Plasmodium falciparum surface antigens thrombospondin-related anonymous protein (TRAP) and circumsporozoite protein (CS) inhibit complement mediated killing

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Sura Saad Hamada Alkhuzaie

Department of Infection, Immunity and Inflammation

University of Leicester

Jun 2019

Statement of originality

This accompanying thesis submitted for the degree of PhD entitled "*Plasmodium falciparum* surface antigens thrombospondin-related anonymous protein (TRAP) and circumsporozoite protein (CS) inhibit complement mediated killing*"* is based on work conducted by the author at the University of Leicester mainly during the period between January 2015 and December 2018.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university.

Signed: Sura Alkhuzaie Date: 03/06/2019

Abstract

Plasmodium falciparum surface antigens thrombospondin-related anonymous protein (TRAP) and circumsporozoite protein (CS) inhibit complement mediated killing

Sura Alkhuzaie

Malaria remains the world's most devastating tropical infectious disease, with as many as 40% of the world population living in perilous areas. It is caused by a parasite called *Plasmodium*, which is transmitted by the bites of infected mosquitoes. In the human body, the parasites multiply in the liver, and then infect the red blood cells. Sporozoites (the stage that infects liver cells) express circumsporozoite (CS) protein and the thrombospondin related anonymous protein (TRAP), which are implicated in recognition of, and entry into, hepatocytes. These proteins contain highly conserved thrombospondin domain motives (TSP domains), structural motifs also found in thrombospondin-1 and properdin. Previous data show that thrombospondin acts as an effective competitive inhibitor of properdin-dependent complement activation, binding firmly to the same target complex as properdin.

My hypothesis is that the TSP domains containing *Plasmodium* TRAP and CS proteins have a similar activity, inhibiting host complement activation and thus protecting the parasite from complement attack.

Recombinant TRAP and CS proteins, and fragments thereof, were produced in prokaryotic expression systems and tested *in vitro* for complement inhibitory activity. The proteins decreased deposition of C3, the central component of complement, via the lectin and alternative pathways. Moreover, adding proteins to serum reduces the lysis of guinea pig RBCs, indicating that the terminal pathway and MAC formation are inhibited. Mouse monoclonal antibody was produced from a C-terminal fragment of CS protein. This mAb could block the inhibition of complement activity by CS protein. This could open up the possibility of new therapies and vaccine targets that exploit this mechanism to interfere with the parasite life cycle at this stage.

Acknowledgements

Firstly, I would to give my thankful gratitude to my supervisor Dr. Nicholas Lynch, for his supervision, support, and for giving me a chance to be a part of this great research project to learn and develop as a scientist. His guidance helped me in all the time of research and writing of this thesis.

I gratefully thank Professor Andrew Tobin, for his support and supervision and continuous help for at least the two years of my PhD from the beginning. Moreover, I express my thanks to Professor Wilhelm Schwaeble, Dr Alan Bevington, Dr Hasan Yesilkaya and, Dr Edouard Galyov for their support

I would like to thank my colleague in lab228, Mohenned Alsaadawi for his support and help; also wish to thank Dr Sadam Yaseen, a Post-doctoral research associate working with Omeros in the USA, for his help along my PhD study.

I would like to thank Eurogentic Company (Seraing, Liège, Belgium) for helping me to produce antibody.

I thank my government (Iraq), University of Al-Qadisiyah for giving me this great opportunity in providing a scholarship to continue my education.

I would like to express my gratitude to my family back home in Iraq for their emotional support (love) and prayers to finish this project in peace, in particular my parents, for their prayers and love. No words can be enough to thank my parents for everything they have done for me. I thank my lovely husband Wameedh for love, patience and help, you are the biggest assistance in this achievement. I will not forget of course my kids Dina, Zinah and Ahmed with their pretty smiles; they gave me strength to finish this project and write my thesis.

Above all, I thank Allah for giving me this opportunity to achieve my goals and allow me to become part of a scientific community.

List of content

List of tables

List of figures

List of abbreviations

aa	Amino acid
AP	Alternative pathway
AP_{50}	half maximum of alternative pathway
BBS	Barbital Buffer Saline
bp	base pair
BSA	Bovine Serum Albumin
C1q	the first subcomponent of the complement components 1
C1r	Complement components 1r
C1s	Complement components 1s
C3, C4, C5, C6, C7, C8, C9	Complement components 3, 4, 5, 6, 7, 8, 9
CCP	Complement Control Protein
cDNA	Complementary DNA
CFP	Complement factor P (properdin)
CHO	Chinese Hamster Ovary
COOH	Carboxylic Acid
CP	Classical pathway
CR1	Complement receptor 1
CS	Circumsporozoite protein
Da	Daltons (atomic mass unit 1 g/mol)
dATP	Deoxyadenosine Triphosphate
DDT	Dithiothreitol
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene glycol-bis-N,N,Ń,Ń-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FB	Factor B
FD	Factor D
FH	Factor H

FI	Factor I
GVB	Gelatin Veronal Buffer
HAS	Human Serum Albumin
His	Histidine
HRP	Horse Reddish Peroxidase
iC3b	inactive C3b
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl Thiogalactoside
kDa	Kilodalton
LB	Luria-Bertani
LA	Lectin pathway
mAbs	Monoclonal antibodyies
MAC	Membrane Attack Complex
MASP	MBL-Associated Serine Protease
MBL	Mannan Binding Lectin
MEM	Minimum Essential Medium
M	Molar
µg	Microgram
µL	Microliter
mL	Milliliter
mM	Millimolar
MWCO	Molecular weight cut-off
NH2	Amino-terminus
NHS	Normal Human Serum
nM	Nanomolar
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PfGAP50	*P. falciparum* Glideosome Associated Protein-

50

PNACL	the Protein Nucleic Acid Chemistry Laboratory University of Leicester
RBCs	red blood cells
rpm	round per minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
TAE	Tris-base/Acetic acid/EDTA
TBS	Tris Buffered Saline
TBST	TBS Tween-20
TEMED	Tetramethylethylenediamine
Tfb	Transformation buffer
TSP	Thrombospondin type I Repeats
w/v	weight/volume
WT	Wild Type
xg	units of times gravity
Xgal	5-bromo-4-chloro-3-indolyl-beta D-galactopyranoside

1 Chapter One

General Introduction

1.1 General introduction

Malaria is a global disease that affects about 500 million people and causes more than one million deaths a year (Snow *et al*, 2005). The disease is transmitted to humans and animals from infected females of the Anopheles mosquito (Weekley and Smith, 2013).This disease is widespread in the tropical and some temperate areas of the world, and is caused by protozoan parasites of the genus Plasmodium, which infect the red blood cells (RBCs). Where transmission occurs in endemic areas in regular long seasons, death rates are highest in children who do not yet have enough immunity to the disease. Where malaria spread occurs in short seasons, or intermittently in epidemic areas in the form of epidemics, it is likely to cause severe mortality in all age groups (Martens and Hall, 2000). Four major parasite species are known to cause disease in humans, namely *Plasmodium falciparum*, *P. vivax, P. ovale and P. malaria. Plasmodium falciparum* is one of the most risky types of parasite, the utmost risk of disease and mortality in *P. falciparum* endemic areas happens in children under 5 years and women pregnant for the first time (and their foetuses) (Snow *et al*, 2005; Boddey and Cowman, 2013).

1.2 Plasmodium falciparum (P. falciparum):

1.2.1 General characteristic of the life cycle

The malaria parasites are apicomplexan unicellular obligate intracellular organisms of the genus Plasmodium, transmitted by female mosquitoes of the genus Anopheles. Malaria parasites have a complex life cycle that takes place within two hosts, an intermediate host (vertebrate) and a final host (insect vector)(Miller *et al*, 2002). Generally, the human infective sporozoite stage develops within the mosquito gut and is injected via the salivary glands into the blood stream when the mosquito takes a blood meal. Sporozoites invade hepatocytes within an hour and start to divide into exoerythrocytic merozoites (tissue schizogony) after 6-10 days. Once merozoites leave the liver, they enter erythrocytes and develop into early trophozoites, which are ring shaped, vacuolated and uninucleated. When the parasite begins to divide, the trophozoites are called schizonts, containing many daughter merozoites (blood schizogony). Finally, the infected erythrocytes are lysed by the merozoites, which later invade other erythrocytes, beginning a new cycle of schizogony (Trampuz *et al*, 2003;

Collins and Jeffery, 2007; Curtidor *et al*, 2008). The period of each cycle of schizogony in *P. falciparum* is about 48 hours. In non-immunized humans, the infection is amplified about 20-fold each cycle. After several cycles, some of the merozoites will develop into gametocytes. These forms pass into the Anopheles gut when the mosquito feeds on infected human blood and develop into male and female gametes. The fertilized female gametes (zygotes) subsequently develop into actively moving ookinetes which can penetrate the mosquito's midgut wall and form oocysts on the exterior surface. Inside the oocyst, thousands of active sporozoites develop. The oocyst eventually bursts, releasing sporozoites into the body cavity that travel to the mosquito's salivary glands) (Bousema and Drakeley, 2011). The cycle of human infection begins again when the mosquito bites another person (Figure 1-1). The sexual stage of malaria (i.e. the gametocytes) causes no symptoms in humans, but is infective for mosquitoes (Roberts and Janovy, 2005).

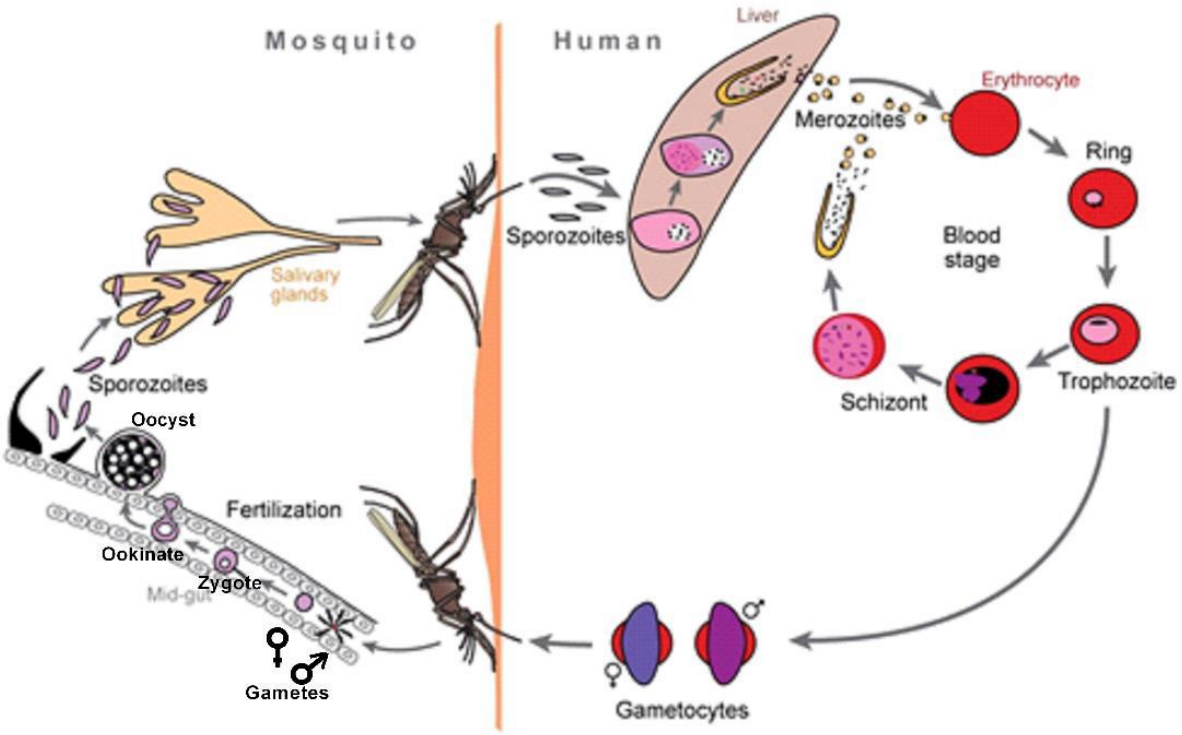


Figure 1-1: The life cycle of *P. falciparum*.

The Anopheles mosquito bites a human and injects sporozoite forms. These move to the liver and invade hepatocytes, and then they develop to produce exoerythrocytic merozoite forms that are released into the blood stream. Merozoites invade erythrocytes and grow into trophozoites and mature schizonts. Merozoites are released that reinvade new erythrocytes. Gametocytes, formed from the asexual blood stage, are taken up by a feeding mosquito into the gut where they mature to form male and female gametes. The fertilized zygote develops to an ookinete and an oocyst and finally sporozoites that migrate to the salivary glands. And the life cycle will be repeated when the sporozoites get injected from its salivary glands into the human bloodstream during mosquito blood feeding. This figure was taken from (Scherf, Lopez-Rubio and Riviere, 2008).

1.2.2 Pathogenesis of *P. falciparum* malaria

All of the pathology and clinical symptoms of malaria is due to parasites multiplying in erythrocytes. Malaria symptoms typically develop within 10 days to four weeks following the infection, but in some cases symptoms may not develop for several months. Some of these symptoms are shaking chills that can range from moderate to severe, high fever, headache, nausea, vomiting, abdominal pain, diarrhoea, anaemia, muscle pain, convulsions and coma. Malaria pathogenesis occurs in two distinct phases: (the liver phase exoerythrocytic) and the erythrocytic phase, in the red blood cells (Rowe *et al*, 2009). In the erythrocytic phase, the parasite adheres to the erythrocytes by several mechanisms which include: invasion the erythrocyte membrane to form the parasitophorous vacuole in which Plasmodium develops, interruption of the erythrocyte membrane and invasion of blood cell by gliding in intracellular fluid, and cytoadherence (adhere to the capillary and post capillary venular endothelium in the deep microvasculature). Infected red cells also adhere to uninfected red cells, resulting in the formation of red cell rosettes (rosetting), which is mediated by parasite adhesion proteins, providing protection from the host immune response by avoidance of clearance by the spleen (Anstey *et al*, 2009; Rowe *et al*, 2009; Malpede and Tolia, 2014). The major complications of severe malaria include cerebral malaria, pulmonary oedema, acute renal failure, severe anaemia, and/or bleeding. Any of these complications can develop rapidly and progress to death within hours or days (Trampuz *et al*, 2003).

1.2.3 Malaria treatment and prevention

Falciparum malaria is caused by *P. falciparum*. In most parts of the world *P. falciparum* is now resistant to chloroquine which should not therefore be given for treatment. Meanwhile quinine can be given for patient with falciparum malaria orally as tablets if the patient can swallow for 5-7day. Quinine should be given by intravenous infusion if the patient is seriously ill or unable to take tablets (World Health Organization, 2015). Other types of Plasmodium can treat with chloroquine (World Health Organization, 2015).

The main malaria prevention plan should be operationalize on the ground including insecticides-treated mosquitos nets and indoor residual spray, also avoid being bitten by protective clothing and cream (Guyatt *et al*, 2002).

1.2.4 Immunity to Malaria

The mechanisms underlying protective immunity to parasite infection and clinical disease in humans are not fully understood. Studies conducted in *vitro,* in animal models and in humans point to the involvement of both cellular and humoral immune effectors in a parasite stage-specific manner (Doolan, Dobano and Baird, 2009), and include both innate and adaptive immune mechanisms (Good and Doolan, 1999). Natural innate immunity against falciparum malaria protects millions of people from the severe disease (Doolan, Dobano and Baird, 2009). Some conditions make it more difficult for malaria parasites to infect red blood cells, for example beta-thalassaemia and sickle cell anaemia, which cause distortions in red blood cells and these are common in people from endemic areas (Pombo *et al*, 2002; Snow *et al*, 2005). In addition, some people have red blood cells that lack proteins called Duffy antigens on their surfaces. These proteins act as receptors for *Plasmodium vivax* merozoites, so people without Duffy antigens are resistant to infection by this parasite (Carvalho and Carvalho, 2011). The acquired immunity to malaria of people living in malaria endemic areas develops through natural exposure to malaria parasites (Doolan, Dobano and Baird, 2009). Children living in regions of steady malaria transmission become infected early in life, and develop stronger disease signs during the first five years of life. Then, as immunity progresses the disease becomes less severe and the number of parasites circulating in the blood decays. The acquired immune response to malaria is strain specific and is lost if an individual moves away from a malaria endemic area.

Antibodies against schizont and merozoite antigens bind to infected red blood cells and to merozoites, and make them easier for macrophages and other immune cells to attack. In addition, there are other immune mediators activated by antibodies, called complement proteins, that destroy parasites and help to prevent the merozoites from infecting fresh red blood cells (Ramasamy, Ramasamy and Yasawardena, 2001; Ghani *et al*, 2009). Macrophages that have taken up Plasmodium present parasite antigens on their surface, and other immune cells called T cells, identify these antigens and attach to them. The T cells become stimulated and release pro-inflammatory cytokines, such as interferon gamma (IFNγ) and tumour necrosis factor (TNF) and additional as yet unknown complementary factors that are present in infected serum (Mendis *et al*, 1990; Stevenson and Riley, 2004; Sun *et al*, 2012) that encourage further cell activation like CD8+ T lymphocytes. Some of the disease symptoms are a result of the body's response to the malaria parasite. For example, malaria fever is related to high levels of TNF. This cytokine is released by macrophages when infected red blood cells break down, and large numbers of parasites go into the blood stream (Trampuz *et al*, 2003). Furthermore malaria anaemia is caused when the red blood cell ruptures, leading to lower haemocrit and possibly severe anaemia (Beare *et al*, 2006).

1.2.5 Thrombospondin related anonymous protein (TRAP) and Circumsporozoite protein (CS)

In malaria there are a number of proteins required for infectivity, motility and invasion (Elsworth *et al*, 2014). Malaria sporozoites have the specific ability to invade two entirely different types of target cell in the mosquito vector and the vertebrate host during the course of the parasite's life cycle (liver stage) (Hodgson *et al*, 2014). Two sporozoite proteins, circumsporozoite protein (CS) and thrombospondin-related adhesive protein (TRAP), have been shown to play important roles in the invasion of both cell types (Soldati, Foth and Cowman, 2004; Combe *et al*, 2009). CS protein is a

multifunctional protein that is involved in sporozoite development in mosquitoes, and invasion into mosquito salivary gland and into human liver cells (Nussenzweig and Nussenzweig, 1985).

Thrombospondin-related anonymous protein (TRAP) is one of the main surface proteins of *P. falciparum* (Robson, Kathryn JH *et al*, 1988). It is located on chromosome 13 and conserved in all *Plasmodium* species. It is necessary for sporozoite gliding on substrates and within tissues and cell invasion (Sultan *et al*, 1997; Combe *et al*, 2009). Furthermore, it is localized to the parasite micronemes (Tomley and Soldati, 2001). TRAP is also released onto the substrate during gliding movement (Sultan *et al*, 1997). The structure of TRAP protein contains two conserved adhesive domains: a von Willebrand factor A-domain ('A-domain') (McCormick *et al*, 1999) and a thrombospondin type I repeat (TSP) (Tucker, 2004) . There is a stretched repeat region located in the centre of protein structure (Gantt *et al*, 2000) (Figure 1-2).

The other most important protein on the sporozoite surface is circumsporozoite (CS) (Sinnis *et al*, 1996). It is located on chromosome 3. CS is comprised of a central repeat region that is diverse across Plasmodium species, and flanking the repeats are two conserved domains: region I, at the N terminus of the repeats, and a known cell-adhesive motif C-terminal to the repeats termed the type I thrombospondin repeat (TSR) or region II (Figure 1-2) (Peterson *et al*, 2002; Bermúdez, Vanegas and Patarroyo, 2008). Region II is similar to the type I repeat of thrombospondin (TSR) (Lawler and Hynes, 1986). The TSR is a ~ 60 residue-long module found in a superfamily of adhesive proteins that includes thrombospondin and properdin (Robson, KJ *et al*, 1995). Due to high immunogenicity of CS and its essential role in hepatic cells invasion, this molecule has been considered an excellent target for antimalarial vaccine development (Kumar *et al*, 2006). Several monoclonal antibodies have been raised against CS of various plasmodia species by different investigators, CS vaccine development initially focused on the central repeated region of CS because this region has immunodominant B cell epitopes (Herrington *et al*, 1987). An immunogen combining the central repeated region (containing the B cell epitopes and the C-terminal, containing the TSR domain, includes T cells epitopes leading to a more effective vaccine (Stoute *et al*, 1997; Nardin, Elizabeth H. *et al*, 2000; Nardin, E. H. *et al*, 2001). Furthermore, monoclonal antibodies

can recognise unfixed *P.falciparum* sporozoites and inhibit sporozoite invasion of HepG2 (hepatic) cells (Plassmeyer *et al*, 2009a).

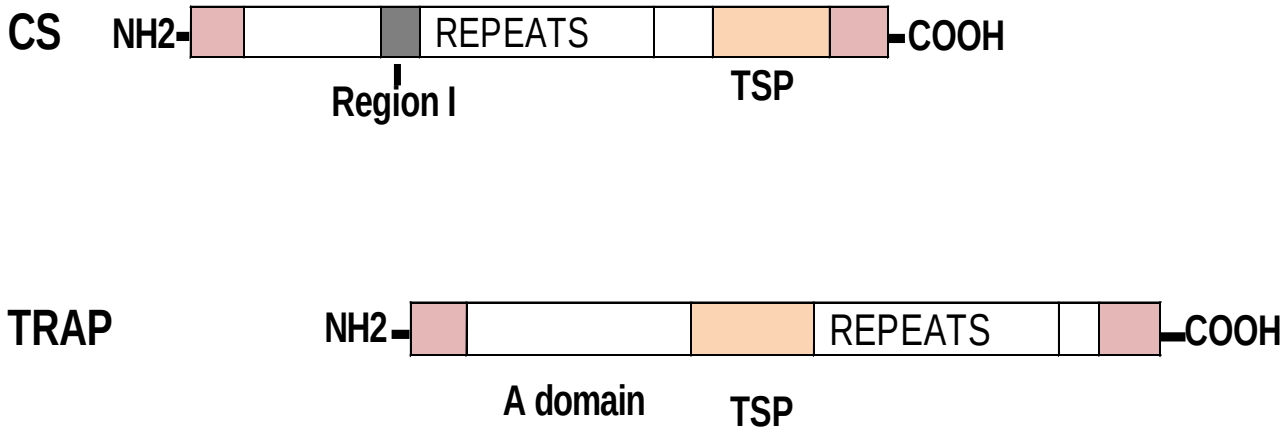


Figure 1-2: Schematic representation of the CS and TRAP proteins

The CS protein starts with a leader sequence and ends by a hydrophobic sequence (pink boxes). CS contains a central repeat domain flanked by two conserved regions, region I and a thrombospondin type I repeat (TSP) region II. The TRAP protein is a type I transmembrane protein containing an A domain, a TSP and a repeat region.

1.3 Complement system

The complement system is part of the body's immune system and consists of a series of protein molecules (Zipfel and Skerka, 2009; Chen, Daha and Kallenberg, 2010). These proteins exist in the blood stream and in the tissue fluid. They are activated when harmful microorganisms enter the body (Walport, 2001).

The complement system contains more than 35 humoral and cell-associated proteins forming three converging enzymatic cascades: the classical pathway, the lectin pathway and the alternative pathway (Figure 1-3); all three pathways lead to the cleavage of C3, the central component of complement, which functions as an opsonin, labelling pathogens and immune complexes for recognition and removal, mediated by specific complement receptors on phagocytic cells (Reid and Porter, 1981; Taylor, Philip, Botto and Walport, 1998a; Carroll, 2004). The most important opsonin of the complement system is C3b (Ajees *et al*, 2006). C3b plays an important role in the formation of the C3 convertase (Ariki *et al*, 2008). In the alternative pathway the C3 convertase is (C3bBb), while in classical and lectin pathways it is C4b2a. Both C3 convertases stimulate the proteolytic cleavage of C3 into C3a and C3b (hence the name "C3-convertase") (Zipfel and Skerka, 2009). The formation of the C3 convertases is the central event of the complement system which leads to stimulation of the rest of the complement cascade and creation of the macromolecular membrane attack complex and cytolysis (Pangburn, M. K., Schreiber and Muller-Eberhard, 1981). C3b is in turn a part of the C5 convertase that cleaves C5 which eventually leads to the formation of the Membrane Attack Complex (MAC) which is the final stage in the complement system (Pangburn, M. K., Schreiber and Muller-Eberhard, 1981; Rawal, Rajagopalan and Salvi, 2008). There are other functions for the complement system like, the complement system clears immune complexes from the circulation and deposits them in the spleen and liver, and the complement proteins, C5a, C4a, and C3a (the anaphylatoxins) induce acute inflammation by activating mast cells and neutrophils. When these complement proteins bind to mast cells, it will release of vasoactive mediators such as histamine (Kinoshita, 1991; Markiewski *et al*, 2009).

1.3.1 Complement pathways

1.3.1.1 The classical pathway (CP)

This pathway involves complement components C1, C2 and C4. The pathway is activated by antibody-antigen complexes (immune complexes) binding to C1, which itself has three subcomponents C1q, C1r and C1s (Kang *et al*, 2009). When activation happens, C1r is auto-activated and it cleaves and activates its only substrate C1s. Then activated C1s cleaves C4 into C4a (9 kDa) and C4b (185 kDa). In a second cleavage step, C1s cleaves C4b-bound C2 into C2a (60 kDa) and C2b (30 kDa). C4a and C2b are released into the microenvironment, while C4b and C2a stay together bound to the complement activating surface (immune complexes or microorganisms) to form the C3 convertase C4b2a (Sim *et al*, 1993; Zundel *et al*, 2004; Schwaeble, W. J. *et al*, 2011). The C3 convertase, C4b2a, splits C3 into two portions; the large portion, C3b, can covalently attach to the surface of microbial pathogens and opsonise them; the small portion, C3a, stimulates mast cells, causing the release of vasoactive mediators such as histamine (Venkatraman Girija *et al*, 2013) .

1.3.1.2 The lectin pathway (LP)

The lectin pathway has similar steps to the CP but differs in its activation (Holmskov *et al*, 1994). The lectin pathway is activated by the binding of mannose-binding lectin (MBL) (Turner, 1996), or one of several ficolins (M, L and H) (Matsushita *et al*, 1996; Endo, Matsushita and Fujita, 2007) or the recently discovered collectin 11(Selman and Hansen, 2012) to polysaccharides on pathogen surfaces(Turner, 1996). The binding of MBL to mannose residues on the pathogen surface activates the MBL-associated serine proteases, MASP-1, and MASP-2, which lead to the cleavage of C4 and C2. C4 and C2 form the C3 convertase, C4b2a, which converts the abundant plasma protein C3 into C3a and C3b; C3b is the main opsonin of the complement system (Frederiksen *et al*, 2005; Ali *et al*, 2012). Ficolins have a similar way of functioning via MASPs (Matuschewski *et al*, 2002). As the lectin pathway doesn't need specific antibody molecules for activation, it is suggested that lectin pathway of complement activation may be one of the important innate defence mechanisms. MBL recognizes a broad range of clinically significant bacteria, viruses, fungi, and parasites (Endo, Takahashi and Fujita, 2006).

1.3.1.3 The alternative pathway (AP)

The alternative pathway involves many factors, B, D, H & I which interact with each other, and with C3b, to form a C3 convertase, C3bBb, that can catalyse more C3 activation (Zipfel and Skerka, 2009). Hence the pathway also acts as an amplification loop, producing more C3b, whichever pathway was initially activated. Activation of the AP is promoted in the presence of bacterial and fungal cell walls, but is inhibited by molecules on the surface of normal mammalian cells (Zipfel and Skerka, 2009). The C3 convertase is stabilized by attachment of a fluid phase regulator, properdin, to C3 and C3b. Alternative pathway activation begins with the spontaneous hydrolysis of C3. This hydrolysis of C3 changes the conformation of C3 and allows the binding of factor B to form a C3 (H_2O) B zymogen complex. Subsequently factor B can be cleaved by factor D to form the C3 convertase C3 (H_2O) Bb, which converts C3 to C3a and C3b (Thomas *et al*, 2000) C3b binds to the surface of pathogens where is serves as an opsonin enhancing the uptake of pathogens by C3 receptor bearing cells. Membrane bound C3b can also bind factor B to initiate the generation of a C3 convertase complex (Fearon and Austen, 1975a; Pangburn, MK and Muller-Eberhard, 1986; Ferreira, Cortes and Pangburn, 2010). C3bBb is a transient complex with a half-life of approximately 90 seconds (Medicus, Gotze and Muller-Eberhard, 1976). Properdin binds to this complex, increasing the half-life by forming a relatively stable complex, C3bBbp (Fearon and Austen, 1975a). The activity of the C3 convertase is regulated by several serum proteins, including properdin, which stabilises it, and factor H, which has the opposite effect. Activation of any of the pathways finally leads to the formation of a C5 convertase, which starts the lytic pathway. C5b attaches to the activating surface then C6, C7, C8 and C9 unite with C5b to form C5b-9, the membrane-attack complex (MAC), which mediates cell lysis and death of some targets (Taylor, Philip, Botto and Walport, 1998b; Chen, Daha and Kallenberg, 2010).

