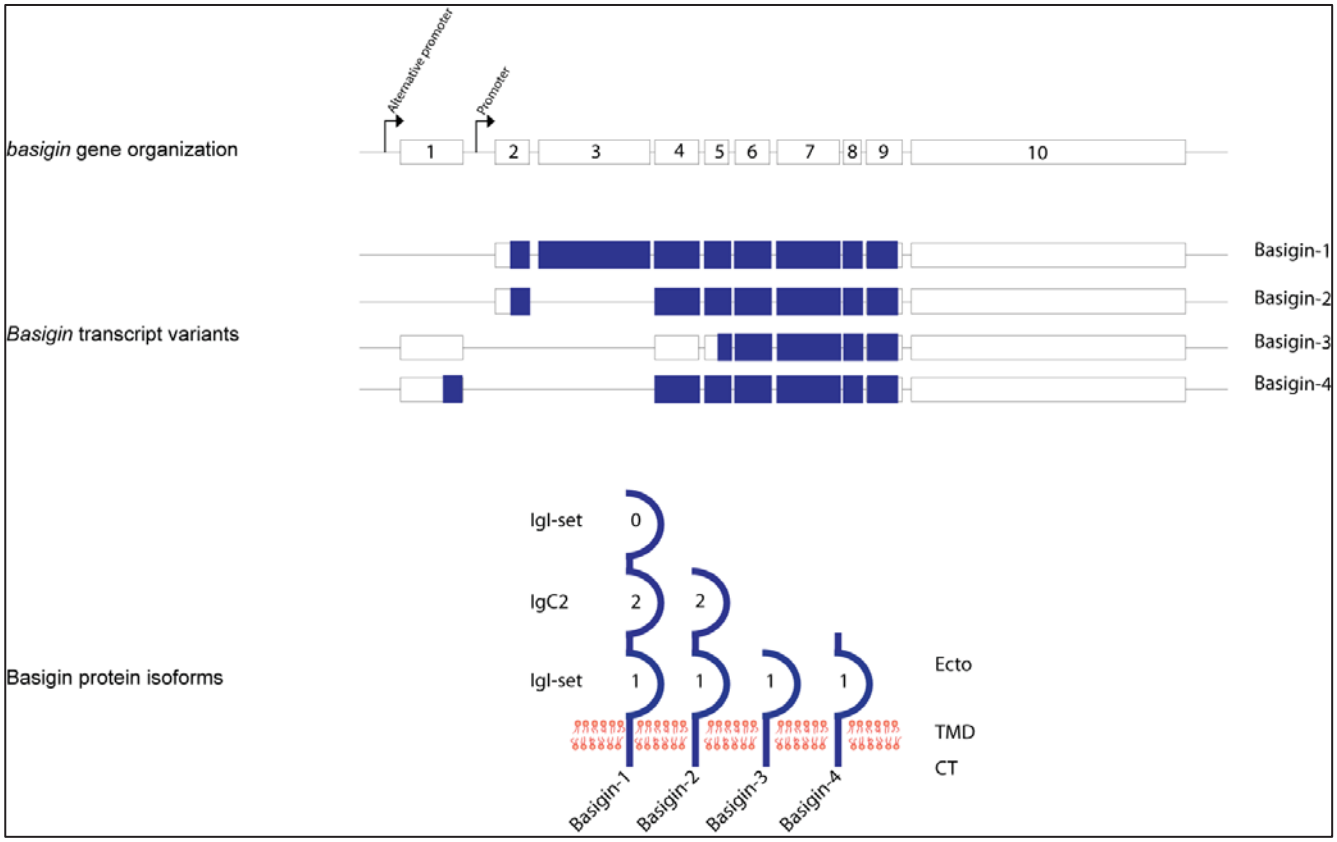


Figure 1.7 Structure of Basigin gene and protein isoforms. The top a diagram shows the *basigin* gene organization in human. The gene consists of 10 exons (1-10) and is regulated by 2 promoters. Alternative splicing events and the use of an alternative promoter, result into transcript variants which are translated into the four Basigin isoforms which are shown at the bottom of the figure. Zero and 1 are IgI-set domains whereas domain 2 is an Ig-C2 domain. Blue shaded boxes represent coding sequence. White boxes represent non-coding sequence. Abbreviations: Ecto, Ectodomain; TMD, Transmembrane Domain; CT, Cytoplasmic Tail.

due to defects in embryo implantation (Igakura *et al.*, 1998; Muramatsu and Miyauchi, 2003).

Bsg deficient mice also exhibit various disorders related to defects of the neural system. They have defects in learning, and in memory and water finding tasks, and are more sensitive to painful stimuli such as electric shock (Muramatsu and Miyauchi, 2003). *Bsg* null mice are also less sensitive to irritating odors and are virtually blind (Muramatsu and Miyauchi, 2003). The loss of vision has been associated with the function of Basigin as a molecular chaperone for monocarboxylate transporters (MCT) MCT1, MCT3 and MCT4 (Muramatsu and Miyauchi, 2003; Iacono *et al.*, 2007). The association of Basigin with MTCs facilitates the cell surface targeting of the MCTs thereby acting as a chaperone for correct localisation. In mice lacking Basigin expression, the cell surface localisation of MCT1, MCT3 and MCT4 is either lost or greatly reduced in both the retinal pigment epithelium and the retina (Muramatsu and Miyauchi, 2003). Of great importance is the loss of MCT1 and MCT4 at the surface of the Müller glial cells and the photoreceptor cells themselves. The loss of MCT activity blocks the transport of lactate from Müller glia to the photoreceptor cells, depriving them of a crucial energy source (Muramatsu and Miyauchi, 2003). Interactions of Basigin with Cyclophilin A, integrins and caveolin-1 have also been described (Muramatsu and Miyauchi, 2003; Iacono *et al.*, 2007).

