Studies on *Plasmodium falciparum*

asexual blood stage antigens:

RAP-2/RSP-2 and Pf332 in focus

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About the Cover

The cover illustration depicts a red cell traversing from the splenic cord to the splenic sinus through the narrow endothelial slit separating the cord from the sinus. Also shown is an image of a camel struggling to go through the eye of a needle. The human red cell, with a diameter of approximately 7-8 μm, is characterised by its discoid shape and an ability to undergo extensive deformations during passage through the narrow capillaries of the microvasculature, during its lifespan of 120 days in the blood stream. If for some reason the red cell loses its ability to squeeze through these tiny slits e.g. during infection with malaria, then it becomes difficult for them to meander their way in circulation. In which case, they will get stuck like the camel.

Personally, the similarity of these images reminds me of the amazing presence of God in nature, with whom, all things are possible (Mathew 19:23-26).

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To Godson,
With lots of love
Curiosity unsatisfied dies
a death of boredom
Adventures of Don Quixote
SUMMARY

Malaria remains a challenging health problem in malaria endemic regions. The life cycle of the malaria parasite is very complex and provides a number of potential targets for vaccination. Several antigens have been studied and characterised in this context. Existing data suggest that antibodies are instrumental in the control of infection, but could also be responsible for pathogenesis. The mechanisms by which the antibodies mediate their effects are known to differ, largely depending on the target antigen. In this thesis, data on two plasmodial asexual blood stage antigens (RAP-2/RSP-2 and Pf332-C231) are presented and their putative roles in disease manifestation and/or control.

Infection with malaria invariably leads to anaemia, involving the destruction of both normal and infected erythrocytes by various mechanisms. The groups most at risk of developing malarial anaemia include children below the age of five years and pregnant women. A partial aim of the work presented herein was to investigate the mechanisms responsible for the destruction of erythroid cells during anaemia, and more specifically to define the role of the rhoptry associated protein (RAP/RSP)-2 and other members of the low molecular weight rhoptry associated protein (RAP) complex, RAP-1 and -3 in processes resulting in anaemia. Antibodies to the RAP complex were shown to have the potential to mediate the destruction of RAP-2/RSP-2-tagged erythroid cells by phagocytosis or by complement activation and lysis. In addition, antibodies to RAP-1 and RAP-2/RSP-2 could induce the apoptotic death of RAP-2/RSP-2-tagged erythroblasts. The frequency and functionality of naturally occurring RAP-2/RSP-2 antibodies in the sera of anaemic and non-anaemic Cameroonian children were also investigated. All sera tested contained RAP-2/RSP-2 reactive antibodies by both immunofluorescence and flow cytometry. The anaemic group of children had significantly higher levels of antibodies of the IgG isotype than the non-anaemic individuals, while the levels of IgM were similar in both groups. With respect to IgG subclasses, low levels of IgG-1 and -3 antibodies were detected. Higher levels of IgG3 were seen in the non-anaemic individuals as compared to anaemic subjects. With regard to antibody functionality, the non-anaemic individuals recognised a greater proportion of RAP-2/RSP-2-tagged erythrocytes and activated complement to a greater extent than the anaemic individuals. These data suggest a role for RAP antibodies in both pathogenesis and protection during malaria.

Earlier studies observed that humans continuously exposed to malaria, recognised Pf332 extensively. Further studies revealed that Pf332 antibodies were able to inhibit parasite growth and cytoadherence in vitro. How these antibodies mediate their effects is not fully known and remains a question undergoing further investigations. Making use of Pf332-C231, a subfragment of the Pf332 antigen, we studied the effects/mode of action of Pf332-C231 specific antibodies, induced upon natural infection on P. falciparum parasite growth and development in in vitro cultures. Our data indicate that, antibodies acted mainly on late stage parasites by two main mechanisms; 1) through the induction of abnormal/pyknotic parasites, and, 2) red cell lysis (disintegration of red cells), thus limiting parasite growth and development as evidenced by a reduction in parasite numbers. The antibody isotype in this context was IgG. Following the removal of immune pressure, parasites were able to resume growth, albeit at a much slower rate compared to control cultures without previous antibody pressure. The results suggest that during natural infections, antibodies to Pf332-C231 could play a role in parasite control. However, the induction of red cell lysis could result in anaemia, especially in acute infections characterised by high parasite densities, although this speculation warrants investigation.

Taken together, these data suggest that, antibodies to both antigens could be instrumental in immune responses leading to disease control. However, a highly regulated/balanced immune response is required as they could also mediate pathology. As such, caution is required if antigens are to be part of components constituting a subunit vaccine.
LIST OF PAPERS

This doctoral thesis is based on the following original papers, which will be referred to by their Roman numerals.


*Equal authorship
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<tr>
<td>CM</td>
<td>Cerebral malaria</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin sulphate A</td>
</tr>
<tr>
<td>DBL</td>
<td>Duffy binding ligand</td>
</tr>
<tr>
<td>EBL</td>
<td>Erythrocyte binding ligand</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophil cationic protein</td>
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<tr>
<td>EDN</td>
<td>Eosinophil derived neurotoxin</td>
</tr>
<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystalisable</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc Receptors</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HGMB1</td>
<td>High mobility group 1</td>
</tr>
<tr>
<td>Hz</td>
<td>Haemozoin</td>
</tr>
<tr>
<td>ICE-IPAF</td>
<td>IL-1β-converting enzyme protease-activating factor</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iRBC</td>
<td>Infected red blood cells</td>
</tr>
<tr>
<td>KAHRP</td>
<td>knob-associated histidine-rich protein</td>
</tr>
<tr>
<td>LP</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>MA</td>
<td>Malaria anaemia</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MBP</td>
<td>Major basic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>MIF</td>
<td>Migratory inhibitory factor</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite surface protein</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptors</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>nRBCs</td>
<td>Normal red blood cells</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>Pf332</td>
<td><em>Plasmodium falciparum</em> antigen 332</td>
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<tr>
<td>PfEMP-1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein-1</td>
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<tr>
<td>PRRs</td>
<td>Pathogen recognition receptors</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<tr>
<td>RAP</td>
<td>Rhotry associated protein</td>
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<tr>
<td>RBCs</td>
<td>Red blood cells/erythrocytes</td>
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<td>RDS</td>
<td>Respiratory distress syndrome</td>
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<td>RIG</td>
<td>Retinoic acid inducible gene I</td>
</tr>
<tr>
<td>riRBCs</td>
<td>Ring-infected red blood cells</td>
</tr>
<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediates</td>
</tr>
<tr>
<td>ROI</td>
<td>Reactive oxygen intermediates</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSP-2</td>
<td>Ring surface protein-2</td>
</tr>
<tr>
<td>SM</td>
<td>Severe malaria</td>
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<tr>
<td>SMA</td>
<td>Severe malaria anaemia</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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Faith is taking the first step even when you don't see the whole staircase.

Martin Luther King, Jr.
INTRODUCTION: AN OVERVIEW

The immune system

The immune system in humans consists of three levels of host defence, including anatomic and physiologic barriers, innate immunity and adaptive immunity. The anatomic and physiologic barriers provide a crucial first line of defense against pathogens.

Innate immunity

Innate immunity augments the protection offered by anatomic and physiologic barriers and provides the next first line of host defence before the adaptive immune response sets in. During the onset of an infection, the host must launch a rapid innate response to control pathogen proliferation and spread until the adaptive response of specific T and B cells has fully developed. The detection of pathogens is first carried out by sentinel cells of the innate immune system, such as macrophages and dendritic cells, located in tissues that are in contact with the host’s natural environment, and then by circulating granulocytes and monocytes that are rapidly recruited to the site of infection.

This innate system recognises highly conserved microbial components (usually carbohydrates, lipids, and proteins) essential for the viability and virulence of microbes, and thus less prone to modifications (reviewed Turvey & Broide, 2010). Generally, innate recognition is based on three broad strategies as follows:

- In the first strategy, innate immunity relies on a limited repertoire of germ-line encoded receptors, known as pathogen recognition receptors (PRRs), that recognise conserved molecular structures, known as pathogen associated molecular patterns (PAMPs) that are expressed by a large variety of microbes.

- A second approach is to detect immunologic danger in the form of danger-associated molecular patterns (DAMPs), which represent a common metabolic consequence of infection
and inflammation, (including alarmins such as the high mobility group box 1 protein (HMGB1), heat shock proteins (HSP) and uric acid).

- In the third, recognition involves the natural killer cell (NK) detection of “missing self”, molecules expressed by normal healthy cells but not by infected cells or microbes (reviewed Turvey & Broide, 2010).

The germ-line encoded Toll-like receptors (TLRs) allow the direct recognition of distinct microbial structures that are shared by microorganisms, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), flagellin and bacterial DNA. Toll-like receptors (TLRs) are central components of the innate immune system. Upon ligand binding, they activate signaling cascades leading to the synthesis of proinflammatory molecules and chemokines. Chemokines recruit innate immune effector cells, such as granulocytes, monocytes, macrophages, and NK cells, to the site of infection (Parroche et al., 2007). The mannose, scavenger and dectin receptors mediate binding and phagocytosis of microorganisms and foreign particles. Fc-, complement- or scavenger receptors further facilitate the clearance of microorganisms that are coated with opsonic proteins, such as immunoglobulins, complement fragment C3b or mannose-binding lectin.

The nucleotide-binding oligomerization domain (NOD-like; NLR) and the retinoic acid inducible gene I (RIG-I-like; RLR) receptors constitute important components of innate immunity (Coban et al., 2005). NLR engagement leads to the production of pro-inflammatory cytokines and chemokines resulting in, for example neutrophil recruitment. Unlike the TLRs, which either detect microbial signatures in the extracellular milieu or engulfed into the lumen of endocytic vesicles, NLRs survey the intracellular environment. In addition to sensing microbial products, NLRs also are able to sense metabolic stress related to infection and sterile inflammation, as demonstrated by the NLR family member, pyrin 3 (NALP3: also known as cryopyrin). NALP3 can be activated by common metabolic signals, such as potassium efflux, which occurs during inflammation due to disruption of the plasma membrane or increased extracellular ATP released by injured cells (reviewed Turvey &
Broide, 2010). Other members of the NLR family include NOD1 and NOD2, that detect distinct substructures generated during the synthesis, degradation and remodeling of bacterial peptidoglycan (PGN), ensuring the recognition of PDG from both gram-positive and gram-negative bacteria. The IL-1β-converting enzyme (ICE) protease-activating factor (IPAF) is another member of the NLR family known to detect bacterial pathogens. Together with TLR5 IPAF detects infection by flagellated bacteria (intracellular flagellin) (reviewed Turvey & Broide, 2010). Not much is known about the RLRs group of receptors. However, data indicate that they recognize and respond to viruses, activating signaling cascades that result in the induction of type I interferons (reviewed Ulevitch, 2004, reviewed Creagh & O’Neill, 2006).

In addition to recognition of PAMPs, it has been suggested that the immune system responds to other signals commonly associated with infection. In particular, it has been proposed that cells dying by necrosis may release molecules (DAMPS), such as DNA, ATP, uric acid and DNA binding proteins (HMGB1), into the extracellular milieu (reviewed Vance et al., 2009).

The activation of the innate immune response can in addition, serve as a prerequisite for triggering the adaptive immunity. Here, the antigen-specific lymphocytes of the adaptive immune response are activated by co-stimulatory molecules that are induced on cells of the innate immune system during their interaction with microorganisms. The cytokines produced during these early phases also have an important role in stimulating the subsequent adaptive immune response and shaping its development (reviewed Takeda & Akira, 2005, Colonna 2003).

**The complement system**

Complement is part of the innate immune system and underlies one of the main effector mechanisms of antibody-mediated immunity (humoral immunity). This system represents a complex pathway of more than 30 serum proteins and cell surface receptors that interact in a range of functions from direct cell lysis to the enhancement of B- and T-cell responses. Its physiologic activities include defense against bacterial infection, bridging innate and adaptive immunity and disposing of immune complexes and products of inflammatory injury (Walport, 2001, reviewed Carroll, 2004). In addition to antibodies, most recognition structures involved in complement activation include PRRs, such as the mannan-binding lectin (MBL), ficolins,
C-reactive protein, C1q and natural immunoglobulin M (IgM) (reviewed Carroll, 2004). The complement system consists of the sequential activation of many pro-enzymes that, in turn, can catalyze the activation of other enzymes.

The complement system is activated by 3 pathways namely: 1) the classical pathway, which is activated by antibodies; 2) the lectin pathway, which is activated by lectin proteins (MBL, ficolin H and ficolin L) and 3) the alternative pathway, which is continuously turned on, due to the spontaneous activation of C3 and its promiscuity in binding a wide range of suitable receptors. All three pathways share the common step of activating the central component C3, although they differ according to the nature of recognition (reviewed Carroll, 2004). A key part of the complement cascade is the formation of C3b from C3 following cleavage by enzyme complexes called C3 convertases. C3b can then bind to cell surfaces (foreign and non-self antigens) opsonising/tagging them for uptake by phagocytic cells (macrophages) (Walport 2001, reviewed Carroll, 2004) or retention on follicular dendritic cells (FDCs) for recognition by cognate B lymphocytes (reviewed Carroll, 2004). Phagocytic complement receptors include Complement receptor 1 (CR1) expressed on erythrocytes, B cells, monocytes, neutrophils, eosinophils and dendritic cells; CR3 (CD11b/CD18 or Mac 1), found on monocytes, macrophages, neutrophils, DCs and NK cells; and CR4 (CD11c/CD18) is not as well characterised like the others (Underhill & Ozinsky, 2002). CR1 binds a broad spectrum of microbial opsonins, including C1q, C4b and C3b as well as MBL, while CR3 and CR4 recognise iC3b.

Complement activation also leads to the generation of C5a, which is inflammatory and chemotactic, as well as the activation of C5 through C9, forming the membrane attack complex, (MAC) responsible for cell lysis (reviewed Beutler, 2004). It is noteworthy that the activation of the complement cascade is carefully regulated by several proteins in the plasma and on cell surfaces, including Factor I, CR1 (CD35), decay-accelerating factor (DAF, also known as CD55) and membrane inhibitor of reactive lysis (MIRL, also known as CD59). These three proteins are found on the erythrocyte surface and are responsible for its complement-regulatory properties. Thus, in addition to functioning as oxygen carriers, erythrocytes perform an important function as regulators of the complement cascade to prevent autologous complement attack (Stoute, 2005). CR1 on erythrocytes serves to remove
immune complexes (ICs) from the circulation by binding ICs containing C3b. In the liver and spleen, these ICs are removed by macrophages, and in this process, CR1 molecules are continuously lost while the erythrocytes are recycled. In addition, CR1 accelerates the degradation of C3 convertases and promotes the inactivation of C3b. CD55 functions mainly to accelerate the decay of C3 convertases. CD59 prevents the assembly of the MAC that forms a pore in the cell membrane, leading to lysis, by binding to the C5b–8 complex and preventing the polymerization of C9 (Walport, 2001, Stoute, 2005).

**Cells of the innate immune system**

**Natural killer Cells**

The natural killer (NK) cell is an important component of the innate immune system representing 10-20% of the lymphocytes in peripheral blood. NK cells mediate cellular cytotoxicity and produce chemokines and inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF). They are important in attacking pathogen-infected cells, especially during the early phase of an infection. They are also efficient killers of tumor cells or virally infected cells primarily through perforin/granzyme mediated cytolysis, or induction of target cell apoptosis by Fas ligand, surface TNF, or TRAIL (TNF-related apoptosis-inducing ligand). Even though classified as innate cells, NK cells also participate directly in adaptive immune responses, mainly by interacting with dendritic cells (reviewed Raulet, 2004). The recognition of target cells by NK cells is regulated by a balance of signals transmitted to NK cells through combinations of activating and inhibitory cell surface receptors (reviewed Lanier, 2003).

**Mononuclear phagocytes**

Mononuclear phagocytes including monocytes, macrophages, and dendritic cells are intrinsically involved in innate immunity. They play a role in phagocytosis, antigen processing and presentation, T-cell activation and cytokine secretion (reviewed Goerdt &
Orfanos, 1999). As the designation denotes, the chief role of these cells is phagocytosis, whereby they engulf and destroy pathogens, apoptotic cells, and other targets. This occurs either through employing opsonin receptor-dependent mechanisms via complement- and Fcγ receptors, or opsonin receptor-independent mechanisms via lectin-receptors, scavenger receptors, or CD14 (reviewed Kelly, 2011).

**Monocytes** represent 5-10% of the peripheral blood leukocytes in human blood. Circulating monocytes give rise to a variety of tissue resident macrophages throughout the body, as well as to specialized cells such as DCs and osteoclasts. They differentiate from hematopoietic stem cells, specifically granulocyte/macrophage progenitors in the bone marrow and enter the periphery (reviewed Gordon & Taylor, 2005) where they circulate for several days before entering tissues and replenishing the tissue macrophage population. Peripheral blood monocytes show morphological heterogeneity in size, and granularity and are identified by the expression of CD14, CD16 or CD64 (reviewed Gordon & Taylor, 2005).

