INVESTIGATIONS OF ANTI-ADHESION AND ENDOTHELIAL ENVIRONMENT FOR *Plasmodium falciparum* CYTOADHERENCE

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy.

by

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ABSTRACT

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A unique feature of mature *Plasmodium falciparum* (*P. falciparum*) parasitized RBC (pRBC) is that they bind to surface molecules of microvasculature endothelium via the parasite-derived surface protein PfEMP1. This ligand is associated with the cytoadherence pathology seen in severe malaria (SM) and recently our group has shown that even when treated with effective anti-malarial drug, pRBC are still able to cytoadhere, therefore, there is a need to find an adjunct treatment (in addition to antimalarial drugs) that can inhibit and reverse the adhesion process. Previous reports have suggested that sulphated glycoconjugates are highly effective at disrupting *P. falciparum* pRBC rosettes. Here, we investigate that effect by using sulphated polysaccharides and modified heparin for their effect to interrupt pRBC sequestration. We found that not all sulphated compounds or modified heparins were able to interrupt the sequestration process. Consideration of the inhibitory compounds generated some 'rules' fore exhibition of inhibitory properties: Sulphate position either at 6-O, or/and 2-O sulphate and N-sulphate is necessary for each compound. In addition, the multivalent effect and drug exhibit low anticoagulant activity also determined an active response to inhibit and de-sequestered *P. falciparum* pRBC on protein and endothelial cells. Here, we provide evidence that polysaccharides that possess a different level of sulphate, conformational structure and sulphate position act differently. This study also addressed the importance of pH host environment and extracellular matrix (glycocalyx) on the surface of endothelial cells on mediating pRBC binding. It found that pRBC bind significantly higher at pH 7-7.2 to CD36 and ICAM-1. Meanwhile, glycocalyx might interact as an instantaneous binder before pRBC reached ICAM-1 or CD36, unfortunately we cannot prove this due to methods and antibody chosen. The work reported in this thesis opens up new possibilities for therapeutic strategies targeting binding interaction of pRBC to host cells.

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ABBREVIATIONS

SM	Severe Malaria
CM	Cerebral Malaria
HUVEC	Human Vascular Endothelial Cells
HDMEC	Human Dermal Endothelial Cell
HBEC	Human Brain Endothelial Cell
FACS	Fluorocence-activated Cell Sorting
CHL	Sodium Chlorate
*Pf*EMP1	*Plasmodium falciparum* Erythrocyre Membrane Protein 1
ICAM-1	Intercellular Cell Adhesion Membrane 1 Protein
ICAM-1^{ref}	Intercellular Cell Adhesion Membrane 1 Reference Protein
ICAM-1^{S22A}	Mutant ICAM-1 at amino acid position 22
ICAM-1^{Kilifi}	Mutant ICAM-1 at amino acid position 29
GAGs	Glycosaminoglycans
PG	Proteoglycan
CS	Chondroitin Sulphate
CSA	Chondroitin Sulphate A

HA	Hyaluronic Acid
HS	Heparan Sulphate
HSPG	Heparan Sulphate Proteoglycan
HS^{EC}	Heparan Sulphate Endothelial Cell
Hep	Heparin
Hep I	Heparatinase I
Hep II	Heparatinase II
Hep III	Heparatinase III
C_{ABC}	Chondroitinase ABC
LMW hep	Low Molecular Weight Heparin

CHAPTER 1: INTRODUCTION

1.1 Research background

Malaria is a serious burden, particularly to low and middle-income countries, and a major contributor to morbidity and mortality. The aetiology agents of malaria to humans are recognized as six distinct protozoa species of *Plasmodium*: *Plasmodium falciparum* (Perlmann & Troye-Blomberg 2000), *Plasmodium vivax* (Sharma & Khanduri 2009), *Plasmodium malariae* (Siswantoro et al. 2011), two species of *Plasmodium ovale* (*P. ovale curtisi* and *P. ovale wallikeri*)(Sutherland et al. 2010) and *Plasmodium knowlesi* (Sabbatani, Fiorino & Manfredi 2010), which was recently recognized as the sixth human-infecting *Plasmodium* species after reported cross infection from long tail Macaca monkey to human in Malaysia (Cox-Singh 2010; Cox-Singh et al. 2008; Cox-Singh et al. 2010; Cox-Singh & Singh 2008). *P. falciparum* has often been seen as the most clinically significant infection due to an association with mortality and the intensity of infection in some regions of sub-Saharan Africa, but *P. vivax* has a wider geographical distribution and its categorization as benign has been challenged (Anstey et al. 2009). An episode of *P. falciparum* malaria in a non- or semi-immune host can lead to severe malaria (SM) if untreated, with a high risk of death. Recently, a study from Papua New Guinea (Indonesia) and Malaysia Borneo extended the pattern of severe disease by showing a strong association of *P. vivax* (Genton et al. 2008) and *P. knowlesi* (Cox-Singh et al. 2010) infection respectively to SM and death. Two Italian pathologists Marchiafava and Bignami started studies of the pathology of SM in the late of 19th century, where they found post-mortem the presence of high parasite load in a comatose malignant blood fever patient compared to the benign fever (Marchiafava, Bignami & Mannaberg 1894). They saw high parasite levels and parasite pigment predominantly retained in the tissue microvessels compared to the peripheral circulation, and the existence of necrosis and alterations in the endothelium of the cerebral vessels. This discovery has led to a suggestion that the preferential accumulation of parasitized red blood cells (pRBC) in tissues might be linked to disease severity. The manifestations of SM are highly variable and are determined by factors from both the human host and the parasite. The most common clinical features of SM are high fever, respiratory complications, vascular

obstructions, metabolic disturbances (e.g acidosis), multi organ dysfunction (e.g renal failure), severe anaemia, intracranial preassure and cerebral malaria (CM), which differs between areas of varying transmission intensity and between adults and children. This creates problems in comparing studies as the clinical definitions can vary. Some aspects of SM occur because the parasite has developed mechanisms to escape the host immune system, which we will discuss later.

It is still unclear how infection with *P. vivax* and *P. knowlesi* lead to SM, and it is possible that research on understanding *P. falciparum*-derived SM may help us to understand and predict how *P. vivax* and *P. knowlesi* act. Once thought to be unique to *P. falciparum*, the ability of the mature (pRBC) to undergo a range of adhesive interactions (cytoadhesion) such as binding of pRBC with endothelial cells (sequestration), interaction of pRBC with non-infected RBC (rosetting) and other pRBC (auto-agglutination) is now thought to be shared with other species. One of the big questions in *P. falciparum* research is "is parasite adhesive behaviour linked to SM"? This question can now be extended to *P. vivax* and *P. knowlesi* with the discovery of adhesion to endothelial receptors by RBC infected with these species (Carvalho et al. 2010; Cox-Singh et al. 2010) (Fatih *et al* unpublished data). In addition, how does *P. falciparum* cause such high levels of morbidity and mortality among those infected humans? Why don't other species contribute to disease severity to the same extent?

One molecule unique to *P. falciparum* identified on the surface of pRBC, known as PfEMP-1 (*P. falciparum* erythrocyte membrane protein-1) encoded by *var* genes, has been correlated with the property of *P. falciparum* cytoadherence (Flick & Chen 2004; Pasternak & Dzikowski 2009; Scherf, Lopez-Rubio & Riviere 2008), and it has been suggested that antigenic switching between different PfEMP1s constitutes an important virulence factor by facilitating parasite escape from the host's immune response and the establishment of chronic infection (Newbold et al. 1999; Newbold et al. 1997b). With the harmful effect of *P. falciparum* cytoadherence to the host, early treatment or even prophylaxis would be highly desirable in preventing cytoadhesion and the progression of disease. Unfortunately, falciparum malaria has become increasingly refractory to chloroquine the cheapest and most widely available antimalarial (Krishna & White 1996; Zucker et al. 1996) and this emergence of drug resistance in Southeast Asia

and Africa was closely associated with the increased incidence of SM (Giha et al. 2006) . The World Health Organization (WHO) advises all countries experiencing anti-malarial drug resistance, including mono-therapies, such as chloroquine, amodiaquine or sulfadoxine-pyrimethamine, should use combination therapies, preferably those containing artemisinin derivatives (ACTs – artemisinin-based combination therapies).

Recent clinical trials in Asia and Africa using ACTs show improved recovery of SM patients, but mortality reported shortly after hospital admission (within 48 hours) is still high despite the administration of highly effective anti-parasite drugs. This finding is consistent with our recent data showing that after exposure to drugs, killed pRBC are still able to cytoadhere (Hughes, Biagini & Craig 2010), which has led us to suggest that this persistent mortality may be due to the effects of adherent pRBC in the microvasculature. Is there any way of reducing mortality related cytoadherence? Perhaps adjunct therapies that can block and reverse the pathogenic effect of pRBC adhesion will lighten the disease burden. However, before embarking on this course, what evidence is there that cytoadherence is involved in SM and is it associated with parasite stages?

1.2 *P. falciparum* life cycle

The *Plasmodium* life cycle is very intricate, including a liver stage, blood stage, and mosquito stage (Figure 1.1). Initially, *Plasmodium* sporozoites are transmitted to a person by a mosquito bite. Once in the bloodstream, sporozoites are carried to the liver. Then they pass through several hepatocytes, until receptors on their surface adhere with heparin sulfate proteoglycans (Weatherall et al. 2002). After sporozoites invade the liver cells, there is a period of development that lasts 7-10 days. This time is not associated with symptoms of the disease, but is a time of cell division that culminates in the release of 20,000-40,000 merozoites. Since infection is initiated with only a few sporozoites, this period is essential for the progression of the disease and is a prime target for possible anti-malaria drugs (Smith & Kain 2004).

Released merozoites travel through the bloodstream, and adhere to host erythrocytes through a set of adhesion proteins. They then invade the cell by

inducing a vacuole in the cell membrane, and entering by formation of a moving junction. Once a merozoite invades an erythrocyte, it undergoes a cycle of asexual division, which lasts approximately 48 hours, and can release from 10-24 new merozoites into the vascular system once completed. The erythrocyte stage of the *Plasmodium* life cycle is responsible for the pathology of malaria. The use of intracellular replication by *Plasmodium* as well antigenic variation reduces the host's ability to eradicate parasites. In some individuals, a chronic infection is produced that can last over a year (Smith & Kain 2004).

In a number of erythrocytes, the merozoites differentiate into male or female gametocytes, which are the sexual form of the parasite. *P. vivax* produces gametocytes before the symptomatic stage of the disease, while *P. falciparum* produces its gametocytes much later (Miller et al. 2002). Currently, it is unknown why some merozoites differentiate into gametocytes, while others do not. The life cycle is completed when gametocytes infect female mosquitoes, and undergo sexual reproduction in the gut of the mosquito to produce sporozoites that migrate to the salivary glands.

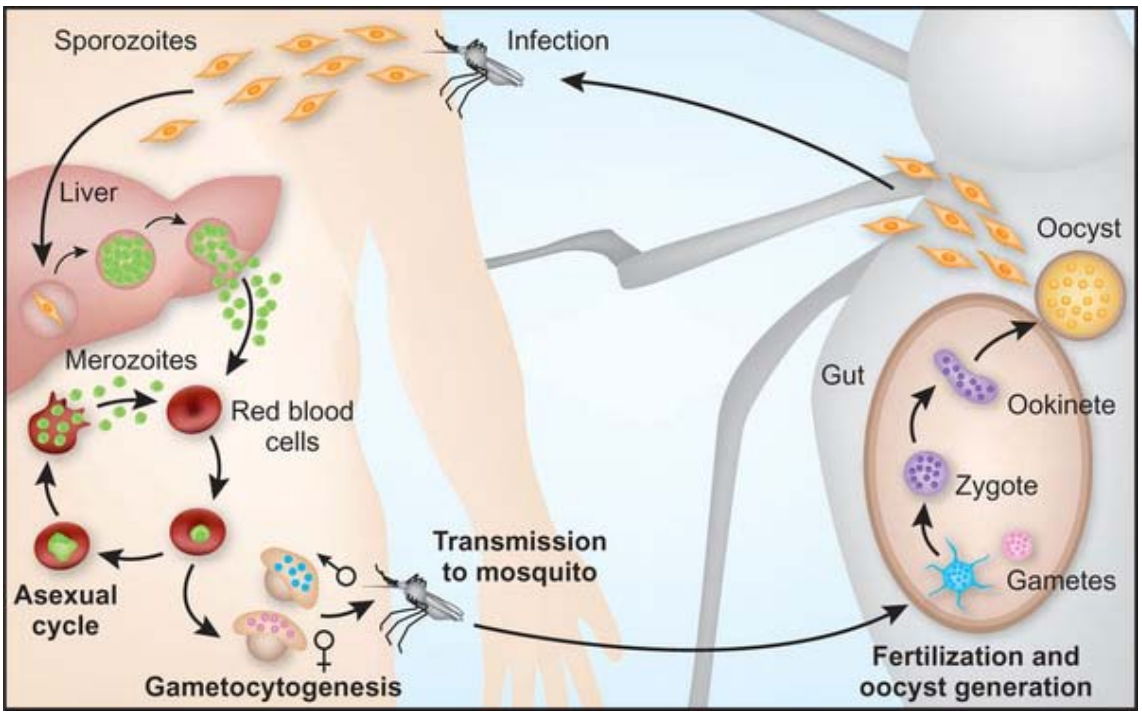


Figure 1.1: Schematic diagram of the various life cycle stages of *Plasmodium falciparum*. Diagram was adopted from Pasvol, G. (2010) 'Protective hemoglobinopathies and *Plasmodium falciparum* transmission', *Nat Genet*, vol. 42, no. 4, pp. 284-285(Klegeris & McGeer 2001).

1.3 Pathophysiology of malaria

Pathogenesis of malaria is initiated when parasites are released from the liver to the blood circulation. This is due to red blood cell (RBC) invasion and consequent rapid parasite replication. The pathogenesis of malaria can range in severity, with SM affecting multiple systems and organs. Malaria generally can divide into two types' mild (or uncomplicated) and severe. Classic symptoms of mild malaria include fever, chills, sweating, and muscle pains. In certain condition malaria patients with SM sometimes exhibit anaemia, kidney failure, respiratory distress, and CM (Weatherall & Clegg 2002).

Many symptoms of malaria are highly interconnected. For example, respiratory distress is believed to be due to metabolic acidosis (Shahmanesh, Cartledge & Miller 2002). Metabolic acidosis is a condition when the blood pH becomes too low (< 7.35), and seems to be due to a combination of several factors that reduce oxygen delivery and increase lactic acid accumulation. Destruction of uninfected RBC and infected erythrocytes during RBC rupture due to increased rigidity contributes to anaemia, which reduces the overall ability of blood to transport oxygen. In severe cases, abundant accumulation of lactic acid can lower the blood pH, leading to a reduced ability of the blood cells to bind oxygen, and it has been found that the ability of parasite to adhere in specific tissues can block blood flow, which subsequently reduces oxygen transport (Zougbede et al. 2011). This is just one example that shows that a single clinical symptom may be due to one or several factors leading to disease severity, making the treatment of malaria difficult.

It has been thought that parasite cytoadherence plays a major role indeveloping SM and mortality due to unique characteristic of mature P. *falciparum* (trophozoites and schizonts). At this stages pRBC are absent from the peripheral blood flow, due to binding of pRBC to adhesion receptors on macrovascular endothelial cells (sequestration), binding of pRBC to uninfected RBC (rosettes) and pRBC binding to other pRBC (clumping) (Figure 1.2).

The sequestration of pRBC in the microvasculature of vital organs is a prominent feature of *P. falciparum* malaria and is likely to be the one of most

important factors in the pathogenesis of the disease (Turner et al. 1994). Sequestration is defined as the removal of pRBC from the peripheral circulation by binding of the pRBC to vascular endothelium, predominantly in post-capilary venules of the deep tissues. Impaired oxygen delivery due to occlusion of blood flow in the vessel may result in organ dysfunction. Massive sequestration in the brain is believed to be the cause of coma in CM (Turner 1997). In the human host the spleen attempts gets rid of all abnormal RBCs from circulation. pRBC are deformed due to parasite development as such they are targeted by the spleen and are removed in a process known as splenic clearance. Most literaly *P. falciparum* sequester in blood vessels to avoid splenic clearance.

Parasite sequestration is an integral part of pRBC cytoadherence to endothelium, and extended by clustering of cells through either rosetting or clumping. Both clumping and rosetting have been independently associated with SM and anemia (Carlson 1993; Rowe et al. 2009).

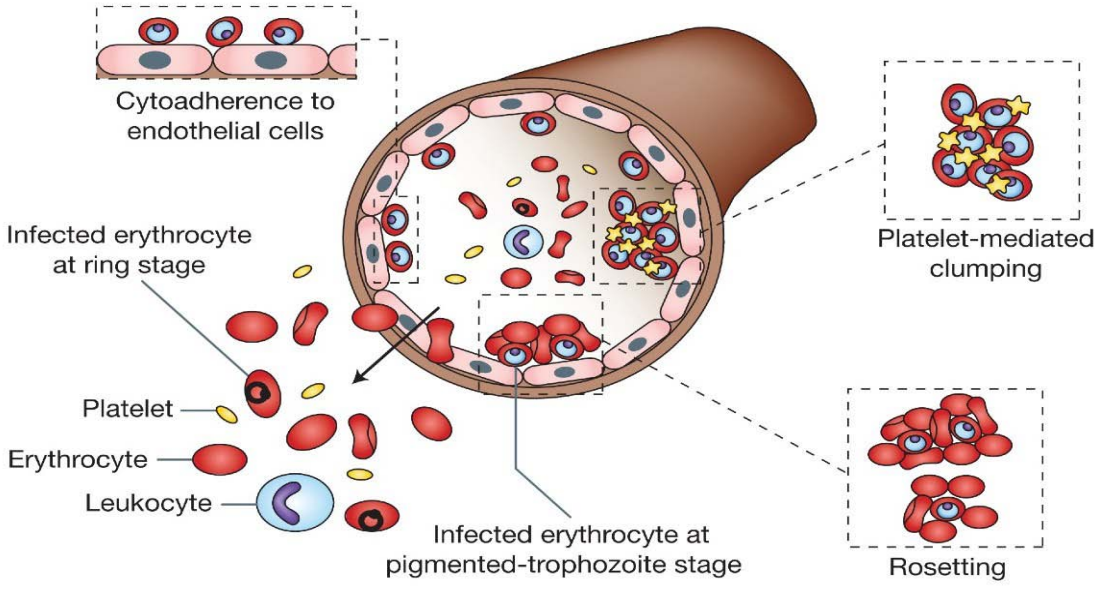


Figure 1. 2: Schematic diagram of malaria cytoadherence to microvascular endothelium. PfEMP1 expressed on the surface of mature RBC with *P. falciparum* is involved with antigenic variation that can bind to many host receptors through its multiple adhesion domain. The different properties of PfEMP1 able to results in three type of cytoadherence; sequestration, where it contribute the survival of the parasite by adhesion to endothelial cells, simultaneously binding to several receptors, binding of uninfected RBC (Rosetting), and Clumping of pRBC to another pRBC through platelet. All types are associated to pathogenesis of malaria.

1.4 Parasitized red blood cell (pRBC)

As a parasite matures inside a host red blood cell, the cell becomes capable of cytoadherence, including adhesion to the endothelial cells that line blood vessel walls. Infected red blood cells are significantly more rigid than the highly deformable red blood cell (Cranston et al. 1984). Sequestration in the microvasculature is a mechanism by which the rigid pRBC can escape from circulation and avoid passage through, and destruction by the spleen. For *P. vivax*, pRBC cytoadherence is not a major feature in this parasite and the mature asexual forms are often seen in peripheral circulation during intraerythrocytic development. However, *P. vivax* infection does not lead to increased rigidity of the pRBC. It has been suggested that the *P. vivax* pRBC is more deformable than uninfected RBC (Suwanarusk et al. 2004). An alternative mechanism for avoidance of splenic clearance of *P. vivax* pRBC has been proposed based on adhesion of *P. vivax* pRBC to barrier cells in the spleen preventing entry into the spleen and subsequent clearance (del Portillo et al. 2004), but there is little evidence to support this.

The many modifications that a parasite makes to the host red blood cell might contribute to the decreased deformability of the pRBC (Cooke, Mohandas & Coppel 2001). Changes made by the parasite to the RBC include: i) expression of parasite proteins on the cytoplasmic face of the RBC membrane. ii) Expression of parasite proteins on the surface of the pRBC and insertion of novel, or modification of existing channels in the RBC membrane to form the new permiability pathways (NPP). iii) The modification of RBC membrane cytoskeletal components. These structural changes made by the parasite occur alongside the increasing physical size of the parasite within the RBC, and the increase in components of the RBC cytoplasm such as solutes being taken up into the cell by the parasite (Kirk 2001). The resulting increased rigidity may be a factor in impairing microcirculatory blood flow (Dondorp et al. 2008), and determination of disease severity (Dondorp et al. 2000).

1.5 pRBC cytoadherence

Why and how does parasite cytoadherence-related mortality take place? Several hypotheses have been proposed and reviewed elsewhere trying to associate binding of pRBC in the microvasculature and mortality, such as; i) changes of RBC and pRBC rigidity (Dondorp et al. 1999; Dondorp et al. 2000; Dondorp et al. 2002); ii) pro-inflammatory induction of adhesion receptor expression (Armah et al. 2005a; Armah et al. 2005b); iii) binding on pRBC to specific adhesion receptors on endothelial cells (Ochola et al., 2011); iv) endothelial activation (Chakravorty, Hughes & Craig 2008; Conroy et al. 2010; Garcia et al. 1999) and v) malaria toxins (Ferreira et al. 2008; Schofield et al. 2002), with various levels of evidence to support them. However, there are also more recent discoveries such as the relevance of platelets and microparticles, as well as a role for the coagulation cascade in mediating pRBC binding on endothelial cells and association with disease severity (Francischetti 2008; Francischetti et al. 2007).

A major question is how *P. falciparum* has adapted to bind in the microvasculature to such an extent that mature pRBC are rarely seen in the peripheral circulation, unlike another human- invading malaria parasite species? An important difference in *P. falciparum* is the modification to the surface of the host erythrocytes to become rigid and inflexible, through exporting specific proteins to the RBC membrane during the intra-erythrocytic stages. This reduction of flexibility of RBC makes their circulation through the microvasculature difficult and favours pRBC adhesion to endothelial cells (Dondorp, Pongponratn & White 2004).

In 1985, MacPherson *et al.* reported higher levels of pRBC in the cerebral vessels of adults post-mortem dying from CM compared to non-CM (NCM) (MacPherson et al. 1985), demonstrating preferential accumulation of pRBC in the brain being linked to CM and consistent with the findings of Marchiafava and Bignami (Bignami 1954; Marchiafava, Bignami & Mannaberg 1894). The MacPherson study identified the contact point for pRBC *in vivo* as a knob-like structure, which had previously been demonstrated by *in vitro* studies. Knobs are distortions on the surface of *P. falciparum* pRBC caused by deposition of a knob-

associated Histidine-rich protein (KAHRP) at the cytoplasmic side of the pRBC me brane (Howard et al. 1990), and containing several other parasite proteins, including PfEMP-1, RESA and MESA (Craig & Scherf 2001). It is generally accepted that PfEMP-1 is largely responsible for pRBC adhesion in *P. falciparum* and various associations between *var* gene expression and complicated/uncomplicated disease have been reported. However, are knobs essential to establish an interaction in the microvasculature? Some other *Plasmodium* species such as *P. brasilianum*, *P. vivax* and *P. malariae* also have knobs but do not exhibit cytoadherence properties. Biggs *et al*, demonstrated that knobless *P. falciparum* could bind to host receptors (Biggs et al. 1989), although later work showed that a KAHRP knockout line (without knobs) could not bind under more physiological flow conditions (Rug et al. 2006) A few research groups have shown that abnormality of PfEMP1 existed on haemoglobin C and sickle cell RBC, which produce fewer knobs and are associated to reduced cytoadherence (Cholera et al. 2008; Crompton et al. 2008; Fairhurst et al. 2005).

1.6 RBC membrane cytoskeleton

A red blood cell needs to be highly deformable to allow passage through small blood vessels throughout its typical 120-day lifespan. This deformability is a result of a highly specialized membrane cytoskeleton structure with elastic properties. *P. falciparum* can maintain a chronic infection due to the sequential immune clearances of parasites, followed by an emergence of isogenic parasite populations expressing different variant antigens on the surface of the pRBC.

More than 16 hours after they infect red blood cells, malaria parasites secrete and export proteins onto the surface of pRBC and produce knobs, which contain a group of proteins including RIFIN, STEVOR, PfMC-2TM, PfEMP-1 and SURFIN. PfEMP-1 is thought to play a significant role in malaria pathogenesis through attachment to host endothelium in a range of tissues in a process called sequestration and has been implicated in the malaria parasite ability to evade host immune responses via it antigenic variation and shown to be essential for the ability of pRBC to cytoadhere under flow conditions (Crabb et al. 1997). Knobs are

formed from the interaction with parasite proteins PfEMP3 and knob associated KAHRP with host spectrin, actin and ankyrin.

Spectrin is the main component of the cytoskeleton, and is composed of α and β chains that form antiparallel heterodimers, which then associate to form tetramers. Junctional complexes connecting spectrin tetramers comprise mainly of protein 4.1, and actin. These are connected to the membrane through interactions between protein 4.1 and ankyrin to the integral membrane protein's band 3 and glycophorin C. In pRBC this membrane cytoskeleton is altered by parasite proteins that interact with the host membrane or cytoskeleton, as well as a parasite induced modifications to endogenous host proteins (Cooke, Mohandas & Coppel 2001). Some of these changes may contribute to the increased rigidity of pRBC compared to uninfected RBC (Cranston et al. 1984).

Protein 4.1 anf MESA

Protein 4.1 appears to be essential for the proper development of *P. falciparum*. It has been shown that protein 4.1 is phosphorylated to a greater extent in pRBC than in uninfected RBC, and the parasite protein, mature parasite-infected erythrocyte surface antigen, (MESA), is known to interact with protein 4.1 (Bennett, Mohandas & Coppel 1997). Failure to interact with protein 4.1 leads to accumulation of MESA in the RBC cytoplasm and reduced parasite viability. However, the precise role of MESA is unknown and MESA is not thought to be important for the formation of knobs or cytoadherence (Lustigman et al. 1990; Magowan et al. 1995).

KAHRP

KAHRP has binding sites for Spectrin, Ankyrin and PfEMP-1. The presence of KAHRP is essential for a formation of knobs on the surface of pRBC (Figure 1.3), and also for the display of PfEMP-1, and efficient cytoadherence. In the absence of KAHRP, whether through chromosomal deletion or targeted genetic deletion, the level of PfEMP-1 on the surface is reduced, the distribution of the protein is altered, and cytoadherence under flow is impossible (Crabb et al. 1997; Horrocks

et al. 2005). The formation of knobs has been shown to be a major determinant in the reduced rigidity of pRBC (Paulitschke & Nash 1993), and KAHRP specifically has been shown to have an important role in RBC reduced rigidity (Glenister et al. 2002).

PfEMP-3

PfEMP-3 has been shown to interact with spectrin and actin (Waller et al. 2007), destabilizing the RBC membrane cytoskeleton (Pei et al. 2007) and playing a role in the reduced deformability of pRBC (Glenister et al. 2002). PfEMP-3 is located in the pRBC membrane cytoskeleton and can be found in knobs (as well as distributed throughout the cytoskeleton) but is not essential for their formation. A truncated mutant of PfEMP-3 fails to reach the surface and also disrupts proper PfEMP-1 trafficking (Waterkeyn et al. 2000).

Band 3 and Glycophorin

Parasites also induce changes to erythrocyte proteins. Components of the pRBC cytoskeleton Band 3 and Glycophorin show restricted mobility within the membrane as compared with uninfected erythrocytes. This could be due to the effect of parasite proteins or due to oxidative stress induced by the parasite (Parker, Tilley & Klonis 2004). Modified band 3, (also known as Pfalhesin) may have a role in cytoadherence to CD36 (Crandall, Land & Sherman 1994).

NPP

Proteins inserted into the erythrocyte membrane form, or partly make up, components of the new permeability pathways (NPP). These pathways induced by the parasite enable it to take up the nutrients needed for rapid growth and differentiation. The NPP increases flux of many solutes, including amino acids, sugars, nucleosides, vitamins and other inorganic and organic anions and cations. (Kirk 2001). Although precise components of the NPP are unknown, and they may

consist of modified host pathways (Kirk et al. 1994), the involvement of parasite proteins in these pathways has been shown (Baumeister et al. 2006).

Surface Exposed Proteins

Parasite proteins, including the protein responsible for cytoadherence, PfEMP-1 (see below), are known to be exposed on the surface of a pRBC (Fig. 1.2) (Craig & Scherf 2001). Other major proteins exposed on the surface of the pRBC are another family of variant proteins, Rifins, as well as the modified host Band 3.

Rifins

Rifins are encoded by the clonally variant *rif* family of genes. There are an estimated 200 copies of *rif genes* per haploid genome and these are located subtelomerically, close to *var* genes. mRNA encoding rifins is detectable in trophozoite-stage parasites from around 18 – 23 hours post-invasion, i.e. slightly later than seen for *var* genes. Rifins are 27 –34 kDa proteins and are detectable in the RBC cytoplasm as well as associated with the mature pRBC membrane (Kyes et al. 1999). Rifins were originally identified as being involved in rosetting (Helmby et al. 1993), a virulence associated phenotype involving the adhesion of a pRBC with one or more uninfected RBC through binding to, amongst others is the complement receptor CR1 (Cockburn et al. 2004). However, it is now thought that PfEMP-1 is the major ligand mediating rosetting (Rowe et al. 1997).

SURFIN

SURFIN, a schizont and merozoite associated protein encoded by 10 surf genes, is transported to the surface of pRBC together with PfEMP1 (Mphande et al. 2008). The protein is mimicly related to a *P. vivax* transmembrane protein, PvSTP1 (*Plasmodium vivax* subtelomeric transmembrane protein 1), the *VIR* protein of the *P. vivax* external cysteine rich domain and the ATS (acidic terminal sequence) of PfEMP1.

PfMC-2TM

P. falciparum Maurer's cleft – 2 transmembrane **(**PfMC-2TM) is a protein which comprises 13 gene members and is localized to Maurer's cleft of *P. falciparum* and transported to erythrocyte surface (Sam-Yellowe et al. 2004). Expression profiles of this protein are not completed yet but a restricted expression in mid-trophozoites stage has been suggested (Lavazec, Sanyal & Templeton 2006, 2007; Tsarukyanova et al. 2009).

STEVOR

STEVOR is a third large family of clonally variant proteins which although expressed in many life cycle stages is now not thought to be exposed on the surface of asexual stage pRBC and gametocytes (McRobert et al. 2004). Recently, these protein were shown to be expressed at the apical end of merozoites and they might be associated with erythrocytes invasion (Blythe, Surentheran & Preiser 2004).

PfEMP-1

The sequestration of pRBC is due to adhesion molecules that the parasites place on the surface of the red blood cell membrane. These adhesion molecules are called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is a family of variant antigen proteins encoded by *var* genes. There are approximately 60 *var* genes that vary between *P. falciparum* strains (Su et al. 1995).

The PfEMP-1 molecules adhere to various host receptors on the surfaces of the endothelial cells (Figure 1.6) in the microvasculature, brain, placenta, and other organs. The most characterized host receptor molecules are CD36, CSA (Chondroitin Sulfate A), and ICAM-1 (Intercellular Adhesion Molecule – 1).

PfEMP-1 is a heterogeneous protein ranging from 200 to 400 kDa. Apart from cytoadhesion and antigenic variation, PfEMP-1 has also been found to mediate the attachment of infected red blood cells to uninfected red blood cells (rosetting) and in addition, the ability of pRBC to attach to other pRBC (agglutination). PfEMP-1 protein is anchored to knobs through association

between the PfEMP-1 cytoplasmic C-terminal acidic terminal segment (ATS) and KAHRP (Figure 1.4) (Oh et al. 2000; Pei et al. 2005; Rug et al. 2006).

Var genes, although very diverse, have a similar basic structure consisting of two exons. The first exon codes for multiple extracellular domains that are homologous to the cysteine-rich domains of *P. falciparum* erythrocyte binding antigens (EBA 175) (Rodriguez et al. 2000) and the *P. knowlesi* duffy binding proteins (DABP) (Adams et al. 1992) and have therefore been termed Duffy binding-like domains (DBL). A short trans- membrane region precedes the second exon coding for a conserved sub- membrane acidic terminal segment (ATS) that probably anchors PfEMP1 at the knob (Oh et al. 2000; Voigt et al. 2000). The first extra cellular domain (-DBL1-) is relatively conserved and is next to another semi-conserved region, the cysteine-rich inter domain region 1 (CIDR-1) (Smith et al. 2000a; Smith et al. 2000b). The relative conservation of these two regions is suggestive of functional constraint and these two regions have been shown to be the binding sites for CD36, PECAM/CD31, blood group antigens and glycosaminoglycans (Figure 1.5) (Baruch et al. 1997; Chen et al. 2000; Smith et al. 1998). PfEMP1 from different parasite isolates have different numbers of extracellular domains, and this appears to influence the parasite isolate's binding phenotype.

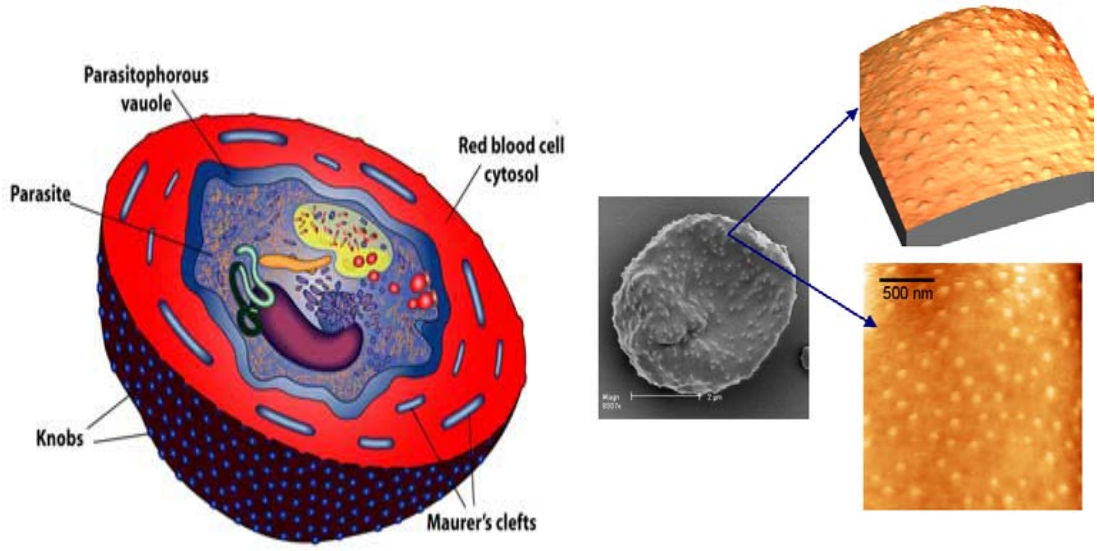


Figure 1. 3: Schematic diagram of pRBC, SEM and AFM images of KAHRP knobs on *P. falciparum*-parasitized red blood cells. Knobs appear on *P. falciparum* pRBC in early trophozoite stages of the asexual life cycle of the parasite. They are protuberances of the RBC membrane that contain various malarial proteins, with the structural component beng the knob-associated histidine rich protein (KAHRP). Picture was taken from Rug, et al (2006).

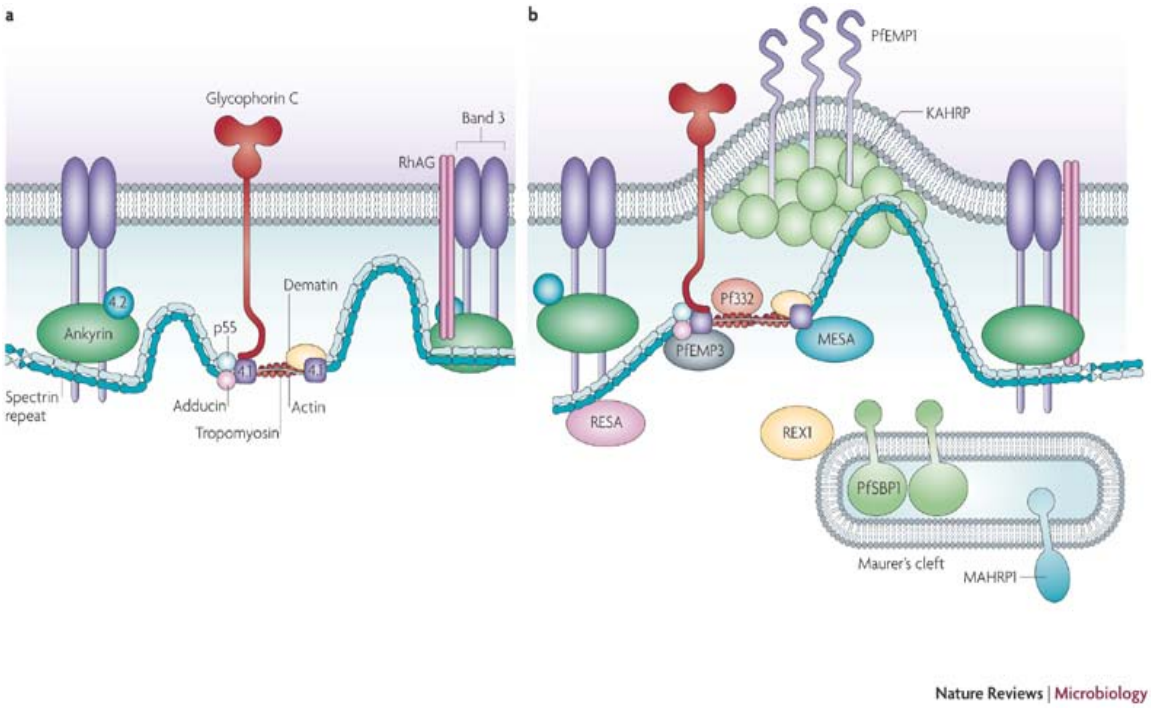


Figure 1. 4: Schematic surface of pRBC; a) surface of non-infected RBC, Spectrin heterodimers, comprising repeat units linked by flexible hinges, can expand and unfold in response to deformation stress. Spectrin molecules are linked head to head to form tetramers and their tails by junction complexes comprising actin oligomers that are stabilized by protein 4.1R and other molecules. b) Knobs on the *P. falciparum* pRBC. In ring-stage pRBC, ring infected erythrocyte surface antigen (RESA) associates with spectrin and stabilizes the membrane skeleton. In mature-stages pRBC, KAHRP molecules self associate to form a conical structure that interact with spectrin. The cytoadherence-mediating protein PfEMP1 is concentrated in the region of the knobs by interaction of its cytoplasmic domain with KAHRP. Diagram was taken from Maier et al 2009.

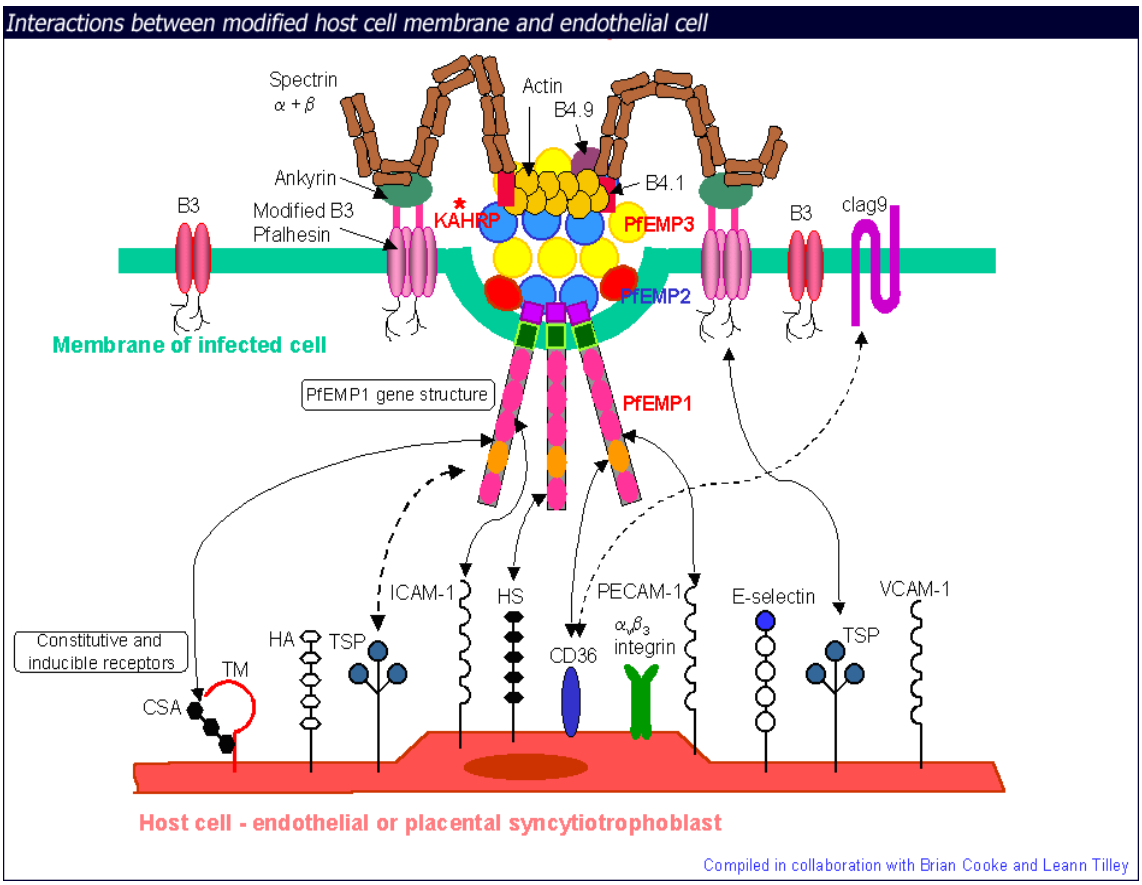


Figure 1. 5: Schematic diagram of interaction of PfEMP1 on the surface endothelial cells. Diagram was adapted from Cooke (2000) (Koehler, Birkelund & Stephens 1992).

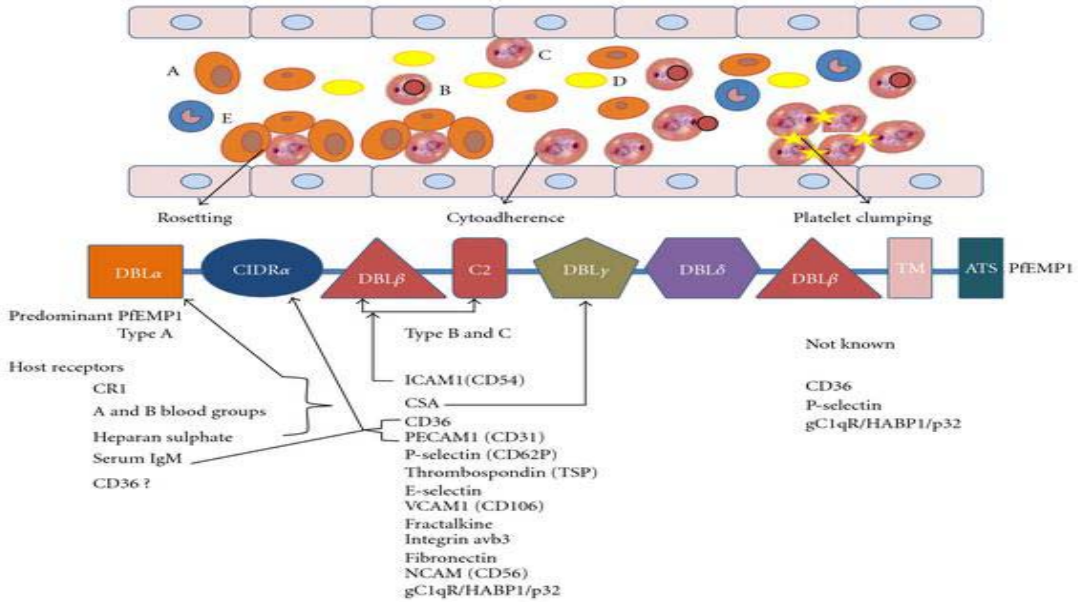


Figure 1.6: Schematic diagram of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1); protein architecture and host receptors binding domains. The intracellular domain ATS is highly conserved and anchors PfEMP1 to parasite-induced knobs that facilitate infected erythrocyte sequestration. The extracellular domain is highly variable but is predominantly assembled from four building blocks: NTS, DBL, CIDR and C2 domains. Based on sequence similarity, DBL domains group as five types (α– ε) and CIDR domains as three types (α–γ). The prototypical PfEMP1 extracellular region (a) consists of an NTS and DBL1α–CIDR1 'semiconserved head structure' followed by a DBL2δ–CIDR2 tandem. Larger PfEMP1 proteins also include the DBLβ, γ and εtypes arrayed differently. CD36 is considered to be the major endothelial sequestration receptor; other receptors are recognized less frequently but might have important roles in disease. A: Erythrocyte, B: Infected erythrocyte, C: Infected erythrocyte at pigmented trophozoite stage, D: Platelet, and E: Leukocyte. Abbreviations: CR1, complement receptor 1, ATS, acidic terminal segment; CIDR, cysteine-rich interdomain region; DBL, Duffy-binding-like domain; ICAM-1, intercellular adhesion molecule-1; NTS, N-terminal segment; TM, transmembrane domain. Figure adapted from Smith 2001 (Smith et al. 2001).

1.7 Antigenic variation

Foreign antigens are quickly and efficiently targeted by the host immune system, so in order to survive in this harsh environment pathogens have evolved various ways to circumvent or disarm these defences. Phenotypic variation will eventually result in the generation of antibodies against the protein displayed on the pRBC surface. These antibodies successfully clear the parasite from the blood circulation resulting in a reduction of parasite density. However, before complete clearance can be achieved, subpopulations of parasite arise that express another variant of PfEMP1 on their surface, a called process antigenic variation. By switching the expressed *var* gene, the parasite changes it antigenic properties and antibodies produced against *var* protein variant become ineffective. This change in *var* gene expression was correlated with functional changes resulting in altered adhesive phenotypes (Smith et al. 1995a) which in turn might influence the virulence of the parasite isolate during the course of an infection. Therefore, antigenic switching allows the parasite to subvert the host immune response and to eventually establish persistent chronic infections (Figure 1.7).

Information about the sequence of *var* gene switching e.g. whether it occurs randomly or follows a defined order is still missing. However, Horrocks et al (Horrocks et al. 2004) propose that the switching history might influence the ability to switch to certain *var* genes and the presence of short-lived cross-protective antibodies might lead to the sequential dominance of *var* gene (Recker et al. 2004). Parasite switching of expression to different PfEMP-1 has been measured *in vitro* at rates up to 2.4% per generation, with immune selection presumably restricting the expressed PfEMP-1 repertoire *in vivo* (Roberts et al. 1992a). Switching between expressed *var* genes generate progeny that differs from parental parasites. However, in another study much slower rates between 0.25% and 0.0025% have been reported (Horrocks et al. 2004). How does *P. falciparum* switch *var* genes on and off? Researchers have come out with antigenic variation models, which explain how *var* gene transcription is controlled. The first model mentioned, the promoter which existed upstream and downstream of the *var* gene can regulate the expression of PfEMP-1 and independent of the 5'UTR of *var* gene (Horrocks et al. 2004). Second model said the *var* gene will express PfEMP-1 during the cell cycle. Mutually exclusive expression and *var* gene expression

switching are thought to be controlled epigenetically, referring to the fact that activation and silencing of individual gene are not accompanied by changes in either DNA sequences or the presence or absence of specific transcription factor or promoter. Meanwhile, the third model said the location (expression site) of the *var* gene is important associated to activation of the promoter to be transcript. It said *var* genes at location euchromatin (internal region of the nucleus where chromatin is loose and open for transcription) is more active compared, to those in heterochromatin region (chromatin is condensed) (Frank et al. 2007).

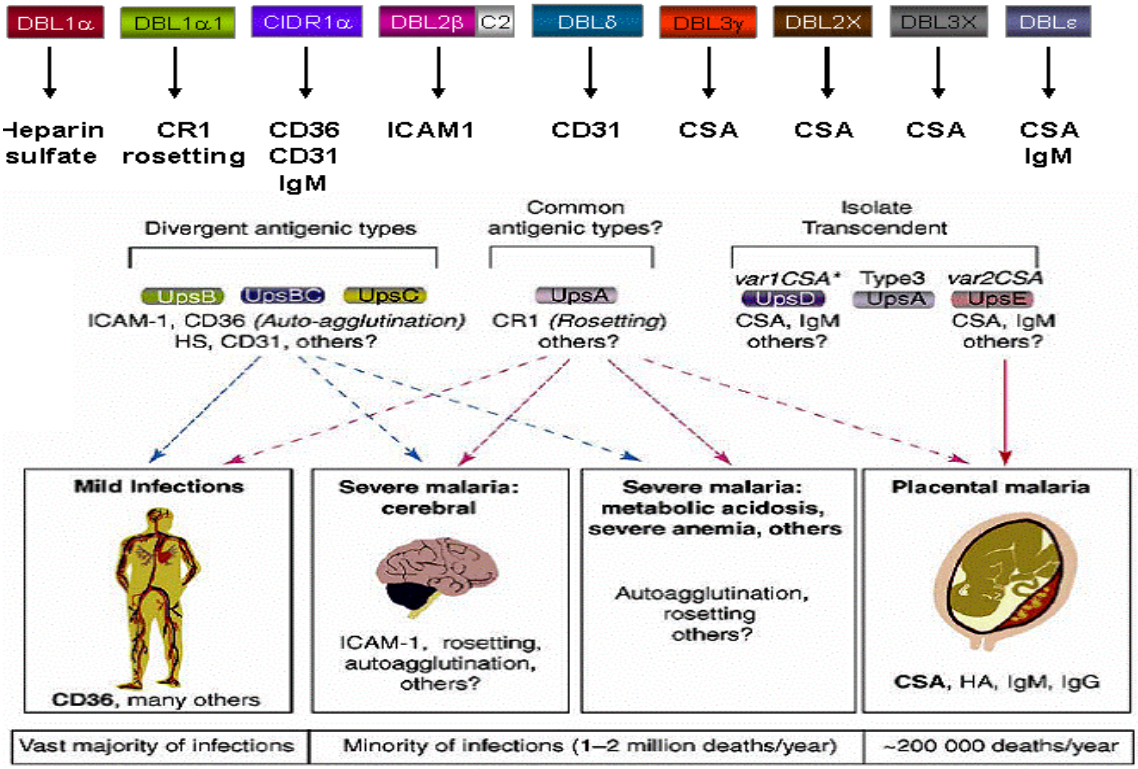


Figure 1. 7: Schematic diagram on association of PfEMP1 domain and adhesion receptors on endothelial cell to confer severe disease. Diagram was taken from Kraemer et al (2006).