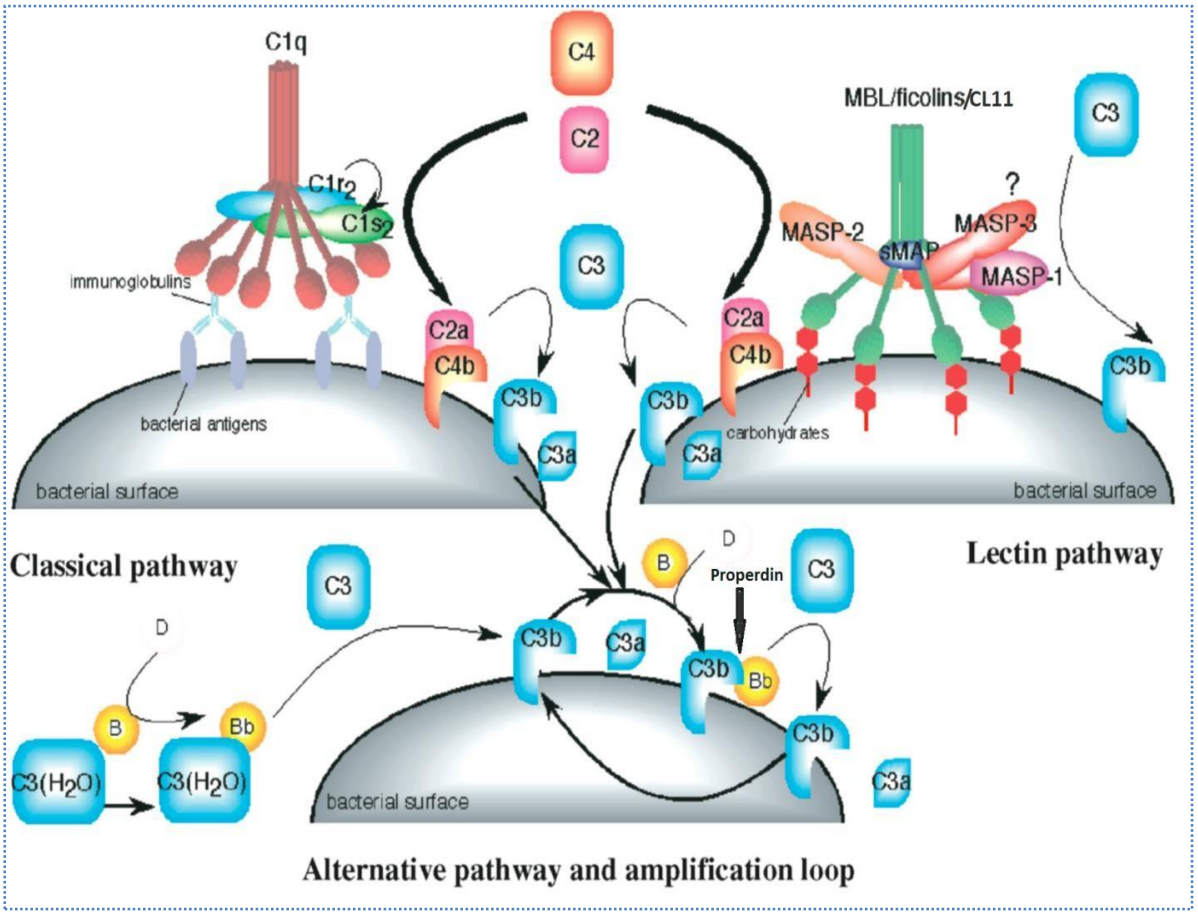


Figure 1-3: Three pathways of the complement system: Classical, Lectin and Alternative pathways (CP, LP and AP).

A schematic presentation of different complement activation pathways (the classical pathway, the lectin pathway and the alternative pathway). Classical pathway activation is initiated by the binding of the C1 complex to antibodies bound to antigen on the pathogen surface. Within CP activation, C1r cleaves C1s. Upon activation C1s cleaves C4 to C4b. C1s then cleaves C4b2 to yield the C3 convertase (C4b2a). Lectin pathway is initiated by binding of either of MBL or ficolin, associated with MASP-1, MASP-2, and MASP-3. As with C1s, MASP-2 is responsible for the C4 and C2 activation, leading to generation of the same C3 convertase as the classical pathway. The alternative pathway is initiated by hydrolysed C3 (H_2O) and activated factor B (Bb). The activated C3b binds factor B which is cleaved into Bb by factor D to form the alternative pathway C3 convertase, C3bBb. (Fujita, 2002)

1.3.2 Regulation of complement system

The complement system must be tightly regulated to prevent injury to self-cells, and this is achieved by a system of both cell-surface regulators and fluid phase regulators (that recognise ligands on the surface of host cells). This prevents self-tissue from damage by unwanted complement activation, and allows activation of the complement system on hazardous surfaces, including pathogens and apoptotic cells.

1.3.2.1 Factor H

Factor H is the main fluid phase regulator of the complement system, also known as complement control proteins. It is the main inhibitor of the alternative pathway C3 convertase, C3bBb. It is a soluble glycoprotein that circulates in human plasma at typical concentrations of 200–300 micrograms per millilitre and a size of nearly 155 kDa. Factor H accelerates the decay of C3bBb and is a co-factor for the factor I mediated decay of C3b to iC3b, which can no longer form a C3 convertase. It distinguishes between self- and non-self surfaces by recognizing specific markers on host cells such as glycosaminoglycans (e.g. heparin) and sialic acid (Schwaeble, Wilhelm *et al*, 1987; Ferreira, Pangburn and Cortés, 2010; Ricklin *et al*, 2010).

1.3.2.1 Properdin

Properdin or factor P is a positive regulator of complement activation that stabilizes the alternative pathway C3 convertase and lectin/classical pathway C5 convertases (i.e. C3bBb(C3b)n and C4b2a(C3b)n, respectively). Properdin, a 53 kDa plasma protein, is found in the blood serum at 5-15 μg/ml (Schwaeble, Wilhelm J. and Reid, 1999; Ferreira, Cortes and Pangburn, 2010). Properdin encourages the binding of C3b with factor B and provides a main point for the association of C3bBb on a surface. In addition, properdin binds to alternative pathway C3convertase, which is quite an unstable product (its life expectancy about 90 seconds) and leads to a rise in life expectancy by making a comparatively stable complex, C3bBb properdin (C3bBbP) (Farries and Atkinson, 1989). In this manner, properdin expands the intensification part of the alternative pathway by tying more C3b to shape more steady complexes (Fearon and Austen, 1975b; Hourcade, 2006). Therefore, properdin and factor H have opposite functions as factor H accelerates the breakdown of C3bBb and inactivates C3b to iC3b

(Skerka and Zipfel, 2008), whilst properdin has the opposite effect stabilising the C3bBb complex (Bhakdi and Tranum-Jensen, 1991).

1.3.2.2 Factor I

Factor I is one of the important fluid phase regulators of C3. It is a serum glycoprotein of 88 kDa and is concentration about approximately 35 µg/mL (Nilsson *et al*, 2011). Factor I controls all the complement pathways by inactivation of C3b and C4b (Davis III, Mejia and Lu, 2008). This protease inactivates complement components C3b with the contribution of the cofactor factor H, and C4b with the contribution of the cofactor C4BP (Seya *et al*, 1995).

1.3.2.3 C4b-binding protein (C4BP)

C4 binding protein (C4BP) is an inhibitory protein that is an essential regulator of the complement system for both the classical and lectin pathways, and is present in plasma at a concentration of 200 µg /ml (Blom, Villoutreix and Dahlbäck, 2004). In CP and LP the role of C4BP is inhibition of C3 convertase formation by binding to C4b and preventing C2a binding. Moreover, C4BP is a cofactor for serine protease factor I, which cleaves C4b and C3b (Jurianz *et al*, 1999).

1.3.2.4 Complement receptor CR1

There are a number of surface proteins exhibited by host cells which prevent further activation of complement and formation of MAC such as complement receptor 1 (CR1) (Zipfel and Skerka, 2009). CR1 is encoded by the CR1 gene on chromosome 1(Rey-Campos, Rubinstein and Rodriguez de Cordoba, 1988). CR1 accelerates the decay of C3 and C5 convertases (Medof *et al*, 1982), and serves as a cofactor in the Factor I-mediated inactivation of C4b and C3b to C4bi and C3bi (Medof and Nussenzweig, 1984; Naik, Sharma and Quigg, 2013). It is found on the surface of many cells such as erythrocytes, white blood cells and visceral epithelial cells (Podocytes) (Ahearn and Fearon, 1989).

1.3.3 *P. falciparum* and complement activation

Some researchers demonstrated that deposition of the Membrane Attack Complex (MAC) increases during malarial infections (Roestenberg *et al*, 2007). Complement activation against blood stages may start at the time that infected RBCs rupture, releasing merozoites. Merozoites can survive in non-immune sera, which suggest that they might possess a complement evasion mechanism (Dasari *et al*, 2011; Dasari *et al*, 2012). Some studies showed the phagocytosis of merozoites, but without explaining the role of complement (Hill *et al*, 2013). Therefore, more studies are needed to explain the role of the complement system during malaria.

There are many discussions about which complement pathway is activated by plasmodium. Some studies mention that the classical pathway might be the main pathway through formation antigen (asexual blood stages of malaria)-antibody complexes (Adam *et al*, 1981; Stoute *et al*, 2003). However, *in vitro* studies showed that the alternative pathway may attack food vacuoles of the parasite (Dasari *et al*, 2011; Dasari *et al*, 2012). The MBL-activated lectin pathway was also implicated, as MBL bound to parasitic proteins (Klabunde *et al*, 2002; Garred, P. *et al*, 2003). The importance of MBL in malarial infections was shown in some infected Africans, as the risk of malaria was increased in patients who carried a particular MBL deficiency allele (MBL2 C) that leads to decreased serum levels of MBL (Lipscombe *et al*, 1992; Madsen *et al*, 1998). However, other researchers could not confirm the association between MBL deficiency and the infection (Garred, P. *et al*, 2003; Thevenon *et al*, 2009). This could be because MBL deficiency renders the patient slightly more susceptible to disease, but the severity is lower because they do not get the MBL-driven component of the inflammatory response, so fever and other symptoms are lower.

The differences between the results of the research could arise from many factors, including the design of the experiments, geographical differences, and the gender and ages of the study population. A clear example is the opposite results of Garred *et al*. (2003) and (Holmberg *et al*, 2008). Garred *et al*. (2003) found that in the seasonally infected places of Ghana, there is no relation between the low concentration of MBL and sever malaria, while Holmberg *et al*. (2008) mentioned that children with MBL deficiency in hyperendemic regions are more susceptible to malaria.

The role of complement system in malaria infections was studied *in vivo*. Two experiments showed no relationship between complement activity and parasitaemia (Atkinson *et al*, 1975; Ramos, Bullard and Barnum, 2012). In the first, parasitaemia in Rhesus monkeys infected with *Plasmodium coatneyi*, was not changed when the complement was depleted by adding cobra venom factor. The same result was shown in C3-depleted infected mice with *Plasmodium berghei* compared with wild type mouse serum. However C1q deficient mice infected with *Plasmodium chabaudi* showed a significant increase in parasitaemia (Taylor, P. R. *et al*, 2001), and *P. berghei* infected rats with complement deficiency had high parasitaemia and mortality rate (Ward *et al*, 1981). Furthermore, there are other studies that have shown that the complement cascade is activated during malaria infection. Both humans studies and animal models report evidence of general complement activation, either reduced serum C1, C2, C3, C4, and C1q levels (Biryukov and Stoute, 2014), or increased TCC (sC5b- 9), in malarial paroxysms(Roestenberg *et al*, 2007).

There are several types of surface proteins expressed by *P. falciparum* that bind to complement regulatory proteins on erythrocytes or in plasma and thus can control or inhibit host complement activation (Zipfel, Würzner and Skerka, 2007). Asexual blood stages of *P. falciparum* can express *P. falciparum* Glideosome Associated Protein-50 (PfGAP50), which can bind to factor H promoting the inhibition of the alternative pathway (Simon *et al*, 2013).

1.4 Distinguishing self and non-self-targets

It is vital that the complement system can distinguish between self and non-self surfaces to prevent autoimmune attack (Meri *et al*, 2013). Some pathogens have different strategies to avoid complement killing by mimicking host cells, which ensure that the immune system cannot recognise them as strangers, thus dealing with them as self-structures. Understanding these mechanisms can be helpful in producing new vaccine to enhance the immune killing of pathogens (Serruto *et al*, 2010).

1.4.1 Role of the lectin pathway in distinguishing self- and non-self surfaces

The lectin pathway could be mediated by the binding of MBL and specific carbohydrate structures on the pathogen surfaces (Garred, Peter *et al*, 2006). The MBL discrimination depends mainly on CRD recognition to molecular pathogens patterns including mannose, N-acetylglucoseamine (GlcNAc) or focus in the presence of Ca^{++} (Eisen and Minchinton, 2003; Thiel and Gadjeva, 2009a; Takahashi, 2011). The oligomerization state of MBL and the antigenic surface patterns structure are the main factors in the MBL-mediated lectin pathway (Gadjeva, Takahashi and Thiel, 2004). However, in the self-cells, surface pattern structures are hidden by sialic acid and not exposed to MBL except in the case of cell alteration such as during apoptosis (Gadjeva, Takahashi and Thiel, 2004). Many bacteria, fungi and viruses activate the lectin pathway by MBL mediated mechanisms like *Escherichia coli, Neisseria meningitidis, Neisseria gonorrhea, Salmonella typhimurium, Pseudomonas aeruginosa, Haemophilus influenzae, Staphylococus aureus, Human Immunodeficiency* Virus (HIV), Herpes Simplex Virus (HSV), Influenza A Virus (IAV), *Saccharomyces cerevisiae*, *Candida albicans* and *Aspergillus fumigatus* (Thiel and Gadjeva, 2009b).

1.4.2 Resistance of microbes to complement attack

Some pathogens developed strategies to escape from the effects of the complement system through interfering with the self and non-self-discrimination mechanisms. Mimicking factor H SCR domains is one of these strategies. There are a lot of examples of recruitment of factor H by different kinds of bacteria such as *Borrelia burgdorferi spirochetes*, the etiological cause of the Lyme disease (Alitalo *et al*, 2002), *Neisseria meningitidis* and *Streptococcus pneumonia* (Dave *et al*, 2004; McNeil *et al*, 2013) by expressing specific types of surface proteins, which can bind to factor H and so inhibit complement activity. Another example is *P. falciparum* which could recruit factor H by expressing surface protein; *P. falciparum* Glideosome Associated Protein-50 (PfGAP50), which could bind also to factor H (Simon *et al*, 2013).

1.5 Hypothesis:

Numerous human pathogens have evolved strategies to control or inhibit complement activation, often by sequestering or mimicking host complement regulatory proteins. The gamete stage of *P. falciparum* expresses a surface protein (PfGAP50) that binds complement factor H, a fluid-phase regulator, which limits the activity of the C3 convertase (C3bBb) or (C4b2a) (Kennedy *et al*, 2016). Two other surface components of *P. falciparum*, thrombospondin-related anonymous protein (TRAP) and circumsporozoite protein (CS), both of which are implicated in immune evasion by malaria, contain highly conserved thrombospondin domain motives (TSP domains), structural motifs also found in thrombospondin-1 and properdin (Robson, Kathryn JH *et al*, 1988). Our laboratory has previously expressed recombinant human thrombospondin-1 and characterised its biological effect on various cells and on the functional activity of the complement system. Thrombosponin-1 binds to complement factor B and C3b bound factor B with much higher affinity than recombinant properdin (unpublished data).

Thrombospondin acts as an effective competitive inhibitor of properdin-dependent complement activation, binding firmly to the same target complex as properdin. My hypothesis is that the TSP domain containing Plasmodium proteins has a similar activity, inhibiting host complement activation and thus protects the parasite from complement attack.

1.6 Aim and Objectives

This work aims to increase the understanding of how *P.falciparum* protects itself from complement attack. My task was to explore to what extent the TSP domains in sporozoite surface proteins protect the parasite from complement attack.

My specific objectives are therefore:

I. To produce recombinant of TRAP and CS fragments.
II. Study the effect of recombinant TRAP and CS proteins on complement activation *in vitro.*
III. Test the role of monoclonal antibodies that block CS on the parasite to restore complement activation.

2 Chapter two

Materials and Methods

2.1 Materials

This section provides information about the commercial chemicals and media materials, buffers, kits, vectors, antibodies and proteins used for the current study.

2.1.1 Chemical materials

Material	Supplier
1kb plus DNA ladder	Invitrogen
2-mercaptoethanol	Sigma-Aldrich
6X DNA loading dye	Thermo Scientific
Acetic acid glacial	Fisher Scientific
Acrylamide bis	Sigma-Aldrich
Agarose, electrophoresis grade	Melford
Ammonium persulphate	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Arginine	Sigma-Aldrich
Barbital	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Brilliant Blue R-250	Fisher Scientific
Bromophenol Blue	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Deoxyadinosine triphosphate (dATP)	Thermo Scientific

Deoxyribo nucleotide PCR grade (dNTPs)	Promega
D-galactopyranoside (Xgal)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Ethanol	University of Leicester, Chemistry Department
Ethidium bromide	Sigma-Aldrich
Ethylene glycol-bis-*N*, *N*, *N', N'*-tetraacetic acid (EGTA)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal bovine serum	Invitrogen
Fuji X-ray film	Fisher Scientific
Gelatine	Serva
Glycerol	Fisher scientific
Glycine	Fisher scientific
Heparin sodium salt	Sigma-Aldrich
High fidelity polymerase	New England Biolabs
HisGravi Trap column	GE Healthcare
Hygromycin B	Invitrogen
Isopropanol	Sigma-Aldrich
Imidazole	Sigma-Aldrich
Lipofectamine™ LTX reagent	Invitrogen

Luminata crescendo Western HRP substrate	Millipore
Magnesium sulphate	Sigma-Aldrich
Manganese chloride	Sigma-Aldrich
Mannan	Sigma-Aldrich
Methanol	University of Leicester Chemistry Department
Oxidized Glutathione	Sigma-Aldrich
Penicillin/0.1 mg/ml streptomycin	Sigma-Aldrich
Potassium acetate	Sigma-Aldrich
Potassium chloride	Sigma-Aldrich
Potassium phosphate	Sigma-Aldrich
Potassium phosphate an hydrous	Sigma-Aldrich
Purified BSA 100X	New England BioLabs
Reduced Glutathione	Sigma-Aldrich
Sample Buffer, Laemmli 2× Concentrate	Sigma
Sigmafast p-Nitrophenyl phosphate tablets	Sigma-Aldrich
Skimmed milk	Sigma-Aldrich
Sodium azide	Sigma-Aldrich
Sodium bicarbonate	Sigma-Aldrich
Sodium carbonate, an-hydrous	Fisher Scientific

Sodium chloride	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium hydrogen carbonates	Fisher Scientific
Superose 6 increase 10/300 GL column	GE Healthcare
T4 DNA ligase	New England Biolabs
T4 DNA ligase 10X buffer	New England Biolabs
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Thermoprime plus DNA polymerase	Thermo Scientific
Tris-HCl	Sigma-Aldrich
Triton X-100	BDH laboratory
Trizma base	Sigma-Aldrich
Trypsin-EDTA	Sigma-Aldrich
Tween-20	Sigma-Aldrich
Urea	VWR international LTD
Zymosan	Sigma-Aldrich

2.1.2 Complement proteins

	Supplier
Human C3b	Complement technology

2.1.3 Antibodies

Antibody	Supplier
FITC Polyclonal rabbit anti human C3c antibody	Dako
Goat anti-rabbit IgG alkaline phosphatase Conjugated antibody	Sigma-Aldrich
Goat Polyclonal IgG COLLECT 11	Santa Cruz
Mouse anti-polyhistidine antibody	Sigma-Aldrich
Monoclonal mouse anti-polyhistidine	Sigma-Aldrich
Monoclonal mouse IgG L-ficolin	Santa Cruz
Monoclonal mouse IgG MBL-C	Santa Cruz
Monoclonal mouse anti-polyhistidine conjugated with HRP (Horse Reddish Peroxidase)	Sigma-Aldrich
Polyclonal rabbit anti-mouse immunoglobulins with HRP (Horse Reddish Peroxidase)	Dako
Rabbit anti-Goat IgG alkaline phosphatase Conjugated antibody	Sigma-Aldrich
Rabbit anti-human C3c	Dako
Rabbit polyclonal anti-mouse alkaline-phosphatase conjugated	Sigma-Aldrich

2.1.4 Kits	Supplier
BamH I restriction enzyme	New England BioLabs
Enterokinase EKMax™	Invitrogen
pGEM-T easy vector	Promega
Pierce™ Coomassie Plus (Bradford) Assay Kit	Thermo Fisher Scientific
pRSET A	Thermo Fisher Scientific
pRSET B	Thermo Fisher Scientific
pSecTag 2/ Hygro A	Thermo Fisher Scientific
pSecTag 2/ Hygro B	Thermo Fisher Scientific
QIAquick gel extraction kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Wizard plus SV minipreps DNA purification kit	Promega
Wizard plus SV midipreps DNA purification kit	Promega
XhoI restriction enzyme	New England BioLabs

2.1.5 Blood and sera	Supplier
Guinea pig blood	ENVIGO

2.1.6 Media Medium	Supplier
Agar	Oxoid
CHO-S-SFM II	Gibco
F-12 Nutrient Mix	Gibco
Opti-MEM® I Reduced Serum	Invitrogen
RPMI-1640	Sigma-Aldrich

2.2 Methods

2.2.1 Molecular Biology Techniques

2.2.1.1 Primer Design

In order to amplify four *P.falciparum* TRAP constructs including the TSP domain, N-terminal and C-terminal fragments (including the TSP domain) and full-length sequence, different sets of primers were designed (Table 2-1) based on Genbank accession number XM_001350052. Another set of primers were designed (Table 2-2) to amplify two *P.falciparum* CS constructs the full length and C-terminal (including TSP domain) and these were based on Genbank accession number XM_001351086.

Table 2-1: Primers used for PfTRAP PCR genotyping

Fragment	Primer name	Sequence 5´--> 3´
N-terminal	PFT-NT-BamF	**AGGATCC**TAGAGA TGTGCAAAAC AATATAGTGG A
	PFT-TSP-XhoR	**CTCGAG**C CCG TTT TGG AAG ACA TCT TTC
TSP domain	PFT-TSP-BamF	**AGGATCC**AACAGCAAGTT GTGGTGTTTG GG
	PFT-TSP-XhoR	**CTCGAG**C CCG TTT TGG AAG ACA TCT TTC
C-terminal	PFT-TSP-BamF	**AGGATCC**AACAGCAAGTT GTGGTGTTTG GG
	PFT-CT-XhoR	**CTCGAG**C CCG TTT TGG AAG ACA TCT TTC TTC
Full length	PFT-NT-BamF	**AGGATCC**TAGAGA TGTGCAAAAC AATATAGTGG A
	PFT-CR-XohR	**CTCGAG**C CCG TTT TGG AAG ACA TCT TTC TTC

Table 2-2: Primers used for PfCS PCR genotyping

Fragment	Primer name	Sequence 5´--> 3´
Full length	PFCS-FL-BamF	**GGA TCC** TTA TTC CAG GAA TAC CAG TGC TA
	PFCS-FL-XhoR	**CTCGAG** AAT TAA GGA ACA AGA AGG ATA ATA CCA
C-terminal	PFCS-TSP-BamF	**GGATCC**TCTCTTTCAACTGAATGGTCCCCA
	PFCS-FL-XhoR	**CTCGAG** AAT TAA GGA ACA AGA AGG ATA ATA CCA

*BamH*1 and *Xho*1 restriction sites are in bold.

2.2.1.2 Polymerase Chain Reaction (PCR)

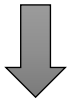
Plasmodium falciparum gDNA was kindly provided by Dr Mahammad Alam, MRC, University of Leicester and used as a template to generate four different constructs encoding for four fragments of TRAP and two of CS protein, as mentioned above. TRAP and CS are single exons genes, so the coding sequence can be amplified from gDNA. Phusion high-fidelity DNA polymerase and a fixed molar concentration of $MgCl_2$ in high-fidelity polymerase phusion buffer 5x and also dNTPs are needed for DNA template production (Thermo Scientific), which were used here to amplify the coding sequences (Table 2-1and Table 2-2). The PCR reaction mixture consisted of the following:

Table 2-3: PCR reaction mixture

Material	Amount
gDNA 10 ng	1 µl
High fidelity phusion buffer (5x)	5 µl
dNTP mix. (10 mM)	0.5 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
Phusion high-fidelity-DNA polymerase (5U/µl)	0. 25 µl
Nanopure distilled water	13.25 µl

The cycling programme was:

Table 2-4: The programme process used to amplify the DNA of TRAP and CS using the thermocycler machine.

Cycle	Temperature/ ºC	Time	Manner
Initial denaturation	95	3 minutes	
Denaturation	95	30 seconds	7 Cycles 
Annealing	72 (-1 ºC / cycle)	30 seconds	
Extending	72	2 minutes	
Denaturation	95	30 seconds	29 Cycles 
Annealing	65	30 seconds	
Extending	72	2 minutes	
Final extending	72	10 minutes	
Cooling down	4	∞	

The products were analysed by 1% agarose gel electrophoresis for the presence of the desired DNA constructs.

2.2.1.3 Purification of the PCR products

The PCR products were purified from the PCR product using QIAquick Nucleotide Removal Kit (QIAGEN, Cat. No 28304) according to the manufacturer's instruction. The purified DNA was analysed in 1% agarose gel.

2.2.1.4 A-Tailing of PCR products

A 3´ terminal A-overhang was added to the purified PCR products. As a result of A-tailing, the T-overhang in the pGEM-T Easy vector (Promega) can be ligated with the PCR product. Taq-polymerase (Thermo Scientific) was used to add A-overhangs to the DNA construct according the following protocol (Table 2-5)

Table 2-5: Protocol used for A-tailing the purified PCR product

Material	amount
100mM dATP	0.5µl
Gel extracted DNA	7µl
Taq-polymerase reaction buffer 10x (containing MgCl2)	2µl
DNA Taq-polymerase	0.5µl

The reaction mixture from the A-tailing was PCR run at 72ºC for 25 minutes.

2.2.1.5 Cloning of the PCR products into pGEM-T Easy Vector

The A-tailed PCR product was ligated into pGEM-T easy (Promega Cat. No. A1360), using T4 DNA ligase. The reaction mixture consisted of 1 µl of pGEM-T Easy vector (50ng/µl) which was then mixed with the insert in a 3:1 molar ratio (insert: vector) (x) µl of the PCR product (x ng), 1 µl of T4 DNA ligase (5 units) (Promega) and 5 µl of T4 DNA ligase buffer (2x) (Promega), after which the ligation reaction mixture was completed to a final volume of 10 µl with Nano pure water. The reaction mixture was then incubated overnight at 4ºC.

2.2.1.6 Preparation of chemically competent cells

Top 10F' *E. coli* was streaked from a frozen stock onto an LB agar plate using a full loop of sterile platinum wire. The plate was incubated for 16h at 37°C.The next day, one colony was transferred to 5ml of LB medium (Table 2-6) containing 20mM $MgSO_4$ and incubated overnight at 37°C. After that, 1ml of the overnight culture was transferred into 100ml of LB broth and incubated at 37°C for 2.5 to 3h until the

OD550nm was 0.7- 0.8. After centrifugation of the culture for 10 minutes at 2000xg, the cells were re-suspended in 30ml of sterile ice-cold TFB1 buffer (Table 2-7) and incubated on ice for 5-30 min. After that, cells were harvested by centrifugation for 10 minutes at 2000xg and 4°C and re-suspended in 4ml of TPF II (Table 2-7), 50 µl single-use aliquots were made and stored at -80°C.

Table 2-6: Media used for bacterial culture

Medium	Composition
Luria-Bertani (LB) broth	1% tryptone (w/v), 0.5% yeast extract (w/v), 0.5% NaCl (w/v).
Luria-Bertani (LA) agar	1% tryptone (w/v), 0.5% yeast extract (w/v), 0.5% NaCl (w/v), 1.5% agar (w/v).

Table 2-7: Buffers used for competent cell preparation

Buffer	Composition
Transformation buffer (Tfb) I, pH 7.4	3 mM $C_2H_3KO_2$, 50 mM $MnCl_2$, 100 mM KCl, 15% glycerol (v/v)
Transformation buffer (Tfb) II, pH 7.4	10 mM Na-MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15% glycerol (v/v)

2.2.1.7 Transformation of Chemically Competent *E. coli* Top10F Strain

Two microliters of the ligation product was transferred into 50 µl of competent *E. coli* (Top10F' strain), mixed gently, and incubated in ice for 20 minutes. Cells were heat shocked at 42°C for 5 minutes and immediately incubated on ice for 2 minutes. Cells were moved to a universal tube containing 450 µl of non-selective medium (LB broth) and incubated at 37C° for 1 hour with gentle shaking. Meanwhile, 40 µl of 20 mg/mL X-gal and 40 µl of 0.1 M IPTG were spread on an LB agar plate containing 100 µg/ml of ampicillin. Chemically transfected competent cells were streaked onto the LB agar plates and incubated overnight at 37°C. The next day, the white colonies were selected and inoculated in 5 ml of LB broth medium containing 100 µg/ml of ampicillin and incubated for overnight at 37°C with gentle shaking. The next day, 5 ml culture was used for Miniprep.

2.2.1.8 Mini-scale Isolation and Purification of Plasmid DNA (vector) (Miniprep)

Plasmid DNA was isolated and purified by using a Wizard plus SV Miniprep DNA Purification system (Promega). 5 ml of the overnight culture was centrifuged for 10 minutes at 13,000 xg and all the other steps were done as the instruction book of kits.
The purified plasmid DNA was digested with *BamH1* and *Xho1* restriction enzymes for 2 h at 37°C in a water bath, and the digested products were analysed in agarose gel.

2.2.1.9 Midi-scale Isolation and Purification of Plasmid DNA (vector) (Midiprep)

The QIAfilter plasmid Midi kit (QIAGEN) was used for large scale purification of plasmid DNA from the competent cells (TOP10F' *E.coli*). The plasmid DNA pellet was reconstituted or dissolved in a desired volume (500 µl) buffer TE (Promega) or sterile deionized water for transfection usage.

2.2.1.10 DNA purification from agarose gel (band prep)

In some cloning steps was necessary to purify DNA that had been separated on agarose gels, e.g. to isolate digested cDNA inserts from a cloning vector. To do this, the desired bands were identified using a UV transluminator and cut out using a sterile, sharp

scalpel. DNA was then purified from the gel using the QIAquick Gel Extraction Kit (QIAgen), according to the supplier's instructions.

2.2.1.11 Restriction digestion of Plasmid DNA

BamHI and *XhoI* restriction enzymes were used to digest the DNA constructs from pGEM-T easy as per the following:

Table 2-8: Protocol used for DNA digestion from pGEM-T easy vector

Material	Amount
DNA sample 1 µg	3 µl
Restriction enzyme buffer 2 10X	2 µl
BSA 10 µg/µl	2 µl
Restriction enzyme 1 10 µg/µl	1 µl
Restriction enzyme 2 10 µg/µl	1 µl
Deionized distilled water	11 µl

The reaction mixture was incubated at 37 °C for 2 hours.

The digested products were run on 1% agarose gel to detect the desired DNA.

2.2.1.12 Sub cloning the DNA constructs into the expression *vectors* pSecTag 2/ Hygro and pRSET

The DNA constructs for all TRAP fragments in the pGEM-T easy vector (Promega) were digested by the same restriction enzymes and evaluated by agarose gel electrophoresis. Then, the purified DNA constructs were ligated into pSecTag2/Hygro A and pRSET-B vectors that had also been linearized with the same enzymes (Invitrogen) (Table 2-9).The ligated constructs were transformed into Top10F' *E. coli* and the plasmid DNA. DNA midipreps prepared as described above. Before transfection of plasmids into prokaryotic cells and mammalian cells for protein expression, all DNA constructs were sequenced at The University of Leicester (PNACL), to ensure no mutations had occurred during the PCR amplification steps or due to the effect of UV-light. CS DNA constructs were sub cloned into the expression vectors pSecTag 2/Hygro B and pRSET A using the same procedures.

Table 2-9: The protocol for sub-cloning TRAP and CS constructs into pSecTag 2/ Hygro A, B and pRSET B, A vectors

Material	Amount
pSecTag 2/ Hygro A,B or pRSET B,A	1 µl
10X ligase Buffer	1 µl
Purified DNA	3 µl
T4 DNA ligase	1 µl
Deionized water to a final volume of	10 µl

2.2.2 Protein Expression and Analysis Methods

Two systems were used for protein expression, a mammalian and a bacterial system (CHO-K1 cell and BL21 (DE3) pLysS cell).