**Macrophages (Mφ)**, are polarized following activation into classic (M1) and alternative (M2) Mφ (Chernykh et al., 2010). M1 Mφ, are characterised by expression of bactericidal effector molecules and opsonic receptors including FcγRI, II and III (Chernykh et al., 2010). M1 macrophages are activated in response to microbial products, such as lipopolysaccharide (LPS) or cytokines like IFN-γ and TNF, and are characterised by a strong propensity to present antigen. These cells have the ability to produce large amounts of proinflammatory cytokines such as IL-12, IL-23 and TNF, reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), high expression of MHC II, efficient antigen presentation and microbial or tumoricidal activity (reviewed Biswas & Mantovani 2010). M2 activation of Mφ is characterised by their expression of non-opsonic receptors, e.g. the macrophage mannose receptor, by their up-regulation of Th2-associated cytokines and the production of extracellular matrix components. M2 Mφs are promoted by various signals, such as IL-4, IL-13, glucocorticoids, IL-10, and immune complexes. In general, these cells function in parasite clearance, the resolution of inflammation, as well as in the promotion of tissue remodeling and tumour progression. They could also have immunoregulatory functions (reviewed Biswas & Mantovani 2010). However, the M1 and M2 classes are able to change from one phenotype
to another depending on the cytokine milieu (Chernykh et al., 2010).

**Dendritic cells** (DCs) are derived from haemopoietic bone marrow progenitor cells. DCs are professional antigen-presenting cells, specialized in capturing, processing and presenting antigen to other immune cells. DCs display a great capacity in stimulating naive T cells and initiating primary immune responses. They play an important role in tolerance induction as well. They are distributed throughout the body, particularly at sites of pathogen encounter, such as the skin and the mucosal surfaces. They are also found in the circulation as immature cells. Immature DCs constantly sample the surrounding environment for pathogenic microbes. Upon antigen encounter, they become activated and thereafter travel to secondary lymphoid tissues, such as the spleen and lymph nodes, where they stimulate naive T-cells to differentiate into functionally competent effector T cells. Fully mature DCs express high levels of several molecules on the cell surface, such as, MHC class I and II, and accessory molecules, such as CD40, CD80, CD86 and early activation markers such as CD83. Both mice and humans have two major types of DCs, recognized based on the expression of the β2-integrin CD11c: CD11c+ myeloid DCs (mDCs, also called conventional or classical DCs), and CD11c- plasmacytoid DCs (pDCs). pDCs express TLR 7/9 and are considered the front line in anti-viral immunity, as they rapidly produce abundant type I interferons including IFNα/β/ω (but mostly IFNα) in response to viral infection. Meanwhile, mDCs expressing TLR2/3 mainly recognise bacterial components and produce TNF, IL-6 and IL-12 (reviewed Banchereau & Steinman, 1998, Ito et al., 2005).

**Granulocytes**

Neutrophils, basophils, eosinophils and mast cells are classified as granulocytes based on their cellular morphology and cytoplasmic staining characteristics. **Neutrophils** are an essential part of the innate immune system. They make up approximately 50-70% of the circulating white blood cells. They represent the first line of defense and rapidly accumulate at sites of infection and injury in response to chemotactic factors such as
IL-8. The function of neutrophils is mainly that of phagocytosis, as such, they are known as ‘professional’ phagocytes. Once ingested, the foreign particles are destroyed by proteolytic enzymes stored in special granules, hence named granulocytes, and by the production of reactive oxygen species (ROS) (reviewed Nathan, 2006). They also have important roles in the regulation of inflammation, in wound healing and tissue repair mechanisms (Appelberg, 2007).

**Basophils** are the least common type of granulocytes, accounting for less than 1% of peripheral blood leukocytes. They are characterised by the release of chemical mediators, such as histamine, after stimulation. They represent an important source of the Th-2 cytokine IL-4, and have been shown to play a role in allergic reactions and in helminthic infections. Basophils, normally circulating in the bloodstream, become recruited in small numbers to peripheral tissues, under pathological conditions e.g. allergic/parasitic diseases (reviewed Karasuyama et al., 2009, Nakanishi, 2010).

**Mast cells** are found in almost all of the major organs and tissues of the body, particularly in association with connective tissue structures such as blood vessels, lymphatic vessels and nerves. They are also found in proximity to surfaces that interface the external environment, such as those of the respiratory and gastrointestinal system and the skin (reviewed Crivellato et al., 2010). Although best known for their role in allergy and anaphylaxis, mast cells play an important protective role as well, being intimately involved in wound healing and defense against pathogens. Their heavily granulated cytoplasm contains preformed inflammatory mediators, including histamine, heparin and other active substances that are released upon activation. The cytokine profile produced by mast cells is quite wide; including mainly TNF-α, IL-4, -5 and -6 (reviewed Crivellato et al., 2004).

**Eosinophils** represent approximately 1-3% of the blood leucocytes. They are motile phagocytic cells that can migrate from blood into tissue spaces. They have been implicated in the pathogenesis of inflammatory processes, such as parasitic helminthic infections and allergic diseases. They mediate their functions by secreting their granular contents that damage parasite membranes. The array of cytotoxic granules they secrete include major basic
protein (MBP: the predominant cationic protein of human eosinophil specific granules), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil derived neurotoxin (EDN) which are capable of inducing tissue damage and dysfunction. In addition, ECP has been implicated in a number of additional noncytotoxic activities, including suppression of T-cell proliferative responses and immunoglobulin synthesis by B-cells, induction of mast cell degranulation, and stimulation of airway mucus secretion (Rothenberg & Hogan, 2006).

Cell death

The outcome of host pathogen interactions has been known to result in host cell death, either as a consequence of infecting host cells or by producing toxic products. Cell death may occur by apoptosis, autophagy or necrosis.

Apoptosis, is a form of programmed cell death, and is involved in development by the elimination of damaged cells and the maintenance of cell homeostasis. However, apoptosis as a strategy for parasitism, in which phagocytes (neutrophils/macrophages) serve a “Trojan horses” for intracellular pathogens in “silent” phagocytosis, has also been described (reviewed van Zandbergen et al., 2007). A subfamily of cysteine proteases known as caspases, irreversibly commit cells to die by apoptosis. As such, caspases are the central components of the apoptotic response. Once an initiator caspase is activated, it can trigger a cascade to activate downstream executioner caspases. Subsequently, the activated executioner caspases cleave numerous cellular targets to destroy/disrupt normal cellular functions, activate other apoptotic factors, inactivate anti-apoptotic proteins, eventually leading to apoptotic cell death (reviewed Jiang & Wang 2004). The final stage of apoptosis is associated with distinct morphological and biochemical changes, including cell shrinkage, extensive plasma membrane blebbing, altered plasma membrane permeability, exposure of phosphatidylserine (PS), loss of mitochondrial integrity, chromatin condensation (pyknosis) and DNA fragmentation (karyorrhexis) (reviewed Maiuri et al., 2007, Bruchhaus et al., 2007). Apoptotic bodies are subsequently phagocytosed by macrophages, parenchymal cells, or
neoplastic cells and degraded within phagolysosomes, thus preventing any inflammatory reactions (reviewed Elmore, 2007). In mammalian cells, the apoptotic response is mediated through either the intrinsic pathway (mitochondrial) or the extrinsic pathway (death receptor pathway), depending on the origin of the death stimuli. The intrinsic pathway is triggered in response to a wide range of death stimuli that are generated from within the cell, such as oncogene activation and DNA damage. The extrinsic pathway is initiated by the binding of an extracellular death ligand, such as FasL, to its cell-surface death receptor, such as Fas. An additional pathway is the perforin/granzyme pathway (reviewed Riedl & Shi, 2004).

**Autophagy** (self-eating) is a form of programmed cell death essential for survival, differentiation, development, and homeostasis. It is an important eukaryotic response during conditions of cellular stress. The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation. At the onset of autophagy, portions of the cytosol and cytoplasmic organelles become sequestered within double- or multi-membrane autophagosomes, which later fuse with lysosomes and their contents degraded, thus, generating new metabolic substrates to meet the needs of the cell. Apart from this, it also plays a role in getting rid of intracellular pathogens, damaged organelles and potentially toxic proteins. It is important for the survival of mammalian cells as a reduction in autophagic capacity can lead to oncogenic events (Levine & Kroemer, 2008).

**Necrosis** represents an accidental death of cells in tissues or organs, which takes place because of interruption of cellular processes. Features of necrosis include cytoplasmic swelling, rapid plasma membrane rupture, and organelle breakdown, which results in the release of lysosomal enzymes causing an inflammatory response (reviewed Denecker *et al.*, 2001).

Depending on the availability of activated caspases, the same cell death stimulus can result in either apoptotic or necrotic cell death, known as secondary or apoptotic necrosis (reviewed Fink & Cookson, 2005). In the absence of phagocytosis, apoptotic bodies may lose their membrane integrity and proceed to necrosis (apoptotic necrosis). Thus, cells in the end stages of apoptosis show features reminiscent of necrosis.
**Phagocytosis**

Phagocytosis plays an essential role in host defense mechanisms through the uptake and destruction of invading infectious pathogens, as well as contributing to inflammation and the immune response. Phagocytosis is triggered by the interaction of opsonins, bound to the intruding pathogen, with specific receptors on the surface of the phagocytes. Following phagocyte-pathogen contact, intracellular signals trigger cellular processes, leading to the production of proinflammatory signals and activation of antimicrobial mechanisms. Receptors that participate in pathogen recognition and internalization include: the Fc receptors (FcRs), which bind to the Fc part of immunoglobulins, complement receptors (CRs) which bind to complement components on opsonised particles, lectin receptors that bind specific sugar residues on pathogens, and scavenger receptors (SRs), which recognize altered lipids, sugars, or proteins on the surface of apoptotic cells (Garcia-Garcia & Rosales, 2002).

As mentioned earlier, complement components have multiple host defense functions, including chemoattraction, formation of membrane attack complex and opsonisation (see previous section on the complement system).

**Fc receptors** for Ig are expressed on many cell types of the immune system and the FcγRs have been the most widely studied. This is further discussed ahead (See section on Fc receptors).

**Adaptive immunity**

Adaptive immunity is usually triggered when an infection eludes or overpowers the innate defense mechanisms. Adaptive immunity is mediated by B and T lymphocytes.

**T lymphocytes** are primarily responsible for cell-mediated immunity. Based on their antigen receptors, they are further divided into αβ T cells and γδ T cells (reviewed Chien & Bonneville, 2006). The different T cells are either CD4+ or CD8+ T cells. In both mice and
humans, the CD4\(^+\) subset can be differentiated into Th1 and Th2 helper T cells, which are characterised by their different cytokine profiles. Th1 cells produce interleukin (IL)-2 and, IFN\(\gamma\) whereas Th2 cells produce IL-4 (reviewed Murphy & Reiner, 2002). While \(\alpha\beta\) T cells recognize processed protein antigens in the form of peptides associated with MHC molecules, there is no known antigen processing and presentation requirement for ligand recognition by \(\gamma\delta\) T cells (reviewed Chien & Bonneville, 2006).

**B lymphocytes** are essential in the generation of humoral immune responses through the production of immunoglobulins (Igs). B-cell development from hematopoietic cells occurs before birth in the foetal liver and subsequently in the bone marrow. In the bone marrow, B cells develop from pro-B to pre-B cells and eventually to membrane-bound IgM-expressing immature B cells. Naïve mature B cells (expressing both IgM and IgD) leave the bone marrow and travel to secondary lymphoid tissues, where they become activated. This activation is initiated following specific antigen engagement of the B-cell receptor (BCR). Activated B cells then differentiate to form extrafollicular antibody-secreting plasmablasts (still dividing), that are essential for rapid antibody production and early protective immune responses. Alternatively, they can enter germinal centers, where they differentiate into plasma cells, secreting high affinity antibodies following affinity maturation or into memory B cells, which confer long-lasting protection from secondary antigenic challenge, and long-lived plasma cells (reviewed Batista & Harwood, 2009).

**Somatic hypermutation and isotype class switching**

After interacting with antigen and T cells, B cells differentiate into centroblasts and migrate into germinal centers where their Ig genes undergo somatic hypermutation (SHM) and class switch recombination (CSR).

**In CSR**, the immunoglobulin heavy chain (H) constant (C) region gene (C\(_H\)) is altered from expressing the C\(_\mu\) region to one of downstream C\(\gamma\), C\(\epsilon\), or C\(\alpha\) segments, resulting in a switch from IgM isotype to generate IgG, IgE, and IgA antibodies, respectively. Thus, the isotype of the antibody is changed and its effector functions, without a change in antigen specificity (Honjo et al., 2002).
SHM takes place in the variable (V) region of both H and light (L) chain genes, where millions of point mutations occur in the V region to generate high affinity antigen binding sites (Li et al., 2004, Honjo et al., 2002). Generally, on first encounter with a foreign antigen, the B cells that recognize it do so with low affinity. During proliferation in germinal centers, the antigen-recruited B cells undergo a period of somatic hypermutation, giving rise to a small proportion of cells expressing high-affinity receptors. These B cells, expressing receptors of improved affinity, are selectively expanded in an antigen-mediated process, giving rise to affinity maturation (McHeyzer-Williams & McHeyzer-Williams, 2005).

**Immunoglobulins**

In humans, there are five classes of immunoglobulins (IgM, IgG, IgA, IgE and IgD). The serum levels vary considerably for Igs of the different classes.

IgG is the most abundant, making up about 85% of total serum Ig levels in humans (reviewed Manz et al., 2005) probably due to its longer half life (Zinkernagel & Hengartner, 2001). IgG is the predominant isotype in secondary immune responses that are characterised by high affinity isotype-switched antibodies (Liu et al., 2010). IgG comprises four subclasses, composed of IgG 1, 2, 3 and 4 (Schroeder and Cavacini 2010) according to their decreasing serum concentrations (IgG1:66%, IgG2:24%, IgG3:7%, and IgG4:3%). The subclasses of IgG display substantial differences in their ability to mediate effector responses, contributing to variable activities of antibodies against microbes, e.g. IgG1 and -3 are more efficient at complement fixation (IgG3>IgG1>IgG2), while IgG4 does not fix complement. In addition, responses against different antigens differ with respect to the nature of the antigen. In humans, anti-carbohydrate specificities tend to be IgG2, while anti-protein as well as anti-viral antibodies often are IgG1 and IgG3 (reviewed Manz et al., 2005).
**IgM** is the first isotype expressed during B cell development, and is the major isotype in primary antibody responses, thus frequently used as a maker of infection (Schroeder & Cavacini, 2010). It constitutes about 10% of human plasma Igs, and is the second most common isotype in the mucosa after IgA. Owning to its pentameric structure and high avidity, it is efficient at opsonising pathogens and mediating their destruction by fixing complement (Schroeder & Cavacini, 2010). IgM is particularly effective at neutralizing and agglutinating blood pathogens, especially those residing within cells, thus preventing cell-to-cell transfer (Racine & Winslow, 2009).

**IgA** exists in two forms (IgA1 and 2) and accounts only for about 7-15% antibody in human serum, but it is the predominant class of antibody in extravascular secretions (Schroeder & Cavacini, 2010).

**IgD** is rare, making up about 0.3% of serum Igs, owing to its low rate of synthesis and its high susceptibility to degradation. Its function remains poorly understood, even though it serves as one of the receptors on mature B cells (reviewed Manz et al., 2005).

**IgE** is the lowest in concentration of all the serum Ig classes, accounting for 0.02% of total serum antibodies and with the shortest half-life. Despite this, it is a very potent Ig. It is associated with hypersensitivity and allergic reactions as well as with the response to parasitic worm infections (Schroeder & Cavacini, 2010).