1.8 Host adhesion receptors

Endothelial cells can express many different adhesion molecules on their cell surface, many of which are highly glycosylated proteins, which have roles in the immune response, often acting as receptors for other immune cells. The expression of many receptors is increased in response to immune stimuli, including TNF, which is known to be upregulated in malaria. pRBC have been shown to be able to bind to many of these receptors (Figure 1.6), and in some cases there is a link with the disease phenotype, notably between adhesion to ICAM-1 and CM, and CSA and placental malaria (Ho et al. 1991). In most cases, the adhesion has been shown to be mediated by the parasite protein PfEMP-1. The variant properties of PfEMP-1 enable different variant PfEMP-1 proteins to be able to have different cytoadherent properties, resulting in a large range of adhesion receptors to which pRBC expressing different PfEMP-1 proteins can bind. Numerous host molecules have been identified which can act as receptors for pRBC. CD36, thrombospondin (TSP), ICAM-1, VCAM-1, CD31, P-and E-selectin serve as receptors on endothelial cells (Barnwell et al. 1989; Ho & White 1999; Ockenhouse et al. 1991a; Roberts et al. 1985). Chondroitin sulphate A (CSA) and hyaluronic acid (HA) (Beeson et al. 1999; Reeder et al. 1999; Rogerson & Beeson 1999) have been shown to be involved in placental malaria. For rosetting, the following molecules have been implicated: IgM (Scholander et al. 1996), heparan sulphate (HS) (Chen et al. 1998), complement receptor 1(CR1) (Rowe et al. 2009), blood antigens A and B (Barragan et al. 2000b) and complement factor D (Luginbuhl et al. 2007). Meanwhile, via CD36, pRBC can also adhere to monocytes and dendritic cells with effects on phagocytosis and clearance of pRBC (McGilvray et al. 2000) and immunosuppression (Urban et al. 1999).

Thrombospondin (TSP)

Thrombospondin (TSP) is a large adhesive glycoprotein that is associated with endothelial cell surfaces and has binding sites for a wide range of ligands, from calcium to glycolipids (Lawler & Hynes 1986). TSP has been implicated in binding under both static (Roberts et al. 1985) and flow conditions (Rock et al. 1988). However, binding to TSP alone is thought not to be sufficient to mediate cytoadherence (Cooke et al. 1994; Ho & White 1999). The modified band three

protein has been implicated in adhesion to TSP contrary to the major adhesion ligand PfEMP-1 which is thought to be responsible for adhesion to most other identified receptors (Eda, Lawler & Sherman 1999; Lucas & Sherman 1998).

CD36

CD36 has been associated with microvascular adhesion of pRBC (Barnwell et al., 1989). CD36 is an 88 kDa integral membrane glycoprotein that is found on the surface of endothelial cells, platelets, and monocytes (Greenwalt et al. 1992). Besides being a receptor for *P. falciparum* pRBC, it has been shown to play many roles in the body including: a platelet collagen receptor, a possible receptor of thrombospondin (TSP) to mediate the TSP-dependent adhesion of monocytes to macrophages and platelets, and may participate in signal transduction in the activation of platelets (Greenwalt et al. 1992).

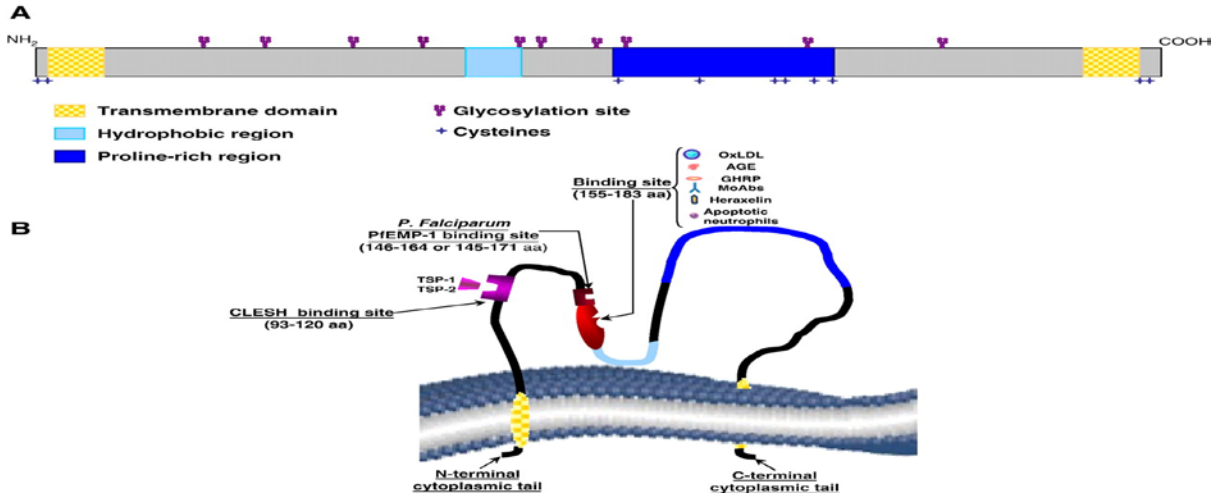


Figure 1. 8: Schematic representation of CD36 protein. (A) Primary structure of CD36 with transmembrane domain (yellow/orange box), hydrophobic region (light blue) and proline-rich region (dark blue). The ten predicted N-linked glycosylated sites, and ten cysteines are shown. (B) 3D representation of CD36 receptor on cellular membrane. The CLESH (CD36 LIMP-II Emp sequence homology) motif and PfEMP-1 sites are the binding sites for thrombospondin-1/-2 (TSP-1 and TSP-2) and *P. falciparum* erythrocyte membrane protein-1 (*P. falciparum* PfEMP-1) respectively. 155–183 amino acid region is the binding site for OxLDL, AGE, growth hormone-releasing peptide heraxelin (GHRP), monoclonal antibodies (mAb), and apoptotic neutrophils. (Adapted from Collot-Teixeira S et al 2007; Cardiovascular Research 75 (2007) 468-477).

CSA

CSA is the best characterized and studied receptor of pRBC and has been associated with placental malaria (Rogerson et al. 1995). CSA is a glycosaminoglycan composed of N-acetyl-galactosamine and glucuronic acid, and is sulfated mainly at carbon 4 of the repeating disaccharide. It is commonly found as part of a proteoglycan, and is a major structural component of the extracellular matrix and cartilage. In placental malaria, large amounts of pRBC sequester in the maternal circulation of the placenta. pRBC taken from women with placental malaria not only bound to purified CSA, but the binding of these pRBC to freshly frozen sections of human placenta was competitively inhibited by CSA (Fried & Duffy 1996).

During the first pregnancy, women may contract placental malaria even though they often have clinical immunity to other forms of malaria. This is due to the fact that the PfEMP1's involved in placental malaria are a subset of PfEMP1's that bind CSA but do not seem to bind to other receptors such as CD36, for which the women have already acquired antibodies (Kraemer & Smith 2003). An example of one such PfEMP1 is var2csa, which binds to CSA but not CD36 or ICAM-1 (Buffet et al. 1999). After several pregnancies, women in endemic areas begin to produce antibodies that block CSA binding to pRBC (Fried & Duffy 1998; Fried et al. 1998).

ICAM-1

ICAM-1 is believed to be the primary host receptor molecule linked to CM (Ochola et al. 2011; Weatherall & Clegg 2002). ICAM-1 is a 90–115 kDa glycoprotein that is found in endothelial cells, including brain, and hematopoietic cells (Chakravorty & Craig 2005). ICAM-1 is a member of the immunoglobulin-like (Ig-like) superfamily, and is comprised of five different extracellular Ig-like domains (Chakravorty & Craig 2005). It also contains a transmembrane domain, and a cytoplasmic tail (van de Stolpe & van der Saag 1996). In the body, ICAM-1 exists in a membrane-bound form as well as in a soluble form, which comes from ICAM1-1 that has been released from the surface of cells through proteolytic

cleavage. ICAM-1 functions as a receptor for several molecules, including leukocyte function associated molecule-1 (LFA-1), and the human rhinovirus (HRV) (Figure 1.9). ICAM-1/LFA-1 interaction plays a role in leukocyte recruitment and subsequent cell-cell adhesion reactions (Piela-Smith, Aneiro & Korn 1991). Endothelial expressed ICAM-1 is up-regulated in response to many inflammatory mediators (Chakravorty & Craig 2005).

There is much evidence to support the idea that ICAM-1 is involved in the pathogenesis of SM. First, ICAM-1 is up-regulated in response to inflammation, and is thought to be up-regulated in brain in response to *P. falciparum* infection (Turner 1997). It is believed that this up-regulation may increase sequestration of pRBC in the brain, which would contribute to the pathogenesis of the disease (Chakravorty & Craig 2005). Second, in one study, autopsy samples taken from people that died of CM showed that brain vessels with sequestered parasites also contained ICAM-1 (Turner et al. 1994). Third, people who are homozygous for the ICAM-1^{Kilifi} mutation have an increased susceptibility to CM, which shows a correlation between ICAM-1 and severity of malaria (Fernandez-Reyes et al. 1997), although this observation was not replicated in other studies. Fourth, adhesion of pRBC to ICAM-1 was higher in isolates from patients with clinical malaria than in parasitized community controls and tended to be higher in those with CM than in controls. Meanwhile Ochola et al found that pRBC-CD36 interaction was higher in uncomplicated malaria (UM) (Newbold et al. 1997a; Ochola et al. 2011).

Tse et al. (2004) characterized the PfEMP1 binding region of ICAM-1 under both static and flow conditions. Their studies suggested the ICAM-1 binding region for pRBC was near the BE and D beta sheet 'side' of the molecule. These authors choose amino acid residues G14-L30 and K39-K50 for alanine substitution. Their assays were performed on 3 different parasite lines A4, IT-ICAM, and JDP8. A4 has a lower avidity for ICAM-1, while IT-ICAM and JDP8 are high avidity binders. Interestingly IT-ICAM and JDP8 share common amino acids on ICAM-1 that are important for binding (Tse et al. 2004)

Tse et al., (2004) found that in JDP8 and IT-ICAM pRBC bind to ICAM-1 under static conditions, and they found cysteine 21, and serine 22 on the ICAM-1 (Figure 1.9 b) were involved in that binding, while A4 pRBC bind strongly to ICAM-

1[Kilifi] with position lysine 29 particularly important (Figure 1.10 b). All lines also require amino acids Glycine 14- leucine18, and leucine 42-44 on ICAM-1 for maximum binding on pRBC. Under flow conditions, an alanine substitution to these previously mentioned amino acids usually caused a greater decrease in binding. Furthermore, threonine 20, cysteine 21, lysine 39, lysine 40, asparagine 47, and lysine 50 were shown to decrease the binding of pRBC to ICAM-1. The similarities between the lines with the exception of lysine 29, implies that the binding interaction between ICAM-1 and PfEMP1 has a similar mechanism across variants. These results also hint at a possible common evolutionary origin of ICAM-1 binding in PfEMP1 (Tse et al. 2004).

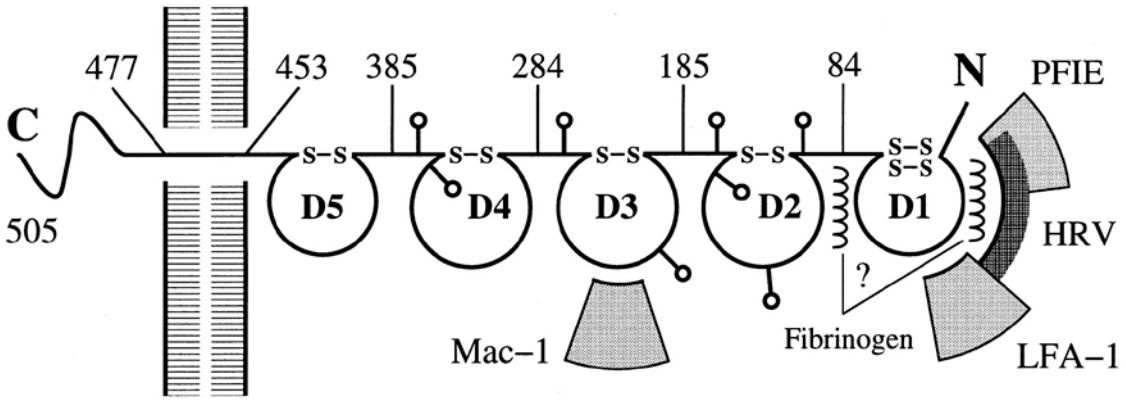


Figure 1.9: Diagram of ICAM-1 molecule. A diagram of an ICAM-1 molecule showing site of glycosylation (lollipop-shaped structure) and the approximate location of binding sites of LFA-1, Mac-1, human rhinovirus, fibrinogen and PfEMP1 (PFIE).

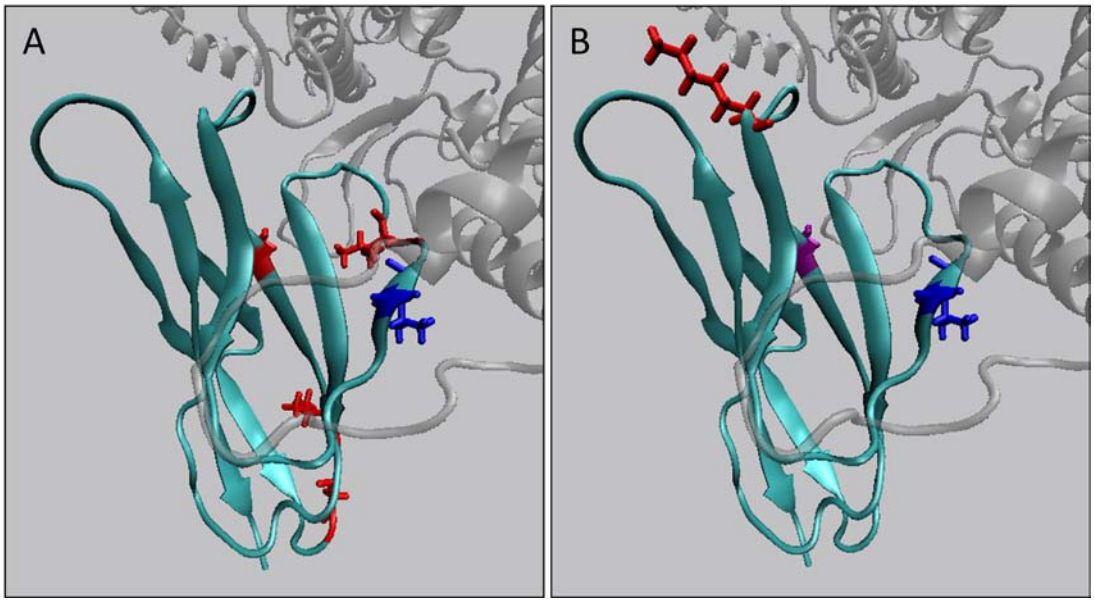


Figure 1. 10: Domain 1 of ICAM-1. Domain 1 of ICAM-1 (light blue) shown with part of modelled PfEMP-1 (translucent grey), Model PM0074932 (Bertonati 2007) (Bertonati & Tramontano 2007) see below. Annotated using VMD. Highlighted residues in A are residues implicated in adhesion of ItG PRBC to ICAM-1 in both static and flow conditions as determined by mutagenesis in (Tse et al. 2004). Highlighted resides in B are: Red - K29 implicated in adhesion of A4 PRBC to ICAM-1; Purple – S22 implicated in adhesion of ItG PRBC to ICAM-1 and Blue L42 implicated in adhesion of general blocking antibody mAb 15.2 to ICAM-1. Orientation of ICAM-1 D1 is shown as in (Tse et al. 2004).

VCAM-1

VCAM-1 is a member of the Ig superfamily of adhesion receptors, along with ICAM-1 and PECAM-1, and is upregulated on endothelium in response to inflammatory mediators. VCAM-1 consists of 6 Ig domains and is predicted to form a bent rod structure protruding out from the endothelial cell surface similarly to ICAM-1. pRBC have been shown to bind to VCAM-1, although VCAM-1 binding under flow conditions may be less important than adhesion to ICAM-1 or CD36 (Ockenhouse et al. 1992b; Yipp et al. 2007).

PECAM/CD31

PECAM-1/CD31 is another glycosylated member of the Ig superfamily. The protein is 130 kDa consisting of 6 extracellular Ig domains with 9 N-linked

glycosylation sites. PECAM is expressed on endothelial cells and intravascular cells, i.e. granulocytes, monocytes and platelets, and is thought to act as a homing receptor for leukocytes to pass through junctions of endothelial cells (Newman et al. 1990). The protein is normally localized to cell junctions but on stimulation with IFN-γ can be relocalised all over the cell surface, increasing binding. Binding of pRBC to CD36 negative but PECAM positive endothelial cells have been shown, as has adhesion to immobilized soluble PECAM (Treutiger et al. 1997a).

NCAM

Recently, the identification of an adhesion receptor on chicken brain tissue, suggested to be NCAM, (another member of the Ig superfamily) illustrates the increasing number of endothelial adhesion molecules known to be important for cytoadherence (Pouvelle et al. 2007). Due to the nature of the PfEMP-1 variation it is possible that variants exist that can adhere to most endothelial cell adhesion receptors. NCAM is known to be expressed on endothelium in two sites where sequestration is commonly observed in severe disease, skin and brain, tempting speculation that it may be an important receptor in severe disease (Pouvelle et al. 2007).

gC1qR/HABP1

Another recently identified receptor for pRBC adhesion is gC1qR/HABP1. This is a 32 kDa membrane protein that is expressed on resting endothelial cells and resting platelets, as well as other cell types. Three out of 8 field isolates tested, and two out of three lab isolates showed the ability to bind to this receptor, independent of ability to bind to ICAM-1 or CD36. This receptor was also able to mediate platelet clumping (as can CD36) so may prove to be an important receptor for different types of pathogenesis (Biswas et al. 2007). The existence of gC1qR/HABP1 on resting endothelium may indicate a role in adhesion early on in infection before the cytokine stimulated up-regulation of other widely used receptors such as ICAM-1.

P-Selectin & E-selectin

Selectins are characterized by N-terminal domain of 117 – 120 amino acids that is homologous to Ca^{2+} dependent animal lectins, and P-Selectin has been shown to be involved in pRBC cytoadherence (Udomsangpetch et al. 1997). The binding site for pRBC on P-selectin is thought to be on the lectin domain but distinct from that of a natural ligand PSGL-1 (Ho et al. 1998). A significant point about P-selectin is the distance from the endothelial cell surface that the molecule protrudes. This enables it to be potentially above the glycocalyx, an important consideration in leukocyte recruitment (Patel, Nollert & McEver 1995) which may well also be relevant for pRBC adhesion. E-selectin is another member of the selectin family that has been suggested to be able to be involved in adhesion (Ockenhouse et al. 1992b), although this has not been seen frequently in patient isolates.

1.9 What factors to mediate adhesion of the pRBC in host microvasculature?

Several receptors on endothelial cells have been shown to support interactions with pRBC, including thrombospondin; CD36; Ig superfamily cell adhesion molecules e.g intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), platelet endothelial cell adhesion molecule (PECAM) and neural cell adhesion molecule (NCAM); selectins e.g P-selectin and E-selectin; integrin αvβ3; globular C1q receptor (gC1qR); and Glycosaminoglycans e.g chondroitin sulphate A (CSA) and heparin sulphate (HS) (for a review see (Chakravorty, Hughes & Craig 2008)). With such a diverse collection of host receptors, how might one investigate associations between disease and specific adhesion phenotypes? It has been reported that ICAM-1 and CD36 (Chilongola et al. 2009) are the most commonly used adhesion receptors by the patient isolates (except in placental malaria), and correlations with severe and uncomplicated disease have been suggested, which has often been a starting point for clinical studies (Cojean et al. 2008). It has been proposed that synergism (or at least cooperation) between these two receptors make the binding of pRBC stronger (Gray et al. 2003; Yipp et al. 2000). Therefore, it is not unusual that clinical studies

examining the association between receptor usage and disease have concentrated on these two proteins (see Table 1).

In addition to ICAM-1 and CD36, chondroitin sulphate A (CSA) is also one of the most common and successfully studied adhesion receptors. CSA provides the clearest example of an interaction of pRBC with an adhesion receptor causing disease, but this does not come from endothelial cytoadherence but rather adhesion of pRBC to CSA in the placenta of pregnant women through a set of semi-conserved PfEMP-1 proteins (Higgins 2008). The restricted variation in this important facet of malaria pathology provides one of the most hopeful cases for the development of a disease-specific vaccine in malaria (Schofield 2007). In the case of placental malaria, the association of a specific *var* gene (*var2csa*) with adhesion and disease has been possible (Dahlback et al. 2011), but this has been much harder to define in other syndromes of SM, such as CM.

Another factor that has been postulated to have an association with host mediated cytoadherence is the role of the host pro-inflammatory cytokines. Pro-inflammatory cytokines have long been implicated in the pathogenesis of SM (Karunaweera et al. 1992) where changes in cytokine plasma levels have paralleled the rise of temperature during fever paroxysms in SM (Brown et al. 1999) and an increase of pro-inflammatory cytokines, especially TNF, in CM in children, especially from Africa and correlated with mortality (Grau et al. 1991; Kwiatkowski 1990) has been observed. Nevertheless, how are these pro-inflammatory cytokines regulated and how might they mediate parasite adhesion and SM, or is this just a general effect? Other studies have challenged the correlation of cytokine, especially TNF towards malaria disease severity and claimed it is quite poor in predicting SM (Conroy et al. 2010; Erdman et al. 2011). It is thought that pro-inflammatory cytokines are central to the pathophysiology of systemic disease caused by infectious and non-infectious agents, and cytokines such as TNF and IL-10 have been proposed to have a protective role to clear the infections but also to avoid inappropriate host responses that might lead to cell destruction and be harmful to the host. In the case of malaria and SM, high levels of pro-inflammatory cytokines TNF, IL-1, IL-6, IL-12, and INF-γ have been observed in patients and low levels of IL-10 and TGF-beta have been correlated with the fatal outcome (Day et al. 1999). It is thought that these cytokines are produced by

activated macrophages and dendritic cells (DC) and potentially endothelial cells during the host response to pRBC and schizont rupture (Richards 1997; Schofield et al. 1996).

Several studies show that pRBC stimulate antigen-presenting cells (APC) macrophages and DC probably through interaction of PfEMP-1 directly or indirectly via CD36 (Patel et al. 2004; Serghides et al. 2003) and it is known that CD36 is expressed on macrophages and DC surfaces. These findings in the literature are variable due to the use of different host species and parasite strains, at pre-erythrocyte or blood stages of infection (Wykes et al. 2007). How might cytokines support pRBC binding on microvascular endothelium? One thought is that it is through endothelial activation. For example, TNF is known to act by increasing the expression of host adhesion molecules. TNF binding on TNFR2 induces a recruitment signal transduction that actives effectors and transcription factors, leading to a strong increase in the level of expression of ICAM-1, VCAM-1 and E-selectin. The involvement of TNF in the upregulation of adhesion molecules has been clearly demonstrated in different *in vitro* and *in vivo* studies. However, does TNF induction alone do enough to exacerbate SM? If pathology of malaria was the direct result of a high level of TNF, patients with SM could be treated using TNF specific antibody. However, a trial using a monoclonal antibody against TNF did not show any protection and in fact, worsened neurological sequelae in patients (van Hensbroek et al. 1996).

The pattern of pathology in malaria is variable and the profound cytokine-mediated changes and tissue oedema seen in other infections are not characteristic of this disease, although some signs of these pathologies are available. Thus, it seems that malaria pathology can be linked to a pro-inflammatory response, but this is not enough to explain it. This is consistent with recent studies that have shown in children that TNF levels are a poor discriminator of severity of disease, whereas proteins associated with endothelial activation (e.g Ang 1/2 vWF; sICAM-1) are relatively good markers (Erdman et al. 2011).

Endothelial activation, responding to inflammatory mediators, collectively increases the expression of adhesion molecules, including E-selectin, ICAM-1 and VCAM-1 on the cell surface through activation of NF_kB signaling transduction.

Following activation, increases of P-selectin on EC have also been reported. P-selectin is different from other adhesion molecules as it is stored in EC specific storage vesicles called Weibel-Palade bodies together with other molecules such as Von Willebrand Factor (vWF). How Weibel-Palade bodies are activated in malaria infection is still unknown; the parasite has a protein, which is known as *P. falciparum* translationally controlled tumor protein (PfTCTP), that can cause basophils to release histamine (Beghdadi et al. 2008), but some reports have also suggested that activated platelets and fibrin might mediate the release of P-selectin and vWF (Bridges et al. 2010). VWF recently has been found to be a good prognostic marker for SM in children, and it has been thought that vWF might mediate pRBC binding on EC via ultra-large vWF multimer by producing a bridge via platelets (Bridges et al. 2010; Hollestelle et al. 2006; Larkin et al. 2009; Lowenberg et al. 2010; Tripathi et al. 2008).

People have speculated that the febrile temperatures seen as part of malaria infection might enhance cytoadherence. We know that fever is due, in part, to the increase in TNF seen during infection but does temperature elevation help pRBC to bind to endothelial cells? Udomsangpetch *et al* (Udomsangpetch et al. 2002) showed that PfEMP-1 expression is accelerated by febrile temperature and increases cytoadherence. However, this is contrary to other findings where febrile temperature affected intra-erythrocyte growth and up-regulation of PfEMP-1 was not seen (Oakley et al. 2007). Recently, Pattanapanyasat et al. (Pattanapanyasat et al. 2010) showed that febrile temperature induced and enriched expression of phosphatidylserine (PS) on the pRBC membrane surface. Several studies have reported that PS promotes pRBC binding to CD36 and thrombospondin (Eda & Sherman 2002; Ho & White 1999; Sherman, Eda & Winograd 2003; Sherman & Prudhomme 1996). Febrile temperature can also lead to EC disruption (Riedel & Maulik 1999). A clinical trial using antipyretic intravenous ibuprofen (IV-ibuprofen) was able to control fever but delayed parasite clearance (Krudsood et al. 2010). This finding suggests that ibuprofen and fever reduction does not act to reduce cytoadherence as might be expected from previous work, and there is some evidence that fever temperatures might act in the opposite way to reduce EC binding. There is also evidence showing that febrile temperatures may increase pRBC rigidity (Marinkovic et al. 2009), and this finding might explain at least some

of the reduced deformability leading to vascular flow obstruction seen in SM, but there is a need for further studies to confirm this. In the absence of a consistent association with inflammation and malaria pathology, and the observation of preferential pRBC accumulation in microvessels in SM, people have turned to cytoadherence and many clinical studies have attempted to correlate adhesion with disease (Table 1.1), particularly with CM where pRBC sequestration in the brain is an invariant feature of disease.

Table 1. 1: Clinical studies on cytoadherence-related pathology in malaria.

Study size and site	Pathological feature	Adhesion molecule tested	Observation technique	Correlation with diseases	References
51 children, Gambia	CM, Uncomplicated malaria	CD36	Static cell binding assay on C32 melanoma cells	Suggested that pH6.9 mediates optimal binding of pRBC to CD36 but no correlation between parasite adhesion with disease was observed.	Marsh et al. 1988 (Marsh et al. 1988)
27 adults, Thailand	CM, Acute renal dysfunction, Acute hepatic dysfunction	CD36 and ICAM-1	Static cell assay (C32 melanoma cells and CHO cells expressing CD36 or ICAM-1) and protein binding	All patient isolates bound to CD36 purified protein and cells but no association with disease.	Ockenhouse et al. 1991 (Ockenhouse et al. 1991a)
59 adults, Thailand	CM, SM (without CM) or anemia, Uncomplicated malaria	CD36	Static cell binding assay on C32 melanoma cells incubated with TNF, IL-1 and IFN-γ	Severe malaria (non-CM) patient isolates show higher binding to C32 melanoma cells compared to uncomplicated and CM isolates. Cytokines did not enhance pRBC cytoadherence on C32 cells	Ho et al. 1991 (Ho et al. 1991)
56 adults,	SM with organ	CSA, CD36,	Static cell assays (C32	All isolates bound to C32 melanoma	Chaiyaroj et al. 1996

Thailand	dysfunction	ICAM-1, E-selectin, VCAM-1-1	melanoma cells and CHO cells expressing E-selectin or VCAM-1-1) and protein binding	cells; a small number of isolates adhered to ICAM-1, CSA and TSP purified protein, but not to E-selectin and VCAM-1-1 on CHO transfected cells. No correlation between severity and level of adhesion observed.	(Chaiyaroj et al. 1996)
60 adults, Thailand	SM with acute organ dysfunction, CM	CD36, ICAM-1, E-selectin, VCAM-1-1	Static cell assays (mouse L cells expressing CD36 or E-selectin or ICAM-1 or VCAM-1-1)	Patient isolates bound to CD36 ten-fold higher then ICAM-1 and no binding to VCAM-1-1. Therefore, CD36 binding could be associated with disease severity through allowing adhesion of a larger proportion of the parasite population..	Udomsangpetch et al. 1996 (Udomsangpetch et al. 1996)
150 children, Kenya	CM, Severe anemia, non-severe	CD36, ICAM-1, VCAM-1-1 and E-selectin	Static protein binding assay	CD36 was quantitatively the major receptor for all isolates but some isolates bound strongly to ICAM-1, but less to VCAM-1-1 and none to E-selectin. Binding to ICAM-1 was associated with disease, but not CM.	Newbold et al. 1997 (Newbold et al. 1997a)
158 children, Malawi	SM; CM; severe anaemia	CD36, ICAM-1, CSA and	Static protein binding assay	Varied cytoadherence profiles from patient isolates; showed all isolates	Rogerson et al. 1999 (Rogerson et al.

		TM		bound to CD36 and low binding to ICAM-1 when using severe anaemia isolates. No correlation with severe disease observed.	1999)
111 children, Kenya	SM with non CM or severe anaemia (n=18), CM (n=11), severe anaemia (n=21), uncomplicated malaria (n=45), control (n=16)	PECAM, CD36, TSP and ICAM-1	Static cell binding assays (mouse L cells expressing PECAM-1 and CHO cells expressing CD36 or ICAM-1) and protein binding assays using FACS technique	Rosetting associated with blood group A and heparin-type receptors (e.g heparin sulfate) are prone to SM; binding to multiple receptors promoted pRBC sequestration in the SM group	Heddini et al. 2001 (Heddini et al. 2001)
22 adults, France	uncomplicated malaria, CM, SM with non CM	ICAM-1 and CD36	Static cell binding assays (CHO cells expressing CD36 or ICAM-1)	Binding of isolates from SM showed no significant difference compared to uncomplicated malaria pRBC	Cojean et al. 2008 (Cojean et al. 2008)
155 children,, Tanzania	Uncomplicated malaria	CD36	Static protein binding assay	CD36 deficiency is protective in malaria anaemia	Chilongola et al. 2009 (Chilongola et al. 2009)
101 children, Kenya	CM, Severe anaemia, Uncomplicated malaria	CD36 and ICAM-1	Static and flow protein binding assays	High pRBC binding to CD36 associated with uncomplicated malaria; High ICAM-1 binding under flow correlated with CM	Ochola et al 2011 (Ochola et al.)

46 children, Mozambique	SM (cerebral; severe anemia, respiratory distress, prostration); uncomplicated malaria	CD36, ICAM-1 and gC1qR	Static protein binding assays	Higher levels of adhesion to gC1qR in isolates from children with multiple seizures.	Mayor et al 2011 (Mayor et al. 2011)

pRBC, parasitized red blood cells; ICAM-1, Intercellular Adhesion Molecule 1; CSA, Chondrointin Sulphate-A; TSP, Trombospondin; VCAM-1-1, Vascular Cell Adhesion Membrane 1; CHO, Chinese Hamster Ovary; TNF, Tumor Necrosis Factor; IL, Interleukin; IFN-γ, Interferon gamma; gC1qR, globular C1q Receptor; SM, Severe Malaria; CM, Cerebral Malaria.

1.10 How might cytoadherence cause severe malaria?

SM is often associated with cytoadhesion, i.e. where pRBC interact with non-infected RBC (rossettes) or adhere to endothelial receptor molecules (sequestration). The sequestration of pRBC is thought to have evolved in order for the parasites to avoid clearance by the spleen, since the spleen in combination with antimalarial drugs rapidly clear non-adherent pRBC.

Only *P. falciparum* and none of the other *Plasmodium* species that infect humans uses this mechanism of sequestration to this extent (Pongponratin et al, 1991). Since *P. falciparum* is also the most pathogenic of the *Plasmodium* species, this supports the link between cytoadherence and pathogenicity of disease.

As mentioned earlier, complex interactions, including the host inflammatory response and endothelial activation may contribute to SM, but how does cytoadherence itself modulate the severity of the disease?

When *P. falciparum* infects red blood cells (RBC), the parasite expresses proteins that are transported to the RBC membrane causing changes in rigidity and shape of the infected RBC. This may lead to difficulties in flowing through the microvasculature, and studies in Thailand and Bangladesh have shown that increased rigidity and reduced flow through blood vessels are associated with severe disease (Dondorp, Pongponratn & White 2004). Other studies on the retinal vasculature (Chang-Ling, Neill & Hunt 1992; Maude et al. 2009; Medana, Chan-Ling & Hunt 2000) have shown micro-haemorrhages and vessel changes, thought to reflect blockage, are highly predictive of CM.

The hypothesis is that adhesion of pRBC in the deep vasculature leads to organ dysfunction. What evidence do we have to support this? As stated earlier, MacPherson et al (1985) showed that there is preferential pRBC accumulation in the brains of people who died of CM compared to NCM (MacPherson et al. 1985). One way that this might be taking place is that in some malaria infections there is higher recruitment of pRBC to cerebral vessels, due to the increased levels of receptors such as ICAM-1 (Turner et al. 1994) and the presence of parasites able to bind efficiently to this receptor (Ochola et al.). This clearly over-simplifies the potential mechanisms contributing to the preferential recruitment of pRBC in the

brain and there are likely to be several pathways by which this can be achieved. The role of the infecting parasite variant should not be ignored in this equation, and data from the analysis of pRBC in post-mortem tissues have shown the enrichment of specific PfEMP1 variant types in the brains of children eho died of CM (Janes et al. 2011; Montgomery et al. 2006).

How might the accumulation of pRBC in tissues lead to pathology? A simple explanation might be that localised ischemia damages the endothelium leading to disease but the histological evidence only partially supports an impact of EC destruction and the relative reversibility of SM on treatment would argue against profound tissue damage. PRBC cytoadherence is known to activate oxidative cascades (SAPK/JNK pathway) which can regulate gene transcription (Adams, Brown & Turner 2002), rho-kinase (Taoufiq et al. 2008) and NFκB (Tripathi, Sullivan & Stins 2006) signalling via radical oxygen species (ROS) to induce local endothelial activation (Jenkins et al. 2007).

Trans-endothelial electrical resistance (TEER) experiments showed that when pRBC adhered to human brain microvascular endothelial cells (HBMECs), the integrity of the human blood-brain barrier (BBB) reduced 3-fold causing increased permeability (Wassmer et al. 2006). The leakage of BBB leads to serum protein penetrating into the central nervous system (Tripathi, Sullivan & Stins 2007). This influx of foreign substances activates the microglial cells that release proinflamatory cytokines damaging astrocytes and glial cells that are crucial for BBB maintenance (Medana & Turner 2006). It has also been suggested that interaction of serum protein with TGF-β receptors TGFBR1 and TGFBR2 could result in astrocyte dysfunction, followed by seizures and neuronal death (Rao et al. 2010).

The binding of pRBC in the brain EC has also been reported to induce EC apoptosis (Pino et al. 2003; Toure et al. 2008). Pino *et al* demonstrated pRBC modulated endothelial cell gene expression such as TNF superfamily genes (Fas, fas L, DR-g) and apoptosis-related genes (Bad, Bax, Caspase-3, SARP2, DFF45/ICAD, IFN-γ Receptor 2, Bcl-w, Bik and iNOS). Toure *et al*, subsequently showed for the first time that clinical isolates can sometimes induce EC apoptosis (Toure et al. 2008) and the presence of apoptotic cells might up-regulate the

expression of cellular adhesion molecules, resulting in hyperadhesiveness (Hebert et al. 1998), leading to greater accumulation of pRBC and subsequent EC apoptosis.

1.11 Aims and outline of the thesis

P. falciparum has been called the most virulent amongst other human infectious *Plasmodium* species (*P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*). Clinical episodes of *P. falciparum* malaria in a non-immune patient will lead to unrestricted multiplication of the parasites and would lead to heavy parasitemia, which can cause vital organ dysfunction with high risk of death. The main question here is why antimalarial drugs and human immunity are not able to fully control disease. To answer this question we will need to understand the mechanism of how parasite evolves in the host, how the parasites escapes from beeing cleared by the spleen and how it survives in such stress environment (pH changes and higher temperature).

The ability of the parasite to 'disappear' from the circulation is associated with pRBC cytoadherence to host cells, leading to pathophysiology of SM (CM and placenta malaria) and almost 50% of malaria deaths are reported 24 to 48 hrs after patients have been hospitalized despite being given effective antimalarials (e.g artemisinine).

It has been reported that intercellular adhesion molecule-1 (ICAM-1) and CD36 are important host receptors for malaria parasites, and most recent data have suggested an association between strong ICAM-1 binding and CM, whilst high levels of CD36 adhesion were observed in parasites from uncomplicated cases. Therefore, our hypothesis is, if we disturb the binding complex (pRBC-ICAM-1 or pRBC-CD36) we might see a reversal of binding and this would help to reduce mortality caused by cytoadherence.

Therefore, this thesis will discuss potential anti-cytoadherence 'leads' that can effectively inhibit and reverse pRBC adhesion to endothelial cells. In addition, this thesis will also discuss co-factors mediating the pRBC cytoadherence.

1.12 Objective

1. INHIBITION AND REVERSAL OF *P. falciparum* CYTOADHERENCE

a) To screen the effect of a chemically-modified polysaccharide library to inhibit and reverse sequestered pRBC on protein and various endothelial cell (HUVEC, HDMEC and HBEC) targets.

b) To investigate the effect of a modified heparin library to inhibit sequestered pRBC on endothelial cells.

c) To investigate the effect of monoclonal antibody to endothelial receptors to inhibit and reverse binding of sequestered pRBC on protein and endothelial cells.

2. HOST ENVIRONMENTAL EFFECTS ON P*. falciparum* CYTOADHERENCE

a) To investigate the role of endothelial glycocalyx on mediating pRBC cytoadherence.

b) To investigate Effect of pH on *P. falciparum* cytoadherence.

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

This chapter contains the details of materials and commonly used methods. Details of methods used in more specific instances and modification or supplements to standard methods are provided at appropriate locations within the experimental chapters.

2.2 Materials

Details of reagents, monoclonal antibodies, equipment, media, endothelial cells and solutions are listed in appendix I, II, III, IV and V respectively.

2.3 Methods

2.3.1 General parasite culture

Parasites strains

Parasite lines used were ItG (Ockenhouse et al. 1992a), A4 (Roberts et al. 1992b), and C24 (Roberts et al. 1992b). All have been previously characterized for their adhesion phenotypes to ICAM-1 and CD36 (Gray et al. 2003). ItG is a strong ICAM-1 binder which also adheres to CD36, A4 binds purified ICAM-1 moderately and CD36 strongly, and C24 only binds to CD36.

Parasite culture

Plasmodium falciparum parasite lines were cultured using a modified method described by Trager and Jensen, 1976. Parasites were grown in RPMI-Blue at 37^{o}C and were maintained at 3% parasitemia in 1% hematocrit, under a gas mixture of 1% CO_2, 1% O_2 and 98% N_2, with fresh RPMI-Blue provided every 48 hours. The amount of media required was estimated using the following equation:

$$\mathbf{B_1 = (A_2 B_2 C_2) / (A_1 C_1)}$$

A_1= Percentage of parasitemia in an initial culture flask

B_1= Volume of culture to be taken (ml) from initial culture

C_1= Haematocrit (HCT) of parasite culture obtained.

A_2= Percentage of desired parasitemia to be maintained

B_2= Final volume media needed in a new culture flask (ml)

C_2= Haematocrit (HCT) of parasite culture required.

Fresh washed RBC were added every 48 hours to maintain a parasitemia between 3 - 5%.

In order to add washed RBC, the following equation was used;

$$\mathbf{D = (B_2 - E)*1000*(C_2/F)}$$

D = Volume of washed RBC to be added (µl)

E = (A2*B2)/A1

F = washed RBC HCT obtained.

For example;

If you need to prepare 10 ml of parasite suspension containing 3 % parasitemia culture in 1 % HCT from 10 % parasitemia initial culture, which contains 0.8 % HCT with 50% washed RBC HCT is;

B1 = (3%*10 ml*1%)/(10%*0.8%) = 3.75 ml

D = (10 ml – (3%*10 ml)/10%)*1000*1/50% = 140 µl

Therefore, to get 3% parasitemia and 1% hematocrit in new culture, 3.75 ml of initial culture were taken and added 140 µl of washed RBC (50% HCT). The culture suspension was spin under 1800 rpm for 5 minutes. The supernatant was removed, and 10 ml of RPMI-Blue were added.

Blood smear

Thin blood films of culture were made to assess parasite maturity and parasitemia. To prepare a thin blood film, 200 µl of culture were taken and centrifuged. The pellet then smeared on the microscope slide, air dried and fixed with 100% methanol. Fixed erythrocytes were stained for 10 minutes using 10% giemsa before washing the slide with water and allowing it to dry. Slides were examined microscopically at 1000 x magnification under an oil immersion lens. Parasitemia was determined by counting the number of infected and uninfected red blood cells up to a total of 500 cells.

Preparation of washed RBC

Human group O^+ red blood cells, collected in acid-citrate-dextrose (15% (v/v)) and with excess plasma removed, were obtained from Blood Transfusion service, The Royal Liverpool Hospital. The cells were stored up to three weeks at 4^{o}C before processing for use in parasite culture. Prior to use in parasite culture, the blood was resuspended to 50% (v/v) in RPMI-Yellow and layered on an equal volume of HISTOPAQUE. After centrifugation at 3000 rpm for 15 minutes, residual plasma and leukocyte aggregates were removed by aspiration; the remaining packed erythrocyte pellet was washed twice with RPMI-Yellow and then resuspended to 50 % hematocrit in RPMI-Yellow. Washed red blood cells were stored up to one week at 4^{o}C.

Cryopreservation of parasites

Parasites were frozen by resuspending a pellet of washed parasite culture at 3 – 5% parasitaemia at ring stage in glycerolyte freezing medium in a ratio of 5 volume (vol) freezing medium to 3 vol cell pellet. One volume of freezing medium was added and cells allowed standing for 5 min before slow addition of the remaining 4 volumes. The solution was gently mixed and transferred to cryovials (0.5 ml per vial) before wrapping in tissue and polystyrene containers to allow slow freezing at -80°C overnight before transfer to liquid nitrogen for long term storage

Reconstitution of frozen parasites

After careful removal of the stabilate from liquid nitrogen, the solution was allowed to thaw rapidly at 37°C. As soon as the stabilate was thawed the 0.5 ml contents were transferred to a 15 ml falcon tube and 100 µl 12 % NaCl was added. After 5 min standing at room temperature, 2.5 ml 1.8% NaCl was added dropwise over 10 min, followed by 2.5 ml 0.9 % NaCl containing 0.2 % glucose. After 10 min standing the mix was centrifuged at 1600 rpm in a bench top centrifuge for 4 min then washed in RPMI-Yellow) parasite medium. The cell pellet was resuspended in 8 ml RPMI-Blue medium, transferred to small culture flask and gassed for 30 seconds before transfer to a 37°C incubator. The following day parasitaemia was assessed and washed red blood cells added as appropriate.

Trophozoite enrichment – Plasmagel flotation

One method used to enrich for trophozoite stage parasite was plasmagel flotation which is based on the ability of knob positive pRBC to float in 3 % gelatin solution (Jensen 1978). We used Plasmion, a physiological saline solution containing 3 % gelatine, as the production of Plasmagel had been discontinued. The parasite pellet was resuspend in a ratio of 2 vol pellet to 3 vol RPMI-Yellow media and 5 vol plasmion, and this mixture transferred to 15 ml of falcon tube and allowed to settle for 20 - 30 min. Trophozoite stage pRBC in the top layer were carefully transferred to a clean tube then washed three times in incomplete medium and parasitaemia assessed by geimsa stained smear. For most experiments the potential effect of plasmion on the pRBC was controlled for by exposing uninfected RBC to plasmion in a similar way, however, the cells were retrieved from the bottom layer as no uninfected cells remain in the upper phase.

Parasite selection using magnetic beads (Dynabeads)

All parasite lines are subject to antigenic switching and so were used for only up to 3 weeks post-selection to minimise the effect of mixed populations. ItG was selected on ICAM-1 protein and expression of the expected *var* gene confirmed

by cloning and sequencing a DBLα tag from cDNA. A4 was selected for binding to the monoclonal antibody BC6 (Horrocks et al. 2002; Smith et al. 1995b) followed by immunofluorescence analysis of expressed PfEMP-1 and sequencing of the expressed *var* tag. Populations used were ~80% homogeneous for the expected *var* tag.

Selection of parasite on ICAM-1^{Ref} purified protein

To increase the homogeneity of the ItG parasite population which expresses a PfEMP-1 protein with high affinity for ICAM-1^{Ref}, the population was subjected to selection on ICAM-1^{Ref} protein. 25 µg of ICAM-1^{Ref} protein was coated to 50µl of protein A Dynabeads (Invitrogen) in 200 µl of 1 % Bovine Serum Albumin (BSA) diluted in PBS (filter sterilised) in a sterile 1.5 ml eppendorf tube and incubated for 1 hour at room temperature with gentle rotation (15 rpm) using a rotator (Stuart Rotator). Dynabeads were washed by gentle pipetting in 1% BSA/PBS then the eppendorf placed next to the magnetic stand for a few seconds before careful removal of the BSA/PBS. 50 µl of ItG parasite culture synchronized and enriched using the Plasmagel technique were incubated to the coated bead in 200 µl BSA/PBS for another 45 minutes at room temperature by gentle rotation. Unbound parasites were washed by 3 x wash with BSA/PBS using the magnetic stand. Beads were resuspended in 5 ml of blue media and transferred to culture in T25 culture flask with addition of 100 µl of washed red blood cells (50 % HCT).

Selection of parasite on BC6 antibody

BC6 antibody given by Mr. B Pinches from Oxford University (Horrocks et al 2002., Smith et al., 1995) was used to coat protein G Dynabeads (Invitrogen). 50 µl of Dynabeads were washed twice in 400 µl of 1 % BSA/PBS (filter sterilised) in a 1.5 ml eppendorf tube. Beads were resuspended by gentle pipetting in BSA/PBS the the eppendorf placed next to the magnetic stand for a few seconds before careful removal of the wash solution. Beads were gently resuspended in 380 µl of BSA/PBS and 20 µl of BC6 monoclonal antibody was added and incubated for 1 hour at room temperature with gentle rotation (15 rpm). Unbound antibody was

removed by 1 x wash with 1 ml BSA/PBS and beads resuspended in 200 µl of BSA/PBS. 50 µl of synchronized and enriched A4 parasite culture (using the Plasmagel technique) were incubated with the coated beads in 200 µl BSA/PBS for another 45 minutes at room temperature by gentle rotation. Unbound parasites were washed by 3 x wash with BSA/PBS using the magnetic stand. Beads were resuspended in 5 ml of blue media and transferred to culture in T25 culture flask with addition of 100 µl of washed red blood cells (50% HCT).

2.3.2 General endothelial cell culture

All procedures were carried out using standard aseptic techniques under laminar flow. A class II microbiology safety cabinet was used to prevent culture contamination. Contaminated plastic ware, tissues and blood products were decontaminated by placing them in a disinfectant solution of 2% (v/v) virkon and then sending them for autoclave disposal.

HUVEC culture

Liquid nitrogen stabilate (1ml) of Human Umbilical vein Endothelial Cells (HUVEC) from Promocell (C-12200) was cultured using warmed HUVEC medium (Promocell medium kit without MV (C-22120)) supplemented with Endothelial cell Growth Medium (C-39215) in gelatin treated vented T25 culture flask until 80% confluent before splitting them by trypsinization technique to another flask for maintenance. The media was changed every alternate day.

HDMEC culture

One liquid nitrogen stabilate (1ml) of Human Dermal Endothelial Cells (HDMEC) from Promocell (C-12210) was cultured using warmed HDMEC medium (Promocell medium kit) with MV (C-39220) supplemented with Endothelial cell Growth Medium (C-39215) in gelatin treated vented T25 culture flask until 80%

confluent before splitting them by trypsinization technique to another flask for maintenance.

HBEC culture

One liquid nitrogen stabilate (1ml) of Human Brain Endothelial Cells (HBEC) was cultured using warmed MCDB 131 (Gibco no. 10372-019) or DMEM (high glucose with sodium bicarbonate, without L-Glutamine and sodium pyruvate) supplemented with 10% fetal bovine/calf serum, L-Glutamine (20 mM), 10 ng/ml EGF (recombinant, human), 1 ug/ml hydrocortisone, 100 units.ml of penicillin G and 100 ug/ml of streptomycin in gelatin treated vented T25 culture flask until 80% confluent before splitting them by trypsinization technique to another flask for maintenance.

Trypsinization

Trypsinization of endothelial cells was done using Promocell detach kit (C-41220). All media in the flask was removed prior to trypsinization. For one T25 flask of confluent cells, 1.5 ml of HEPES was added for washing remaining media and then was discarded and 1.5 ml of trypsin was added to detach the adhered cells. Cell detachment was confirmed by viewing under an inverted microscope. Trypsin neutralized saline (TNS) was added immediately once cells have detached. This was to neutralize the trypsin effect and to reduce harm to the cells.

Preparation of purified proteins

Recombinant protein chimeras of wild-type ICAM-1 ($ICAM^{Ref}$) (Berendt et al. 1992) were prepared by Tadge Szestak (LSTM), by using COS cells and transfection technique, as full-length Fc proteins and purified to concentrations of 200 – 700 µg/ml. $ICAM\text{-}1^{Ref}$ was diluted to 50 µg/ml in Dulbecco's phosphate buffer saline (PBS) for binding assays. Meanwhile, CD36 protein was obtained from R&D systems was diluted in PBS to 25 µg/ml.

2.3.3 General adhesion assay

Static protein adhesion assay

Static protein assays were carried out as described by McCormick ((McCormick et al. 1997). Briefly, 2 µl of purified recombinant protein chimeras of wild-type ICAM-1 (ICAM-1^{Ref}) and CD36 proteins were spotted onto 60 mm diameter bacteriological Petri dishes and incubated in a humidified chamber for 2 hr at 37^{o}C. Proteins were aspirated off, and dishes were blocked overnight at 4^{o}C in 1% BSA/PBS. Blocking solution was removed, the dish washed in binding buffer and 2 ml of parasite suspension at 3% parasitemia and 1% hematocrit added to each dish. Dishes were incubated at 37^{o}C for 1 hr, with re-suspension for every 10 min. Unbound pRBC and RBC were removed by repeated washing, bound cells fixed with 1% glutaraldehyde for 1 hr and then stained with 5% giemsa for 20 min. Level of adhesion were viewed under 10 x 100 magnification fields microscopically, and photo was taken and was analyzed using Image-Pro Plus image analyzer software version 5.1. From this the number of adherent pRBC per mm^2 was calculated.