The role of Basigin in human erythrocytes is not fully understood. MCT1 is also expressed in human erythrocytes where it functions as an L-lactate-protein transporter (Bartholdson *et al.*, 2013). Therefore, Basigin it is likely to act as chaperone, escorting MCT1 to erythrocyte plasma membrane. Moreover, studies demonstrated that blockade of Basigin by using an anti-Basigin $F(ab)_2$ fragment, interferes with the migration of erythrocytes out of the spleen (Coste, 2001). Thus, erythrocyte Basigin is likely act as an adhesion molecule, which plays a critical role in the recirculation of mature erythrocytes from the spleen into the blood stream (Coste, 2001).

1.10.6.2.2 Basigin based anti-cancer therapies

Basigin has attracted a great deal of interest as a cancer biomarker (Weidle *et al.*, 2010). It has been shown that Basigin is shed from tumor cells and stimulates

matrix metalloprotease (MMPs) synthesis and release in peritumorial cells, and the tumor cells themselves (Gabison *et al.*, 2005; Iacono *et al.*, 2007). Homophilic interactions between secreted and membrane tethered Basigin have been proposed to be important for the induction of MMP expression and secretion (Belton *et al.*, 2008). Various studies showed that Basigin is capable of inducing expression of MMP1-3, MMP9 and MMP11 (Muramatsu and Miyauchi, 2003; Iacono *et al.*, 2007), and thereby facilitates tumor invasion during metastasis. Apart from stimulating MMP expression in neighbouring cells, evidence suggests that Basigin induces its own expression through a positive feedback mechanism and also induces the production of vascular endothelial growth factor (VEGF) though which tumor metastasis is promoted (Iacono *et al.*, 2007).

The critical role of Basigin in tumor progression has led to the evaluation of anti-Basigin monoclonal antibodies as anti-cancer agents. In 2005, the Chinese Food and Drug Administration licenced the use of an anti-BSG iodine-131-labeled $F(ab)_2$ fragment under the brand name Licartin (also known as metuximab), for the treatment of patients suffering from hepatocellular carcinoma (HCC) (Yu *et al.*, 2008; He *et al.*, 2013). Licartin has been proved to be safe and effective for targeted treatment in clinical trials, and has been approved as a new radioimmunotherapeutical drug for clinical therapy of primary HCC (Yu *et al.*, 2008). This suggests that despite the fact that Basigin is broadly expressed on a number of cell types, anti-Basigin monoclonal antibodies do not have intrinsic safety issues when used as therapeutics.

1.10.6.2.3 The value of Basigin as a target for the treatment of Graft versus host disease

Besides erythrocytes, other circulating blood cells, like activated T and B lymphocytes, dendritic cells, monocytes and macrophage also express Basigin (Iacono *et al.*, 2007). Basigin was identified as an activation associated antigen on phytohemaglutinin (PHA) activated T cells (Koch *et al.*, 1999) and has been implicated in T cell chemotaxis at areas of inflammation (Iacono *et al.*, 2007). The important role of Basigin on activated T cells has been exploited for the development of therapeutics in the treatment of steroid-refractory acute graft-versus-host disease (GVHD).

GVHD is a T cell driven disease where donor lymphocytes recognise host tissues as foreign and initiate immune responses against them. As a result, acute GVHD is an important complication following hematopoietic stem cell transplantation. (Iacono *et al.*, 2007). Because Basigin is highly expressed on activated T cell populations, those cells that would mediate GVHD can be selectively depleted without affecting the entire T cell repertoire (Iacono *et al.*, 2007). Indeed, treatment of patients with steroid refractory, acute GVHD with an anti-Basigin antibody (a mouse IgM; known as CBL-1 or ABX-CBL) was shown to be very effective, in part due to decreased leukocyte activation (Heslop *et al.*, 1995; Deeg *et al.*, 2001; Macmillan *et al.*, 2007).

1.11 The importance of antibodies in malaria immunity

The role of antibodies in immunity against malaria became clear from the very early days. Initial studies which involved passive transfer of malaria immunity to animals and human provided solid evidence for the critical role of naturally occurring antibodies in protection against the blood stages of the disease. In 1937, Coggeshall and colleagues demonstrated that the transfer of serum from *Macacus rhesus* monkeys with chronic *P. knowlesi* infection to non-infected monkeys, confer partial or full protection against a subsequent parasite challenge, even after inoculation with large numbers of parasites (500 millions; Coggeshall & Kumm, 1937).

Later, in 1961, Cohen and colleagues were the first to recapitulate these experiments in man (Cohen *et al.*, 1961). Twelve Gambian children aged four months to 2.5 years old (weight 5.4-12.6 Kg) who had been diagnosed with clinical malaria were injected with gamma-globulin prepared from pooled serum obtained from apparently malaria immune adults living in Gambia, a malaria hyperendemic area. The total dose of immunoglobulin was 1.2-2.5 grams per child and it was administered over three days. After the initiation of gamma-globulin therapy, parasitemia levels dramatically decreased with concomitant disappearance of clinical symptoms. By the fourth day after inception of treatment, parasite levels dropped to less than 1% of the initial value and by day nine there was no detectable parasitemia in eight out of 12 cases. The gamma-globulin treatment appeared to be effective both against *P. falciparum* and *P. malariae* blood stage parasites but with no obvious action against gametocytes. Due to the absence of morphological abnormalities in the intra-erythrocytic forms of the parasite in peripheral blood, it was suggested that

the protective antibodies acted either on mature intracellular forms of the parasite or on merozoites liberated from infected erythrocytes (Cohen *et al.*, 1961).