The primary function of Igs is to bind antigen, resulting in a direct effect, e.g. neutralization. However, binding to antigen can also be ineffective, unless secondary effector functions are recruited, including Fc-receptors (FcRs), and complement components amongst others (reviewed Daëron, 1997).
**Fc Receptors**

Receptors for immunoglobulins (Fc-receptors: FcRs) are widely spread throughout the immune system. They are expressed on a wide range of immune cells of both myeloid and lymphoid origins, as well as on non-immune cells, including endothelial cells, placental tissue and synovial and mesengial cells (van der Pol & van de Winkel, 1998). By binding to the antibody Fc-portion, FcRs serve as key immune modulators in bridging the innate and adaptive immunity, through linking specific antigen recognition by antibodies with cellular effector mechanisms triggered by innate immune cells, such as mast cells, neutrophils, monocytes and macrophages. FcRs exist for every class of antibody: FcγR binds IgG, FcαR binds IgA, FecR binds IgE, FcµR binds IgM, and FcδR binds IgD. They recognize the Fc portions of antibodies, and the affinity with which they bind can either be of low or high affinity. Generally, high affinity FcRs bind noncomplex monomer Igs, while low affinity FcRs bind aggregated Igs or antibodies complexed to multivalent antigens with a high affinity. However, high- and low-affinity FcRs trigger cell responses with equal efficiency, even though the order of events differs. Monomeric antibodies bind high affinity FcRs before they are complexed by multivalent antigens while antibodies are complexed to antigen before they bind low affinity FcRs (reviewed Daëron, 1997, reviewed Flesch & Neppert, 2000). The receptors for IgG have been the most studied and fall into three main classes, each consisting of several individual isoforms: FcγRI also known as CD64, FcγRII (CD32), and FcγRIII (CD16). FcγRI and FcγRII are coded for by three different genes (A B, and C) and FcγRIII (CD16) by two genes (A and B). FcγRs in humans are expressed on a variety of cell types derived from the bone marrow, such as B cells, monocytes, macrophages, neutrophils and platelets. On a functional level, FcRs receptors are classified based on the affinity of interaction with their ligands or the type of signaling pathway initiated on FcR cross-linking (reviewed Nimmerjahn & Ravetch, 2008). FcγRIIA/B/C and FcγRIIIA/B have a low affinity for IgG while FcγRI displays a higher affinity. FcγRI binds monomeric IgG1, -3 and -4 whereas the other 2 classes interact mainly with immune complexed IgG (reviewed Flesch & Neppert 2000). With regard to their signaling pathways, FcγIIB is inhibitory, while the rest trigger activating signaling pathways, except for FcγRIIIB, which has no signaling function (reviewed Nimmerjahn & Ravetch, 2008). Interaction between antibodies of a given class and the corresponding FcR results in a variety of cellular responses, such as phagocytosis,
antioxidant production, antibody-dependent cellular cytotoxicity (ADCC) and the release of inflammatory mediators. Phagocytic FcRs include FcγRI, IIA and IIIA, while IIB negatively regulates phagocytosis. The paired expression of activating and inhibitory FcRs (with the exception of NK cells) on the same cell is of crucial importance in the generation of balanced immune responses (Nimmerjahn & Ravetch, 2007, Schroeder & Cavacini, 2010).

**Antibody Affinity/ avidity**

Generally, the development of the humoral immune response is characterised by affinity maturation (the increase in the affinity of antigen-specific antibodies during the course of immune responses), a central feature of humoral immunity. Affinity is the binding strength between an antigenic determinant in an antigen (epitope) and an antigen-binding site in an antibody (paratope) (Kumagai & Tsumoto, 2001). Antibodies elicited in humans or experimental animals by infection or immunization, tend to be heterogeneous with regard to affinity. As such, polyclonal antibodies are considered to be a mixture of antibody subpopulations of different affinities; with low affinity subpopulations predominant during acute infections, but as time after infection elapses, high affinity B-cell clones dominate the population of responding cells (Ferreira & Katzin, 1995). Antibody avidity, also referred to as functional affinity, is an important feature of the humoral immune response (Leoratti et al., 2008, Ferreira et al., 1996). This is related to both the affinity of the interaction and the valency of the antibodies (Kumagai & Tsumoto, 2001). The measurement of the force between the antigen and the antibody can reflect the quality of the antibodies involved in protection or in different phases of disease. Increased avidities and on-rates of antibodies have been postulated to provide increased *in vivo* effectiveness and protection (Bachmann et al., 1997). The avidity of antibody-antigen binding has been shown to vary according to the time of infection and the class of antibody produced. In fact, the valency of the antibody can compensate for low affinity, as is the case with monomeric IgM versus its pentameric form.
Human red blood cells and membrane architecture

The erythrocyte membrane primarily consists of a phospholipid bilayer contributing to the bending resistance while maintaining the cell’s surface area and an underlying two-dimensional network of spectrin molecules with shear elastic properties (Lim & Li, 2011). The membrane skeleton is organized as a hexagonal lattice of junctional complexes; each composed of the central component of short actin filaments, which are cross-linked by long, flexible spectrin molecules (reviewed An & Mohandas, 2008).

![Diagram of the red cell membrane](image)

Figure 1: A schematic representation of the red cell membrane (Mohandas & Gallagher, 2008).

Spectrin heterodimers constitute the crossbeams of the molecular architecture and self-associate head-to-head, to form tetramers. The tails of the spectrin heterodimers are linked into a junctional complex that contains actin oligomers, protein 4.1R, tropomyosin, adducin, dematin (protein 4.9), p55 and tropomodulin. The membrane skeleton is connected to the overlying plasma membrane via a series of vertical interactions with different integral membrane proteins, including ankyrin, and band 3. Protein 4.2 and Rhesus proteins help in
stabilizing the vertical interactions between spectrin, ankyrin and band 3. Additional support is provided by protein 4.1R and p55, which form a complex with glycoporphin C (An & Mohandas, 2008, Maier et al., 2009). This membrane skeleton network, which underlies the cytoplasmic surface of the plasma membrane, confers flexibility and strength to the membrane. With a diameter of approximately 8 µm, a RBC is capable of transiting through the 1-2 µm of microcapillary spaces in venules and spleen. Analyses of normal and modified RBCs indicate that the unfolding and refolding of distinct spectrin repeats accounts for the remarkable elasticity of the normal red cell membrane, and hindrance of the unfolding and refolding results in increased membrane rigidity. While the vertical linkages between bilayer and membrane skeleton play a critical role in maintaining membrane cohesion, the lateral linkages between spectrin dimers and between spectrin-actin-protein 4.1R are the dominant regulators of membrane mechanical stability. Thus, the maintenance of both membrane cohesion and membrane mechanical stability is critical for the red cell to maintain its redundant surface area that is critical for it to undergo extensive deformations (reviewed An & Mohandas, 2008). Any disruptions or perturbations to the lipid bilayer, spectrin network and/or membrane proteins would lead to a change in cell morphology and deformability (Lim & Li, 2011). This would in turn result in rigid and less deformable membranes that are fragile and thus prone to fragmentation and subsequent clearance by the spleen. Alternatively, an increase in the number of linkages because of mutations would also increase membrane rigidity (reviewed An & Mohandas, 2008). Changes in the rheological properties of RBC membrane are often associated with various hemolytic anaemias (Diez-Silva et al., 2010).
Malaria

Malaria, caused by protozoan parasites of the genus *Plasmodium* is an ancient disease that continues to plague the world and take a toll on human existence. It remains one of the world's greatest public health problems today. An estimated 225 million cases are reported annually globally, resulting in approximately 781 thousand deaths, 85% of which occurred in children below the age of five. Overall, 91% of these deaths are reported in the African region (WHO, World Malaria Report, 2010). Five species of *Plasmodium* infect man namely: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Cox-Singh et al., 2008, Prudencio et al., 2006). In addition to these, there are over a 100 *Plasmodium* species that infect a variety of other hosts, including reptiles, rodents, birds, primates and other mammals. *P. falciparum* is so far the deadliest of the five species, and is responsible for most of the morbidity and mortality associated with malaria (Prudencio et al., 2006). *P. knowlesi* could become potentially dangerous if control measures are not taken.

The parasite and its life cycle

Malaria parasites are obligate intracellular parasites with a complex life cycle involving both a vertebrate (e.g. man) and an invertebrate host (mosquito vector), where they undergo different developmental stages. The life cycle of *P. falciparum* (and other plasmodia) starts when sporozoites enter the mammalian host through the bite of an infected female *Anopheles* mosquito (Fig. 2). Sporozoites enter the blood vessel at the site of the bite and travel through the blood stream to the liver where they invade parenchymal cells and develop into a liver stage parasite.

For two of the five human species (*P. vivax*, *P. ovale*), dormant hypnozoite forms can develop in the liver, leading to delayed clinical attacks months or years later, but for *P. falciparum*, there are no dormant forms. In the liver, parasite replicates within the hepatocytes and upon maturation, several thousands of merozoites are released into the blood stream.
The erythrocytic cycle is initiated upon merozoite invasion of erythrocytes, initiating a replication cycle of 48 h (P. falciparum, P. vivax, and P. ovale), 72 h (P. malariae) or 24 h (P. knowlesi), during which a single invaded merozoite develops into “ring” trophozoite, mature trophozoite, and finally a schizont. Each erythrocytic schizont, depending on the species, releases approximately 6 to 32 merozoites, which then in turn initiate a new asexual cycle by infecting other RBCs. This is the phase of the parasite’s life cycle responsible for the observed clinical symptoms and the resulting mortality due to malaria. At this stage, the parasite is known to cause a number of changes on the red cell membrane including the transport of parasite proteins across the parasites’ plasma membrane into the parasitophorous vacuole and beyond (Berzins & Perlmann, 1996, Maier et al., 2009). Some of these proteins are inserted into the erythrocyte membrane or become associated with the erythrocyte cytoskeleton. The surface-exposed proteins are important in host-parasite interactions and
constitute important immune targets. In addition, antibodies might reach the parasite via the parasitophorous duct to mediate their effects (Kirk, 2001, Berzins & Perlmann, 1996). Some of these changes are responsible for the virulence of the parasite and the associated pathology seen during falciparum infections (Fig. 2). The intra-erythrocytic parasite at this stage is shielded by the parasitophorous vacuole membrane and the RBC membrane and is thus protected from direct attack by cells of the immune system (Berzins & Perlmann, 1996). In addition, at about this time, parasitised RBCs are detectable microscopically, and symptoms of disease occur, which are often characterised by fever, chills, headache, lassitude, and gastro-intestinal symptoms.

The *Plasmodium* life cycle continues when some merozoites develop into the sexual parasite stages, the male and female gametocytes, which can be taken up by mosquitoes during subsequent blood meals. The gametocytes undergo fertilization and maturation in the mosquito midgut, forming an infective ookinete form that migrates through the mosquito midgut into the hemocele and develops into an oocyst in which sporozoites are formed. When fully matured, the oocysts burst and release sporozoites that migrate into the mosquito's salivary glands, ready for the next transmission step (Sargeant *et al.*, 2006, Prudencio *et al.*, 2006).

**The disease: clinical manifestations of malaria/ Pathogenesis of malaria**

The signs and symptoms of malaria and their gravity in humans are caused by the asexual blood stage of the malaria parasite and are quite diverse with a wide range of outcomes and pathologies (Weatherall *et al.*, 2002).

In fact, the spectrum of the disease may range from mild asymptomatic infections, through symptomatic (fever), to severe disease, and this could be greatly influenced by the infecting parasite, host genetic, geographic, nutritional and social factors (Miller *et al.*, 2002). The fact
that some individuals remain asymptomatic reflects the ability of adaptive immune mechanisms to prevent disease.

The initial symptoms of the disease are also quite variable, particularly in children, and may include irregular fever, malaise, headaches, muscular pains, sweats, chills, nausea and vomiting (Odongo-Aginya et al., 2005). However, the principal symptom is fever. The classical fever paroxysms last 8-12 hours and occur in three stages:-
- The ‘cold stage’, which is characterised by marked vasoconstriction, lasts for 30 minutes to 1 hour. The patient feels intensely cold and uncomfortable and there is marked shivering though the temperature rises rapidly, often as high as 41°C.
- The ‘hot stage’ follows abruptly and lasts for 2-6 hours. The patient feels intensely hot and uncomfortable with intense headache and delirium may be present.
- The sweating stage then occurs with profuse sweating and a drop in temperature. The patient feels fatigued and exhausted, but otherwise well and often sleeps off. Headache and other pains are relieved until the next rigour. However, in some instances, there might be an overlap in symptoms and signs between malaria and other acute febrile illnesses such as influenza or gastro-intestinal infections (Rooth & Björkman, 1992, Font et al., 2001). Redd et al. (1992) observed that 95% of children with a clinical definition for pneumonia also met the malaria case definition. In a small fraction of children, the disease may present with more severe disorders (severe malaria).

**Severe falciparum malaria**

Annually, severe malaria (SM) causes close to one million deaths, most of which occur in African children less than five years of age (WHO, 2000). It encompasses the major life-threatening clinical syndromes in malaria patients and includes cerebral malaria (CM), severe malarial anaemia (SMA), respiratory distress syndrome (RDS) and placental associated malaria (PAM) in pregnant women (Miller et al., 2002, Weatherall et al., 2002, Schofield and Grau, 2005). Only *P. falciparum* causes the above-mentioned complications and, in some cases, more than one syndrome may be present in a single individual. Hence, the disease might be accompanied by single organ, multi-organ and or a systemic involvement.
**Cerebral malaria**

Cerebral malaria is defined by an unarousable coma not attributable to other causes, with any level of parasitaemia. It is a major cause of death in *falciparum* infection. Approximately 1% of the infection progresses to CM. The greater majority of cases occur in sub-Saharan Africa, and approximately 10-20% of individuals who develop CM die. Meanwhile, despite adequate treatment, survivors develop neurological sequelae. These sequelae are more common in children.

A key feature of this manifestation is the sequestration of parasitised red cells, mediated by PfEMP1, in the microvasculature of various organs, most notably in the brain (reviewed Good et al., 2005). The massive parasite sequestration in the brain microcapillaries, largely through binding to intercellular adhesion molecule 1 (ICAM-1) (Smith et al., 2000) causes local hypoxia, vascular occlusion, inflammation and damage of the blood brain barrier (Miller et al., 1994).

Rosetting is another adhesion property demonstrated by *P. falciparum* isolates, mediated by PfEMP1 (Kyriacou et al., 2007). Rosetting is the spontaneous binding of uninfected erythrocytes to erythrocytes infected with mature asexual parasites. Rosette formation has been associated with cerebral malaria (Carlson et al., 1990).

**Respiratory distress syndrome**

The pathogenesis of respiratory distress syndrome (RDS) in malaria is poorly understood. It is defined as the presence of tachypnea (abnormally rapid breathing) and one or more of nasal flaring, in drawing, grunting or deep breathing in life-threatening malaria (Menendez et al., 2000). It is most commonly associated with metabolic acidosis, usually involving lactic acidosis, which has been shown to be a poor prognostic sign in children with severe malaria (Menendez et al., 2000, Marsh et al., 1995). The main clinical sign of RDS is deep breathing caused by attempts to release CO₂ to compensate for acidosis. There are several causes of
lactic acidosis in children with severe malaria ranging from: - (1) Reduced oxygen delivery to tissues as a result of anaemia, causing increased tissue production of lactate; (2) Decreased clearance of lactate as a result of reduced liver function in malaria; (3) Effects of pro-inflammatory cytokines, e.g. TNF may contribute to increased lactate production; (4) Lactate production by *P. falciparum* (through direct stimulation by cytokines) represents a minor contribution in this context (Miller *et al.*, 2002, reviewed Good *et al.*, 2005).

**Pregnancy associated malaria and anaemia**

Pregnant women are particularly vulnerable to the effects of malaria, especially primigravid women, despite previously acquired protective immunity (reviewed Hviid & Salanti, 2007). They are more likely to be infected with *P. falciparum* than their non-pregnant counterparts, and once infected; there is a tendency towards increased disease severity. This was once speculated to be caused in part by the transient immuno-suppression or –modulation that is characteristic of pregnancy, in order to sustain the foetal allograft (Menendez, 1995, reviewed Hviid & Salanti, 2007). However, pregnancy-induced immune-modulation has been shown to mainly affect the cellular arm of immunity, whereas the humoral immunity is largely unaffected (reviewed Hviid & Salanti, 2007). The effects of malaria on pregnant women may differ with regard to the level of acquired immunity, gravidity, trimester of pregnancy and the presence or absence of co-morbidity (reviewed Duffy, 2007, reviewed Lagerberg, 2008). An adverse consequence of infection with *P. falciparum* during pregnancy (pregnancy-associated malaria-PAM) is the sequestration of malaria parasites in the placenta resulting in placental malaria. Placental malaria is thus defined as the accumulation of *Plasmodium* infected erythrocytes in the intervillous spaces in the placenta, causing histological changes, including leukocyte induced damage to the trophoblastic basement membrane (Uneke, 2007). One major outcome of infection is maternal anemia, which may lead to maternal death. Globally, each year, out of an estimated 50 million women living in malaria-endemic countries who become pregnant, 10000 die because of PAM, and over half of these deaths are due to SMA (http://www.who.int/malaria/high_risk_groups/pregnancy/en/index.html). The effects on the developing fetus and newborn include intrauterine growth retardation, infant anaemia and low birth weight (LBW: due to preterm delivery). Infact, LBW is an important determinant of
infant mortality (Rogerson et al., 2003, Duffy, 2007). Around 19% of infant LBWs are due to malaria, 6% of whom die (Guyatt & Snow, 2005). An estimated 200 000 infants are believed to die as a direct result of PAM each year (Steketee et al., 2001, http://www.who.int/malaria/high_risk_groups/pregnancy/en/index.html). The accumulation of infected erythrocytes in the maternal circulation or intervillous spaces of the placenta occurs through adhesion of infected erythrocytes to placental receptors, such as chondroitin sulfate A (CSA) on the syncytiotrophoblasts surface or within the intervillous space. The clonally variant parasite protein, PfEMP1, which is expressed on the surface of infected erythrocytes, mediates infected erythrocyte adhesion. The main ligand for placental sequestration is the PfEMP1 variant, Var2CSA, which mediates strong binding to CSA (reviewed Rogerson & Boeuf, 2007).