2.2.2.1 Transformation and Protein Expression in Prokaryotic Cells

Transformation of competent *E. coli* BL-21 (DE3) pLysS carrying the desired pRSET B and pRSET A construct was performed using chemical transformation (Hanahan, 1983). The cells were removed from a -80°C freezer and thawed slowly on ice. Then, 2 µl of the plasmid DNA were added to 50 µl of the competent cells mixed gently and incubated in an ice bath for 20 minutes. After that, the cells were heat shocked in a water bath at 37°C for 4 minutes and then again transferred into ice for a further 2 minutes, then 50 µl of transformation mixture were added to 450 µl of LB broth (without any antibiotic) in sterile culture tubes and incubated at 37°C for 1-2 hours with gentle shaking, at 220 rpm. Two different volumes, 70 µl, and 300 µl were placed on an LA plate containing ampicillin, 50 µg/ml, and chloramphenicol 35 µg/ml, final concentrations and the plate was incubated overnight at 37°C. The next day, one colony was picked using a sterile loop then inoculated into a universal tube containing10 ml of LB medium, and incubated at 37°C overnight with shaking at 220 rpm. To achieve the protein expression, 1000 ml of LB medium containing 50 µg/ml ampicillin and 35 µg/ml chloramphenicol were inoculated with BL21 (DE3) pLysS carrying pRSET B construct or pRSET A and incubated until the OD600 reached 0.6–0.7, at which time protein expression was induced by addition of IPTG to a final of concentration of 1mM. After 5 h incubation, bacteria were harvested by centrifugation (4000 rpm for 10 min). After that, lysates were centrifuged, and the pellets stored at -80°C.

2.2.2.1.1 Soluble protein purification

A cell pellet of C-terminal, TSP and full-length TRAP was taken from -80 ºC storage and melted on ice. The cell pellet was then re-suspended in 25 ml of 1xTBS (50 mM Tris–Cl, 150 mM NaCl, pH 7.6) containing 1 mM Pefabloc (Roche). Cells were lysed by three cycles of freezing, thawing and sonication and the lysate cleared by centrifugation. After sonication, the lysate was centrifuged at 20,000 xg for 30 min and the supernatant collected. The lysate was loaded into a 5 ml HisGravi Trap column (GE Healthcare). The column was washed with 10 ml of 1xTBS containing 25 mM imidazole and the protein eluted using 2ml of elution buffer (1, 2, and 3 Table 2-10)containing different concentrations of imidazole; 100 mM, 200 mM and 400 mM The protein fractions were then analysed via SDS-PAGE and confirmed by western blotting.

Table 2-10: Buffer used for eluting the C-terminal, TSP domain and Full-length TRAP protein

Buffer	Composition
Elution buffer 1, pH 7.6	50 mM Tris–Cl, 150 mM NaCl and 100 mM Imidazole
Elution buffer 2, pH 7.6	50 mM Tris–Cl, 150 mM NaCl and 200 mM Imidazole
Elution buffer 3, pH 7.6	50 mM Tris–Cl, 150 mM NaCl and 400 mM Imidazole

2.2.2.1.2 Solubilisation of inclusion bodies and recombinant protein refolding process

Once the expression of the protein is complete for the N-terminal of TRAP and C-terminal and full-length CS, the cell pellet was taken from storage at -80º C and defrosted on ice. It was then dissolved in 20 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl). This was followed by sonication (amplitude 8, macro-probe; 8 to 9 pulses, 30 seconds per pulse with 1-minute break between each) and then centrifugation at 20,000 xg, 4º C, for 20 minutes. The lysate was collected and supernatant was discarded.

The pellet was then re-suspended in 20 ml of washing buffer (50 mM Tris-HCL pH7.4, 150 mM NaCl, 1M Urea) then centrifuged at 20,000 g, 4º C, for 20 minutes to remove soluble impurites. Inclusion body samples were dissolved in a 30 ml solubilisation buffer (50 mM Tris-HCL pH 7.4, 8 M Urea, 150 mM NaCl, and 0.1 mM Pefabloc) on a rotating wheel at 4º C for 20 minutes. After that, the solution was centrifuged at 21,000 xg at 4ºC for 20 minutes. The refolding process was achieved by taking the supernatant and dialysed it against two refolding buffers; the first 2 L refolding buffer (50 mM TrisCl, pH 7.4, 250 mM NaCl, 2 M urea, 0.4 M arginine, 3 mM reduced glutathione, 0.9 mM Oxidized glutathione) was corned out overnight at 4ºC with gentle stirring, then the protein was dialysed again overnight at 4º C against 2 L buffer 2 (50 mM Tris, pH 8, 250 mM NaCl, 0.1 M arginine, 3 mM reduced glutathione, 0.9 mM Oxidized glutathione) overnight at 4º C with gentle stirring to remove the remaining urea. The final dialyzed protein solution was cleared by centrifugation at 21,000 xg at 4ºC for 20 minutes. The refolding protocol above was modified from (Thomson *et al*, 2012).

2.2.2.1.3 Purification with HisGravi Trap column (GE Healthcare)

To facilitate the purification process, the sequence of the DNA construct was designed with a histidine-tag in the N-terminus. HisGravi Trap columns (GE Healthcare) provided rapid purification of the histidine-tagged proteins. After refolding the protein, to obtain maximum purity, the solution containing the refolded protein was run through a HisGravi Trap column (GE Healthcare), allowing the solution to flow through the column by gravity. The column was then washed with 20 ml washing buffer (TBS buffer pH 7.4, containing 25 mM imidazole). Finally, the protein was eluted using 3 ml of the elution buffer (TBS buffer pH 7.4, containing 200 or 400 mM imidazole). Proteins were analysed on SDS-PAGE and western blot.

2.2.2.1.4 Purification of recombinant C-terminal CS using Gel filtration FPLC

The Superose 6 increase 10/300 GL column from GE Healthcare was used. The principle of gel the filtration column depends on the porous beads that are able to separates molecules based upon their molecular weight, as large molecules pass faster than smaller molecules via the beads in the column (Stellwagen, 1990). Therefore, the large molecules emerge first from the column through the external volume while the smaller size proteins access the internal volume through the beads and emerge later. The column was equilibrated with filtered buffer (50 mM Tris-HCl pH 7.4, 145 mM NaCl). When the equilibration process was completed, 500 µl of the concentrated protein (C-terminal of CS), after refolding process (2.2.2.1.2) and protein purification with HisGravi Trap column (see section2.2.2.1.3), was injected into the loading loop and the sample run over the column at a flow rate of 1 ml/min. The OD280nm was monitored and recorded, and that fractions (21-23 ml) were collected. Peaks folded protein was compared to an elution profile with standard molecular weights provided by the manufacturer. To confirm the sizes of the eluted fractions, samples were analysed using SDS-PAGE gel

2.2.2.2 Cell culture techniques

2.2.2.2.1 Chinese Hamster Ovary (CHO-K1) cell cultivation

CHO-K1 cells were collect from liquid nitrogen and the freezing medium was removed by suspension of the cells in a Ham's F12 Nutrient Mix, after which cells were centrifuged at 1500rpm for 10 minutes. Cells were cultivated in Ham's F-12 Nutrient Mix, GlutaMax (Gibco) culture medium supplemented with 10% Foetal Bovine Serum (FBS) (Gibco) in the presence 500U/L penicillin / 0.1 mg/ml streptomycin (Sigma-Aldrich) and incubated at 37˚C in CO_2 incubator.

2.2.2.2.2 Transfecting CHO-K1 cells with TRAP and CS construct

When the CHO-K1 culture was approximately 80% confluent, it was split into six well plates and incubated at 37 ºC in a CO_2 incubator until it become 50-80% confluent.

Lipofectamine™ LTX reagent (Invitrogen) was used to transfect CHO-K1 cells with *Plasmodium falciparum* TRAP and CS plasmid DNA. 3 µL of plasmids DNA (500 ng/µL) of C-terminal, N-terminal, TSP domain and full-length of TRAP and C-terminal and full-length CS) were diluted in 100 µL of Opti-MEM I reduced serum medium (Invitrogen). After that 3 µL of Lipofectamine™ LTX were added to the diluted DNA solution. The solution was mixed gently and incubated for 25 minutes at room temperature to form DNA-Lipofectamine™ LTX complexes. Before transfecting the cells with plasmid DNA, the growth medium was discarded and changed with 1500 µL of new growth medium. Then 100 µL of the DNA-Lipofectamine™ LTX mixture were added directly to each well containing cells and mixed by swinging the plate back and forth. Cells were incubated at 37˚C in a CO_2 incubator overnight.

The following day, Ham's F-12 Nutrient Mix serum medium containing 300 µg/mL hygromycin B (Invitrogen) was added into the wells after the transfection mixture had been removed. After 48 hours of transfection, cells were washed three times with phosphate buffered saline (PBS) (pH 7.4, 2 mM KH_2PO_4, 10 mM NaH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) then, 100 µL of trypsin-EDTA (Sigma-Aldrich) were added to each well and the plate placed into an incubator at 37˚C for one minute. The cells were then re-suspended immediately with Ham's F-12 Nutrient Mix serum medium containing 300 µg/mL hygromycin B and distributed into 24 well plates.

When the cells were 80-90% confluent, the serum medium was replaced with serum-free medium (CHO-S-SFM II) (Gibco) supplemented with 300 µg/ml of hygromycin B and 100U penicillin/ 100µg/ml streptomycin. After two days of incubation at 37˚C, the supernatant was collected and protein expression was detected by dot blot.

2.2.2.3 Protein Methods

2.2.2.3.1 Dot blot

Dot blot is a simple technique used for identifying positive clones expressing recombinant protein. After 48 hours of incubation of transfected cells in serum-free medium, the culture supernatant was collected into Eppendorf tubes and spun down to remove cell debris. 30 µl of the culture supernatant were spotted onto nitrocellulose membrane (Bio-Rad) and allowed to dry. The membrane was then blocked for one hour at room temperature with blocking buffer (5% skimmed milk dissolved in 1xTBS buffer) with shaking. Monoclonal mouse anti-polyhistidine HRP conjugated antibodies (Sigma-Aldrich) were diluted to a ratio of 1:10000 in blocking buffer. The diluted antibody was next applied on the nitrocellulose membrane and incubated for another 1 hour with shaking. After washing three times with 1xTBS containing 0.05% Tween-20, luminata crescendo western HRP substrate (Millipore) was added onto the entire surface of the nitrocellulose membrane and the membrane was then exposed to Fuji film (Fisher Scientific) for 1 to 2 minutes.

2.2.2.3.2 SDS Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

The recombinant proteins were studied via SDS-PAGE under reducing conditions. The protein was boiled with loading dye containing SDS and a strong reducing agent (β-Mercaptoethanol) (Table 2-11). The denatured polypeptides bind to SDS and become negatively charged, thus the protein only migrates according to the molecular weight. Hence, by using a marker, PageRuler, (Thermo Scientific) of known molecular weight, the size of the protein can be estimated.

Samples (25 µl) were denatured by heating for 10 minutes at 95ºC (20 µl of 0.1 mg/ml recombinant proteins + 5 µl loading buffer containing β-Mercaptoethanol). Then proteins were loaded onto a 10% or 15% SDS-gel that was prepared as described in (Table 2-12). The gel was run in 1x SDS running buffer (Table 2-11) at 150V for 1 hour. The protein first migrated through a stacking gel of high porosity and was placed as a thin layer on the resolving gel where it was resolved according to molecular weight. The size of the fragments was compared to a broad range molecular weight standard. After that, the SDS-PAG was fixed for 15 minutes with fixing buffer (Table 2-12). Then the gel was stained using Coomassie blue (Table 2-11) for 30 minutes and finally de-staining solution (Table 2-11) was used until the protein bands become visible

Table 2-11: Protocol used to prepare SDS-PAGE buffers

Buffer	Composition
5X SDS protein-loading buffer	5.8 ml Tris-HCl pH 6.8, 2.5 ml glycerol, 0.83g SDS, 1 mg bromophenol Blue
5X SDS protein-loading buffer for reducing protein	0.4 M of 2-mercaptoethanol in 5X SDS protein-loading buffer
Ammonium per-sulphate	10% $(NH_4)_2S_2O_8$ (w/v) in distilled water
Coomassie Blue dye	1.2 g Coomassie brilliant Blue, 50% methanol, 10% acetic acid glacial (v/v), 40% Milli-Q water
De-staining solution	45% methanol, 10% acetic acid glacial (v/v), 45% Milli-Q water
Fixing solution	50% methanol, 10% acetic acid glacial (v/v), 40% Milli-Q water
SDS solution	10% SDS (w/v) in distilled water
Tris solution, pH 6.8	1 M Tris-HCl
Tris solution, pH 8.8	1.5 M Tris-HCl
Tris-Buffer Saline (TBS), pH 7.4	10 mM Tris-HCl, 140 mM NaCl

Tris/glycin/SDS running buffer, pH 8.3	25 mM trizma base, 192 mM glycin, 0.1% SDS (w/v)

Table 2-12: Protocol used to prepare SDS-PAGE gel

Gel concentration	Composition	Amount (ml)
10% SDS-PAGE	30% acrylamide mix	5
	1.5 M Tris (pH 8.8)	3.8
	10% SDS	0.15
	10% ammonium persulfate	0.15
	TEMED	0.006
	H_2O	5.9

Gel concentration	Composition	Amount (ml)
15% SDS-PAGE	30% acrylamide mix	25
	1.5 M Tris (pH 8.8)	12.5
	10% SDS	0.5
	10% ammonium persulfate	0.5
	TEMED	0.002
	H_2O	11.5

Gel concentration	Composition	Amount (ml)
Stacking gel (5%)	30% acrylamide mix	0.83
	1 M Tris (pH 6.8)	0.63
	10% SDS	0.05
	10% ammonium persulfate	0.05
	TEMED	0.005
	H_2O	3.4

2.2.2.3.3 Western blotting

Western blotting is a technique conducted to recognize and confirm the recombinant proteins and their size. The SDS-PAGE-separated proteins were transferred electrophoretically at 300 mA for 90 minutes in transfer buffer onto nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in 1xTBS for 1 hour with shaking. After that, the membrane was washed three times with 1xTBS containing 0.05% Tween-20. Monoclonal mouse anti-polyhistidine HRP conjugated antibodies were diluted to a 1:10000 ratio, and monoclonal mouse anti-circumsporozoite 10B10 or 8C6 to a diluted 1:1000 ratio in blocking buffer were added to the membrane and incubated for 1 hour at room temperature with shaking. A 1:1000 dilution of alkaline phosphatase conjugated goat anti -mouse antibodies (Sigma-Aldrich) was added to the anti-CS antibody (10B10 AND 8C6) for 1 hr. After that, monoclonal rabbit anti-goat HRP conjugated antibodies diluted to 1:10000 was added to anti-CS antibody (10B10and 8C6) for 1 hr. Then, the membrane was washed three times with 1xTBS containing 0.05% Tween and luminata crescendo western HRP substrate was added onto the entire nitrocellulose membrane surface and exposed to Fuji film for 5 minutes or the membrane was visualised using ChemiDoc™ Touch Imaging System (BIO-RAD).

2.2.2.3.4 Protein dialysis

After protein purification confirmation, and in order to eliminate imidazole within the purified protein, dialysis was undertaken. Dialysis is a separation method which can be used to remove unwanted molecules. Snake skin membrane (10 kDa MWCO) was used to remove the imidazole. The membrane allows the high concentration of salts (imidazole) to exchange into a low concentration medium. Thus, the protein elution was loaded into a dialysis bag and the membrane was closed. After that, the membrane was placed into a beaker containing 1X TBS pH 7.4 and incubated overnight at 4ºC on a stirring machine. The following day, the entire dialysed sample was concentrated using a centrifugal concentrator with a 10 kDa cut-off (Millipore) and measured using Bradford assay.

2.2.2.3.5 Calculation of Protein Concentration

Bradford assay is one of the most commonly used methods for measuring protein concentrations. This assay is used to measure the total concentration of protein in a sample in comparison to standard protein. 300 µl of Coomassie plus reagent was placed in a 96-well flat plate. A known concentration of standard BSA was serially diluted starting from 2 mg/ml (Table 2-13). Then, an equal volume, for example 5 µl, from each dilution was added into separate wells in duplicate. Whilst the same amount of the purified protein was added, and the final well was considered a blank. Then, the plate was gently swayed to achieve for mixing and then incubated for 10 minutes at RT. The protein-dye complex formed absorbed at a wavelength of 595nm using a Bio-Rad iMark microplate reader (Kruger, 2009).

To prepare a dilution of bovine serum albumin (BSA) standard, the contents of one BSA ampule was diluted into several clean vials (Table 2-13) used as a guide to prepare a group of protein standards.

Table 2-13: Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA concentration
A	0 µl	300 µL of stock	2000 µg/ml
B	125 µl	375 µL of stock	1500 µg/ml
C	375 µl	375 µL of stock	1000 µg/ml
D	175 µl	175 µL of vial B dilution	750 µg/ml
E	375 µl	375 µL of vial C dilution	500 µg/ml
F	375 µl	375 µL of vial E dilution	250 µg/ml
G	375 µl	375µL of vial F dilution	125 µg/ml
H	400 µl	100 µL of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

2.2.2.4 Protein functional assays

2.2.2.4.1 Complement assays

2.2.2.4.1.1 Preparation of human sera for functional complement assay

Some sera were obtained from healthy individuals. The human blood was obtained, after signing an informed consent form, and places it in sterile tubes and left at room temperature for 15-30 minutes for clotting. Each tube was centrifuged at 1800xg for 10 minutes at 4˚C (a general lab handbook). Serum was collected, aliquoted and stored at -80˚C. The same protocol was used for pooled human serum which was applied by SERALAB.

2.2.2.4.1.2 Lectin pathway 50 assay

Serial dilutions normal human serums (NHS) in barbital buffered saline (Ca^{2+}/Mg^{2+}) (2 mM CaCl, 1 mM $MgCl_2$, 5 mM barbital and145 mM NaCl, pH 7.4) were added to a mannan-coated plate**. A** Nunc MaxiSorp 96 well plate was coated with 100µl of 10 µg/ml mannan in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The next day, the plate was blocked with 250 µl of blocking buffer composed of 1% bovine serum albumin BSA in TBS. The diluted sera were added to the plate and plate was incubated for 60 minutes at 37˚C to allow C3b to deposit on the plate. After that, the plate was washed three times with 1X TBS containing 0.05% Tween-20, and 100 µl of polyclonal rabbit antihuman C3c antibody (Dako) diluted to 1:5000 in washing buffer was added to the plate and incubated for 60 minutes at 37ºC. After washing three times with washing buffer, 100µl of alkaline phosphatase goat anti-rabbit IgG antibody (Sigma-Aldrich) diluted to 1:5000 in washing buffer was added. After 1 h incubation at room temperature, the plate was washed as above and 100 µl of colorimetric substrate solution (Sigma Fast *p*-Nitrophenyl phosphate tablet) was added and the plate was incubated at room temperature for a few minutes, and the absorbance of substrate was measured by a Bio-Rad microtiter plate reader at a wavelength of 415 nm.

2.2.2.4.1.3 Enzyme Link Immuno-Sorbent Assay (ELISA)

ELISA is a simple technique which is used to measure the complement activation activity. C3b deposition through the lectin and alternative pathways were verified on mannan and zymosan-coated ELISA plates, respectively. To assay the complement inhibitory activity, serial dilutions of the recombinant proteins were added to the sera before incubation on ELISA plates.

2.2.2.4.1.3.1 Binding assays

2.2.2.4.1.3.1.1 Binding assays for heparin and C3b

Microtiter ELISA 96-well plates were coated with heparin in final concentration 25 of μg/ml, and C3b or CS proteins (FL and C-terminal) at a final concentration of 5 μg/ml. After overnight coating, the free residual protein binding sites in the plates were blocked with 250 μl of 1% (BSA) in (TBS) for 2 h at room temperature. After incubation, the plates were washed three times with 250 μl of washing buffer. Meanwhile, four-fold dilutions of recombinant FL.CS, C-terminal of CS and N-terminal and C-terminal of TRAP were added onto both heparin and C3b coated plates. After 1 h of incubation at 37°C, wells were washed three times using washing buffer. Then, 100 μl of mouse polyclonal anti-polyhistidine (Sigma) was diluted to 1:10000 in washing buffer and added as primary antibody. Next, the plates were incubated for 1 h at 37°C followed, by three washing steps. After that, in order to detect any recombinant protein binding, 100 μl of diluted rabbit polyclonal anti-mouse alkaline phosphatase conjugated (Sigma) (1:5000) in washing buffer was added as secondary antibodies, respectively. Next, the plates were incubated for 1 h at room temperature followed by a final three washing steps. After these final washing steps, tablets of alkaline phosphatase substrate (Fast *p*-Nitrophenyl Phosphate tablet sets, Sigma) were prepared in 20 ml distilled water. Then, 100 μl of the prepared substrate was added to each well in order to determine the presence of the conjugated ALK-phosphatase. Lastly, the colour intensity was measured at a wavelength of 415 nm using a Bio-Rad iMark microplate reader. All ELISA assays were repeated three times using fresh buffers each time.

2.2.2.4.1.3.1.2 Binding assay to anti-circumsporozoid antibody

ELISA plates were coated with 5μg/ml CS protein in coating buffer overnight at 4ºC. Next day, the plates were blocked as described above. Serial dilution of mouse monoclonal anti CS antibody (10B10 and 8C6) in TBS/Ca_2 (washing buffer) were added starting at 1:10. The remaining steps are identical to those described above, except that the detection antibody was AP-conjugated anti-mouse IgG.

2.2.2.4.1.3.1.3 TRAP and CS recombinants binding to different lectin pathway ligands

Microtiter ELISA plates were coated with 1 μg/ml of N-terminal of TRAP, C-terminal and full-length CS, or with 10 μg/ml mannan, 10 μg/ml zymosan or 5 μg/ml *N*-acetyl BSA as positive controls for MBL, collectin C-11 and ficolins, respectively then incubated overnight at 4˚C. All these ligands were suspended or dissolved in coating buffer and 100 μl was added to each well. After that, the protein-binding sites were blocked using 250 μl of 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS), and the plate incubated for two hours at room temperature. Then, the plate was washed three times with 250 μl of washing buffer (TBS with 0.05%, Tween 20 and 5 mM $CaCl_2$) and serial dilutions of NHS serum in MBL-binding buffer (20 mM Tris, 1 M NaCl, 10 mM $CaCl_2$, 0.05% (v/v) Triton X-100, 0.1% HSA, pH 7.4) were added in duplicate to the plate, starting from 20%. The plate was incubated at 37˚C for 1 h. After washing the plates three times, primary antibodies were added, 100 μl monoclonal mouse anti-human MBL, 100 μl polyclonal goat anti-human Collectin 11 and 100 μl monoclonal mouse anti human L-ficolins (all three antibodies from Santa Cruz) were diluted to 1:5000 in washing buffer and added to plates that had mannan, zymosan and *N*-acetyl BSA as positive controls, respectively, and incubated for 90 minutes. In order to detect the binding to those ligands, the wells were washed three times and 100 μl of alkaline *phosphatase*-conjugated goat anti-mouse antibodies (Sigma-Aldrich) for MBL and L-ficolins added, while 100 μl of alkaline phosphatase conjugated rabbit anti-goat antibodies (Sigma-Aldrich) for Collectin-11 diluted to 1:10.000 in washing buffer and added as secondary antibodies, respectively. The plates were then incubated at 37ºC for

1 hr. After three washing steps, the alkaline phosphatase substrate tablets (Fast *p*-Nitrophenyl Phosphate tablet sets, Sigma) were prepared in 20ml distilled water. Then, 100 µl of the colorimetric substrate pNPP was added and the absorbance at 415 nm was measured with a Bio-Rad microtitre plate reader.

2.2.2.4.1.3.2 The Lectin pathway assay

Inhibitory activity of CS and TRAP to the complement pathway was measured by C3 deposition on mannan-coated plate. A Nunc MaxiSorp 96 well plate was coated with 10 µg/ml of mannan in coating buffer pH 9.6. Then, the plate was incubated overnight at 4ºC. The next day, the plate was blocked with 250 µl of blocking buffer composed of 1% bovine serum albumin (BSA) in TBS (145 mM NaCl, 50 mM Tris-base pH 7.4) and incubated for 2 h at room temperature. The recombinants FL.CS,C-terminal of CS and N-terminal, C-terminal of TRAP were added as two fold or four fold dilution starting at 20 µg/ml or 10 µg/ml to the 1% normal human serum in barbital buffer saline (Ca^{2+}/Mg^{2+}) (2 mM CaCl, 1 mM $MgCl_2$, 5 mM barbital and145 mM NaCl, pH 7.4)(lectin pathway buffer). The diluted sera were added to the plate in fixed concentrations and the plate was incubated for 90 minutes at 37˚C to allow C3b to deposit on the plate. After that, the plate was washed three times, and 100µl of polyclonal rabbit antihuman C3c antibody (Dako) was diluted to 1:5000 in washing buffer, added to the plate and incubated for 90 minutes at 37ºC. After washing three times with washing buffer, 100 µl of alkaline phosphatase goat anti-rabbit IgG antibody (Sigma-Aldrich) diluted to 1:5000 in washing buffer was added. After 1 h incubation at room temperature, the plate was re-washed and 100 µl of colorimetric substrate solution (Sigma Fast *p*-Nitrophenyl phosphate tablet) was added and the plate was incubated at room temperature for a few minutes, then the absorbance of the substrate was measured by a Bio-Rad microtiter plate reader at a wavelength of 415 nm.

2.2.2.4.1.3.3 The alternative pathway assay

The alternative pathway of complement activation in NHS for TRAP and CS-treated sera was measured on zymosan. A Nunc MaxiSorp 96 well plate was coated with 10 µg/ml of Zymosan in coating buffer at pH 9.6. Then, the plate was incubated overnight at 4ºC. The next day, the plate was blocked with 250 µl of blocking buffer composed of 1% bovine serum albumin (BSA) in 1xTris (TBS) (145 mM Nacl, 50 mM Tris-base pH 7.4) and incubated for two hours at room temperature. The recombinants C-terminal and full-length CS and N-terminal of TRAP was added in serial dilution starting at 10µg/ml concentration to a fixed concentration of 10% normal human serum in barbital buffer saline (GVB/Mg^{2+}/EGTA) (10 mM EGTA, 5 mM $MgCl_2$, 5 mM barbital and 145 mM NaCl, pH 7.4), which blocks activation of the classical and lectin pathways (both of which are calcium-dependent), but allows activation via the alternative pathway. The remaining steps were as described for the lectin pathway above2.2.2.4.1.3.2).

2.2.2.4.1.3.4 Reactivation of complement system by mouse monoclonal anti CS antibody

ELISA plates were coated with 10µg/ml of mannan or zymosan in coating buffer overnight at 4º C. Next day, the plate was blocked with 250 µl of blocking buffer composed of 1% bovine serum albumin (BSA) in 1xTris (TBS) for 2 h. After washing three times with washing buffer, 100 µl of mixture containing 1% NHS, 10 µg/ml of Fl or C-terminal CS protein five-fold serial dilutions of anti-CS antibody, starting at 1/10 were added to the plate and incubated for 90 minutes at. C3b deposition was detected as above.

2.2.2.5 Haemolysis assays

In order to prepare the RBC for haemolysis assay, guiana-pig blood was ordered from ENVIGO. Whole blood was immersed in ice; subsequently 2 ml of blood was taken to spin down in the 4˚C centrifuge at 2000 xg for 1-2 minutes. After that, the plasma and buffy coat were removed. The RBC was washed three times with the ice-cold lectin pathway buffer. Then it was again centrifuged, and when the last wash was finished the RBC was re-suspended with 4 ml lectin pathway buffer. This 4 ml was split to 2 ml aliquot, one of which was set up as an uncoated control and the other was prepared by adding 2 ml each of $CrCl_3$ (0.5 mg/ml in lectin pathway buffer) and 2 ml mannan (100 µg/ml in lectin pathway buffer) then incubated with gentle mixing at room temperature for 5 minutes. After that, 7.5 ml lectin pathway buffer was added to stop the reaction. The cells were then centrifuged as above then cells were re-suspended in 2 ml lectin pathway buffer and washed an additional two times as above and stored at 4˚C. The next day, in order to detect the RBC concentration, a group of dilutions in the lectin pathway buffer were arranged (as 5x, 10x, 15x, and 20x). Then, 100 µl of each of these dilutions was placed in a round-bottomed plate. Thereafter, 100 µl of Nano pure water was added to lyse the cells and the plate was spun down for 5 minutes at maximum speed. To read the result on the reader, 100 µl of the fluid after centrifugation was transferred to a 96-well plate and readied at OD 540. The dilution that gives an OD 540 for maximum lysis of approximately 0.7-0.9 was chosen (An OD 540 of 0.9 should correspond to approx. 10^8 cells/ml). 100 µl sera and protein was added in 96 round-bottomed plates. Then, 100 µl of right diluted RBC preparation was added and incubated at room temperature for around 60 minutes to detect the lysis. Finally, the plate was centrifuged at maximum speed for 5 minutes, then 100 µl of the solutions were aspirated off and the OD at 540 nm was noted.

2.2.2.6 Protein preparation to produce antibody

2.2.2.6.1 Enterokinase enzyme

For antibody production, the N-terminal fusion partner was removed from recombinant proteins by digestion with EKmax. Enterokinase is a highly specific serine protease that can be used to digest fusion proteins to release the fusion partner or tag from the desired protein. The enzyme recognizes the sequence (Asp) 4 Lys and cleaves after the lysine residue. This cleavage sequence is present in many expression vectors available from Invitrogen. Genes cloned into the multiple cloning sites of these vectors express recombinant N-terminal fusion proteins. The native proteins can be released from the N-terminal fusion peptide or protein by digesting with enterokinase. 120 µg of fusion protein C-terminal CS prepared for the pilot reactions. The ratio of enzyme to fusion protein used to achieve complete digestion may vary depending on the protein expressed. To determine the optimal units of EKMax™ needed for complete digestion, five different amounts of EKMax™ were used (4 units, 1 unit, 0.1 units, 0.01 units, and 0.001 units). For 4 units of EKMax™, 4 µl of undiluted EKMax™ was used. At the same time, 1X EKMax™ buffer was used to make serial 10-fold dilutions of the enzyme. After that, six reactions were undertaken, including a reaction without EKMax™ to control for proteases in C-terminal CS protein solution. The C-terminal CS was digested by10X EKMax™ Buffer 3 µl, EKMax™ 4 units and made up with deionized water to 29 µl in order to get native C-terminal CS, as well as using 30 µl with no EKMax™ as a control. The solution were mixed well and incubated at 37°C overnight (~16 hours). The following day, the digested protein was analysed via SDS-PAGE gel. 1 mg/ml of EKMax purified C-terminal CS was sent to Eurogentic company to produce monoclonal mouse antibodies. The same amount of recombinant SCR3-5 (the C-terminal part of complement factor H related protein 1, kindly provided by Mohenned Alsaadawi, PhD student) was used as negative control.