Around the same time, Edozien and colleagues performed a similar study which involved the injection of gamma-globulin purified from peripheral or cord blood of malaria immune adult Nigerians, to six Nigerian children who suffered from *P. falciparum* or *P. malariae* malaria (Edozien *et al.*, 1962). The gamma-globulin was administered by intramuscular route at intervals of 8-24h for three days with a total dose of 1.2-2.8 grams per child. The group of children treated with adult gamma-globulin there was displayed a constant decrease in parasite-counts by the fourth day, and by day eight there was no detectable parasitemia in four out of five children. Concurrently, clinical symptoms were eliminated. Similar results were obtained in another case where a 12 months old child was treated with cord blood gamma-globulin. It was not however clear whether immune gamma-globulin is equally effective when administered to patients living in a different hyperedemic area, where possibly different *P. falciparum* strains are transmitted.

To address this question, McGregor and colleagues tested the therapeutic effect of West African immune gamma-globulin in malaria *P. falciparum* infected East African children (McGregor *et al.*, 1963). For this purpose gamma-globulin purified from pooled immune adult serum of healthy Gambia residents was administered via intramuscular route to nine children aged 6-20 months old who belonged to the M'bondei or M'samba tribes living in the area around Magila, Tanzania, and all showed clinical signs of malaria infection. Gamma-globulin therapy was associated with alleviation of pyrexia and clinical illness and by day four, the average parasite count was less than 1% of the mean initial count. However, in four cases parasite density relapsed on day seven to nine, probably representing rapid multiplication of a serologically distinct strain of *P. falciparum* which was resistant to antibody present in West African gamma-globulin. Collectively, these results suggested that gamma globulin from West African immune adults contained antibodies which, at least in part, are effective against *P. falciparum* malaria in people living in East Africa.

More recently, Sabchareon and colleagues used IgGs purified from immune serum obtained from 178 young African adults (age range 19-23) living in Cote d'Ivoire, which were then administered intravenously to eight *P. falciparum* infected Thai children, aged 5-12 years old, originated from Prachiburi province, and who

previously failed to be treated with quinine (Sabchareon *et al.*, 1991). While gametocyte counts fluctuated during the study, the asexual parasitemia fall significantly. In certain cases, parasitemia remitted by day nine to 12, but following a second round of gamma-globulin injection it decreased again, without however achieving a sterilizing effect. Nevertheless, these data established the critical role of antibodies in immunity against *P. falciparum* malaria, and simultaneously confirmed McGregor and colleagues' observations that the transferred antibodies conferred, at least in part, immunity against *P.falciparum* malaria to people living in different geographical region that the antibody donors.

1.11.1 Merozoite cell surface proteins are targets of protective antibodies of the naturally acquired malaria immunity.

The studies involving passive transfer of immunity clearly demonstrated that serum contained antibodies significantly contribute to protective immunity against malaria. However, the targets of protective antibodies had been unclear until Cohen and colleagues provided evidence that at least a fraction of antibodies is directed against erythrocyte invasion ligands (Cohen *et al.*, 1969). Cohen's group demonstrated that while immune serum antibodies had little effect on the *in vitro* intra-erythrocytic growth of *P. knowlesi* they severely affected re-invasion of red blood cells (Cohen *et al.*, 1969), suggesting that merozoite cell surface proteins are targets of protective antibodies.

Subsequent studies demonstrated that the presence of antibodies in serum directed against specific merozoite cell surface proteins is correlated with protection against severe disease. In 1993 Dziegiel and colleagues showed that asymptomatically infected Gambian children, aged five to eight years old, had significantly higher levels of IgG antibodies against the carboxy terminal domain of GLURP than clinically ill children of the same age, suggesting that antibodies targeting GLURP c-terminal domain may contribute to immunity against *P. falciparum* malaria (Dziegiel *et al.*, 1993). The role of GLURP as a target of protective immunity was also investigated by Dodoo and colleagues in two subsequent studies where they showed that the presence of IgGs targeting the non-repeat region of GLURP were significantly correlated with clinical protection from *P. falciparum* malaria in Ghanian children (Dodoo *et al.*, 2000, 2008). In the latest of Dodoo's studies, it was also reported that higher levels of IgG targeting $MSP1_{19}$,

MSP3 and IgM against $MSP1_{19}$, MSP3 and AMA1 were associated with decreased malaria incidence (Dodoo *et al.*, 2008).