Flow protein adhesion assay (microslide)

Flow reversal assays were carried out on microslides (3-aminopropyltriethoxysilane (APES)-treated) coated with ICAM-1^{Ref} at 50 µg/ml (Berendt et al. 1992). Microslides were pre-prepared by coating with APES following nitric acid treatment to allow for protein/cell adherence, then autoclaved. Before the experiment, a small piece (1.5 cm) of thin wall rubber tubing was attached to one end of the microslide, marked to indicate orientation of the coated surface, and slides were autoclaved. Slides were then coated with ICAM-1^{Ref} at 50 µg/ml by drawing up 65 µl of solution using a pipette tip connected to the tubing. Protein was allowed to adhere to the microslide by incubation in a humid 37°C chamber for 2 hours then washed by drawing through 4 x 65 µl of PBS with 1% BSA Slides were blocked in 1 % BSA overnight at 4°C. Parasites were prepared as for static protein assay and everything warmed to 37°C before use. The flow system was based on that described by Nash et al (Nash et al. 1992). A

suspension of pRBC at 3% parasitemia and 1% hematocrit were flowed through the microslide for 5 min to allow for pRBC adhesion. Flow was continuous throughout the experiment at 0.05 Pa (0.186 ml/min) shear stress. After 5 min PRBC flow, flow was switched to binding buffer alone to remove unbound pRBC for another 2 minutes. The number of adherent pRBC was counted in six separate fields under 20 x 100 magnification fields. From this the number of adherent pRBC per mm^2 was calculated. To enable re-use of the slide parasites were removed by washing the slide through 2 x 2 ml with water then 2 x 2 ml binding buffer. To ensure this had no effect on binding, a positive control was carried out as the last run on a slide in each experiment. Slides were not re-used more than 6 times.

Static cell cdhesion assay

Static cell assays were carried out as described by Gray et al 2003. Endothelial cells, HUVEC and HDMEC (2-6^{th} passage) at 5 x 10^{-3} cells were seeded onto 1% gelatin coated 13 mm Thermanox coverslips (Nalgene, Nunc). After leaving overnight for cells to adhere the medium was changed and cells cultured until confluent (2 – 5 days). Once confluent, the cells were incubated overnight at 37°C with or without 1 ng/ml TNF (Biosource International). Cells were washed with binding buffer and incubated with 0.5 ml of parasite suspension (3% parasitemia, 1% HCT) for 1 h at 37°C, with gentle resuspension every 10 min. A one hour gravity wash was used to remove unbound cells. Adherent cells were fixed using 1% glutaraldehyde for 1 hr and then stained with 5% giemsa for 20 min. Coverslips were dried and mounted on slides using DPX mountant. Level of adhesion was quantitated microscopically under 20 x 100 magnification fields. From this the number of adherent pRBC per mm^2 was calculated.

Flow cell adhesion assay (chamber slide)

Binding assays under flow conditions were carried out as described by Gray et al 2003. This type of assay attempts to mimic the conditions seen in the post capillary venule by allowing infected RBC to flow over chamber slides coated with endothelial cells with constant flow rate at 0.24 ml/min (0.05Pa). The slides were

coated with 1% gelatin for 1 hr at 37^{o}C and further seeded with 5 x 10^{-3} cells and were kept in a CO_2 incubator overnight. One confluent T25 flask of endothelial cells was trypsinised and used to seed 3 (for next day assay) or 6 (For 2-4 days growth before assay) chamber flasks. 2.5 ml trypsinised cells were added per flask. After settling overnight the medium was changed and cells cultured until confluent. Confluent chamber slides were then incubated overnight with or without 1 ng/ml TNF prior to use. Parasite suspensions (3% parasitemia and 1% HCT) were flowed over for a total 5 min, and flowed by binding buffer for another 2 min to remove the unbound pRBC. The flow rate yielded a wall shear stress of 0.05 Pa (o.24 ml/min), which has been used widely to mimic wall shear stresses in the microvasculature. The number of adherent pRBC was counted in six separate fields under 20 x 100 magnification fields. From this the number of adherent pRBC per mm^2 was calculated.

2.3.4 Fluorescence-activated cell sorting (FACS)

Cells were grown in six-well plates until confluent at 37^{o}C in a CO_2 incubator. Confluent cells then incubated with or without 1ng/ml TNF overnight. The cells then were washed with HEPES at once and trypsinized. Monoclonal antibody (α –ICAM-1, α –CD36, and α –ICAM-1+ α –CD36) were then added to the cells. Negative and isotype controls were also prepared by either not adding primary antibody or replacing the specific antibody with an irrelevant, isotype matched antibody.

Flow rate calculation for Shear Stress on chamber slides (Cooke, Coppel & Nash 2002).

$$\mathbf{Q = t.Wh^2/6n}$$

t = Shear Stress (Pa)

Q = Flow rate (m^2/sec)

n = media viscosity (Pa.sec/Nm^{-4}.sec)

W = Width (m)

h^2 = Depth (m)

For example (flow rate for chamber slide)

n = 0.71 mPa.sec = 7.10E-04 Pa.sec

W = 5.5 mm = 0.006 m

h^2 = 0.25 mm = 0.0003 m

t = 0.05 Pa

Therefore, Q = 4.03E-09 m.sec = 0.2420775 ml.min

CHAPTER 3: INHIBITION AND REVERSAL OF *P. falciparum* CYTOADHERENCE

3.0 Introduction

An important aspect of the pathogenesis of SM results from the ability of infected red blood cells to sequester in the microvasculature (as described in chapter 1). Post-mortem studies of SM show high levels of infected red blood cells (pRBC) bound to microvasculature (Taylor et al. 2004; Turner 1997). The involvement of sequestration in pathogenesis could be directly a result of blocking of the blood vessels, and/or downstream effects caused by the interaction between pRBC and the endothelium, including local inflammatory responses (Chakravorty, Hughes & Craig 2008).

Cytoadherence by pRBC on vascular endothelial cells happens when PfEMP1, a parasite derived molecule present on the surface of pRBC, adheres to several different adhesion molecules on the surface of host endothelium. Previous studies showed that parasite isolates from children with SM bind to several receptors (Heddini et al. 2001), which suggested that synergistic effects between adhesion molecules may contribute to malaria pathophysiology. Yipp et al and others (Yipp et al. 2000) indicated that in some cases more than one receptor may be involved in adhesion, but ICAM-1 seems to be identified critical in cerebral malaria (Armah et al. 2005a; Ochola et al. 2011; Tripathi, Sullivan & Stins 2006).

If cytoadherence, as well as causing the adhered pRBC to escape from spleen destruction, results in SM, there is a need to find anti-adhesive compounds in order to inhibit or reverse this process. However much of this work has concentrated on inhibiting cytoadherence, whereas for acute cases it is likely that the agent needs to be able to reverse existing adhesion.

As described in chapter 1, cytoadherence, which we believe may lead to some aspects of disease severity, is a process where mature pRBC erythrocytic stages escape from splenic clearance by binding to endothelial cells and promoting parasite growth in a relatively hypoxic environment. Therefore, can we use this information to devise treatments to prevent death or neurological sequelae?

The choice of anti-parasite drug will still be the main treatment to reduce mortality in patients with malaria, and preferentially these should kill early (in terms of the erythrocytic cycle) stages as destruction of non-adhesive ring stages will prevent the next wave of pRBC from sequestering. Therefore artemisinin is a good choice as it kills ring stages and this might explain the reduced mortality seen in the field studies from Thailand (SEQUAMAT) (Dondorp et al. 2005) and Africa (AQUAMAT) (Dondorp et al. 2010) comparing artemisinin and quinine (which only kills mature pRBC). However even with this welcome progress, there is still over 50% of the mortality recorded during first 48 hours after hospital admission that is largely unaffected by the use of ACTs. This may be due to the pRBC already sequestered to the endothelium. Therefore, there is a need for adjunct therapies to support the critically ill patient to be used in combination with antimalarials such as artemisinin to remove the sequestered pRBC mass or reduce its effects on the host, whilst the standard drugs kill the parasite effectively.

To date several compounds have been explored and screened for their potential to improve SM. N-acetylcysteine (NAC) is an antioxidant drug that is widely used in humans for the treatment of paracetamol overdose and had been shown to be able to reverse almost 72% of pRBC binding to CD36 (Treeprasertsuk et al. 2003). In addition, it also reduced rigidity of pRBC (Nuchsongsin et al. 2007). A pilot clinical trial study in Thailand showed that it was able to normalise serum lactate (an indicator of SM) significantly in SM patients (Watt, Jongsakul & Ruangvirayuth 2002). NAC inhibits TNF release, impedes cytoadherence and is a potent scavenger of free oxygen radical, which produced in response to TNF and mediates some of its toxic effects. However, despite these encouraging results NAC has recently been shown to antagonise the action of artesunate (Arreesrisom et al. 2007), and clinical trials have been disappointing (Charunwatthana et al. 2009).

Levamisole is an alkaline phosphatase inhibitor that has been used as an anti-helminth drug. Using levamisole for treatment of EC *in vitro* showed that it was able to reduce the binding of *P. falciparum* pRBC to CD36 (Dondorp et al. 2007). A clinical trial of Levamisole in combination with artesunate is currently underway and so far treatment with Levamisole has been shown to be safe and to cause the release of mature pRBC into the peripheral circulation (Dondorp et al. 2007).

Epigalloyl-catechin-gallate (EGCG), a naturally occurring polyphenol compound from green tea, was identified as being able to inhibit pRBC binding to ICAM-1 by 50% at micromolar concentrations (Dormeyer et al. 2006) and has been postulated to synergise the effect of artemisimin on malaria by lowering the IC_{50} from 14nM to 8.4nM (Sannella et al. 2007), but unfortunately this compound does not appear to be able to reverse adhesion. This highlights the need to test potential anti-cytoadherence agents for inhibition and reversal.

Interventions based on adhesion-related pathology are not limited to attempts to modulate direct interactions of pRBC to specific adhesion molecules but include inhibiting EC dysfunction during cytoadherence. L-arginine is a substrate for Nitric oxide (NO) synthesis by NO synthase. The rationale for the use of L-arginine follows on from studies in SM patients showing low NO production and low plasma arginine (Lopansri et al. 2003). In normal conditions NO mediates host resistance to a wide variety to infectious microorganisms and some *in vitro* studies have shown that it possesses anti-parasitic effects by killing pRBC, as well as an anti-adhesion effect. NO is also a potent inhibitor of TNF production and other pro-inflammatory cytokines implicated in malaria immunopathology (John et al. 2010). Therefore, L-arginine is a good candidate to be used as an adjunct therapy for SM by improving endothelial function. Clinical studies measuring reperfusion parameters have been encouraging (Yeo et al. 2008) and a clinical trial is currently underway.

Erythropoietin (EPO) is a hormone produced by the kidney, which modulates survival of developing erythroid precursors and production of new erythrocytes in the bone marrow. In SM patients, low EPO has been detected and correlated with severe anaemia. Injection of high doses of EPO in mice infected with *P. berghei* showed a significant reduction of proinflamatory cytokines (TNF and IFN-γ) (Kaiser et al. 2006) and showed increased survival rate when EPO was used in combination with artesunate (Bienvenu et al. 2008). Preliminary clinical trials of EPO in combination with quinine in CM children in Mali showed that it is safe and did not show any side effects (Picot et al. 2009).

As mentioned before, apoptosis has been postulated to be one way in which cytoadherence can cause disease. Therefore, the use of anti-apoptotic agents

should be advantageous. Fasudil is a Rho kinase inhibitor and widely used in humans for cardio and neuro-vascular diseases. An *in vitro* study using clinical isolates showed that fasudil has the potential to inhibit apoptosis mediated by *P. falciparum* pRBC adhesion to endothelial cells, but showed no effect on reversing or inhibiting pRBC cytoadherence (Zang-Edou et al. 2010). It appears to be a promising adjunctive therapeutic approach for reducing neurological sequelae because it could reduce NFκB activation and endothelial apoptosis (Taoufiq et al. 2008). The use of statins to control levels of cholesterol in the blood has also been shown to be able to restore endothelial damage caused by pRBC cytoadherence (Taoufiq et al. 2011). Atorvastatin appears to improve endothelial function through increased production of NO, protecting endothelial barrier integrity, reducing oxidative stress and inhibiting inflammatory responses (Laufs 2003) through activated anti-apoptotic Akt cascade. There is also evidence that statins decrease ICAM-1 expression in stimulated EC and monocytes (Romano et al. 2000), but there is no evidence to show that they are able to reverse established pRBC adhesion.

Even though many studies have been done, few of them showed any potential to reverse sequestered pRBC or to reduce pathology. Therefore, more research and more validation is needed before being able to introduce adjunct therapies to patients. In other words, we still lack a potential effective reversing agent without giving any harm to patients.

This chapter will describe some other approaches in the search of potential and effective adjunct compounds to reverse *P. falciparum* cytoadherence such as the use of monoclonal antibody (mAb) (Chapter 3.1), modified polysaccharides (Chapter 3.2), and modified heparin (Chapter 3.3) on inhibiting and reversing pRBC sequestration *in vitro*.

3.1: Effect of monoclonal antibody on reversing *P. falciparum* sequestration *in vitro*

Hypothesis: Cytoadherence, resulting in sequestration of pRBC from the peripheral circulation, is caused by a receptor-ligand interaction between the parasite protein PfEMP1 and receptors on the surface of endothelial cells. Therefore, it is assumed that blocking the adhesion receptor using a specific mAb to the host receptors will be a good target to inhibit pRBC sequestration, but is it able to reverse binding by already adhered pRBC?

3.1.1: Introduction

With the understanding of cytoadherence mechanisms, we are now able to conclude that the parasite adhesions on vascular endothelial cells are associated with interaction of surface a membrane protein of pRBC, PfEMP1 with various surface adhesion receptors on microvascur beds.

Endothelial cells-expressed receptors that support adhesion of pRBC includ CD36, ICAM-1, TSP, VCAM-1, E-selectin, CSA and PECAM-1, which discussed in chapter 1. The role and relative importance of these receptors in sequestration is still not clear, but receptor synergism may enhance the binding.

It has been demonstrated that interaction of PfEMP1 with ICAM-1 is critical in developing cerebral malaria while adhesion to CD36 is associated with uncomplicated malaria and non-CM, SM (Ho et al. 1991; Ochola et al. 2011; Ockenhouse et al. 1991b; Ringwald et al. 1993). Therefore blocking and disrupting pRBC adhesion to host receptors will be the main strategy to reduce a probability to develop SM. The use of a specific blocker or inhibitor such as monoclonal antibodies (mAbs) would be the best choice because it able to identify specifically the receptor target, although translating this approach to the clinic would be challenging.

Antibody-therapy is one of the best approaches due to its ability to identify specific protein or cells. An antibody can be effective against microorganism without mediating a direct effect to host (Casadevall, Dadachova & Pirofski 2004). There is now considerable evidence to indicate that it is possible to generate mAb that are protective against microorganisms such as *Mycobacterium tuberculosis* (Teitelbaum et al. 1998) (Pethe et al. 2001a; Pethe et al. 2001b), *Listeria monocytogenes* (Edelson, Cossart & Unanue 1999), *Leishmania mexicana* (Anderson, David & McMahon-Pratt 1983) and *Histoplasma capsulatum* (Nosanchuk et al. 2003). Antibodies can be effective in controlling disease by reducing the damage that results from excessive host inflammatory response. This has been shown by Verbon et al (2001), they showed that IC14, which is anti-CD14 (innate immune system) that recognise LPS-component of gram-negative and gram-positive bacteria able to inhibit LPS-induced proinfalmmatory cytokine release, and capable of delaying release of soluble TNF and IL-1 (Verbon et al. 2001).

Immunoglobulins (Igs) have an important role in immunity to malaria, and the passive administration of human Igs in the form of immunoglobulins from malaria hyperimmune sera can be effective at controlling disease (Singh et al. 1988). However, the difficulty in finding immune donors, the amount of reagent needed and the risks associated with the use of human blood products have necessitated the introduction of recombinant antibody against important parasite molecule for use in therapeutic studies.

Monoclonal antibodies (mAbs) are monospecific immune agents, which are capable of identifying a unique epitope on specific proteins or cells. Antibody-therapy has been proposed by Paul Ehrlich at the beginning of 20th century to target disease associated-organisms (Ehrlich et al. 1988; Ogata et al. 1993). Since then, monoclonal antibodies have been used extensively in clinical settings and for research in molecular and cell biology.

If we target PfEMP1, the culprit on the surface of pRBC associated with binding, we might able to lessen the development of cytoadherence. We have known that PfEMP1 consists of multiple domains of CIDR, DBL and ATS. Researchers also have shown anti-CIDR antibody able to block binding of pRBC to

immobilized CD36 protein, unfortunately it is just only tested in one strain (Mo et al. 2008). Antibody to recombinant protein rC1-2, which is a sub-fragment of PfEMP1, blocked pRBC adhesion in a strain specific manner (Cooke et al. 1998), unfortunately it is now has been shown to be just an artefact. Cytoadherence of pRBC to placenta in pregnant patients is an example of receptor-specific interaction of PfEMP1 to CSA, which has not found in other organ (e.g brain and lung) associated with SM. Var2csa-PfEMP1 has been found on pRBC associated with placenta malaria. Recently, anti-NTS-DBLγ region domain of the var2csa shown able to cross-reactive and inhibit pRBC binding to CSA (Bigey et al. 2011).

Expression of *var* genes has been shown to be regulated at the level of transcription initiation (Kyes et al. 2007; Scherf et al. 1998; Schieck et al. 2007). Switching between expressed *var* genes occurs at this level by turning on or off *var* promoters. Switching rates have been estimated at up to roughly 2% per generation (Roberts et al. 1992b) but are thought to vary between different *var* genes, with different promoters having different "off" rates (Horrocks et al. 2004). For example, centrally located *var* genes appear to have slower switching rates than telomeric *var* genes (Dzikowski et al. 2007). Given the high degree of variability in this system of antigenic variation, the use of antibody to various PfEMP1 fragment will not simple and will require further work to define key binding signatures.

Therefore, blocking adhesion receptor-target strategies is currently thought to be the best way to solve the problem, instead of blocking PfEMP1, and it has been shown previously that some mAbs effectively inhibit the interaction of pRBC to specific receptors on endothelial cells (table 3.1).

It has been found that MAb OKM5, where the epitope is the immunodominant region at amino acids 139-184 of CD36, is able to block cytoadherence of pRBC to CD36 (Ockenhouse, Magowan & Chulay 1989; Panton et al. 1987; Wright et al. 1991). Adhesion to ICAM-1 can be inhibited using several different mAbs against ICAM-1 (Berendt et al. 1992; Tse et al. 2004). mAb 15.2 against the L42 loop of domain 1 of ICAM-1 is commonly used as control antibody to inhibit ICAM-1 mediated cytoadherence (Craig et al. 1997; Keizer et al. 2003).

Although many experiments have addressed inhibition of adhesion of pRBC, meaning the prevention of de-novo adhesion, there have been very few studies looking at the potential to reverse existing pRBC cytoadherence.

Reversing pRBC sequestration has been investigated as an attractive contribution strategy to the management of SM (Taylor et al. 1992). The rationale for reversing sequestration was based on the beneficial effects of administration of antimalaria immunoglobulins to children with mild malaria to adults with malaria in Thailand by Maneerat's group. Maneerat et al. found all the sera used variably inhibited rosette formation of the parasites but showed no association with the antibody titers. The results suggested that the antibodies to cytoadherence and rosette formation can be elicited and sustained in the malaria experienced host while living in the endemic area. This may be a natural preventive mechanism against the severity of *P. falciparum* infection in the infected host (Maneerat et al. 1999), in animal models (David et al. 1983) and in *in vitro* adhesion studies (Goldring et al. 1992; Singh et al. 1988). In a squirrel monkey model of malaria (David et al. 1983), the administration of hyperimmune serum was rapidly followed by the swift appearance (30 min after injection) of previously adherent infected erythrocytes in the peripheral circulation and an impressive recovery from sickness. *In vitro*, adhesion of Thai isolates to C32 melanoma cells was reversed with Thai immune sera (Singh et al. 1988), and the adhesion of isolates from Malawi was reversed with a pool of local immune sera (Goldring et al. 1992). Adherence to individual receptors can be reversed with monoclonal antibodies (Iqbal, Perlmann & Berzins 1993; Johnson et al. 1993; Sherwood et al. 1989; Udomsangpetch et al. 1989) or ligand peptide segments (Crandall et al. 1993).

The reversal of cytoadherence by serum containing antibodies, both *in vitro* and *in vivo,* suggests that a pool of high-titre malarial antibodies, shown to contain antibodies to the surface of infected erythrocytes (Goldring et al. 1992), could reverse adhesion *in vivo*. However, a double blind, placebo-controlled administration of the antibodies as an adjunct to quinine (the best available antimalarial at the time) had no measurable observed effect on adherence, and did not affect patient recovery (Taylor et al. 1992).

Can mAbs reverse binding of sequestered pRBC? Here, we would like to look at the ability of mAbs to inhibit and reverse adhesion of pRBC to ICAM-1 and CD36, well-known and common adhesion receptors.

Table 3. 1: Monoclonal antibodies used to block specific ligands to inhibit cytoadherence.

Target receptor	**Antibody**	**References**
ICAM-1	84H10	(Johnson et al. 1993)
	15.2	(Adams et al. 2000; Berendt et al. 1992; Craig et al. 1997; Smith et al. 2000a)
	1G12	(Duperray et al. 1997)
	2D5	(Duperray et al. 1997)
CD36	OKM5	(Barnwell et al. 1989; Panton et al. 1987; Wright et al. 1991)
CSA	1A7, 5C11, 3D3, 1A7F, 2H5	(Avril et al. 2006; Lekana Douki et al. 2002)
TSP	A61	(Cox et al. 2008)
PECAM-1	JC70A	(Fernandez et al. 1998; Rosso & Lucioni 2006; Treutiger et al. 1997b)

3.1.2 Material and methods

3.1.2.1 Parasites

Lab-adapted parasite isolates; ItG and C24 were used as described in methods chapter 2. Parasites were synchronised at ring stage using sorbitol lysis and at mature stages using plasmionsl as described in methods chapter 2.

3.1.2.2 Endothelial cells

Endothelial cell lines HUVEC and HDMEC were seeded and cultured as described in methods chapter 2. All binding assays were performed on 70 – 80 % confluent monolayers endothelialcells.

3.1.2.3 Monoclonal antibody

Monoclonal antibody 15.2 (αICAM-1) and FA6-152 (αCD36) were prepared at 5 µg/ml and 10 µg/ml respectively in binding buffer solution (pH 7.2).

3.1.2.4 Adhesion assays

Static and flow binding assays were carried out as described in general methodology in chapter 2, with slight modifications based on the specific experiments to be performed.

3.1.2.4.1 Blocking assays - Static protein

Petri-dishes were pre-incubated with 1.5 ml of binding buffer with or without antibody (αICAM-1; 5 µg/ml or αCD36; 10 µgml), at 37°C for 30 minutes, before proceeding with adhesion assay. The solutions were aspirated, parasite suspension added and incubated for one hour at 37^{o}C rotating every 10 minutes. The petri-dish was washed using binding buffer (pH 7.2) for 3 – 5 times and fixed

using 1 % glutaraldehyde and further stained by 5 % Giemsa as described details in chapter 2.

3.1.2.4.2 Reversal assays – Static protein

For reversal assays, controls were always carried out in the same experiment such that in a given experiment there were at least four conditions. Dishes 1 and 2 were treated as a standard binding or inhibition assay. Dishes 3 and 4 were treated as a standard binding assay along with dishes 1–2 up to the end of 8 x 2 ml binding buffer washes. At this point dishes 1–2 were fixed in 1% glutaraldheyde in PBS while dishes 3–4 were incubated for a further 1 hour with either binding buffer alone, or mAb 15.2 (5 μg/ml in binding buffer), or DBL-β protein (when used), with gentle mixing every 10 min. After the further incubation, dishes were washed 4 X 2 ml binding buffer and fixed in 1% glutaraldehyde in PBS. All experiments were carried out with duplicate dishes each containing triplicate spots. After overnight fixing at room temperature, dishes were stained for 30 min in 5% Giemsa, washed with water and air dried before counting.

3.1.2.4.3 Reversal assays – Flow protein

Flow reversal assays were carried out on microslides coated with ICAM-1 ref at 50 μg/ml. Slides were prepared and assays carried out as described in Methods Chapter 2. pRBC at 3% parasitaemia and 1% haematocrit were flowed through the microslide for 5 min to allow for pRBC adhesion. Flow was continuous throughout the experiment at 0.05 Pa shear stress. After 5 min pRBC flow, flow was switched to binding medium alone to remove unbound pRBC and clear flow lines of pRBC. Timing was started at the start of this wash, which continued for 2 min before the binding medium was swapped for medium containing mAb 15.2 or FA6-152. The number of bound cells in six fields along the slide was counted at 2 min, 5 min, 10 min 15 min and 20 min time points.

3.1.2.4.4 Reversal assays – Static endothelial cell

HUVEC were maintained and static cell assays carried out as described in methods chapter 2. PRBC were allowed to bind as for normal assays then, following two dip washes, coverslips were placed in a gravity wash for 30 min. After 30 min coverslips were placed cell-side up into a well containing binding buffer with (and control without) mAb 15.2 at 5 μg/ml. After 30 min coverslips were transferred to a second gravity wash for 10 min then fixed in 1% glutaraldehyde. As with static protein assays, controls were carried out which were fixed before the second incubation. All cell assays were carried out in triplicate.

3.1.2.4.5 Reversal Assays –Flow Endothelial Cell

Reversal of adhesion to HUVEC or HDMEC under flow conditions was carried out in a similar way on TNF (1 ng/ml 16h) stimulated HUVEC or HDMEC grown overnight on chamber slides. Chamber slides were prepared and assays were carried out as described in Chapter 2. pRBC at 3% parasitaemia and 1% haematocrit were flowed through the slide for 5 min to allow for pRBC adhesion. Flow was continuous throughout the experiment at 0.05 Pa shear stress (0.24ml/min). After 5 min pRBC flow, flow was switched to binding medium alone to remove unbound pRBC. Timing was started at the start of this wash, which continued for 2 min before the binding medium was swapped for medium containing mAb. The number of bound cells in six fields along the slide was counted at 2 min, 5 min, 10 min 15 min and 20 min time points.

3.1.3 Results

3.1.3.1 Effect of mAb inhibiting cytoadherence on protein

P. falciparum lab isolates ItG and C24 were selected for static and flow ICAM-1 and CD36 inhibition studies respectively. Results show that ItG (Figure 3.1 and figure 3.3) and C24 (Figure 3.2 and 3.4) binding were highly significantly (P = 0.001) inhibited to bind to the protein ($ICAM^{Ref}$ and CD36 respectively) under static and flow conditions. The abilities of mAb to inhibit adhesion of the isolates to ICAM-1 (15.2; αICAM-1) and CD36 (FA6-152; αCD36) were determined using 5 µg/ml and 10 µg/ml respectively of mAb diluted in adhesion buffer (pH 7.2).

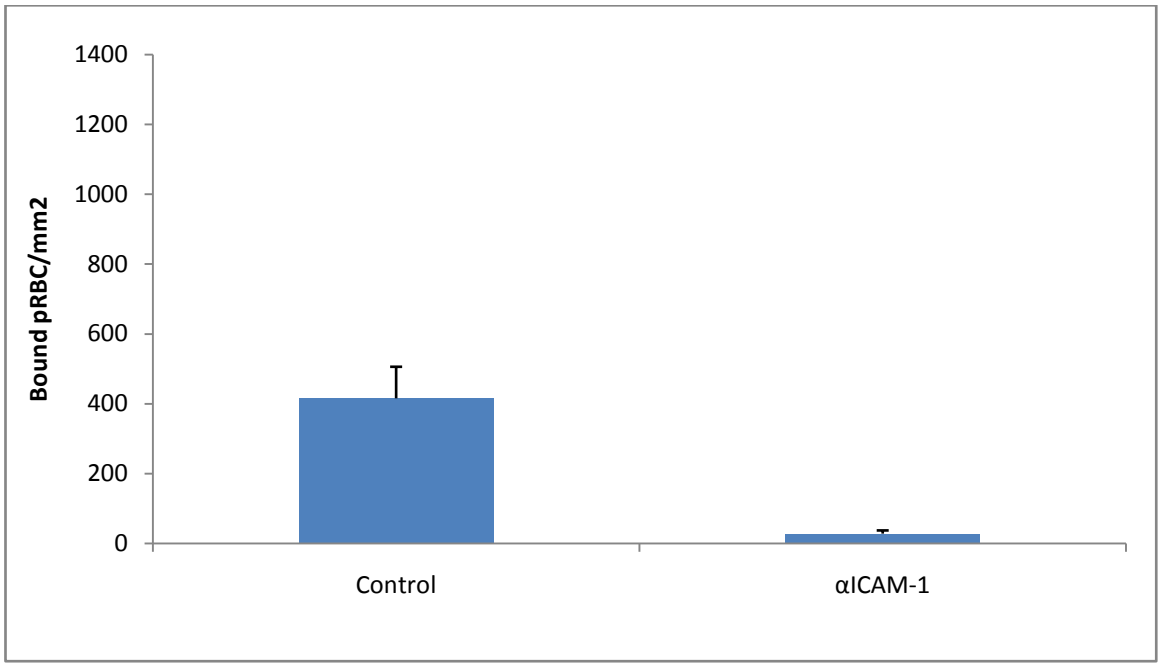


Figure 3. 1: Inhibition of ItG to $ICAM\text{-}1^{Ref}$ under static conditions. Binding of ItG (3% parasitemia; 1% HCT) observed after incubation with an antibody $ICAM\text{-}1^{Ref}$; 15.2 (αICAM-1) at 5 µg/ml for one hour. The remaining bound ItG after 3 - 5X wash was calculated using Image-Pro Plus software and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody.

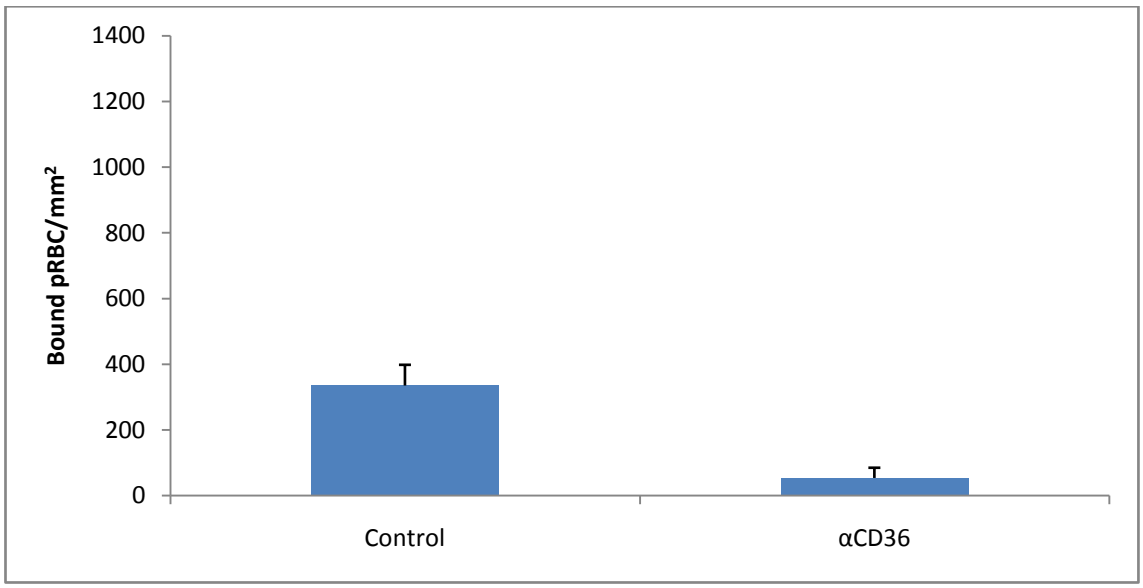


Figure 3. 2: Inhibition of C24 to CD36 under static conditions. Binding of C24 (3% parasitemia; 1% HCT) observed after co-incubation of an antibody CD36; FA6-152 (αCD36) at 10 µg/ml for one hour. The remaining bound C24 after 3 – 5X wash was calculated using Image-Pro Plus software and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody..

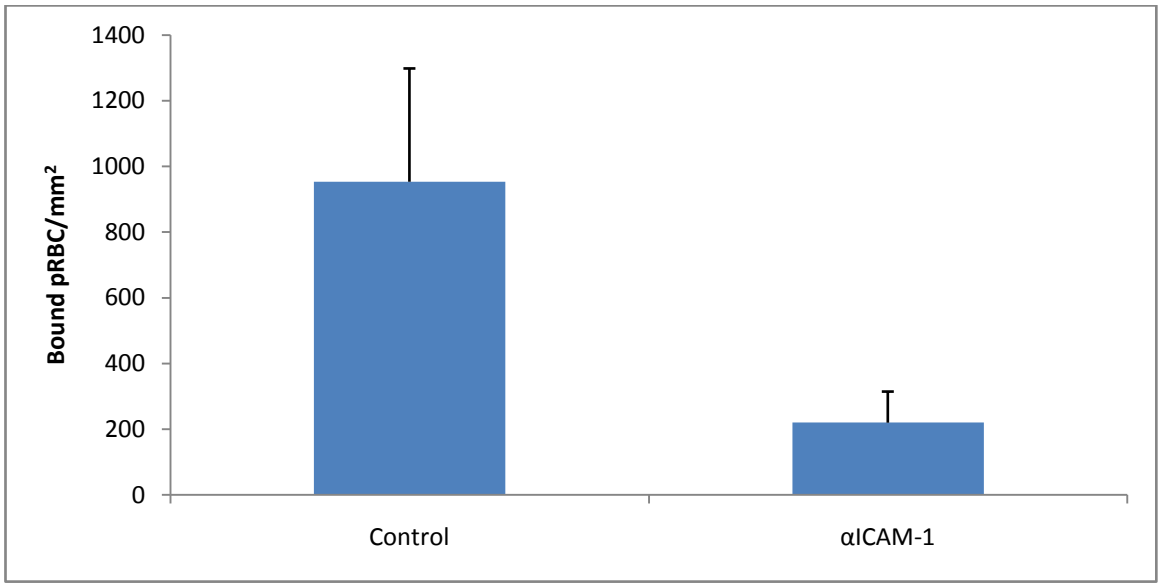


Figure 3. 3: Inhibition of ItG to $ICAM\text{-}1^{Ref}$ under flow conditions. Binding of ItG (3% parasitemia; 1% HCT) observed after co-incubation with an antibody $ICAM\text{-}1^{Ref}$; 15.2 (αICAM-1) at 5 µg/ml for one hour. The remaining bound ItG after 3-5X wash was calculated using Image-Pro Plus software and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody.

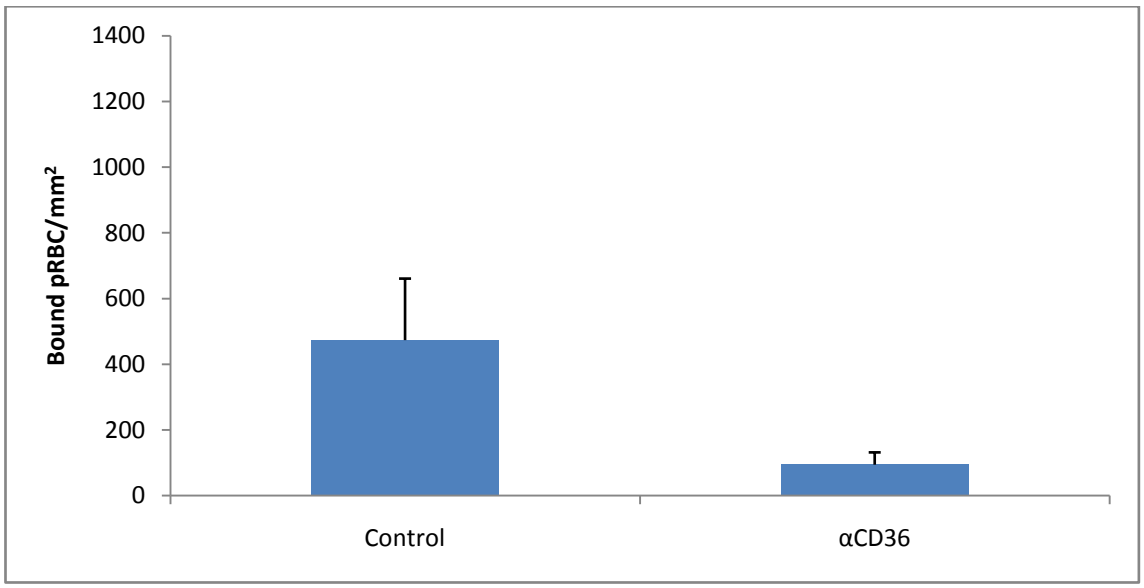


Figure 3. 4: Inhibition of C24 to CD36 under flow conditions. Binding of C24 (3% parasitemia; 1% HCT) observed after co-incubation of an antibody CD36; FA6-152 (αCD36) at 10 µg/ml for one hour. The remaining bound C24 after 3 – 5X wash was calculated using Image-Pro Plus software and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody.

3.1.3.2 Effect of mAb reversing cytoadherence on protein

Adhesions of both parasites were completely blocked by **αICAM-1** and **αCD36**, showing that adherence of ItG and C24 was specific to ICAM-1^{Ref} and CD36 respectively. Based on blocked results, we tried using these two antibodies for their ability to de-sequeter bound parasites on the protein (ICAM-1^{Ref} and CD36). Results show that the mAb 15.2 reverses binding of ItG (Figure 3.5 and Figure 3.6), and FA6-152 (Figure 3.7 and Figure 3.8) reverses binding of C24 under static and flow conditions.

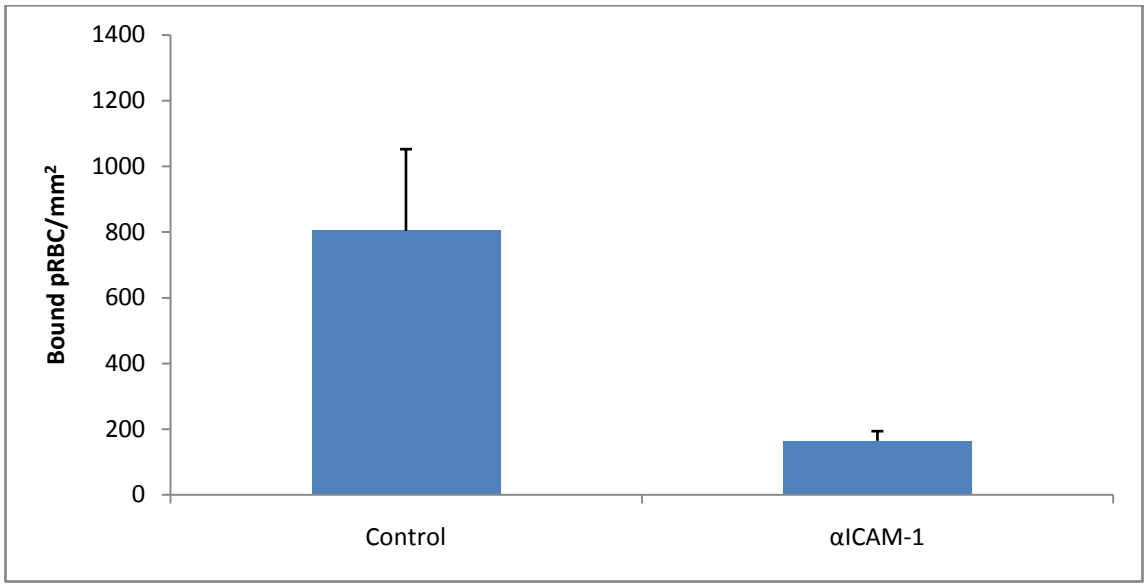


Figure 3. 5: Reversal of ItG to ICAM^{Ref} under static conditions**.** Binding of ItG (3% parasitemia; 1% HCT) observed after incubation with an antibody ICAM-1^{Ref}; 15.2 (αICAM-1) at 5 µg/ml for one hour. The remaining bound ItG after 3-5X wash was calculated using Image-Pro Plus software and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody.

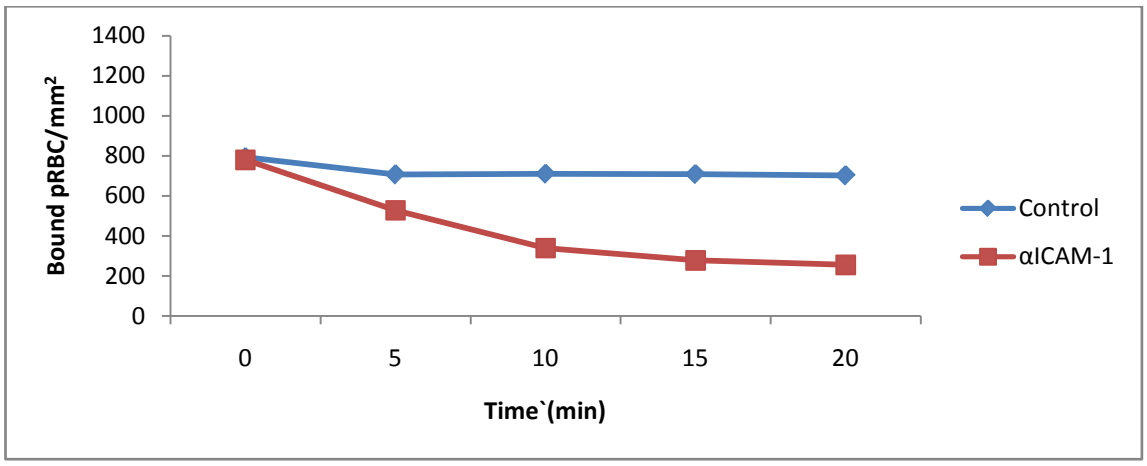


Figure 3. 6: Reversal of ItG to ICAM-1^{Ref} under flow conditions. Binding of ItG (3%, parasitemia; 1% HCT) observed after flowing through with an antibody ICAMRef: 15.2 (αICAM-1) at 5 µg/ml for 20 minutes. Parasite bound were calculate for every five minute exposure and expressed as bound pRBC/ mm^2. Control, without antibody.

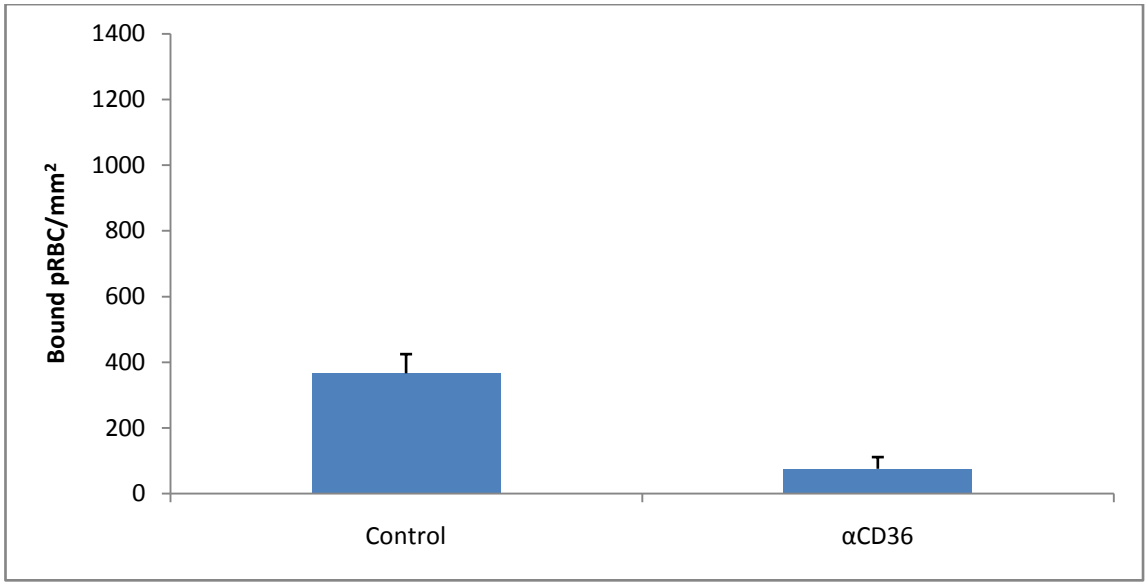


Figure 3. 7: Reversal of C24 to CD36 under static conditions. Binding of C24 (3% parasitemia; 1% HCT) observed after incubation with an antibody CD36 ; FA6-152 (αCD36) at 10 µg/ml for one hour. The remaining bound C24 after 3-5X wash was calculated using Image-Pro Plus software and expressed as bound pRBC/ mm^2. Control, without antibody.

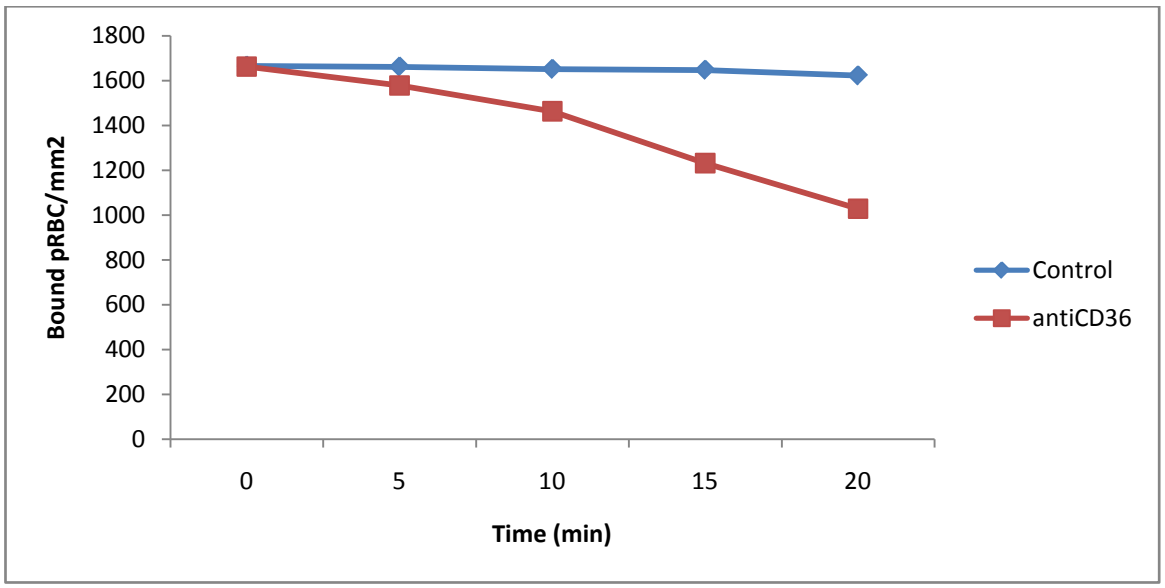


Figure 3. 8: Reversal of C24 to CD36 under flow conditions. Binding of C24 (3%, parasitemia; 1% HCT) observed after flowing through with an antibody CD36: FA6-152 (αCD36) at 10 µg/ml for 20 minutes. Parasite bound were calculate for every five minute exposure and expressed as bound pRBC/ mm^2. Control, without antibody.

3.1.3.3 Effect of mAb inhibiting cytoadherence on endothelial cells

After 18 h TNF stimulation of HUVEC and HDMEC, 3% parasitaemia ItG pRBC were co-cultured with stimulated HDMEC in the presence and absence of mAbs (αICAM-1; 5 µg/ml and αCD36; 10 µg/ml). Figure 3.9 and 3.10 shows binding of ItG, which has strong binding to ICAM-1^{Ref}, was inhibited significantly using both antibodies ($p<0.0001$) in comparison with ItG control (without antibody). Meanwhile C24 pRBC, that only bind to CD36 protein and not ICAM-1, shows a significant reduction of binding after αCD36 exposure (Figure 3.11). Results show that either αICAM-1 or αCD36 individually or together produce the same level of pRBC inhibition. Therefore, synergistic binding-interaction of pRBC is dependent to ICAM-1 and CD36 receptors in mediating efficient adhesion pRBC on the host endothelial cell.

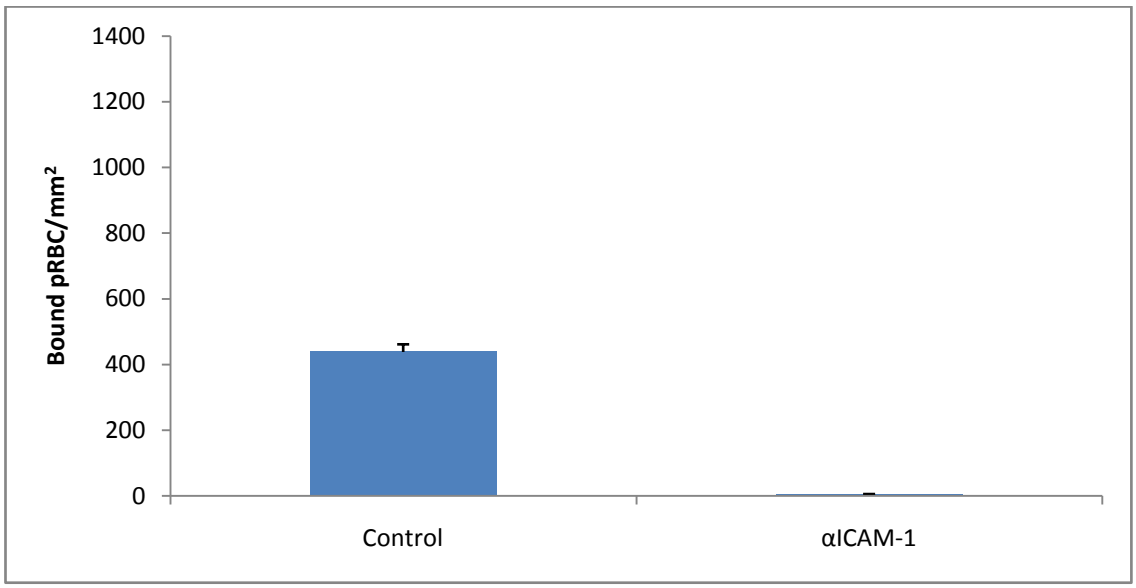


Figure 3. 9: Inhibition of ItG on TNF-stimulated HUVEC under static conditions. Binding of ItG (3% parasitemia; 1% HCT) observed after monoclonal antibody ICAM^{Ref} ; 15.2 (αICAM-1) at 5 µg/ml treatment for one hour. The remaining bound ItG after gravity wash was counted and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without αICAM-1; HCT, haematochrit.