2.2.2.6.2 Anti-CS Antibody Purification Using Protein G

Antibody purification involves isolation of an antibody from the culture supernatant of a hybridoma cell line (monoclonal antibody). Purification methods range from very crude to highly specific. Purification was accomplished through Nab Spin Column with protein G. The column was the column and equilibrated using 5 ml binding buffer (PBS). The sample was diluted in a 1:1 ratio with binding buffer, and the diluted sample added to the column and the solution allowed to drain into a 15 ml collection tube. Next, the column was washed by adding 15 ml of binding buffer. 100 µl neutralization buffer (1 M Tris, pH 8-9) was added to five collection tubes. After that, the antibodies were eluted with 5 ml of elution buffer (0.1 M glycine, pH 2-3), collecting 1 ml in fractions in each of the buffer-containing tubes, each was studied at a wavelength of 280 nm then analysed via SDS-PAGE and western blot. Next, the column was regenerated by adding 8 ml of elution buffer and the solution allowed to flow through the column.

2.2.3 Statistics

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, USA). The results represent the means ± SD of two or three independent experiments. For comparison, we used t-test and one-way ANOVA, P-values <0.05 and $p< 0.0001$ were considered as significant.

3 Chapter three (Results) Production of recombinant Thrombospondin- related anonymous and circumsporozoite proteins

3.1 Introduction

Two surface molecules that play significant roles in liver cell infection are the circumsporozoite protein (CS) and the thrombospondin-related anonymous protein (TRAP) (Robson, K. J. *et al*, 1995). TRAP contains an acidic cytoplasmic C-terminal tail, a transmembrane domain, a proline-rich area and two adhesive domains, i.e. a TSR (thrombospondin type-I repeat) domain and a vWA (von Willebrand factor A) - like N-terminal A domain. This design provides a double role for TRAP in the invasion process (Tucker, 2004; Robson, Kathryn JH *et al*, 1988; Morahan, Wang and Coppel, 2009). CS can be approximately segmented into three portions, namely (i) the amino terminus region containing region I, (ii) a central repeat region, and (iii) a carboxyl terminal segment having a TSR domain. Region I (N-terminal) has been implicated in the invasion of salivary glands in mosquitoes and in the inhibition of protein synthesis in liver cells. The central repeat segment is a low complexity region inclosing multiple duplicates of species-specific repeat peptides (NANP in case of *P. falciparum*), with no clear sequence homology to any known protein. The carboxyl terminus (C-terminal) of CS demonstrates sequence conservation across the species and its region II-plus has been implicated in liver cell binding and subsequent inhibition of protein synthesis. Region II plus is an 18-amino acid region and is a part of a larger TSR domain, which was originally discovered in thrombospondin (Rathore *et al*, 2002; Bermúdez, Vanegas and Patarroyo, 2008; Bongfen *et al*, 2009; Roggero *et al*, 1995). The TSR contains the consensus sequence Cys-Ser- Val-Thr-Cys-Gly-x-Gly-x-x-x-Arg-x-Arg/Lys, which is conserved in all CS proteins (Holt *et al*, 1989) . In addition, homologies were found with the complement protein properdin and with TRAP (Tewari *et al*, 2002). These sequences, known as TSR1 (TSP) domains are likely to be inhibitory for the complement system, and are the focus of this work. To investigate the effect of *Plasmodium falciparum* TSP domains on complement activation, TRAP and CS proteins, and fragments of the proteins containing the TSP domain, were expressed and purified. Four fragments of TRAP were expressed in a bacterial system: an N-terminal polypeptide, the TSP domain, a C-terminal fragment and the full length protein (minus the signal peptide); all fragments contain the TSR (Figure 3-1 A). Two fragments of *Plasmodium falciparum* CS protein were expressed in the bacterial system: The full length protein (minus the signal peptide) and a C-terminal fragment, both of which contain the TSR (Figure 3-1 B). All fragments were expressed as 6xHis-tagged

proteins. Furthermore, TRAP and CS fragments were expressed in CHO cells using a eukaryotic system. These eukaryotic and prokaryotic systems were initially chosen to investigate whether post translational modification and correct folding are necessary to get functional protein.

3.2 Production of recombinant Thrombospondin- related anonymous and circumsporozoite proteins

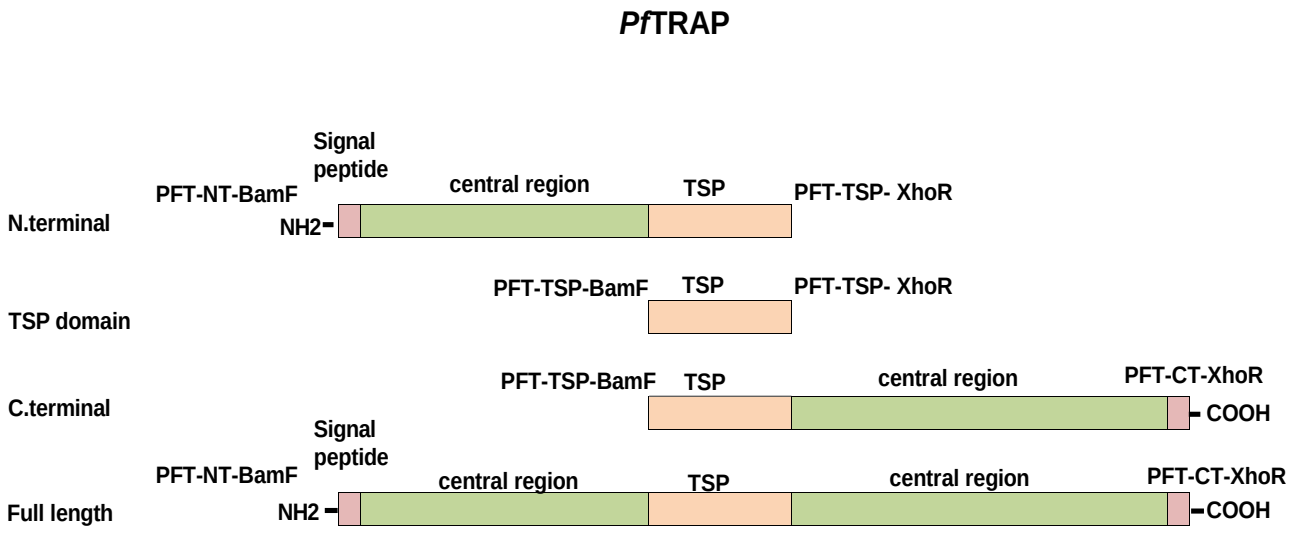
3.2.1 Cloning and expression of TRAP and CS protein

3.2.1.1 Polymerase chain reaction (PCR)

Four fragments of TRAP and two of CS were amplified by PCR from *P. falciparum* using genomic DNA. TRAP and CS are single exon genes, so they can be amplified from gDNA, using the primers listed in (Table 2-1 and Table 2-2), and illustrated in (Figure 3-1 A and B). Phusion high fidelity polymerase was used for the amplification. The primers were modified with *XhoI* and *BamHI* sites to facilitate in-frame sub-cloning into pSecTag2 /Hygro A and pRSET B. PCR products were then visualised on 1% agarose gels. Comparing with the DNA ladder, the DNA bands were: full length, 1647bp; C-terminal, 1002 bp; N-terminal, 795bp; and TSP domain, 150bp of TRAP. Full length CS protein was 1140bp and the C-terminal 210bp (

Figure 3-2 A and B). All products were approximately the size anticipated from the known cDNA sequence.

A



B

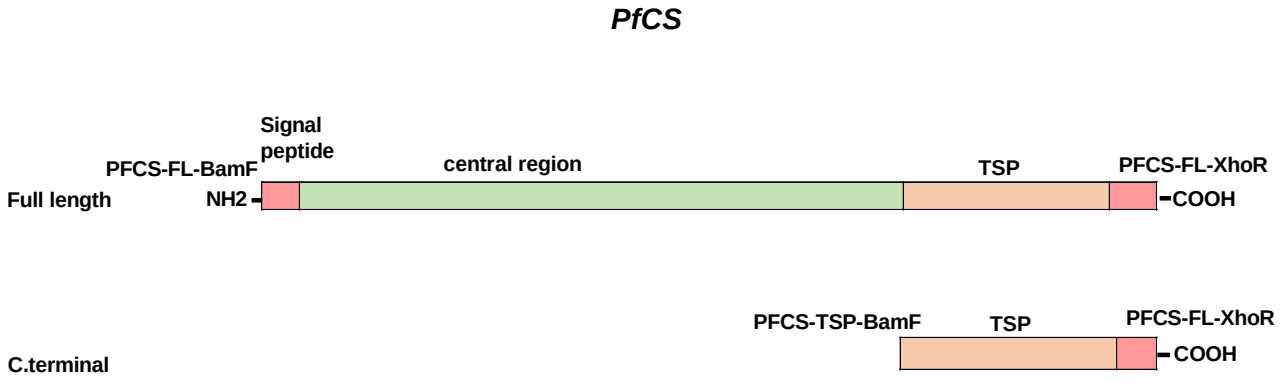


Figure 3-1: Schematic representation of TRAP and CS fragments design

(A) Four fragments of *P. falciparum* TRAP protein were expressed in prokaryotic and eukaryotic systems: N- terminal, TSP domain, C-terminal and the full-length protein (minus the signal peptide); all fragments contain the TSP. All fragments were expressed as 6xhis-tagged proteins

(B) Two fragments of *P. falciparum* CS protein were expressed in Prokaryotic and Eukaryotic systems: The full length protein (minus the signal peptide) and a C-terminal fragment, both of which contain the TSR. Both were expressed as 6xhis-tagged proteins

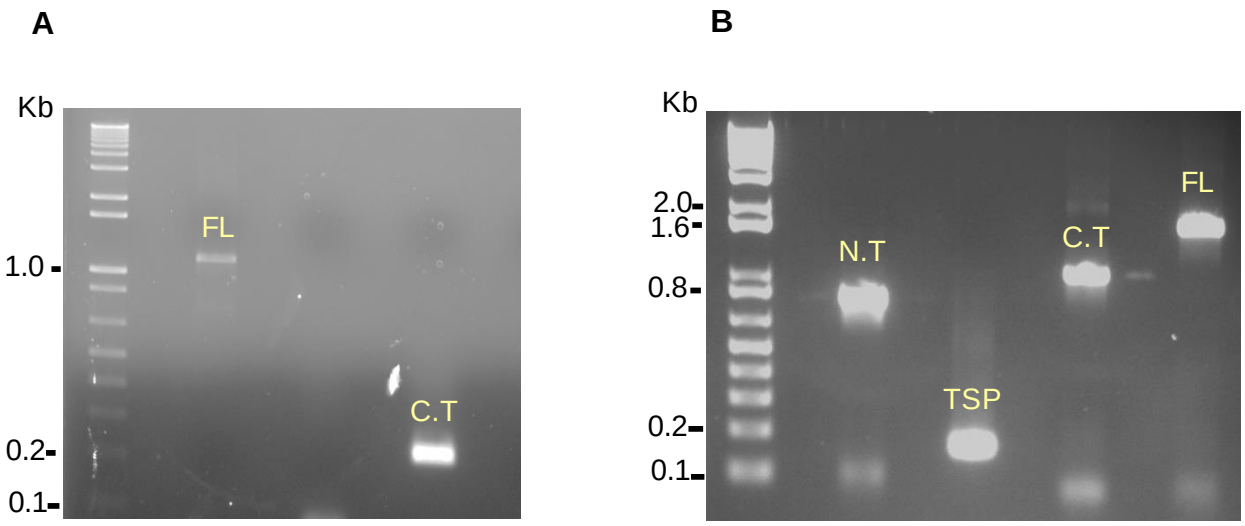


Figure 3-2: Agarose gel of electrophoresis of *P. falciparum* CS protein and TRAP PCR products

(A) Generation of full length (FL) and C-terminus (C.T) coding sequence of CS, showing bands of approximately 1140 and 210bp respectively.

(B) The N-terminus (N.T), TSP domain, C-terminus (C.T) and the full length (FL) coding sequences of TRAP were PCR amplified using Phusion DNA polymerase and showed bands approximately 795bp, 150bp, 1002bp and 1647bp respectively.

3.2.1.2 Cloning the DNA constructs of TRAP and CS into pGEM-T Easy vector

The DNA PCR products of TRAP and CS were cloned into pGEM-T Easy vector which simplifies the cloning of the PCR products. T4 ligase was used to ligate TRAP and CS DNA constructs, with the open pGEM-T Easy vector, which has T overhangs complementary to the A-tailed PCR products. After that these DNA constructs were transfected into *E.coli* TOP10 competent cells, cultured, and the plasmids were extracted using Wizard plus SV Minipreps (Promega) (see section 2.2.1.8). The TRAP and CS constructs were digested from the pGEM-T Easy vector with restriction enzymes *BamHI* and *XhoI*. Following that, digested samples were separated on 1% agarose gel, showing the expected bands of approximately 1002bp for C-terminus, 750bp for N-terminal, 150bp for TSP and 1647bp for the full length coding sequence of TRAP (figure). CS constructs show bands of approximately 1140bp for full length and 210bp for C-terminus, while the pGEM –T Easy band was 3018bp (
Figure 3-3 A, B and C).

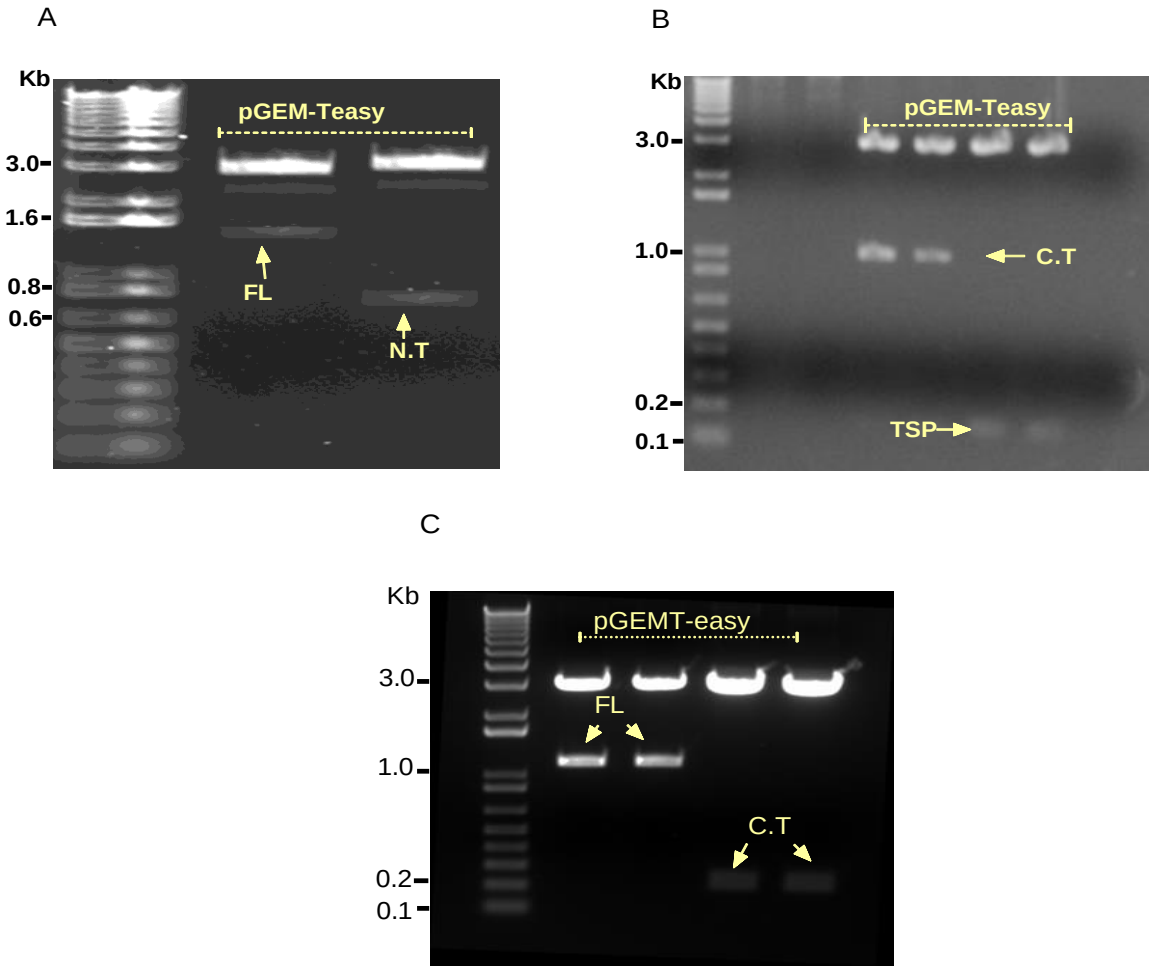


Figure 3-3: Cloning of TRAP and CS coding sequences fragments into pGEM-T easy vector

(A) Agarose gel electrophoresis of the TRAP DNA constructs in pGEM-T easy, after digestion with *BamH I* and *Xho I* restriction enzymes. Full length and N-terminus coding sequences show bands of approximately 1647bp and 750bp, respectively, while pGEM T easy vector shows a band of around 3000 base pairs**. (B)**The C-terminus and TSP coding sequences of TRAP show band of 1002pb and 150bp, respectively **(C)** The full length and C-terminus coding sequence of CS shows a band of approximately 1140bp and 210bp, respectively. All TRAP and CS constructs bands were in the right size.

3.2.1.3 Sub-cloning of DNA constructs of TRAP and CS into expression vectors

3.2.1.3.1 Sub-cloning of the DNA constructs of all TRAP's fragments into pSecTag2 /Hygro A and pRSET B expression vectors of TRAP.

DNA constructs were removed from the pGEM-T Easy vector by double digestion using *BamHI* and *XhoI* restriction enzymes, and then ligated and sub-cloned into pSecTag2 /Hygro A and pRSET B expression vectors for TRAP and pSecTag2/Hygro B and pRSET A expression vectors for CS. The successful sub-cloning into the expression vectors was confirmed by restriction digestion of the purified plasmid DNA and visualized on agarose gels. All the bands of TRAP and CS were the expected sizes (Figure 3-4 A, B, C and D).

All DNA constructs were sequenced in the DNA sequencing unit at the University of Leicester (PNACL), which conformed that no mutations had been introduced by PCR amplification steps or by the effect of UV-light emission (Figure 3-6 A).

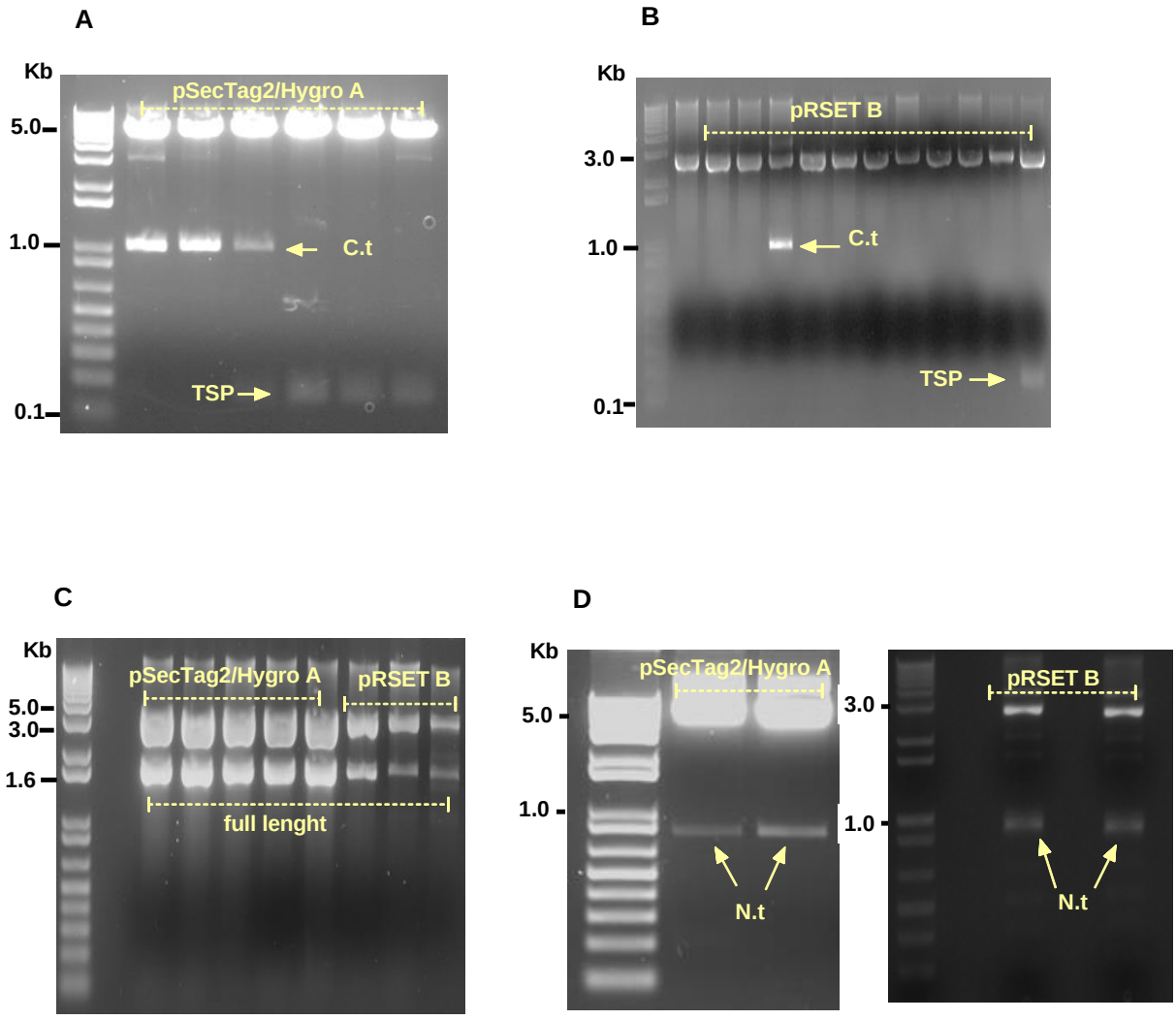


Figure 3-4:Sub-cloning of C-terminus, TSP and full length TRAP coding sequences into pSecTag2/Hygro A and pRSET B expression vectors

(A) Sub-cloning of the DNA construct of C-terminus and TSP coding sequences into pSecTag2/HygroA. Plasmid minipreps were prepared, digested with *BamH1* and *Xho1* and separated on 1% agarose gels. The vector shows a single band of around 5166bp and constructs show the expected size of 1002bp for the C-terminus and 150bp for the TSP coding sequence. **(B)** The C-terminus and TSP constructs were also sub-cloned into pRSETB (circa 2.9kb).

(C) The full length cDNA was sub-cloned into pSecTag2/HygroA and pRSETB and digested with *BamH1* and *Xho1* restriction enzymes. The coding sequence of the full length TRAP shows a band of around 1647bp. **(D)** N-terminus of TRAP was sub-cloning into both mammalian and bacterial expression vectors, and shows bands of around 750bp.

3.2.1.3.2 Sub-cloning of the DNA constructs of CS fragments into pSecTag2/Hygro B and pRSET A expression vectors.

Full length CS DNA and the C-terminal fragment were sub-cloned in eukaryotic and prokaryotic expression vectors (pSecTag2/HygroB and pRSETA, respectively). All sub-cloning were confirmed by restriction enzyme digestion with *BamHI* and *XhoI*. The full length and C-terminal coding sequence of CS digested from pSecTag2/Hygro B and pRSET A vectors shows a band of approximately 1140bp and 214bp, respectively (Figure 3-5 A and B). Before the transfection of the eukaryotic and prokaryotic cell lines, all of the constructs were sequenced to confirm that all clones were inserted in frame and to ensure that there were no mutations (Figure 3-6 B)

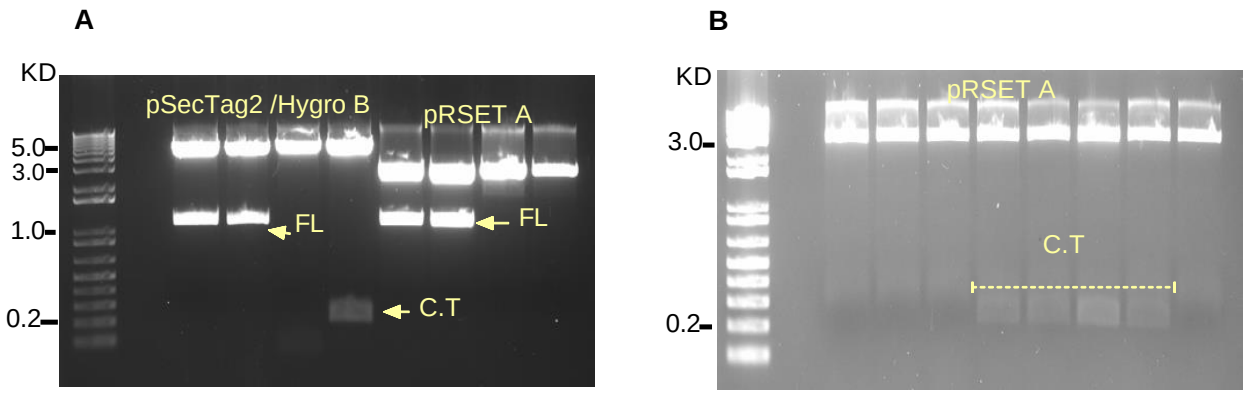


Figure 3-5: Sub-cloning of C-terminus and full length CS coding sequences into pSecTag2/Hygro B and pRSET A expression vectors

(A) Sub-cloning of the DNA construct of C-terminus and full length coding sequences into pSecTag2/Hygro B. The vector shows a single band of around 5166bp and constructs show bands of 210bp for C-terminus and 1400bp for FL coding sequence, in the same figure A, the full length was sub-cloned into pREST A vector, the vector shows a single band of 3000bp. (B) The C-terminus was sub-cloned into bacterial expression vector, pREST A and digested with BamH1 and Xoh1 restriction enzymes. The coding sequence of the C -terminus CS shows a band of around 210bp.

A

CLUSTAL O (1.2.4) multiple sequence alignment

```
TRAP XP_001350088.1  MNHLGNVKYLVIVFLIFFDLFLVNGRDVQNNIVDEIKYREEVCNDEVDLYLLMDCSGSIR
C.terminal           ------------------------------------------------------------
TSP                  ------------------------------------------------------------
Full length          ----------------------------------------RDVQNNIVDEIKYREEVCNDEVDLYLLMDCSGSIR
N.terminal           ----------------------------------------RDVQNNIVDEIKYREEVCNDEVDLYLLMDCSGSIR

TRAP XP_001350088.1  RHNWVNHAVPLAMKLIQQLNLNDNAIHLYASVFSNNAREIIRLHSDASKNKEKALIIIKS
C.terminal           ------------------------------------------------------------
TSP                  ------------------------------------------------------------
Full length          RHNWVNHAVPLAMKLIQQLNLNDNAIHLYASVFSNNAREIIRLHSDASKNKEKALIIIKS
N.terminal           RHNWVNHAVPLAMKLIQQLNLNDNAIHLYASVFSNNAREIIRLHSDASKNKEKALIIIKS

TRAP XP_001350088.1  LLSTNLPYGKTNLTDALLQVRKHLNDRINRENANQLVVILTDGIPDSIQDSLKESRKLSD
C.terminal           ------------------------------------------------------------
TSP                  ------------------------------------------------------------
Full length          LLSTNLPYGKTNLTDALLQVRKHLNDRINRENANQLVVILTDGIPDSIQDSLKESRKLSD
N.terminal           LLSTNLPYGKTNLTDALLQVRKHLNDRINRENANQLVVILTDGIPDSIQDSLKESRKLSD

TRAP XP_001350088.1  RGVKIAVFGIGQGINVAFNRFLVGCHPSDGKCNLYADSAWENVKNVIGPFMKAVCVE VE
C.terminal           ------------------------------------------------------------
TSP                  ------------------------------------------------------------
Full length          RGVKIAVFGIGQGINVAFNRFLVGCHPSDGKCNLYADSAWENVKNVIGPFMKAVCVEVE
N.terminal           RGVKIAVFGIGQGINVAFNRFLVGCHPSDGKCNLYADSAW ENVKNVIGPFMKAVCVEVE

TRAP XP_001350088.1 TASCGVWDEWSPCSVTCGKGTRSRKREILHEGCTSELQEQCEEERCLPKREPLDVPDEPE
C.terminal           TASCGVWDEWSPCSVTCGKGTRSRKREILHEGCTSELQEQCEEERCLPKREPLDVPDEPE
TSP                  TASCGVWDEWSPCSVTCGKGTRSRKREILHEGCTSELQEQCEEERCLPKR
Full length          TASCGVWDEWSPCSVTCGKGTRSRKREILHEGCTSELQEQCEEERCLPKREPLDVPDEPE
N.terminal           TASCGVWDEWSPCSVTCGKGTRSRKREILHEGCTSELQEQCEEERCLPKR

TRAP XP_001350088.1 DDQPRPRGDNFAVEKPNENIIDNNPQEPSPNPEEGKGENPNGFDLDENPENPPNPPNPPN
C.terminal           DDQPRPRGDNFAVEKPNENIIDNNPQEPSPNPEEGKGENPN GFDLDENPENPPNPPNPPN
TSP                  ------------------------------------------------------------
Full length          DDQPRPRGDNFAVEKPNENIIDNNPQEPSPNPEEGKGENPNGFDLDENPENPPNPPKPPN
N.terminal           ------------------------------------------------------------

TRAP XP_001350088.1  PPNPPNPPNPDIPEQEPNIPEDSEKEVPSDVPKNPEDDREENFDIPKKPENKHDNQNNLP
C.terminal           PPNPPNPPNPDIPEQEPNIPEDSEKEVPSDVPKNPEDDREENFDIPKKPENKHDNQN NLP
TSP                  ------------------------------------------------------------
Full length          PPNPPNPPNPDIPEQEPNIPEDSEKEVPSDVPKNPEDDREENFDIPK KPENKHDNQNNLP
N.terminal           ------------------------------------------------------------

TRAP XP_001350088.1  NDKSDRYIPYSPLSPKVLDNERKQSDPQSQDNNGNRHVPNSEDRETRPHGRNNENRSYNR
C.terminal           NDKSDRYIPYSPLSPKVLDNERKQSDPQSQDNNGNRHVPNSEDRETRPHGRNNENRSYNR
TSP                  ------------------------------------------------------------
Full length          NDKSDRYIPYSPLSPKVLDNERKQSDPQSQDNNGNRHVPNSEDRETRPHGRNNENRSYNR
N.terminal           ------------------------------------------------------------

TRAP XP_001350088.1  KHNNTPKHPEREEHEKPDNNKKKAGSDNKYKIAGGIAGGLALLACAGLAYKFVVPGAATP
C.terminal           KHNNTPKHPEREEHEKPDNNKKKAGSDNKYKIAGGIAGGLALLACAGLAYKFVVPGAATP
TSP                  ------------------------------------------------------------
```

B

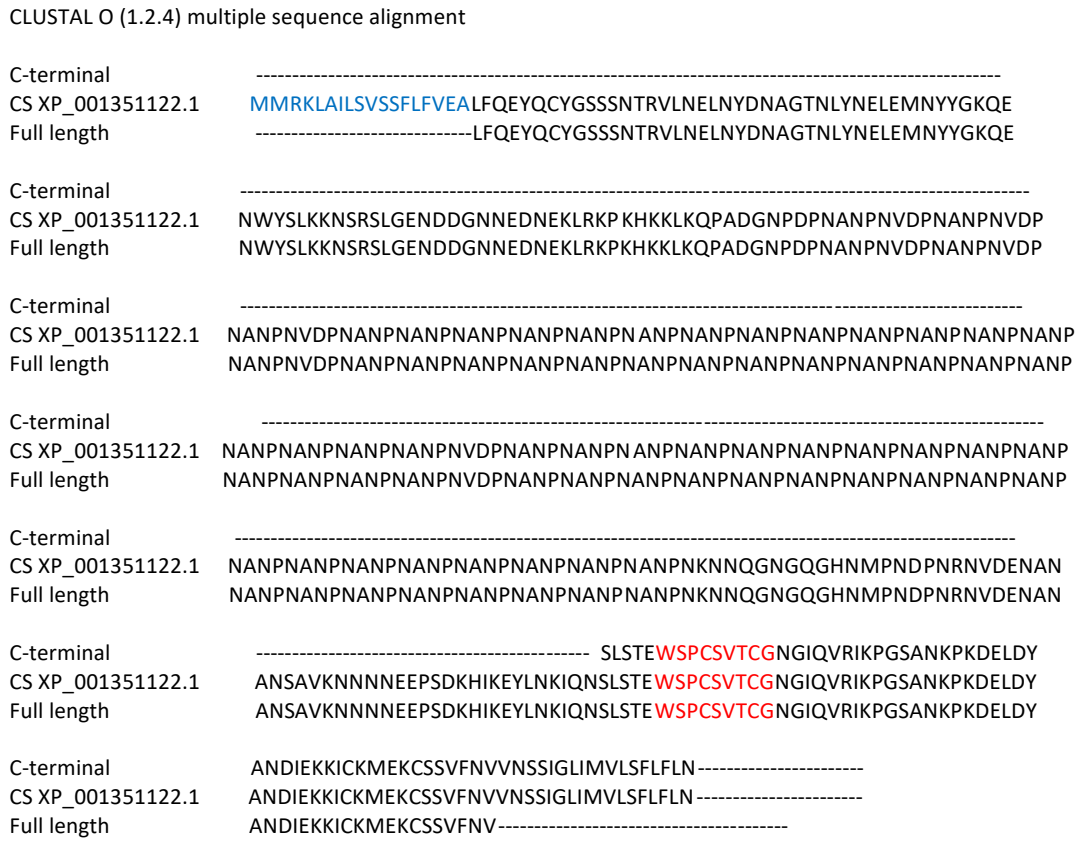


Figure 3-6: Alignment of cDNA-derived amino acid sequences for TRAP and CS protein

(A) Reference amino acid sequences of TRAP (derived from XP_001350088.1**)** and **(B)** Reference amino acid sequence of CS (derived from XP_001351122.1) compared with the cDNA-derived amino acid from obtained by sequencing the TRAP and CS constructs in pGEM-T easy. Sequences of TRAP and CS fragments were checked at the University of Leicester proteomic unit (PNACL). The sequences all agreed with the anticipated amino acid sequences. Colour arranged as following: Signal peptide blue, TSR red, and agreement alignment black.