**Malarial anaemia**

The blood stages of *P. falciparum* inevitably cause life-threatening anaemia (Miller et al., 2002). Compared to CM, not much is known about the host and parasite factors that contribute to malarial anaemia. A haemoglobin value of less than 5 g/dL is considered to represent severe disease (Weatherall et al., 2002, reviewed Good et al., 2005). Several factors may contribute to malaria anaemia. Since anaemia is the running theme for this piece of work, it will be discussed in detail in the following sections (see the section “Related Background”).

**Immunity to malaria**

The immune response in humans against natural infections due to malaria parasites is complex and varies with the level of endemicity, genetic makeup, age of the host and parasite stage and species. In addition, repeated infections and continued exposure are required to achieve clinical immunity (Marsh, 1992). In endemic areas, children born to immune mothers rarely are infected during the first 6 months of life. They are presumed to be protected by transplacentally transferred maternal antibodies. However, this protection is lost during early childhood, rendering infants susceptible to severe forms of the disease with high mortality until the age of about 3-5 years. Once this stage of high parasitaemia infection is passed,
acquired immunity gradually develops. Nevertheless, this acquired “anti-disease” immunity is not stable and requires exposure to repeated infections over many years. However, despite life-long exposure, this developed immunity is not sterile, as individuals usually show low-grade parasitaemia without apparent clinical symptoms. In these circumstances, the protective immune response is hypothesised to only limit the parasitaemia, but not clear the infection completely. Clinical immunity is usually lost during pregnancy, especially among primigravidae women, or after migration to areas where the disease is not endemic (Ferreira et al., 2004, Marsh, 1992).

The mechanisms of acquired immunity are poorly understood, but are thought to be cell mediated as well as humoral. Both antibodies and T cells are required for naturally acquired immunity; CD4+ and CD8+ T-cell responses are particularly effective against intracellular liver-stage parasites, while antibodies may block host cell invasion by sporozoites and merozoites. In addition, antibodies to parasite-derived antigens expressed on the surface of RBCs may facilitate phagocytosis (opsonisation) of infected cells or their complement-mediated lysis. Such antibodies may also inhibit or revert the adhesion of infected RBCs to endothelial receptors/sequestration as well as inhibit the intra-erythrocytic parasite growth (reviewed Beeson et al., 2008, Ferreira et al., 2004, Ahlborg et al., 1996).

An early response by monocytes or macrophages to parasite toxins is known to induce fever and other non-specific immune mechanisms, which lead to reduction of blood stage parasite densities (Marsh, 1992). Extensive antigenic variation, immune-dominance of non-protective antigenic structures together with low immunogenicity of epitopes critical for protection, could probably explain the inability of the host to develop a sterilising immunity (Ferreira et al., 2004, Anders, 1986).

**Acquired immunity to malaria**

Acquired immunity to malaria involves both the humoral and cell mediated arm of the adaptive immune system. The specific immune response to malaria may limit the rising
parasitaemia, and with exposure to a sufficient number of parasite strains, it may eventually confer resistance to disease but not infection. Consequently, asymptomatic parasitaemias are commonly found in older children and adults living in hyper- or holoendemic areas.

Endemicity is traditionally defined in terms of the spleen rate and parasite rate and has been classified by the WHO as hypo-, hyper- and holoendemic areas (Wickramasinghe & Abdalla, 2000). In holoendemic areas, the spleen rate in children is > 75% and that in adults is low. Transmission is intense and continuous, and there is a considerable morbidity and mortality from malaria in the first 2-3 years of life, followed by the acquisition of immunity. In hyperendemic areas, spleen rates are >50% in children and >25% in adults. Transmission is intense but seasonal, immunity within the population is low, and symptomatic disease can be observed in all age groups. In hypoendemic areas, the spleen rate in children is less than <10% and transmission is weak (unstable). Epidemics can occasionally occur in such areas.

**Humoral immunity to malaria**

Antibody responses against erythrocytic stages in malaria are a crucial component of naturally acquired immunity, being instrumental for the clearance of infection. Evidence for the importance of antibody responses in malaria was earlier demonstrated by classic experiments in which passively transferred sera or purified immunoglobulins (IgG) from adult residents in hyperendemic regions of malaria conferred protection in malaria-naive recipients (Cohen et al., 1961, McGregor et al., 1963, Sabchareon et al., 1991). In malaria, acute infections are usually associated with a non-specific polyclonal B cell activation. It has been estimated that only 6% of the IgG and 11% the IgM are specific against malaria antigens, and a high proportion of those antibodies are unrelated to protection (White & Plorde, 1991).

Protection has been mainly associated with IgG, and this seems to be largely dependent on the balance between cytophilic (IgG1 and IgG3) and non-cytophilic (IgG2 and IgG4) antibodies (Bouhroun-Tayoun & Druilhe, 1992, Aribot et al., 1996). The predominance of cytophilic IgG1 and IgG3 in individuals living in endemic areas has been associated with either lower
parasitaemia or a lower risk of malarial attacks (Sarthou et al., 1997, Tangteerawatana et al., 2001). However, IgG2 antibodies to certain antigens have been associated with protection in some studies (Aucan et al., 2000). IgG4 antibodies, on the other hand, are non-protective, and might instead compete with cytophilic antibodies, thus inhibiting certain effector mechanisms (Warmerdam et al., 1991, Garraud et al., 2003).

Anti-malarial IgE antibodies seem to mediate both pathogenic and protective responses during malaria (Duarte et al., 2007). Both the levels of total IgE and of IgE anti-plasmodial antibodies are elevated in human and experimental malaria infections, but their role in protection and/or pathogenesis in this context is not yet well-established. Total and anti-malarial IgE have been shown to be higher in patients with cerebral malaria than in patients with uncomplicated malaria (Perlmann et al., 1994, Perlmann et al., 2000). However, high levels of anti- \textit{falciparum} IgE have also been associated with a reduced risk of acute malaria in Tanzania (Bereczky et al., 2004). IgE-containing immune complexes are thought to be pathogenic via cross-linking of IgE receptors on monocytes/macrophages, with subsequent overproduction of local TNF-\textgreek{a}, a major pathogenic factor in malaria (Perlmann & Troye-Blomberg, 2000).

The mechanisms by which \textit{Plasmodium}-specific antibodies mediate their effects is by either specifically binding to antigens on the surface of RBCs or on the merozoites, thereby blocking invasion, or inhibiting schizont development, either alone or in cooperation with ancillary cells (e.g. monocytes in ADCI) (Bouharoun-Tayoun et al., 1995). Parasite neutralization may also be mediated by antibody-dependent phagocytosis, involving monocytes or polymorphonuclear leucocytes or by initiating complement-mediated damage. Antibodies may also prevent the adherence of infected cells to endothelial surfaces or rosetting of parasitised RBCs to uninfected RBCs, thereby preventing severe disease (reviewed Wipasa et al., 2002, Jafarshad et al., 2007). Since protective immunity to malaria seems to be associated with different antibody classes and subclasses, the antibody quality and effector function will therefore be important in defining the antibody immune response in infected individuals.
**Cell- mediated immunity to malaria**

Cell-mediated immunity (CMI) plays a very important role in malarial immunity. The CD4$^+$ T-cell subset is of major importance for the induction of blood-stage immunity, while the CD8$^+$ subset has been shown to be cytolytic against liver stages of the parasite (Oliveira-Ferreira & Daniel-Ribeiro, 2001). Both Th1 and Th2 cells are involved in protective immunity against blood stage malaria, and the balance of cytokines produced by these two subsets appears to be crucial in determining the disease outcome. During the initial phases of infection, Th1 cells are the main players, being important in the initial control of primary parasitaemia, while a shift to Th2 later during the course of infection is crucial for the eventual resolution of infection via B cell/T cell cooperation and subsequent antibody production (reviewed Troye-Blomberg et al., 1994, Wipasa et al., 2002). A potential role for $\gamma\delta$ T cells in protection has also been described, involving a granulysin mediated mechanism (Farouk et al., 2004). Although the CD4$^+$ T cells are critical for protection, they are also implicated in the pathogenesis of lethal complications (reviewed Wipasa et al., 2002).

**Innate immunity to malaria**

Innate immune responses are the first line of defense against pathogens, and have an important role in malaria. Recent evidence suggests that Toll like receptors (TLRs) are involved in the innate immune responses to malaria infections. *P. falciparum* blood-stage parasites have been shown to activate human plasmacytoid dendritic cells (DCs), as well as murine DCs, through MyD88- and TLR9-dependent pathways (Coban et al., 2005). Furthermore, glycosylphosphatidylinositol (GPI) from *P. falciparum* has been reported to interact with immune cells through the activation of TLR2 and TLR4 (Parroche et al., 2007).

Variation in the host response to infection is known to have a genetic basis. Studies have also shown that, individuals with certain red blood cell disorders are to some extent protected from malaria.
Genetic disorders such as sickle cell trait, thalasaemia, and glucose-6-phosphate dehydrogenase (G6PD) affect parasite survival and provide resistance to the host, probably by impeding the intraerythrocytic development of the parasite. West African children with the African form of G6PD had a 46-68% reduction in the risk of severe malaria, and HbS carriers had more than 90% protection from severe malaria (Yuthavong & Wilairat, 1997, Weatherall et al., 2002). Persons with ovalocytosis have lower parasite densities following infection with *P. vivax* and *P. falciparum*, due to the elliptical red blood cells resisting parasite invasion.

There is also evidence that HbC protects against severe *falciparum* malaria. Studies performed in Burkina Faso showed HbC to be associated with a 29% reduction in the risk of clinical malaria in HbAC heterozygotes, and with 93% in HbCC homozygotes (Modiano et al., 2001). In addition, HbF in new borns inhibits *P. falciparum* development (Miller et al., 1994).

Associations between the severity of *P. falciparum* malaria and the antigens of the ABO blood group have also been reported. In a Gambian study, a weak but significant association of blood group O with resistance was found (Hill, 1992). Indeed, rosetting has been shown to be reduced with blood group O erythrocytes compared with the non-O blood groups (A, B, and AB) in *P. falciparum* laboratory strains and field isolates (Barragan et al., 2000). Rosetting is a known parasite virulence factor that is thought to contribute to the pathogenesis of severe malaria by obstructing the microvascular blood flow (Rowe et al., 2007).

Non-specific phagocytosis and clearance of parasitised red cells from the circulation by the action of the reticulo-endothelial system in the liver and the spleen may also contribute to innate resistance to malaria infection (Looareesuwan et al., 1991).

Also, there is evidence that both *P. malariae* and *P. vivax* can provide some protection against subsequent *P. falciparum* episodes, or at least prevent severe symptoms, suggesting that cross-species immunity between *falciparum* and *vivax* might be capable of controlling *P. falciparum* parasite densities (May et al., 1999, Smith et al., 2001). In contrast, the *P. malariae*, effect is thought to be a consequence of down regulation of the cytokine production by this parasite (Black et al., 1994).
RELATED BACKGROUND

General introduction to anaemia

Compared with available knowledge on the pathogenesis of CM, relatively little is known about the host/parasite factors that contribute to the etiology of anaemia during malaria infection and the mechanisms involved. Severe anaemia can be observed in patients despite low levels of parasitaemia during infection, or as a result of chronic subclinical infection, and can persist for weeks following complete therapeutic clearance of parasites (Abdalla et al., 1980; Weatherall & Abdalla, 1982; Phillips & Pasvol, 1992; Camacho et al., 1998; Weatherall et al., 2002).

The *P. falciparum* life cycle includes a non-pathogenic, asymptomatic hepatic stage (extrerythrocytic), which is followed by the invasion of mature erythrocytes by infective forms (merozoites) and the initiation of the pathogenic intra-erythrocytic stage. As the parasite develops within the host’s erythrocytes, a number of changes take place, including the remodeling of the red cell membrane. Host cell remodeling includes the development of electron dense protrusions on the infected RBC surface called knobs, which are composed of the knob-associated histidine-rich protein (KAHRP). Apart from knob formation, the remodeling process facilitates the import of nutrients into the parasite, as well as asexual reproduction within the host cell. Knob formation also results in the rigidity of the cell, as they serve as anchors for the insertion of surface-exposed parasite antigens (reviewed Craig & Scherf, 2001, Sargeant et al., 2006). The significance of these changes has not been fully dissected, but the development of immunity to malaria is thought to involve responses to malaria antigens expressed at the surface of the infected erythrocytes. A number of such proteins have been described to date, some of which have been implicated in the pathogenesis of severe disease, such as PfEMP-1, which is involved in antigenic variation, cytoadherence and sequestration, leading to different outcomes depending on sequestration site (reviewed Craig & Scherf, 2001).
More recently, the parasite protein RAP-2/RSP-2, on which part of this thesis is based, has been implicated in the development of SMA. Studies by Layez et al. (2005) demonstrated that the immune response against RAP-2/RSP-2 could lead to the destruction of normal RBCs and bone marrow erythroid precursor cells through mechanisms such as opsono-phagocytosis and complement activation. The immune mechanisms underlying severe malaria anaemia are poorly understood, but apparently involve both humoral (e.g. antibody and/or complement) as well as cellular (e.g. cytokines) immune responses. Hence, the immunologic basis of SMA is of particular interest because of its potential relevance to malaria vaccine development. This is especially so, following observations from vaccine studies, which suggest that immunized monkeys which acquired protection from acute infection, may succumb to anaemia during a subacute or chronic phase of infection (Egan et al., 2002, Jones et al., 2002). In addition, data by Layez et al. (2005), suggesting the possible involvement of the RAP-2/RSP-2 protein in the development of SMA, also emphasizes the importance of studies on the immunologic basis of SMA. Hence, an improved understanding of the pathogenic basis of severe malarial anaemia and the mechanisms involved will have important implications in the design, development and deployment of safe and effective interventions against malaria, including vaccines.

The epidemiology/prevalence of malaria-related anaemia

As earlier mentioned, malarial anaemia is a common complication during malaria infections in endemic areas. Its prevalence and/or severity is determined by a number of interacting factors, including: the species of the infecting parasite; the intensity of transmission (endemicity); age and pregnancy status of the host; associated host genetic factors; and causes of anaemia other than malaria, e.g. hookworm infections (Abdalla & Pasvol, 2004). SMA varies geographically (Nagel, 2002), and is most frequently seen in areas with very high malaria transmission, and most commonly in young children and pregnant women (Greenwood, 1997). Anaemia is defined as a haematocrit <0.33 as measured in community surveys in malaria endemic areas of Africa. Its prevalence varies between 31 and 91% in children and between 60 and 80% in pregnant women (Menendez et al., 2000, Schellenberg et al., 2003, Casals-Pascual & Roberts, 2006). The severity, incidence and predominant age for
the onset of SMA are dependent on the intensity of malaria transmission. In areas of high and stable malaria transmission, children between the ages 1 and 3 years present with SMA as a result of early and frequent exposure and quicker development of immunity. Meanwhile, in less endemic areas, it is common in older children due to less frequent infections and a delay in the acquisition of immunity (Menendez et al., 2000, Schellenberg et al., 2003). The mortality rate of malaria-related anaemia is between 5.6% and 16% for children 4-6 years of age and 6% for pregnant women, especially in primigravidae (Chang et al., 2004). Children born in endemic areas are largely protected from severe malaria in the first 6 months of life by the passive transfer of maternal immunoglobulins and by fetal haemoglobin. Also, the rate at which anaemia develops and its severity is dependent on the level of *P. falciparum* parasitaemia (Achidi et al., 1996). However, asymptomatic low-density parasitaemia may also result in anaemia, particularly if the parasitaemia persists for prolonged periods upon lack of, or ineffective treatment (McElroy et al., 2000). Price et al. (2001) observed that even a single episode of uncomplicated malaria, diagnosed and treated properly, could still cause anaemia, which resolves slowly. In malaria endemic regions, coexistent helminthic infections are common, a major impact of which is aggravated anaemia (Degarege et al., 2010). Therefore, establishing the relative contribution of malaria infection to anaemia is essential for both clinical management and development of prevention strategies.

**Features of malarial anaemia**

**Severe malarial anaemia**

SMA is usually observed at low parasitaemia levels during chronic infections, and even after complete chemotherapeutic clearance of infection (Abdalla et al., 1980, Phillips et al., 1986). According to the WHO, SMA is defined as a haemoglobin (Hb) concentration <5g/dL or a haematocrit (Hct) <0.15 in the presence of a *P. falciparum* parasitaemia >10,000 parasites per µl, with a normocytic blood film. This definition is however considered specific but of low sensitivity, and of limited use both for the clinical management of patients and for epidemiological studies of anaemia. Over a third of the children admitted with severe anaemia
had blood smears negative for malaria parasites, but responded to anti-malarial treatment (reviewed Casals-Pascual & Roberts, 2006). In addition, SMA may occur in acute or chronic infection, accompanied by a background of normal or low haemoglobin. Children with SMA may present with malaise, fatigue and dyspnoea or respiratory distress, defined by tachypnoea and deep gasping breathing (Menendez et al., 2000).