In another report Conway and colleagues showed that serum IgG antibodies against MSP1 block 2 were strongly associated with protection from *P. falciparum* malaria in Gambian children (Conway *et al.*, 2000). Similarly, the presence of serum anti-RH5 antibodies has been shown to predict protection to malaria in Malian children (Tran *et al.*, 2013). Finally, a number of other studies have reported that the presence of circulating antibodies against the erythrocyte invasion ligands AMA1, MSP2, MSP3, EBA-175 and SERA5 is correlated with reduced malaria risk (Okech *et al.*, 2006; Fowkes *et al.*, 2010). Together, all the aforementioned publications clearly demonstrated that at least a fraction of malaria protective antibodies is directed against merozoite adhesins and invasins

1.12 Malaria prevention and control

Several intervention measures have been applied to control malaria**.** Tactics used to prevent the spread of disease, or to protect individuals from getting infected, include prophylactic drugs (e.g. quinacrine, chloroquine, primaquine), mosquito eradication by using insecticides, and prevention of mosquito bites by using insecticide-treated bed nets (ITNs) (Crompton *et al.*, 2010; Geels *et al.*, 2011; Greenwood and Targett, 2011; World Health Organization, 2012). Moreover, artemisinin-based combination therapy (ACT) is currently being used for malaria treatment (Miller *et al.*, 2013).

The general increases in funding during the last decade, enabled a wider application of anti-malarial intervention measures, which in turn resulted in a decrease in malaria deaths by 32%, between the years 2004-2010 (Murray *et al.*, 2012). However, there are still serious problems towards the goal of efficient malaria control worldwide. The emergence of parasite drug resistance is a major impediment for malaria control (Verdrager, 1986; Phyo *et al.*, 2012). Mosquito insecticide resistance is another important problem that is emerging (Riley and Stewart, 2013). From a financial point of view, the global funding requirement for malaria control was estimated by the Global Malaria Action Plan (GMAP) in 2008, to exceed US$ 5.1 billion annually between the years 2011 and 2020, with Africa alone needing US$ 2.3 billion each year. However, the total international and domestic resources directed

towards malaria control was estimated to be US$ 2.3 billion in 2011, less than half the total amount needed to reach the global goals (World Health Organization, 2012).

Taken all together, the development of a cost effective (US$ 1-2 per dose) (Moorthy *et al.*, 2012) and highly efficacious malaria vaccine is clearly imperative for the control, elimination, or even possible eradication of malaria (Crompton *et al.*, 2010).

1.13 The development of an effective malaria vaccine is a challenging problem

The development of a highly efficient malaria vaccine has been a long standing goal for the global control of malaria. The key fact underpinning the efforts for the development of a malaria vaccine is that adults in malaria endemic areas can naturally become immune to malaria after repeated exposure to the parasite (Marsh and Kinyanjui, 2006). This immunity affords protection against symptomatic disease, high-density parasitemia and death (Richards and Beeson, 2009). Nevertheless, even in adults who have had decades of exposure to *P. falciparum*, sterile immunity to blood-stage infection is infrequently observed, and an occasional episode of fever can occur (Crompton *et al.*, 2010). Infants and young children who have never been exposed to *P. falciparum* are more vulnerable in developing severe malaria and therefore, it is these age groups that particularly require the development of a vaccine that protects against malaria (Crompton *et al.*, 2010; Greenwood and Targett, 2011).

The completion of the *P. falciparum* genome project in 2002 (Gardner *et al.*, 2002) identified thousands of novel proteins, providing new opportunities for vaccine development. However, the function of the vast majority of these proteins is still unclear and therefore, targeting proteins essential for parasite survival becomes a challenging task (Richards and Beeson, 2009). Vaccine development is further complicated by the functional redundancy of several *P. falciparum* proteins, especially the proteins involved in erythrocyte invasion (Drew *et al.*, 2008; Bannister and Mitchell, 2009) (section 1.10.4.1). The latter suggests that multiple antigens should be targeted at the same time to induce an effective response. Additionally, the extensive naturally occurring antigenic polymorphisms between different parasite

strains is a major obstacle for the formulation of a cross-protective vaccine (Richards and Beeson, 2009).

The problems faced with recombinant expression of *P. falciparum* proteins is another important impediment (Richards and Beeson, 2009). Such recombinant protein expression is invaluable for the construction of a protein based, subunit malaria vaccine but also as tools to address fundamental biological questions. The technical challenges that prevent the high level expression of *P. falciparum* proteins in a recombinant form and in their native conformation have been attributed to the unusually high (~80%) A+T content of the parasite genome with the consequent non-optimal codon bias in heterologous expression systems. Also, *Plasmodium* proteins are typified by the prevalence of repetitive amino acid stretches (Tsuboi *et al.*, 2008). Moreover, the high molecular weight (> 56 kDa) and presence of export motifs and atypical signal peptides are all protein characteristics that were associated with the problematic recombinant expression of *P. flaciparum* proteins (Birkholtz *et al.*, 2008).

Malaria vaccine approaches can be grouped as pre-erythrocytic, transmission blocking and blood stage (Richards and Beeson, 2009). All three approaches are analysed below.

1.13.1 Transmission blocking vaccines

The principle of transmission blocking vaccines is to immunise people with *P. falciparum* antigens that are expressed while the parasite is in mosquito, before the formation of oocyst. Antibodies against such antigens would be taken up by the mosquito during a blood meal and would prevent parasite development in the mosquito midgut thereby preventing malaria transmission (Crompton *et al.*, 2010).

Vaccines based on the gamete surface antigens, P230, P48/45 and HAP2, abolish fertilization in the mosquito midgut, whereas those based on the ookinete antigens, P25 and P28, elicit antibodies that prevent ookinetes from traversing the wall of the mosquito midgut (Greenwood and Targett, 2011). To the best of my knowledge, there is only one reported clinical trial of a transmission blocking vaccine: vaccination with Pfs25 generated significant transmission-blocking activity in vaccinated individuals, even though the trial was not completed because of the high reactogenicity of the adjuvant that was used (Wu *et al.*, 2008).