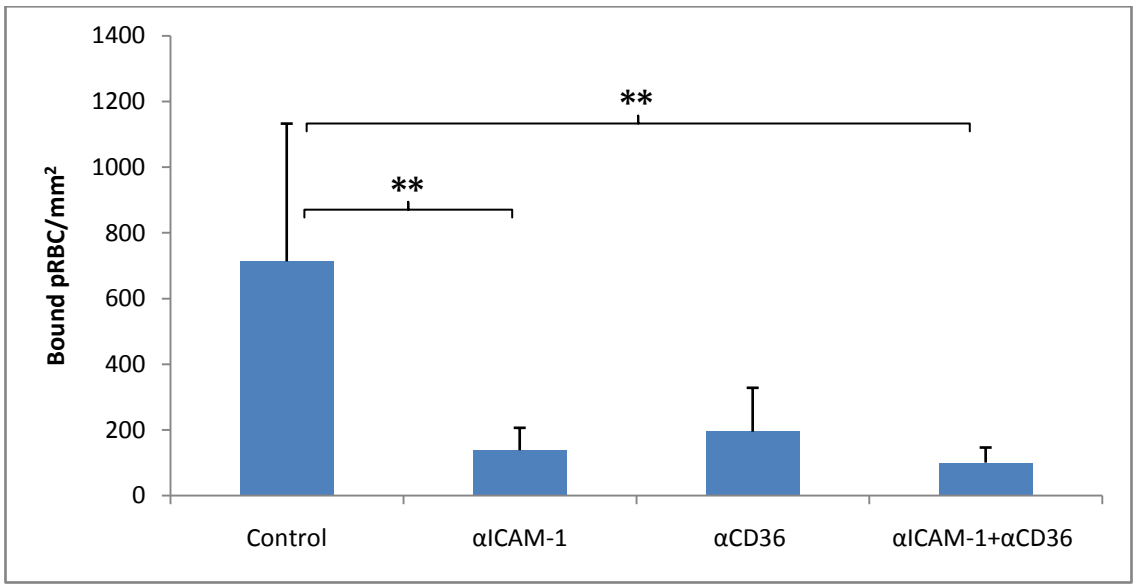


Figure 3. 10: Inhibition of ItG on TNF-stimulated HDMEC under static conditions. Binding of ItG (3% parasitemia; 1% HCT) observed after monoclonal antibody ICAM^{Ref} ; 15.2 (αICAM-1) at 5 µg/ml and FA6-152 (αCD36) at 10 µg/ml treatment for one hour. The remaining bound ItG after gravity wash was counted and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody. **; P < 0.01.

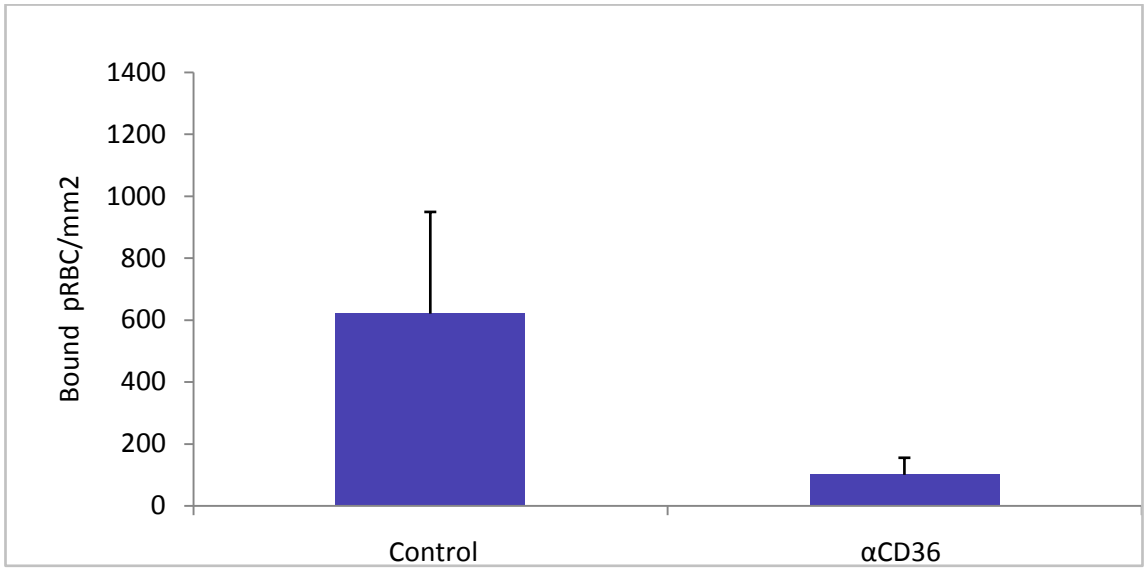


Figure 3. 11: Inhibition of C24 on resting HDMEC under static conditions. Binding of C24 (3% parasitemia; 1% HCT) observed after monoclonal antibody CD36; FA6-152 (αCD36) at 10 µg/ml treatment for one hour. The remaining bound ItG after gravity wash was counted and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody.

3.1.3.4 Effect of mAb reversing cytoadherence on endothelial cells

With the successful inhibition of the ICAM1^{Ref} and CD36 binding with the antibodies, we proposed that SM might be alleviated by reversing the adhesion of pRBC on HDMEC by using antibodies (αICAM1 and αCD36). Therefore, to examine this effect we co-cultured 3 % parasitaemia pRBC with the resting HDMEC and then treated with the antibody for another 1 h in the 37^{o}C / 5 % CO_2 with continuously rotating every 10 min (as mentioned in 2.3.3). Figures 3.13 and 3.14 show the binding of ItG and C24 respectively was reduced after treatment with αICAM1; 5 µg/ml and αCD36; 10 µg/ml in comparison with the control.

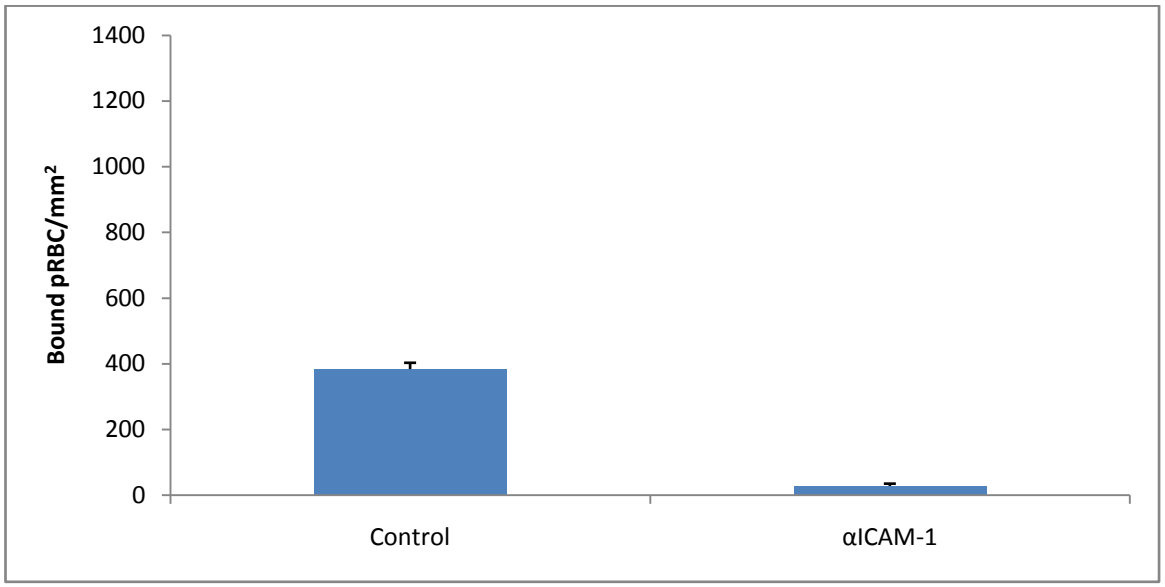


Figure 3. 12: Reversal of ItG on TNF-stimulated HUVEC under static conditions**.** Binding of ItG (3% parasitemia; 1% HCT) observed after monoclonal antibody ICAM^{Ref} ; 15.2 (αICAM-1) at 5 µg/ml treatment for one hour. The remaining bound ItG after gravity wash was counted and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody.

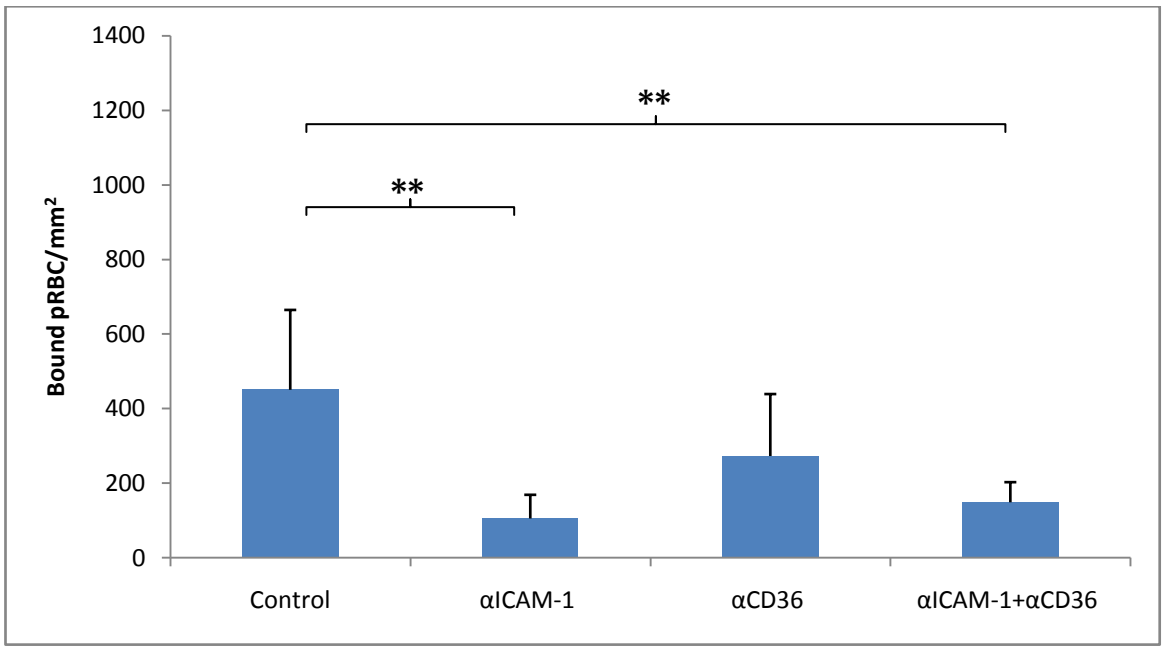


Figure 3. 13: Reversal of ItG on TNF-stimulated HDMEC under static conditions. ABinding of ItG (3% parasitemia; 1% HCT) observed after monoclonal antibody ICAM^{Ref} ; 15.2 (αICAM-1) at 5 µg/ml and FA6-152 (αCD36) at 10 µg/ml treatment for one hour. The remaining bound ItG after gravity wash was counted and expressed as bound pRBC/ mm^2. Control, without antibody. ** ; $P < 0.01$.

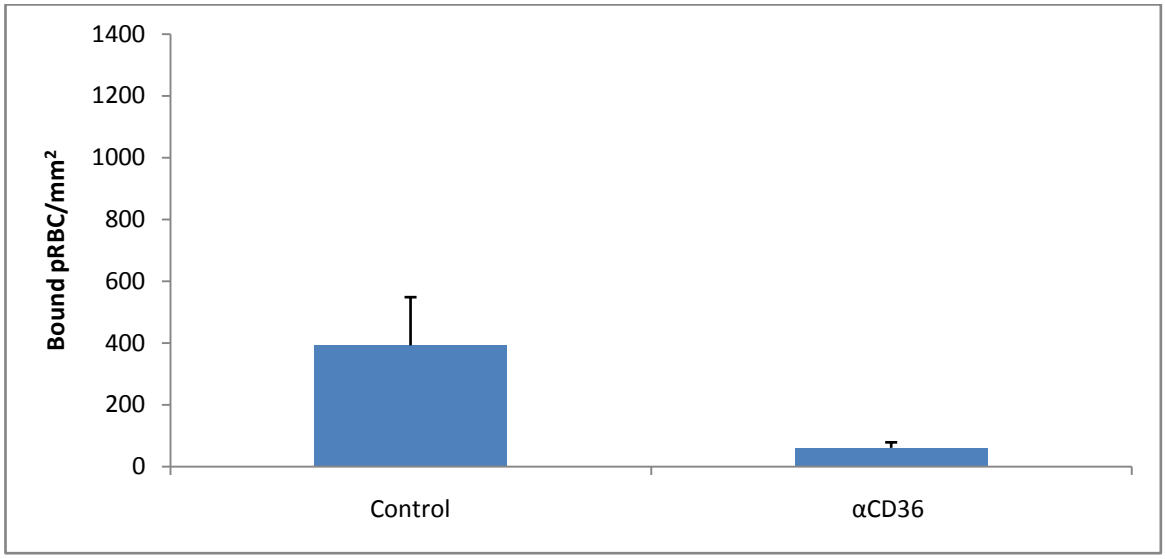


Figure 3. 14: Reversal of C24 on resting HDMEC under static conditions. Binding of C24 (3% parasitemia; 1% HCT) observed after monoclonal antibody FA6-152 (αCD36) at 10 µg/ml treatment for one hour. The remaining bound C24 after gravity wash was counted and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody.

With the successful reversal of the sequestered pRBC on static cell based assays, endothelial cell flow based assays were performed as described in section 2.3.5. Following 20 min exposure, a large reduction on binding of ITG and C24 was seen in comparison with control (Figure 3.15, 3.16 and 3.17) .

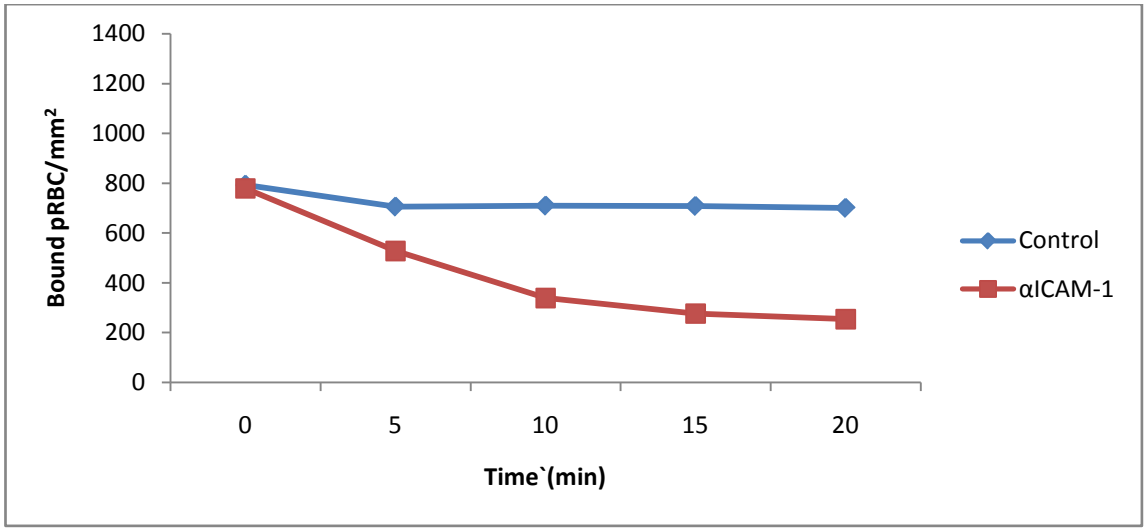


Figure 3. 15: Reversal of ItG on TNF-stimulated HUVEC under flow conditions. Binding of ItG (3%, parasitemia; 1% HCT) observed after flowing through with monoclonal antibody ICAM-1: 15.2 (αICAM-1) at 5 µg/ml for 20 minutes. Parasite bound were calculate for every five minute exposure and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody.

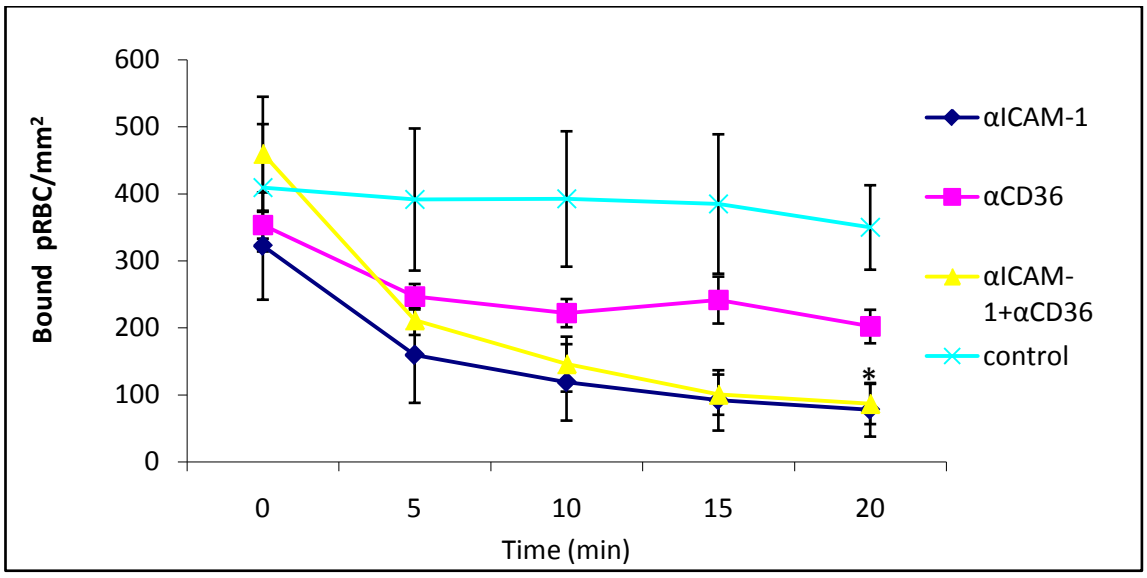


Figure 3. 16: Reversal of ItG on TNF-stimulated HDMEC under flow conditions. Binding of ItG (3%, parasitemia; 1% HCT) observed after flowing through with monoclonal antibody CD36: FA6-152 (αCD36) at 10 µg/ml and αICAM-1; 15.2 at 5 µg/ml for 20 minutes. Parasite bound were calculate for every five minute exposure and expressed as bound pRBC/ mm² ± standard deviation. Control, without antibody. *; P<0.05 (compare to 0 minute)

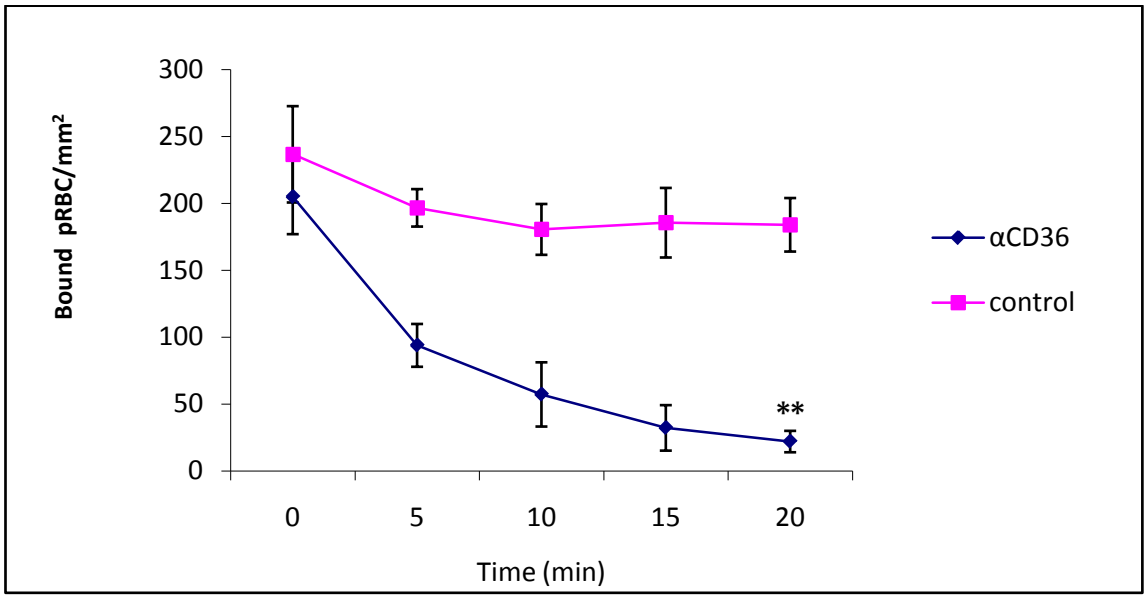


Figure 3. 17: Reversal of C24 on resting HDMEC under flow conditions. Binding of C24 (3%, parasitemia; 1% HCT) observed after flowing through with monoclonal antibody CD36: FA6-152 (αCD36) at 10 µg/ml for 20 minutes. Parasite bound were calculate for every five minute exposure and expressed as bound pRBC/ mm² ± standard deviation. Control, without antibody. **; *P*< 0.01 (compare to 0 minute).

3.1.3.5 Effect of mAb reversing clinical isolates on EC

Reversing effects of αICAM-1 and αCD36 were further investigated by using lab-adapted clinical isolates (GL6, 8146 and P069) which have been characterized for their binding properties on ICAM-1[Ref]. Our results show that αICAM-1 alone is able to reverse P069 and GL6 binding, meanwhile αCD36 alone does show slight decrease on all isolates binding to TNF-stimulated HDMEC. However, there is no doubt that when both of the antibodies (αICAM-1 + αCD36) are combined, it shows similar level of binding reduction when αICAM-1 alone is given on all parasites except 8146. All patient isolates are tested on static and flow as refer to Figures 3.18- 3.21.

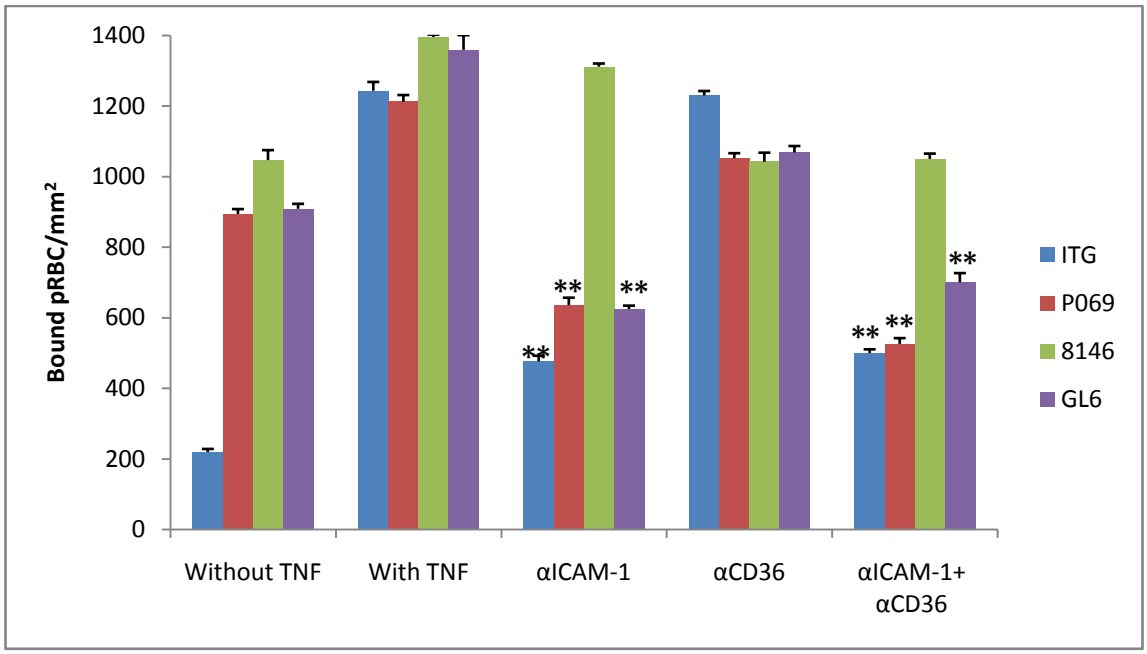


Figure 3. 18: Reversal of clinical isolates on TNF-stimulated HDMEC under static conditions. Binding of lab-adapted clinical isolates (3%, parasitemia; 1% HCT) observed after monoclonal antibody ICAM-1; 15.2 (αICAM-1) at 5 µg/ml and CD36: FA6-152 (αCD36) at 10 µg/ml for one hour. The remaining bound parasite after gravity wash was counted and expressed as bound pRBC/ mm^2 ± standard deviation. Control, without antibody. **; $P<0.01$ (compare with group "with TNF").

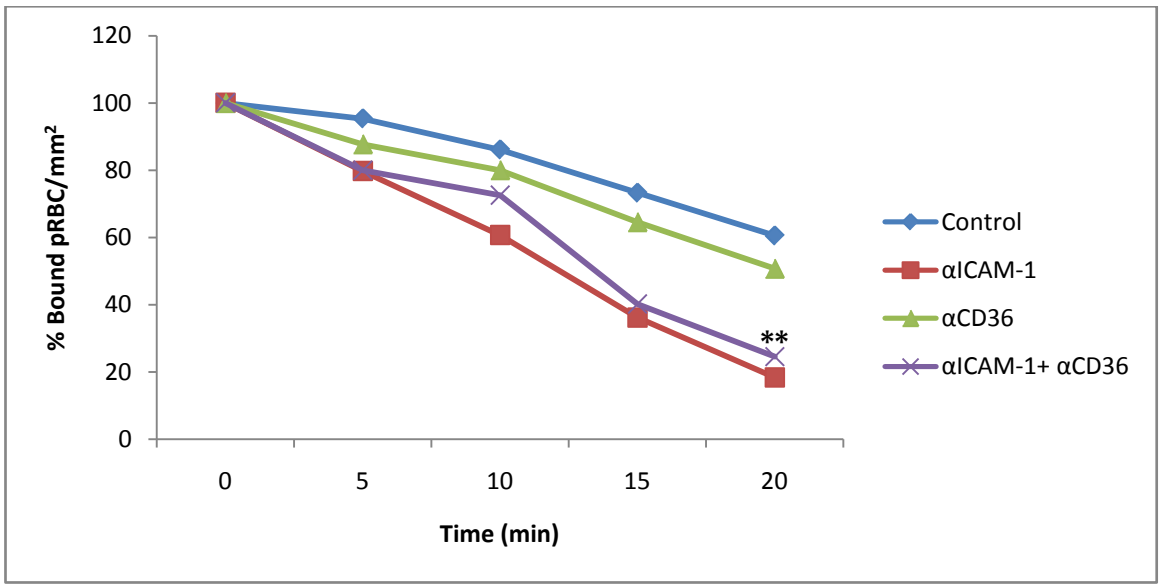


Figure 3. 19: Control study on reversing ItG on TNF-stimulated HDMEC under flow conditions. Binding of ItG (3%, parasitemia; 1% HCT) observed after flowing through with monoclonal antibody CD36: FA6-152 (αCD36) at 10 µg/ml and αICAM-1; 15.2 at 5 µg/ml for 20 minutes. Parasite bound were calculate for every five minute exposure and expressed as percentage (%) bound pRBC/ mm². Control, without antibody.**, P< 0.01 (compare to 0 minute)

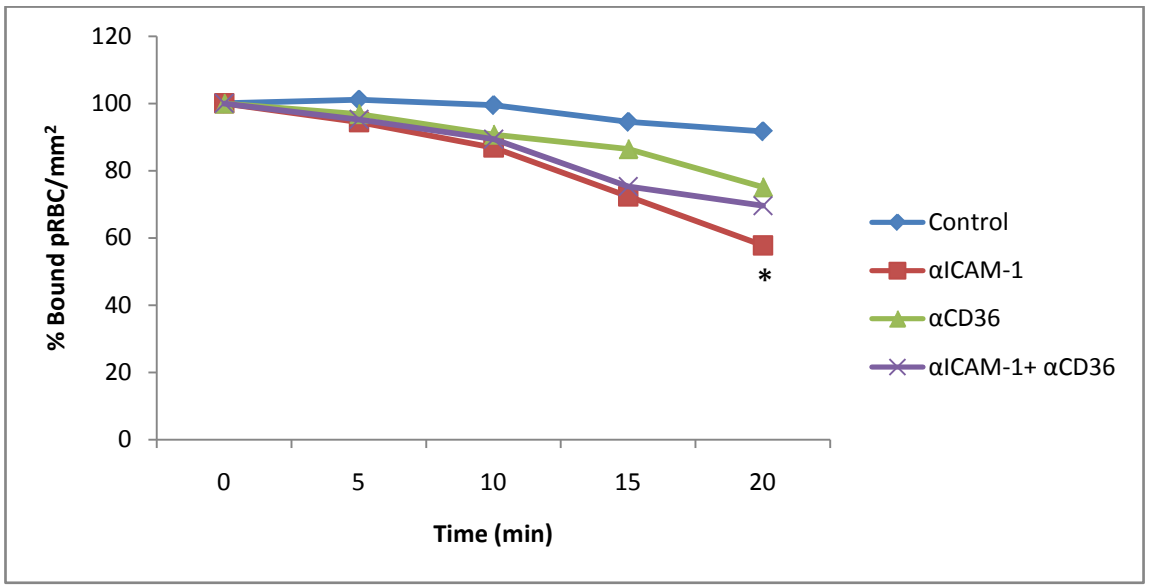


Figure 3. 20: Reversal of P069 on TNF-stimulated HDMEC under flow conditions. Binding of P069 (3%, parasitemia; 1% HCT) observed after flowing through with monoclonal antibody CD36: FA6-152 (αCD36) at 10 µg/ml and αICAM-1; 15.2 at 5 µg/ml for 20 minutes. Parasite bound were calculate for every five minute exposure and expressed as percentage (%) bound pRBC/ mm² Control, without antibody. *; *P*< 0.05.

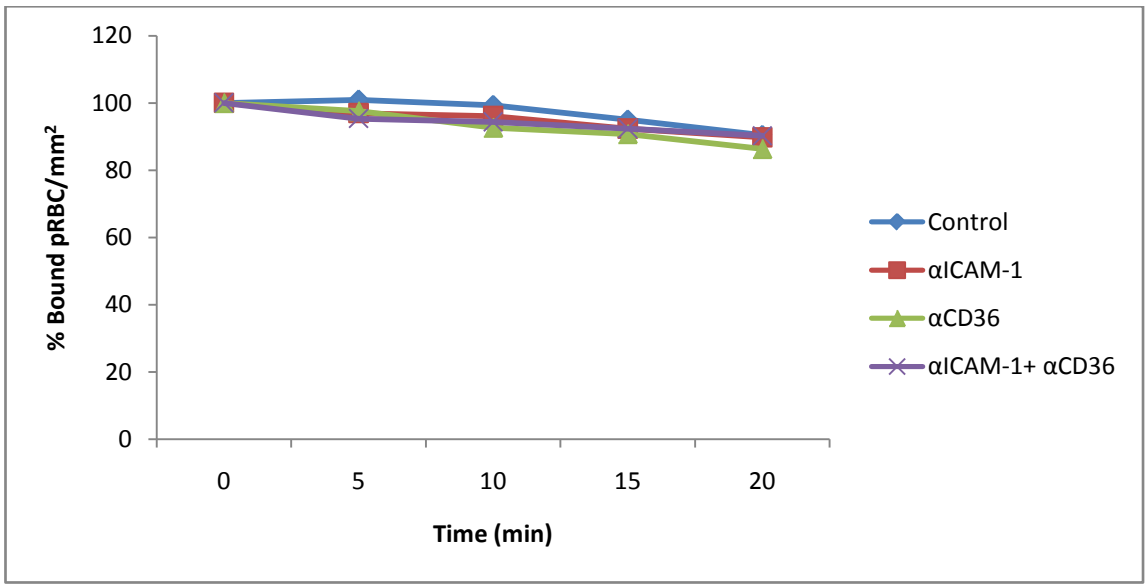


Figure 3. 21: Reversal of 8146 on TNF-stimulated HDMEC under flow conditions. Binding of 8146 (3%, parasitemia; 1% HCT) observed after flowing through with monoclonal antibody CD36: FA6-152 (αCD36) at 10 µg/ml and αICAM-1; 15.2 at 5 µg/ml for 20 minutes. Parasites bound were calculate for every five minute exposure and expressed as percentage (%) bound pRBC/ mm^2. Control, without antibody.

3.1.4 Discussion

Cytoadherence is thought to be a major virulence factor involved in the cause of death in SM. It has been observed that most of the malarial deaths occur in the first 24-48 hrs of hospitalization, in spite of effective antimalarial regimes (quinine or artimisinine) given immediately once admitted (Dondorp et al. 2005; Mohanty et al. 2003). Hence, reversing the sequestration of pRBC could be an important approach in the treatment of the acute phase of severe disease.

The ability of anti-adhesion to not only inhibits, but also to reverse adhesion has important therapeutic implications. Hughes *et al* have shown cytoadherence of pRBC to endothelium can continue for several hours after administration of anti-malarial drug treatment (Hughes, Biagini & Craig 2010) despite the pRBC being dead.

The continuing sequestered parasite load has the potential to still be contributing to disease severity by impeding blood flow and contributing to endothelial damage, as well as enhancing local inflammatory response (Chakravorty, Hughes & Craig 2008).

In this study, we have assessed the ability of mAbs to inhibit and reverse sequestered pRBC on purified protein (ICAM-1^{Ref} and CD36), HUVEC and HDMEC, an important process in at least part of the pathophysiology of SM. Our data demonstrate that mAbs 15.2 (αICAM-1) and FA6-152 (αCD36) not only highly significantly inhibit but also significantly reverse *P. falciparum* pRBC cytoadherence on protein and EC *in vitro*.

Complex interactions of pRBC to ICAM-1 and CD36 are thought to occur through DBL and CIDR domains of PfEMP1 to the N-terminal (domain 1) of ICAM-1 (Berendt et al. 1992) and phosphorylated ectodomain of CD36 respectively (Ho et al. 2005). The binding of pRBC to ICAM-1 is similar to that between ICAM-1 and human rhinovirus (Smith et al. 2000a; Xiao et al. 2004), using a similar region of the host receptor for the pathogen to interact with but having variation in the contact residues used. The ability of anti-LFA-1 mAbs to inhibit and reverse sequestered leucocytes from ICAM-1 in murine models shows the ability of these reagents to inhibit and reverse binding, and is applicable to pRBC cytoadherence

(Berendt et al. 1992). Therefore, by postulating the same mechanism on how pRBC would be able to be inhibited and reversed from ICAM-1 binding, the use of mAbs to the surface protein PfEMP1 would seem to be the best approach. Unfortunately, it is not the case. This is due to rapid changes in expression of *var* genes, which encode PfEMP1, and the high variability seen in this gene family. In addition, because not all PfEMP1 act identically and cause SM especially in CM (Ochola et al. 2011), finding the appropriate *var* sequences to use for a PfEMP1-based vaccine could be very difficult. Therefore, an approach to inhibit pRBC adhesion based on the host receptor is the best approach for now.

Anti-ICAM-1 15.2 was chosen because it has been used previously and shown able to inhibit binding of pRBC to ICAM-1 through interaction at loop 42 domain of ICAM-1. While FA6-152 antibody was chosen based on its ability to inhibit pRBC adhesion on CD36, recognising an epitopes within the region 155-183. The facts that, mAb directed against the 155-183 domain of CD36 can inhibit adhesion suggest that this domain is directly involved in CD36-ligand binding (Daviet et al. 1995).

Based on the ability of these antibodies, this experiment was carried out on two laboratory *P. falciparum* lines, ITG and C24, which are lab adapted parasite's ability to interact with the ICAM-1 and CD36. ItG is a *P. falciparum* line expressing a PfEMP1 encoded by ITvar16, which binds to ICAM-1 strongly, but less to CD36, while the C24 line has a PfEMP1 that is encoded by ITvar24 and has the ability to bind to CD36 stronger than ItG but does not bind to ICAM-1 (Ochola et al. 2011).

Our data demonstrate that both mAbs (αICAM-1 and αCD36) significantly inhibit and reverse pRBC adhesion to ICAM-1 and CD36 respectively under static and flow conditions. These findings are consistent with other studies. Following successful reversal and inhibition of binding to purified protein with both parasite strains, assays were carried out using EC (HUVEC and HDMEC). Adhesion assays showed that ItG binding was greatly increased following stimulation with TNF, whilst C24 binding was unaffected, under static and flow conditions, as expected due to increased expression of ICAM-1. Assays using ItG were carried out using both antibodies individually, and in-combination to observe any synergistic activity between these receptors.

Cytoadherence under flow conditions is thought to involve multiple adhesion receptors acting cooperatively, shown with CD36, ICAM-1 and P-Selectin (Gray et al. 2003; McCormick et al. 1997; Yipp et al. 2007). A model has been widely proposed where pRBC are captured from flow by one receptor, roll along endothelial cells before firm adhesion, possibly being mediated by a different receptor. This may separate out roles for different receptor families, where a selectin (i.e. P-selectin) or Ig superfamily receptor (i.e. ICAM-1) may be required to capture PRBC from flow followed by firm adhesion to a different class of receptor i.e. CD36. Firm adhesion may also require or be enhanced by interactions with more than one receptor concurrently (i.e. ICAM-1 and CD36).

Under static conditions, binding of C24 to HDMEC was inhibited and reversed by 84 – 85% by αCD36. Binding of ItG was inhibited by both antibodies, with the greatest level seen when both antibodies were used in combination. This suggests that both receptors (ICAM-1 and CD36) are required for binding for a population of pRBC. Reversal assays were then carried out on 24 under flow conditions using resting-HDMEC. Assays were run with antibody for 20 minutes, after which significant reduction in the number of C24 adhered could be seen (using αCD36). As with reversal assays, little difference was observed between the use of αICAM-1 alone, and a combination of both αICAM-1 and αCD36. However, αCD36 on its own was also quite efficient in reversing adhesion but not well as αICAM-1.

Analysis of αCD36 was effective at inhibiting and reversing binding of C24 to HDMEC under static and flow conditions. This is expected as it was shown that C24 binds only to CD36 and αCD36 especifically inhibits the interaction. The results from reversal experiment using ItG illustrated that αICAM-1 and in-combination (αICAM-1+ αCD36) give similar efficacy, where αCD36 is less effective, suggesting that ICAM-1 plays a more significant role for binding of this ItG line. This was also shown in the flow adhesion assays; adhesion more than doubled following stimulation with TNF, which up regulates ICAM-1. This results supports McCormick et al, where CD36 was found at lower densities than ICAM-1 on stimulated HDMEC (McCormick et al. 1997).

These characteristics were further explored by seeing the ability of these antibodies to reverse lab-adapted patient isolates (8146, 8026 and P069) in comparison with long-term lab-adapted parasites (ItG). Results show that the antibodies were only capable of reversing 20 – 30% of pRBC binding compared to the level of 50% seen with ItG. This suggests that the clinical isolates tested might be expressing PfEMP1 with different, possibly more extensive, repertoires of host receptor binding. This might support other findings, which show that not all SM parasite express similar *var* gene, and confer SM especially CM.

It is well known how these antibodies work through inhibiting the binding of pRBC to specific receptors by binding to specific domains of the receptor, but how does it work to reverse the binding. Here, we propose that the affinity of mAbs to interact with the receptor or blocking the functional site of the receptor is higher than that of the PfEMP1/host receptor interaction thereby reducing their chances to bind or interact with ICAM-1^{Ref} and CD36. Meanwhile, the equilibrium of antibody, receptors and pRBC ligand under flow conditions allows the antibody to compete for the functional site on the receptors (Figure 3.22). With the strong affinity towards a specific receptor, mAbs will bind more strongly, and free the bound pRBC, effectively allowing the pRBC to circulate again in the microvasculature (Goldring 2004).

This study assessed the ability of mAbs against EC receptors to inhibit and reverse cytoadherence. The data demonstrate that mAbs can both inhibit and reverse binding under static and flow conditions. It is thought that anti-adhesion therapy, which could minimize the severe complications of falciparum malaria, would be ideal in hyperendemic areas, since it would reduce both mortality and morbidity in those individuals who were most susceptible (young children and non-immunized persons).

Importantly, antibody-based therapies have 'proof of concept' in clinical settings, ensuring that the development time from laboratory to patient would be significantly reduced and the costs constrained, a luxury unavailable to some other prospective therapies.

With SM patients, the intravenous introduction of adhesion-blocking substances could not only lead to reversal of sequestration, but may also prevent the onset of cerebral malaria through reducing pRBC-induced endothelial activation. Successful anti adhesion therapy would also be practical to lessen SM, since it would displace infected cells bearing the more-mature parasite stages which would promote their selective removal from the circulation by the spleen system.

Here, our experiments suggest that malaria might be managed through mAb-therapy, but we need to bear in mind, that as well as being receptors for parasite adhesion, ICAM-1 and CD36 play major roles in leucocyte trafficking and normal human immune system responses. It has been shown that in blocking the receptor protein for the pathogen, this might inhibit the normal function of the protein and probably affect the host (Kelly & Younson 2000). Therefore, while this experiment might show the concept of anti-adhesion development based on receptor competition, further work should be considered for the development of a future anti-adhesion therapy.

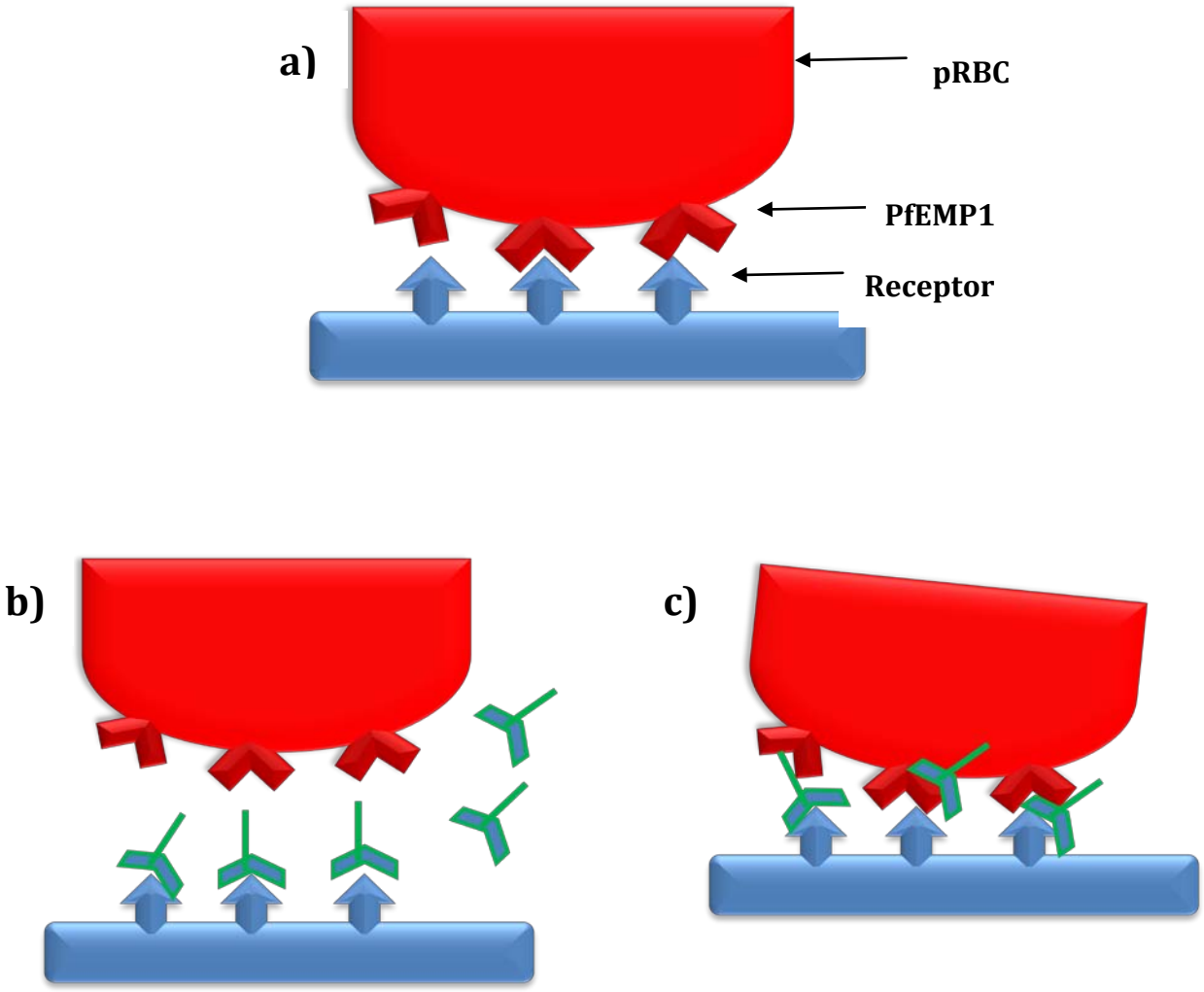


Figure 3. 22: Hypothesis of how mAb interact with endothelial adhesion receptors. a) Normal pRBC binding to receptor; b) mAb block receptor binding site; c) mAb challenge or compete for binding site. pRBC; parasitized red blood cells, PfEMP1; *P. falciparum* membrane protein 1.

3.2 Screening of modified polysaccharides on inhibiting and reversing *P. falciparum* sequestration *in vitro.*

Hypothesis: It has been shown that modified polysaccharides are able to inhibit parasite invasion and reverse parasite rosetting, but few have been tested for pRBC sequestration. Here, we would like to see if modified polysaccharides are able to inhibit and reverse *P. falciparum* sequestration to ICAM-1 and CD36.

3.2.1 Introduction

Carbohydrates are found throughout the animal kingdom and in plants. They have diverse functions in their tissues of origin, and have more or less complex and often heterogenous structures. Carbohydrate on their large acidic groups (e.g sulphate and carboxyl) dominates the physical characteristics of the compounds and can be equally dominant in their effects in biological systems.

Previous studies have indicated that carbohydrates may also play a role in mediating the cytoadherence of pRBC to microvascular endothelial cells (Clark, Su et al. 1997). Specific interactions of parasite with physiological carbohydrate receptors such as glycosaminoglycans (GAGS) have shown that carbohydrates are important as potential and future anti-adhesion therapeutic interventions because of their participation in many significant biological processes. This has been initiated by a group of company in Sweden, which using GAGs to be anti-adhesion for leukocytes (Ulbrich, Eriksson & Lindbom 2003).

During the asexual stage of malaria life cycle, *P. falciparum* parasites interact with human host cells in a number of ways and many of these interactions have been shown to be carbohydrate mediated. After being injected into the host's blood circulation, sporozoite-stage parasites travel to the liver and migrate into hepatocytes (Frevert et al. 1993). It has been shown that involvement of GAGs such as heparin sulphate proteoglycan (HSPG) on hepatocyte surface membranes interact with circumsporozoite surface protein (CSP) on the sporozoite surface

(Frevert et al. 1993; Nussenzweig & Nussenzweig 1985). Invasion of merozoites into RBC during erythrocytic stages has also shown involvement of carbohydrates (sialoglycophorin A) (Duraisingh et al. 2003; Maier et al. 2003; Ockenhouse et al. 2001).

Interactions of pRBC with host receptors have also been shown involving carbohydrate interactions, especially sequestration of pRBC in placenta during maternal malaria with chondroitin-4-sulphate A (CSA) and possibly hyaluronic acid (HA) during SM (Beeson et al. 1999; Fried & Duffy 1996; Rogerson et al. 1995), while interaction of pRBC with HS has been found confer formation of rosettes, a phenotype associated with SM.

Given that many of the interactions between *P. falciparum* and host cells are carbohydrate mediated, agents that interrupt these interactions may help to define these biological processes and may contribute to development of anti-malaria therapies. It is inevitable that acidic and negatively charged biological macromolecules will be attracted to, and affect the activity of, any basic protein. This is no doubt that such interactions can lead to potent but non-specific biological activities. Moreover, there is an increasing body of evidence that differences in patterns of structure and charges along polysaccharides or carbohydrates are involved in recognition events (Rudd et al. 2010a; Rudd & Yates 2010).

Modified polysaccharides are capable of binding with several proteins at several levels of specificity. As highly acidic macromolecules, they can bind non-specifically to any patch on a protein surface at low ionic strength, and such interactions are not likely to be physiologically significant. Several highly negatively charged sulphated polysaccharides, such as heparins (hep), chondroitin sulphate (CS), dextran sulphates, fucoidan and the non-sulphated glycosaminoglycan HA, confer high affinity for particular proteins and have been reported to inhibit invasion of erythrocytes by plasmodial merozoites and cytoadherence of pRBC to host cells as well as disrupting rosette formation of parasite-infected erythrocytes with uninfected erythrocytes (Rowe et al. 1994).

Modified polysaccharides with different levels and patterns of sulphation have been shown to be able to inhibit the growth of *P. falciparum* and interfere with adhesion of pRBC to host endothelial receptor CD36 (Butcher, Parish & Cowden 1988; Clark, Su & Davidson 1997; Xiao et al. 1996). Modified cellulose was able to inhibit adhesion to CSA expressed on CHO cells and placental tissue (Andrews et al. 2005). Cellulose sulphate with unusually high sulphation in positions 2 and 3, showed the best inhibitory capacity and was able to reverse bound pRBC to CHO cells and placenta tissue (Andrews et al. 2005).

As well as being directly involved in adhesion, it has been reported that sulphated soluble CSA is also able to inhibit and reverse adhesion of CSA-adherent parasites *in vitro* (Andrews et al. 2005) and in splenectomised monkeys *in vivo* (Pouvelle, Meyer et al. 1997). Furthermore altering selected functional groups, especially sulphate of the saccharide branches, showed that it is possible to reduce binding, and in some cases augment the attachment of the pRBC to endothelium, all mediated by sulphated carbohydrates (McCormick, Newbold et al. 2000). In this latter study, a variety of different levels and patterns of sulphated modified plant polysaccharides from important industrial polysaccharides were tested for their capacity to inhibit and reverse malaria cytoadherence, which may contribute to the development of novel therapeutics targeting adhesion of pRBC to receptors such as ICAM-1 and CD36.

Therefore, here in this sub-chapter we would like to investigate a group of chemically modified polysaccharides to inhibit and reverse lab adapted pRBC on protein and endothelial cells.

3.2.2 Material and methods

3.2.2.1 Parasites

The isogenic lab-adapted parasite lines ItG and A4 were used as described in methods chapter 2. Parasites were synchronised at ring stage and mature stages using sorbitol lysis and Plasmagel respectively as described in chapter 2.

3.2.2.2 Endothelial Cells

Endothelial cell lines HUVEC and HDMEC were used and grown as described in methods chapter 2. All assays were performed on 70-90% confluent monolayers of cells.

3.2.2.3 Compounds

A panel of modified polysaccharides were tested (Appendix VI; table 3.2). These were kindly provided by Dr Mark Skidmore (School of Biological Sciences, Keele University).

3.2.2.4 Adhesion assays

3.2.2.4.1 Inhibition assay – static endothelial cells

HUVEC were cultured and static cell assays were carried out as described in methods chapter 2. Parasite suspension of 3% parasitaemia pRBC and 1% HCT containing 1 mg/ml of compound were allowed to bind as for normal static assay for 1 hour with mixing every 10 minutes, then following two dip washes, coverslips were placed in a gravity wash for 30 min. After 30 min, coverslips were transferred to a second gravity wash for 10 min then fixed in 1% glutaraldehyde. As with static endothelial cell assays, controls were carried out which no contained additional compounds. All cells assays were carried out in triplicate.

3.2.2.4.2 Inhibition assay – flow endothelial cells

Inhibition of adhesion to HUVEC or HDMEC under flow conditions was carried out in a similar way on TNF (1 ng/ml 16h) stimulated HUVEC or HDMEC grown overnight on chamber slides. Suspensions of 3% parasitaemia pRBC and 1% HCT containing modified compounds were allowed to bind as for normal flow assay.