**Hematologic features**

The anaemia of *P. falciparum* is typically normocytic, with a notable absence of reticulocytes. It may be accompanied by changes in white cell and platelet counts. The leukocyte counts are usually low to normal in most cases of malaria. Increased leukocyte counts indicate either a severe infection or secondary bacterial infection. Reduction in the leukocyte counts is attributed to hypersplenism or sequestration in the spleen. Relative lymphocytosis and/or monocytosis are also seen in acute infections. The presence of neutrophils containing pigment is associated with severe disease and unfavourable outcome (reviewed Casals-Pascual & Roberts, 2006).

**Blackwater fever**

Black water fever (BWF) received attention in early studies of expatriates living in endemic areas with low or no immunity against malaria. The syndrome is characterised by severe intravascular hemolysis, haemoglobinemia and haemoglobinuria (Rogier et al., 2003). Its symptoms include a rapid pulse, high fever and chills, extreme prostration, a rapidly developing anaemia, and the passage of urine that is black or dark red in colour (hence the disease’s name). Abdominal pain, jaundice, hepatosplenomegaly, vomiting, and renal failure, especially in adults have also been reported. The pathogenesis of BWF remains unclear, but has been largely associated with the irregular use of quinine for chemoprophylaxis in non-immune individuals and patients with severe *falciparum* malaria, as well as with other related drugs. BWF is also occasionally observed in malaria patients with G6PD deficiencies that are treated with quinine, artemisinin or primaquine (Rogier et al., 2003, Gobbi et al., 2005). BWF
is most prevalent in Africa and Southeast Asia. Individuals with increased susceptibility, such as non-immune immigrants or individuals who are chronically exposed to malaria, are classic sufferers from the complication. BWF seldom appears until a person has had at least four attacks of malaria and has been in an endemic area for six months (Weatherall *et al.*, 2002 Rogier *et al.*, 2003, Gobbi *et al.*, 2005).

**The pathogenesis of malarial anaemia**

Pathogenesis relates to various host and parasite factors that are responsible for causing pathology. Several mechanisms have been implicated in the pathogenesis of severe anaemia including erythrocyte lysis and splenic phagocytosis, increased sequestration of parasitised red blood cells, and immune-mediated erythrocyte destruction, bone marrow dyserythropoiesis and ineffective erythropoiesis, and lower erythroblast proliferative rates and numbers (Wickramasinghe & Abdalla, 2000). The relative contributions to anaemia by the various mechanisms differ according to the age, pregnancy state, anti-malarial immune status and genetic constitution of infected individuals, and the local endemicity of malaria. In general, haemolysis is considered to be of greater importance in non-immune children experiencing acute malaria, whereas dyserythropoiesis is seen in persons experiencing recurrent or frequent *falciparum* malaria, although it is thought that several mechanisms are likely to operate in any one individual. In summary, SMA is thought to arise from mechanisms involving increased destruction of non-parasitised and parasitised RBCs as well as a decreased production of RBCs (Menendez *et al.*, 2000).

**Clearance of infected RBC**

*Plasmodium* parasites reside within host erythrocytes, schizogony and the subsequent release of merozoites invariably leads to intravascular haemolysis. This may suggest that hemolysis of the infected erythrocytes by the parasite is the most likely cause of anaemia. However, the destruction of infected RBCs alone cannot explain the dramatic drop in haemoglobin levels frequently observed in anaemic malarious children. In humans, SMA is typically associated
with parasite burdens that are considerably lower than those required for marked, direct destruction of RBCs (Jakeman et al., 1999).

RBC surface changes are commonly observed in malaria following parasitisation. Normal RBCs have an average diameter of approximately 7.5 µm and possess an amazing ability to elongate, allowing them to squeeze through capillaries with a patent lumen much smaller than their own diameter (Mokken et al., 1992, Dondorp et al., 2004). However, maturation of the parasite inside the RBC progressively abolishes this deformability, whereby the normally flexible biconcave disc becomes progressively more spherical and rigid and the surface becomes irregular with the presence of electron dense knobs. RBCs that become rigid and inflexible because of malaria infection, are held up in the splenic microvasculature (and might contribute to tissue hypoxia) and are subsequently cleared up by the spleen (Burchard et al., 1995, Dondorp et al., 2004).

The pitting of malaria parasites from RBCs has been shown to have a role in controlling malaria infection. Pitting is a phenomenon wherein parasites are removed from erythrocytes and the once infected erythrocyte returned into circulation. On the down side, pitting alters the membrane of the red cells left behind, cells becoming more spherical and less deformable, hence susceptible to removal by stromal cells. Pitting of parasitised RBCs has been proven responsible for the presence of ring-infected erythrocyte surface antigen (RESA/Pf155) on non-parasitised red blood cells (Angus et al., 1997, reviewed Casals-Pascual & Roberts, 2006).

**Band 3** is a RBC transmembrane protein known as the anion channel. It marks senescent RBC for death by triggering the binding of specific auto-anti-band 3 antibodies, with subsequent complement activation. The antigenicity of band 3 is thought to result from its cleavage, clustering and/or from exposure of cryptic epitopes (Santos-Silva et al., 2001). The growth of *P. falciparum* induces profound modifications in the host erythrocyte membrane that lead to increased binding of autologous IgG and complement. Further, the parasite development induces in sequence, deposition of haemichromes and oxidative aggregation of band 3, which become sites for deposition of autologous IgG and complement and hence...
subsequent recognition by phagocytes, a phenomenon typical of what may be seen with normally senescent RBCs (Giribaldi et al., 2001).

In addition, *P. falciparum* development within infected RBCs alters the distribution of phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (reviewed Nagel, 2002). Cells with exposed phosphatidylserine at their surface, probably due to oxidative stress, are hence readily recognized by macrophages, engulfed and degraded (Koka et al., 2007).

**Clearance of normal RBC**

During malaria, many uninfected RBCs are destroyed in the spleen and liver. Uninfected RBC destruction is a major contributing factor to the onset of malarial anaemia. Both mathematical modeling and clinical studies have shown that approximately 12 uninfected RBCs are lost per every parasitised RBC, thus implicating the destruction of normal RBCs as a significant cause of the observed anaemia in malaria (Jakeman et al., 1999, Price et al., 2001). Different mechanisms that target uninfected RBCs for destruction by intravascular hemolysis or for clearance mediated by the reticulo-endothelial system (extravascular hemolysis) remain unclear, but are thought to include increased oxidative damage (Greve et al., 1999, 2000), phosphatidylserine externalisation (Haldar et al., 2007) and reduced deformability of RBCs (Dondorp et al., 1999). The reduction in RBC deformability has been shown to parallel disease severity and to correlate with anaemia, possibly due to splenic clearance. At hospital admission, a severe reduction in cell deformability is seen as a strong predictor of mortality, both in adults and children with severe malaria (Dondorp et al., 1997, 1999, 2002).

However, other mechanisms leading to destruction of normal RBCs may also involve IgG antibodies targeting parasite antigens, such as RAP-2/RSP-2 (Layez et al., 2005) or the *P. falciparum* glycosylphosphatidylinositol (GPI) (Brattig et al., 2008) bound at the RBC surface and which may cause cell destruction by phagocytosis or complement mediated lysis.
Furthermore, during malaria infection, macrophages release reactive oxygen intermediates and nitric oxide, which can cause damage to the parasites as well as to ‘innocent bystanders’, such as uninfected erythrocyte membranes and contribute towards erythrocyte destruction and subsequent anaemia in the host (Kremsner et al., 2000, Griffiths et al., 2001). Increased oxidation of normal RBC membranes has been reported in children with severe P. falciparum malaria (Griffiths et al., 2001).

Normally, human blood cells express complement regulatory proteins such as complement receptor 1(CR1: CD35) and decay accelerating factor (DAF: CD55). A lack of suppression of these proteins has been reported, thus implicating complement mediated phagocytosis of normal red cells as an important contributor to severe malarial anaemia (Stoute et al., 2003, Owuor et al., 2008). Stoute et al. (2003), observed an association of reduced CR1 and an increase in immune complexes on the surface of RBCs and malarial anaemia.

**Decreased production of RBCs**

Although RBC destruction plays a major role in anaemia of acute malaria, reduced production of erythrocytes in the bone marrow seems to be an important factor in maintaining anaemia, which usually takes 3-4 weeks to resolve. In a study of Malawian children, among several aetiologic factors studied, red cell production failure came out to be the predominant pathway leading to severe anaemia (Boele van Hensbroek et al., 2010). Decreased erythrocyte production is thought to involve bone marrow hypoplasia/suppression, dyserythropoiesis, ineffective erythropoiesis, and inappropriately low erythropoietin levels (Nagel, 2002).

RBCs normally stay in circulation for about 120 days and during normal homeostasis, RBC numbers are balanced through the destruction of old RBC by the reticulo-endothelial system (RES) and the production of new RBCs through erythropoiesis. The ability of the bone marrow to compensate for a sudden increase in RBC loss is termed “effective erythropoiesis”. Under the influence of factors such as erythropoietin, haematopoietic stem cells in the bone marrow or spleen (during effective erythropoiesis) multiply and differentiate to produce young and fully functional RBCs, i.e. reticulocytes, which are the earliest RBCs to be released.
into circulation (Schofield & Grau, 2005). Thus, the reticulocyte count reflects an increased bone marrow output to replace the red cell losses. Otherwise, anaemia will inevitably develop when the accelerated removal of erythrocytes is not compensated for by an adequate bone marrow response, as is often the case in malaria. Inadequate erythropoiesis can result from reduced production of erythroblasts (erythroid hypoplasia) or from normal or increased production of erythroblasts, but with an intramedullary cell death (ineffective erythropoiesis).

In malarial anaemia, bone marrow suppression has been described in all malaria patients as well as in asymptomatic infections (Helleberg et al., 2005), and is thought to be responsible for both the degree and the delayed recovery from anaemia (Abdalla et al., 1980). In acute malaria, there is a reduced total erythropoietic activity, as indicated by a normal or reduced marrow cellularity, combined with reduced erythroblast proportions. Meanwhile, in chronic malaria, there is an increase in total erythropoietic activity, as evidenced by an increase in marrow cellularity (erythroid hyperplasia) together with an increased proportion of erythroblasts. The accompanying inappropriately low levels of reticulocytosis, suggest that chronic malaria is associated with a greater ineffectiveness of erythropoiesis than in acute malaria (Wickramasinghe & Abdalla, 2000, reviewed Chang & Stevenson, 2004). RBC iron utilisation, which is a measure of effective erythropoiesis, is reduced in both acute and chronic malaria. During malaria, there is thought to be a shift of iron distribution from functional compartments, comprising metabolically active iron that is required for normal function, towards storage compartments, that constitute an iron reserve. Thus, this suggests the presence of a relative deficit in erythropoietin production or bone marrow unresponsiveness to erythropoietin (Verhoef et al., 2002). Functional studies on dyserythropoietic bone marrows confirmed that there was an abnormal cell cycle distribution of early polychromatic erythroblasts in both acute and chronic malaria. This was characterised by an increased proportion of cells in the G2 phase of the cell cycle, and a reduction in the ratio of the number of cells in the DNA synthesis (S) phase to the number in G2 (S/G2 ratio) (Wickramasinghe et al., 1982). Furthermore, kinetic studies of erythroblasts suggested that all classes of erythroblasts had a prolonged S phase, with a 50% reduction than normal of the rate of production of polychromatic erythroblasts. This was thought to be as a result of a high death rate in this cell compartment (Dörmer et al., 1983). Based on morphological abnormalities of erythroblasts in the bone marrow of patients, the basis for the ineffective erythropoiesis was thought to be due to apoptosis (Abdalla & Wickramasinghe, 1988).
Morphological abnormalities of erythropoiesis (dyserythropoiesis) include multi-nucleated erythroblasts, rupture of the cell nucleus with disintegration of chromatin (karyorrhexis), as well as incomplete mitosis. In a study of Gambian children with malaria, the disturbance in erythropoiesis was found to be confined to the morphologically recognisable erythroblast population (early polychromatic erythroblasts). The prevalence of these abnormalities was more marked in children with chronic than acute malaria (Abdalla & Wickramasinghe, 1988, reviewed Wickramasinghe & Abdalla, 2000, reviewed Chang & Stevenson, 2004).

The mechanisms underlying the perturbation of erythropoiesis and ineffective erythropoiesis are not fully clarified. However, several factors have been implicated, including both direct and indirect effects of factors produced by the parasite.

Effect of soluble mediators (cytokines /chemokines)

Following clinical observations, the nature of the host’s response critically influences the pathologic manifestations of malaria infection. Severe malaria is known to be associated with an acute inflammatory response, characterised by elevated levels of pro-inflammatory cytokines. Moreover, such elevated responses have been linked to the etiology of severe malarial anaemia (Lyke et al., 2004; Cordery et al., 2007).

The macrophage migration inhibitory factor (MIF) is produced by activated T cells and macrophages, and has a wide range of biological activities, including the induction of TNF-α.

MIF also inhibits the anti-inflammatory activity of glucocorticoids. It is released from macrophages in response to Plasmodium infected red cells. Due to its prominent expression in plasma, spleen and bone marrow during experimental malaria, MIF has been implicated in the development of malarial anaemia through erythropoietic suppression (Martiney et al., 2000, McDevitt et al., 2006). In in vitro studies, MIF was found to inhibit the formation of burst forming unit-erythroid (BFU-E) cells (Martiney et al., 2000). MIF-knockout mice infected with P. chabaudi developed less severe anaemia, had better erythroid development (as
evidenced by increases in colony forming unit-erythroid (CFU-E) and BFU-E), and demonstrated an improved survival relative to controls (McDevitt et al., 2006).

TNF-α is an important immunoregulatory molecule in malaria, which on one hand plays an important role in limiting malaria parasitaemia; but on the other hand is responsible for the development of the life-threatening complications of severe malaria. In patients with malarial anaemia, high levels of serum TNF have been reported, and which correlated with the severity of the anaemia (Gandapur & Malik, 1996). Children with severe anaemia were observed to have a depressed reticulocyte response and gross morphologic abnormalities of erythropoietic cells in the bone marrow. In rheumatoid arthritis patients, an increased local production of TNF-α is associated with a low frequency and increased apoptosis of bone marrow erythroid progenitor and precursor cells, and the cytokine is responsible for the anaemia seen in these patients (Papadaki et al., 2002).

The chemokine, Regulated on Activation Normal T-cell Expressed and Secreted (RANTES: CCL5), has been implicated in malarial anaemia. Known roles of RANTES include, promotion of the migration of erythroid precursors into hematopoietic tissues as well as prevention of erythroid progenitor cell apoptosis. Hence, suppression of RANTES may lead to an ineffective erythropoietic response. In a study of Kenyan children, RANTES was observed to decrease during severe malaria anaemia and was associated with the suppression of erythropoiesis (Were et al., 2006).
Haemozoin

Malaria pigment, also known as haemozoin (Hz), is produced by Plasmodium parasites during haemoglobin proteolysis (reviewed Shio et al., 2010). During a malaria infection, the concentration of Hz after erythrocyte rupture may be as high as 100 µg/mL, but it is rapidly cleared from the circulation by the liver and spleen because of its particulate nature. Because of its high concentration in immune tissue, Hz has been suggested to contribute to the systemic inflammatory immune responses seen during malaria infection. Immune cells, including monocytes, macrophages, neutrophils, DCs, and endothelial cells, can interact with and internalize Hz as well as iRBCs, resulting in the up or down regulation of immune-modulatory molecules (reviewed Shio et al., 2010). The Phagocytosis of Hz promotes overproduction of pro-inflammatory mediators, such as TNF-α and NO that may be involved in the suppression of erythropoiesis (Casals-Pascual et al., 2006). In addition, it has been shown that Hz purified from P. falciparum activates macrophages to produce pro-inflammatory cytokines, chemokines, and nitric oxide, as well as enhance maturation of human myeloid dendritic cells (DC) (Coban et al., 2005, Parroche et al., 2007). In children residing in holoendemic areas with high levels of transmission, SMA is associated with high concentrations of circulating Hz, as well as elevated levels of Hz containing monocytes (Casals-Pascual et al., 2006). In a study of Kenyan children with malaria, circulating free and intraleukocytic Hz was found to be associated with anaemia and ineffective erythropoiesis, probably as a consequence of lipid peroxidation attributed to the oxidative properties of heme. Furthermore, this association was independent of levels of the proinflammatory cytokines TNF-α and IFN-γ, which have been implicated in the suppression of erythropoiesis during anaemia (Casals-Pascual et al., 2006). In vitro, 4-hydroxynonenal (4-HNE), a final product of lipid peroxidation generated by haemozoin, was found to inhibit the growth of progenitor erythroid cultures. Thus, 4-HNE, in addition to playing a role in erythrocyte deformability and consequent cell destruction, may also be involved in dyserythropoiesis and anaemia (Giribaldi et al., 2004). In a previous study, it was shown that Hz may inhibit erythroid precursors in vitro at concentrations found in the peripheral blood of children presenting with malaria and anaemia (Casals-Pascual et al., 2006), albeit by an unknown mechanism. Since Hz is also deposited intracellularly in the bone marrows of children with SMA (Wickramasinghe & Abdalla, Chang et al., 2004), the mechanism(s) responsible for decreased erythropoietic
responses are thought to be either direct, or indirect, through the release of soluble mediators from Hz-containing cells (Awandare et al., 2011). However, studies by other investigators showed that in the absence of inflammatory cytokines, Hz could promote the apoptosis of erythroid precursors (Lamikanra et al., 2009). This thus indicates that the accumulation of Hz in the bone marrow could contribute to the severity of anaemia in children with chronic malarial infection, as a result of ineffective erythropoiesis in the bone marrow. Ingested Hz by human monocytes fills up to 30% of their total volume, and can persist unmodified within the monocytes for long periods of time (reviewed Shio et al., 2010).