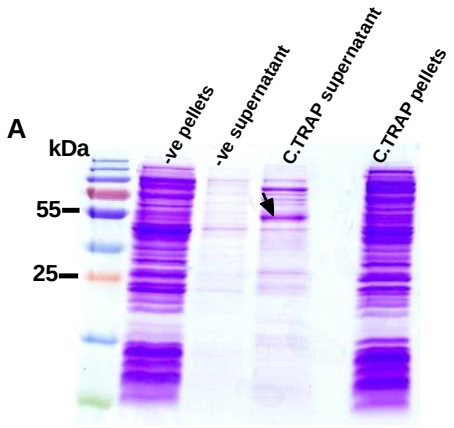
3.2.2 Protein expression in a prokaryotic system

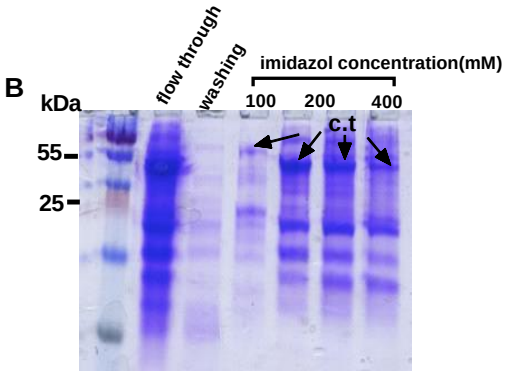
BL21 (pLysS) *E. coli* competent cells were transfected with pRSET A and B expression vector containing the constructs of *Plasmodium falciparum* TRAP (full length, C-terminus, N-terminus and TSP domain) and CS of *Plasmodium falciparum* (full length and C-terminus).

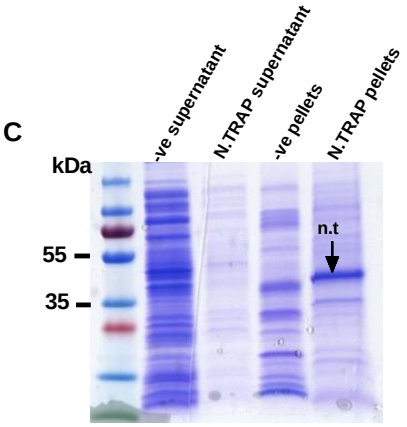
For protein expression, a large culture of transfected bacteria was grown-up, and when the OD_{550nm} reached 0.6-0.8, expression was induced with 1 mM IPTG. The cells were harvested after 3-5 hours, sonicated to release the protein and the sonicate centrifuged to clarify it. Some recombinant proteins were soluble (full-length TRAP, C-terminal TRAP and TSP domain), and others were insoluble (inclusion bodies in the pallet), (N-TRAP, full-length CS and C-terminal CS). Cells containing the inclusion bodies were lysed using 8M urea lysis buffer, as detailed in Chapter 2 (see section2.2.2.1.2), the inclusion bodies were then purified, and washed to reduce the contaminants. The recombinant proteins were purified by nickel-affinity chromatography using His GraviTrap columns (GE Healthcare). Protein was eluted with TBS buffer containing different concentrations of imidazole; 100mM, 200mM and 400mM. 200mM imidazole was found to be the best concentration for most recombinants, since almost all of the protein was eluted without contaminants. This concentration was therefore used for eluting the recombinant protein from the His GraviTrap column. The eluted proteins were analysed by SDS-PAGE under reducing conditions and Coomassie stained.

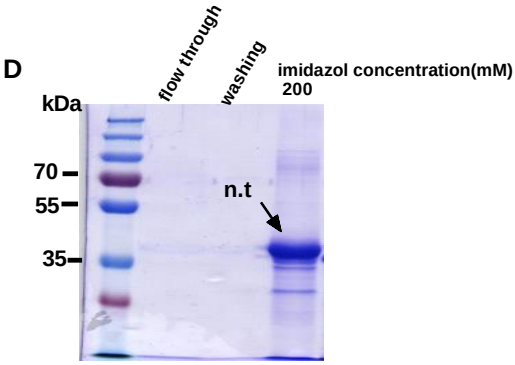
All recombinant proteins showed bands of approximately the expected sizes, and were absent from the supernatant and pellets of non-transfected BL21 *pLysS* (negative control). Recombinants of TRAP showed bands at 69 kDa (Fl), 48 kDa (C-terminal), 41 kDa (N-terminal) and 17 kDa (TSP domain) (Figure 3-7 A, B, C, D, E and F). Furthermore, recombinants of CS showed bands at 60 kDa (Fl) and at 18 kDa (C-terminal) (Figure 3-8 A, B and C)

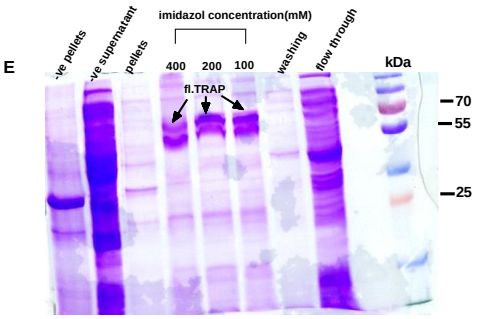
After checking the concentration of protein with Bradford assay, the purified recombinant proteins were separated in SDS-polyacrylamide gel in 1µg/well concentration and blotted into nitrocellulose membrane for TRAP (Figure 3-9 A, B, C and D). The membrane was blocked and the proteins were then probed with anti poly Histidine antibody (
Figure 3-10 A and B).


A
kDa
-ve pellets
-ve supernatant
C.TRAP supernatant
C.TRAP pellets
55
25


B
kDa
flow through
washing
imidazol concentration(mM)
100
200
400
c.t
55
25


C
kDa
-ve supernatant
N.TRAP supernatant
-ve pellets
N.TRAP pellets
n.t
55
35


D
kDa
flow through
washing
imidazol concentration(mM)
200
n.t
70
55
35


E
-ve pellets
-ve supernatant
pellets
imidazol concentration(mM)
400
200
100
washing
flow through
kDa
fl.TRAP
70
55
25

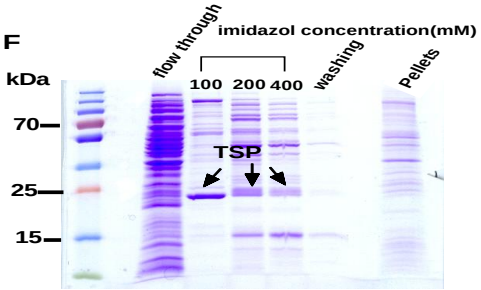

F
kDa
flow through
imidazol concentration(mM)
100
200
400
washing
Pellets
TSP
70
25
15

Figure 3-7: SDS-Polyacrylamide gel electrophoresis of the four different recombinant segments of TRAP.

After expression, cells were lysed and separated into lysates and insoluble pallets. Twenty microliters of the protein were mixed with 5 µL of 5X protein loading buffer containing 5% 2-mercaptoetanol and samples were boiled at 95˚C for 10 minutes. Proteins were separated in 12% SDS-polyacrylamide gel and gel was stained with Coomassie Blue as follows **(A)** Supernatant and pellets of non-transfected BL21 as a negative control, compared with supernatant and pellets of BL21 expressing C-terminal TRAP, C-terminal recombinant (approximately 48 kDa) was found in the supernatant transfected BL21. **(B)** Shows a band of purified C-terminal TRAP. 200mM imidazole was the ideal concentration to elute the recombinant protein**.(C)** Supernatant and pellets of non-transfected BL21 as a negative control, compared with supernatant and pellets of BL21 expressing N-terminal TRAP, N-terminal recombinant (approximately 41 kDa) was found in the pellets (insoluble) of transfected BL21. **(D)** Shows a band of purified N-terminal TRAP. The 200mM imidazole was the ideal concentration to elute the recombinant protein. **(E)** Shows a band of the full length of TRAP. The 200mM imidazole was ideal concentration to elute the recombinant protein which appears at 69 kDa (soluble protein) compared with supernatant and pellets of non-transfected BL21 as negative control. **(F)** The recombinant TSP domain of TRAP protein was eluted with 200mM imidazole and the protein ran at a molecular weight of 17 kDa as soluble protein.

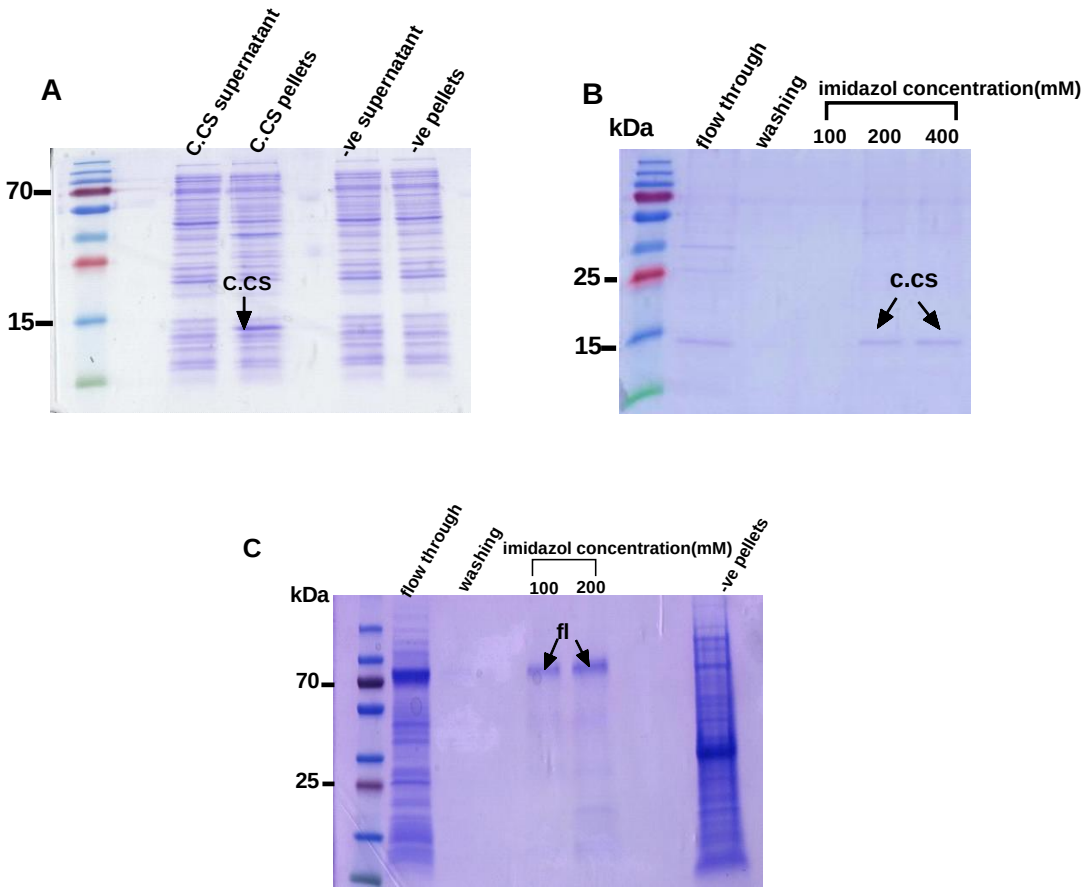


Figure 3-8: SDS-PAGE analysis of purified Full length and C-terminus of CS expressed in BL21 (DE3) *pLysS*.

Recombinant CS proteins were loaded onto nickel columns after expression in BL21 cells and were as insoluble proteins (inclusion bodies). **(A)** Supernatant and pellets of non-transfected BL21 as a negative control, compared with supernatant and pellets of BL21 expressing C-terminal CS, C-terminal recombinant was found in the pellets transfected of BL21. **(B)** 15% SDS- polyacrylamide gel was used to run the C-terminus protein of CS with Coomassie Blue which shows flow-through, column washing (25mM imidazol in TBS). C-terminal was purified by using His GraviTrap columns eluted with different concentrations of imidazole (100 mM, 200 mM and 400mM) in the right size. **(C)** Full length His tag was purified by using His GraviTrap columns eluted with different concentrations of imidazole (100 mM and 200 mM) and was in the expected size comparing with non-transfected BL21 pellets at proximately 60 kDa. Protein was separated in 12% SDS-polyacrylamide gel and gel was stained with Coomassie Blue which shows Flow-through, washing column with 25mM imidazol in TBS.

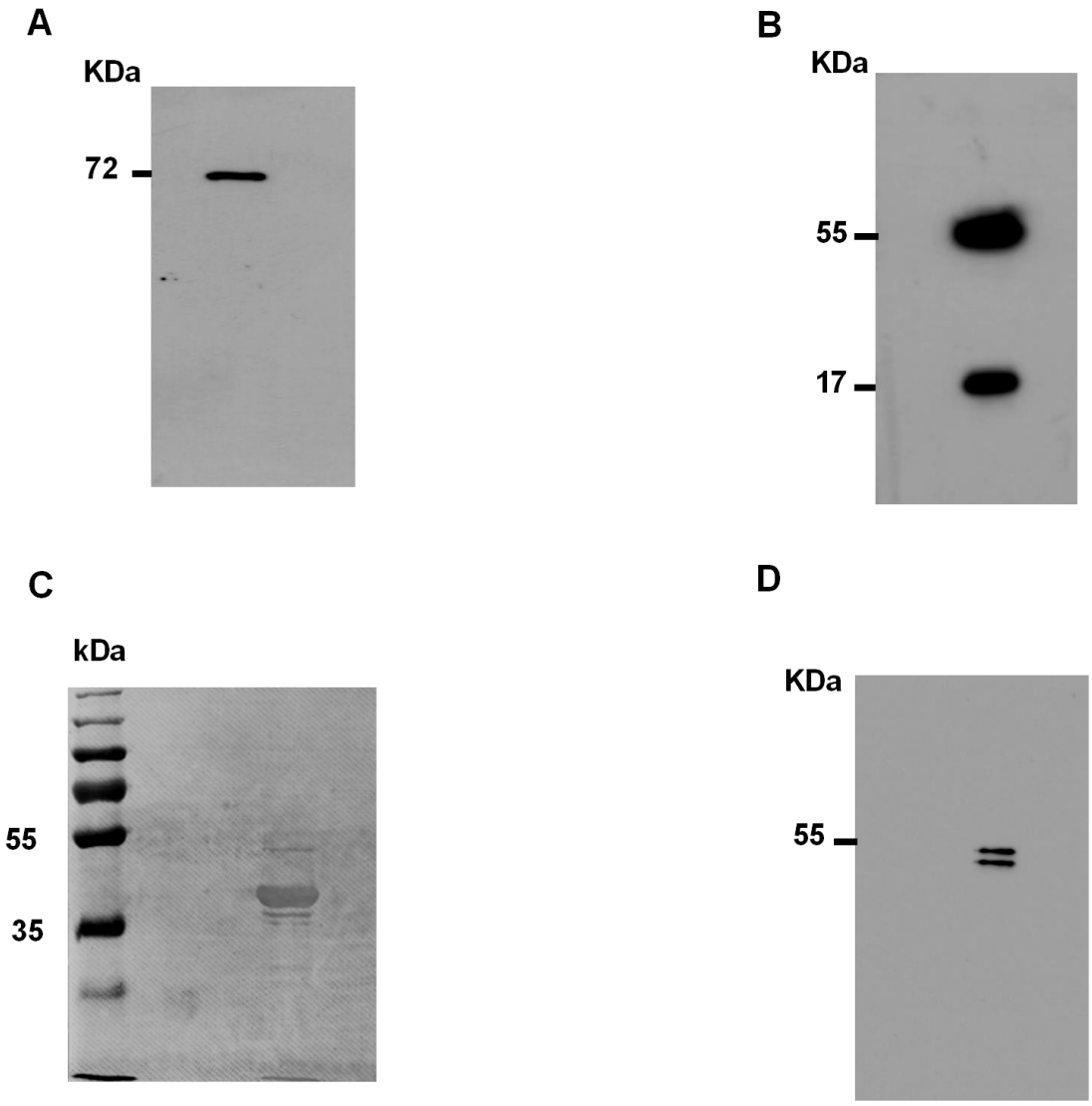


Figure 3-9: Western blotting analysis of TRAP recombinant proteins

1µg of four different forms of recombinant TRAP were separated in SDS-polyacrylamide gels and electrophoretically blotted onto nitrocellulose membrane. Proteins were probed with anti-poly histidine antibody and showed: **(A)** FL.TRAP shows a single band at 69 kDa of the recombinant protein. **(B)** The recombinant TSP domain of TRAP shows a single band of 17 kDa. **(C)** N-terminus TRAP shows a single band at 41 kDa. **(D)** The purified recombinant C-terminal TRAP shows band of 48 kDa.

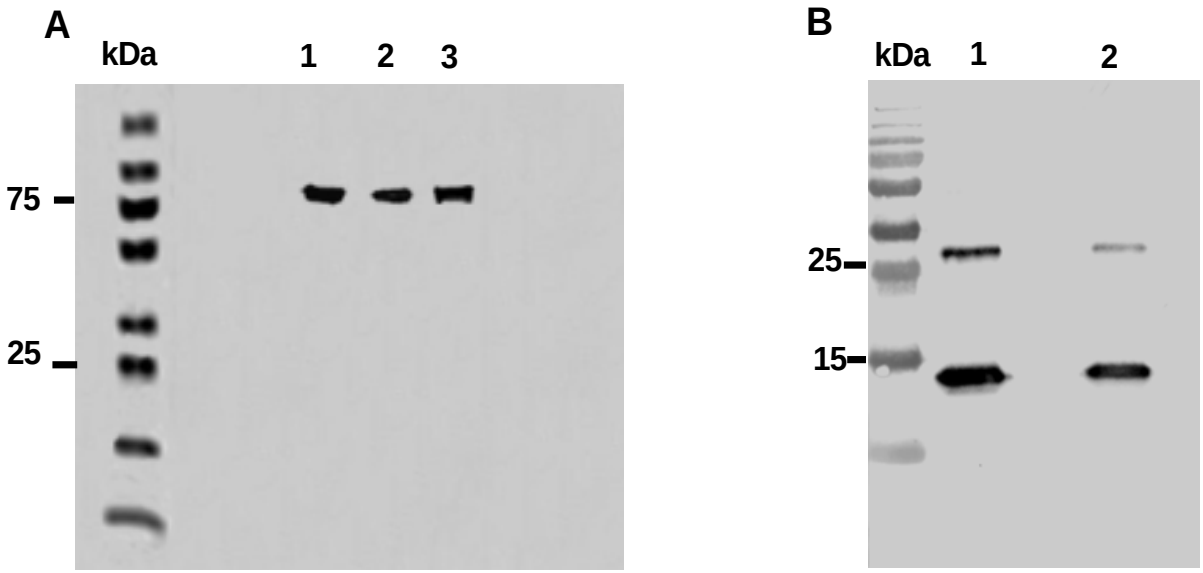


Figure 3-10: Western blotting analysis of CS recombinants protein.

(A) 1μg of the recombinant proteins were separated on SDS polyacrylamide gel then electrophoretically transferred onto a nitrocellulose membrane (lane 1, 2 and 3) were for the same elution but different tubes. The blot was probed with monoclonal mouse anti poly histidine antibodies show bands of FL.CS approximately 60 kDa. **(B)** The recombinant C-terminus of CS shows a band of 18 kDa. Both recombinants band were the expected size

3.2.3 Purification of the recombinant C-terminus of TRAP and C-terminus of CS using Gel filtration by Fast Performance Liquid Chromatography (FPLC)

Gel filtration was carried out using Superose increase 6 10/300 on a Fast Performance Liquid Chromatography (FPLC) system in order to separate the recombinant C-terminus of CS from other contaminants in order to detect the two bands above in western blotting belong to C-terminal of CS. The column works depending on the protein size, thus, the largest molecules pass through the column first, and vice versa, as mentioned in methodology. The column was calibrated by using the Gel Filtration Markers Kit for Protein Molecular Weights 29,000-700,000 Da from Sigma, (Figure 3-11 A). 500 µl of C-terminal CS recombinant protein was loaded to the column and two peaks appeared at the end of column elution's between 20 -24 ml (Figure 3-11 B) these two peaks were collected and shown to contain C-terminal of CS by SDS-PAGE and Western blotting (Figure 3-11 C). This gel filtration was done to confirm that two bands in Western blot for C-terminal CS in (Figure 3-11 D) belong to this recombinant protein and the other big peak at 15 ml was considered contamination.

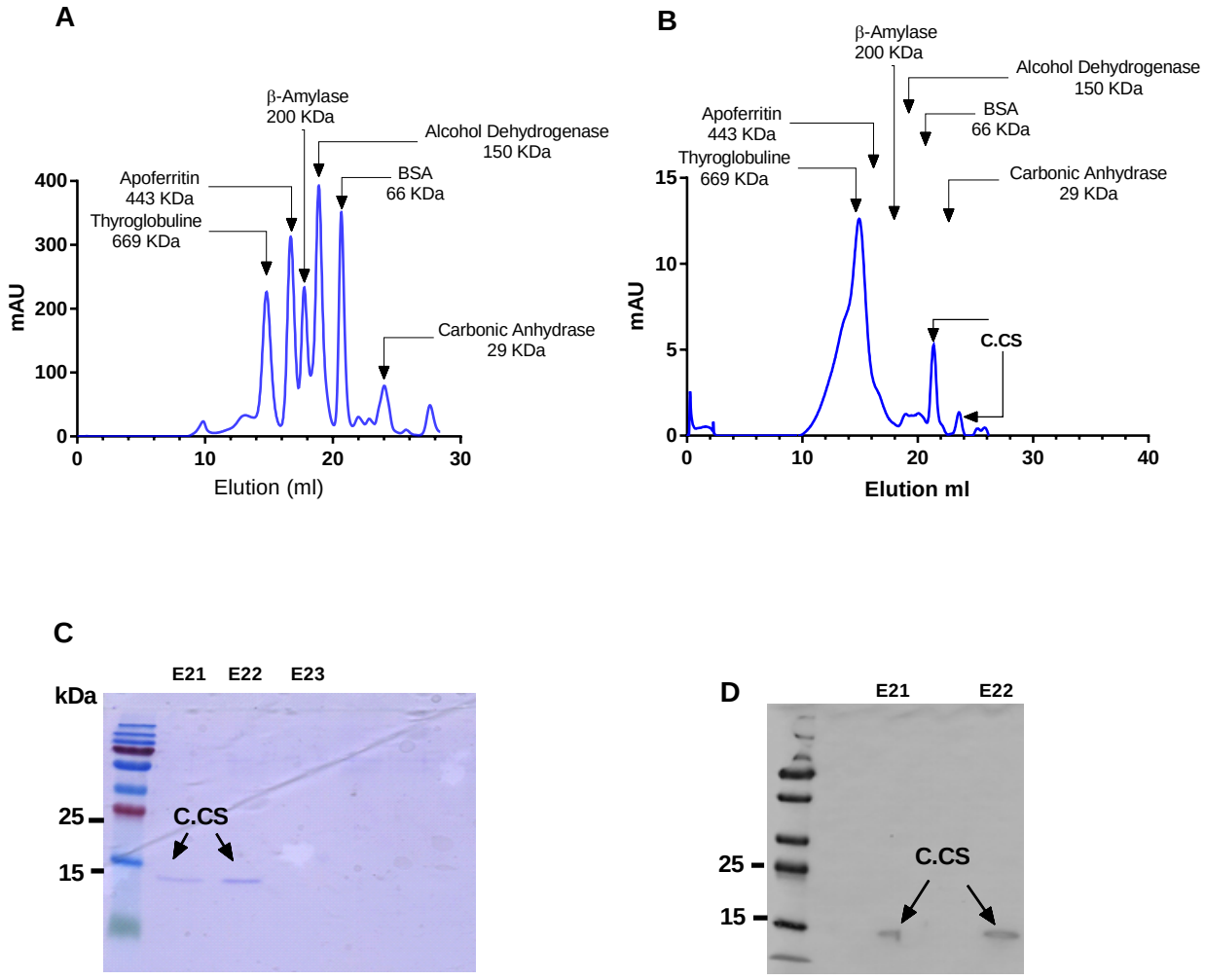


Figure 3-11: Analytic of FPLC for C-terminal CS

(A) Superose 6 Increase 10/300 GE healthcare column was loaded with 0.5ml of various concentration of marker proteins which are (Thyroglobulin, horse spleen, T9145 8 mg/ml; Apoferritin, bovine, A3660 10 mg/ml; β-Amylase, sweet potato, A8781 4 mg/ml; Alcohol dehydrogenase, yeast, A8656 5 mg/ml; Albumin, bovine serum (BSA), A8531 10 mg/ml; Carbonic anhydrase, bovine erythrocytes, C7025 3 mg/ml). After that the column was eluted with three column volumes of TBS. Multiple peaks of marker protein were eluted between 14 and 20 ml. The molecular weights of the standard proteins are marked by arrows **(B)** Purification of the C-terminal fragment of CS on the same column. The C-terminus comes out the end from Superose 6, whereas the other peaks consider as contaminant proteins. **(C)** The purified C-terminus of CS was also run under reducing conditions in 15% SDS-PAGE. After staining one single band of approximately 18 kDa is seen. **(D)** 1µg each of two fractions (21 and 22) was separated on a SDS polyacrylamide gel then transferred onto nitrocellulose membrane. The blot was probed with HRP-conjugated monoclonal mouse anti-polyhistidine antibodies and the recombinant shows a single band of 18 kDa in the right size.

3.2.4 Protein expression in mammalian cells

The mammalian cell line CHO-K1 was transfected with pSecTag2/HygroA containing three different TRAP DNA fragments (full length, C-terminal and TSP domain), and pSecTag2/ HygroB containing two CS cDNA fragments (The full length and the C-terminal), as described in section (2.2.2.2.2). The culture supernatant was supplemented with 300µg/ml 300µg/ml of Hygromycin-B to select for successfully transformed cells.

The transfected CHO-K1 cells were grown to 80% confluency in three well plates, washed with PBS and serum free medium was added and incubated for 48 hours. Samples of supernatant were collected from each well and directly spotted onto nitrocellulose membrane using the BioRad Bio-Dot microfiltration unit. The positive clones were visualized on X-ray film, after probing the recombinant protein with anti-polyhistidine antibody. The three different forms of recombinant TRAP and two of recombinant CS were successfully expressed in the mammalian expression system (Figure 3-12 A, B and C, Figure 3-13 A and B). A frozen stock was made from the three different transfected cells and protein assays, and dot blotting analyses were done. Unfortunately, when transferred to large scale expression, most of the TRAP and CS recombinants when could only be found in very low concentrations (Figure 3-14).