3.2.2.4.3 Reversal assay – flow endothelial cells

Reversal of adhesion to HUVEC or HDMEC under flow conditions was carried out in a similar way on TNF (1 ng/ml 16h) stimulated HUVEC or HDMEC grown overnight on chamber slides. Slides were prepared and assays were carried out as described in Chapter 2. PRBC at 3% parasitaemia and 1% haematocrit (hct) were flowed through the slide for 5 min to allow for pRBC adhesion. Flow was continuous throughout the experiment at 0.05 Pa shear stress (0.24ml/min). After 5 min pRBC flowwas switched to binding medium alone to remove unbound pRBC. Timing was started at the beginning of this wash, which continued for 2 min before the binding medium was swapped for medium containing modified compound. The number of bound cells in six fields along the slide was counted at 2 min, 5 min, 10 min 15 min and 20 min time points.

3.2.3 Results

3.2.3.1 Screening of inhibitory effect of modified polysaccharides

To determine the effect of these compounds to inhibit sequestration, a number of modified polysaccharides (MS compounds) were screened for their anti-adhesion properties on static based endothelial cell binding assay. Compounds at 1 mg/ml were incubated for about 5 min in pRBC suspension prior to the binding assay. Out of 42 compounds screened, 11 compounds appeared to show a potential adhesion inhibitory effect by giving a cut-off point below 50% binding reduction. These were tested further by comparing responses of ItG and A4 to TNF-activated endothelial cells (HUVEC (Figure 3.23 and 3.24 respectively) and HDMEC (Figure 3.25)) using flow binding assays. From the second screening, we identified only two compounds (MS34 and MS40) showing a significant adhesion inhibitory effect towards laboratory parasite strains by reducing the binding of A4 pRBC strain up to 50% and at least 20% reduction of ItG pRBC strain to TNF activated HDMEC in comparison with control (non-treated HDMEC).

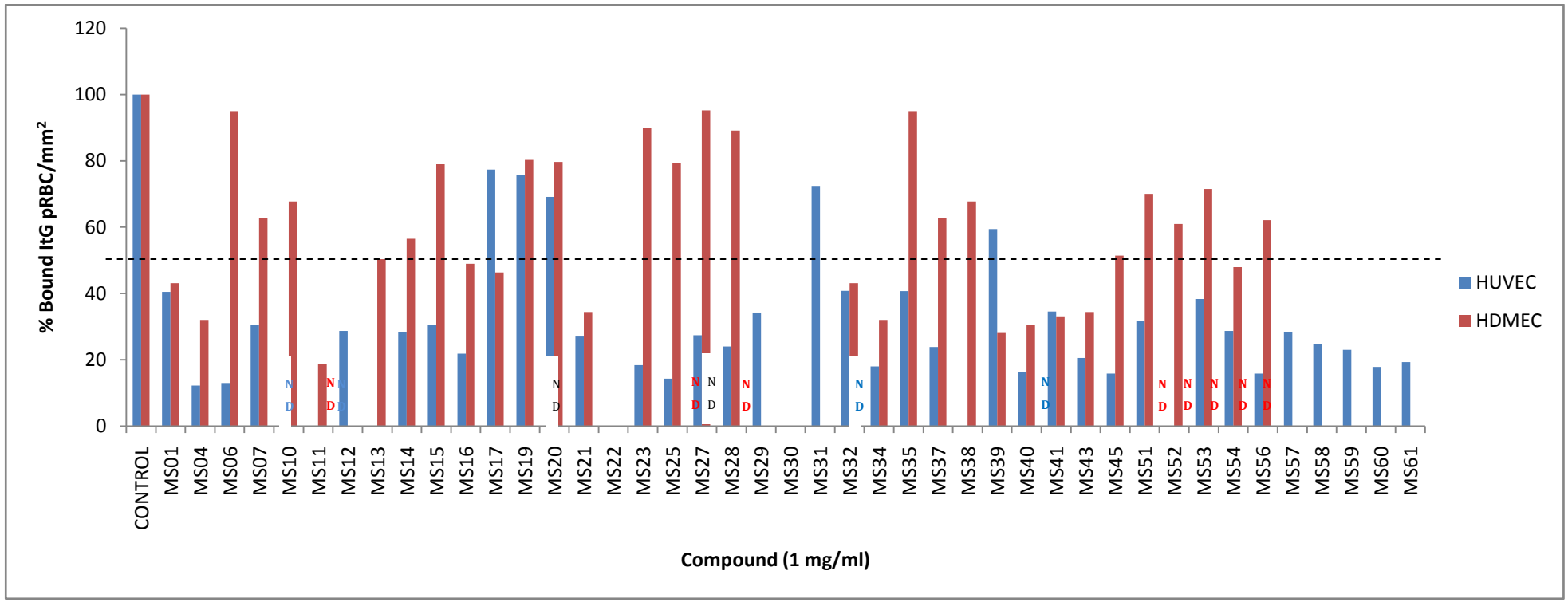


Figure 3.23: Effect of chemically-modified polysaccharides on ItG binding to TNF-stimulated HUVEC and HDMEC under static binding conditions (single screening). A binding of ItG (3%, parasitemia; 1% HCT) observed after polysaccharide treatment for one hour. The remaining bound parasite after gravity wash was counted and expressed as% bound pRBC/ mm² (N=1). Control, without compounds. ND, not done, Red; HDMEC, Blue; HUVEC.

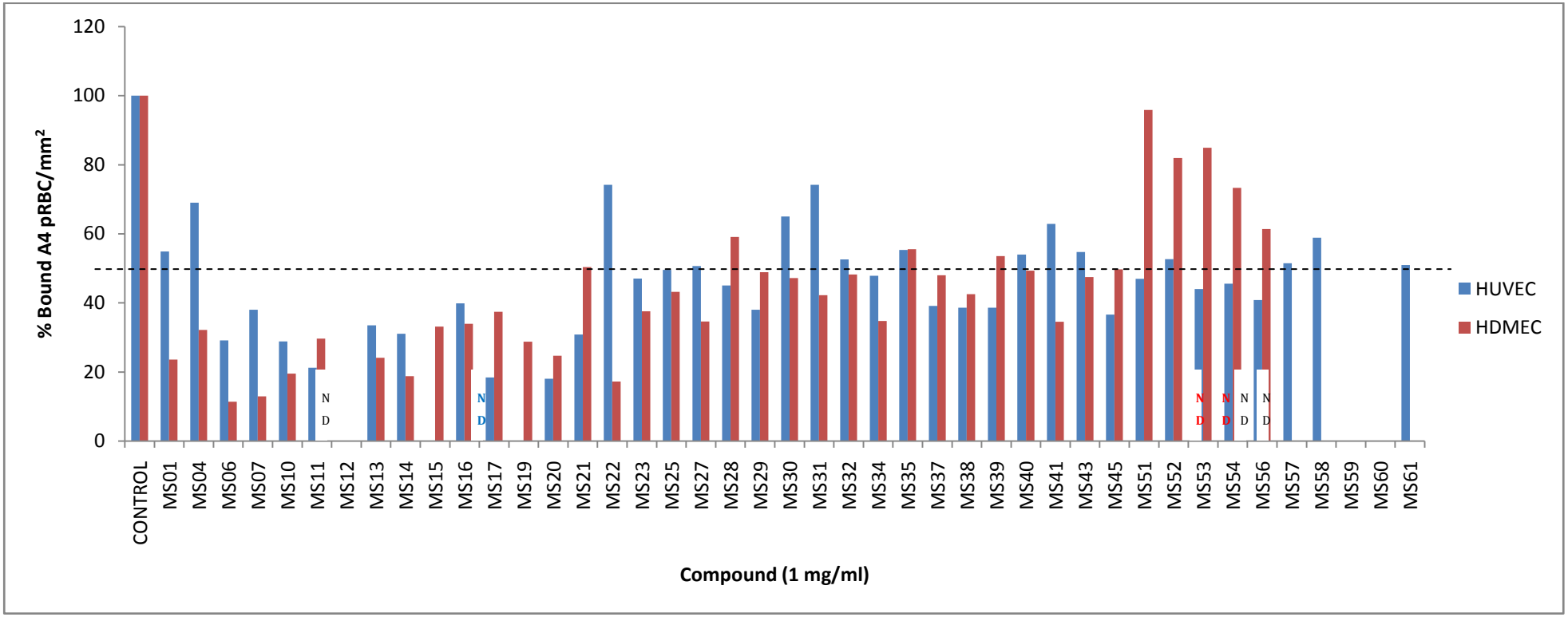


Figure 3.24: Binding response of A4 to screened modified polysaccharide compound at 1 mg/ml on TNF-stimulated HUVEC and HDMEC under static condition (single screening). Binding of A4 (3%, parasitemia; 1% HCT) observed after polysaccharide treatment for one hour. The remaining bound parasite after gravity wash was counted and expressed as % bound pRBC/ mm² (N=1). Control, without compounds. ND; not done, Blue; HUVEC, Red; HDMEC.

Table 3. 2: Percentage of reduction on static parasite binding after treatment with modified polysaccharide at 1 mg/ml relate to Figure 3.23 (ItG) and 3.24 (A4).

Compounds	A4		ITG	
	HUVEC (%)	HDMEC (%)	HUVEC (%)	HDMEC (%)
CONTROL	0	0	0	0
MS01	45.07	76.37	59.47	10.94
MS04	30.98	67.85	87.74	46.15
MS06	70.83	88.58	86.98	ND
MS07	61.97	87.03	69.35	42.90
MS10	71.12	80.45	84.37	40.14
MS11	52.57	70.32	ND	18.614
MS12	ND	ND	71.32581	ND
MS13	54.02	75.88	ND	50.31
MS14	53.66	81.18	71.77	56.47
MS15	ND	66.87	69.53	78.94
MS16	35.67	66.04	78.11	48.94
MS17	74.64	62.54	77.32	46.30
MS19	ND	71.19	75.71	80.25
MS20	78.30	75.31	69.153	79.63
MS21	14.084	49.63	72.983	34.43
MS22	25.81	82.75	ND	ND
MS23	52.95	62.40	81.58	89.85
MS25	50.46	56.76	85.68	79.42
MS27	49.29	65.39	72.58	95.21
MS28	54.92	40.87	75.96	89.14
MS29	61.97	51.08	65.72	ND
MS30	34.97	52.81	ND	ND
MS31	25.81	57.72	72.44	ND
MS32	47.42	51.75	59.21	56.86
MS34	52.12	65.22	81.98	67.98
MS35	44.64	44.47	59.27	4.99
MS37	ND	51.99	76.15	37.28
MS38	60.87	57.43	ND	32.25
MS39	61.40	46.41	40.59	71.91
MS40	40.01	50.63	83.66	69.44
MS41	37.11	65.43	65.45	66.90
MS43	45.23	52.517	79.43	65.56
MS45	63.38	50.26	84.13	48.61
MS51	53.05	4.12	68.22	29.90
MS52	47.32	18.00	ND	39.07
MS53	55.95	15.06	61.69	28.47
MS54	54.39	26.66	71.29	52.061
MS56	59.15493	38.60857	84.13952	37.86317
MS57	48.50704	ND	71.50565	ND
MS58	41.08451	ND	75.40323	ND
MS59	ND	ND	77.01613	ND

MS60	ND	ND	82.12339	ND
MS61	49.05634	ND	80.64516	ND

ND; not detected, **Red highlight**; compounds chosen for further screening (second screening).

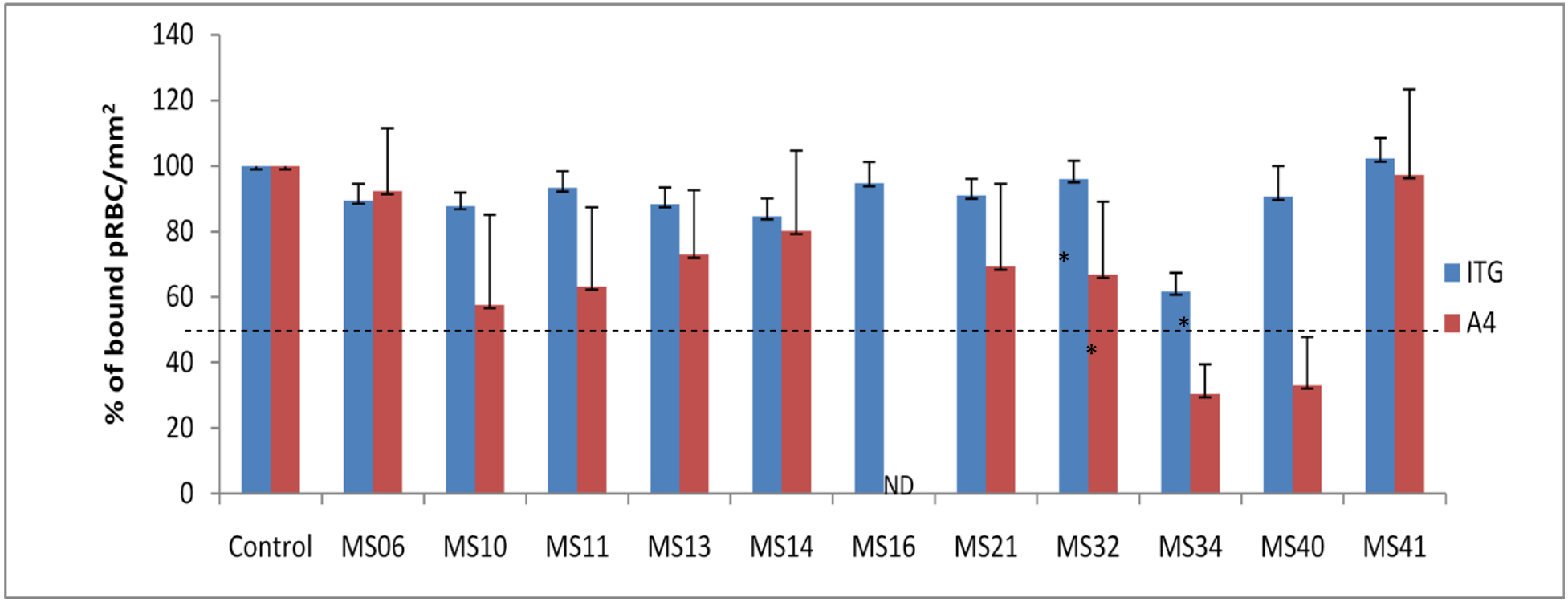


Figure 3.25: Binding response of ItG and A4 to screened modified polysaccharides compound at 1 mg/ml on TNF-stimulated HDMEC under flow condition. A binding of ItG and A4 (3%, parasitemia; 1% HCT) observed after polysaccharide treatment for 5 minute flow through. The remaining bound parasite after 2 minute wash was counted and expressed as % bound pRBC/ mm^2 ± standard deviation. Control, without compounds. ND; not done. *, $P < 0.05$ (compare to control).

3.2.3.2 Reversal effect of modified polysaccharides

Having identified MS34 and MS40 as being able to inhibit parasite adhesion, we further analysed these compounds for their reversal activity towards these two-laboratory parasite strains to different endothelial cells line (HUVEC, HDMEC and HBEC). We found that MS34 effectively disrupted sequestration with 40% to 60% reduction of pRBC on these cells, comparing 0 min with 20-minute exposure to the compounds (Figure 3.26 (a) and 3.27 (a)), meanwhile MS40 did not show any reversal effect or at par towards the endothelial cells (Figure 3.26 (b) and Figure 3.27 (b)).

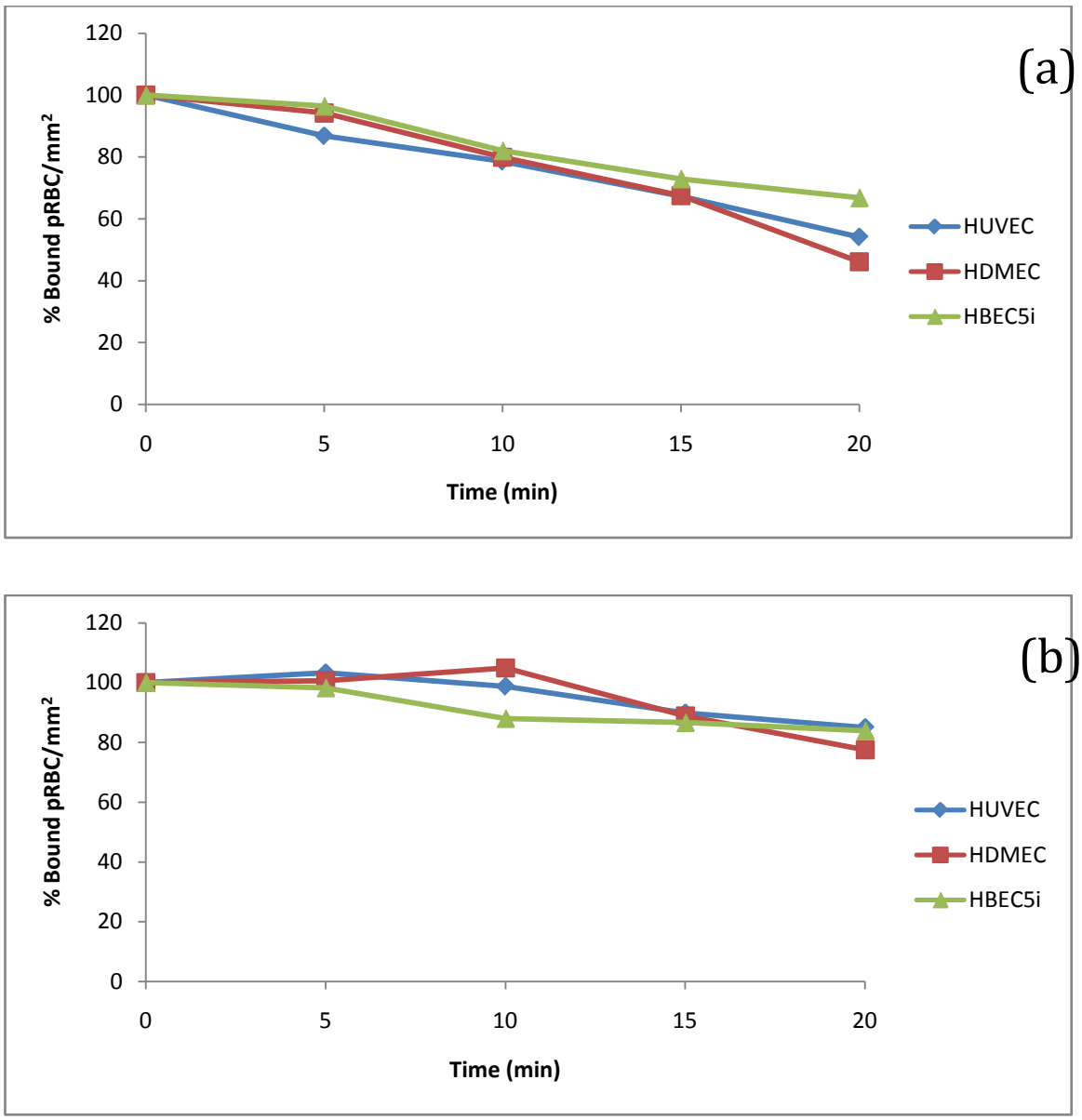


Figure 3.26: Reversal effect on ItG pRBC to different TNF-stimulated endothelial cells (HUVEC, HDMEC and HBEC) after flowing through 1mg/ml of (a) MS34 and (b) MS40 for 20 minutes under flow conditions. Parasite bound were calculate for every five minute exposure and expressed as bound pRBC/ mm^2. Control, without compounds.

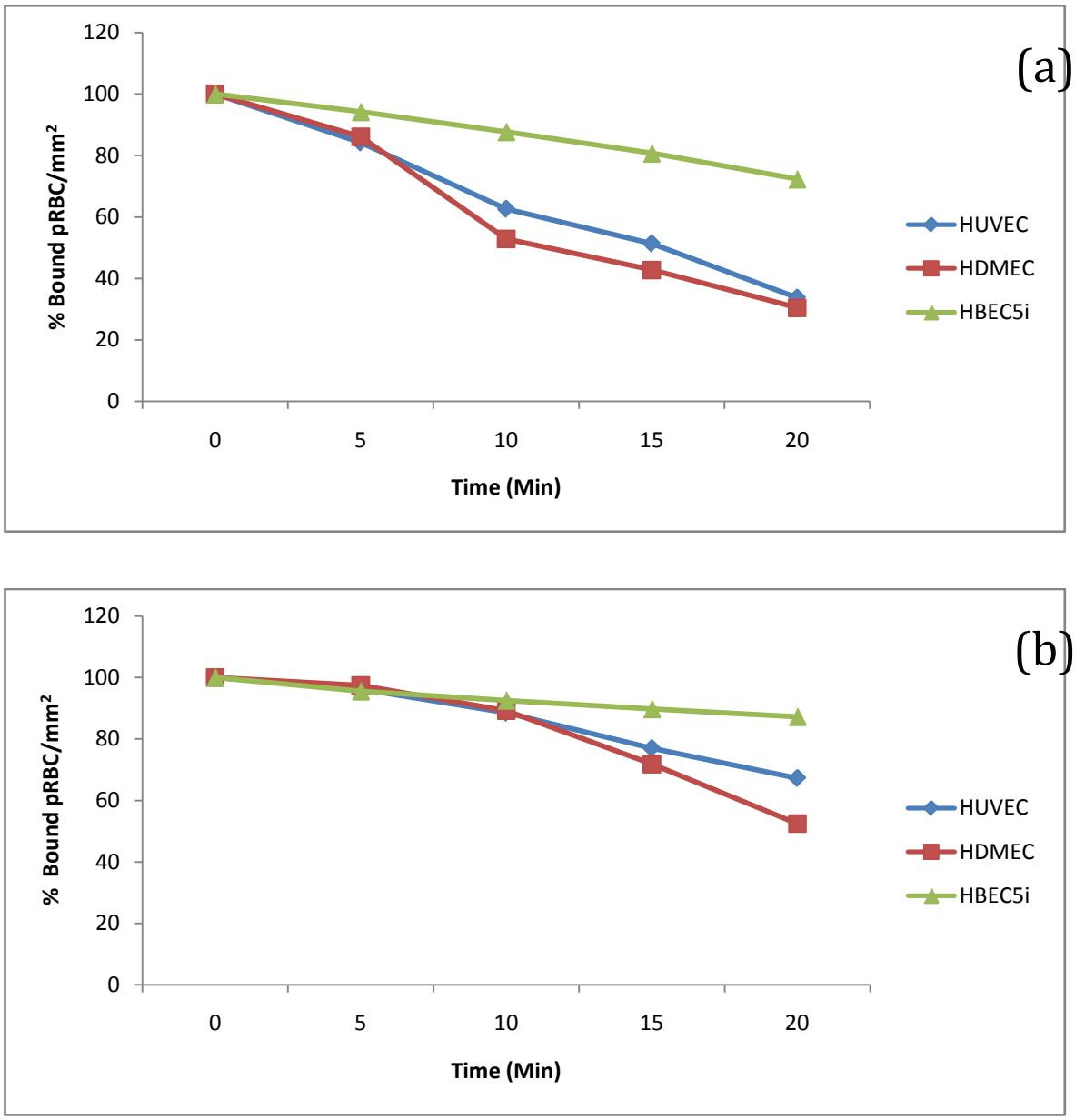
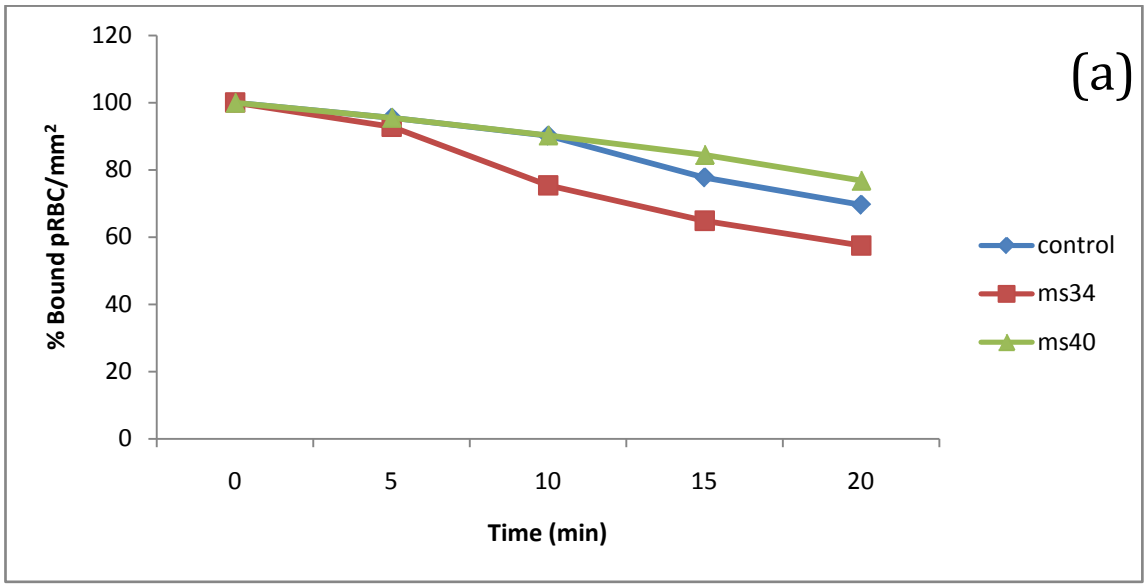
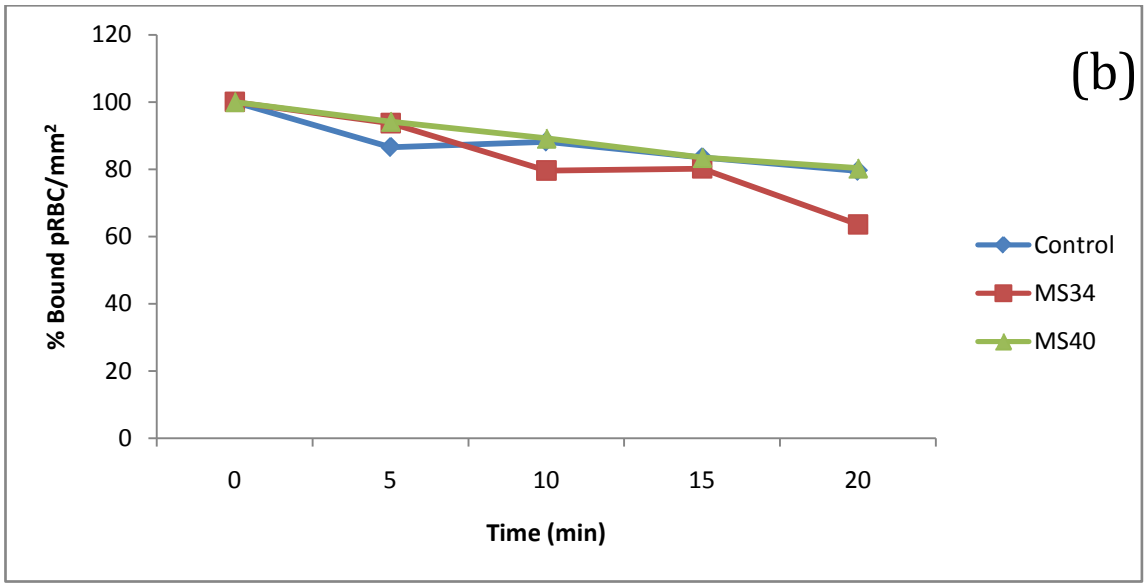


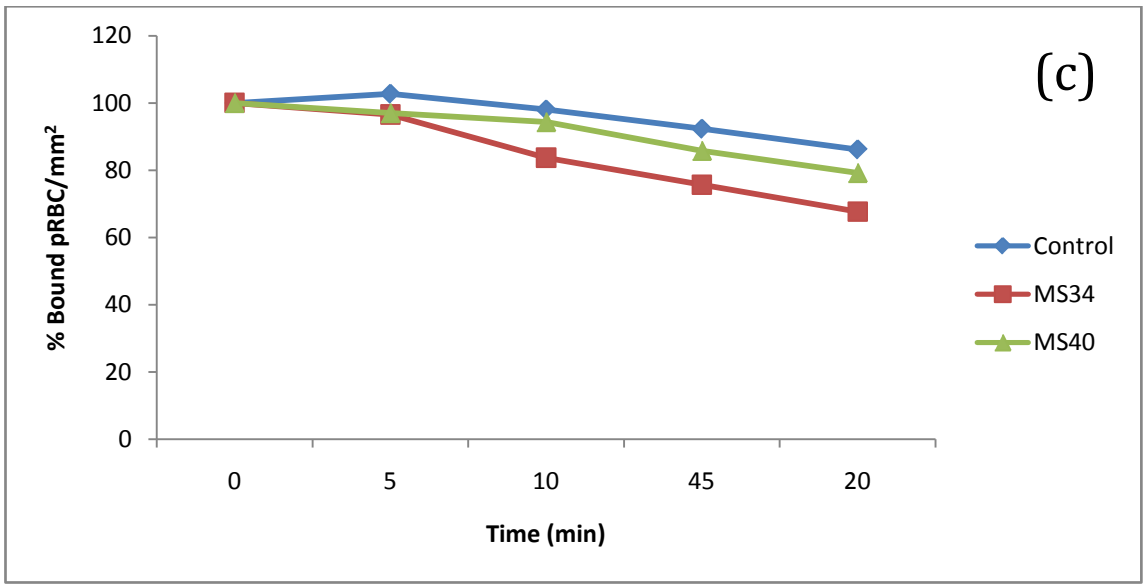
Figure 3.27: Reversal effect on A4 pRBC to different TNF-stimulated endothelial cells (HUVEC, HDMEC and HBEC) after flowing through 1mg/ml of (a) MS34 and (b) MS40 for 20 minutes under flow conditions. Parasites bound were calculate for every five minute exposure and expressed as percentage (%) bound pRBC/ mm². Control, without compounds.

3.2.3.3 Reversal effect of selected compounds on clinical isolates

To examine the effect of MS34 and MS40 using a broader range of parasite variants, three recently lab-adapted clinical patient isolates (P069, 8146 and 8026) were chosen. The first task was to test the compounds on already bound pRBC on ICAM-1-ref protein. These showed that each isolate responded differently towards the compounds, but overall similarly flushing through around 20-30% pRBC from the system in comparison to 0 min (initial reversal time) (Figure 3.28). Furthermore, the reduction in binding was significant (p- value = 0.001) when using TNF activated HUVEC cells (Figure 3.29 and Figure 3.30).







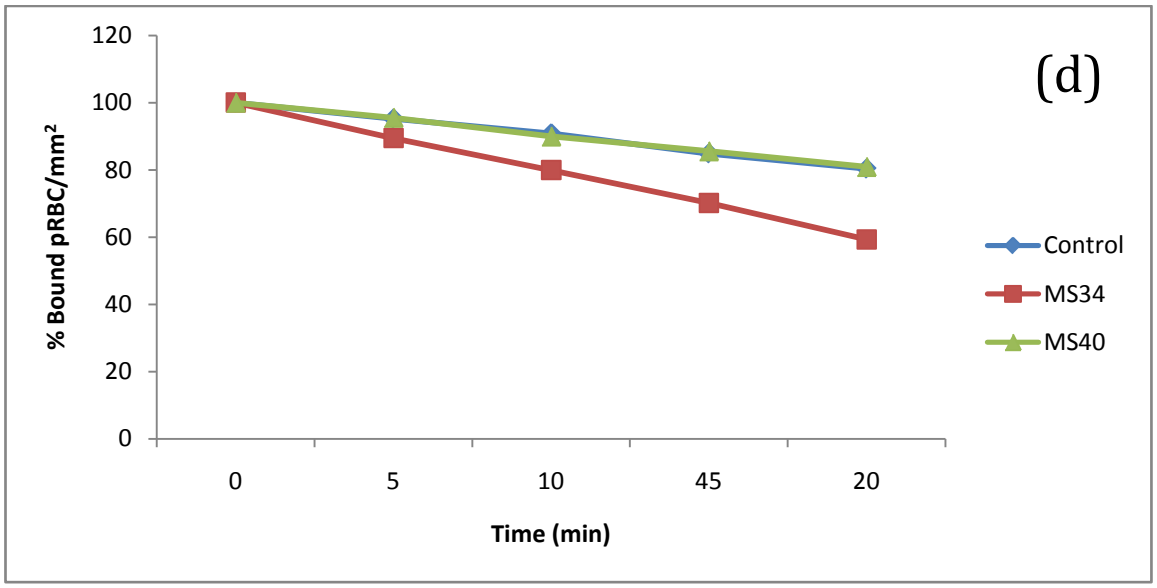


Figure 3. 28: Effect of MS34 and MS40 to lab-adapted clinical isolates a) Control, ItG on ICAM-1^{Ref} ; b) P069 on ICAM-1^{Ref}; c) 8146 on ICAM-1^{Ref}; d) 8026 on ICAM-1^{Ref}. Parasites bound were calculate for every five minute exposure and expressed as percentage (%) bound pRBC/ mm^2. Control, without compounds.

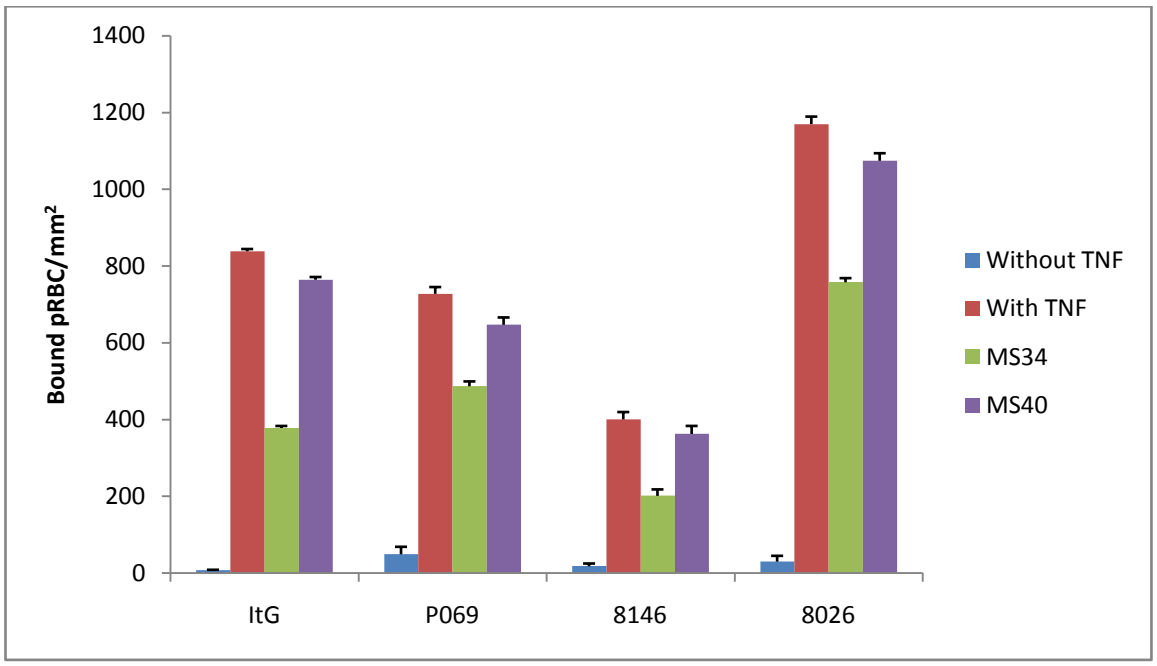
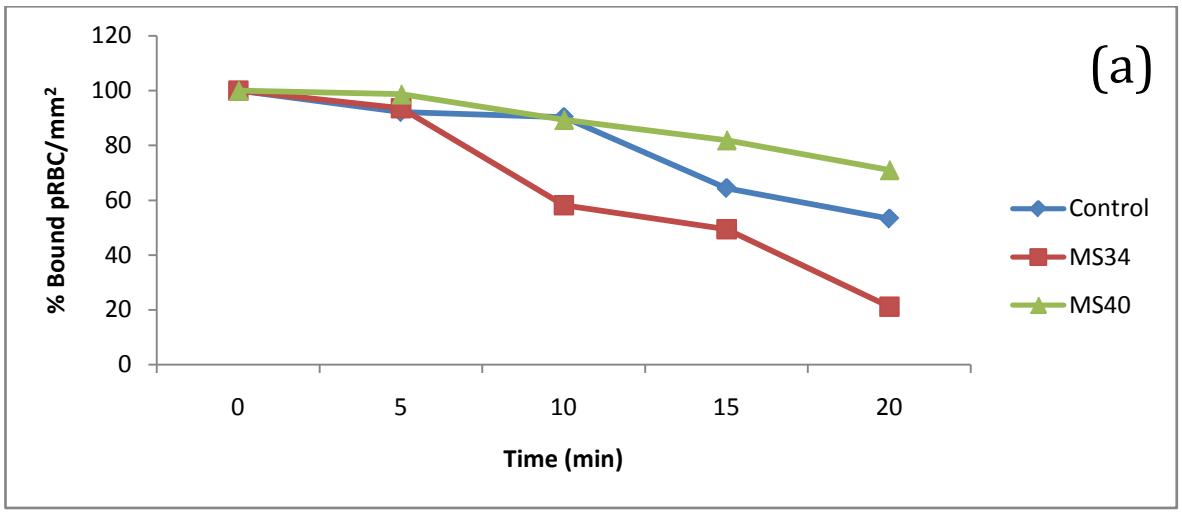
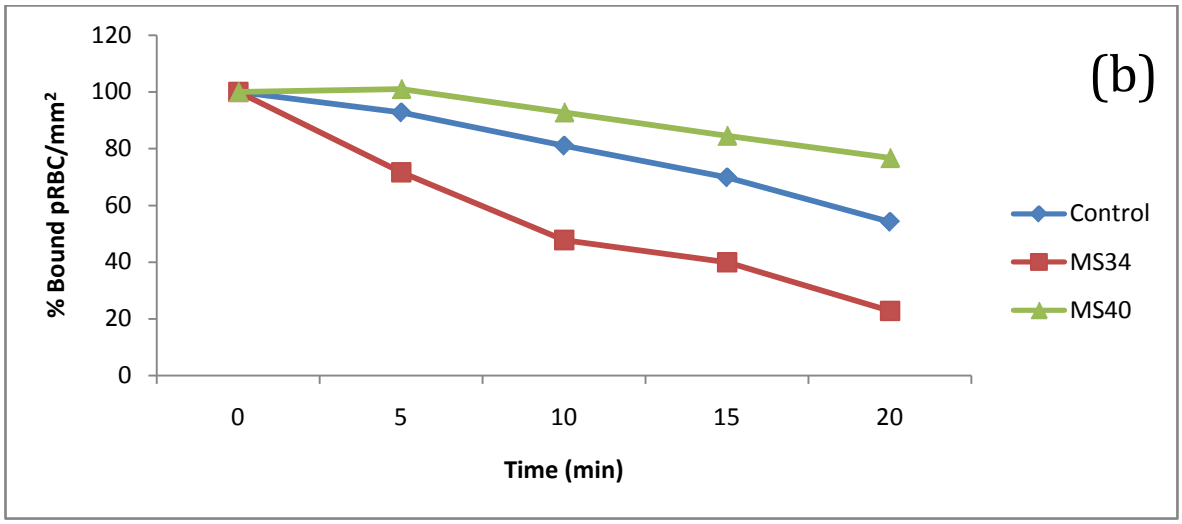
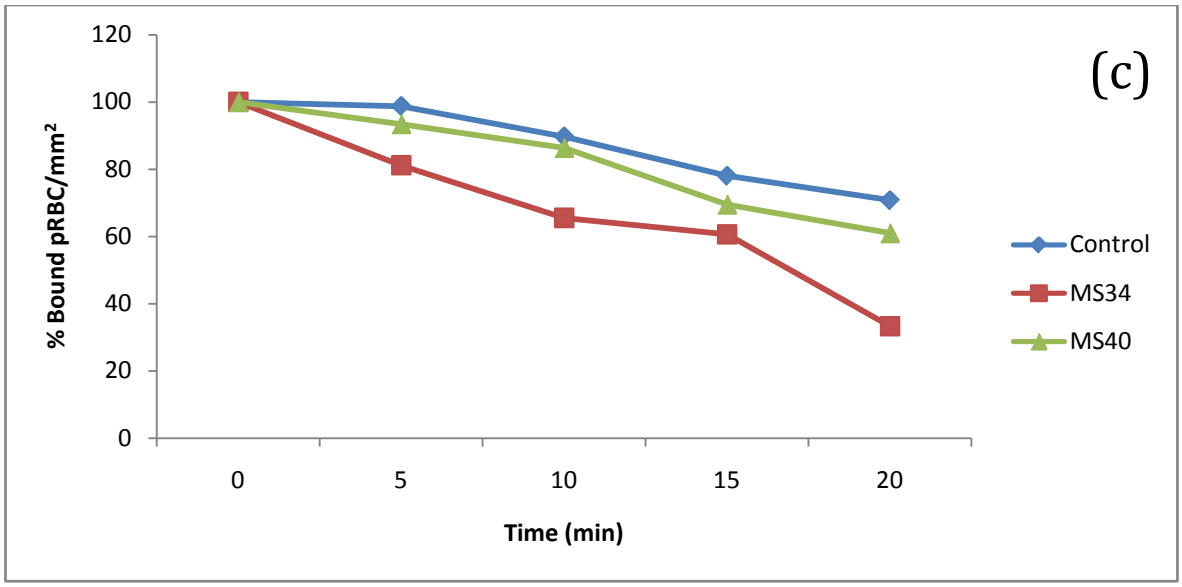


Figure 3.29: Effect of modified compounds on binding to HUVEC using lab-adapted clinical isolates parasite line (P069, 8146 and 8026) under static assay conditions. ItG was used as a control. The remaining bound parasite after gravity wash was counted and expressed as bound pRBC/ mm^2 ± standard deviation.


(a)
% Bound pRBC/mm²
120
100
80
60
40
20
0
0
5
10
15
20
Time (min)
Control
MS34
MS40


(b)
% Bound pRBC/mm²
120
100
80
60
40
20
0
0
5
10
15
20
Time (min)
Control
MS34
MS40


(c)
% Bound pRBC/mm²
120
100
80
60
40
20
0
0
5
10
15
20
Time (min)
Control
MS34
MS40

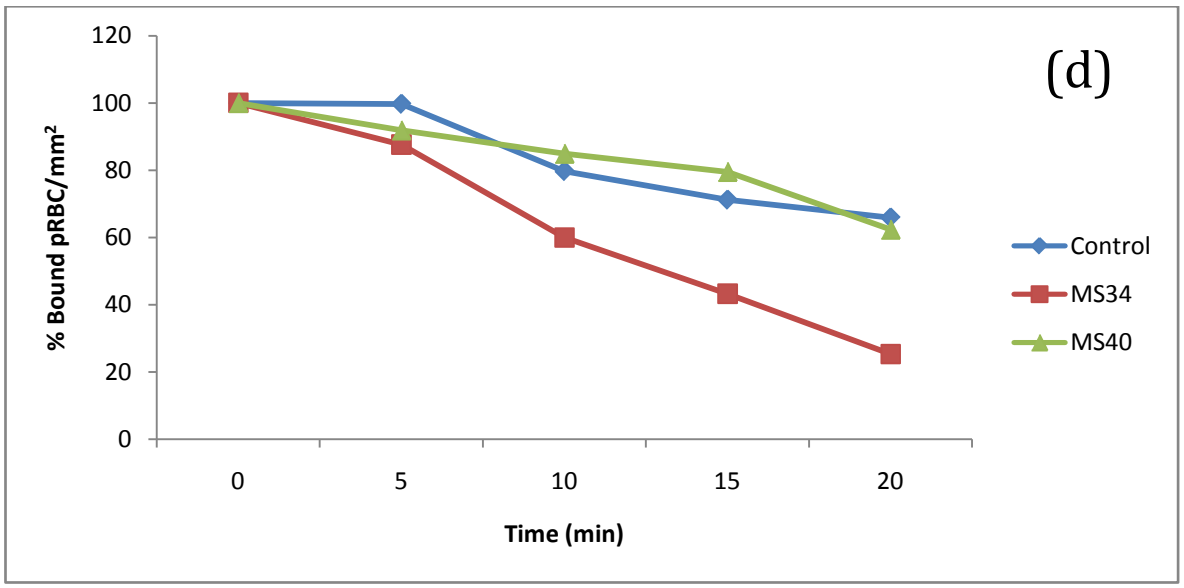


Figure 3.30: Testing compounds under flow conditions for their ability to reverse existing binding. a) ItG reversal on HUVEC; b) P069 reversal on HUVEC; c) 8146 reversal on HUVEC; d) 8026 reversal on HUVEC (all HUVEC cultures were activated with TNF). Parasites bound were calculate for every five minute exposure and expressed as percentage (%) bound pRBC/ mm^2. Control, without compounds.

3.2.4 Discussion

A distinct characteristic of *P. falciparum* is the ability of matured parasite to escape spleen clearance. This is associated with a unique ability of surface parasite protein PfEMP1 to interacts and bind to endothelial receptors such as ICAM-1 which leads to clinical manifestations associated with SM such as hypoxia and coma. Therefore, molecules that are able to inhibit or interrupt these interactions may have a role in understanding host-parasite biology and as well developing new therapies.

Studies into the adhesive interaction between the pRBC and CSA in pregnancy malaria have demonstrated that highly sulphated polysaccharides not only inhibit binding of pRBC to CSA (Andrews et al. 2005; Clark, Su & Davidson 1997; Xiao et al. 1996), but they can also be utilized to reverse sequestered parasites from the placenta (Fried & Duffy 1996; Pouvelle, Fusai & Gysin 1998).

In this study, we have screened a panel of chemically-modified plant polysaccharides (Table 3.2) for their ability to inhibit and reverse ICAM-1^{Ref} and CD36-mediated pRBC adhesion of *P. falciparum* and found two compounds MS34 and MS40 inhibiting binding of pRBC under static and flow conditions.

Parasite line ItG shows different responses to each compound on different EC. Most compounds show some reduction of binding at more than 50% on TNF-activated HUVEC compared to TNF-activated HDMEC, while A4 does not show such dramatic effects on TNF-activated HUVEC and TNF-activated HDMEC (Table 3.3). We do not know why this happens, it probably due to receptors expressed on both EC. TNF stimulates expression of ICAM-1 on HUVEC and HDMEC (Chakravorty, Hughes & Craig 2008). While HDMEC contain CD36 receptors on its surface, HUVEC does not. Therefore, it is possible that these modified polysaccharide responses to both lab-adapted isolates are based on compound-specificity in blocking the interactions of PfEMP1 with certain receptors such as wild type ICAM-1(ICAM-1^{Ref}) on HUVEC and, both ICAM-1 and CD36 on HDMEC.

In order to see a clear result after the first screen, eleven compounds (MS06, 10, 11, 13, 14, 16, 21, 32, 34, 40 and 41) which showed higher binding inhibitions were chosen. We used flow binding based assays to investigate

potential of these eleven modified polysaccharide compounds in inhibiting binding on these two lab-adapted strains. Flow assays are the best way to mimic the situation in host vasculature. We used non-activated HDMEC that does not express ICAM-1 but only CD36 on its surface in second screening, so by that we can see whether this interaction is strain-specific or based on compound specific interaction to ICAM-1 or CD36 protein. It was found that out of the eleven compounds coming through the primary screen, only two compounds, MS34 and MS40 strongly gave more than 50% reduction in binding when using A4, but not ItG. We know that A4 possesess higher CD36 avidity binding compared to ITG, while ItG possesses higher binding to normal ICAM-1 but lower binding with CD36. From our observations, here, we presumed that MS34 and MS40 probably interact to CD36 and less with ICAM-1.

Many studies had shown that polysaccharides such as dextran sulphate and fucoidan are able to inhibit adhesion of *P. falciparum* to host receptors such as CSA and CD36 (Andrews et al. 2005; Clark, Su & Davidson 1997; Xiao et al. 1996). There are also studies showing regioselective modified polysaccharides and modified carrageenans able to inhibit binding of pRBC to CD36 (Adams et al. 2005; Schwartz-Albiez et al. 2007). This discovery may put MS34 and MS40 on the list as potential compounds that are able to inhibit *P. falciparum* adhesion on endothelial cells especially on CD36. Our real challenge is, however, whether these compounds would be able to reverse bound pRBC, which would be the likely situation found clinically in SM, especially in CM patients.

To answer either MS34 or MS40 able to reverse bound pRBC, three different EC (HUVEC, HDMEC and HBEC) have been prepared in order to see MS34 and MS40 properties on reversing bounded lab-adapted *P. falciparum* A4 and ItG. To our knowledge ICAM-1 is highly expressed on human brain and associated with CM (Conroy et al. 2010; Tripathi, Sullivan & Stins 2006). The use of brain EC is very important here, it may be able to show us what really happens during CM and whether these MS34 and MS40 can help to lessen sequestrations.

The reversal results support our inhibition results under flow using lab-adapted parasites A4 and ITG, and show that MS34 gave a better response compared MS40 on different EC (HUVEC, HDMEC and HBEC) and they also show

that MS34 is more effective in reversal of A4 binding on HUVEC and HDMEC compared to HBEC. We can also see similar reversal effects of MS34 on ItG binding similarly on all three of the EC. Meanwhile, MS40 does not show any reversing effect on ItG but giving slightly better on reversing A4 binding on HDMEC and HUVEC. Although MS34 and MS40 showed different effects on reversing both lab-adapted strains, here we can see that these compounds are unable to affect or gave little effect on HBEC.

These two compounds were further investigated, by testing a panel of new parasite isolates from SM patients provided by Kiliffi group (8146, 8026 and P069). It is found that 8146 is more ICAM-1^{Ref} binder followed by 8206 and P069 from our lab observation (different experiment). It is found that 8146 binds more to ICAM-1^{Ref} compared to two mutant ICAM-1 proteins (ICAM-1^{Kilifi} and ICAM-1^{S22A}). ICAM-1^{Kilifi} has polymorphism at position 29 which replaces a lysine residue with methionine and is associated to SM incidence (Adams et al. 2000; Craig et al. 2000; Jenkins et al. 2005), while ICAM-1^{S22A} has replace serine to alanine at position 22 (Howell et al. 2008). Here, we observed that each patient strain does show different reversal responses when the compounds were tested under static condition to TNF-activated HUVEC, which expresses high levels of normal ICAM-1 receptors (Gray et al. 2003).

To support our static reversal results, we tested the reversal effect of MS34 and MS40 on TNF-activated HUVEC under flow condition toward these three patient isolates. We found that all patient isolate flow responses were similar to static responses and we found that MS34 reverses better compared to MS40 on both static and flow. Therefore, our flow results proved our static binding observations.

Why MS34 shows higher reversal effects compare to MS40 is not known. Does it relate to structure and charges of the compounds? However, here is the question, if all the compounds work non-specifically we should see more compounds working at the same level as MS34 and MS40 but this is not the case and there appears to be no major structural or charge correlation with the ability to block pRBC binding. We also see other related compounds that contain cellulose (MS26, 35 and 40) and carrageenan (MS20, 52, and 53) backbones, which had

previously been shown to have activity to inhibit and reverse pRBC binding (Adams et al. 2005; Andrews et al. 2005; Baba et al. 1988a; Baba et al. 1988b; Utt & Wadstrom 1997) but do not work in our assays. This might support that not all compounds containing the same carbohydrate backbone do act similarly, but rather it might be based on sulphate positions or conformational structure of the compound (Rudd et al. 2010b).