The spleen and reticuloendothelial hyperactivity

The spleen plays a very important role in the pathophysiology of malaria. One of its functions is in the removal of infected, opsonised or damaged cells in the circulation (Urban et al., 2005). A rapid splenic enlargement in malaria has been associated with its increased capacity to clear both damaged and infected red cells from the circulation, both by Fc-receptor mediated immune mechanisms and by recognition of the reduced deformability, thus limiting the acute expansion of infection. In addition, splenomegaly has been associated with macrophage hyperplasia during malaria (Buffet et al., 2011).

Red cell invasion by Plasmodium

Entry of a malaria parasite into its host RBC initiates the intra-erythrocytic asexual cycle that is responsible for the clinical manifestations of malaria. The process by which a merozoite invades RBCs is crucial for the survival of the parasite and this ensures the maintenance of the blood stage cycle. Interference with this process would therefore prevent disease, since merozoites are short-lived outside the host cell. In fact, the development of rapid and efficient entry into the host cell has been important in the evolutionary success of this P. falciparum (e.g. immune escape).
RBC invasion by the merozoite is comprised of several sequential steps. This involves the initial attachment of any part of the merozoite to RBC membrane, followed by re-orientation to allow the apical end of the parasite to gain contact with the RBC membrane, release of the contents of the apical organelles, junction formation, membrane invagination, and finally parasite entry (Aikawa et al., 1978, Bannister & Mitchell, 2003, Cowman & Crabb, 2006).

At release from the schizont, merozoites have a dense surface coat of proteins including the merozoite surface protein (MSP)-1, which it uses to make initial contact with a red cell. Apical contact following reorientation then triggers the secretion of the contents of the micronemes and rhoptries, resulting in invagination of the red cell surface. The parasite moves into the depression it has created, and the tight junction, the ring of contact where the two membranes are associated, migrates from the anterior to the posterior end of the parasite. The parasite is now enclosed within the parasitophorous vacuole, and during the final phase of invasion, the contents of the dense granules are released, causing further expansion of the membrane lining the vacuole (reviewed Preiser et al., 2000). The molecular mechanisms by which these processes occur remain poorly understood. Malaria proteases have been implicated in the invasion process, but their specific cellular functions remain unclear. The invasion process is thought to involve multiple proteolytic cleavages of *P. falciparum* MSP-1, an abundant protein on the surface of the invading merozoite, and the shedding of apical membrane antigen-1 (PfAMA-1), a microneme protein. Prior to erythrocyte entry, a series of serine proteases and cysteine proteases mediate the invasion process (Blackman & Holder, 1992, Howell et al., 2003, Kitjaroentham et al., 2006).

**Malaria and the red cell**

The surface of the erythrocytes displays several dramatic morphological changes upon infection. In the first few hours of intraerythrocytic development, there is no noticeable change outside the PV or in the erythrocyte membrane. Noticeable changes start appearing around 12-14 hours post invasion, with alterations of the infected erythrocyte membrane. First, there is an increase in membrane rigidity as well as a reduction in red cell deformability.
As the parasites mature, the red cell becomes more permeable allowing nutrient uptake, metabolite removal and regulation of cytosolic ion concentration. During parasite development, other profound structural and morphological changes occur in erythrocytes as well. These changes contribute in the remodeling of infected RBCs by the trafficking of parasite proteins into the red cell and their deposition either beneath the surface or onto the RBC membrane. The binding of proteins discharged during invasion (Maier et al., 2009, Layez et al., 2005) also modifies uninfected RBC membranes. Proteins inserted in the red cell membrane contribute in altering the architecture of the membrane skeleton as well as in facilitating the delivery of virulence proteins (PfEMP1) to the membrane of the red cell. During host cell remodeling, electron dense protrusions called knobs are induced by the developing parasite to appear on the infected red blood cell surface. The knobs, which are composed of the knob-associated histidine-rich protein (KAHRP) bound to spectrin and actin, serve in anchoring of the major virulence factor PfEMP1 (Glenister et al., 2002). Other proteins trafficked to the red cell membrane, that play a role in membrane alteration and deformability, include the ring-stage-infected erythrocyte surface antigen (RESA), the mature parasite infected erythrocyte surface antigen (MESA; also known as PfEMP2), the P. falciparum erythrocyte membrane protein 3 (PfEMP3) and Pf332 (reviewed Maier et al., 2009). KAHRP, PfEMP3 and Pf332 are responsible for late stage stiffness of the iRBC membrane, while RESA modifies the stiffness of the early stages. The exported proteins have been shown to interact and embed themselves in the spectrin network or by binding to actin (Glenister et al., 2009, Glenister et al., 2002, Maier et al., 2009, Waller et al., 2010) in the RBC membrane skeleton (Fig. 3).
Figure 3: The membrane skeleton in uninfected (a) and *Plasmodium falciparum*-infected red blood cells (b) showing parasite proteins inserted into the membrane skeleton. Adapted and modified from Maier et al. (2009).

This leads to an alteration in the mechanical properties of the infected erythrocyte, rendering them stiffer and more adhesive to the endothelium (Glenister et al., 2009, Glenister et al., 2002), possibly serving as a survival strategy by the parasite to escape clearance by the spleen (Buffet et al., 2011). The human RBC, characterised by its discoid shape, is endowed with an ability to undergo extensive passive deformation during repeated passage through the narrow capillaries of the microvasculature during its 120 days life span in the circulation. Loss of cellular deformability, not only compromises the ability of the red cell to optimally perform its oxygen delivery functions to the tissues, but can also lead to its premature removal from circulation by the spleen. During malaria, in addition to the parasite-encoded proteins that associate with the RBC membrane skeleton, the overall increase in RBC rigidity is also due, in part, to the presence of the large, non-deformable intracellular parasite (reviewed Maier et al., 2009). Other antigens, such as the low molecular weight rhoptry-associated proteins (RAPs), shown to interact with the surface of both infected and normal RBCs have been
suggested to play a role in RBC deformability (Layez et al., 2005). These proteins probably do this through binding to the glycoporphins and/or band 3 (Garcia et al., 2010), although their exact receptor on the RBC skeleton has not yet been confirmed. Overall, the reduced deformability of normal and P. falciparum iRBCs have a direct effect causing impaired flow velocity and consequent flow obstruction. Hence, the mechanopathologic consequence of red cell deformability is the massive accumulation of infected and normal RBCs in the capillary beds of the brain, kidney and other organs. This may eventually block the blood flow and lead to organ failure and ultimately death. Red cell deformability can also lead to enhanced red cell destruction by different immune mechanisms leading to anaemia (Layez et al., 2005). In fact, when measured on admission, RBC deformability serves as a predictor for subsequent mortality in adults and children presenting with severe malaria (Dondorp et al., 1999, 2002).

Another change induced by the infecting parasite is the establishment of new permeation pathways on the erythrocyte membrane for nutrient acquisition from the bloodstream (Goldberg & Cowman, 2010).

**The rhoptry and rhoptry proteins**

To date, several *P. falciparum* antigens belonging to the intraerythrocytic stages of the parasite have been identified and studied as vaccine candidates. Most of these antigens are mainly located on the merozoite surface or in the apical organelles, i.e. the rhoptries, micronemes and dense granules (Mongui et al., 2007). The rhoptries are a pair of organelles at the apical end of the parasite. Ultrastructural and biochemical studies suggest that the rhoptries play an important role in the process of erythrocyte invasion (López et al., 2004). They contain many lipids and proteins, which are released during the merozoite invasion of erythrocytes. The rhoptries are connected to the surface of the apical end of the merozoite by a duct-like structure (López et al., 2004), and they discharge their content onto the erythrocyte membrane at the point of initial contact between the merozoite and the erythrocyte (Aikawa et al., 1981).

The described constituents of this organelle include, the non-covalently associated low-molecular weight complex (LMW, designated Rhop-L), the high-molecular weight rhoptry protein complex (HMW, Rhop-H), the apical membrane antigen-1 (AMA-1), MAEBL, a

In *P. falciparum*, at least 20 rhoptry proteins have been characterised. These proteins are involved both in binding to the exterior of RBCs and in the later stages of invasion in the formation of the parasitophorous vacuole (reviewed Kats *et al*., 2006). The rhoptry-resident proteins appear to be predominantly secreted; with soluble proteins present in the rhoptry neck and lumen. However, a small number of proteins are membrane-associated, either by glycoposphatidylinositol (GPI) anchors e.g. rhoptry associated membrane antigen (RAMA) (Topolska *et al*., 2004a), or integral membrane anchors-Rhop148 (Lobo *et al*., 2003).

### The low molecular weight rhoptry complex (Rhop-L)

The Rhop-L complex is formed by RAP-1, -2 and -3 proteins. The precise role of the RAPs is not clear, but it has been speculated that the complex may be important in the erythrocyte invasion of merozoites (Baldi *et al*., 2000, reviewed Preiser *et al*., 2000, Garcia *et al*., 2010). RAP-1, -2 and -3 exist in complexes, and these complexes are maintained even after erythrocyte invasion and early parasite maturation (Howard *et al*., 1984, reviewed Preiser *et al*., 2000). Proteins of the Rhop-L complex are, to a varying degree, quite immunogenic, as observed from several studies, and considerable amounts of data exists on the efficacy of *in vivo* human humoral/cellular immune responses to these antigens as well as from *in vitro* and experimental animal studies (reviewed Preiser *et al*., 2000). Hence, antibodies to the RAP complex may play a role in immunity to *P. falciparum* infections.

Merozoite proteins are known to be exposed to the immune system both as surface proteins on merozoites and as exoantigens circulating in blood. Although the contents of the rhoptries are only transiently accessible to antibodies, seroepidemiological studies have demonstrated the development of antibody responses to RAP-1, RAP-2 and RAP-3 following infection in different endemic regions (Alifrangis *et al*., 1999, Fonjungo *et al*., 1999, Johnson *et al*., 2000, Howard *et al*., 1993, Jakobsen *et al*., 1993, Jakobsen *et al*., 1997).
Monoclonal antibodies directed against epitopes of *P. falciparum* RAP-1 inhibited merozoite invasion of RBCs *in vitro* (Schofield *et al*., 1986). In that study, one of the epitopes detected was highly conserved while the other was more variable between different *P. falciparum* isolates. *In vitro* growth inhibition assays have indicated that antibodies directed against RAP-1 and RAP-2 proteins have inhibitory effects on *P. falciparum* growth (Moreno *et al*., 2001).

Experimental immunisation of *Saimiri* monkeys with a mixture of RAP-1 and -2 induced specific merozoite invasion inhibitory antibodies, which protected the animals against challenge with *P. falciparum* (Ridley *et al*., 1990). Although denatured RAP-2 seemed to be relatively immunogenic in *Saimiri* monkeys, human serum antibodies only weakly recognised the denatured protein (Ridley *et al*., 1990, Stowers *et al*., 1997). This implies either that, the conformational epitopes were destroyed, or that the assays used to screen for reactivity were of low sensitivity. Mice immunised with a recombinant form of RAP-2 produced antisera, which recognised the native protein by indirect immunofluorescence and immunoblotting (Stowers *et al*., 1997). Since both RAP-1 and RAP-2 are relatively non-polymorphic antigens, they are considered good vaccine candidates. Immune responses to RAP-3 following natural infections in humans and in experimental models have to the best of our knowledge, not been elucidated.

**Rhoptry-associated protein- 1 (RAP- 1)**

RAP-1 is a soluble protein with an apparent molecular weight of 82 kDa. It is expressed during schizogony and it is first detected at 38 hrs post infection (Jaikaria *et al*., 1993). It exhibits minimal genetic polymorphism and is synthesized as an 86 kDa precursor, which subsequently is N-terminally cleaved to generate an 82 kDa molecule (p82) (Moreno *et al*., 2001). In late schizogony, a fraction of p82 is further processed at amino acid residue 119 to yield a 67kDa molecule (p67). As part of their maturation, the processed RAP-1 products associate non-covalently with either p39 (RAP-2) or p37 (RAP-3) to form hetero-oligomeric complexes (Jacobson *et al*., 1998, Moreno *et al*., 2001). Both p82 and p67 are present in schizont/segmenter stages, and in free extracellular merozoites, while the p82 protein is also
detected in ring stage parasites (Moreno et al., 2001). Merozoites containing a form of RAP-1 truncated from residue 345 are equally viable. They display normal erythrocyte invasion and grow at similar rates as the wild-type parasites. This truncated form still expresses the N-terminal region and is detected in rhoptries, suggesting that normal trafficking signals exist in this region (Baldi et al., 2000). However, it is not detected in newly invaded rings. Here, RAP-2 is detected in the endoplasmic reticulum rather than in the rhoptry organelles, suggesting that the C-terminal region is important for binding and trafficking of RAP-2. This suggests that RAP-2 lacks its own rhoptry-targeting signal and is probably not involved in the invasion process (Baldi et al., 2000). Antibodies to RAP-1 have been shown to block merozoite invasion in vitro. In addition, monkeys immunized with RAP-1 and -2 are partially protected against parasite challenge (Clark et al., 1987, Ridley et al., 1990).

**Rhoptry-associated protein-2 (RAP-2)**

RAP-2 is a protein of 42 kDa, although a size of 39 kDa has also been reported (Ridley et al., 1990, Baldi et al., 2002). Its expression pattern coincides with that of RAP-1. Subsequent to erythrocyte invasion, RAP-2 is detected in ring-stage parasites along with RAP-1, but the rhoptry localization of RAP-2 is considered not to be essential for RBC invasion (Baldi et al., 2000). The ring surface protein (RSP)-2 has been shown to be identical to RAP-2 (Douki et al., 2003) and henceforth the protein will be referred to as RAP-2/RSP-2. RAP-2/RSP-2 is present on the surface of normal RBCs (nRBCs) and ring-infected RBCs (riRBC) for 16-20 h post merozoite invasion. Between approximately 16-20 h, it progressively becomes replaced by PfEMP-1 (Douki et al., 2003). The protein has been identified as a target of ring stage-reactive monoclonal antibodies (Douki et al., 2003). Monoclonal antibodies produced against RAP-2/RSP-2 have been shown to specifically bind to conformational epitopes located in the first 200 amino acids of the N-terminal region of the protein (Sterkers et al., 2007). The presence of the protein on nRBCs possibly reflects a delayed or aborted invasion. The protein appears first at the point of contact with the merozoite where after it gradually spreads on the RBC surface (Layez et al., 2005). The number of RAP-2/RSP-2-tagged riRBCs depends on the parasite load and is always much lower than the number of RAP-2/RSP-2-tagged nRBCs.
RAP-2/RSP-2 has been noted as a key molecule in the sequestration of young blood stage forms of the CSA phenotype and nRBCs to endothelial cells, thus evading possible destruction in the spleen (Douki et al., 2003). Previous and recent data suggest that the antibody response against RAP-2/RSP-2 can induce phagocytosis and complement binding to RAP-2/RSP-2-tagged RBCs, leading to the destruction of nRBCs, which could be partly responsible for the anaemia observed in *P. falciparum* infected individuals (Layez et al., 2005).