The idea of developing a transmission blocking vaccine has been questioned. It has been characterised as an altruistic vaccination strategy because there is no direct benefit for the immunised individuals (no protection against disease) but there is benefit for the whole community instead (Hill, 2011; Vaughan and Kappe, 2012). Hence, it might be difficult to convince governments, health care workers or communities to implement such vaccination programmes. Moreover, because all age groups of a population can potentially transmit malaria, this strategy would require mass vaccinations and this could be logistically challenging (Hill, 2011; Vaughan and Kappe, 2012).

Another problem associated the development of vaccines against parasite antigens which are exclusively expressed during mosquito stages, is that there will be no natural boosting by malaria infection and thus, unless boosting injections are provided, the antibody responses in immunised individuals might be short lived (Carter *et al.*, 2000; Vaughan and Kappe, 2012). Others argue that if the complete blockade of parasite development in the mosquito is not achieved, transmission blocking vaccines are likely to paradoxically increase transmission because of the decrease in parasite load within the mosquito, which will reduce the impact of the parasite on mosquito mortality. Therefore, the competence of the mosquito as a vector will be enhanced (Sinden, 2010).

During the last few years the concept of developing a transmission blocking vaccine has regained ground. Recently, there has been a renewed call for global malaria eradication (World Health Organization, 2008). An effective transmission blocking vaccine will probably be needed to achieve eradication, in addition to other existing measures (Richards and Beeson, 2009). The combination of a pre-erythrocytic or blood stage vaccine, to prevent infection (see below), and a transmission blocking vaccine, may be an ideal vaccine strategy because, if effective, it would reduce transmission and provide some protection to vaccinated individuals (Crompton *et al.*, 2010).

1.13.2 Pre-erythrocytic vaccines

It is currently unclear whether protective immunity to the pre-erythrocytic stages of the parasite can be naturally acquired (Marsh and Kinyanjui, 2006). However, it is possible to evoke, by vaccination, pre-erythrocytic immune responses which might

be in place to prevent the blood-stage infection from occurring (Crompton *et al.*, 2010). The idea of a pre-erythrocytic vaccine took shape with the work of Nussenzweig and colleagues, who demonstrated that vaccination of mice with irradiated sporozoites resulted in protection against *P. berghei* malaria (Nussenzweig *et al.*, 1967; Crompton *et al.*, 2010).

Early trials in humans confirmed the initial observations of Nussenzweig. In 1973 Clyde and colleagues showed that immunisation of human volunteers with the bites of *P. falciparum* infected, irradiated mosquitoes could protect humans against later challenge with fully infectious *P. falciparum* sporozoites (Clyde *et al.*, 1973). Later studies demonstrated that ~92% protective efficacy, in the form of sterilizing immunity, can be achieved in humans, after immunisation with >1000 bites of mosquitoes carrying live, radiation attenuated *P. falciparum* sporozoites (Hoffman *et al.*, 2002). A more recent study reported that 100% of subjects exposed on three occasions to bites of 12 to 15 *P. falciparum* infected mosquitoes and concomitantly treated with a chloroquine prophylactic regiment, were protected from later challenge (Roestenberg *et al.*, 2009).

Despite the previously reported high efficacies of immunisation regimes which utilize whole live sporozoites, this approach has raised several concerns regarding the practicalities of its implementation. The requirement of manufacturing and scaling up the product, as well as the cost and feasibility of delivering a vaccine that is cryopreserved, are major logistic challenges (Schwartz *et al.*, 2012). On the other hand, such a vaccination approach is likely to require intravenous administration which could prove clinically impractical on a large scale (Hoffman *et al.*, 2002). Indeed, vaccination with cryopreserved, live attenuated sporozoites, using the intradermal route, conferred protection to only the 5% of vaccinees (Epstein *et al.*, 2011). In contrast, in a similar study where sporozoites were administered intravenously, all subjects (100%) who received five doses of the vaccine were protected against later challenge (Seder *et al.*, 2013).

Besides immunising with sporozoites, other vaccination strategies have been examined for their ability to elicit immune responses to pre-erythrocytic *P.falciparum* stages. One such strategy utilizes vectored vaccines, aiming mainly to induce cellular immunity against the parasite liver-stage (Hill, 2011). The ME (multiepitope) – TRAP (thrombospondin – related protein) vaccine is an example of a

multicomponent viral vectored vaccine (Hill, 2011). The ME-TRAP construct, encodes for a fusion antigen consisting of 17 B-cell, CD4 and CD8 T cell epitopes from six *P. falciparum* antigens (LSA1, CSP, STARP, LSA3, Exp1, TRAP) fused to the T9/96 allele of TRAP. It also includes a single *Plasmodium berghei* CD8+ T cell epitope (Pb9) for potency studies in mice, and a T-helper epitope (FTTp) from tetanus toxoid protein (McConkey *et al.*, 2003; Graves and Gelband, 2006; Draper and Heeney, 2010; Schwartz *et al.*, 2012). In a Phase Ib clinical trial, priming humans with a construct of chimpanzee (Ch) adenovirus 63 encoding ME-TRAP (AdCh63- ME-TRAP), followed by a boost with a modified vaccinia Ankara (MVA) construct, MVA–ME-TRAP, induced strong CD8 T cell responses (Ogwang *et al.*, 2013).