We know that MS34 contains low charge and low sulphate levels from other studies by Skidmore's group (data not shown). This finding might support McCormick et al, where they showed low sulphated glycoconjugates able to modulate binding of pRBC to CSA protein receptors (McCormick, Newbold & Berendt 2000). What about other modified polysaccharides compound tested as well as with MS34 and MS40, do they contain higher charges? Unfortunately, there is still lack of information regarding these compounds. We still do not know the exact structure or the level of sulphation that exists in them. Therefore, we cannot be sure or predict the mechanism of inhibition, but here we propose that this compound interaction probably could work a few ways based on other studies related to polysaccharides as adhesion inhibitors.

Our first hypothesis is the modified polysaccharides might work through multivalent effects. A multivalent effect is a good practical application of interference strategies as it can lead to large rate enhancement, even for systems with relatively low valence, such as a divalent system (Dam et al. 2005; Peterson 1998). The multivalent effect has been much studied regarding on interaction of polysaccharide compounds with protein, such as interaction of sulphated polysaccharides with fibroblast growth factors (Guimond et al. 1993; Spivak-Kroizman et al. 1994; Walker, Turnbull & Gallagher 1994), inactivation of thrombin by polysaccharide-like antithrombin (Bourin & Lindahl 1993), and use of dendritic polyglycerol sulphate to inhibit binding of leukocytes to L –selectin (Weinhart et al. 2011). A multivalent effect is a phenomenon which relies upon the formation of non-covalent bonds. In the multivalent interactions, the receptor binding mechanism may occur simultaneously or independently, depending on structural of polysaccharides (glycoconjugates) and nature of receptors (such as ICAM-1). We know that ICAM-1 is highly glycosylated, so it tends to accept many compounds especially negatively charged compounds. The enhanced effectiveness

of multivalent competitors as inhibitors of biospecific interactions between cells has been widely reported (Adler et al. 1995; Hosoya et al. 1991). The concept of multiple carbohydrate binding sites providing the basis for strong adherence to carbohydrate receptors evolved from studies of lectin binding. Those studies indicated that even though binding of a lectin to a single saccharide might be weak, adherence to multiple saccharides resulted in a strong association with ligand.

Our second hypothesis to explain that carbohydrate-therapy involves conformational structure of the modified polysaccharides. There is no doubt it a certain polysaccharide structure is required to interact with receptors giving high inhibitory activity and leading to potent inhibitory compounds. It has been postulated that if a compound contains heparin/HS or GAG analogues it has potential inhibitory actions leading to researcher claims that important physiological processes may depend on subtle and specific distinctions between structures found in sulphated polysaccharides (Jones, Smith & Polasek 2004). Rudd et al 2010 found that several but not all sulphated plant polysaccharides are able to interact with FGF-1 and FGF-2 through capacity to support signalling through FGFR1c in combination with heparin. This demonstrated that structural requirements were not solely restricted to heparin/HS or GAGs analogue, but they could be satisfied by sulphate ion underlying in the structure sequence (Rudd et al. 2010a). Andrews *et al* found that distinct patterns of sulphation rather than high overall and even distributions of sulphate groups govern interactions with pRBC (Andrews et al 2005).

Therefore, the last hypothesis to explain on how carbohydrate can work as drugs or anti-adhesion is through charge distribution in the modified-polysaccharides scaffolds, although in this case not all inhibitors need to be highly charged, because MS34 is a low negative charge compound. This supports another previous study, which showed low sulphated modified CSA able to inhibit binding of pRBC to CSA (Andrew et al 2005). This however contradicts the McCormick et al finding, where they showed low sulphated CSA mediating pRBC binding to placenta receptors. Conformational studies by NMR and molecular modelling have shown that the polysaccharide chain which has relative heparin/HS shape, regardless of its pattern of sulphate substitutions might interact similarly like heparin/HS (Mulloy et al. 1993). It is found that as long as a minimal specific motif

of sulphate substitutions is present it still can accommodate the same heparin/HS binding site (Ashikari-Hada et al. 2004). Meanwhile, placenta parasites do not adhere to CSB and CSC, glycans which are close to CSA, indicating fine specificity of pRBC binding interaction. The GAGs CSA and CSC share the same disaccharide repeat of D-glucuronic acid (GlcUA) linked to amino sugar N-acetyl-D-galactosamine (GalNAc). CSA carries a sulphate group at the position C4 of GalNAc and CSC carries sulphate group C6 positions of GalNAc, which could dictate the sulphate position to support adhesion of *P. falciparum*. On the other hands, it found sulphate at position 6-O GalNAc of CSA was able to inhibit binding of pRBC to CSA (Andrews et al 2005).

The ability of sulphated polysaccharides with distinct primary sequences from HS and heparin yet still able to mimic their biological activities, is well known, but is not caused solely by them possessing high charge density. Indeed, the most active compounds are not usually the most highly charged and this has also been borne out in this study. An alternative explanation is that particular sulphated sequences exhibit similar conformational *and* charge characteristics to HS and heparin, while others do not, despite their sometimes higher charge density, and are able to better fit and bind to the complementary protein surface. This phenomenon is well documented for a range of different biological activities and arises partly as a consequence of the somewhat relaxed selectivity normally seen in HS-protein interactions. This is itself evident in the degree of redundancy shown among HS and heparin derivatives. It is also interesting that sulphated (non-glycosaminoglycan) polysaccharides, not only show comparable activities to HS and heparin in interactions with particular proteins, but can also induce comparable protein stabilisation (Uniewicz et al. 2010) and secondary structural changes in proteins (Rudd et al. 2010).

Here, this work has demonstrated the potential of sulphated polysaccharides to both inhibit and reverse cytoadherence in malaria and offers considerable potential for the future development of pharmaceutical agents based on these materials.

3.3 Screening of chemically modified heparin, modified GAGs and low molecular weight (LMW) heparin on inhibiting *P. falciparum* sequestration *in vitro*.

Hypothesis: The use of modified heparin, GAGs and LMW heparin to interrupt rosetting and ligand-selectin interactions is well studied, but not much work aimed at trying to disturb parasite sequestration, especially the interaction of PfEMP1 and ICAM-1. Therefore, we have tested these classes of compounds to see if they produce a similar effect to inhibit parasite sequestration on ICAM-1, which is thought to contribute to the development of cerebral malaria.

3.3.1 Introductions

Polysaccharides such as Glycosaminoglycans (GAGs) comprise diverse populations of negatively charged, linear polysaccharides, which are important components of the extracellular matrix (Rudd et al. 2010a). Several pathogenic microorganisms such as *Trypanosoma cruzi* (Ortega-Barria & Pereira 1992b), *Clamydia trachomatis* (Zhang & Stephens 1992), Herpes simplex virus (Spear et al. 1992), and cytomegalovirus (Neyts et al. 1992) bind to host cells through GAGs and these interactions can be inhibited by heparin.

Heparin is best known as an inhibitor of the blood coagulation system and is thus widely used as an anticoagulant but has a number of other biological activities (Jaques 1979). It is a heterogeneous mixture and can be fractioned according to size or affinity for anti-thrombin (Andersson et al. 1976). The interaction of heparin and heparan sulphate (HS) with adhesion proteins has implications in various physiological and pathological processes, including inflammation (Ley, Cerrito & Arfors 1991; Matzner et al. 1984; Sasaki, Herd & Page 1993), nerve tissue growth, tumour cell invasion, leucocyte trafficking, and plaque formation in the brain. Heparin has been shown to inhibit neutrophil adherence to resting (Bazzoni et al. 1993) and stimulated EC (Silvestro et al. 1994).

Heparin also has been shown to interact with L- and P-selectin but not to E-selectin (Norgard-Sumnicht, Varki & Varki 1993; Wang et al. 2002). The interaction of heparin with L-selectin is calcium-dependent and requires micro-molar levels of free calcium. L-selectin binds oligosaccharides that contain highly modified, heavily sulphated, iduronate-rich regions and endothelial tissue-derived HS that is enriched in free amino groups (Norgard-Sumnicht & Varki 1995).

In malaria, Heparin, HS, chondroitin sulphate A and hyaluronic acids have been well studied for their interactions to disturb *P. falciparum* asexual growth, invasion and binding of pRBC to non-infected RBC (rosettes) (Barragan et al. 1999a). Heparin also has been used for treatment of patients with *P. falciparum* malaria (Mitchell 1974; Munir et al. 1980; Smitskamp & Wolthuis 1971), with ambiguous results. In studies of Rhesus monkeys infected with *Plasmodium knowlesi*, treatment with heparin has, according to some authors (Dennis & Conrad 1968), efficiently cured the monkeys, while others have found it to be inefficient (Howard & Collins 1972; Reid & Sucharit 1972). *In vitro* heparin has been shown to inhibit invasion and development of *P. falciparum* (Butcher et al. 1992) (Sivaraman & Rai Chowdhuri 1983) and in one of these studies the 50% inhibitory dose of heparin and of heparin fractions with high or low affinity for antithrombin III were reported to be the same at 1 mg/ml (Butcher, Parish & Cowden 1988).

Heparin was initially discovered because of its profound effect on blood coagulation, and it was in that capacity that in 1935, it was used in clinical trials and subsequently in the clinics (Linhardt et al. 1986). Heparin binds to antithrombin III, causing conformational changes within the protein, which enhanced the neutralization of thrombin leading to anticoagulant effects (Whisstock et al. 2000).

Heparin, which is cheap and easy to use, is an interesting compound to be chosen as an adjunct or anti-adhesive treatment despite being an anticoagulant. It is related to heparan sulphate (HS), which is composed of the same building blocks (glucosamine and glucuronic or iduronic acid). Heparin that contains negatively charged sulphate and carboxyl groups has been previously used in the adjunct treatment of SM with some success (Munir et al. 1980) (Rampengan 1991), but was discontinued due to the occurrence of serious side effects such as intracranial

bleeding and thrombocytopenia (Jaroonvesama 1972) (Munir et al. 1980) (Rampengan 1991) (Smitskamp & Wolthuis 1971).

Recently, several attempts have been made to modify heparin chemically and other naturally-occurring polysaccharides, particularly with the aim of producing antithrombotic drugs with better biological properties. These modified compounds possess very low anticoagulant properties, and these types of modified heparin and polysaccharides have more predictable pharmacological actions, sustained activity, improved bioavailability and a better therapeutic index, and so have successfully displaced heparin as the major anticoagulant regime.

Modified heparins, which are sulphated glycoconjugates with distinct disaccharide compositions, have been used successfully in the prevention and treatment of venous thromboembolism (Pineo & Hull 1997) while, Rowe *et al* found a sulphated glycoconjugate that could disrupt rosettes (Rowe et al. 1994).

Sulphation patterns present on sulphated glycoconjugates define physiologically important specificity in their interaction with proteins. Subtle variations in patterns of sulphation of GAGs or polysaccharides can modulate relative affinities for extracellular proteins (Mulloy 2005).

A group of researchers found that a highly negative charged modified heparin, which has low coagulation properties and variable levels of sulphation could inhibit and disrupt rosettes, which consist of a cluster of RBC around pRBC, and have been associated with SM (Skidmore, Dumax-Vorzet et al. 2008)(Vogt, Pettersson et al. 2006) (Carlson 1993).

It has been shown that both heparin and HS bind directly to DBL1α of PfEMP1 (Chen et al. 1998; Vogt et al. 2003) and avid interaction requires at least a 12-mer (3.6-kDa) fragment of heparin as well as N-sulphation and 6-O and 2-O-sulphation (Barragan et al. 2000a). Baraggan's also concluded that the N-sulphate group is of particular importance in maintaining high rosette disruption potential, and their data showed that N-acetylated heparin derivatives only possessed minimal effect to disrupt rosetting (Barragan et al. 1999b).

Vogt *et al* has shown the ability to disturb parasite rosettes and sequestration on EC using a group of modified GAGs (depolymerised GAGs). Unfortunately, Vogt used a parasite which is a rosetting variant (FCR3S1.2) which was characterized specific and sensitive to heparin and HS. Meanwhile, the clinical isolates chosen in that study were from SM Uganda children that exhibit high rosette formation (Vogt et al. 2006). Therefore, parasites used in that studies were HS and Hep specific binders and therefore, Vogt et al. does show that endothelial glycocalyx mediates pRBC sequestration on EC.

Modified heparin also has been shown to potentially inhibit pRBC binding to CSA in placental malaria in Thailand (Chaisavaneeyakorn et al. 2004). It shows that modified heparin possesses more sulphate level compared to unfractionated heparin leading to inhibition of pRBC-CSA binding on placenta. Interestingly, PI-88 mimetic of HS is a hyper-sulphated oligosaccharide compound, which is currently undergoing phase II cancer trials and has shown activity towards inhibition of adhesion to CSA (Rosenthal et al. 2002). It possesses decreased anticoagulant properties compared to heparin alone.

Therefore, based on the Skidmore group's work, the ability of chemically modified heparin compounds (that have variable sulphate substitutions), LMW heparins (that have low anticoagulant properties) and modified GAGs to inhibit pRBC adhesion to ICAM-1 and CD36 on the endothelial cells was further investigated.

3.3.2 Materials and methods

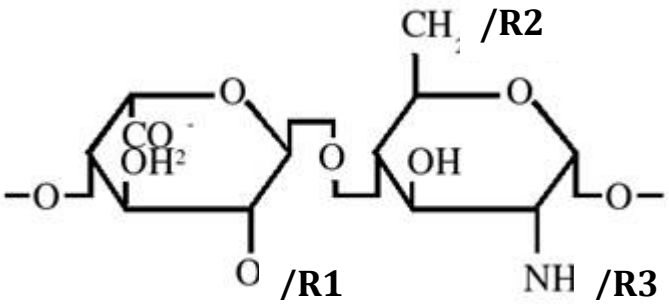
3.3.2.1 Parasite

Lab-adapted ItG strain, which is known to have strong avidity binding to ICAM-1 and less to CD36, was used for all assays. Plasmagel synchronization of mature asexual stages and magnetic bead selection (for ICAM-1^{Ref} binding) was performed prior to binding assays.

3.3.2.2 Compounds

All compounds were purified and provided by Mark Skidmore from School of Biology, Keele University, UK.

Table 3.3: Structure of chemically-modified heparin derivatives 1-9 showing the predominant disaccharides based on the repeating disaccharide -4 L-Iduronic α(1-4)-D-glucosamine are shown;



R1 = SO_3 or H R2= SO_3 or H R3 = SO_3, $COCH_3$ or H

MH	Compounds	R1	R2	R3
1	BLH heparin	SO3-	SO3-	SO3-
2	NAc heparin	SO3-	SO3-	COCH3
3	2-desulphated heparin	H	SO3-	SO3-
4	6-desulphated heparin	SO3-	H	SO3-
5	2-desulphated NAc heparin	H	SO3-	COCH3
6	6-sulphated NAc heparin	SO3-	H	COCH3
7	NS only heparin	H	H	SO3-
8	NAc only heparin	H	H	COCH3
9	Per sulphated heparin	SO3-	H	H

Adapted from (Skidmore et al. 2008).

Table 3.4: List of MK compounds

MK	Compounds
1	BLH heparin free amine
2	Dermatan sulphate free amine
3	PMH heparin free amine
4	Chondroitin sulphate A free amine
5	Chondroitin sulphate A
6	De N sulphated enoxaparin
7	Per sulphated N acetylated heparin (PMH)
8	Curdlan sulphate PSA (3a/3c)
9	Curdlan sulphate SPC (1a)
10	Curdlan sulphate CSA (2a)
11	N-butylated heparin (PMH)
12	N-propylated heparin (PMH)
13	BLH
14	Chondroitin sulphate 4S

Table 3.5: List of ML compounds

ML	Compounds	Weight (kDa)	Anti-Xa:Anti-IIa Ratio
1	Dalteparin	6.0	2.5[1]
2	Tinzaparin	6.5	1.6[1]
3	Reviparin	4.4	4.2[1]
4	Certoparin	5.4	2.4[1]
5	Ardeparin	6.0	1.9[2]
6	Enoxaparin	4.5	3.9[1]
7	Danaparoid	6.0	10.0[1]
8	N-acetylated Enoxaparin	ND	ND
9	Sulodexide	ND	ND
10	PMHS-heparan sulphate	ND	ND

Molecular weight data and anticoagulant properties. Adapted from (Gray, Mulloy & Barrowcliffe 2008)[1] and Alastair et al 1997[2]. The ratios were calculated by dividing anti factor Xa activity (anti-Xa) by the antithrombin (anti-IIa) activity. ND, not detected.

3.3.2.3 Endothelial cells

HUVEC was seeded and cultured as described in chapter 2. TNF at 1 ng/ml was used to activate expression of ICAM-1 on the cells.

3.3.2.4 Adhesion assays

The binding assays with modified heparin, LMW heparin and modified GAGs were performed under static and flow conditions essentially as previous described in chapter 2. The compounds were incubated with parasite suspension at 0.4 mg/ml for modified heparin and 1 mg/ml for LMW heparin and GAG compounds. Compounds were co-incubated with parasite suspensions (3 % parasitemia; 1 % HCT) for 5 minutes or less at 37^{o}C prior to the binding assays.

3.3.3 Results

3.3.3.1 Inhibition effect of modified heparin compounds

Modified heparins differing by sulphation pattern and size were tested for their ability to affect pRBC sequestration under static binding assays on TNF-activated HUVEC. Skidmore *et al* showed some of these compounds were able to disrupt rosettes (Skidmore et al. 2008). Figure 3.31 shows that most of the modified heparin compounds were able to inhibit sequestration of ItG to TNF-activated HUVEC to some extent, and the compounds were compared for their effectiveness with standard unfractionated heparin at 0.4 mg/ml. Results show that compound 8 and 9, which are N-acetyl (NAc) only heparin and Per sulphated heparin compounds respectively, did not effectively inhibit pRBC sequestration.

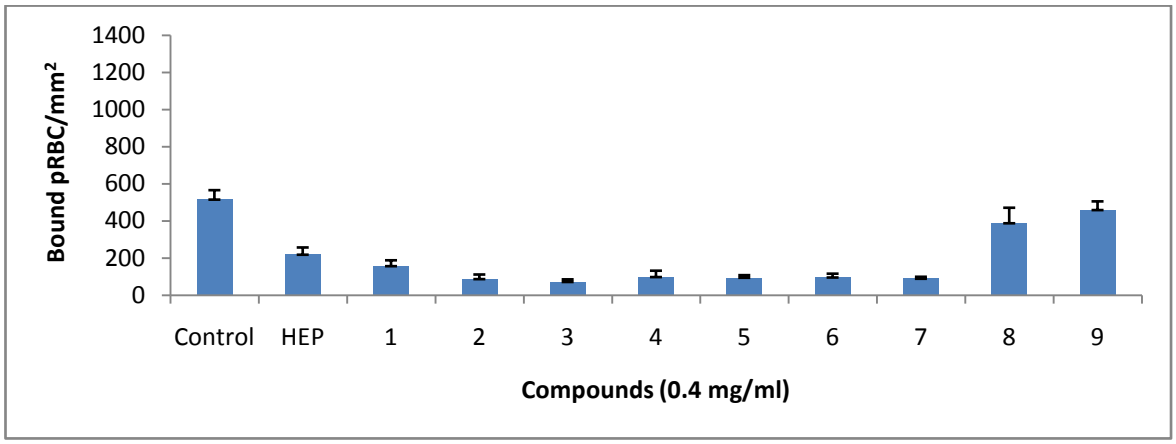


Figure 3.31: Effect of modified heparin at 0.4 mg/ml on ItG binding to TNF activated HUVEC under static adhesion assay. Results were expressed as bound pRBC/ mm^2. Experiment was done in duplicate or triplicate independent assays.

3.3.3.2 Inhibition effect of MK compounds on pRBC cytoadherence

Modified GAG compounds, which contain different level of sulphates, have been screened on TNF- activated HUVEC for their properties to inhibit pRBC binding to ICAM-1. As shown in figure 3.32, it was found that all MK compounds tested at 1 mg/ml were able, to some extent, to inhibit binding of ItG to HUVEC. For comparison purposes, a standard, highly sulphated heparin was screened for it adhesion inhibition capacity towards lab-adapted strains ItG, a parasite well known characteristic to bind to ICAM-1[Ref]. As previously described, the effect of standard heparin significantly inhibited adhesion of pRBC by more than 50%.

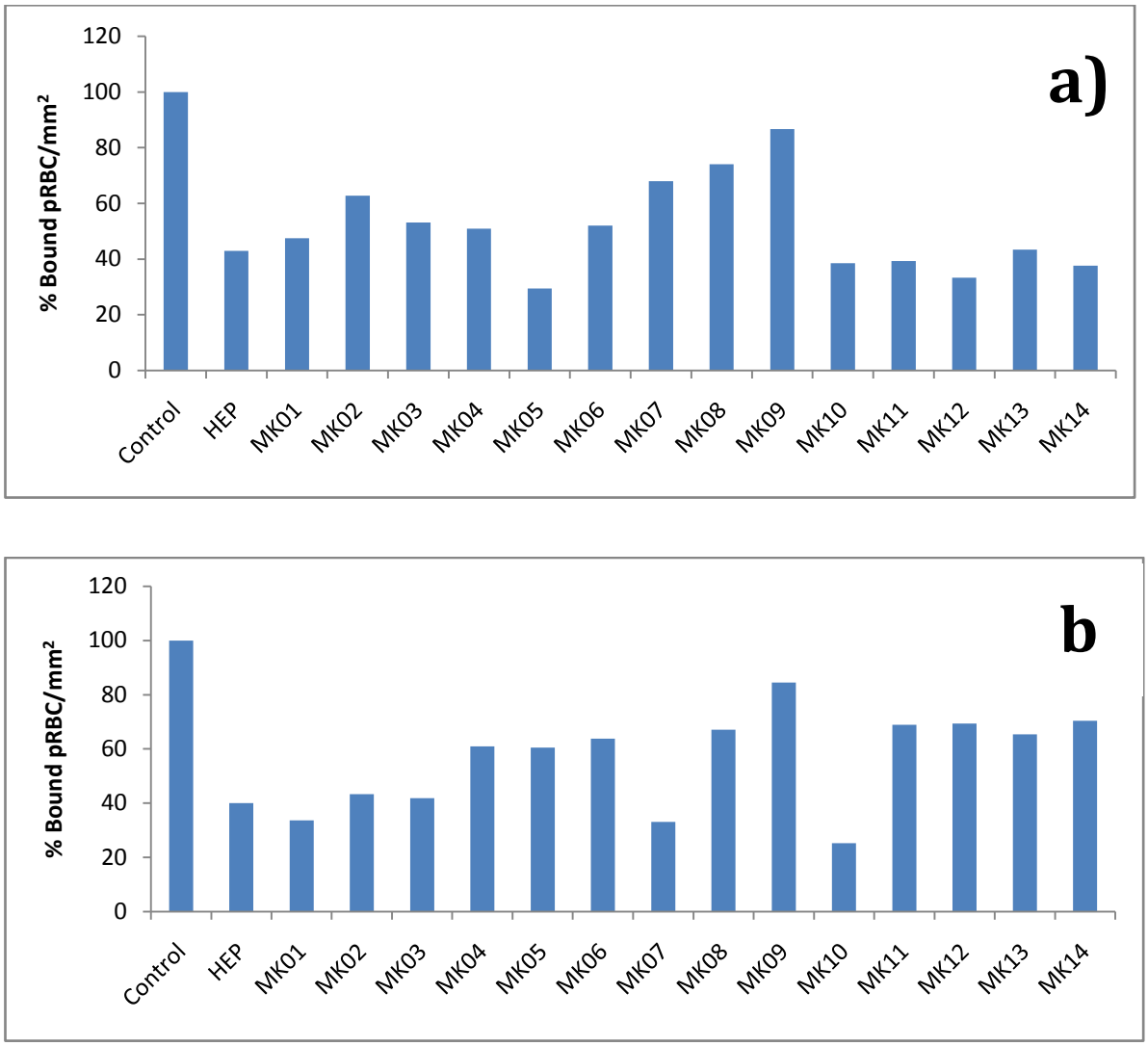


Figure 3.32: Effect of modified GAGs on inhibiting ItG binding to TNF-activated HUVEC, a) under static conditions and b) flow conditions. Results were expressed as percentage (%) bound pRBC/ mm². Control, without compounds.

3.3.3.3 Inhibition effect of ML compounds on pRBC cytoadherence

Low molecular weight (LMW) heparin compounds were screened for their potential to inhibit ItG binding to TNF activated HUVEC. It found that only some compounds showed inhibitory action (Figure 3.33).

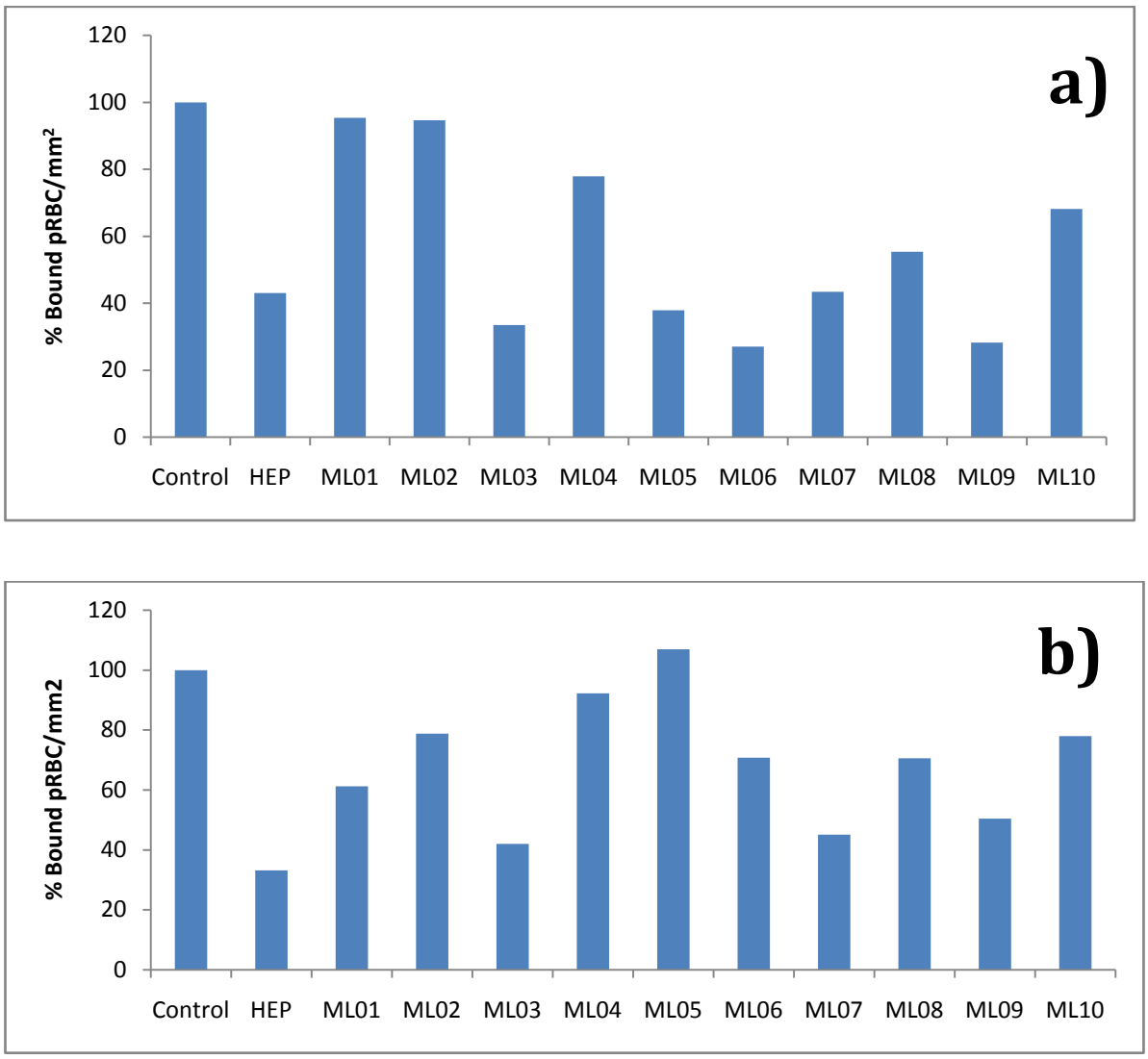


Figure 3.33: Effect of LMW heparin on inhibiting ItG binding to TNF-activated HUVEC, a) under static conditions and b) flow conditions. Results were expressed as percentage (%) bound pRBC/ mm². Control, without compounds.

3.3.4 Discussion

The use of polysaccharides to disturb rosettes has been well characterised. Here we report our observations on the ability of modified heparin, modified GAGs and LMW heparin for their ability to disturb pRBC sequestrations on EC.

In our modified heparin studies, compounds MH1 (BLH heparin), MH2 (NAc heparin), MH3 (2-desulphated heparin), MH4 (6-desulphated heparin) and MH5 (2-desulphated NAc heparin) have show potential to inhibit pRBC binding to EC at 60-80%. Our findings extend the resetting disruption data of Skidmore et al by showing that the NAc derivative heparin and 6-desulphated derivative heparin are able to inhibit pRBC sequestrations. Here, we showed consistent potential anti-adhesion properties of two compounds MH2 and MH4, which are able to inhibit rosette formation (Skidmore et al 2008) and pRBC sequestration. This suggests that these two compounds should be further investigated for their malaria adhesion (rosettes and sequestration) inhibitory properties. Unfortunately, this finding goes against the results from Barragan et al, in which they showed N-acetylated heparin had a low effect in disturbing rosettes. In contrast, my findings suggesting that either position at 6-0 or 2-O-sulphate being required for inhibitory action of heparin derivatives (Barragan et al. 1999b). This indicates that overall charge density and substitution pattern of the heparin are correlated with inhibition of pRBC binding. Our results provide evidence that the modified heparins screened here have the ability to inhibit adhesion of ItG to EC and might interact with ICAM-1.

The observed effects of modified GAGs on pRBC sequestration showed that all the modified GAGs tested were able to inhibit the binding at 1mg/ml. This supports the Vogt *et al* finding, where they found that modified GAGs were able to inhibit and desequester pRBC binding (Vogt et al. 2006). Most of the GAG compounds studied here are free amine compounds (MK01, 02, 03 and 04) and we observed that the amine group on GAGs is not necessary for inhibiting pRBC binding to HUVEC. I also found that compound MK07, which is a per sulphated N-acetylated heparin, MK11 (N-butylated heparin) and MK12 (N-propylated heparin) were able to inhibit binding of pRBC to HUVEC cells. This finding also supports the results of Barragan *et al*, which showed that 6-O and 2-O sulphation is necessary

for inhibitory action, and yet we also provided information that substitution on the N-site does not affect heparin action of inhibiting pRBC binding. Meanwhile, our results using curdlan sulphate and chondroitin sulphate also supported previous studies that showed that curdlan sulphate (Evans et al. 1998; Havlik et al. 2005; Kyriacou et al. 2007) and CSA (Adams et al. 2005) were able to inhibit pRBC cytoadherence to EC.

The LMW heparins screened were able to inhibit pRBC binding to EC but ML03 (Reviparin) and ML07 (Danaparoid) showed more potency by inhibiting at 50% of ItG binding on HUVEC expressing ICAM-1 protein at 1 mg/ml under static and flow assay conditions. Reviparin (4.4 kilodalton (kDa)) had previously been shown to be able to diminish tumour cell adhesion but the effect was dose-dependent (De Giorgi et al. 2005; Vitale et al. 2011). Danaparoid sodium (DA) is a low-molecular-weight heparinoid with a mean molecular weight of approximately 6.0 kDa. It consists mainly of heparan sulfate (HS) (83%) and dermatan sulfate (12%) which suggests strongly that HS and dermatan sulphate are able to inhibit pRBC binding (Barragan et al. 2000a).

It is known that heparin, GAGs and LMW heparin inhibit rosettes by interaction with the PfEMP-1 domain DBL1-α, which can be specific for HS binding. But how do these compounds inhibit pRBC sequestration? This question is particularly relevant because our lab-adapted ItG-ICAM-1 strain does not bind to HS and I will discuss this further in Chapter 4.

Therefore, here I would like to propose that these compounds act non-specifically and are not parasite variant specific, as described previously in chapter 3.2. From our observation, here I conclude that sulphate substitutions in core structure are required to exhibit their potent pRBC cytoadherence inhibitory properties. Due to higher charge it can interact with various proteins, such as ICAM-1 or VCAM-1, which are highly expressed on HUVEC cells (Wu et al. 2011).

However, ionic contacts are not sufficient to explain optimal structural interaction. Therefore, we also cannot exclude the multivalent effects of these compounds on their effect on inhibiting pRBC binding to EC. To establish a proper or stable binding, pRBC might interact with other binding receptors.

In the LMW heparin study, we found only two compounds showed very potent effects. As described by Rudd et al. size and conformational structure can determine action of heparin derivatives to interact with proteins (Ahn et al. 2010). Heparin polysaccharides and oligosaccharides are known to have complex conformational characteristics, in which sulphation and acetylation pattern influence the conformation of the derivatives (Ferro et al. 1995; Ragazzi et al. 1987) as well as altering the geometry of the glycosidic linkages (Yates et al. 2000). These factors result in complex overall conformational changes in which appended groups, such as hydroxyl group, N-acetyl, N-, O-sulphate and carboxylic acid groups, are presented in distinct spatial orientation. Our data support that conformational structure of compounds gave effect on inhibiting pRBC sequestration. Unfortunately, our data go against Rudd's finding regarding compound size, as Reviparin and Danaparoid have a very big gap of molecular size (4.4 and 6.0 kDa respectively). It is also found that Dalteparin, which has a molecular weight approximately 6.0 kDa similar to Danaparoid, does not show similar action, but we do note that anti-Xa/Anti-IIa activity ratio of Reviparin (4.2) and Danaparoid (10) are higher compared to others. The overall anti-Xa over anti-thrombin ratio of Danaparoid is over 10 as compared with ratio of 1 of heparin (Fujishima, Yokota & Sukamoto 1998).

All factors taken together, distinct molecular structures of heparin derivatives or heparin-like compounds and high anti-Xa: anti-thrombin ratio activities seem necessary to enable these polysaccharide compounds to interact with host receptors and modulate their coagulation cascades. This study provides intriguing possibilities to developing new therapeutic strategies for SM patients who suffer from sequestered parasite mass, without risk of thrombocytopenia (Davenport 1998).

CHAPTER 4: HOST ENVIRONMENTAL EFFECTS ON P*. falciparum* CYTOADHERENCE

4.0 Introduction

Cytoadherence involves adhesion between knobby pRBC and adhesion receptors (e.g ICAM-1 and CD36) on the ECs. *Var* gene encoded PfEMP1 protein is the only parasite factor so far known to be directly involved in the *P. falciparum* cytoadherence process; however host factors also play an important part in determining disease severity. There is still missing information that needs to be addressed in what mediates the adhesion.

In early stages of infection although some indication of inflammation has been noted in human experimental infection, elevated levels of adhesion molecules, such as ICAM-1, have not been observed. Thus the parasite faces a challenge to modify the host environment to support pRBC adhesion during the early stages of infection in erythrocytic cycle. The ability to modulate the host environment to produce efficient cytoadherence would be of great benefit to parasite survival and transmission.

Until now few studies have tried to associate cytoadherence with host environment factors based on clinical symptoms and potential prognostic markers seen in patients with severe malaria, such as temperature elevation, decreasing nitric oxide (NO), accumulation of lactic acid (acidosis) and elevation of proinflamatory cytokines; are all these factors necessary and contribute to the cytoadherence process?

We do know that proinflammatory cytokines such as TNF and IL-1 play a significant role in mediating cytoadherence by elevating expression of adhesion receptors such as ICAM-1 and VCAM-1 through activating endothelial cells (Clark & Schofield 2000) . The response of EC to several cytokines, especially TNF, has been considered as one of important determinants of pathology during infection with a number of pathogens (Kita et al. 2000; Lawson & Wolf 2009) (Qureshi et al. 2003) . However, *in vitro* and *in vivo* studies do not always find a consistent correlation between TNF level and disease (and TNF is implicated in mediating both

protection and pathogenicity during malaria infection (Clark & Cowden 1992; Kwiatkowski 1990; Richards 1997). However, a trial using mAb against TNF did not show a beneficial effect on either mortality or coma duration (Kwiatkowski et al. 1993). Thus TNF may play some role, such as ensuring sufficient receptor expression for efficient cytoadherence, but the progression to SM, such as cerebral malaria, requires other factors for its aetiology.

How about nitric oxide (NO)? It has been shown that low production of NO is associated with severe malaria (Clark & Cowden 2003; Clark & Schofield 2000). NO might be a key effector for TNF in pathogenesis of malaria. NO is involved in host defence by killing intracellular organisms, in maintenance of vascular status and in neurotransmission. Cytokines may upregulate inducible nitric oxide synthase (iNOS) in brain endothelial cells, increasing production of NO. The associations found between disease and NO activity and iNOS are not consistent. Results have varied with age, endemicity and geographical location. Post-mortem staining of brain specimens in African children and South East Asian adults have revealed increased iNOS in vessel walls associated with sequestered pRBC in CM (Clark & Cowden 2003), whereas in other studies NO is associated with protection (Anstey et al. 1996; Cramer et al. 2005). However in some individuals, production of NO happens too slowly to down-regulate the primary wave of cytokine induction, so that slow production of iNOS-induced NO allows iNOS and NO to reach the harmful concentrations seen in CM (Clark et al. 2004). SM is characterised by marked activation of microvascular endothelium (Wassmer et al. 2005). One prominent feature of endothelial activation is the exocytosis of intracellular Weibel-Palade bodies (WPB), causing release of vWF, P-selectin and Ang2 into the circulation, and has been found to support pRBC cytoadherence (Erdman et al. 2011). Therefore, NO might be needed to inhibit the exocytosis of WPB content through S-nitrosylation regulatory enzymes (Matsushita et al. 2003). Recently, L-arginine was found to enhance production of NO in SM and able to reverse adhesion of sequestered pRBC (Yeo et al. 2008).

The earliest symptom of malaria is usually elevation of body temperature due to release of inflammatory cytokines, especially TNF (Kwiatkowski et al. 1997). As fever is a universal symptom in malaria, knowing whether fever affects cytoadherence could have implications for patient management, as well as being

interesting in terms of host-pathogen interactions. There is one study that shows that febrile temperature could help the process of adhesion between pRBC to endothelial receptors (Udomsangpetch et al. 2002), but the findings are questionable based on the early stages parasite (ring) does not express PfEMP-1 on the membrane surface and so the increase seen in adhesion may merely reflect a larger mature population of parasites. Therefore, there is still a need to show whether febrile temperature regulates pRBC cytoadherence. Antipyretic treatment is standard adjunct therapy for malaria (Winstanley & Ward 2006), however, studies assessing the effects of antipyretic treatments have not shown evidence for an advantage in terms of parasite clearance or disease outcome (Meremikwu, Logan & Garner 2000). Oakley *et al* showed that febrile temperatures stop the growth of pRBC, which lead to mature pRBC sticking to EC (Oakley et al. 2007).

It is well known that synergism of binding receptors to strengthen the binding process of pRBC is needed to establish cytoadherence (Chen, Schlichtherle & Wahlgren 2000) . The endothelial cell surface consists of a huge amount of extracellular matrix (glycocalyx) such as heparan sulphate (HS), chondroitin sulphate (CS) and hyaluronan that has been ruled-in in terms of their contribution to binding properties in developing SM, especially in non-EC contexts such as mediating rosettes and pRBC sequestration in the placenta. Identification at the molecular level of binding involving PfEMP1 and other host receptors may facilitate the development of new therapeutic strategies to reverse cytoadherence and prevent SM especially CM. However, there it is still not clear what role glycocalyx plays in mediating pRBC binding, if any.

Several studies have pointed out that sequestration of pRBC results in microcirculatory obstruction, decreased oxygen delivery, tissue hypoxia and metabolic acidosis. This phenomenon has been reported to be higher in the brains of patients dying from CM than in other organs in the same patients and also higher than in patients without CM (MacPherson et al. 1985; Pongponratn et al. 2003). Recently Zougbede *et al* found that slightly acidic environment and metabolic acidosis triggered BBB permeability alterations in human brain endothelial cells (hcMEC/D3) *in vitro* by activating ICAM-1 expression through modification of tight junction and disorganization of *zonula occluden* protein 1, which lead to pRBC sequestration (Zougbede et al. 2011). Unfortunately, Zougbede

did not shows the optimum host environmental pH that mediates pRBC binding to hcMEC.

In this chapter I will try to investigate a number of host environment factors such as host pH, and endothelial matrix (glycocalyx) that may contribute to the process of pRBC adhesion to EC.

4.1 Effect of pH on *P. falciparum* cytoadherence *in vitro*

Hypothesis: Studies have shown that metabolic acidosis (pH < 7.3) or raised blood lactates are features of major prognostic important in severe malaria. Physiological pH changes also have been associated with sepsis. Elevated pH in circulated blood might interrupt normal function and structure formation of certain proteins. This might enhance protein-protein or cell-protein interaction through its ionic phase.

4.1.1 Introduction

It is clear that SM encompasses a complex syndrome affecting many organs resulting in biochemical and haematological derangements that have many features in common with the pathophysiological derangement seen in children with sepsis syndrome (Proulx et al. 1996a; Proulx et al. 1996b; Saez-Llorens et al. 1995) . Among these metabolic acidosis has emerged as a central feature of SM, and is widely recognized as the best predictor of a fatal outcome both in adults and children (Agbenyega et al. 2003; Allen et al. 1996; Day et al. 2000; Krishna et al. 1995; Taylor, Borgstein & Molyneux 1993).

Physiological conditions characterized by low pH in body tissue and blood are considered a distinct clinical diagnosis in the form of metabolic acidosis. It has been found that acidosis is one of several important clinical manifestations of patients with SM (Dondorp 2008) . Although dehydration is likely to contribute to the development of acidosis in some children, the role of cytokines in the pathophysiology of acidosis and/or a raised blood lactate remain questions (Krishna et al. 1995; Matsushita et al. 2003; Taylor, Borgstein & Molyneux 1993). The active replication and RBC invasion of *P. falciparum* uses a lot of nutrient sources (e.g. glucose), with resulting production of lactate as an end product of metabolic glycolysis. However, it also apparent that the presence of elevated plasma lactate need not be an essential nor a major component of metabolic acidosis in African children (English et al. 1997). A further contribution is likely from ketoacids which are detectable in the urine testing of more than 50 % of

children with SM (English et al. 1997). Numerous other factors might contribute to acidosis in SM, including fever; severe anemia (English 2000); hypovolaemia (English & Williams 2004); altered rheological properties of non-pRBC (Dondorp et al. 1997); recent seizure activity; the end product of parasite metabolism; decreased elimination through impaired hepatic blood flow and function (Day et al. 2000; Molyneux et al. 1989) and, more recently, a Reyes-like syndrome has been proposed (Clark et al. 2001). In the case of SM anaemia RBC destruction further compromises oxygen delivery to host cells. When the oxygen supply is not adequate, it leads to mitochondria that are unable to sufficiently supply ATP to cells. In this situation, cell glycolysis is increased to provide more ATP, and the excess metabolic product (D-lactate and L-lactate) is released into the blood stream where it accumulates over time. The excess ATP produce more H^+, and the detrimental rise of H^+ concentration causes external pH changes. As defined by WHO, metabolic acidosis is a situations in which the pH of the blood serum is less than normal serum specifically pH 7.4 (English et al. 1997; Maitland et al. 2003). Meanwhile, skeletal muscle and the kidney have been shown to be net lactate producers in SM (Davis et al. 1990).

The degree of metabolic acidosis in *P. falciparum* correlates positively with disease severity, and both admission venous lactate and standard base deficit have strong predictive values for mortality in both adults and children with SM (Krishna et al. 1995; Wassmer et al. 2005). It is also found that pH changes are able to regulate vascular endothelial growth factor binding to fibronectin, which is probably a good model for how pRBC bind to binding receptors on EC (Goerges & Nugent 2004).

A number of researchers have assessed adhesion of parasite lab lines or clinical isolates at variable pH conditions. Newbold *et al* 1997 and Rogerson *et al* 1996 have tried using pH 6.8 while others assessed adhesion at pH 7.2 (Newbold et al. 1997a; Rogerson et al. 1996). Craig *et al* examined the adhesion of ITO4-A4 (A4ultra) to ICAM-1 and CD36 under varying pH concentrations (6.8 to 7.5) and observed, no difference in adhesion of A4ultra to platelet-purified CD36 across all pH values, but found that at pH values above 7.3, adhesion to ICAM-1 decreased considerably (Craig et al. 1997). Meanwhile, Crandall *et al.* showed that cytoadherence of pRBC to the C32 melanoma EC was pH dependent and greatest

between pH 6.6 and 6.8. Unfortunately, the Crandall study used only using resting C32 melanoma cells which are known to express higher CD36 and not ICAM-1 (Crandall, Land & Sherman 1994). CSA-binding pRBC are able to cytoadhere to host cells almost equally well over a range of pH values from 6.4 to 7.4 In contrast, in their study the binding of pRBC to CD36 and ICAM-1 was most efficient in the range pH 6.4-6.8 and was weak at physiological pH values between 7.2 and 7.4 respectively (Pouvelle, Fusai & Gysin 1998). While many researchers have identified the pH needed for pRBC binding to the receptors such as ICAM-1 and CD36, no studies have yet concluded the optimum pH needed for pRBC binding especially to be used in the binding assays *in vitro*.

Therefore, in this chapter, we investigated the optimum pH mediating the binding of lab-adapted *P. falciparum* pRBC using static and flow adhesion assays on ICAM-1Ref and CD36 protein and tried to determine whether adhesion of pRBC is pH dependent or not.

4.1.2 Material and Methodology

4.1.2.1 Parasite

P. falciparum lab-adapted ItG (ICAM-1 selected), A4 (BC6 selected) and C24 (CD36 selected) were cultured using well established culture system with slight modifications (Chapter 2).

4.1.2.2 Preparation of binding buffer

Binding buffer was prepared as described earlier in chapter 2 but pH was adjusted to pH ranges 7.0 – 7.4.

4.1.2.3 Binding assays

Static and flow binding assays was done as described in chapter 2 general methods with slight modifications on binding buffer pH ranges 7.0 – 7.4.

4.1.2.4 Endothelial cells

HUVEC and HDMEC were cultures and maintained as described in chapter 2.

4.1.3 Results

4.1.3.1 Protein binding assay

To investigate the optimum pH needed for pRBC cytoadherence, we used three different lab-adapted *P. falciparum* lines which differ in their avidity of binding to ICAM-1 and CD36 proteins. The effects of varying the pH of the binding buffer for the selected parasite lines were observed. Maximum binding of ItG to ICAM-1^{Ref} was observed at pH 7.2, and we observed that A4 and C24 binding to ICAM-1^{Ref} are similar at all pH tested (Figure 4.1) and the number of cytoadherent pRBC slightly dropped off at pH 7.4. Meanwhile, we observed higher C24 binding at pH 7.0 and binding was decreased significantly when pH increased (Figure 4.2).

Optimum pH for pRBC binding to ICAM-1^{Ref} and CD36 was further investigated under flow conditions. Flow binding results confirm our static binding by showing maximum ItG binding to ICAM-1^{Ref} at pH 7.2 compared to other pH, while A4 showed similar binding at all pH, with only slightly higher at pH 7.2 (Figure 4.3). Meanwhile, we also observed the C24 highly binding on CD36 at pH 7.0 and 7.1 (figure 4.4).

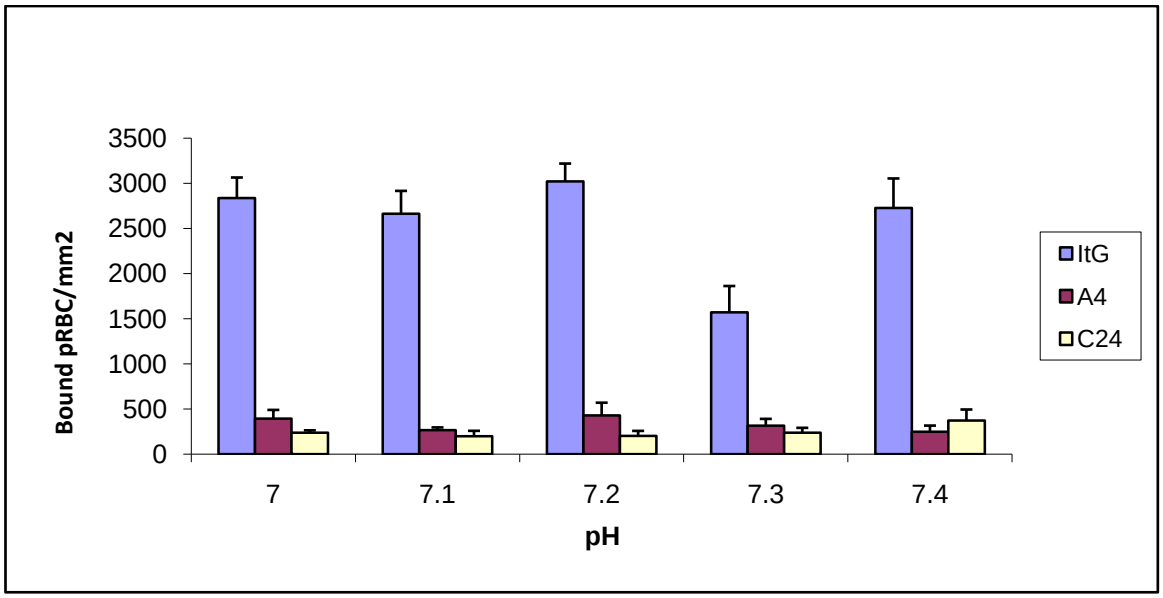


Figure 4. 1: The effect of pH on binding of pRBC to ICAM-1^{Ref} (50 µg/ml) protein under static conditions. Plotted bars are the means ± standard deviation for triplicate or duplicate independent binding assays for three representative lab isolates.