**Rhoptry-associated protein-3 (RAP-3)**

RAP-3 has a molecular weight of 40 kDa (37 kDa has also been reported). RAP-3 is highly conserved in all parasite strains studied so far and shares 68% identity and 44% similarity with RAP-2/RSP-2 (Baldi et al., 2002, Garcia et al., 2010). It has been observed to co-precipitate with RAP-1 and RAP-2/RSP-2. Furthermore, peptides from RAP-1 and RAP-2/RSP-2 showing high-affinity binding to the RBC membrane, were able to inhibit such binding of the corresponding peptides from RAP-3 to different extents (Garcia et al., 2010), suggesting the recognition of similar binding sites on the RBC membrane. The expression of RAP-3 coincides with that of RAP-1 and RAP-2/RSP-2, during schizogony and RAP-3 can be detected in ring-stage parasites after erythrocyte invasion. Targeted gene disruption experiments have demonstrated that RAP-3 is not essential for parasite survival, as the loss of RAP-3 had no effect on parasite growth or on trafficking of RAP-1 and RAP-2/RSP-2 into rhoptry organelles. Thus lending credence to the notion that the loss of RAP-3 can be complemented for by RAP-2/RSP-2. This is consistent with the fact that RAP-2/RSP-2 and RAP-3 form separate heterodimers with RAP-1 rather than a trimeric complex (Baldi et al., 2002). However, this remains to be elucidated.
Antigen Pf332

Pf332 is a high molecular weight protein in the megadalton range (Mattei & Scherf, 1992). It is so far the largest known malaria protein that is exported to the RBC membrane. It comprises a Duffy binding-like (DBL) domain at the N-terminus of the protein followed by a predicted transmembrane domain (Moll et al., 2007). The rest of the protein is dominated by highly degenerated glutamic acid-rich 11 amino acid repeats in the C-terminal domain (Moll et al., 2007, Balogun et al., 2009). The Pf332 gene, located in the subtelomeric region on chromosome 11, is present in all strains of P. falciparum investigated, but shows an extensive restriction-fragment length polymorphism (RFLP) (Mattei & Scherf 1992). Antigen Pf332 is present in vesicular structures in the cytoplasm of parasitised red blood cells at the trophozoite stage and is later expressed at the parasitised red cell surface at late schizogony (Mattei & Scherf, 1992, Hinterberg et al., 1994). To date, the function of Pf332 in the infected erythrocyte is not fully known. Pf332 co-localizes with other parasite antigens such as the RIFINS and PfEMP1 during its transport, occupying a central location within the transport vesicle (Haeggström et al., 2004). Functional deletion studies further showed that this protein is important in the trafficking of PfEMP1 to the surface of the infected RBC, suggesting an indirect role for Pf332 in red cell cytoadhesion and thus in the pathogenesis of the disease (Glenister et al., 2009). Data also suggest that Pf332 may contribute to the overall deformability of the P. falciparum infected erythrocyte by regulating the extent of parasite-induced membrane rigidification, and this may have important implications with regard to pathogenicity. A consequence of red cell deformability/rigidification has been previously reported for other red cell interacting proteins such as PfEMP3 and KAHRP (Hodder et al., 2009, Glenister et al., 2009). A potential role for Pf332 in merozoite invasion has been reported (Moll et al., 2007) and in anchoring and scaffolding (Hodder et al., 2009). Given that Pf332 signals disappear in very late schizonts and are apparently degraded at the time of cell rupture and merozoite release, a role in red cell rupture had been suggested, albeit by a yet unknown mechanism (Wiesner et al., 1998, Blisnick et al., 1998).

Pf332 is highly immunogenic and antibodies reactive with the antigen have been demonstrated in sera from malaria-exposed individuals (Iqbal et al., 1993, Warsame et al.,
1997, Israelsson et al., 2008, Balogun et al., 2009). In addition, Pf332 reactive antibodies have been shown to inhibit parasite growth in vitro, mainly through interference with intraerythrocytic parasite development and blocking the rupture of mature schizonts (Ahlborg et al., 1996), but also by inhibition of merozoite reinvasion (Ahlborg et al., 1991). The characterisation of Pf332 has so far been hampered by antibody cross-reactivities with other plasmodial antigens. In an attempt to overcome these cross-reactivities (Mattei et al., 1989), and because of its extreme size (Mattei & Scherf, 1992), studies of Pf332 protein have been based on different fragments of the molecule. Data obtained so far seem to suggest that the different regions display different functions (Iqbal et al., 1993, Moll et al., 2007, Glenister et al., 2009, Balogun et al., 2009). However, these regions all display some functional overlap, especially with regard to their immunogenic properties and sero-reactivities/sero-recognition in epidemiological studies. These different fragments, which include Pf332-EB200, Pf332-DBL and Pf332-C231, have all been shown to be widely recognized by sera from different endemic regions (Giha et al., 2009 (Sudan), Balogun et al., 2009 (Senegal, Liberia, Burkina Faso, Mali), Iriemenam et al., 2009 (Nigeria), Balogun et al., unpublished (Senegal for Pf332-DBL). In addition, antibodies targeting these different regions have demonstrated either growth inhibitory or invasion inhibitory functions in in vitro assays (Ahlborg et al., 1993, Ahlborg et al., 1996, Moll et al., 2007, Balogun et al., 2009).

Nevertheless, some of the functional differences observed (invasion versus growth/cytoadhesion inhibition) could imply that antibodies induced by the different parts of the molecule could be instrumental in interfering with the in vivo growth and multiplication of the parasite at different points in the parasites erythrocytic cycle. Thus, observations with the different fragments do not undermine the importance of this antigen as a vaccine candidate. However, further studies are warranted, to clarify the existing data.

**EB200** is a 157 amino acids long central fragment of Pf332. Its features are reminiscent of the antigen as a whole, with arrays of tandemly repeated 11-mer amino acid sequences and a high frequency of glutamic acid residues often occurring in pairs (Mattei & Scherf, 1992). Due to its high number of repeats, there exists a high potential for cross-reactivity of antibodies to
this fragment with other glutamic acid rich *P. falciparum* antigens, such as Pf155/RESA and the gametocyte antigen Pf11.1 (Mattei *et al.*, 1989, Udomsangpetch *et al.*, 1989).

**Pf332-C231** is a 231 amino acids long fragment of Pf332 located in the C-terminal region of the molecule (Balogun *et al.*, 2009). Unlike Pf332-EB200, this region is devoid of the numerous glutamic acid repeats characteristic of Pf332. Hence, the fragment demonstrates limited cross reactivity with other *P. falciparum* antigens. In an earlier study with this fragment, rabbit anti-C231 antibodies were found to inhibit parasite growth *in vitro* (Balogun *et al.*, 2009). In addition, in field studies, high levels of C231 antibodies were observed to be associated with protection from malaria (Israelsson *et al.*, 2008).

**Pf332-DBL**, the DBL domain of Pf332 is highly conserved and is located in the N-terminal region of this molecule. The DBL domain is homologous but with a distinct sequence to the DBL-domains of the erythrocyte-binding ligand (EBL) family of invasion related proteins. Thus, it is thought that this region might exert functions related to the EBLs (Moll *et al.*, 2007). Analysis of this region further revealed its ability to bind to uninfected human RBCs (supporting merozoite invasion) as well as an association with the red cell surface. Furthermore, antibodies raised against this region were able to reduce merozoite invasion efficiency of different parasite strains/clones (Moll *et al.*, 2007). However, other studies failed to confirm this observation (Hodder *et al.*, 2009). Immunization of mice with this fragment in the presence of adjuvant generated a strong IgG response (Du *et al.*, 2010).

**Phagocytosis in malaria**

Phagocytosis has long been recognized to play a crucial role in the defense of the host during malaria infections. It is regarded as an important effector mechanism in malaria. Rapid phagocytosis of parasitised RBCs not only prevents merozoite invasion of RBCs, but also reduces the toxic effects of malaria GPI (Kumaratilake & Ferrante, 2000). However, increased erythrophagocytosis also contributes to the development of severe anaemia. Indirect signs of erythrophagocytosis include splenomegaly and the presence of malaria pigment in
monocytes/macrophages, resulting from the phagocytosis of parasitised erythrocytes and free haemoglobin.

**Complement in malaria**

Studies in human malaria suggest that the complement system, in particular the classical pathway, may play a role in the host defense against malaria and may also be associated with disease pathology (reviewed Chang & Stevenson, 2004). Components of the classical pathway were demonstrated to be depressed, whereas components of the alternative pathway remained unaffected. Complement activation was found to occur during or soon after schizont rupture, if the parasite density was sufficiently high and if complement fixing antibodies were present. The degree of hypocomplementemia was found to correlate with various complications of malaria, such as disseminated intravascular coagulation jaundice, anaemia and cerebral malaria (Wenisch et al., 1997). During childhood malarial anaemia, the RBCs remaining in the circulation show reduced levels of CR1 and CD55 expression and increased C3b deposition, which may result in increased destruction of RBCs and ensuing anaemia (Waitumbi et al., 2000, Owuor et al., 2008, Odhiambo et al., 2008). In addition, a deficiency in CR1 and CD55 expression has been shown to vary with the age of the host, but irrespective of endemicity; being low in young children and increasing with age in both endemic and non-endemic countries (Waitumbi et al., 2004, Stoute, 2005).

**Apoptosis in malaria**

Ferrokinetic studies have shown that as many as 50% of the erythroid precursors die in the bone marrow. In malaria, the bone marrow response is suppressed in anaemic individuals who present with a deficit in erythroid cells numbers, thought to be due to the death of erythroid cells in the erythroblast compartment. The mechanism of cell death in this context is, however, not clear. Based on morphological abnormalities in the bone marrow of patients, apoptosis had been suggested to be responsible for the observed dyserythropoiesis in patients with anaemia (see previous section on Mechanisms of anaemia). Severe malaria represents a
multi-organ disease and is characterised by endothelial damage. Sera from patients with malaria have been shown to induce endothelial cell apoptosis, which is characteristic of fatal malaria, in vitro/ex vivo. The induced apoptosis is further amplified by neutrophils as a result of TNF and TNF-activated neutrophil derived ROS and proteases (elastase) in the sera (Hemmer et al., 2005). The ROS are thought to inactivate protease inhibitors, which would otherwise protect the endothelium from elastase-induced damage. Apoptosis as a mechanism of parasitism has also been reported in *P. falciparum* (reviewed Bruchhaus et al., 2007).
THE PRESENT STUDY

Prelude: The context

Malaria is an ancient disease that continues to exact its toll in many parts of the world especially in tropical countries. Most of the severe morbidity and mortality, which occurs mostly in children and pregnant women, is caused by *P. falciparum*. The outcome of a blood stage infection with *P. falciparum* can be quite diverse in different individuals, with a wide range of clinical presentations and pathologies (Weatherall *et al.*, 2002), often ranging from asymptomatic infections to severe malaria with life threatening complications and death. Severe malaria often times embodies the syndrome of severe malaria anaemia, cerebral malaria and respiratory distress.

Generally, anaemia, which constitutes a major part of this thesis, is a public health problem that affects populations in both rich and poor countries. Although the primary cause is iron (Fe) deficiency, it is a multifactorial condition, particularly in malaria endemic areas. As such, establishing the relative contribution of malaria to anaemia is difficult, as malarial anaemia is seldom present in isolation. Apart from Fe deficiency, it more frequently coexists with a number of other causes, such as other parasitic infections (helminthes), nutritional deficiencies, and haemoglobinopathies. In some cases, several aetiologies might operate in the same individual. Malarial anaemia on its own affects predominantly people in the developing world, with children below 5 years of age and pregnant women, mostly primigravidae, representing the most vulnerable groups. Until recently, malaria-related anaemia has received very little attention compared to other complications of severe malaria, notably cerebral malaria, perhaps due to its less dramatic presentation and the ease of treatment by blood transfusion. With the advent and spread of HIV, blood transfusion is no longer deemed a safe and attractive option, especially in the developing world where malaria is most prevalent, thus warranting alternative strategies for the treatment of malarial anaemia. The observations from vaccine studies which, suggest that following immunisation with blood stage antigens, monkeys developed anaemia even though their parasitaemia had been successfully controlled (Egan *et al.*, 2002, Jones *et al.*, 2002), might explain the recent renewed interest in severe
malarial anaemia and the mechanisms responsible for its aetiology.

Widespread and increasing drug and insecticide resistance further exacerbates the situation of malaria globally, thus undermining the effectiveness of current malaria control strategies, which include at the moment chemotherapy and vector control, with the use of impregnated bed nets and indoor residual spraying, as the main stay (Ridley 2002).

In addition to the enduring presence of malaria in tropical Africa, its rapid resurgence in many parts of the world raises the urgency for the development of novel and cost effective control measures. Major efforts are being made to develop effective vaccines against the disease, and this has been the ultimate goal (priority) of the research community within the last decades. However, concerted efforts by several investigators/groups to develop a vaccine have so far been relatively unfruitful. This is probably due to the complex nature of the *Plasmodium* parasites, amongst several other factors.

Since the asexual erythrocytic stages of the parasite are the major cause of morbidity and mortality due to malaria, interest has been focused mainly on the identification and characterization of antigens originating from this phase of its complex life cycle, which are expected to be instrumental in inducing protective immunity against these stages.

So far, there is foregoing consensus that the major candidates for a vaccine are those antigens present on the merozoites or on the surface of infected erythrocytes. These latter antigens could either be expressed on the RBC membrane (PfEMP1), adsorbed onto the RBC membrane (RAP-2/RSP-2) or interacting with the RBC membrane cytoskeleton (Pf332). In addition, it is also believed that a highly effective malaria vaccine would require a combination of key antigens or epitopes from different stages of the life cycle.

Following unequivocal evidence of the protective role of antibodies in the pioneering and elegant studies of Cohen *et al.* (1961), McGregor *et al.* (1963) and later by others (Sabchareon *et al.*, 1991), there has been a resurged interest over the years in the molecular identification of parasite proteins that could serve as potential targets of vaccine-induced antibodies. The
characterisation of such antigens is of importance in order to identify parasite epitopes and mechanisms of action of antibody-mediated responses to malaria, as well as, to elucidate which antigens and combinations will induce the right types of immune responses conferring protection.

Pf332 and the RAP complex, which are the focus in this thesis, are two asexual blood stage antigens that both have been shown to interact with the RBC membrane at different stages of the erythrocytic cycle, and thus, have been identified as vaccine candidates. Studies on these proteins are continuously being undertaken in order to fully elucidate and understand their role in inducing protective immunity during natural *P. falciparum* infections. Several sero-epidemiological studies indicate that antibodies to these antigens can inhibit parasite growth and adherence to host cells *in vitro* by themselves or as for Pf332 in synergy with monocytes in this context.

In this thesis, the pattern of antibody responses to these two blood stage antigens is reported and their relation to disease, as well as, their possible role in the development of clinical immunity is investigated.

**Scope of the Thesis**

- To determine whether RAP-2/RSP-2 and other members of the low molecular weight rhoptry associated protein complex (RAP-1 & -3) could be associated with erythrocyte destruction and bone marrow suppression associated with malarial anaemia in *P. falciparum* infections.

- To investigate the frequency and functional capacity of naturally acquired anti-RAP-2/RSP-2 antibodies in the sera of anaemic and non-anaemic malaria infected children residing in a malaria endemic region.

- To investigate the immunological capacities of affinity purified human antibodies reactive with the Pf332-C231 fragment of *P. falciparum* antigen Pf332.
**Materials and methods**

The materials and methods for the included investigations are described in the separate papers.

**Results and discussion**

**Paper I**

Severe malaria is an inevitable consequence of a *P. falciparum* infection and anaemia is a constant feature of the disease. The causes of anaemia are complex and multifactorial. Summarily, its pathogenesis is thought to be a result of increased red cell destruction as well as decreased red cell production, probably due to bone marrow suppression. However, the destruction of normal erythrocytes is considered paramount (Jakeman *et al.*, 1997). To date, the mechanisms and molecular components involved in RBC destruction remain elusive. Recently, RAP-2/RSP-2 has been implicated in the destruction of erythroid cells during anaemia (Layez *et al.*, 2005). In this study, we have confirmed and extended these findings. We have shown that antibodies to RAP-2/RSP-2 and other low molecular weight rhoptry proteins recognize the surface of RAP-2/RSP-2-tagged erythroid cells in a parasitaemia-dependent manner, and, furthermore, can induce the destruction of these cells by opsonophagocytosis or complement activation. This was also true for other proteins of the low molecular weight complex. We further showed that antibodies to RAP-2/RSP-2 and RAP-1 mediated the death of erythroblasts in the presence of monocytes/macrophages *in vitro*. Previous studies had shown that in response to acute malaria, there is proliferation and hyperactivity of the reticulo-endothelial system, resulting in the phagocytosis of both parasitised and unparasitised RBCs (reviewed Menendez *et al.*, 2000). It had been suggested that the presence of RAP-2/RSP-2 on the surface of normal and infected RBCs might lead to the rigidification of these cells and hence an enhanced clearance from the circulation (Layez *et al.*, 2005). The deformed membrane may also encourage the deposition of complement components, thus targeting them for clearance by the reticuloendothelial system or lysis (Waitumbi *et al.*, 2000, Waitumbi *et al.*, 2004).
Bone marrow suppression is generally present in all malaria patients (Kurtzhals et al., 1997), and is characterised by ineffective erythropoiesis and dyserythropoiesis (morphological abnormalities of erythropoiesis). Bone marrow suppression is considered an important aggravating factor in the pathogenesis of malarial anaemia (Abdalla et al., 1980, Phillips & Pasvol, 1992, Wickramasinghe & Abdalla, 2000) and is thought to be a result of cell death in the erythroblast cell compartment (Dörmer et al., 1983, Wickramasinghe & Abdalla, 2000)

We showed that antibodies to RAP-2/RSP-2 and RAP-1 could mediate the death of erythroblasts in the presence of monocytes/macrophages in vitro. The mechanism of cell death is not yet known. However, our data indicate that, the cells are to some extent dying by apoptosis. In support of our results, Anantrakulsil et al. (2005) detected some apoptosis in bone marrow aspirates from Thai patients. However, data from each subject could not be interpreted in the same way, as the percentages of apoptotic erythroid cells in bone marrow aspirates from each patient and controls varied from low to high. In addition, there was no association with parasitaemia.