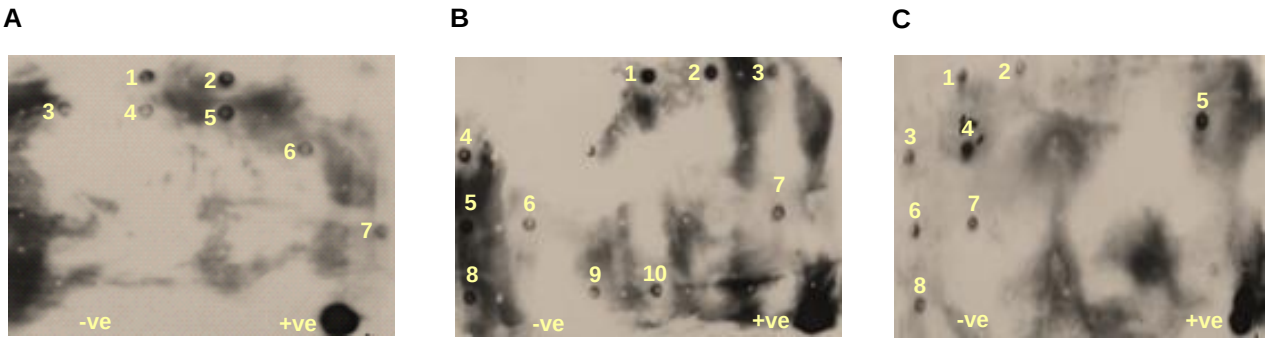


Figure 3-12: Dot blot assay of the supernatant of different clones for expression of TRAP recombinant protein

Eighty microliters of supernatant from the transfected CHO-K1 cells were spotted onto a nitrocellulose membrane, the membrane was blocked with 5% skimmed milk and the expression of the recombinant proteins was detected by anti-poly histidine antibody. **(A)** Supernatant from the cells transfected with the full length TRAP constructs. The positive clones are indicated. Recombinant mouse properdin with 6x histidine tag was used as a positive control. **(B)** Supernatant from the cells transfected with the C-terminal TRAP constructs. **(C)** Screening of recombinant TSP domain expression in the supernatant of the cells transfected with the TRAP TSP construct. Supernatant from non-transfected CHO-K1 cells was used as a negative control (-ve). The numbers in yellow colour refer to positive transfected cells.

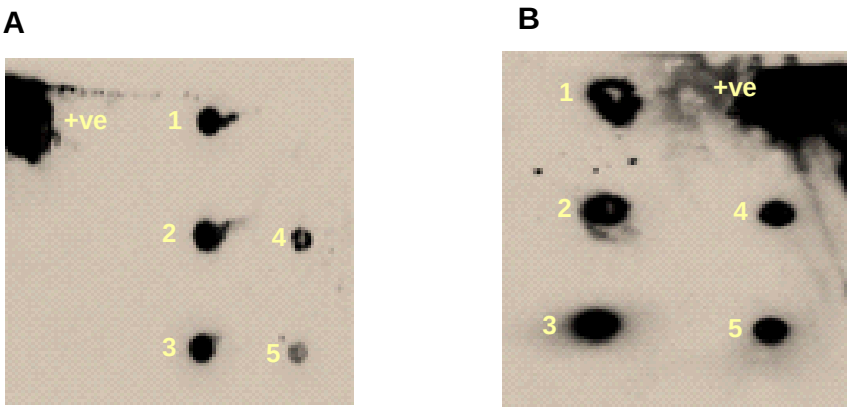


Figure 3-13: Dot blot assay of the supernatant of different clones for expression of CS recombinant protein

Screening of some selected clones for expression of full length and C-terminal after recombinants protein transfected CHO-K1. The protein expression was visualized using HRP conjugated monoclonal mouse anti-polyhistidine antibodies. Recombinant properdin with histidine linker was used as a positive control (+ve). The numbers in yellow colour refer to positive transfected cells.

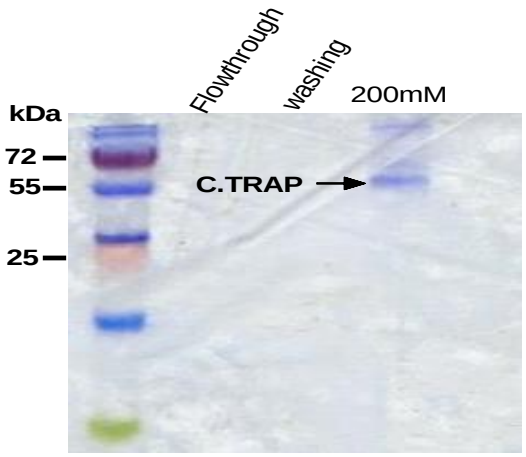


Figure 3-14: SDS- Page analysis of c.terminal of TRAP in CHO-K1 cells

Purified recombinant C-terminal of TRAP shows a band of approximately 58 kDa under reducing condition in the expected size.

3.3 Summary

Different designer primers were set to generate four fragments of TRAP (full-length, C-terminal, N-terminal and TSP domain) and two fragments of the CS protein (full-length and C-terminal); all of the fragments contained a TSP domain. These DNA constructs were visualised on 1% agarose gels and the sizes found to be correct. Two different expression systems (Eukaryotic and Prokaryotic systems) were used to express the polypeptides. The recombinant proteins were successfully expressed in the prokaryotic (pRSET) system by using *BL21 (DE3) pLysS*. The recombinants for the CS protein were insoluble proteins (inclusion bodies) and were refolded using the specific refolding protocol in (2.2.2.1.2). On the other hand, most of the TRAP recombinant proteins were soluble except the N-terminal which was insoluble protein (inclusion body). All these recombinants were size confirmed and visualised by SDS-PAGE and western blot. These recombinant proteins were expressed in CHO-K1 cells, but all the recombinant protein were very low in concentration and that not enough to visualise by SDS-PAGE except C-terminal TRAP was expressed in very small amount in good concentration that was just enough to run on SDS-PAGE. The bacterial expression system was chosen to produce protein for subsequent experiments because it was active, the inclusion bodies refolded easily, and the method is quicker and cheaper.

4Chapter four (Results)

The role of Thrombospondin-related anonymous (TRAP) and circumsporozoite (CS) proteins in the complement system

4.1 Introduction

Transmission of malaria to the human body happens when sporozoites are injected through the mosquito bite. The bloodstream carries the sporozoites and they invade and proliferate rapidly in the liver (Matuschewski *et al*, 2002). Two surface sporozoite proteins, TRAP and CS, are known to play roles in this process (Ménard, 2000). They contain highly conserved thrombospondin domain motifs (TSP domains), structural motifs also found in thrombospondin-1 (Lawler and Hynes, 1986) and properdin (Goundis and Reid, 1988).

Properdin binds to and stabilises the C3 convertase, acting us a positive regulator of complement activation (Farries, Lachmann and Harrison, 1988). The TSP1 in thrombospondin can act as a competitive inhibitor of properdin thus inhibiting the complement pathways (Lynch and Alwashmi, personal communication). To dissect the functional role of the TSP domains of CS and TRAP on the complement system, I generated two recombinant fragments of CS and four of TRAP, all of which contain a TSP domain, as explained in chapter three.

The work presented in this chapter will investigate if both TRAP and CS protect the early sporozoite stages of *P. falciparum* by binding to C3b and inhibiting properdin activity, thus reducing complement activation.

Thrombospondin-1 (TSP-1) is a major endogenous angiogenesis inhibitor binding with high affinity to several heparin-binding angiogenic factors. The heparin binding capacity of TSP-containing proteins was utilised to confirm that the recombinant proteins were correctly folded – heparin was immobilised on ELISA plates and the binding of the recombinant proteins assayed.

After the proteins had been shown to be functionally active, we demonstrated that TRAP and CS inhibit LP and AP activation, using model activators of each pathway.

Complement activation leads to MAC formation which can disrupt the cell membrane of target cells, leading to cell lysis and death (Peitsch and Tschopp, 1991) show that inhibition of early stages of complement activation resulted in inhibition of MAC activity, haemolysis assays were done.

4.2 Results

4.2.1 Binding assay for TRAP and CS

4.2.1.1 TRAP and CS purified recombinant proteins bind to heparin

Heparin is a naturally occurring anticoagulant produced by basophils and mast cells (Guyton and Hall, 2006). The molecular weight of native heparin ranges from 3 to 30 kDa, although the average molecular weight of most commercial heparin preparations is in the range of 12 to 15 kDa (Francis and Kaplan, 2006). The TSP domains in TRAP and CS function as coagulants, by binding heparin. The heparin binding ability of recombinant CS and TRAP polypeptides tested to determine whether the TSP domains within these proteins were functionally active after refolding. ELISA plates were coated with 25 µg/ml of heparin in PBS then blocked with 1% BSA. After blocking serial dilutions of recombinant TRAP and CS were added to the plates. Mouse monoclonal anti-polyhistidine conjugated with HRP antibody was added to determine the binding. Recombinant CS protein clearly shows binding to heparin, but the full-length protein has a much higher affinity for heparin than the C-terminal fragment, probably because the full length protein contains region I and II-plus that preferentially bind to highly sulphated heparin-like oligosaccharides in heparin sulphate(Figure 4-1A and B)(Ying *et al*, 1997). The N-terminal and C-terminal TRAP polypeptides bound to heparin; showing that the proteins are probably correctly refolded (Figure 4-1 C and D). On the other hand, neither the full-length TRAP nor the TSP domain TRAP showed evidence of binding to heparin, suggesting that the proteins were not correctly folded (Figure 4-1 E and F).

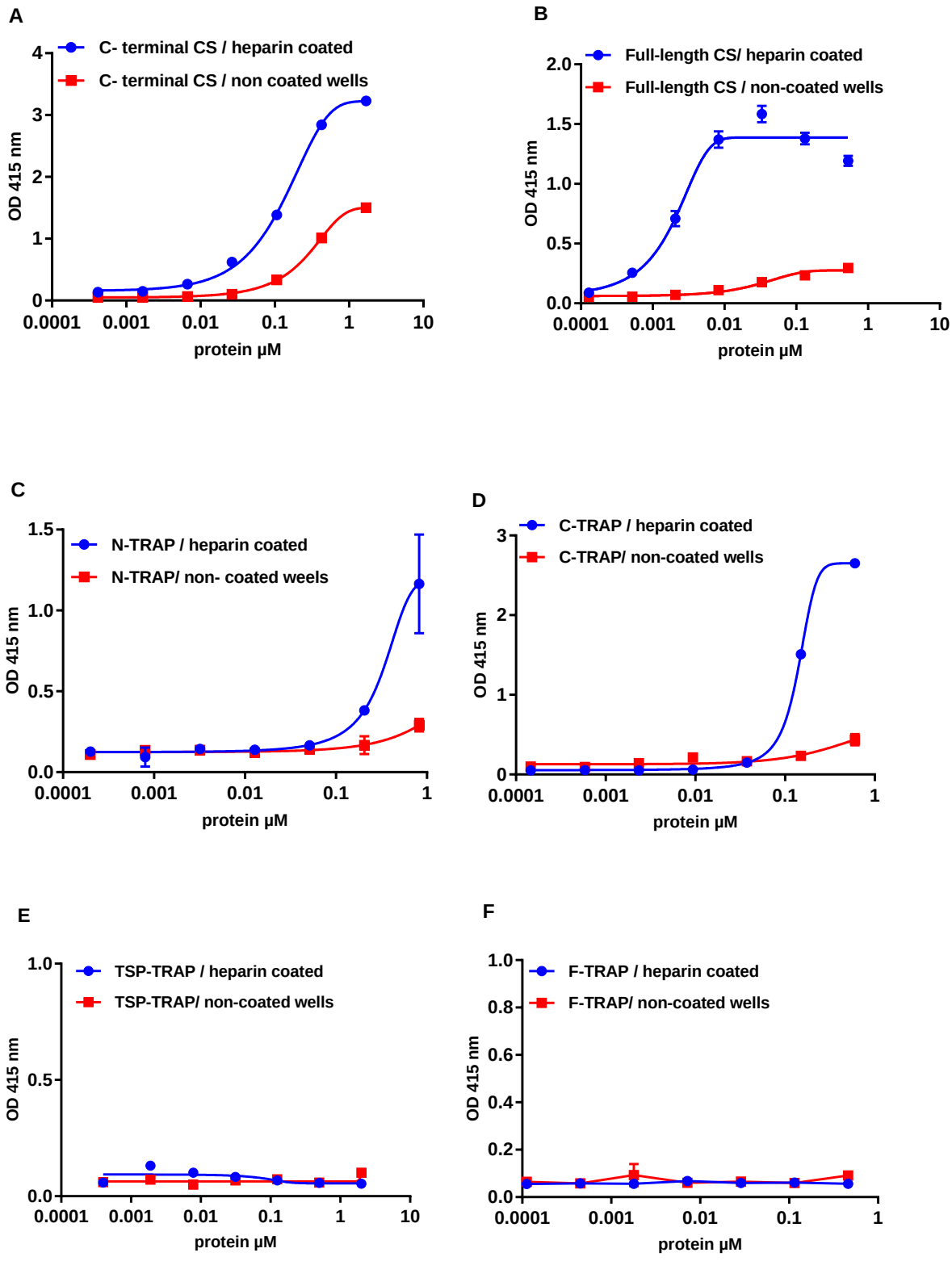

A
C- terminal CS / heparin coated
C- terminal CS / non coated wells
OD 415 nm
protein µM
B
Full-length CS/ heparin coated
Full-length CS / non-coated wells
OD 415 nm
protein µM
C
N-TRAP / heparin coated
N-TRAP/ non- coated weels
OD 415 nm
protein µM
D
C-TRAP / heparin coated
C-TRAP/ non-coated wells
OD 415 nm
protein µM
E
TSP-TRAP / heparin coated
TSP-TRAP/ non-coated wells
OD 415 nm
protein µM
F
F-TRAP / heparin coated
F-TRAP/ non-coated wells
OD 415 nm
protein µM

Figure 4-1: Heparin binding assay of TRAP and CS recombinant proteins

Binding of full length and C-terminal circumsporozoite proteins (CS) and N-terminal, C-terminal, full length and TSP fragments of TRAP to heparin. To show whether the recombinant proteins were correctly refolded, four-fold serial dilutions of recombinant C-terminus CS (A), full-length CS (B), N-terminus (TRAP) (C), C.terminus(TRAP), (E) TSP domain and (F) full length TRAP were added to heparin coated plates (25 μg/ml in PBS). After blocking the plates with 1% BSA, the binding was determined using monoclonal mouse anti poly histidine antibodies. Heparin binding was statistically different when t test was used between C-CS, F-CS C-TRAP and N-TRAP vs C-CS, F-CS C-TRAP and N-TRAP + non heparin coated wells. While heparin binding was no statistically different when t test was used between F.TRAP or TSP domain vs F.TRAP or TSP domain + non heparin coated wells. Results are means (±SEM) of duplicates and are representative of three independent experiments.

4.2.1.2 Binding of TRAP and CS recombinants to C3b

C3b is a key component of all three pathways of complement activation (Charlesworth *et al*, 1979). To probe whether TRAP and CS could bind to human C3b, an assay was designed, by coating microtiter plates with human with 1µg human C3b in coating buffer2.2.2.4.1.2. Serial dilutions of TRAP and CS recombinants were added to the plates. After blocking with 1% BSA, protein binding was detected using a monoclonal mouse anti-polyhistidine antibody. TRAP and CS recombinant shows clear binding to C3b when they were compared to uncoated wells (Figure 4-2 A, B, C and D). The two CS recombinants had nearly the same binding affinity for C3b, suggesting that there is a single binding site near the C-terminal of the protein, where the TSP is located. The results for TRAP were similar; again, the apparent Kd values for the two polypeptides were similar and, since the only shared part is the TSP, this suggests that the C3b binding site is located within the TSP of TRAP. The TSPs in TRAP and CS bind to C3b and so inhibit complement by competing out properdin, which was supported in the sub sequences complement assay.

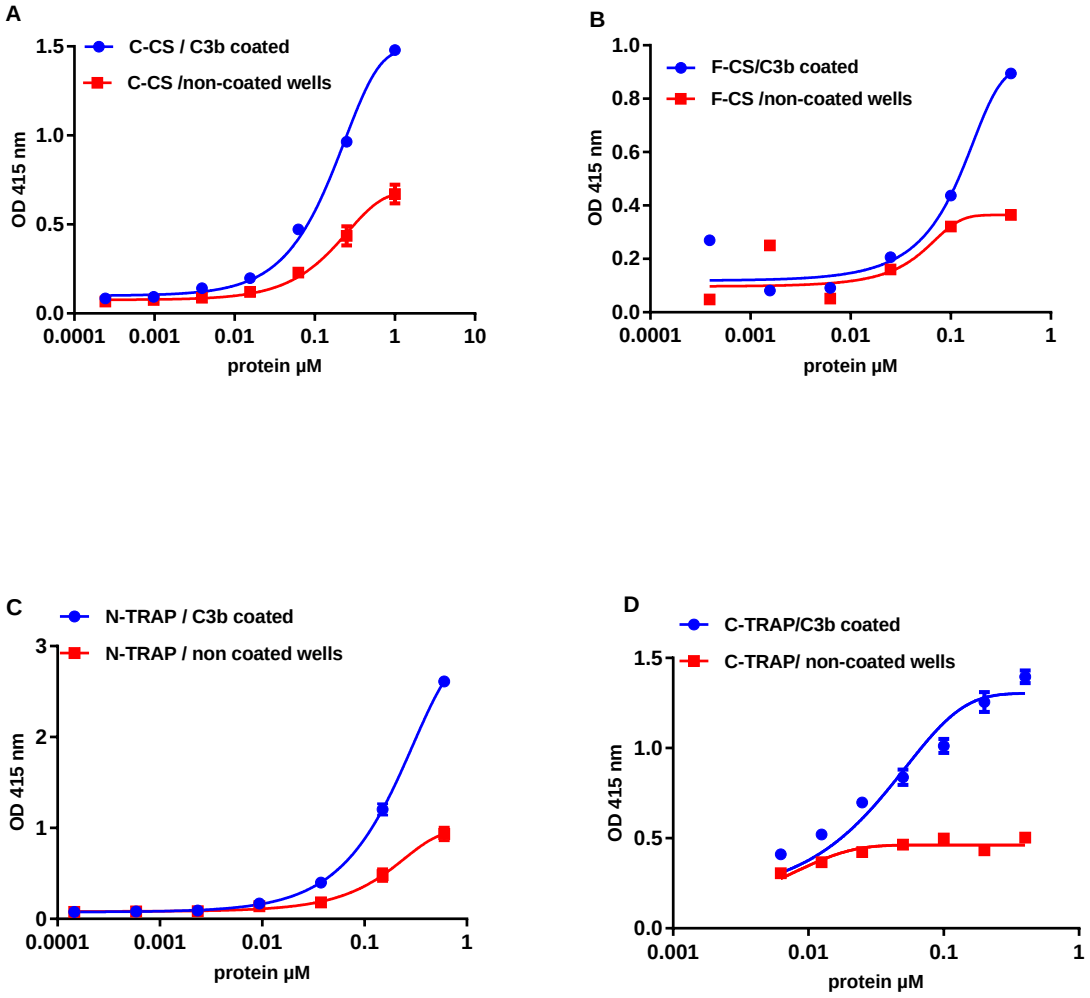


Figure 4-2: Binding of TRAP and CS recombinant proteins to C3b

The C-terminal fragment and full-length CS proteins (A and B), and the N-terminal and C-terminal of TRAP (C and D) were diluted and added to microtiter plates coated with 1µg/ml purified C3b. The wells were blocked with BSA. C3 binding was detected with monoclonal mouse anti-polyhistidine antibody. Wells just blocked with BSA were used as a negative control. The significant P value was statistically calculated by (paired t test) Significant values=$P<0.0001$. Results are means (±SEM) of duplicates and are representative of three independent experiments.

4.2.2 Complement assay

4.2.2.1 C3b deposition on mannan coated plate

C3b can deposit on mannan via the lectin pathway. To determine the effect of CS and TRAP polypeptides on the lectin pathway, we first determined the LP_{50} (the concentration of serum that gives half-maximal C3b deposition through the lectin pathway) of several serum samples, and then used this as a starting point in the subsequent assays that examined inhibition of the lectin pathway. To find out the LP_{50} of serum, serial dilutions of two samples of NHS (commercial pooled NHS from SERALAB & NHS obtained from a healthy person) were added to a mannan coated plate (10µg/ml), starting at a maximum concentration of 2.5%; C3b deposition was detected by using polyclonal rabbit anti-human C3c antibody. The serum concentration needed to give half maximalC3b deposition (LP_{50}) was approximately 1% and 0.6 % for NHS and pooled NHS (Figure 4-3). 1% serum concentration was chosen for the inhibition assay.

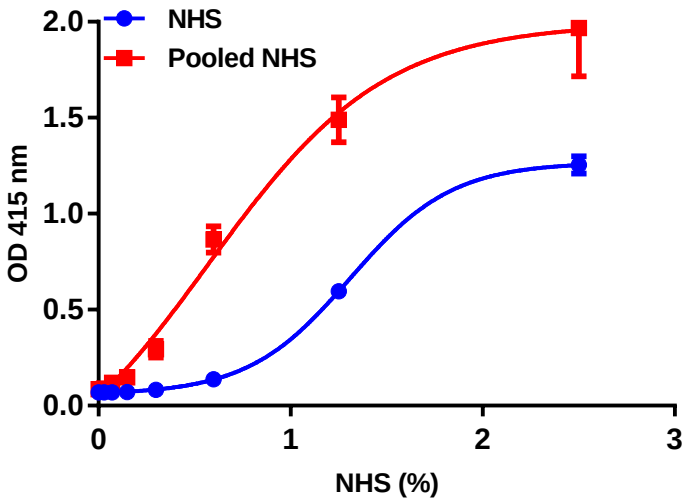


Figure 4-3: Detection of LP 50 on mannan coated plate using two types of serum

Different concentrations of serum (pooled NHS and NHS) were added to mannan coated plate. After 2 hrs blocking with 1% BSA, polyclonal rabbit anti-human C3c antibody was used to determine the LP_{50}. Sigmoid curves were fitted to data and the LP_{50} was calculated. Results are means (±SEM) of duplicates and are representative of three independent experiments.

4.2.2.2 TRAP and CS polypeptides inhibit the lectin pathway

CS and TRAP of *P. falciparum* contain TSP1 domains, as dose properdin (Robson, Kathryn JH *et al*, 1988). It has been shown before in our lab that TSR1 in thrombospondin can act as a competitive inhibitor of properdin, thus inhibiting the complement pathways (unpublished data) (Lynch and Alwashmi, personal communication). Properdin enhances the stability of C3b so increasing the half-life of the C3 convertase (Schwaeble, Wilhelm J. and Reid, 1999). The three complement pathways converge in the formation of a C3 convertase (Kinoshita, 1991). Therefore, inhibition of properdin activity could lead to inhibition of all complement pathways.

The inhibitory effects of the N and C-terminus of TRAP and full-length and C-terminal CS recombinants on the lectin pathway of complement activation were studied. ELISA microtiter plates were firstly coated overnight with 10 mg/ml mannan at 4°C. Serial dilutions of TRAP and CS recombinant proteins, starting at a maximum of 10, or 20 µg/ml, were added to a constant concentration of normal human serum (1%) diluted in Ca^{2+}/Mg^{2+}/BBS buffer. The diluted serum mixture was added to mannan coated plates and the C3 deposition was measured at OD 415 nm by using polyclonal rabbit anti-C3c. All of the recombinants proteins showed clear inhibition of the lectin pathway. C3b deposition was inhibited in the presence of TSP that located each recombinant (Figure 4-4 A, B, C and D).

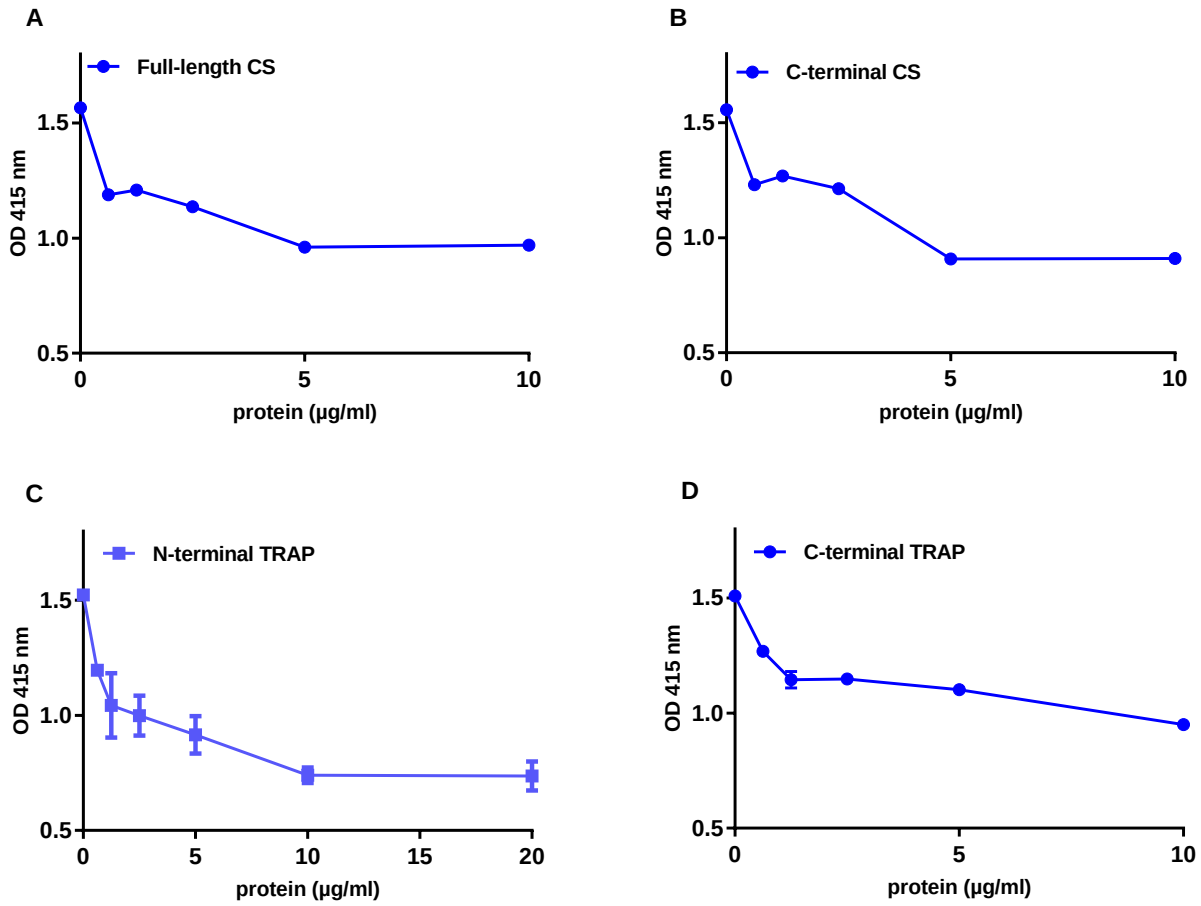


Figure 4-4: Inhibition of the lectin pathway in normal human serum treated with (full length and C-terminal) CS and (N-terminal and C-terminal) TRAP recombinant proteins.

Microtiter ELISA plates was coated with mannan (1 µg /well). 1% NHS was supplemented with recombinant Full length CS. **(A)** C-terminus of CS **(B)** N-terminus of TRAP **(C)** and C-terminal of TRAP (**D).** starting at maximum concentration of **10 µg/mL** (except C-terminal TRAP; **20 µg/mL**), in BBS/ Mg^{2+} /Ca^{2+} buffer and the serum mixture incubated in the mannan-coated wells C3b deposition was determined using polyclonal rabbit anti-human C3c. This experiment shows clear inhibition of the lectin pathway. Results are means (±SEM) of duplicates and are representative of three independent experiments.

4.2.2.3 Alternative pathway

The alternative pathway C3 convertase (C3bBb) is unstable (Schwaeble, Wilhelm J. and Reid, 1999). Properdin has the ability to stabilize the C3 and the C5 convertase, (C3bBb and (C3b)nBb), respectively which increases the half-life of the convertases (Schwaeble, Wilhelm J. and Reid, 1999). Based on the positive role of properdin in regulating the complement alternative pathway, TRAP and CS were assayed using the alternative pathway in order to inhibit this pathway as properdin competitive inhibiter.

The results above for lectin pathway showed inhibition of complement system C3b deposition and by TRAP and CS recombinant proteins (Figure 4-4).

The purified recombinant TRAP and CS proteins were tested for their ability to inhibit the alternative pathway. Serial dilutions of recombinant proteins, starting at 20 µg/ml, were mixed with 10% serum in GVB/Mg^{2+}/EGTA buffer and added to zymosan coated plate. GVB/Mg2+/EGTA buffer blocks activation of the classical and lectin pathways (both of which are calcium-dependent), but allows activation via the alternative pathway. Three samples of serum; NHS1, NHS2, and wild type mouse serum were use. All of the recombinant CS and TRAP proteins showed inhibition of the alternative pathway activity (Figure 4-5 A, B and C). It appears that the TSP that is located each recombinant protein worked as a competitive inhibitor of properdin, hence C3b deposition was inhibited. When comparing the IC50 values on the LP and AP by for all of the proteins (Table 4-1), all the result were very similar (around 0.1-1ug/ml on both pathways). This suggests that mechanism of inhibition could be very likely the same in both cases.