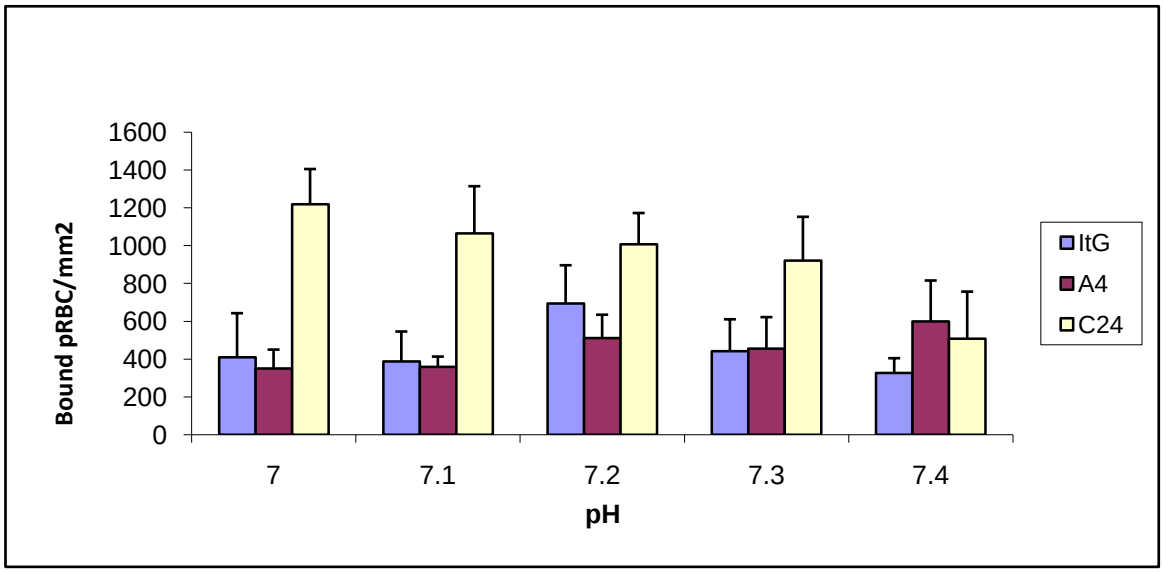


Figure 4. 2: The effect of pH on binding of pRBC (3% Parasitemia;1% HCT) to CD36 (25 µg/ml) protein under static conditions. Plotted bars are the means ± standard deviation for triplicate or duplicate independent binding assays for three representative lab isolates.

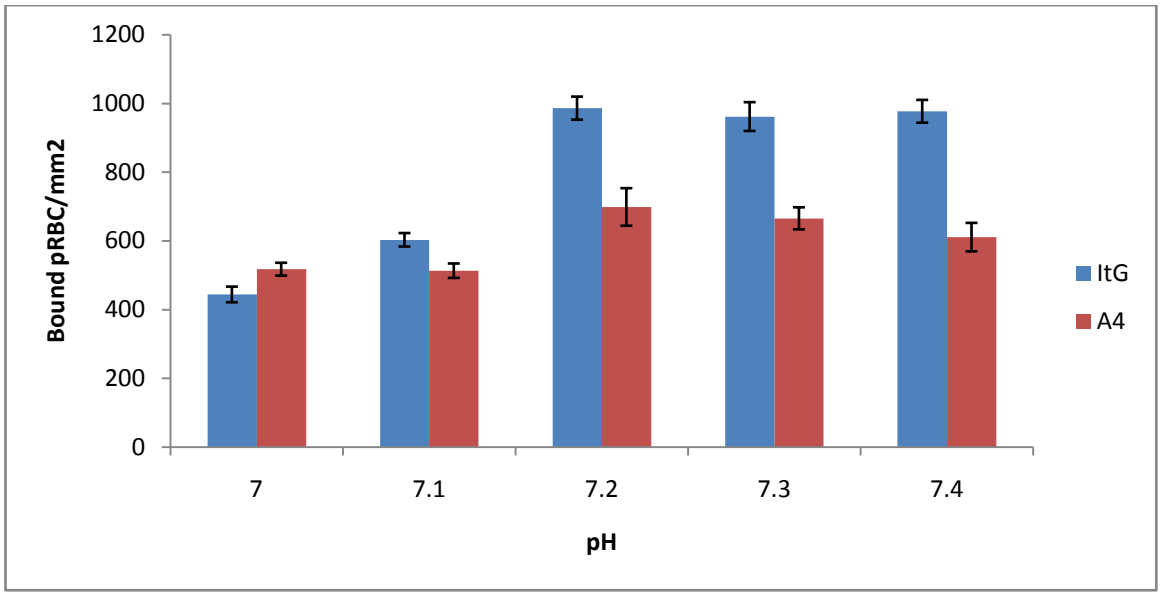


Figure 4. 3: The effect of pH on binding of pRBC to ICAM-1^{Ref} (50 µg/ml) protein under flow conditions (0.186 ml/min). Plotted bars are the means ± standard deviation for triplicate or duplicate independent binding assays for two representative lab isolates.

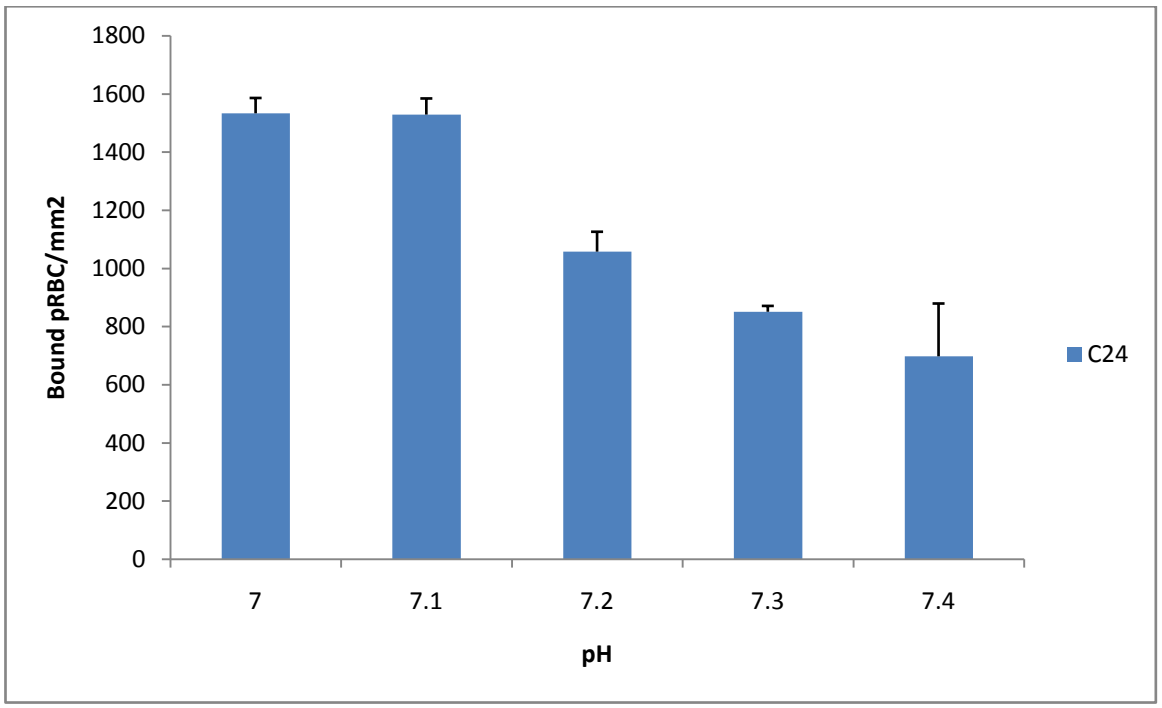


Figure 4. 4: The effect of pH on binding of pRBC to CD36 (25 µg/ml) protein under flow conditions (0.186 ml/min). Plotted bars are the means ± standard deviation for triplicate independent binding assays for the C24 lab isolate.

4.1.3.2 Cell binding assays

The assays were extended using TNF-activated endothelial cells HUVEC and resting HDMEC under static and flow binding assays. Our finding supports our preliminary protein binding assays. Both ItG and A4 show slightly high binding to activated HUVEC at pH 7.2 (Figure 4.5 and 4.7) and C24 highly binding to HDMEC was at pH 7.1 (Figure 4.6 and 4.8).

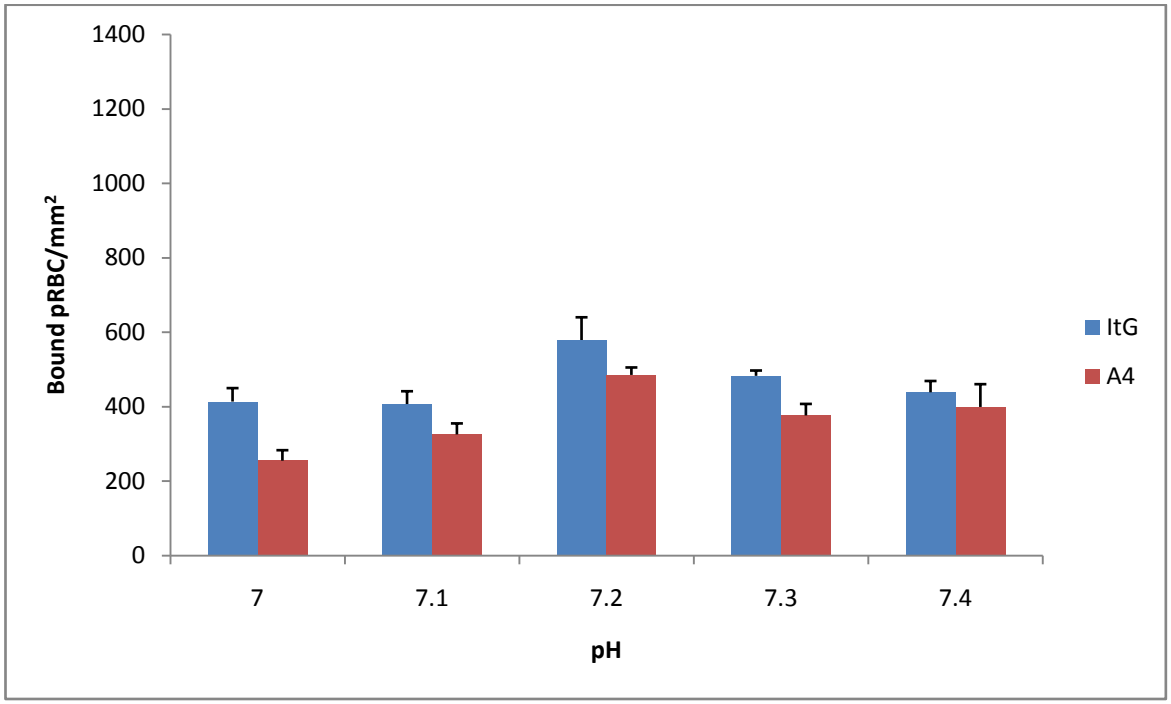


Figure 4. 5: The effect of pH on binding of pRBC to HUVEC endothelial cells under static conditions. Plotted bars are the means ± standard deviation for triplicate or duplicate independent binding assays for two representative lab isolates.

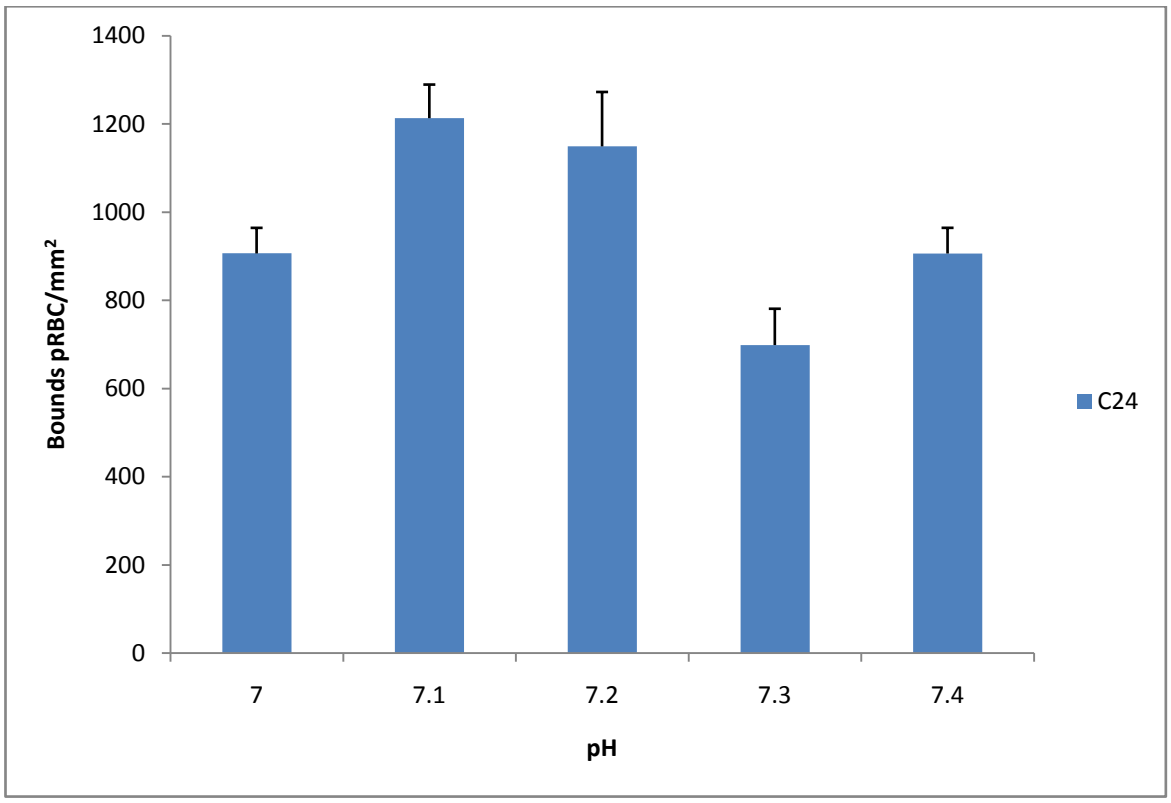


Figure 4. 6: The effect of pH on binding of pRBC to HDMEC endothelial cells under static conditions. Plotted bars are the means ± standard deviation for triplicate or duplicate independent binding assays for the C24 lab isolate.

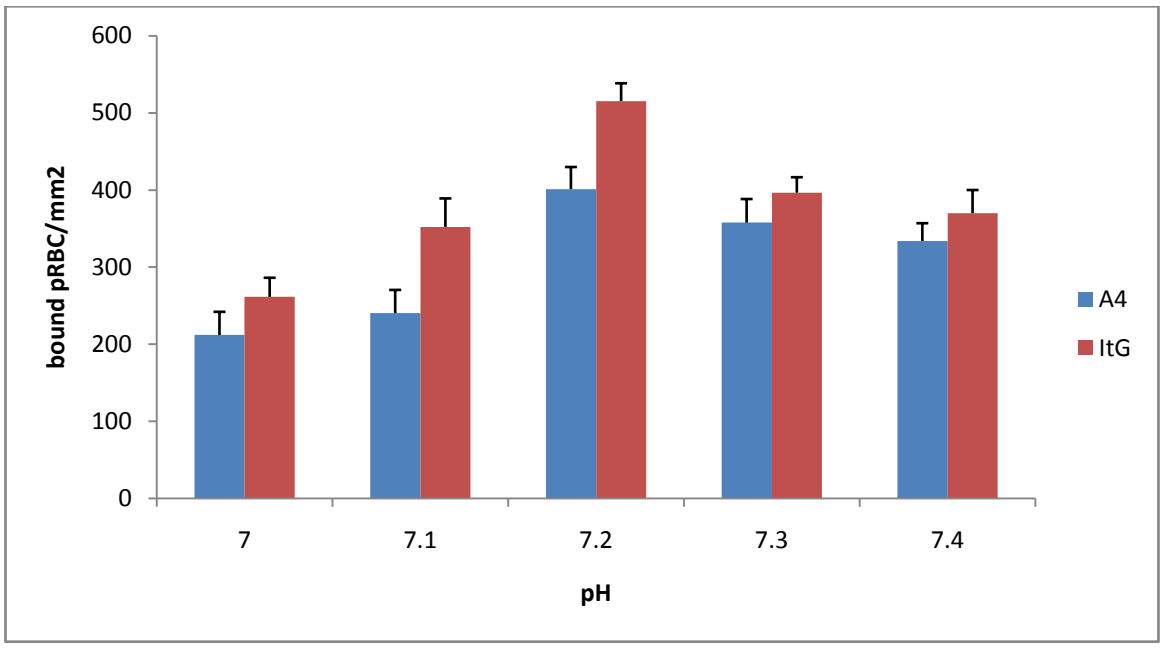


Figure 4. 7: The effect of pH on binding of pRBC to HUVEC endothelial cells under flow conditions (0.24 ml/min). Plotted bars are the means ± standard deviation for triplicate or duplicate independent binding assays for two representative lab isolates.

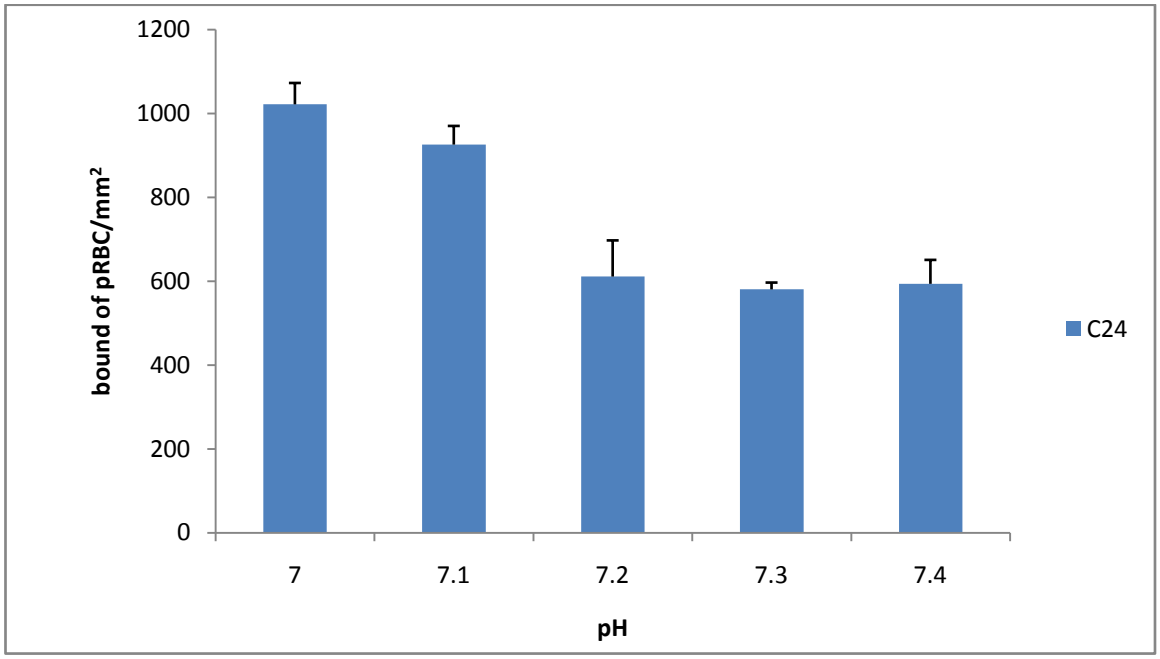


Figure 4. 8: The effect of pH on binding of pRBC to HDMEC cells under flow conditions (0.24 ml/min). Plotted bars are the means ± standard deviation for triplicate or duplicate assays for the C24 lab isolate.

4.1.4 Discussion

Metabolic acidosis is the best prognostic indiator for SM but there as yet no conclusive correlation of host pH environmental changes to pRBC binding. Moreover, there is still debate on what is the best optimum pH for pRBC cytoadherence. The dependence of cytoadherence on the pH of the surrounding environment was first observed by Marsh et al., (1988) by using resting C32 amelanotic melanoma cells towards testing binding of clinical parasite isolates from patients showing variety of malaria severity at pH ranges 6.6 to 7.8 (Marsh et al. 1988). The study was then confirmed by Sherman and Valdez (1989), who used resting C32 melanoma and HUVEC to investigate binding of parasite line FCR-3 (Sherman & Valdez 1989). Though these two observations do agree that pH may enhance parasite binding to endothelial receptors, unfortunately, they did not define what receptors the pRBC were binding to. However, we do know that parasite line used in that study is FCR-3 which is a rosette strain and binds to CD36 and HS, and C32 melanoma cells do express more CD36 along with other receptors such as TSP, but not ICAM-1.

Here in our study we extended these observations by looking at the optimal pH which mediates binding of lab-adapted *P. falciparum* with known binding properties towards ICAM-1 and CD36. Adhesion was assessed using standard static and flow protein and cell binding assays. We found that lab-adapted parasites associated with ICAM-1 (ItG) optimally bound to ICAM-1 protein and ICAM-1 activated endothelial cell (HUVEC) at pH 7.2 when compared with normal blood pH 7.4 and a parasite line associated with CD36 (C24) optimally bound to CD36 protein and endothelial cells at pH 7.0 compared with normal blood pH 7.4. Meanwhile, A4 bound to ICAM-1 reference and CD36 at similar levels through all the pH conditions tested. From our observation, here we conclude that binding of pRBC to specific receptors especially ICAM-1 and CD36 are pH-dependent, but critically the pH dependency was variant specific, which has implications for the design of experiments to measure the adhesion characteristics of patient isolates.

In a healthy human the pH of the blood plasma is rigidly maintained at pH 7.4, a value distinctly higher than the cytoadherence pH optimum≤(pH 7.3); however individuals infected with *P. falciparum* frequently have abnormally low

levels of blood pH (Fisher 1983) as well as high lactate levels (Molyneux et al. 1989; Taylor et al. 1988; White et al. 1983; White et al. 1985). How might pH mediate malaria cytoadherence? Here we hypothesise that it may mediate it through conformational protein structure change. Physiological pH changes are known to affect secondary and tertiary protein structure through changes in protein energetic properties (Berg, Tymoczko & Stryer 2002; Branden & Tooze 1999; Dill et al. 1995; Han & Tamm 2000) such examples of pH driven conformational changes of hemagglutinin trimer, pH dependent on formation changes surface protein of influenza virus (Han & Tamm 2000). It also has been thought that the interaction of pRBC to binding receptors is mediated through protein-protein ionic interactions, and hence could be variably dependent on pH based on the contact residues employed by the variant PfEMP1-receptor combinations.

Crandall *et al* suggested that cytoadherence pH dependency of pRBC to EC was also related to Calcium (Ca^{2+}) content of binding buffer used during the binding assays. Ca^{2+} affected cytoadherence at a much higher concentration (50 mM), which is well beyond any physiological levels (Crandall, Smith & Sherman 1991). The presence of high levels of Ca^{2+} also has been thought to enhance binding by reducing the surface charge on the target cells (e.g high level of Ca^{2+} reduce the surface charge of HUVEC (Vargas et al. 1989). Here, we argue against Crandall's findings because level of Ca2+ used in our binding buffer is fifty-fold lower (1 mM) than Crandall used. Moreover, 50 mM of Ca^{2+} is too high to be found in humans. Therefore, from our observation, we suggested that Ca2+ does not mediate pRBC binding to ICAM-1 and CD36 receptors in a physiological context.

In this present study, we evaluated the effect of various pH (7.0, 7.1, 7.2, 7.3 and 7.4) on *P. falciparum* pRBC interaction to ICAM-1 and CD36 proteins. We found that the optimal pH for pRBC binding to both receptors is in the range pH 7.0 to 7.2 and suggest that optimal pH of binding buffer is critical for successful pRBC binding assays. Adhesion of *P. falciparum* pRBC varies with pH and this variation is different between the three parasite lines used in this study, probably due to the variant PfEMP1 proteins expressed on the surface of infected erythrocytes. These findings might reflect the difficulties in collating adhesion information from

different studies and suggest how malaria pathogenesis might progress and how pH factors might influence pRBC sequestration.

4.2 Effect of endothelial glycocalyx on *P. falciparum* cytoadherence *in vitro.*

Hypothesis: Glycosaminoglycans (GAGs) such as CSA and HS have been confirmed as being associated with parasite cytoadherence phenotypes in SM, but no study has focussed on the role of glycan in the context of the endothelial glycocalyx and parasite sequestration. We do know that synergistic interactions of receptors on the EC with PfEMP1 may enhance and stabilize pRBC binding. Therefore, here we have tested the suggestion that the glycocalyx influences parasite sequestration on the endothelial cells.

4.2.1 Introduction

In recent years, a great deal of attention has been focused on the extracellular matrix (Glycocalyx). The interest was stimulated by active studies of mammalian stem-cell proliferation and differentiation, as well as by the development of new techniques of stem-cell cultivation required for their therapeutic and scientific use. Endothelium glycocalyx plays a number of important roles in the vascular system, including mediation of leukocyte adhesion and inflammatory responses, and the regulation of vascular permeability (van den Berg et al. 2006).

An additional level in the complexity of this biological structure of the glycocalyx arises from its dynamic nature. The interactions between glycocalyx and protein are highly dependent on the conditions of their local microenvironments such as pH (Coombe & Kett 2005; McGee & Liang 2001). *Helicobacter pylori* interact strongly with HS at low pH values around 4.5 (Utt & Wadstrom 1997). Furthermore, ECs actively regulate the content and physiochemical properties of GAGs on their surface by having high rates of continuous metabolic turnover that allows adaptation to changes in the local environment (Turnbull, Powell & Guimond 2001; Turnbull 2001).

The glycocalyx is composed of various sulphated proteoglycans (PG), GAGs, glycoproteins and associated plasma proteins (Pries, Secomb & Gaehtgens 2000). It serves as an interface between the blood flow and endothelial cells, thus providing a structure that senses the fluid shear stress exerted by extracellular flow, and transmits it to the intracellular structure. Located on the apical surface of a blood vessel ECs, the glycocalyx has been implicated in a wide range of mechanisms and pathologies.

Glycocalyx is inevitably the first site of encounter for any kind of cellular recognition and adhesion phenomenon, including microbial attachment. Several pathogenic micro-organisms and viruses such as *Bordetella pertussis* (Dickenson & Hill 1996; Jacob-Dubuisson et al. 1996), *Streptococcus mutans* (Choi & Stinson 1989)), *Leishmania donovani* (Butcher et al. 1992), *Trypnosoma cruzi* (Ortega-Barria & Pereira 1992a), *Plasmodium* circumsporozoites (Sinden 1985)), *Clymamydia trachomatis* (Stephens 1992), human immunodeficiency virus (Baba et al. 1988b; McClure & Dalgleish 1992; McClure et al. 1992), herpes virus (Trybala, Larski & Wisniewski 1991) and cytomegalovirus (Damonte et al. 1994) interact with host cells by binding to sulphated GAG-molecules on cell surfaces and glycocalyx.

In malaria cytoadherence, the GAGs commonly associated with the vasculature are heparan sulphate (HS), chondroitin sulphate (CS) and hyaluronic acid (HA). In previous studies, it has been suggested that HS on non infected-erythrocyte, CS (CSA, which contains 4-O-sulphate glucosamine unit) and HA (non-sulphate GAGs) serves as primary receptors for the adherence of *P. falciparum* to establish rosette and sequestration in placenta respectively. However, as yet there is no study trying to correlate HS with pRBC sequestration in brain. It has been found that the most prominent glycocalyx components on the surface of ECs are HS, accounting for 50-90% of the total GAGs pool, the rest being comprised of CS and HA (Gamse, Fromme & Kresse 1978; Ihrcke et al. 1993; Oohira, Wight & Bornstein 1983).

Owing to the structural analogy with heparin, HS and associated PG (the transmembrane syndecans, the membrane-bound glypicans, and the basement membrane-associated perlecans) have been most extensively studied, along with

the major protein core families of heparan sulphate proteoglycan (HSPG) found on ECs (Rosenberg et al. 1997b).

HS is one such GAG, composed of alternating glucosamine and uronic acid residues in a repeating disaccharide unit (-4GlcAβ1-4GlcNAcα1-). During biosynthesis, this backbone becomes variably modified by N-deacetylation/N-sulfation and C5 epimerization of the glucuronic acid to iduronic acid, followed by O-sulphation at C2 of the uronic acid and at C3 and C6 of the glucosamine unit. Owing to biosynthetic constraints, HS chains are modified to different degrees, and the modified units, especially the sulphate groups, are unevenly distributed along the chain.

Syndecans, which consist of syndecan-1 (33kDa), syndecan-2 (22kDa) and syndecan-3 (22kDa), have three GAG attachment sites that are primarily modified by the attachment of HS and that are close to N-termini of the proteins and distal to apical surface, but not exclusively (Halden et al. 2004; Rosenberg et al. 1997a). The cytoplasmic tails of syndecans associate with the cytoskeleton through various linker molecules that provide the structures required to distribute force throughout the cell. It is likely that syndecans are involved in pRBC adhesion through the CS chain (Kokenyesi & Bernfield 1994).

Of the glypicans, glypican-1 (64kDa) is the only one present on the surface of ECs. The glypican ectodomain is thought to form compact globular tertiary structure distal to the membrane, with three to four GAG attachment sites closer to the membrane that attach exclusively to HS, unlike syndecans (Fransson et al. 2004). The major chondroitin sulphate proteoglycan (CSPG) found on the surface of ECs is biglycan (40kDa), which assumes a compact structure and contains CS chains (Hardingham & Fosang 1992). Thrombomodulin, a 57 kDa transmembrane protein with anticoagulant activities, may also have a CS chain (Malyszko, Malyszko & Mysliwiec 2005). In contrast to HS and CS, HA, which is a much longer disaccharide polymer than HS or CS, is synthesized on the cell surface and is not covalently attached to a core protein. It is not sulphated but obtains its negative charge from carboxyl groups.

It also has been found that the glycocalyx is able to regulate eNOS production responses to shear stress (Goligorsky et al. 2002), the enzyme that catalyses the reaction of L-arginine and oxygen to yield L-citrulline and NO. As mentioned earlier NO is one of the predictors for SM (Dondorp et al. 1998).

Due to the facts that most of the glycans are highly sulphated and highly negatively charged through GAG chains (e.g. HS) attached to the core proteoglycan, many researchers have associated sulphated GAGs with chemokines, cytokines, selectin, integrin and adhesion molecules (Rops et al. 2004b). We showed earlier in chapter 3.2 and 3.3 that modified polysaccharide mimics of heparin and GAGs such as HS and CS were able to inhibit pRBC binding to EC, but did not prove an interaction of pRBC with endothelial HS (HS^{EC}). Here in this chapter, by using a sulphate inhibitor (sodium chlorate) we have investigated whether glycocalyx and glycosaminoglycan HS mediates the binding of lab-adapted *P. falciparum* strains to EC.

4.2.2 Material and methods

4.2.2.1 Parasite

Three lab-adapted *P. falciparum* lines ItG, A4 and C24 have been cultured and selected for their phenotypic binding properties to ICAM-1 and CD36 protein has been performed by magnetic bead procedure. Meanwhile, FCR3 and R29, which are rosetting line, have been cultured and maintained for 4 weeks.

4.2.2.2 Endothelial cells

HUVEC was cultured following manufacturer's recommended protocols and has been described in chapter 2.

4.2.2.3 Preparation of sodium chlorate (Na $(Cl_3)_2$)

Chlorate was prepared by dissolving sodium chlorate in binding buffer (pH 7.2) to 100 mM for stock solution and was filter sterilized.

4.2.2.4 Preparation of TUT buffer

TUT buffer was prepared by adding 240 g of Urea, 5ml of 10% triton X-100, 0.60g of Trizma Base, 0. 07g of Na_2SO_4 to 400 ml of distilled water under heating (45^oC) and pH adjusted to 8. The buffer will go into solution faster by heating to 45^oC while stirring. The buffer was then topped-up to 500 ml with distilled water and kept at room temperature prior to use.

4.2.2.5 Extraction of glycan from HUVEC cells and preparation of the matrix

Extraction of glycocalyx on HUVEC was followed as described by Scott E Guidmond (Centre of Glycobiology, School of Biological Sciences, University of Liverpool (Guimond et al. 2009). Confluent HUVEC cells (70-80%) were trypsinized and washed with cold PBS twice to remove remaining trypsin and to keep the proteins intact. Then TUT buffer was added, which functions to dissolve trypsinized cells and the samples were boiled for 5 minute prior to sonication. DEAE beads were prepared by washing 100 µl of beads with cold PBS twice. The DEAE-sephagel beads were added to the solution and kept in the cold room on a rotator. Next day, the solutions were centrifuged at 5000rpm for 3 minutes. The supernatant was removed, and pellets were washed twice with 10x volume of cold PBS. After that,

10x vol of 0.25M NaCl was added and centrifuged, then the supernatant was removed and additional 10x vol of 2M of NaCl, was added prior to running the sample on a PD10 column.

4.2.2.6 Preparation of PD10 column

The PD10 column was washed through using water HPLC grade under gravity, avoiding using vacuum because it is harsh on the beads.

4.2.2.7 Running samples the on PD10 column

Samples were centrifuged, and the supernatants were run through the column. This process allows the extracted glycan to bind to resin. Once the supernatant (1 ml per run) had run through, the column was eluted by adding 3.5 ml of water HPLC grade. The first elute from water added was collected. The column was then washed once with 30 ml of water before another supernatant added and continue the process as described above. The eluted sample was then freeze dried overnight or kept in the fridge prior to use.

4.2.2.8 HS purification

In the eluted sample there is mixed glycan, so in order to purify HS, 20x Chondroitinase ABC (C_{ABC}) buffer was added, and C_{ABC} lyse enzyme was added (final concentration of buffer should 1x) and the mixture was incubated in the 37^{o}C incubator for overnight or a minimum of 4 hours. The next day, 5x Neuramidase buffer was added prior to the addition of 2.5 mU Neuramidase and further incubated in the 37^{o}C incubator for another 4 hours or overnight. Then, 5x Pronase buffer was added prior to 2.5 mU Pronase and further incubation at 37^{o}C for another 4 hours or overnight. DEAE beads were then added to the digested sample and incubated with rotation for 3 to 4 hours at room temperature. Then, the sample was washed twice using PBS prior to adding 10x vol of 0.25M NaCl. The solution was then further centrifuged and the supernatant was removed. The pellet was then dissolved in 10x vol of 2M NaCl and was centrifuged; the supernatant was kept for purification on the PD10 column. The eluted sample then was freeze dried. The freeze dried sample was dissolved in water and then 40 µl of sample was added to 5x Heparatinase buffer prior to adding 10 mU heparatinase III (hep III) and further incubated for 2 hours in the 37^{o}C incubator. Then 10 mU

heparatinase I (hep I) and Heparatinase II (hep II) were added sequentially with incubation in the 37^{o}C incubator for 2 hours each. The content of the digested sample was measured using a spectrophotometer at UV 232nm.

4.2.2.9 Enzymatic assays

Chondroitinase ABC (C_{ABC}) and Heparatinase I, II, and III to cleave chondroitin sulphate and heparan sulphate respectively were given by Scott E. Guidmond (Biology School, University of Liverpool). For this assay 2.5 mU of C_{ABC} and 10 mU of heparitinase I, II and III were incubated with endothelial cells overnight (16-20 hours) at 37^{o}C prior to the binding assay. The cells were then washed with warmed binding buffer (pH 7.2) once before the parasite suspension (3% parasitemia and 1% haematocrit) was added.

4.2.2.10 Binding assay

Static and flow binding assays were performed as described earlier in chapter 2.

4.2.2.11 HS quantification (FACS)

HUVEC cells were grown in six-well plates until confluent at 37^{o}C in a CO_2 incubator. 70-80% confluent cells were then trypsinized. Monoclonal antibody (F58-10E4) and Alexa-Fluor 488, provided by Scott E. Guidmond (Biology School, University of Liverpool) was then added to the trypsinized cells as a primary antibody and incubated at 37^{o}C for 45 minutes. The cells were further washed 3 times using 1% BSA/PBS and further incubated with anti-mouse–Alexa Fluor 488 secondary antibody for another 45 minute in the dark at room temperature. After that cells were washed 3 times in 1%BSA/PBS and resuspended in 250 µl of 4% paraformaldehyde in PBS (pH 7.2). The fixed cells were stored at 4^{o}C and washed 3 times with PBS prior to FACs analysis using BD™ LSR II. The histograms were then analyzed using BD™FACSDiva software.

4.2.3 Results

4.2.3.1 Effect of sodium chlorate on HUVEC growth

To investigate the effect of sodium chlorate (CHL) on *P. falciparum* binding on endothelial cells, a series of experiments has been done. First, CHL was incubated with growing HUVEC cells at 40, 20, 10, 5 and 2.5 mM overnight at 37°C. The next day, the cells were checked for their viability and growth. HUVEC cell cultures incubated for the total of 16 hours in serum-containing medium with 10, 5 and 2.5 mM chlorate appeared unaffected in terms of the number of cells attached. Meanwhile, 40 and 20 mM CHL did show some cell detachment and rounded cells after overnight incubation (data not shown). From our observations, therefore, 10 mM CHL has been chosen due to its compatibility with cell growth for further investigation on its properties of inhibiting pRBC binding to EC.

4.2.3.2 Effect of 10 mM chlorate on *P. falciparum* binding to HUVEC

The effect of 10 mM CHL was further investigated on inhibiting the binding of two lab-adapted *P. falciparum* (ItG and A4) to TNF-activated HUVEC cells under static and flow conditions. CHL and TNF at 10 mM and 1 ng/ml were co-incubated overnight with 70-80% confluent HUVEC cells prior to binding assays. The cells were then washed a few times with warmed binding buffer (pH 7.2) before parasite suspension (3% parasitemia and 1% haematocrit) was added. Our static (Figure 4.9) and flow binding (figure 4.10) data show that CHL was able to inhibit ItG and A4 binding to TNF-activated HUVEC at 60% binding reduction compared to control (HUVEC/TNF).

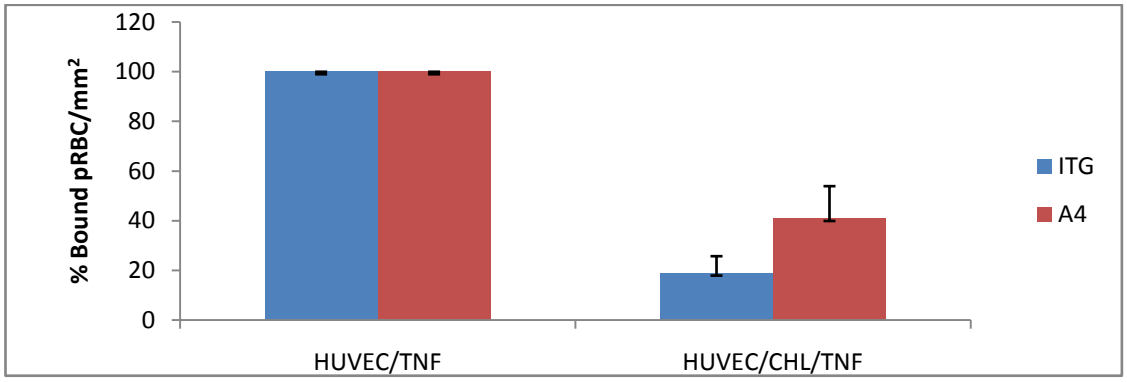


Figure 4. 9: Effect of 10 mM of sodium chlorate (CHL) on the binding of ItG and A4 to TNF-activated HUVEC cells under static conditions.

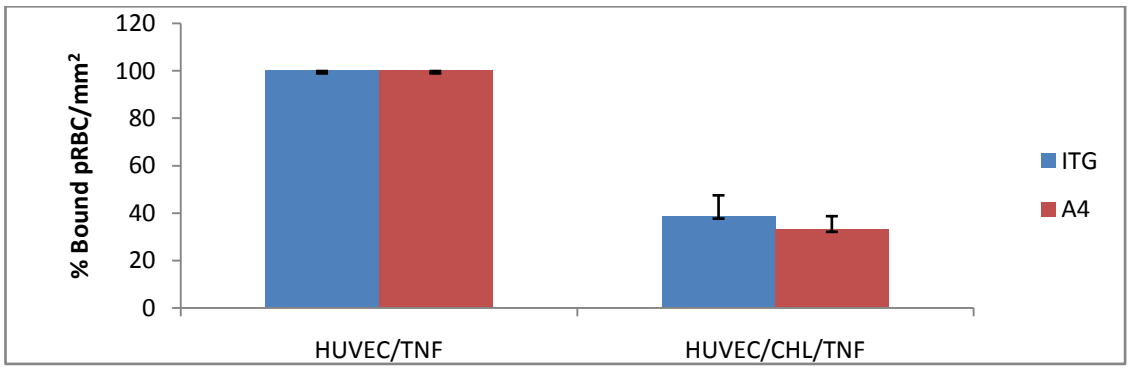


Figure 4. 10: Effect of 10 mM of sodium chlorate (CHL) on the binding of ItG and A4 to TNF-activated HUVEC cells under flow conditions.

4.2.3.3 Effect of sodium chlorate on ICAM-1 expression

Based on these binding results and the knowledge that adhesion of ItG lines to HUVEC is largely ICAM-1-dependent, we would like to see if CHL treatment disturbed the expression of ICAM-1 or not. FACS result shows that even at 40 mM CHL, it does not significantly inhibit or disturb ICAM-1 expression on HUVEC (Figure 4.11). This finding was further supported by our ICAM-1 FACS results (figure 4.12)

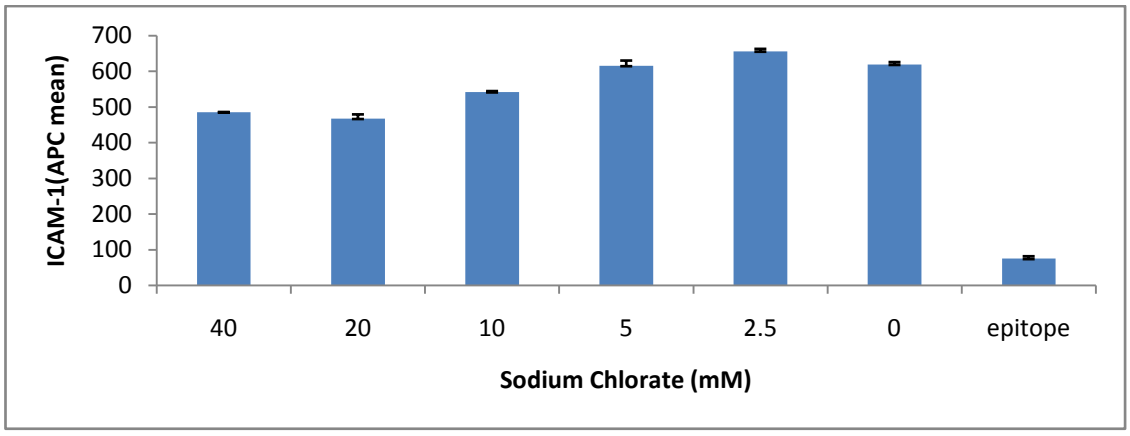


Figure 4.11: FACS results on ICAM-1 expression after sodium chlorate (CHL) treatment on TNF-activated HUVEC for 16 – 20 hours. Results are given as the mean ± standard deviation. Epitope, APC alone without anti-ICAM-1 antibody (anti-CD54); APC, allophycocyanin (secondary antibody conjugates).

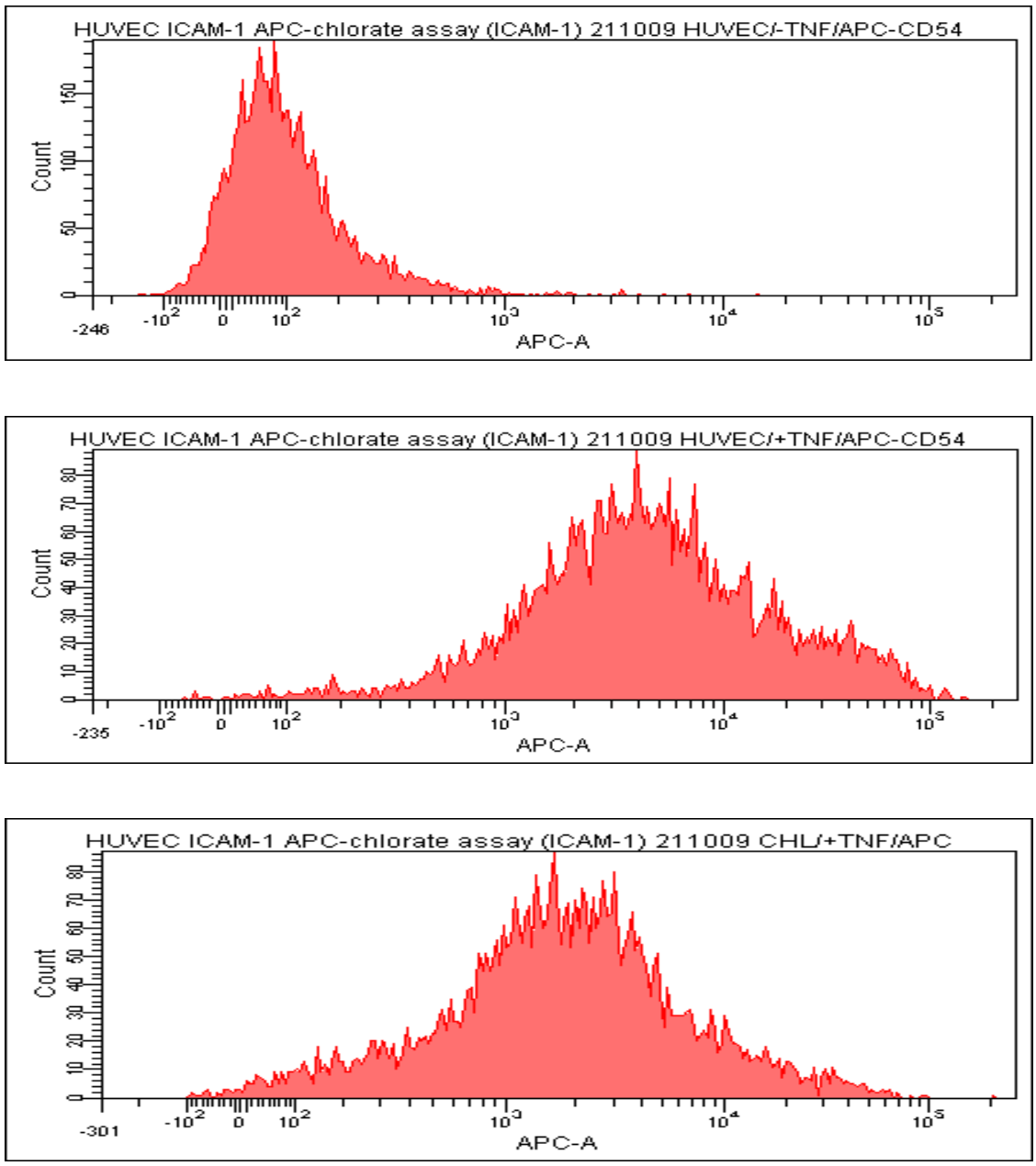


Figure 4.12: FACS result on ICAM-1 (CD54) levels on TNF-activated and non-activated HUVEC cells; a) Representative flow cytometry histogram of emitted APC fluorescence intensity on non TNF-activated HUVEC cells without ICAM-1 antibody (anti-CD54); b) Representative flow cytometry histogram of emitted fluorescence APC (ICAM-1 distribution) on TNF-activated HUVEC (non-treated CHL) with ICAM-1 antibody (anti-CD54); c) Representative flow cytometry histogram of emitted fluorescence APC (ICAM-1 distribution) on 10 mM CHL treated TNF-activated HUVEC cells with ICAM-1 antibody (anti-CD54).

4.2.3.4 Enzymatic assay

Based on our FACS result, it was shown that 10 mM CHL treatment does not interrupt ICAM-1 expression. This means that the 60% reduction of pRBC binding on TNF-activated HUVEC could be associated with other receptors/ interactions, such as the glycocalyx, due to the 10 mM CHL treatment overnight. Therefore, to prove our hypothesis, that glycocalyx might play a role in mediating pRBC binding to EC, we used enzymatic treatments to identify which glycan was associated with the reducing binding of ItG on HUVEC. For this assay, 2.5 mU of Chondotinase ABC and 10 mU of heparatinase (hep) I, II and III were incubated individually with TNF-activated HUVEC cells. From the assays, it is found that Chondoitinase ABC and hep I, II and III do not show any significant reduction of ItG binding (Figures 4.13 and 4.14) compared to control (without enzyme).

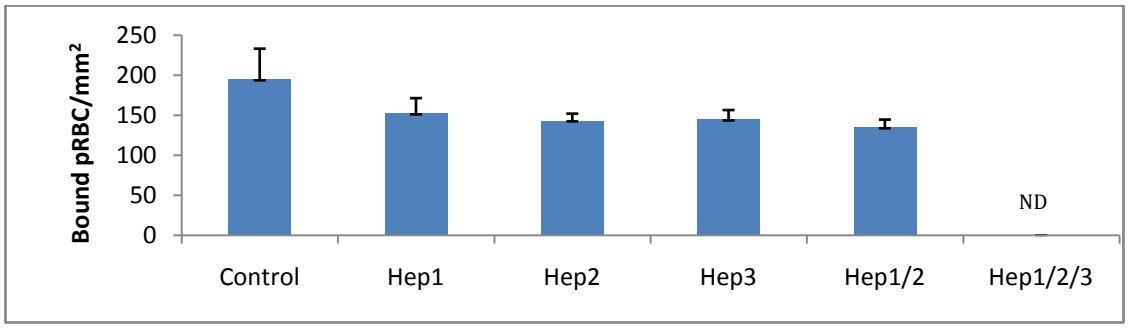


Figure 4. 13: Effect of heparatinase I, II and III on ItG binding to TNF-activated HUVEC under static conditions. ND; not done.

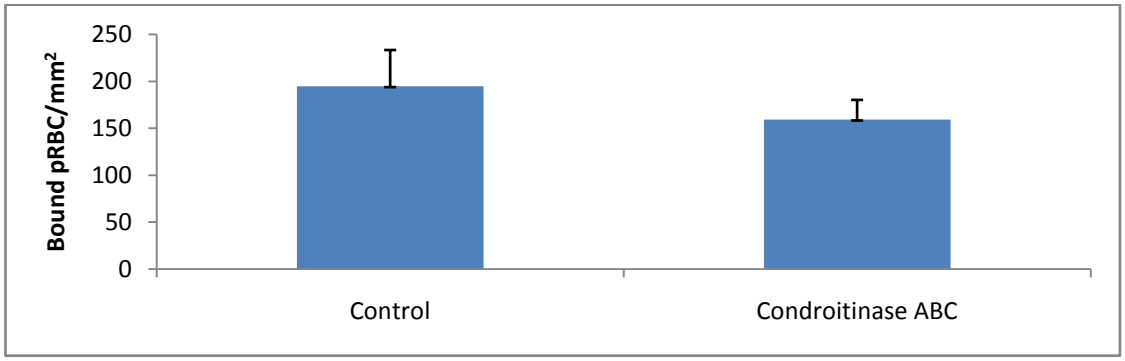


Figure 4.14: Effect of chondroitinase ABC on ItG binding to TNF-activated HUVEC under static conditions.

4.2.3.4 HS binding static protein assays

We also investigated whether our lab-adapted strain did bind to HS (Figure 4.15). Therefore, HS from HUVEC cells was extracted using TUT solution and purified using a PD10 column. From our observations, one T175 confluent HUVEC cell flask able to produced 104 µg/ml of purified HS in PBS. The purified HS^{HUVEC} (50 µg/ml) was then spotted together with ICAM-1 (50 µg/ml), CD36 protein (25 µg/ml) and PBS for control on dishes for static binding assays. The binding results show that none of the parasite lines tested bind to HS^{HUVEC}, this observation was controlled by ItG, A4 and C24, which are known their binding to ICAM-1 and CD36. Unfortunately, we do not have a positive HS-binding control and our attempts to select a HS-binding line were repeatedly unsuccessful.