Proteins in adjuvant, is another vaccine platform that has been investigated for immunisation against pre-erythrocytic *P. falciparum* stages (Duffy *et al.*, 2012). In general, this approach involves the administration of recombinantly expressed pre-erythrocytic antigens as a mixture with adjuvants which can robustly activate the immune responses (Duffy *et al.*, 2012). RTS,S is a recombinant protein based vaccine against parasite pre-erythrocytic stages and is currently in a multicenter Phase III clinical trial in sub- Saharan Africa.

1.13.2.1 The RTS,S vaccine

The initial observation of Nussenzweig that immunisation of mice with the bites of radiation-attenuated, parasite infected mosquitoes, conferred protection against challenge with infectious sporozoites (Nussenzweig *et al.*, 1967), led to the identification of the immunodominant cell surface antigen, the circumsporozoite protein (CSP), which is expressed on sporozoites and liver stage schizonts (Enea *et al.*, 1984; Casares *et al.*, 2010). *Pf*CSP is a 58kDa, GPI-anchored cell surface protein, composed of an N-terminal region (RI) that binds heparin sulfate proteoglycans, a central region containing approximately 41 repeats (range 37–49) of NANP (N, asparagine; A, alanine; P, proline) amino acid sequences and a smaller number of NVDP (V, valine; D, aspartic acid) sequences, and a C-terminal thrombospondin-like domain (RII) (Casares *et al.*, 2010; Crompton *et al.*, 2010). The central repeat region contains immunodominant B cell epitopes whereas the C-terminal region contains both B and T cell epitopes (Plassmeyer *et al.*, 2009).

Initial studies demonstrated that the recombinantly expressed central region of *Pf*CSP was immunogenic to humans (Regules *et al.*, 2011). However, there was little or no protection against sporozoite challenge (Regules *et al.*, 2011). To boost antibody responses, a construct was designed that encodes for a fusion protein consisting of 16 *Pf*CSP NANP repeats fused at the N-terminus with the S antigen from hepatitis B virus (HBsAg) (Casares *et al.*, 2010). The fusion protein, designated R16HBsAg, was expressed in yeast cells and it was capable of self-assembling into virus-like particles similar to native HBsAg (Regules *et al.*, 2011). R16HBsAg elicited high antibody titers in experiments involving immunisation of both animals and humans (Regules *et al.*, 2011).

There was, however, the concern that R16HBsAg was still lacking the T cell epitopes located at the C-terminal region of CSP, which would be required for the induction of T cell responses (Casares *et al.*, 2010). Therefore, the construct encoding for R16HBsAg was redesigned to include 19 NANP repeats from the central region (R), the entire C-terminal region of *Pf*CSP which contains the T cell epitopes (T), and the HBsAg (S). To stabililize the recombinant viral particles, the fusion protein was co-expressed in yeast together with free HBsAg (S). The result is a viral particle, RTS,S, containing RTS and HBsAg in 1:4 ratio (Gordon *et al.*, 1995).

Currently, RTS,S (formulated with AS01 adjuvant) is under a Phase III randomized clinical trial which takes place in 11 centres, in seven African countries (Agnandji *et al.*, 2012). In 2011, it was reported that RTS,S/AS01 had an efficacy of 55.8% and 47.3% against clinical and severe malaria respectively, among children 5 to 17 months of age (Agnandji *et al.*, 2011). The latest data from the currently on-going clinical trial demonstrate that the efficacy of RTS,S in infants 6-12 weeks old, during one year of follow-up, is ~31% against clinical malaria and ~26% against severe malaria (Agnandji *et al.*, 2012). The mechanism, by which a vaccine that targets the liver stages protects against severe malaria, which is caused by blood-stage infection, is still elusive. It is possible that RTS,S induces protection against clinical malaria by reducing the number of merozoites released from the liver. This may lead to prolonged exposure to subclinical levels of asexual blood-stage parasites, which in turn enables the acquisition of blood-stage immunity (Crompton *et al.*, 2010) .

1.13.3 Blood stage vaccines

There is strong rationale for the development of a vaccine targeting the asexual blood stages of the parasite. Parasite blood stages have a central role in disease pathology (Cowman *et al.*, 2012) and naturally acquired immunity is principally directed against asexual blood stage antigens (Richards and Beeson, 2009). Early studies demonstrated that transfer of IgGs from malaria-immune adults to malaria infected children, rapidly reduced parasitemia and fever (section 1.11), suggesting that antibodies directed against blood stage antigens is a key component of the naturally acquired immunity (Richards and Beeson, 2009). Hence, the development of a vaccine, able to elicit in children the antibodies that protect against disease in adults, is likely to have a major impact on malaria morbidity and mortality world-wide. The latter is also supported by the belief that one of the mechanisms by which RTS,S (section 1.13.2.1) confers protection to severe malaria, may be the leaky pre-erythrocytic protection, which results in the continued exposure of the organism to blood stage parasites which in turn induces blood stage immunity (Crompton *et al.*, 2010; Ellis *et al.*, 2010).

The efforts for the development of an asexual blood stage vaccine have been primarily focused on erythrocyte invasion (section 1.10). It is an obligatory step in the *Plasmodium* lifecycle and is essential for survival of the parasite within the human host (Cowman and Crabb, 2006). During invasion, the merozoite is – albeit briefly – directly exposed to the host humoral immune system immune system, and therefore, its cell surface proteins are tractable vaccine candidates (Bartholdson *et al.*, 2013). Indeed, out of the eight blood stage antigens that are under clinical development, seven (all but RESA) are merozoite cell surface exposed: apical membrane antigen 1 (AMA1), merozoite surface protein (MSP) -1, MSP-2, MSP-3, glutamate rich protein (GLURP), ring-infected erythrocyte surface antigen (RESA), serine repeat antigen 5 (SERA5), and erythrocyte-binding antigen 175 (EBA-175) (Ellis *et al.*, 2010).