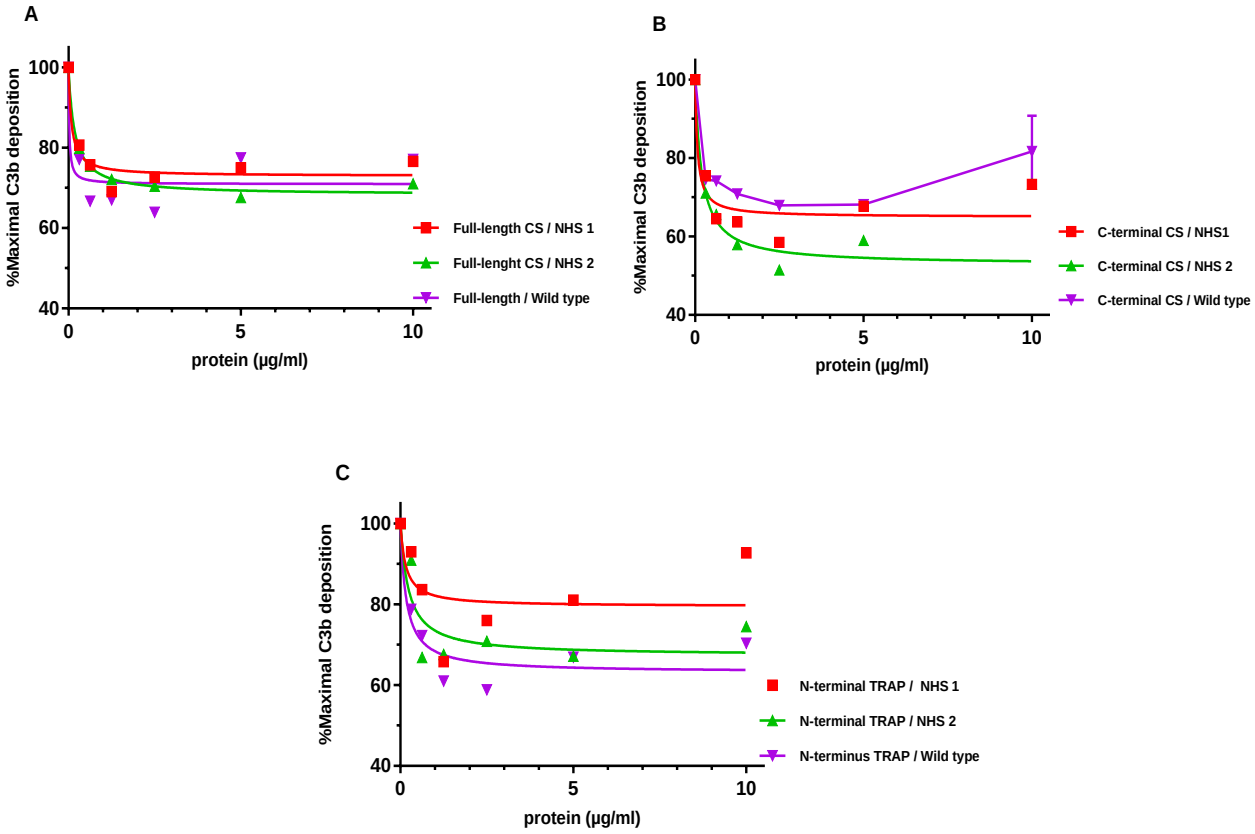


Figure 4-5: Role of TRAP and CS recombinants protein on the alternative pathway

10% of constant concentration for different NHS and wild type serum was treated with serial dilution of proteins which started at 10 ug/ml and diluted in GVB/Mg2+/EGTA buffer. The mixture was added to zymosan coated ELISA plate and C3b deposition was determined. (A) Full-length CS (B) C-terminal CS (C) N-terminal TRAP. Data are the means ± SEM from two independent experiments carried out.

4.2.2.4 Binding of TRAP and CS recombinants to lectin pathway recognition molecules

The first components of the lectin pathway of the complement system are mannose-binding lectin (MBL), the ficolins (Bouwman, Roep and Roos, 2006; Luz *et al*, 2013; Ren, Ding and Zhang, 2014) and the recently discovered the CL-11. These activate the lectin pathway of the complement system through mannose-binding lectin-associated serine proteases, MASP-2 and MASP-1(Selman and Hansen, 2012; Henriksen *et al*, 2013).

The above experiments (Figure 4-4) showed that recombinants protein of TRAP and CS can reduce C3b deposition and inhibition on the lectin pathway. In light of these results, to consider the possibility that TRAP and CS have a hitherto undiscovered effect on the lectin pathway recognition components.

TRAP and CS recombinants were immobilised on microtiter plate together with positive controls to detect the binding of the lectin pathway recognition components (N-acetyl BSA, mannan and zymosan, for L-ficolin, MBL and CL11, respectively). Serial dilutions of NHS were incubated on the plates and captured recognition molecules detected using specific antibodies. Neither MBL nor CL11appeared to bind to any of the TRAP and CS recombinants (Figure 4-6 C, D, E and F), whereas, L-ficolins showed significant binding to full-length and C-terminal CS and N-terminal of TRAP (Figure 4-6 A and B). This suggests another possible mechanism by which the *Pf* proteins could be interfering with the lectin pathway (Figure 4-4).

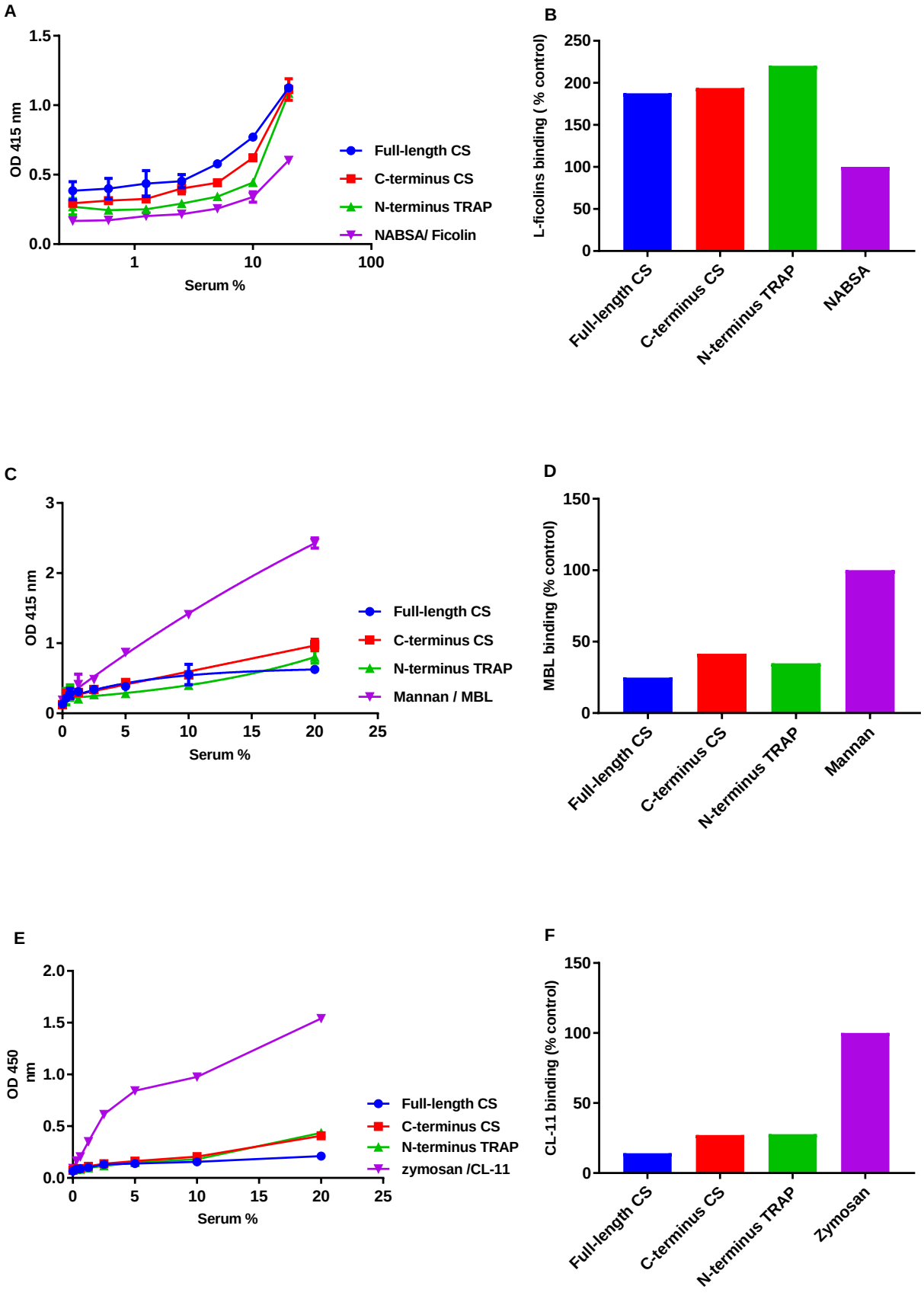

A
OD 415 nm
Serum %
Full-length CS
C-terminus CS
N-terminus TRAP
NABSA/ Ficolin
B
L-ficolins binding (% control)
Full-length CS
C-terminus CS
N-terminus TRAP
NABSA
C
OD 415 nm
Serum %
Full-length CS
C-terminus CS
N-terminus TRAP
Mannan / MBL
D
MBL binding (% control)
Full-length CS
C-terminus CS
N-terminus TRAP
Mannan
E
OD 450 nm
Serum %
Full-length CS
C-terminus CS
N-terminus TRAP
zymosan /CL-11
F
CL-11 binding (% control)
Full-length CS
C-terminus CS
N-terminus TRAP
Zymosan

Figure 4-6: TRAP and CS recombinants protein binding to the lectin pathway recognition molecules

ELISA plates were coated with 5 µg/ml of recombinant proteins as indicated, or N-acetyl BSA mannan or zymosan (used as a positive controls for L-ficolin, MBL and CL-11, respectively). Serial dilutions of NHS starting at 20% were added to the plates. **(A)** A monoclonal mouse IgG anti-L-ficolin was used to detect L- ficolin binding. Recombinant proteins bound L-ficolin compared to positive control. **(B)** The result representing maximum values of proteins bound L-ficolin. The percentage of ficolin showed that statistically difference was seen between NABSA vs full-length CS, C-terminal CS and N-terminal TRAP ($P < 0.5$), L-ficolin = $\text{proteins}/\text{ NABSA}. x\ 100$. **(C)** Monoclonal mouse IgG anti-MBL-C was used to detect MBL binding. None of the recombinant proteins bound MBL. **(D**) The result representing maximum values of proteins bound MBL. **(E)** A specific goat polyclonal IgG anti-CL-11 was used to detect CL-11 binding. Recombinant proteins showed no binding with CL-11 comparing with the positive control**. (F)** The result representing maximum values of proteins bound CL-11. Statistically difference was seen between zymosan vs full-length CS, C-terminal CS and N-terminal TRAP ($P < 0.5$). Data are the means ± SEM from two independent experiments carried out.

4.2.2.5 Haemolysis assay on the complement system

The complement system is a group of proteins that when activated lead to target cell lysis and which also facilitates phagocytosis through opsonisation (Cole and Morgan, 2003). The membrane attack complex (MAC) is the "killer molecule" of complement (Podack, Preissner and Muller-Eberhard, 1984). The previous results showed that recombinant TRAP and CS protein have the ability to inhibit lectin pathway-driven C3b deposition. Haemolysis assays were therefore used to investigate whether the inhibitory effects of the recombinant proteins extend to downstream complement activation and the formation of the MAC. Haemolysis assays exploit the ability of serum from one species to lyse RBC from another species, which are poorly protected from complement attack because of the species-specificity of the surface-bound complement control proteins. RBCs can be rendered more susceptible to complement attack by coating them with specific activators of complement, e.g. immunoglobulin to activate the classical pathway or, in this case, mannan to activate the LP via MBL.

Before recombinant TRAP and CS were tested, a preliminary experiment was done to determine an appropriate concentration of serum to be used in subsequent inhibition experiments. Guinea pigs RBCs were coated with mannan and incubated with serial dilutions of NHS starting at 12.5%, in U-bottomed 96-well plates. After incubation, the plates were centrifuged and the OD_{540nm} of the supernatants determined as a measure of haemoglobin release. Uncoated RBCs and MBL-deficient sera were used as negative controls. Water, instead of buffered serum, was used as a positive control to determine 100% haemolysis. The results were plotted and the LH_{50} (the concentration of serum that gives half-maximal RBC haemolysis through the lectin pathway) was found to be 2 % in NHS on mannan coated erythrocytes (Figure 4-7). The LH_{50} was significantly higher using uncoated erythrocytes and no lysis was observed using MBL-/- serum, confirming that the MAC formation in this system is MBL dependant.

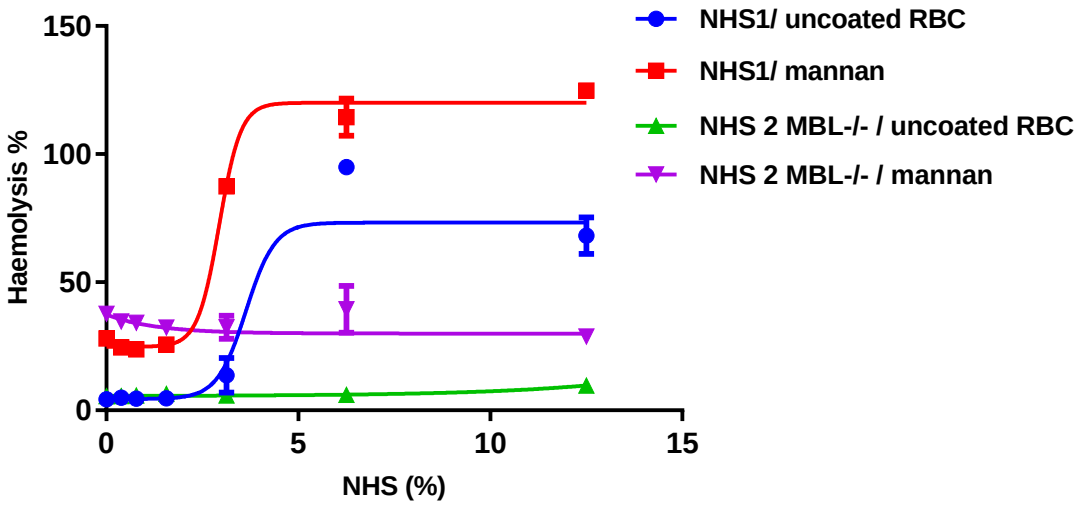


Figure 4-7: Haemolysis assay through the lectin pathway activity (LH50)

In a haemolysis assay, different concentrations of NHS and NHS/MBL-/- in GVB/Mg^{2+}/Ca^{2+} buffer using the lectin pathway initiating to two types of guinea pig erythrocytes, mannan coated and uncoated RBCs. The LH50 was calculated and fitted to the data. Data are the means ± SEM from three independent experiments carried out.

4.2.2.5.1 TRAP and CS inhibit haemolysis of Guinea pig RBC initiated through the lectin pathway

Serial dilutions of recombinant N-terminal TRAP and full length and C-terminal CS protein, starting at 20 µg/ml were added to a constant concentration of 2% NHS serum in (GVB/Mg^{2+}/Ca^{2+}) then the serum mixture was added to mannan coated RBCs $1x10^{8}$ cells/ml and incubated for 1 hour at 37°C under humid conditions and the haemolysis (due to complement activity) was read at OD $_{540nm}$ wavelength. Uncoated RBCs were used as controls. The experiment showed the recombinant proteins inhibit haemolysis via the lectin pathway (Figure 4-8)

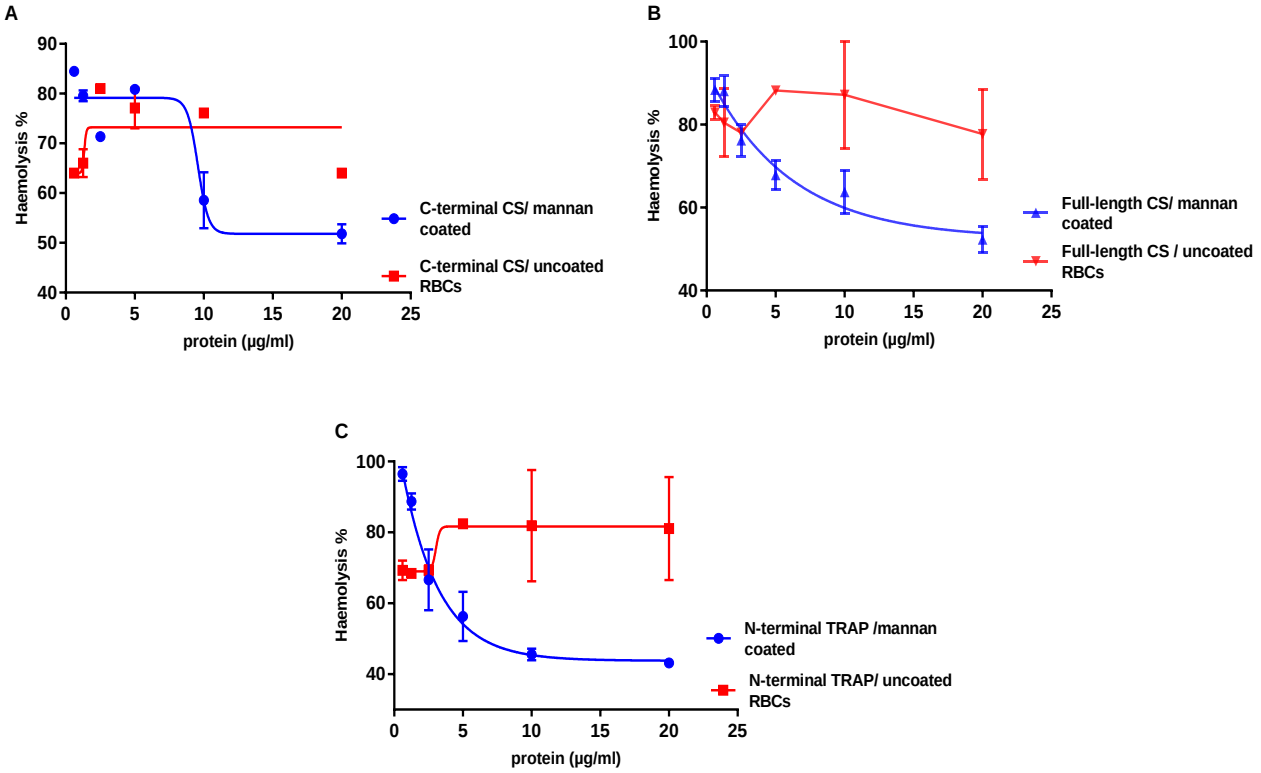


Figure 4-8: TRAP and CS recombinant proteins induced RBC haemolysis through the lectin pathway.

In a haemolysis assay different concentrations of C.CS, F.CS and N.TRAP were added to 2% NHS and the mixture added to guinea pig erythrocytes. The addition CS and TRAP recombinant inhibits the RBC lysis. Statistical significance difference (pairs t test, $P<0.05$) between different C.CS, F.CS and N.TRAP (mannan coated RBCs) vs C.CS, F.CS and N.TRAP (uncoated RBCs) concentrations were statistically different. Results are means (±SEM) of duplicates and are representative of three independent experiments.

Table 4-1: IC 50 values (C3b deposition via LP and AP and haemolysis via the LP)

Fragment	LP	AP	Haemolysis
Full-length CS	0.8 µg/ml	0.08 – 0.1 µg/ml	4.7 µg/ml
C-terminal CS	1.61 µg/ml	0.06 – 0.19 µg/ml	9.5 µg/ml
N-terminal TRAP	0.55 µg/ml	0.14 – 0.22 µg/ml	2.2 µg/ml
C-terminal TRAP	0.31 µg/ml		

4.3 Summary

TRAP and CS recombinants protein were produced successfully in BL21 (DE3) *pLysS* chemically competent *E. coli*. These recombinant proteins were tested for heparin binding to determine whether they were successfully refolded. Full length and C-terminal of CS proteins and the C-terminal, N-terminal of TRAP showed good binding (indicating successful refolding). Meanwhile, the full length and TSP domains of TRAP showed no binding for heparin (thus suggesting refolding for these proteins was not successful). Therefore, they were not using in the following assays. Complement assay was done by using TRAP and CS recombinants protein on both lectin and alternative pathways. The lectin pathway assay showed inhibition of complement by mediated C3b deposition and this seemed to depend on the TSP domain, since this was the only part that all of the recombinant polypeptides had in common. Furthermore, alternative pathway showed inhibition of complement with these recombinants. The IC 50 for both pathway (AP and LP) have nearly similar values and that suggests the proteins have same mechanism of inhibition. TRAP and CS recombinant proteins were tested for binding to lectin pathway recognition molecules and the results showed the ability of recombinants to bind to L-ficolin. In addition, these recombinants were inhibiting the terminal pathway and MAC inhibition. In conclusion, TRAP and CS recombinants protein have TSP1 domain in their structure that TSP1 domain can inhibit the complement pathways, probably by acting as a competitive inhibitor of properdin, and thus protect the parasite from complement attack.

5 Chapter five (Results) The usefulness of anti-CS antibody in the defence against P. falciparum

5.1 Introduction

Circumsporozoite (CS) protein is the most abundant protein on the sporozoite surface. One of its functions is to help malaria sporozoites invade the liver of the vertebrate host via by gliding motility (independent translocation) within minutes after transmission by an infected mosquito (Shin, Vanderberg and Terzakis, 1982). The circumsporozoite protein (CS), contains a conserved C-terminal sequence called the II-plus region which has a thrombospondin repeat (TSR) at the COOH-terminus (Frevert *et al*, 1993).

The results presented in chapter four showed that recombinant CS inhibits the complement system via the lectin and alternative pathways. Anti-CS protein antibodies are therefore likely to be good neutralising antibodies, and CS is a component of the RTS, S/AS02A vaccine, developed by GlaxoSmithKline (Alonso *et al*, 2005). The work presented in this chapter is tests the hypothesis that monoclonal antibodies against CS protein can restore complement activity in serum that has been inhibited with CS proteins.

Monoclonal antibodies were raised against the C-terminal part of CS. The fusion partner (containing the 6x His tag) was removed from the immunogen by proteolytic digest to avoid raising antibodies against this part.

Monoclonal antibodies (mABs) can have specific affinity that means they can bind to one epitope (mAbs) (Marx *et al*, 1997). In contrast, polyclonal antibodies bind to multiple epitopes (Stills, 2012). Monoclonal antibody can be used as therapeutics in medicine, and combine high specificity with generally low toxicity (Keizer *et al*, 2010).

Monoclonal antibodies (mAbs) are produced by introducing an antigen to a mouse and then fusing polyclonal B cells from the mouse's spleen to myeloma cells. The resulting hybridoma cells are cultured and continue to produce antibodies to the antigen (Marx *et al*, 1997) (Figure 5-1)

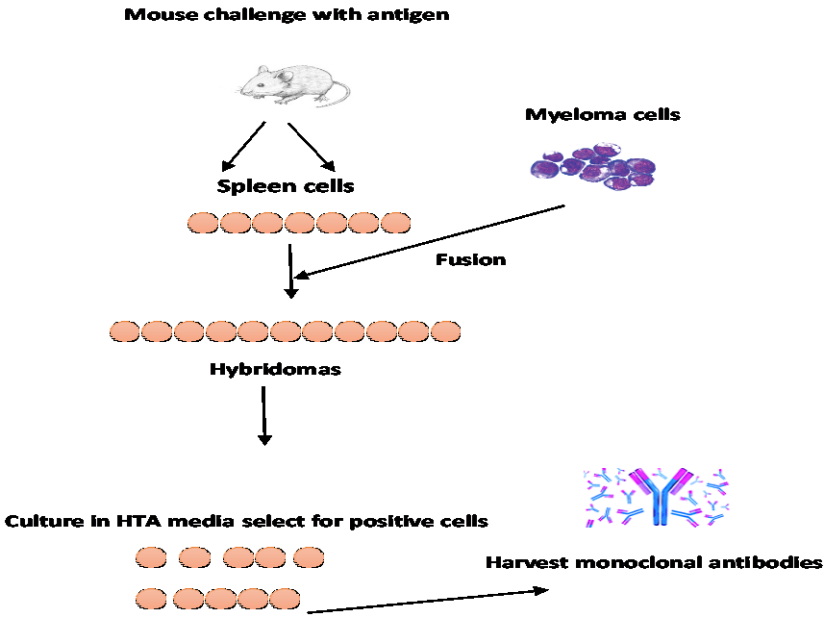


Figure 5-1: A general representation of the method used to produce monoclonal antibodies.

Monoclonal antibodies (mAbs) are produced by introducing an antigen to a mouse and then fusing polyclonal B cells from the mouse's spleen to myeloma cells. The resulting hybridoma cells are cultured and continue to produce antibodies to the antigen. Hybridomas producing the desired mAb are then grown in large numbers on a selective medium that is periodically harvested to obtain the desired mAbs.

5.2 Monoclonal anti C-terminal CS antibody purification and analysis

5.2.1 Enterokinase EKMax digestion of C.terminus of CS

Recombinant C-terminal CS protein without a His-tag was prepared by using Enterokinase enzyme (EKMax)^{Tm} to cleave the N-terminal fusion partner from the recombinant protein, before contracting out production of monoclonal antibodies to a commercial partner (Eurogentec).

In a pilot experiment, the C-terminal of CS was digested with enterokinase enzyme using different concentrations of the enzyme (4, 1, 0.1, 0.01 and 0.001units). SDS-PAGE showed the difference between undigested and digested fusion protein. Four units of EKMax/ 20 µg protein gave complete digestion of the recombinant fusion protein, and this concentration was used for subsequent large-scale digests (Figure 5-2).

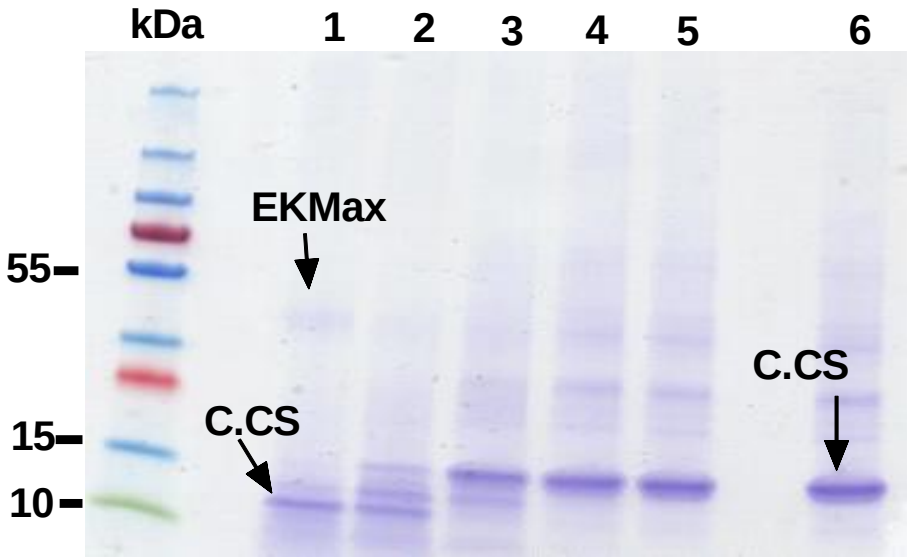


Figure 5-2: C.t-CS protein digested with EKMaxC.t-CS protein digested with EKMax

Each reaction contain 20 µg purifed C-terminal CS protein and varying amounts of EKMax. Reactions were incubated for 16 hrs and analysed on SDS-PAGE**.** Twenty microliters of the protein were mixed with 5 µL of 5X protein loading buffer containing 5% 2- mercaptoetanol and samples were boiled at 95˚C for 10 minutes then separated in a 15% SDS-polyacrylamide gel and the gel was stained with Coomassie Blue. (A) kDa: protein ladder, (1) digested C.T CS recombinant protein with 4 unit EKMax at 10 kDa and EKMax bands show at 43 kDa, (2) digested C.T CS protein with 1unit EKMax, (3) digested C.T CS protein with 0.1unit EKMax (4) digested C.T CS protein with 0.01unit EKMax (5) digested C.T CS protein with 0.001unit EKMax (6) C.T CS protein before digestion as control at 18kDa.

5.2.2 Preparing the recombinant C-terminus of CS for produce antibody

Recombinant C-terminal CS protein was purified using His GraviTrap columns (GE Healthcare) after being digested with 4 unit of EKMax enzyme per 20 µg amount of protein for 16 hourse at 37º C. The fusion partner is retained on the column by its His-tag. The flow through , containing the CS protein without its fusion partner, was collected after the sample was loaded into His GraviTrap columns. Purified recombinant C-terminal CS protein was analysis using SDS-PAGE 15% under reducing conditions and Coomassie stained (Figure 5-3)

This purified digestion recombinant was done to get C.T CS recombinant protein without His-tag in order to send this recombinant to Eurogentic company to produce monoclonal mouse antibody.

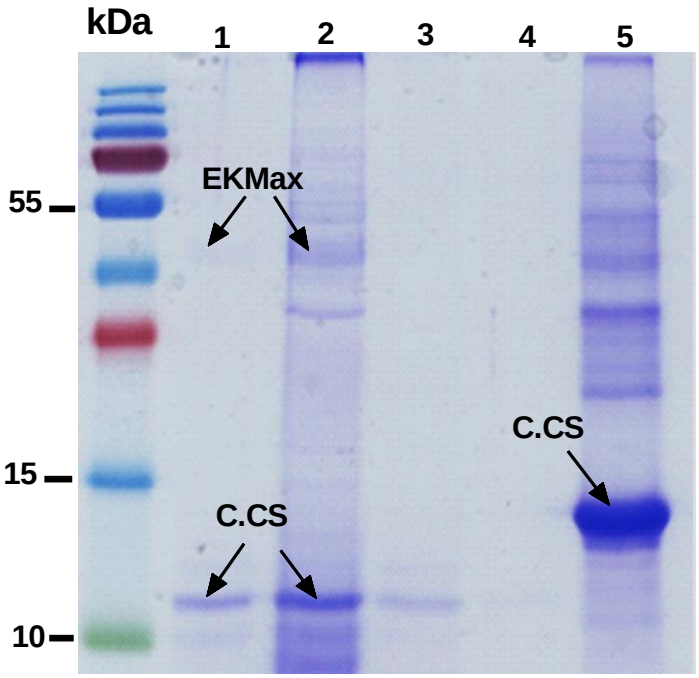


Figure 5-3: SDS-Polyacrylamide gel electrophoresis of recombinant C-terminal CS protein digested with EKMax.

20µg of the recombinant protein was separated in 15% polyacrylamide gel under reducing conditions and stained with Coomassie Blue**.** C.TCS was digested with EKMax enzyme for getting C.T-CS without the his tag (1) His GraviTrap column flow through showed purified C.T CS recombinant protein at 10 kDa and EKMax bands show at 43 kDa. (2) Digested C.T CS recombinant protein with 4 units EKMax at 10 kDa and EKMax bands show at 43 kDa before was loaded in the His GraviTrap column. (3) Washing column with 40 mM imidazol. (4) Column elution with 400mM imidazol. (5) C.T CS recombinant protein before digestion with EKMax.