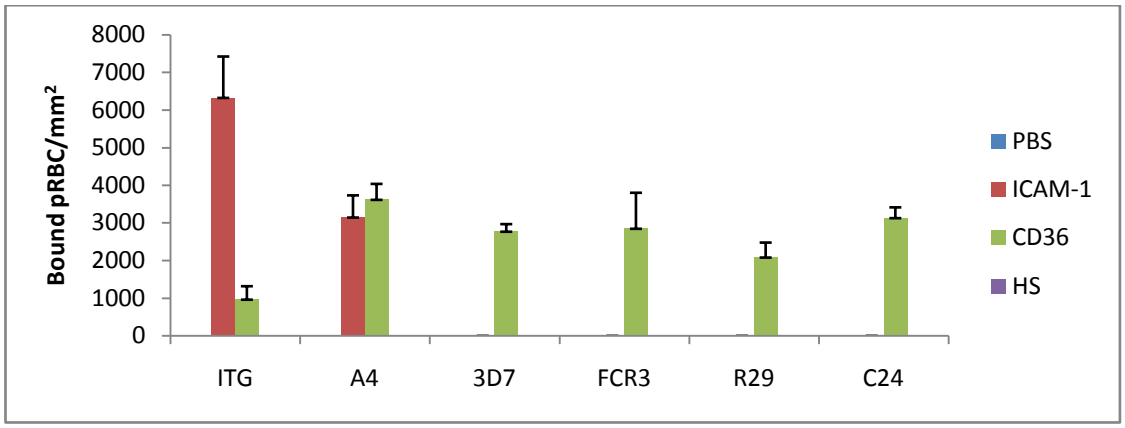


Figure 4. 15: Static binding assay of lab-adapted *P. falciparum* lines to HS^{HUVEC} (50 µg/ml) and other receptors (ICAM-1; 50 µg/ml, CD36; 25 µg/ml). The data are represented as mean ± standard deviation based on three independent experiment.

4.2.4 Discussion

Heparan sulphate proteoglycan (HSPG) are protein which are glycosylated with heavy HS glycosaminoglycan chains and are present on the surface of most vertebrate cells and in many type of extracellular matrix (glycocalyx) (Turnbull et al. 2010). Interest in the role of HS in a variety of cell-cell and cell-matrix reactions has stemmed from specific interactions of HS chains with a variety of proteins and the proven structural heterogeneity of the GAG chain, which provide modulation of cellular mechanisms (Gallagher, Lyon & Steward 1986). The sulphate residues, which may be present on at least 3 different positions (6-O, 2-O and N-position) of the HS polysaccharide backbone (Razi et al. 1995), are of particularly interest since they have been shown to be major factors in the determination of specificity in protein-GAG interactions (Lindahl et al. 1984).

In this study the glycocalyx has been investigated based on its sulphated residues (e.g HS or CS) and their role in mediating pRBC binding to EC. From our previous experiments (chapter 3), we have seen that the sulphate position on modified polysaccharides and heparin is necessary to determine their inhibitory binding properties, and other researchers have found the requirement for specific sulphate position to exhibit binding properties (McCormick, Newbold & Berendt 2000). Therefore, by using the sulphate inhibitor CHL, we have seen a reduction in pRBC binding and thereby rule-in that the glycocalyx might be necessary for malaria cytoadherence, although its direct role is still unknown.

Therefore, in order to investigate whether glycocalyx might help pRBC bind to EC, we used sodium chlorate (CHL) which is functioning to be inhibiting sulphate on the glycan. CHL is known to be an *in vitro* inhibitor of ATP-sulphurylase, the first enzyme in the biosynthesis of 3'-phosphoadenyl 5'-phosphosulphate (PAPS), the high-energy sulphate donor in biological reactions (Lansdon, Fisher & Segel 2004). In previous work, Baeuerle and Huttner (1986) mentioned that treatment of various cell cultures with CHL resulted in inhibition of protein sulphation (Baeuerle & Huttner 1986). Known also as a detergent, CHL was first tested for its toxicity effect on our cultured cells (HUVEC). Therefore, confluent (70-80%) HUVEC cells were incubated for the total of 16-20 hours in the serum-containing medium with various concentrations (40, 30, 20, 10, 5 and 2.5

mM) overnight at 37°C. From our observations, the number of attached cells CHL appeared unaffected in 10 mM. However, at 40, 30 and 20 mM CHL there appeared to be some loss of cells and detachment of the cells, probably due to the excess salt concentration (Humphries & Silbert 1988). This finding was supported by Baeuerle (1986) and Humpries (1988) by showing 10 mM of CHL did not affect cell growth (Baeuerle & Huttner 1986; Humphries & Silbert 1988). In addition, a few researchers reported that cells grown in the presence of CHL produced HS GAG chains containing only about 8% of sulphate normally present and which had lost the ability bind to human plasma fibronectin (Jaikaria et al. 1991; Keller, Brauer & Keller 1989). Furthermore, the iduronic acid content of HS produced in the presence of CHL was reduced to less than 7% as compared to the 36% from untreated cells. Therefore, it was concluded that the use of CHL could be valuable for the study of structure-function relationship of sulphated GAGs with pRBC.

Choosing 10 mM chlorate as a working concentration, we further tested CHL on it properties to inhibit sulphation of GAGs (HS and CS) on HUVEC cells and investigate pRBC binding properties by using ItG and A4. We know that ItG is a stronger ICAM-1 binder, while A4 is a strong CD36 binder but binds slightly less to ICAM-1. Therefore, using these properties, static and flow binding assays were conducted. TNF-activated HUVEC cells were co-incubated with 10 mM of chlorate and 1 ng/ml of TNF overnight at 37°C. This showed a dramatic reduction by chlorate-treated HUVEC for the binding of ItG and A4 at almost 60% when compared to control (no chlorate, TNF-activated HUVEC). This primary finding suggested that glycocalyx is required together with ICAM-1 to establish cytoadherence. Here, we observed for the first time, that glycocalyx might mediate pRBC binding on HUVEC and the sulphate position on glycocalyx is needed for pRBC binding. Our observation might be supported by Rops et al (2007). They showed that glycocalyx especially 6-O sulphated HS is crucials influencing rolling and firm adhesion of leukocyte to TNF-stimulated mouse glomerular EC (Rops et al. 2007; Rops et al. 2004a).

To rule out the possibility that chlorate suppresses ICAM-1 expression, flow cytometry (FACS) analysis has been done on chlorate treated HUVEC. It was found that ICAM-1 expression is not inhibited by CHL treatment. This finding is consistent with other data that chlorate did not inhibit or suppress protein

synthesis on cultured M31 and 81-O4 hybridoma cells (Baeuerle & Huttner 1986). We tried to investigate the sulphated HS population after chlorate treatment on HUVEC by FACS by using anti-heparan sulphate (F58-10E4) antibody provided by Scott E. Guidmond (Biology School, University of Liverpool). Unfortunately, we observed a similar amount of HS on treated and non-treated HUVEC (data not show here). This finding is due to chlorate only removing sulphate at 6-O and 2-O position while leave sulphate at N- position (Safaiyan et al. 1999). Meanwhile, F58-10E4 is sensitive and reacts to an epitope present in HS. The epitope includes N-sulphated glucosamine residue that is critical for the reactivity of the F58-10E4 (Seikagaku specificity recognition). Therefore, we did not observe any HS decreased after chlorate treatment when compared to non-treated chlorate group. Thus the change in pRBC adhesion is not likely due to the amount of HS expressed on treated EC, and our interpretation is that the sulphation pattern is critical for efficient binding, although at this stage we are unable to say whether this is due to a direct interaction or facilitated binding.

Due to the fact that we cannot see any difference in HS by FACS, we looked at another way to prove that the glycocalyx mediates binding of pRBC to HUVEC cells, using enzymatic digestion of specific GAG populations. Heparatinase is a bacterial analogue to heparanase, a mammalian endoglycosidase participating in degradation and remodelling of the glycocalyx and facilitating cell invasion associated with inflammation (Myler & West 2002) and specific shedding of charged sulphated HS (Chappell et al. 2008). Heparatinase I cleaves highly sulfated heparin/HS chains, heparatinase III cleaves less sulfated HS chains, while heparatinase II cleaves domains of both high and low sulfation on both heparin and HS. Heparatinase I, II and III used in combination can produce a near-complete depolymerization of heparin/HS polysaccharide chains to disaccharides. From our observation, 10 mU of heparatinase I, II and III separately do not significantly reduce the binding of ItG to TNF-activated HUVEC. Chappell (2008) had shown that heparatinase selectively shed HS, but not the core protein syndecan-1 off the glycocalyx (Chappell et al. 2008). Meanwhile, chondroitinase ABC from *Protus vulgaris* is a galactosaminoglycan (GalAG) depolymerising lyase that cleaves its substrate at the glycosidic bond via β-elimination and cleaves a broad range of GalAG, including CS, DS and HA (Prabhakar et al. 2005). From our enzymatic

results, similar to heparatinase, chondroitinase ABC also does not inhibit binding of pRBC to HUVEC cells. This finding seemingly contradicts our primary finding (effect of chlorate on pRBC binding on HUVEC), which showed that chlorate reduces by almost 60% pRBC binding to HUVEC, although the total removal of HS by heparatinase I/II/III does not lead to loss of pRBC binding and does not suggest a primary role of HS in adhesion. This study further investigated if the lab-adapted parasite strains used in this experiment were able to bind directly to endothelial HS (HS^{EC}). For this purpose, HS^{EC} from HUVEC cells were extracted. HUVEC were seeded and cultivated on tissue-flask coated with 1% gelatin until confluent for up to two or three passages. After reaching confluence, cells were rinsed three times with phosphate-buffered saline and trypsinised. During the trypsinization process, all the glycan will be cut off from the cell surface along with detached cells, and was dissolved in TUT buffer prior to cell sonication. TUT buffer, which consists of 8M of urea, triton X-100, Trizma base and salt (sodium sulphate) acting as detergent to dissolve all tissue and protein and leave only polysaccharide in the solution. The amount of HS present in the samples was determined at A_{232} using Beer-Lambert law with the extinction coefficients of 5500 $mol^{-1}cm^{-1}$ for the unsaturated bond chromophore generated by heparatinase enzymes. The purified HS was spotted on the dish for static binding assay.

Our static binding assay revealed that none of the parasite lines (ItG, A4, C24, R29, FCR3 and 3D7) used bind to HS^{EC}. Nevertheless, we presumed R29 and FCR3 would bind to HS because they are rosetting strains, which had previously been shown, bind to HS on non-pRBC and EC (Vogt et al. 2003). In contrast to our experiment, Vogt et al 2003 shows that FCR3-2 strain which is a rosetting strain, was de-sequestered from human lung endothelial and HUVEC when using an HS/heparin-derived compound and an enzyme that removes HS from the endothelial surface (Vogt et al. 2003). We do not know why Vogt's results contradict ours. But, this HS binding negative result might be explained by our technique of purifying HS from HUVECs which is suggested by Guidmond's group. Guidmond et al (2009), found that by using the technique to purify HS (as described in methods this chapter), it allowed structural changes in HS by removing 6-O- and 2-O-sulphate from all HS disaccharides, and it is also well known that HS can be N-desulphated when exposed to pH conditions (pH 7-10)

(Guimond et al. 2009). This is supported particularly in that the buffers used to purify the HS were at pH 7.4 and 8 in our technique. Therefore, we assumed that binding ability of the HS^{EC} extracted was lost due to sulphate removal during the purification process. This suggests that neither 6-O nor 2-O and N-sulphate are important to determine HS binding properties.

In this study, we have tried to answer whether glycocalyx mediates pRBC binding, but, we have not been able to fully answer the question. However we have shown for the first time that the sulphation of the glycocalyx can play an important role in pRBC adhesion based on our chlorate and HS purification results. It is thought that ICAM-1 plays a critical role in pRBC sequestration in cerebral vascular sites. However, the underlying mechanism of pRBC sequestration is complex and involves specific domains of PfEMP1 to interact with host adhesion receptors, especially ICAM-1 and CD36, but it is important that we do not disregard the roles of other potential adhesion molecules, especially the extracellular matrix, which has the potential to augment parasite sequestration.

CHAPTER 5: CONCLUSIONS AND FUTURE PERSPECTIVES

Treatment of malaria disease remains the cornerstone of patient management and, in the absence of a fully effective vaccine, will likely remain so for the foreseeable future. One branch which should be given therapeutic emphasis is malaria cytoadherence, which is associated with the development of SM. The unique feature of *P. falciparum* is an ability of mature forms to adhere on endothelial bed through interaction of PfEMP1 surface protein to binding receptors protein such as ICAM-1, CD36, Thrombospondin (TSP), VCAM-1 and Heparan sulphate (HS).

The use of adjunct treatments to inhibit and reverse sequestration of pRBC is a rational approach to reduce disease severity but the release of the large amount of pRBC into the circulation could be damaging. Can the spleen deal with removing the released pRBC or might it lead to side effects such as splenic dysfunction? Further work is needed in this area, such as a better understanding of adhesion-related pathology in malaria. One major difficulty is the need to record mortality as an outcome of an intervention, making the number of patients needed to be recruited relatively large. This means that we need to have better measures of clinical success if this development is to be viable, which means identifying appropriate surrogate markers.

A major obstacle in the development of adjunct compounds aimed at reducing severe malaria pathology has been lack of robust animal models for screening and evaluating drug candidates. Therefore, better animal models would help to address this and identify and test lead compounds. Efforts are underway to develop humanized animal models and transgenic parasites (containing PfEMP1 adhesion domains) that could provide a resource to study the pathophysiology of SM in humans. If we are to preserve some of the advantages gained using ACTs, then the design of new anti-adhesion drugs should incorporate artemisinins, which have the ability to kill ring stages, as well as mature pRBC and gametocytes, and thereby prevent new pRBC from adhering.

As a proof of concept, that *P. falciparum* cytoadherence can be block and reversed, this thesis has used monoclonal antibodies (mAb) specific to host

receptor molecules (ICAM-1; 15.2 and CD36; FA6-152) to inhibit and reverse pRBC binding to protein and EC (Chapter 3.1). Our results have confirmed that inhibition of the PfEMP1/ICAM-1 and PfEMP1/CD36 interaction can be realised by mAbs directed against either protein. Thus, this study has proven that mAbs directed to receptor binding are able not only to reduce or inhibit parasite binding but also to reverse existing binding, an essential feature of any potential therapy. However, antibody therapies have significant deficiencies as therapeutic agents, including scaling up production and potential immunoreactivity, which will limit the utility of these mAb for sequential administration for chronic infection. Furthermore, antibody-based therapeutics is generally expensive to produce. There is, therefore, a need for research aimed at discovering low-cost and low-toxicity inhibitors of the PfEMP1-receptor interactions to provide therapeutic agents with broader application.

Based on an assumption that intravascular coagulation was a central mechanism in malaria pathogenesis, anticoagulant therapy with heparin was previously used with some success in the treatment of human CM. Unfortunately the use of heparin was later abandoned due to the occurrence of severe bleeding and death of some children treated. However, a better knowledge of the sequestration mechanism causing SM, and recent data suggesting that an important role of GAG such as HS and CS as pRBC adhesion receptors, prompted us to screen novel anti-adhesion or anti-sequestration compounds based on heparin, GAGs and modified polysaccharide mimics to heparin with reduced anticoagulant properties.

The inhibitory and reversing effect of sulphated compounds on the cytoadherence of pRBC can be simply because of nonspecific interactions. All the compounds used in this study were thought to consist of highly negative charge. Endothelial cells are covered with sialic acid, and GAGs, which all have net negative charges, and so it is possible that negatively charged sulphated compounds could exert their effect on sequestration of pRBC by increasing the charge repulsive force between cells and sterically interfering with binding of specific ligands to receptors. However, this is not what we observed, as the ability to inhibit cytoadherence was not determined by the overall ionic charge of the compound.

In the experiment on screening a library of chemically modified polysaccharides (Chapter 3.2), only two compounds; glycogen sulphate (MS34) and cellulose sulphate (MS40) potentially inhibit and reverse pRBC binding on endothelial cells but other compounds, which have a similar chemical backbone, do not show any potential. We presumed that these compounds might work due to a multivalent effect (ability of compound to interact with multiple receptors) or sulphate level (charge) of the compound. Unfortunately, we do not have any information to show that MS34 and MS40 have high levels of sulphation or possess larger multivalent effects compared to other, non-inhibitory compounds with the same side-groups or backbone. However, another group (Ed Yates, Biosciences, University of Liverpool and Mark Skidmore, Keele University, UK) showed that MS34 is able to inhibit other microorganism and virus disease pathogenesis (data not published yet). An investigation of compound MS34 is needed for future malaria adjunct work to understand how this compound works, whether it interact with multiple receptors or just to PfEMP1. It needs proper structural identification using NMR on identifying sulphate position and structure conformation. It would also be essential to investigate the minimum inhibitory concentration of MS34 that would reduce binding to 50% (IC_{50}), because most of the modified-polysaccharides were screened at 1 mg/ml of compound, which is relatively very high.

This study further investigated modified GAGs, low molecular weight heparin and modified heparins for their effect on interrupting parasite adhesion, in chapter 3.3. From this experiment, we identified that restricted sulphate position on the compound is needed in order to exhibit inhibitory activity. Here, we proposed that sulphate at position 6-O or/and 2-O of glucuronic acid and glucosamine group and low anti-coagulant effect of the compound is important.

This thesis has emphasized the search for anti-adhesion compounds by using inexpensive and natural polysaccharide sources. Saccharides are ideal for this purpose as they are unlikely to be toxic or immunogenic, in particular, since many of those that inhibit adhesion are normal constituents of plant compounds. The involvement of carbohydrate in a biological process is increasingly acknowledged, and substantial amounts of work to understand, mimic and control these processes have been done. The function of carbohydrate as cell-surface attachment sites for different types of pathogens has led to the ideas that many of

the diseases caused by pathogens may be avoided by prevention of attachment. Carbohydrate-based inhibitors of the attachment process could be an alternative route to disease therapy.

The use of heparin to inhibit and reverse pRBC binding is well acknowledged but the side effects based on its anti-coagulant effect may have led to discontinued use. Therefore, the use of modified and LMW heparin, which have very low anti-coagulant properties, as malaria anti-adhesion 'drugs' is well supported and at the same time it can reduce the risk of thrombocytopenia. Heparin-induced thrombocytopenia is a prothrombotic adverse effect of heparin caused by platelet-activating antibodies that recognise complexes of chemokine platelet factor 4 (PF4) when it is bound to heparin. Therefore, by using low anti-coagulant heparin, it will reduce platelet complex and increase free platelet. An *in vivo* study is also needed before it can be confirmed that LMW heparin and modified versions are able to reverse cytoadherence.

The need for rapid acting adjunct treatment is critical in order to reduce mortality in SM cases. Even though screening for an anti-adhesion therapy forms a part of this thesis, we still need to understand the biology of this interaction. Therefore, this thesis also investigates environmental host factors, which can mediate or modulate binding of pRBC to EC. Here, we found host environment pH contributes to the level of parasite binding to ICAM-1 and CD36 (Chapter 4.1). Therefore, it might be true to say here that parasite mass may contribute indirectly to development of SM by producing more lactic acid, but it may be contrary with a previous study, which shows that parasite mass is not directly associated with SM. However, further study needs to be done before we conclude environmental host factors can be really contributing to enhance pRBC binding. I propose that this can be done by co-culture parasite with EC overnight (and shorter time points) at specific pH, with subsequent analysis of pRBC binding and EC characteristics. Structural conformation and protein-protein interaction studies also need to be done to identify why interaction efficiencies change with pH, e.g. CD36 binding is higher at pH 7 while binding to ICAM-1 higher at pH 7.2. These pH effects have also been seen to alter with different parasite variant, and more work to examine the specific PfEMP1-receptor interactions could help to unravel the molecular mechanisms underlying this behaviour.

Interaction of PfEMP1 with HS on non-infected RBC has been shown by many researchers to be associated with rosette formation, yet there is less study on trying to find an association of PfEMP1 interaction with endothelial HS. Therefore, this thesis attempts to address the roles of an extracellular matrix (glycocalyx) on the EC surface to mediate malaria cytoadherence (Chapter 4.2). However, the study performed here is still at a very early stage and still has not provided definitive information about this complex interaction. We do see that treatment with 10 mM sodium chlorate is able to reduce the pRBC binding to TNF-activated HUVEC with almost 60% reduction but hampered by the enzymatic and HS binding assay. It is thought by Safaiyan et al (1999), that chlorate only removes 6- and 2-O sulphates but not N-sulphate in the HS, and meanwhile, Dull et al (2003) found that heparatinase was able to reduce sulphate at a level of 67% removal. Unfortunately, we are not observed any of the mentioned because HS-antibody (F58-10E4) was dependent on N-sulphate substitute of the polysaccharides rather than O-sulphate (Humphries & Silbert 1988 1380). Safaiyan's study has been supports by Baraggan et al (1999) study, which shows that sulphate position on GAG is necessary for enhancement and de-sequestration of pRBC binding. Therefore, a need to new HS antibody such as JM13 which only recognised 2-O-sulphate substitute of glucoronic acid {van den Born, 2005 #1380) before we can understand the correlation of glycocalyx in mediating malaria cytoadherence.

We cannot exclude the fact that the parasite has to make use of the available structures in a constantly changing host environment, and simultaneously adapt to the immunological pressure set by the host defence. It is therefore not necessarily so, that exclusive highly specific interactions that require strictly defined ligand and receptor structures are always advantageous. The understanding of the glycobiology of malarial infection has increased considerably during recent years; insight into the molecular interactions involving different stages of *P. falciparum* infection will permit the development of new strategies for malaria therapy. It is also of interest to compare different strains of parasite for their adhesion properties at the molecular level to learn about the strategies of the parasite.

Here, in this thesis, we have explored how to control *P. falciparum* pathogenesis by using anti-adhesion compounds as adjunct therapies and a few

factors such as pH and extracellular matrix that might enhance pRBC binding to EC. Elucidating carbohydrate-protein interactions might provide new tools for designing specific adjunct therapy and new intervention strategies might be developed to decrease both tropism and invasion of RBC by *P. falciparum*, as well as cytoadherence (sequestration and rosetting). This in turn might alleviate the occurrence of severe complications, such as CM, severe anaemia or placenta dysfunction, without interfering or affecting the host.

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PUBLICATION AND PRESENTATIONS

Work in this thesis has been presented for publication, or is in preparation for publication, and has been presented at meeting in the following forms;

1. Publications;

a) Review article; Alister G Craig, **Mohd Fadzli Mustaffa, Khairul** and Pradeep R Patil (2012). Cytoadherence and severe malaria. Malaysian Journal of Medical Sciences (Accepted for publication on 15 November 2011).

b) Paton, D., Faragher, B., **Mustaffa, K.M.,** Szestak, T., Barrett, S.D., Craig, A.G (2011). Automated counting for *Plasmodium falciparum* cytoadherence experiments. Malaria Journal 10: 91.

2. Publication in preparation;

a) Inhibition and reversal of *P. falciparum* cytoadherence using monoclonal antibody.

b) Inhibition and reversal of Malaria Cytoadherence by using chemically modified glyco-compounds.

c) Effect of pH on *P. falciparum* cytoadherence.

3. Presentations;

a) **Khairul M. Mustaffa**. Polysaccharides: the next adhesion-based adjunct therapy for *Plasmodium falciparum* severe malaria. 6th Annual BioMalPar Conference on the Biology and Pathology of the Malaria Parasite. 3-5 May 2010. EMBL andvaced training centre, Heidelberg, Germany.

b) **Khairul M. Mustaffa**. Anti-cytoadherent: the next *Plasmodium falciparum* adjunct therapy. Molecular Parasitology Meeting. 11-15 September 2011. Woods Hole, MA, USA.

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Technical Experiences	
Technical and specialized skills	Malaria *in-vitro* culture Endothelial cells *in-vitro* culture Malaria diagnostic Molecular - PCR - Northern blot - RFLP - DNA extraction - Cloning Proteomic - SDS PAGE - Western blot - 1D gel - 2D gel - Protein expression High Performance Liquid Chromatography (HPLC) Liquid chromatography –mass spectrometer (LC-MS) Fluorometer... cell sorting (FACS) Spectrophotometer Light microscope Light inverted microscope Bacterial in-vitro culture Animal/Mice handling Flow and static adhesion assay Drug inhibitory assay MTT assay

Other experienced	1. INFORMM Research assistant - Typhoid diagnostic (DNA and protein diagnostic (IgG/IgM)) - Shiegella diagnostic (DNA diagnostic) - TB diagnostic (dot blot) 2. Treasurer of Postgraduate Students Association Session 2006/07 USM Health Campus 3. Welfare Comitee of Postgraduate Students Associates Session 2004/05 USM Health Campus 4. Malaysia Agriculture Research and Development Institute (MARDI), Department of Horticulture. (Assitant Research Officer 2002/03) 5. Universiti Teknologi, Malaysia (Bio-Informatics Research assistant 2003)
Professional Achievements	
Professional /academic Honours and awards	2008-2012: Academic Staff Trainee Scheme Scholarship (ASTS), Malaysia 2004-2007 : Ministry of Sciences and Technology Scholarship, Malaysia 2002-2003: Dean list for Bachelor of Sciences (Biotechnology), Faculty of Food Sciences and Biotechnology, UPM (CGPA: 4.00) 2001-2002: Dean list for Bachelor of Sciences (Biotechnology), Faculty of Food Sciences and Biotechnology, UPM (CGPA: 3.50) 1998 -1999 : MARA Scholarship
Professional development	Malariologist (summary ----study on Plasmodium parasite) Molecular and Biochemical Parasitology (summary ----host pathogen-interactions) Glycobiologist (summary----use of sugar/carbohydrate for disease therapy)

	Biotechnologist (summary----medical diagnostic)
Professional Activity	
Conference/workshop attended	2011: - 22nd Annual Molecular Parasitology Meeting (Woods Hole, Boston, USA) - GlycoTRIC (Imperial College, UK) - Welcome Trust Advance Course: Malaria Genetic Experimental Course (Cambridge, UK) 2010 : - Career in Academic. Career Skills Workshop (Bristol, UK) - 2010 Drug Discovery Conferences (Coventry, UK) - Sixth Annual BioMalPar Conference on the Biology and Pathology of the Malaria Parasite (Heidelbergh, Germany) 2009: - Postgraduate research seminar (Liverpool, UK) 2008: - Fifth Annual BioMalPar Conference on the Biology and Pathology of the Malaria Parasite (Paris, France) - Statistic and research methodology (Liverpool, UK) 2007: 2006: - The 1st USM-Penang International Postgraduate Convention: 1st Health and medical Sciences Conferences (Malaysia) - Research methodology workshop (Malaysia) - 2nd EndNote Workshop & Manuscript Writing Workshop (Malaysia) - National Seminar on Current Issues in Infectious Diseases (Malaysia) - International 5th Virtual conference on Genomic and Bio-informatics (Malaysia) 2005: - The 10th National Conference on Medical Sciences (Malaysia) - Basic Statistic and Research Methodology - Intermediate Statistic, Quality Thesis & Scientific Writing workshop 2004: - Scientific Writing workshop
Research/scholar activity	2008-2011: An investigation of inhibitors/reversal and

	endothelial environment on Plasmodium cytoadherence. 2004-2007: Effect of P-Glycoprotein Inhibitors on Chloroquine Resistant *in-vitro* and *in-vivo* malaria model: Towards looking chemosensitizers for malaria. 2003: An investigation of polysaccharides effect on Mangosteen growth (field study). 2002: Analysis of Antibiotic Susceptibility, Plasmid Profiling and Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) on Streptococcus group D.
Journal article	1. Paton D, Faragher B, ***Mustaffa KM***, Szestak T, Barret SD, Craig AG (2011). Automated counting for *Plasmodium falciparum* cytoadherence experiments. Malar J. 2011 Apr 16:10:91 2. ***Khairul M.F.M***, *T. H. Min, J. H. Low, C. H. Che Nasriyyah, A. Noor A'shikin, M. N. Norazmi, M. Ravichandran and S. S. Raju* (2006). Fluoxetine potentiates reversing Chloroquine and Mefloquine resistance in *Plasmodium falciparum* in-vitro. J Infect Dis. 2006 Oct; 59(5): 329-31. 3. T. H. Min, ***M. F. M. Khairul***, J. H. Low, C. H. Che Nasriyyah, A. Noor A'shikin, M. N. Norazmi, M. Ravichandran and S. S. Raju (2007). Roxithromycin reverses chloroquine and mefloquine resistance in *Plasmodium falciparum* in vitro. Exp Parasitol. 2007 Apr;115(4):387-92. Epub 2006 Nov 21.
Conference proceeding	1. ***Mustaffa, Khairul M.F*** (2010). Polysaccharides: the next adhesion-based adjunct therapy for severe *Plasmodium falciparum* malaria. Proceeding book Sixth Annual BioMalPar Conference on the Biology and Pathology of the Malaria Parasite,pg 80. 2. Mustaffa, Khairul M.F (2009). 3. Mustaffa, Khairul M.F (2008) 4. ***Khairul MFM***, *and SS Raju*. (2006). Effect of macrolide antibiotic towards in-vitro chemosensitization of chloroquine resistance *P. falciparum*, extended abstract. Proceeding book 1st USM-Penang International Postgraduate Convention: 1st Health and Medical Sciences Conference.

	5. ***Khairul MFM***, *Tan HM, Low JH, Che Nasriyah CH, Noor A'shikin A, Norazmi MN, M. Ravichandran, and SS Raju*. (2005). Effects of Fluoxetine on reversing Chloroquine and Mefloquine resistance *Plasmodium falciparum*. Extended abstract. Proceeding book of 10th National Conference on Medical Sciences. The Malaysian Journal of Medical Sciences, vol 12, supplements 1, pg 113. 6. *Noor A'shikin, Nasriyah CH,* ***Khairul MFM***, *Tan HM, Low JH, Che A, Norazmi MN, M. Ravichandran, and SS Raju*. (2005). Expression of human MDR1 in an outer membrane permeability mutant of Escherichia coli. Proceeding book of 10th National Conference on Medical Sciences. The Malaysian Journal of Medical Sciences, vol 12, supplements 1, pg 64. 7. *Low JH,* ***Khairul MFM***, *Tan HM, Che Nasriyah CH, Noor A'shikin A, Norazmi MN, M. Ravichandran, and SS Raju*. (2005). Effect of Omeprazole-chloroquine combination on chloroquine resistant plasmodia .Extended abstract. Proceeding book of 10th National Conference on Medical Sciences. The Malaysian Journal of Medical Sciences, vol 12, supplements 1, pg 163.
Work currently under submission	1. ***Khairul M.F.M*** and Craig A.G. (2011). Cytoadherence and severe malaria. Malaysia Journal of Medical Sciences (MJMS) (submitted for publication)
Work in progress	1. The effect of Monoclonal Antibody on reversing *Plasmodium falciparum* cytoadherence in-vitro. 2. Reversal effect of modified polysaccharides on *Plasmodium falciparum cytoadherence* in-vitro.
Academic/research interest	Malaria, Host-Pathogen interactions, Medical Diagnostic
Affiliations/memberships	Members of Malaysia Society for Microbiology (2004-present)
Foreign language Abilities/skills	Written: Good in English and excellent in Malay language Spoken: Good in English and excellent in Malay

Career objective	My career objective is to advance and further develop the molecular diagnostic under INFORMM, aiming to make it one of the best at our institution and a referral centre for many other labs. I am determined to develop new ideas, new findings and new technologies in the field of Malaria that will pave way to even more findings. As a scientist, I wish to contribute what I can on my behalf for the benefit of humanity as a whole. I'm also resolute to find new drugs therapy for malaria that will improve the quality of life for those suffering of this ailment all around the world. In addition to all that I would also like to establish a good network among fellow scientists whom I will gain during and after my PhD studies, as sharing of information and expertise is crucial and will lead to fruitful discussion. In all, I just wish to learn all I can whilst my stay in UK and experience the work culture, lifestyles and cultures of the people there, and everything that comes parcelled with it so I can share it with my colleagues back home.
References	Prof Alister Craig Senior lecturer Department of Molecular and Biochemical Parasitology Liverpool School of Tropical Medicine Pembroke Place L3 5QA Liverpool, United Kingdom. agcraig@liverpool.ac.uk Prof Asma Ismail Senior Lecturer/Director Institute for Research in Molecular medicine (INFORMM) Universiti Sains Malaysia Health Campus 16150 Kubang kerian Kelantan Malaysia. asmaismail@usm.edu.my Prof Dr. S. Sivachandra Raju Senior Lecturer Department of Pharmacology Ras Al Khaimah (RAK) Medical and Health Sciences University PO Box 11172, Ras Al Khaimah, United Arab Emirates ssraju@yahoo.com Prof Manikam Ravichandran

	Senior lecturer Faculty of Applied Sciences AIMST University, Malaysia Jalan Bedong-Semeling 08100 Bedong Kedah Darul Aman Malaysia. ravichandran@aimst.edu.my

APPENDIX I

Table 2. 1: List of reagents.

Reagents	**Code**	**Supplier**
Acetic acid	10001CU	BDH Laboratory supplies
Glutaraldehyde (25%)	360802F	
Microscopy lens oil immersion oil	361023N	
Giemsa stain	350864X	
Sodium chloride (NaCl)	102414J	
Sodium hydroxide (NaOH)	104384F	
Hydrochloric acid (HCl)	BDH3203	
Ethanol	E7023	Merck Ltd., Leicester
Formaldehyde (4 % solution)	100496	
Glycerol	356350	
Methanol	270474	
Virkon™ disinfectant or Trigene	TM305	
MCDB 131 1 x liquid ((-)L-glutamine, (-) sodium pyruvate	10372-019	Gibco Europe Ltd., Middlesex
10 x Penicillin/streptomycin		
Tumor necrosis factor (TNFα)	PHC3024	
Plasmagel™		Rhone-Poulenc, Neuilly-

		sur-Seine. France
Gelatin (2%)	G1393	Sigma Chemical Co., Dorset
Gentamycin sulphate (10 mg/ml)	G1272	
RPMI 1640 1x liquid ((+)HCO_3-. (–)L- glutamine)	R0883	
RPMI 1640 powder (-HCO_3-, +L-glutamine, + modified HEPES)	R4130	
HEPES Buffer (1M)	H0887	
Bovine Serum Albumin (BSA) solution (30%)	A8327	
HISTOPAQUE^R-1077 (+polysucrose, + sodium diatrizoate)	H8889	
Dimethylsulphoxide (DMSO)	D8418	
D-sorbitol	S3889	
Water	W3500	
3-Aminopropyl-Triethoxysilane (APES)	A3648	
Dulbecco's phosphate buffer saline	D8537	
D(+) Glucose	G7021	
Trypsin-EDTA	T4174	
L-Glutamine (200mM)	G7513	

3-aminopropyltriethoxysilane (APES)	A3648	
Endothelial cell growth medium Endothelial cell growth medium MV Endothelial cell growth medium supplement Endothelial cell growth medium MV supplement Promocell detach kit (HEPES, Trypsin and Trypsin neutralizer solution (TNS) Cryo-SFM	C-22110 C-39220 C-39215 C-39225 C-41220 C-29910	Promocell GmbH
DPX mounting medium	D/5319/05	Fisher Scientific

APPENDIX II

Table 2. 2: List of monoclonal antibodies.

Antibody	**Code**	**Isotype**	**Antigen**	**Supplier**
F58-10E4	370255-1	IgM, κ-chain	Heparan sulphate	Seikagu Biobusiness corporation
FA6-152	Ab17044		CD36	abcam
15.2	SC-107	IgG1	ICAM-1	Santa Cruz
BC6	-	IgG1	A4 PfEMP-1	Oxford University
APC mouse anti-human CD54	559771	IgG1, κ-chain	ICAM-1	BD Biosciences Ltd.
FITC mouse anti-human CD36		IgG1, κ-chain	CD36	
FITC mouse IgG1, κ-isotype control	555748	IgG1, κ-chain	-	
APC mouse IgG1, κ-isotype control	555751	IgG1, κ-chain	-	

APPENDIX III

Table 2. 3: List of equipment.

Equipment	**Supplier**
Light microscope Nikon phase-contrast Inverted phase-contrast microscope	Nikon
Water-bath	Grant instruments (Cambridge) Ltd.
Centrifuge (Biofuge Prime)	Heraeus instruments
Spectrafuge 24D	Jencons-Pls
PocH-100*i*	Sysmex UK Limited
Coulter Ac.T series analyzer	Beckman coulter™ Inc
Gilson pipette tips 1.5 ml Eppendorf tubes	Alpha Laboratories, Hampshire
Gilson pipettes (P2, P20, P200, P1000)	Anachem Ltd., Bedfordshire
Microscope slides (76 x 26mm)	BDH Ltd., Warwickshire
Vacuum pump (Vacuubrand Model ME4R)	
Falcon 60mm diameter bacteriological plastic petri dish Falcon 15 and 50ml polypropylene centrifuge tubes Falcon 1, 2, 5, 10 and 25ml plastic pipettes	Becton Dickenson Ltd., Oxford

Gas cylinders (1% CO2, 3% O2, 98% N2)	British Oxygen plc., Surrey
FACS machine	BD Biosciences
Parasite 37^{o}C incubator	LTE Scientific/Raven incubator
CO_2 incubator	Binder
24-well flat bottom plate 6-well plate 500ml filter unit T25, T75 and T175 ventilated cap T25, T75 and T175 non-ventilated cap 13mm Thermanox coverslips	Nunc

APPENDIX IV

Table 2. 4: List of Endothelial cells.

Endothelial cells	**Catalogue number**	**Company**
Human Vascular Endothelial Cell (HUVEC)	C-12203	Promocell
Human Dermal Endothelial Cell (HDMEC)	C-12210	
Human Brain Endothelial cell (HBEC; HBEC5i)	-	Provided by Sam Wassmer

APPENDIX V

Table 2. 5: List of media and solution.

All solutions were made up in distilled water and then were filter-sterilised (0.22µm) and kept in 4^{o}C and were pre-warmed prior to use.

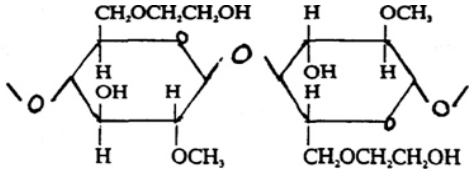
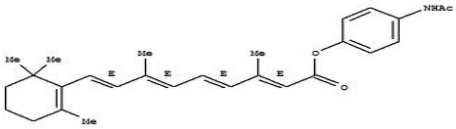
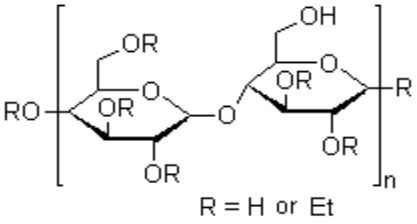
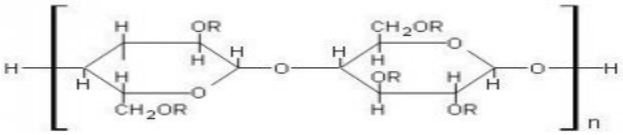
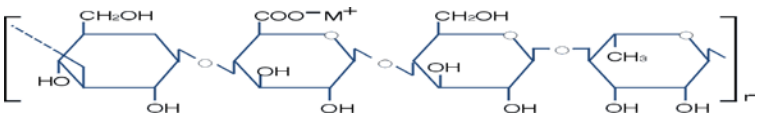
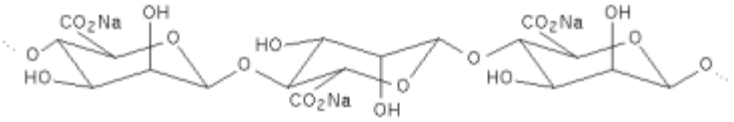
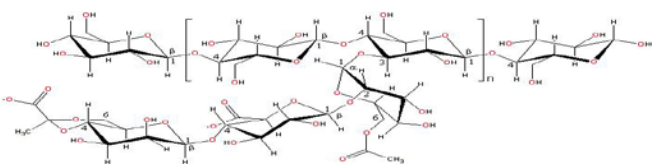
Media	**Solutions**
RPMI-Yellow	1 x RPMI 1640 (+$NaHCO_3$, -L-glutamine) supplemented with - 10 mM D-glucose - 37.5 mM HEPES - 6 mM NaOH - 2 mM L-glutamine - 25 µg/ml gentamicin
RPMI-Blue	RPMI-Yellow supplemented with 10% (v/v) pooled human serum and were filter sterilised
Giemsa stain	10 % or 5 % (v/v) Giemsa diluted in a solution of - 20 mM Na_2HPO_4 - 4 mM KH_2PO_4 - pH adjusted to 7.2 with HCl
Parasite Cryo solution	45.6 % (v/v) glycerol 142 mM sodium lactate 115 mM NaH_2PO_4 4 mM KCl pH adjusted to 6.8 with NaoH

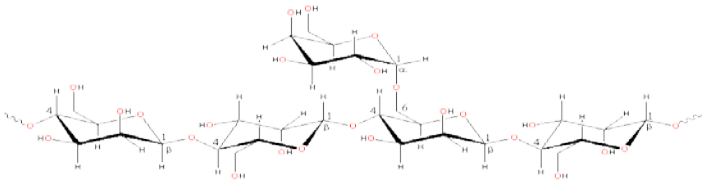
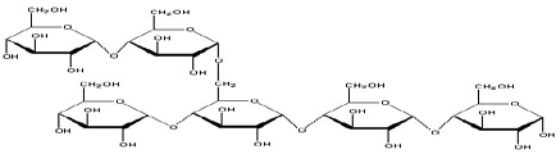
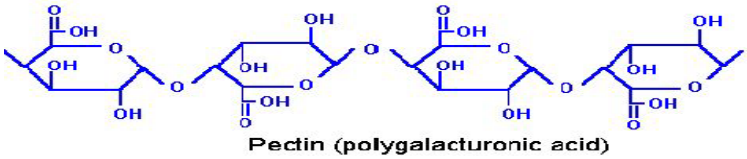
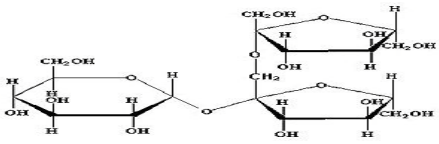
	filter sterilised
HUVEC media	Endothelial cell growth medium supplemented with 1x C-22010 supplement mix to give concentrations of growth factors in the complete medium as follows; - 2 % Fetal Calf serum - 0.1 ng/ml Epidermal Growth Factor - 1 µg/ml Hydrocortisone - 1 ng/ml Basic Fibroblast Factor
HDMEC media	Endothelial cell growth medium MV supplemented with 1x C-39225 supplement mix to give concentrations of growth factors in the complete medium as follows; - 5 % Fetal Calf serum - 10 ng/ml Epidermal Growth Factor - 1 µg/ml Hydrocortisone
HBEC media	MCDB 131 (without L-Glutamine and sodium pyruvate) supplemented with - 10% FCS - 2 mM L-glutamine - 10 ng/ml EGF (recombinant, human) - 1 µg/ml hydrocortisone - 100 unit of penicillin G - 100 µg/ml streptomycin
Binding buffer	RPMI 1640 powder (-HCO_3-, +L-glutamine, + modified HEPES) made up to 1 x concentration and supplemented

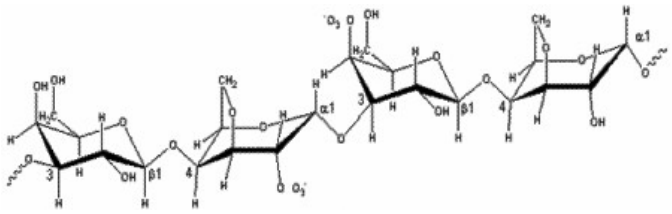
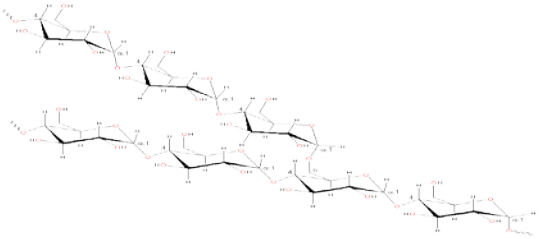
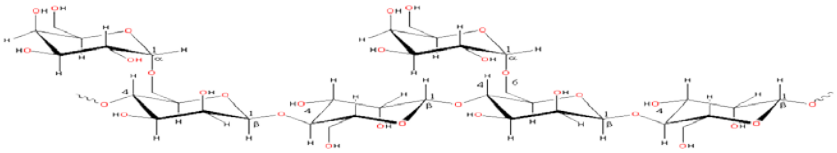
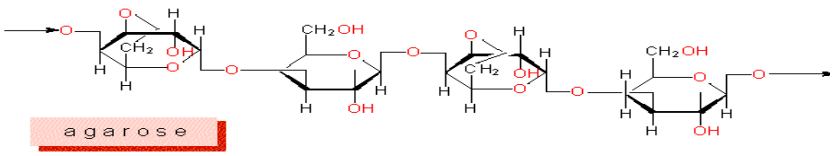
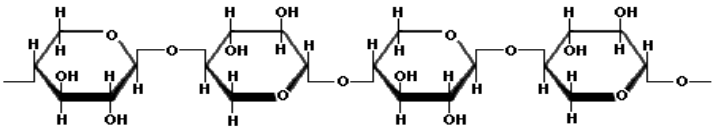
	with - 10 mM G-glucose (2 g in 1L water) pH adjusted to 7.2 with NaOH filter sterilised

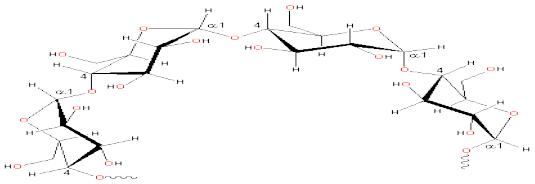
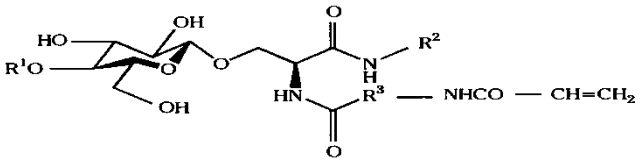
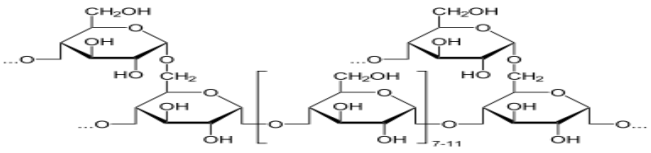
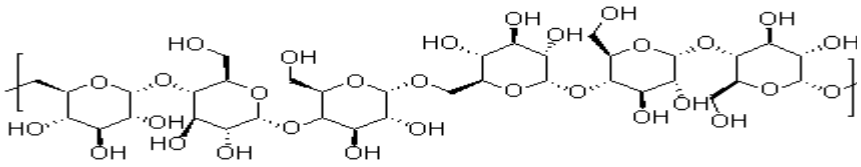
APPENDIX VI

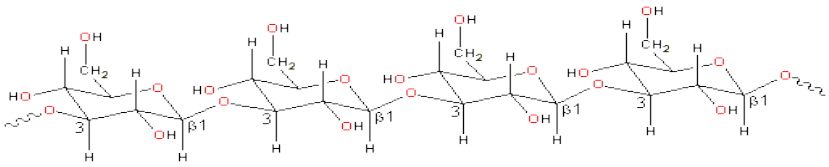
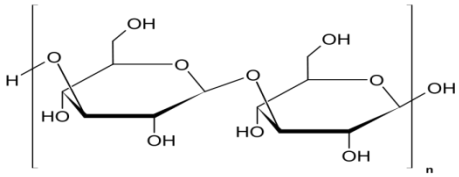
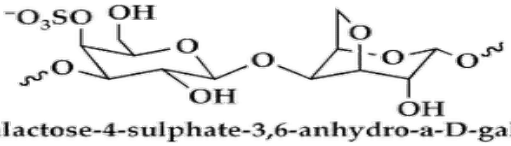
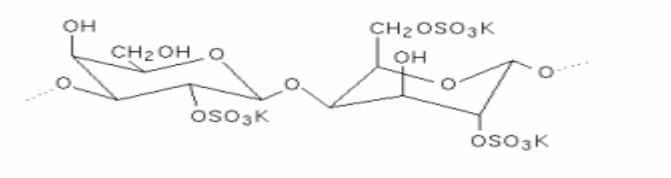
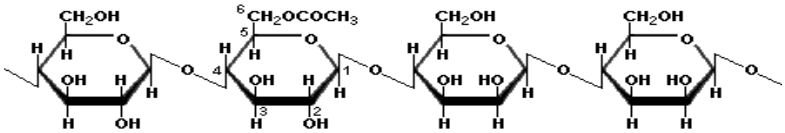
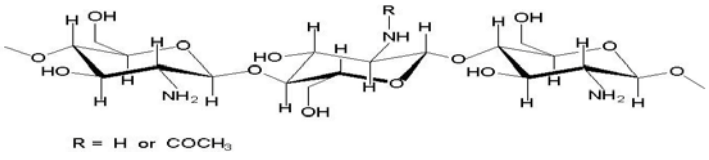
Table 3.6: List of chemically-modified polysaccharides (MS) compound. All compounds were prepared to 1 mg/ml in binding buffer (ph 7.2) prior to assays.

No	Compounds	Gross structure
1	Tylose sulphate	
2	Ghatti sulphate	
3	Gum Accroides	ND
4	Ethyl cellulose sulphate	
5	Propylmethyl sulphate	
6	Gellan sulphate	
7	Alginic sulphate	
8	Xanthan sulphate	

9	Locust bean sulphate	
10	Gum Arabic sulphate	ND
11	Gum rosin sulphate	ND
12	Gum Styrax	ND
13	Starch sulphate	
14	Pectin sulphate	 Pectin (polygalacturonic acid)
15	Mastic gum sulphate	ND
16	Pectin sulphate (Type 2)	ND
17	Inulin sulphate	
18	Storax sulphate	ND
19	Carboxymethyl cellulose sulphate	ND

20	i-carageenan persulphate	
21	Tragacanth sulphate	ND
22	Amylopectin sulphate	
23	Karaya sulphate	ND
24	Guar sulphate	
25	Dextrin sulphate	ND
26	Hydroxyethyl cellulose sulphate	ND
27	Agarose sulphate	
28	Xylan sulphate	
29	Arabic sulphate (Type 2)	ND

30	Amylose sulphate	
31	Dextran persulphate	 (III)
32	Hypromellose sulphate	ND
33	Polygalacturonic acid	ND
34	Glycogen sulphate	
35	Methylcellulose sulphate	ND
36	Cyclodextrin sulphate	ND
37	Pullulan sulphate	
38	Levan sulphate	ND
39	Glucan b1-3 sulphate	ND
40	Penoxylacetyl cellulose sulphate	ND
41	Paramylon sulphate	ND

42	Curdlan sulphate	
43	Stachyose sulphate	ND
44	Laminarin	
51	Fucogalactan sulphate	ND
52	K-carrageenan persulphate	 β-D-galactose-4-sulphate-3,6-anhydro-a-D-galactose
53	Lambda-carrageenan persulphate	
54	Konjac glucomannan sulphate	
55	Chitosan sulphate	 R = H or $COCH_3$
56	Psyllium sulphate	ND
57	Scleroglucan sulphate	ND
58	Welan sulphate	ND

59	Alginic sulphate (Type 2)	ND
60	Propyleneglycol alginic sulphate	ND
61	Taramind sulphate	ND
62	Tara sulphate	ND

All the structure displayed was adapted from google images and not the exact structure represents the compound. ND; not available.