The immunity against the blood stages of the parasite is thought to primarily be mediated by protective antibodies (section 1.11). Therefore, most candidate vaccines against *P. falciparum* blood stages, are recombinant proteins, but peptides, DNA and viral vectored approaches have also been used (Ellis *et al.*, 2010). As mentioned in section 1.13, one major problem that arises from the use of

recombinant proteins for the formulation of blood stage vaccines, is the technical difficulties in expressing *Plasmodium* proteins in a biochemically active recombinant form that are capable of eliciting high antibody titers, and at levels adequate to allow large-scale manufacturing (Hill, 2011; Bartholdson *et al.*, 2013).

Another important challenge towards the development of highly efficient blood stage malaria vaccine, is the functional redundancy observed between the merozoite ligands, which are involved in erythrocyte invasion (section 1.10.4.1; Cowman *et al.*, 2012). Merozoites are believed to express on their surface a set of ligands with overlapping roles, that enables the parasite to access alternative invasion pathways (Duraisingh *et al.*, 2003; Baum *et al.*, 2005; Stubbs *et al.*, 2005). Therefore, it is thought that an efficient blood stage vaccine should simultaneously target multiple merozoite antigens to block a broad array of invasion pathways (Lopaticki *et al.*, 2011).

Besides the ability of the parasite to access alternative invasion pathways, another important obstacle for the development of an effective blood stage malaria vaccine, is the vast allelic polymorphism found across different *Plasmodium* strains (Manske *et al.*, 2012). Vaccine approaches need to account for this, such that they cover the majority of *P. falciparum* antigenic polymorphisms (Richards and Beeson, 2009). The antigenic variation, was the main reason why the two leading blood stage vaccine candidates AMA1 and $MSP1_{42}$ did not demonstrate protective efficacy in African children (Ogutu *et al.*, 2009; Thera *et al.*, 2011). Despite eliciting good antibody titers, there was insufficient cross-protection against diverse malaria strains (Ogutu *et al.*, 2009; Thera *et al.*, 2011).

Recently, however, the identification of the interaction between RH5 and Basigin (section 1.10.6) which has been shown to be essential and universally required for erythrocyte invasion, allowed new hopes for the formulation of a highly efficacious blood stage vaccine (Crosnier *et al.*, 2011). Erythrocyte invasion can be completely blocked using low concentrations of anti-Basigin monoclonal antibodies and this effect was observed in multiple *P. falciparum* laboratory-adapted lines and field isolates (Crosnier *et al.*, 2011). Conversely, polyclonal antibodies raised against the 3D7 variant of RH5 were able to block erythrocyte invasion in nine different *P. falciparum* strains, which, between them, included all of the five most common RH5 polymorphisms (Bustamante *et al.*, 2013). Moreover, virally delivered RH5 can

induce antibodies that inhibit erythrocyte invasion of a wide range of parasite genetic variants (Douglas *et al.*, 2011). Thus, it is likely that the identification of the interaction between RH5 and Basigin will lead to the development of efficient therapeutic interventions targeting the blood stages of the parasite.

1.14 Host oriented approaches for treatment of various infectious diseases

As mentioned in section 1.13.3 the development of highly efficacious interventions targeting merozoite-erythrocyte interactions, has been hampered by the highly polymorphic nature of merozoite ligands, as well as by functional redundancy of the receptor-ligand pairs involved in the invasion process (Cowman and Crabb, 2006). Another fundamental problem towards the development of effective anti-malarial therapeutics targeting merozoite cell surface proteins, is the short timing window during which merozoite antigens are available for binding to a therapeutic agent (e.g an antibody), as merozoites are only extracellular for 1-2 minutes following schizont rupture (Dvorak *et al.*, 1975; Mason and Williams, 1980; Saul, 1987).

One possible strategy to overcome the obstacles is the development of approaches directed against the human host, instead of the parasite. In contrast to *Plasmodium* spp. and pathogens in general, humans have a much longer generation time and therefore, they are not evolving as rapidly as the parasite, minimizing the problems arising from sequence variability (Baillie and Digard, 2013). Furthermore, the development of a therapeutic agent targeting host cell surface molecules overcomes the problem of a restrictive time-window, as unlike merozoite ligands, erythrocyte receptors are constantly exposed on the cell surface and hence, are continuously available for binding. Moreover, the targeting of host factors that are essential for the survival of different *Plasmodium* species and strains, may allow the development of more broad spectrum interventions (Prussia *et al.*, 2011). In addition, it is unlikely that individual point mutations in parasite components could compensate for the loss of essential host factors and thus, the potential for the development of resistance or escape mutants is diminished (Prussia *et al.*, 2011).