Paper II

In order to complement our in vitro findings, we sought to determine the frequency, specificity and functional capacity of naturally acquired anti-RAP-2/RSP-2 antibodies in the sera of infected children. All sera investigated recognised RAP-2/RSP-2 irrespective of the clinical status of the subjects. The anaemic group of children had higher levels of IgG than the non-anaemic controls, while the mean levels of IgM were similar in both groups. With regard to IgG subclasses, there were generally low levels of IgG1 and IgG3 antibodies detected, while the levels of IgG2 and -4 antibodies were below the detection limit in all individuals. A possible reason for the absence of anti-RAP-2/RSP-2- specific IgG subclasses in our study, is that the assay for antibody detection was not sensitive enough. To the best of our knowledge, no previous study has looked at the subclass distribution of anti-RAP-2/RSP-2 antibodies in the sera of patients. However, previous studies with RAP-1 peptides showed that individuals had predominantly IgG1 antibodies and only a few sera contained traces of IgG2, -3, or -4, where only two individuals had IgG3 reactive with one of the peptides used in the study (Fonjungo et al., 1998).
RAP-1 is known to form hetero-oligomeric complexes with RAP-2/RSP-2 and related RAP-3 (Baldi et al., 2000, Baldi et al., 2002). Experimental immunisation of *Saimiri* monkeys with purified RAP-1 and RAP-2/RSP-2 conferred partial protection against *P. falciparum* infection (Ridley et al., 1990). In addition, we do not know much about the dynamics of anti-RSP2 responses in natural infections. At the moment, there is paucity of data regarding the dynamics of antibody responses to RAP-2/RSP-2. In endemic areas, acquired immunity to malaria builds up with age following several repeated attacks and this immunity is not sterile. Previous studies by Johnson et al. (2000) indicated that levels of antibodies to RAP-2/RSP-2 do not reach a maximum until the age of 30 years, while levels of antibodies to RAP-1 increased significantly above the age of 15 years. In another study, of Papua New Guinean natives, maximum levels of antibodies to both RAP-1 and RAP-2/RSP-2 were not reached until after the age of 30 years (Stowers et al., 1997), implying that there might be a slow acquisition or build up of antibodies to RAP-2/RSP-2 during natural infections. This could explain the low levels or absence of antibodies of the different IgG subclasses in our study population of four and five year olds. Another plausible explanation could be that, as for RAP-1, antibodies to RAP-2/RSP-2 are short-lived (Fonjungo et al., 1999), or might be a reflection of exposure, as suggested by several authors with regard to other antigens as well (Fonjungo et al., 1999, Perlmann et al., 2000, reviewed Preiser et al., 2002).

In addition, sera from the non-anaemic group with lower levels of IgG, activated complement more in terms of the proportion of cells binding C3b. This implies that the effector functions of these antibodies are more important than their amounts. Indeed, the non-anaemic individuals showed higher levels of IgG3 antibodies than the anaemic ones and this IgG subclass is the most efficient complement activating IgG. Studies in human malaria suggest that the complement system, particularly the classical pathway, may play a role in host defense against malaria, even though it may also be associated with disease pathology (reviewed Chang & Stevenson, 2004). *P. falciparum* reactive antibodies have previously been shown to play a critical role in immune protection against the asexual blood stages of the parasite (Cohen et al., 1961, McGregor et al., 1963, Sabchareon et al., 1991), and the antibody isotype, and subclass appears to be critical in this context (Warmerdam et al., 1991, Bouharoun-Tayoun & Drulilhe, 1995, Garraud et al., 2003, Tangteerawatana et al., 2007).
Paper III

As previously mentioned, antibodies directed against blood stages of malaria have been shown to be efficacious in the prevention of disease as shown in passive transfer experiments in humans (Cohen et al., 1961). Mechanisms by which these antibodies mediate their effects have been shown to differ and to largely depend on the target antigen. Antibodies could mediate their effects through merozoite/parasite opsonisation/phagocytosis (Groux & Gysin, 1990), prevention of invasion, inhibition of parasite growth and development within the erythrocyte (Ahlborg et al., 1996) as well as interference of merozoite dispersal by agglutination (Woehlbier et al., 2006).

Pf332 is present in the cytoplasm of iRBC at the trophozoite stage and becomes exposed on the surface in late schizogony, thus becoming a target for parasite neutralizing antibodies. The gene codes for a protein of 6094 amino acids, with a predicted molecular weight of approximately 670 kDa (Moll et al., 2007) and a high percentage of glutamic acids of 11 mer repeats. Individuals living in malaria endemic areas show a high prevalence of sero-reactivity with these repeats (Iqbal et al., 1993, Warsame et al., 1997, Israelsson et al., 2008, Balogun et al., 2009).

The interest in Pf332 as a malaria vaccine candidate derives partly for the observation that the Pf332 reactive human monoclonal 33G2 inhibits parasite growth and cytoadherence in vitro (Udomsangpetch et al., 1989). Previous studies have shown that humans continuously exposed to malaria recognize antigen Pf332 extensively and antibodies to Pf332 have been shown to inhibit parasite growth in vitro. How these antibodies mediate their effects is not fully known and remains a question undergoing further investigations. However, interference with intra-erythrocytic parasite development and inhibition of schizont rupture have been suggested as plausible mechanisms by which antibodies targeting Pf332 exert their parasite neutralizing activity (Ahlborg et al., 1996).

In an attempt to further elucidate the function of this protein in the life cycle of the parasite and the mode of action of specific antibodies, we employed a sub-fragment of this antigen
(Pf332-C231) which is almost devoid of the repetitive sequences, which are reminiscent of the antigen as a whole. Thus, this fragment shows a considerably lower cross reactivity with other plasmodial antigens as compared with that of Pf332-EB200 or other peptides based on Pf332 repeats (Balogun et al., 2009).

Here, Pf332-C231 reactive antibodies induced following natural exposure were analysed for their potential protective capacity in terms of their ability to inhibit parasite growth in vitro. Antibodies reactive with Pf332-C231 were affinity-purified from seven different Liberian samples. All seven antibody preparations recognized C231 in ELISA and gave the typical Pf332 pattern of staining of iRBC in immunofluorescence. Furthermore, all but one of the antibody preparations efficiently inhibited parasite growth in vitro. This inhibition was specific, as confirmed by antigen reversal assays. Antibody-mediated inhibition of parasite growth in *P. falciparum* in vitro cultures is thought to reflect the parasite neutralizing potential of antibodies in vivo (Iqbal et al., 1997).

Our data presented herein, clearly show that, in addition to inhibiting parasite growth, antibodies targeting the C-terminal fragment of Pf332 indeed perturbed the development of parasites, as evidenced by the presence of pyknotic parasites, and parasites with abnormal morphologies. In addition, red cell lysis seemed to be another means by which C231-specific antibodies mediated their effects, as a large proportion of external parasites were frequently observed, especially at the trophozoite-schizont stages, which are the stages at which antibodies to Pf332 as a whole largely act (Ahlborg et al., 1996).

More recently, it has been shown that in addition to its role in the trafficking of PfEMP1 to the surface of the red cell, Pf332 was able to interact with the RBC membrane skeleton by binding to F-actin, and thus could play a role in RBC deformability (Waller et al., 2010, Hodder et al., 2009, Glenister et al., 2009). Studies of both normal and abnormal red cell membrane architecture indicated that distortions in RBC cytoskeleton would result in less deformable RBCs with fragile membranes, thus leading to premature removal/destruction by the spleen (An & Mohandas, 2008, Lim & Li 2001). Thus, in the presence of specific antibodies, less deformable and rigid membranes could result in RBC lysis, thus accounting
for the external parasites observed in the present study. However, since it is beyond the scope of the present study to explain fully this observation, detailed structural and functional studies are necessary to provide more insight into the above hypothesis.

Although several isotypes of specific antibodies are often produced during infection with *P. falciparum*, protective immunity seems to be associated predominantly with IgG (Cohen & Butcher, 1970). Even though the affinity purified fractions contained IgG and IgM, the inhibition of parasite growth in this study appeared mainly to be attributable to the IgG fraction.

Microscopically, it was observed that parasites became affected by antibodies as early as 22 hr post invasion through to late stages, when a large proportion of trophozoites and schizonts were observed outside of RBCs. This, together with the high percentage of morphologically abnormal parasites culminated in the reduction of parasitaemia, especially at later time points as recorded in the growth inhibition assays. Data on the surface exposure of Pf332 is still contradictory (Moll *et al*., 2007, Glenister *et al*., 2009, Waller *et al*., 2010). Thus, how antibodies to the Pf332 antigen as a whole and to the different sub-fragments studied so far, gain access to the parasite and mediate their effects, still remains a critical question. It has previously been suggested that antibodies may gain access via the leaky membrane during late stages close to the time of rupture or via the parasitophorous duct (Pouvelle *et al*., 1991, Taraschi 1999). Previously, it was further suggested that parasite induced changes on the red cell membrane not only altered the properties of the erythrocyte membrane (such as deformability, fluidity and permeability), but could also contribute to antibodies gaining access to none-exposed antigens (Cranston *et al*., 1984, Sherman, 1985, Hinterberg *et al*., 1994).

The observed perturbation in parasite development was further accessed by monitoring parasite growth in culture, following the removal of immune pressure. It was noted that parasites were able to regain their growth ability, albeit at a slower rate than untreated controls, implying that under persistent antibody pressure, the effects of the antibodies would have been of a longer duration. However, it is known that individuals remain infected long after developing detectable antibodies to the respective target antigens, thus, the above
observation could probably just be a reflection of the chronic recrudescent nature of malaria in natural infections. Immunity to malaria in endemic regions is non-sterile, develops slowly and probably needs boosting from repeated exposure (reviewed Doolan et al., 2009). Also, antibody responses to malaria are mostly short-lived, as asymptomatic infections are common, wherein clinically immune individuals are often chronically parasitaemic at any one time, albeit at low parasite densities, a common observation in endemic regions for malaria. The chronic nature of asymptomatic parasitaemia is thought to be important in the maintenance of immune responses, as well as in protection against new infections (reviewed Doolan et al., 2009). In addition, asymptomatic infections are often marked by multi-clonality (Färnebo et al., 1999, Liljander et al., 2011).

Given that natural infections are characterised by multi-clonality, it could be speculated that, the growing parasites after removing antibody pressure represented a different dominating parasite clone, as previously reported on the existence of different parasite clones with a differential sensitivity to growth inhibitory antibodies following immune pressure with a Pf332-reactive antibody (Iqbal et al., 1997). Antigenic variation as an immune evasion mechanism exists in malaria infections, and antibody responses to variant antigens are known to be type specific and this may serve as a selective factor for induction of new antigenic variants, even in the absence of immune pressure (Recker et al., 2004, Bull et al., 2005).

Antibodies to Pf332 have largely been studied with regard to different fragments of the molecule in part due to its extreme size and numerous glutamic acid repeats. As such, one could speculate that antibodies to the different fragments could act in an additive manner, capable of inhibiting merozoite invasion (Moll et al., 2007), growth inhibition, cytoadhesion inhibition (Ahlborg et al., 1996, Balogun et al., 2009, Udomsangpetch et al., 1989), as well as inducing red cell lysis as reported herein (Paper III).
Concluding remarks

With regard to paper I and II, we might surmise that during SMA, an inadequate bone marrow response due to erythroblast cell death may either overlap with the accelerated destruction of normal erythrocytes, by opsonisation or complement activation, further aggravating the anaemia, which may become fatal. In addition, high levels of anti-RAP-2/RSP-2 antibodies may be detrimental to the host. This could therefore have implications in the design, development and deployment of further interventions against malaria.

The presence of antibodies to both antigens is potentially important in the control of infection, as antibodies to both antigens have been shown to inhibit reinvasion and growth, respectively, as well as cytoadhesion. However, they could also be involved in the genesis of disease processes and pathology (Fig. 4). As such, the timing of infection and antibody expression could be critical in determining the outcome of infection/disease. Both their involvement in red cell deformability would mean an enhanced destruction of altered red cells by splenic clearance in the presence of specific antibodies (Fig. 4). This would especially be prominent during infections with fulminant parasite densities, in particular, if antibodies produced are not efficient at blocking invasion or inhibiting growth. An additional mode of action of antibodies to Pf332-C231 could be in cell lysis, which could proceed independently of the role of the spleen.

Overall conclusions and future perspectives

One of the difficulties in developing an effective malaria vaccine is due to the antigenic change of the parasite during its complex life cycle. Thus, it is important that vaccine-induced protective immunity be targeted to the different stages of the parasite’s development. Hence, careful analyses of the different components need to be undertaken in order to understand fully the antigen requirements needed in inducing an optimal immune response.

From our studies, the function of RAP and Pf332 antibodies in natural infections seems to be
dependent on the quality (effector functions) rather than quantity (antibody levels). Moreover, the role of IgG3 seemed to be important. Overall, the quality and fine specificities of antibodies generated during an immune response are critical in pathogen control, although, this may depend or act in concert with other factors.

Taken together, one could hypothesize that both proteins may play a role in further exacerbating pathological outcomes during falciparum infections given their roles in red cell deformability and cytoadhesion (Fig. 4 & 5). A putative/suspected role of Pf332 in the development of anaemia warrants further investigations. Further studies of immune responses against these antigens and their relation to disease are thus needed, especially in pregnant women and children in whom the phenomenon of sequestration and anaemia are often reported. PAM has consequences, not only for the mother, but also for the unborn foetus. Since pregnant women and children demonstrate antibody reactivity to RAP-2/RSP-2, it is believed that the presence of RAP-2/RSP-2 in combination with high parasitaemia, anaemia would develop as a consequence of RAP-2/RSP-2-tagged RBC elimination (Douki et al., 2003). However, the quality of antibodies in this context is of paramount importance. Thus, future studies will focus on elucidating the avidities of antibodies induced following natural infection. It would furthermore be of importance to investigate which receptors mediate the binding of RAPs to the erythrocyte membrane.

When measured on admission, RBC deformability serves as a predictor for subsequent mortality in adults and children presenting with severe malaria (Dondorp et al., 2002). RAP-2/RSP-2 and Pf332 are differentially expressed during the parasites’ erythrocytic cycle and both proteins have previously been implicated in the alteration of red cell deformability. Thus, studies of red cell deformability and the relation to disease patterns will be important. In addition, studies on the expression and prevalence of RAP-2/RSP-2 and Pf332 on RBCs of different groups of individuals in endemic regions will be instrumental in further understanding the role of these proteins in falciparum infections.
Figure 4. Mechanisms of anaemia in malaria: Role of RAPs and Pf332

During *P. falciparum* erythrocytic cycle, RAP-1, -2 and/or -3 can be transferred to the surface of both normal and infected RBCs as a result of an aborted or a delayed invasion, respectively. In the absence of a protective immune response (such as parasite invasion/growth inhibition and parasitaemia control by innate cells), the opsonisation of the RBC-bound proteins/protein complex by antibodies and/or complement component C3 would result in their elimination by one of several ways. Protein-tagged RBCs bound by opsonising antibodies are destroyed by phagocytosis. These antibodies may also activate complement, thus leading to the phagocytosis and or lysis of protein-tagged RBCs. Less deformable protein-tagged RBCs would result in fragmentation and subsequent clearance by the spleen. This could either by antibody-mediated or not. In the bone marrow, protein-tagged erythroblasts in the presence of opsonising antibodies and or complement could result either in erythroblast phagocytosis or lysis. Alternatively, antibody opsonised erythroblast could lead to apoptotic cell death. RBCs normally stay in circulation for about 120 days and during normal homeostasis, RBC numbers are balanced through the destruction of old RBCs by the reticulo-endothelial system and the production of new RBCs through erythropoiesis. Under the influence of factors such as erythropoietin, haematopoietic stem cells in the bone marrow multiply and differentiate to produce young and fully functional reticulocytes which develop into mature RBCs. This will therefore control the ensuing anaemia. Due to the potential role of Pf332 on RBC integrity, it is hypothesised that this giant protein could also play a role in the genesis of malaria anaemia.
Figure 5. Model showing a putative role of RAPs and Pf332 in cytoadhesion and pathogenesis of *P. falciparum*

(A) Young RBCs tagged with RAPs are able to adhere to the vascular endothelium or placental syncytiotrophoblasts during the first 20 h post-invasion. After 20 h, cytoadhesion is then mediated by PfEMP1, which is trafficked to the RBC membrane by Pf332. Thus, Pf332 plays an indirect role in cytoadhesion and vascular occlusion. Vascular occlusion could also be due to RBC rigidity as a result of the presence of these proteins on the RBC membrane. (B) Normal erythrocytes carrying RAP on their surface could also mediate adhesion to the vascular endothelium and placental syncytiotrophoblasts. The role of Pf332 in these contexts needs to be investigated. The binding by RAP-tagged RBCs is mediated by an unknown receptor, while for PfEMP1; binding could be mediated by CD36 and ICAM 1.
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Plasmodium falciparum: RAP-2/Pf332 in focus

Nancy Wenjighe Awah

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Above all, Glory and Honor to the LORD ALMIGHTY, who makes all things possible. It is only by His Amazing Grace and Love that this day “came to pass”. 
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PUBLICATIONS

That which we call a rose
by any other name would
still smell as a rose

William Shakespeare