While perhaps at fist counter-intuitive, host-oriented intervention strategies have been successfully applied for the treatment of a number of viral infections, and particularly HIV. For example, ibalizumab, a humanised monoclonal antibody against

human CD4 –the receptor required for HIV entry in human T helper cells- demonstrated high anti-HIV efficacy and was well tolerated by subjects (Bruno and Jacobson, 2010; Fessel *et al.*, 2011). Similarly, PRO 140, a humanised anti-CCR5 antibody, has been shown to be safe for administration, with potent and prolonged antiretroviral activity (Jacobson *et al.*, 2010). Importantly, a CCR5 antagonist (maraviroc) has recently been approved by the US Food and Drug Administration for the same purpose (Tan *et al.*, 2007; Lieberman-Blum *et al.*, 2008). Other examples of host-oriented anti-viral approaches are those proposed against poxvirus (Reeves *et al.*, 2005), West Nile virus (Hirsch *et al.*, 2005) and influenza virus (Pleschka *et al.*, 2001; Morita *et al.*, 2013).

Host-targeted therapeutics have shown considerable promise in the context of other infectious diseases but have not been to date developed for malaria. Previously, we showed that the RH5-Basigin interaction is essential and universally required for erythrocyte invasion by *P. falciparum*, and low concentrations of anti-Basigin monoclonal antibodies were able to potently block erythrocyte invasion *in vitro* (Crosnier *et al.*, 2011). While vaccine targeting the parasite component of this interaction are under active development (Douglas *et al.*, 2011; Bustamante *et al.*, 2013; Reddy *et al.*, 2013), the potent invasion-blocking effect of anti-Basigin monoclonal antibodies also raises the possibility that these could be used as therapeutics to treat *P. falciparum* infected individuals. The previous safe use of anti-Basigin monoclonal antibodies in the clinic (section 1.10.6.2.2 and 1.10.6.2.3) is encouraging that antibodies against Basigin would be safe and very efficient in when administered to treat malaria patients.

In this PhD thesis, I aimed at the development of a recombinant anti-Basigin humanised chimeric monoclonal antibody, as a potential anti-malarial therapeutic. In a parallel project I aimed at the identification of novel erythrocyte-merozoite cell surface protein interactions which are critical for erythrocyte invasion.

1.15 Aims of this thesis

1.15.1 Development of a humanised or chimeric anti-BSG monoclonal antibody as a potential anti-malarial therapeutic

One of the goals of this PhD project was to exploit the essential nature of the RH5-Basigin interaction as a therapy, based on humanised or chimeric anti-BSG

monoclonal antibodies. In pursuit of this aim, I established a versatile plasmid vector system which enables the rapid and efficient recombinant expression of engineered antibodies (Chapter 3). By using this plasmid system, two anti-BSG monoclonal antibodies, MEM-M6/4 and MEM-M6/8 (Koch *et al.*, 1999) were successfully humanised by Complementarity Determining Region (CDR) grafting. The therapeutic potential of huMEM6-M/4 and huMEM6-M/8 was hampered by their low affinity for BSG. To obtain a higher potency antibody, a panel of hybridoma lines secreting anti-BSG antibodies was generated by directly immunising animals. A monoclonal antibody, m6D9, secreted by one of the generated hybridoma clones, demonstrated high efficacy in inhibiting erythrocyte invasion in parasite culture, and was selected for chimerisation. The chimeric antibody, ch6D9, retained its high affinity for BSG and blocked erythrocyte invasion with very low IC_{50} in all parasite lines tested. Furthermore, ch6D9 displayed reduced binding to FcγRIIA and C1q *in vitro*, suggesting that this antibody may have reduced ability to trigger antibody effector functions. Attempts to fully humanise this antibody were unsuccessful.

To directly compare the efficacy between anti-BSG and anti-RH5 antibodies in inhibiting erythrocyte invasion, two anti-RH5 monoclonals (2AC7 and 9AD4;Douglas *et al.*, 2013) were successfully chimerised. Both ch2AC7 and ch9AD4 preserved their high affinity for RH5 and inhibited erythrocyte invasion in parasite culture, but with much higher IC_{50} as compared to ch6D9. Finally, the variable regions of ch2AC7 and ch6D9 were combined in an anti-RH5 and anti-BSG bi-specific antibody: 2AC7-6D9 DVD-Ig. 2AC7-6D9 DVD-Ig was capable of simultaneous binding to RH5 and BSG but its affinity for BSG decreased in comparison to ch6D9. When tested in *P. falciparum* growth inhibition assay, 2AC7-6D9 DVD-Ig was more efficient than ch2AC7, but less than ch6D9 in blocking erythrocyte invasion.

1.15.2 Identification of novel receptor-ligand pairs involved in erythrocyte invasion

During erythrocyte invasion, the merozoite is directly exposed to the host humoral system, and therefore, merozoite cell surface proteins they are of fundamental importance for the development of anti-malarial therapeutics. Merozoites, however, are known to use a functionally redundant set of ligands that enable the parasite to access alternative invasion pathways complicating the development of highly effective therapeutic interventions. To date, only a handful of

receptor-ligand interactions implicated in invasion have been identified, and of those, only the interaction between RH5 and BSG has been shown to be essential and universally required for merozoite invasion.

A goal of this PhD project was the identification of novel receptor-ligand pairs involved in erythrocyte invasion. For this purpose, an existing *P. falciparum* merozoite recombinant protein library was expanded by 26 proteins. The new members of the *P. falciparum* protein library were expressed recombinantly and systematically screened against an equivalent library consisting of erythrocyte receptors. The screen identified a putative interaction (PF13_0125 – P4HB). Further characterisation of the identified interaction provided inconclusive results, and more experiments are required to confirm its validity. The recombinant *P. falciparum* merozoite proteins reported in this project will also be useful for further studies towards the understanding of erythrocyte invasion and *P. falciparum* merozoite biology. .