5.2.3 Antibody purification

Four mice were immunised with C-terminal CS by Eurogentic and two mice which gave a higher titter of antibody in the mouse serum test bleed were selected for hybridoma production (7.1). After hybridoma production screening for positive hybridomas was done; hybridomas that were positive for binding to the CS fragment, but negative for binding to the fusion partner were chosen. Isotyping of the positive hybridoma was done and two supernatant selected (appendix7.2). Supernatants from 8C6 and 10B10 mouse samples supplied by Eurogentic Company. Monoclonal antibodies were purified using IgG protein G kit. Protein G is a recombinant protein expressed in *Escherichia coli* and is recommended for purifying IgG from mouse, human, cow, goat, or sheep serum. Monoclonal antibody for 8C6 and 10B10 were purified by protein G column (Thermo-Pierce) and the antibodies were eluted into five fractions, each of 1ml with elution buffer as in (2.2.2.6.1). mAb concentration was measured and fractions containing Ab were analysed by SDS-PAGE. One fraction of the three shows two bands in the right size that are the heavy chain and light chain at 55 kDa and 25 kDa respectively, under reducing condition (Figure 5-4)

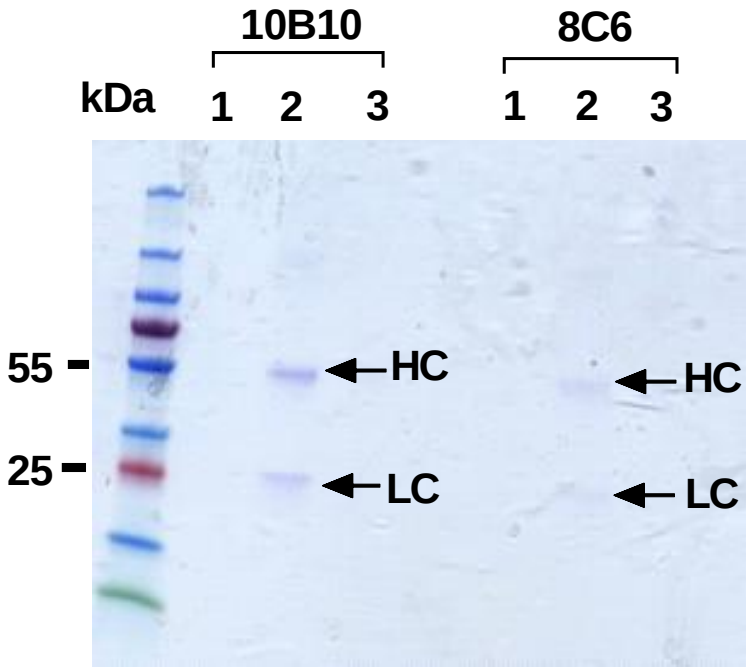


Figure 5-4: SDS-PAGE analysis of purified 8C6 and 10B10 mouse monoclonal anti-CS.

Twenty microliters of the eluted anti-CS antibody were mixed with 5 µL of 5X protein loading buffer containing 5% 2- mercaptoetanol and sample was boiled at 95˚C for 10 minutes. Samples of 8C6 and 10B10 were separated in 12% SDS-polyacrylamide gel and gel was stained with Coomassie Blue. Column eluate with elution buffer show bands in the right size for IgG antibody in the lane 2 for both in heavy chain and light chain at 55kDa and 25 kDa, respectively.

5.2.4 Western blotting of Monoclonal anti CS antibody

The purified recombinant proteins of full length and C-terminal of CS were separated in SDS-polyacrylamide gel and blotted into nitrocellulose membrane. The membranes were blocked and the proteins were then probed with three kind of antibody ; 8C6 mouse monclonal anti CS antibody (Figure 5-5 A), 10B10 mouse monclonal anti CS antibody (Figure 5-5 B) and monoclonal mouse anti-polyhistidin antibody (Figure 5-5 C). The membran that probed with 8C6 mouse monclonal anti CS antibody did not show any bands for full length or C-terminal of CS. On the other hand, the other membranes that were probed with 10B10 mouse monclonal anti CS antibody and monoclonal mouse anti-polyhistidine show bands in the right size for full length and C.T of CS at 68 kDa and 18 kDa, respictively (Figure 5-5 A , B and C), thus confirming that 10B10 recognises CS protein on a Western blot.

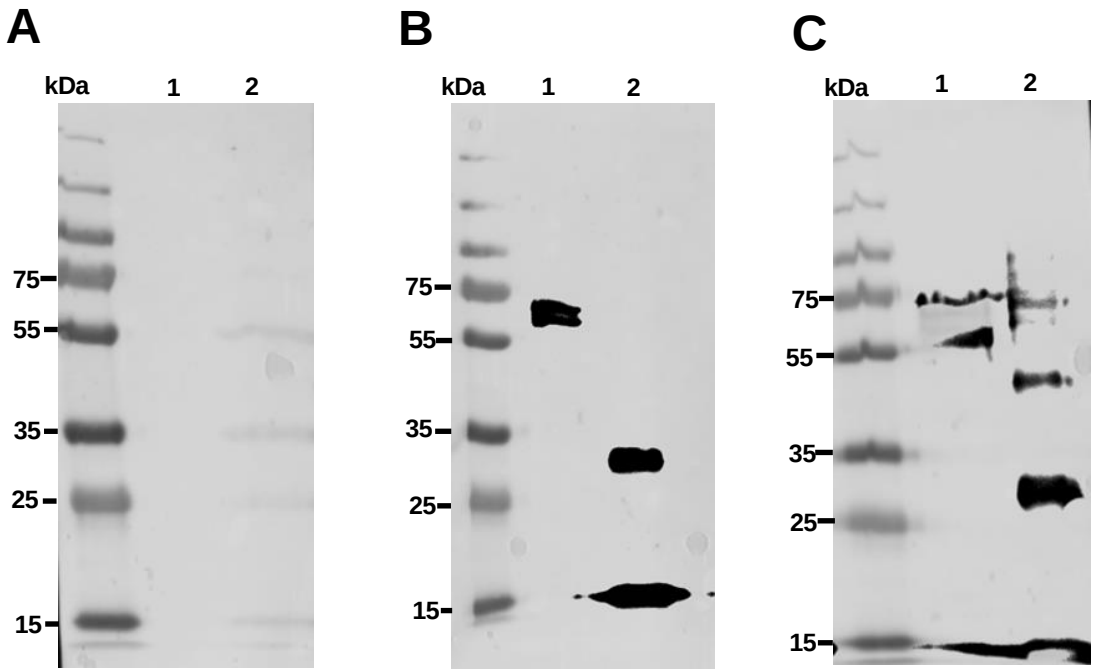


Figure 5-5: Western blotting analysis of CS recombinant protein with different antibodies.

5µg of full length and C.T CS the recombinant proteins of were separated on SDS polyacrylamide gel then electrophoretically transferred onto nitrocellulose membranes. The blot was probed with **(A)** 8C6 mouse monclonal anti-CS antibody and was no bands on the membrane. **(B)** 10B10 mouse monclonal anti CS antibody and shows band for FL.CS and C.T CS in the right size at 68kDa and 18 kDa respectively. **(C)** Monoclonal mouse anti poly histidine antibodies show bands in the right size.

5.2.5 Binding of monoclonal anti CS antibody to CS proteins

Monoclonal anti CS antibody was checked for binding with full-length and C-terminal CS protein after coated the plate with 5µg/ml of CS proteins then checked the binding by serial dilution of anti-CS antibody for both 8C6 and 10B10 antibody. 10B10 anti CS antibody shows strong binding to CS proteins (Figure 5-6 B and Figure 5-7). While 8C6 anti-CS antibody did not work (Figure 5-6 A). This agrees with the Western blot above (Figure 5-5).

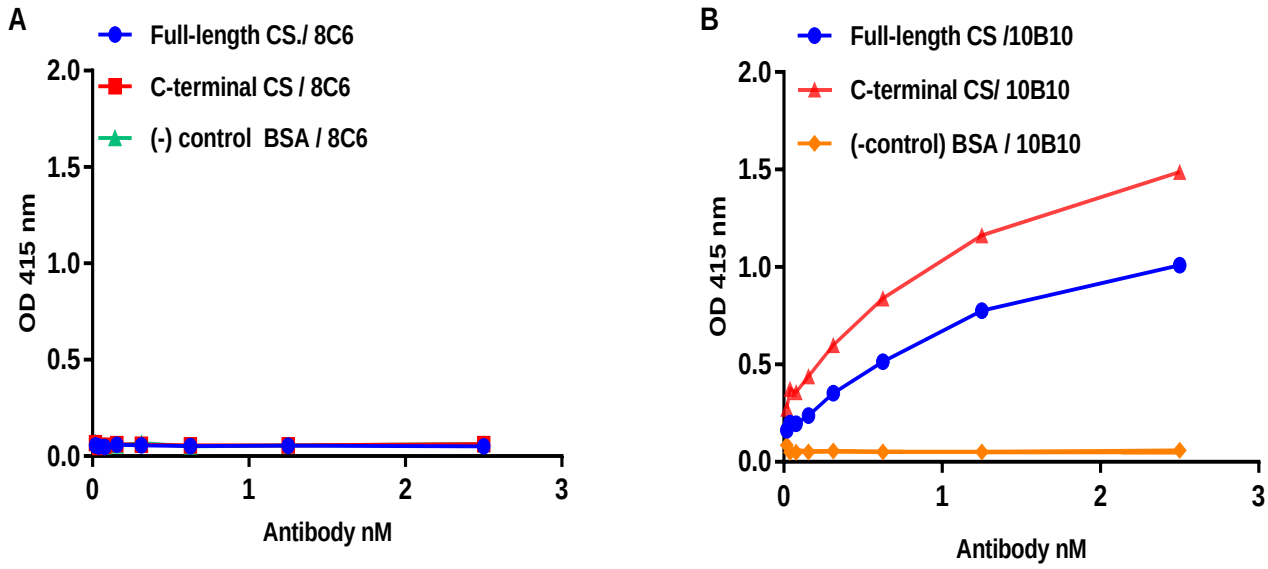


Figure 5-6: Monoclonal anti-CS antibody binding assay on full length and C-terminal of CS.

(A) Monoclonal anti-CS 8C6 antibody binding assay on full length and C-terminal, following coating of microtiter plates with (5 µg/mL in coating buffer) CS proteins and then blocking the plate with 1% BSA. The binding was checked by serial dilution of monoclonal mouse anti-CS 8C6 antibody starting at 2.5nM, 8C6 anti-CS antibody did not bind. (B) The same thing was done for monoclonal anti-CS 10B10 antibody binding. BSA was used as a negative control. The statistical significance of 10B10 was calculated between the full-length, C-terminal wells and (-) control wells by using Bartlett's multiple comparisons test, one-way ANOVA test. The statistical significance is = ($P<0.05$). Results are means (±SEM) of duplicates and are representative of two independent experiments.

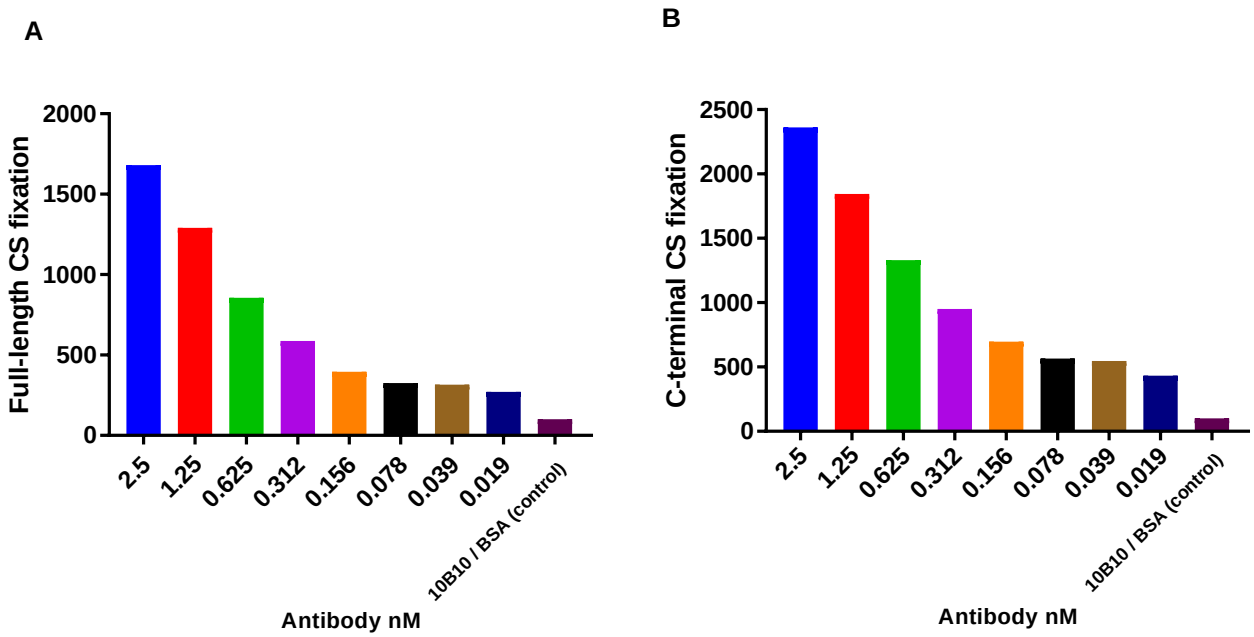


Figure 5-7: Monoclonal anti-CS 10B10 antibody binding assay on full length and C-terminal of CS

The percentage of TRAP and CS binding was determined by plotting the serial dilution of monoclonal mouse anti-CS 10B10 antibody starting at (1/10), the binding were showed clear binding of 10B10 anti CS antibody both full-length and C-terminal of CS comparing with the control BSA + monoclonal 10B10 anti CS antibody. Results are means (±SEM) of duplicates and are representative of two independent experiments anti-CS 10B10 antibody.

5.3 Reactivation of the complement system by monoclonal anti-CS antibody

5.3.1 Monoclonal anti-CS antibodies assay on the lectin pathway.

As mention aboved, recombinant CS proteins inhibit to the lectin pathway of the complement system (Figure 4-4). To investigate whether monoclonal anti-CS antibody can reactive the complement system, serially diluted mouse monoclonal anti-CS antibody, starting from 125nM, was supplemented with 10 µg/ml recombinant CS protein and 1% NHS serum on mannan coated plates. The reactivity was checked by using polyclonal rabbit anti human C3c. It is clear that 10B10 anti-CS antibody reactivates the complement system when it is inactivated by either recombinant FL-CS or C-CS, compared with 8C6 anti-CS antibody that was used as a negative control for anti-CS activity (Figure 5-8).

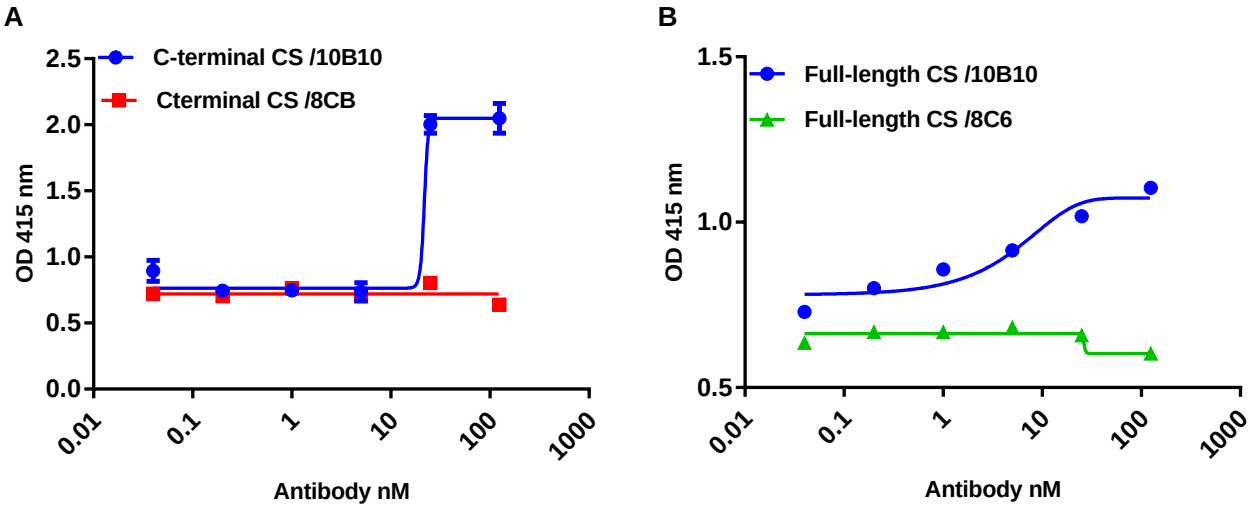


Figure 5-8: Complement reactivation by using monoclonal mouse anti-CS antibody on the lectin pathway.

ELISA plates were coated with (10µg/ ml) mannan and then blocked with 1% BSA. Serum mixture with serial dilution of both 8C6 and 10B10 monoclonal mouse anti-CS antibody and constant CS protein at 10µg/ml were added to plate for reactivation the complement system via lectin pathway. Polyclonal rabbit anti-C3c was used to detect C3b deposition on mannan. This experiment shows that clear reactivation of the lectin pathway by blocking both C-CS (A) and F-CS (B) by 10B10 anti-CS antibody while 8C6 anti-CS antibody was used as negative control. The significant P value was statistically calculated by paired t test. Significant values=$P<0.5$ for both C-terminal and full length. Results are means (±SEM) of duplicates and are representative of three independent experiments.

5.3.2 Monoclonal anti-CS antibodies assay on the alternative pathway.

It was previously shown that recombinant CS proteins inhibit the alternative pathway (Figure 4-5), so anti-CS antibody was checked on the zymosan coated plate. 5% NHS serum with 10 µg/ml of recombinant CS proteins were added with serial dilutions 10B10 and 8C6 in TBS buffer. Both types of anti-CS antibody did not work on the alternative pathway (Figure 5-9). One possible explanation of this result is that the antibody binding might be calcium dependent (there is no calcium in this assay system).

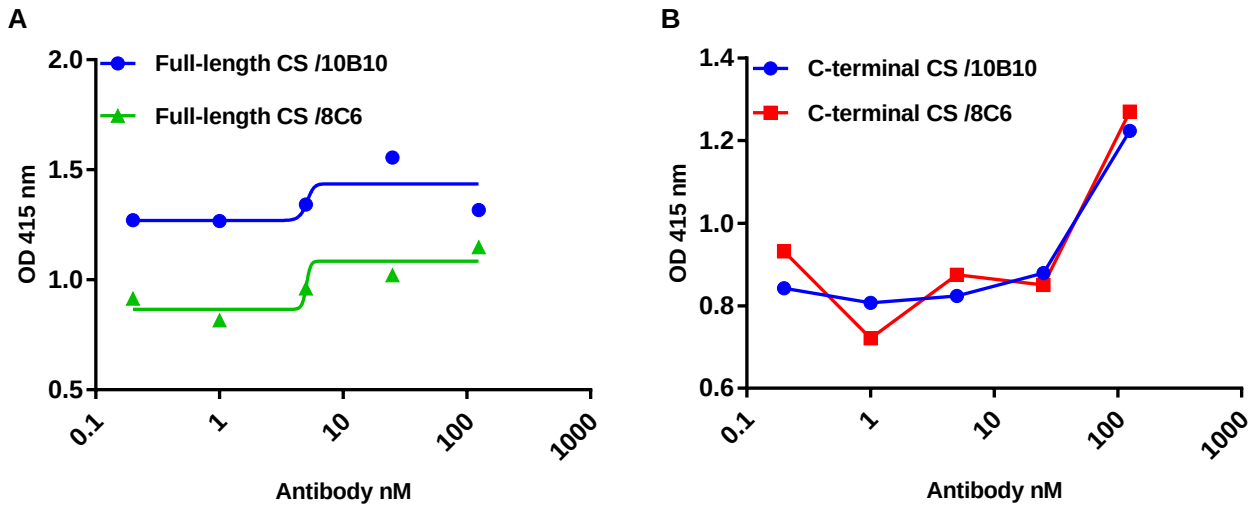


Figure 5-9: Complement reactivation by using monoclonal mouse anti-CS antibody on the alternative pathway

ELISA plates were coated with (10µg/ ml) zymosan and then blocking with 1% BSA. Serum mixture with serial dilutions of both 8C6 and 10B10 monoclonal mouse anti-CS antibody were added to plate for reactivation the complement system via alternative pathway. Polyclonal rabbit anti-C3c was used to detect C3b deposition on zymosan. This experiment shows that clear no reactivation the complement system by blocking both C-CS (A) and F-CS (B) of the alternative pathway by 10B10 anti-CS antibody while 8C6 anti-CS antibody was used as negative control. Results are means (±SEM) of duplicates and are representative of two independent experiments.

5.4 Summary

Enterokinase enzyme was used to digest recombinant C-terminal CS to remove the His-tag preparation for production of mAb by Eurogentic Company to avoid across-reactivity with histidine protein. Two types of supernatant (10B10 and 8C6) were received from the company. After that, these supernatant were purified by using IgG protein G kit to get mAb. These two mAb were used in binding assays with F-CS and C-CS recombinant protein to check their activity. The 10B10mAb was active so, that was used in the complement reactivation assay, while 8C6 mAb was not active and used as negative control. The role of 10B10 mAb in the complement system was tested on both lectin and alternative pathways. The lectin pathway was clearly reactivated by 10B10, presumably by blocking CS protein and allowing for C3b augmentation. On the other hand, the result for complement reactivation on alternative pathway was no different between the 10B10 mAb and the negative control 8C6 mAb and that refer to not work on the alternative pathway. Anti-CP protein could be in future being as a potential therapy against *P. falciparum*.

6 Chapter six

Discussion

.

6.1 General discussion

Plasmodium falciparum is a protozoan parasite that causes malaria. It has different stages in its life cycle. Humans can get infection when female of Anopheles mosquitoes injects sporozoites (hepatic stage) into the skin, which travel to the liver via the peripheral blood circulation. After that, merozoites (blood stages) invade RBCs leading to appearance of clinical symptoms (Scherf, Lopez-Rubio and Riviere, 2008). Different methods are used to control malaria, which include using insecticides, drugs, environmental control and some attempts to produce effective vaccines (Hemingway and Ranson, 2000).

Mordmüller *et al*. (2017) succeeded in producing long-lasting, sterile immunity when they intravenously injected 150,000 whole live attenuated (cryopreserved) sporozoites of *P. falciparum* into human volunteers. The immune response involved in this experiment included T-cells, B-cells and antibodies against sporozoites (Bijker *et al*, 2014). The antibodies produced can neutralise sporozoite infectivity in hepatic cells (Behet, Marije C. *et al*, 2014).

The blood stage of *P. falciparum* (merozoites) is susceptible to antibody-mediated complement killing (Boyle *et al*, 2015). In addition, sporozoites can be targeted by acquired antibody which induces complement activation (Kurtovic *et al*, 2018). Recently, the complement system has been shown to have an important role in sporozoite lysis, mainly mediated by antibody and the classical pathway (Behet, M. C. *et al*, 2018). There is also recent evidence that the other two complement pathways (the AP and LP) contribute to complement activation on *P. falciparum*, as reviewed by (Silver *et al*, 2010)

The sporozoite, the asexual hepatic stage of *P. falciparum* that is also the infectious stage, which enter the human body by mosquito's bite, has two important surface proteins TRAP and CS. Both are involved in the attachment of sporozoites to liver cells.

Both of these surface proteins have TSP domains in their structures (Figure 1-2) that share amino acid sequence motifs with thrombospondin 1 and properdin. Thrombospondin I can work as a competitive inhibiter for properdin, and that leads to destabilization of the C3 convertase, thus inhibiting the complement system. Based on

that, I expect that sporozoites can evade complement attack by inhibiting properdin, TRAP and CS.

6.2 Protein expression

TRAP and CS are important surface proteins of the *P.falciparum* sporozoite (the liver stage). These proteins have a TSP domain in their structure (Naitza *et al*, 1998).

Two expression vectors were used for cloning TRAP and CS, in order to express these proteins in bacterial and mammalian systems (Figure 3-4 and Figure 3-5). The bacterial system is rapid and simple, and proved enough to produce high amounts of active proteins; while the mammalian system gave small amounts of purified protein, so was dropped.

TRAP and CS proteins were expressed as insoluble inclusion bodies (Figure 3-7 D) and (Figure 3-8 B and C), therefore, they were refolded by using a modified refolding protocol, included solubilization by urea. This result agrees with Tero *et al*. (2013) who expressed the A domain of *Plasmodium falciparum* in *E.coli* BL 21, but purified the inclusion bodies in a buffer containing guanidinium chloride. The purified protein was used in crystallization then comparison of the *Pf*TRAP-A structure with other members of the Vwa-family. Furthermore Salas *et al*. (1995) used the same protocol as us for refolding, but the difference that after the first resuspension of the pellets, the protein was incubated at 56ºC for 2h denature the protein. Subsequence refolding steps were the same, and this protein was used for crystallization.

The mammalian expression system (pSecTag2/Hygro) was used to express TRAP and CS. CHO cells were chosen to stably express recombinant proteins and were successfully expressed as assessed by dot blot (Figure 3-12 and Figure 3-13), but the amount of the protein that was harvested after large scale purification using nickel columns was relatively low compared to the amount of recombinant proteins expressed in bacteria. Therefore, the proteins expression was only done in the bacterial systems.

There are many researchers who successfully used HEK 293 cell as cell line for expression of TRAP. Song *et al*. (2012) successfully expressed *P. falciparum* TRAP in HEK 293 cells, as well as (Bauza *et al*, 2014), who expressed *P. vivax* TRAP into HEK 293 cells. I transfected TRAP and CS protein (the same TSP family member) (Thompson *et al*, 2004), to HEK293 and CHO-K1 cells, in order to express proteins in sufficient amount. However, I could not get any expression for other proteins either in CHO-k1 or HEK293. Only small amount of C-terminal TRAP was expressed in CHO-K1 and the purified protein was about amount 0.2 µg/ml compared with Song *et al*. (2012) who expressed *P.falciparum* TRAP in HEK cells and obtained 5mg/ml.

6.3 The role of TRAP and CS in the complement system

Host cells can have heparin on their surface (Meri *et al*, 2013), which helps protect them from the activity of the host's own complement system by binding factor H thus inhibiting the C3 convertase. We used heparin binding as a marker of correct folding. Recombinant full-length of CS has a much higher affinity for heparin than the C-terminal fragment, probably because the full-length protein has regions I and II-plus that preferentially bind to highly sulphated heparin-like oligosaccharides in heparin sulphate (Figure 4-1A and B) (Ying *et al*, 1997). The N-terminal and C-terminal TRAP polypeptides are probably correctly refolded as they bind to heparin (Figure 4-1C and D). However, the full-length and the TSP domain TRAP did not bind to heparin, which probably indicates failure of refolding (Figure 4-1E and F).

C3 convertases, the central component of the complement cascade, are stabilized by properdin. C3 convertases cleave C3 to C3b. This cleavage can occur via three mechanisms (classical pathway, alternative pathway and lectin pathway).

Both recombinant CS fragments showed nearly the same binding affinity for C3b (Figure 4-2 A and B), suggesting that there is a single binding site near the C-terminal of the protein, where the TSP is located. The results for TRAP were similar; as the Kd values for the two recombinant TRAP fragments were similar, since the only shared part is the TSP (Figure 4-2 C and D), this suggests that the C3b binding site is located within the TSP of TRAP. These results showed that TSPs in TRAP and CS can bind to C3b and thus might inhibit complement activity by competing out properdin.

One of the aims my study was to examine whether the TSP domains in TRAP and CS act as competitive inhibiters for properdin. That was done by measuring the activation of C3 in serum via the lectin and alternative pathways.

Mannan coated plates were used to specifically activate the lectin pathway, with a concentration of serum too low to allow activation of the alternative pathway. The alternative pathway assay uses a high concentration of serum, diluted in a buffer (GVB with EGTA and Mg^{2+}) that removes the calcium needed for lectin and classical pathway activation. Zymosan was used as the activator (Smith, Pensky and Naff, 1982). Anti C3c antibodies were used to detect deposited C3b because it can bind and cross react with surface bound C3b.

CS and TRAP fragments inhibit lectin pathway mediated C3b deposition (Figure 4-4 A, B, C and D). This could be because of the TSP1 domains included in CS and TRAP of *P. falciparum* (Robson, Kathryn JH *et al*, 1988); it was proved before in our lab that TSP1 in thrombospondin can act as a competitive inhibitor of properdin, thus inhibiting the complement pathways (Lynch and Alwashmi, personal communication). Furthermore, these recombinant proteins showed inhibition of the alternative pathway (Figure 4-5). When comparing the IC50 values (the concentration of an inhibitor where the response is reduced by half) for the AP and LP results, it seems that the AP IC50 values are quite low compared to the LP IC 50 values (Table 4-1), which suggests that the AP inhibition is more powerful than the LP inhibition. This could be because in the AP the *P.falciparum* proteins work directly on C3bBb, whereas the LP retains some C3 cleavage capacity through C4b2a.

The above experiments showed that recombinant TRAP and CS proteins can inhibit the lectin pathway and the alternative pathway, reducing C3b deposition, suggesting that TRAP and CS act as competitive inhibiter of properdin.

TRAP and CS recombinant proteins did not show any binding to MBL and CL11 (Figure 4-6 C, D, E and F), whilst, full-length and C-terminal CS and N-terminal of TRAP bound significantly to L-ficolins (Figure 4-6 A and B). This might mean that TRAP and CS can activate the lectin pathway, but further experiments looking specifically at activation rather than binding would be needed to confirm this. Equally it is possible that TRAP and CS bind L-ficolins in a manner or conformation that prevents MASP activation, thus inhibiting LP activation. The lectin pathway of complement

activation is a key mechanism for the mammalian acute phase response to infection in innate immunity. This complement activation pathway involves carbohydrate recognition by MBL and ficolins (Liu *et al*, 2005).

The results also showed that the recombinants protein inhibited the final pathway and MAC formation via the lectin pathway using haemolysis assay (Figure 4-8). These results confirm that the recombinant fragments can inhibit the complement pathways in the results above. The IC50 values in haemolysis via lectin pathway were higher than IC values of LP. This suggests that the inhibitors are less effective at inhibiting of C5 convertase than C3convertase. When inhibiting complement activation, the parasite ability to survive will increase. This result is in agreement with Goto and Sanchez, (2013) who say failure the complement system to destroy the parasite is because the parasite has the ability to develop effective mechanisms to avoid complement attack.

6.4 Reactivation of the complement system by anti-CS antibody

There are many studies about producing monoclonal antibody against TRAP and CS for different species of Plasmodium in order to reduce the infection with malaria.

Polyclonal antibody against TRAP including the TSP was prepared by (Sharma *et al*, 1996); this antibody was produced with an 18-mer polypeptide containing TSP domain sequences (the most conserved sequence in TRAP), in order to inhibit merozoite invasion of erythrocyte. The authors measured RBC invasion by culturing the parasite in 10% human serum and added 5% normal rabbit serum, and then adding 0.05% rabbit anti-nonapeptide antiserum to this culture. The results were 26% inhibition of parasite growth using rabbit anti-nonapeptide serum compared with parasite culture without anti-nonapeptide. Polyclonal antibody, which contains the nonapeptide sequence (TSP), caused significant inhibition of the merozoites invasion of erythrocytes. That suggests this motif, or other similar motifs in different proteins, might be involved in some vital biological process of the parasite.

Recently, there have been numerous of trials to examine the effectiveness of RTS,S, a CS-based-vaccine. Reported efficacy rates from 30 to 66% for infection, and 30% for medical malaria, in children 1–4 years (Alonso *et al*, 2004; Aponte *et al*, 2007).

RTS,S CS vaccine is against two regions; repeat region and C terminus but not for N-terminal (Aponte *et al*, 2007). Plassmeyer *et al*. (2009) succeed in generating mAbs toward all CS domains which gives complete insight into the role of CS. To get monoclonal anti-TSP domain, a purified recombinant piece of CS corresponding to the TSR domain sequences of CSP was boosted in mice. Thereof, mAbs produced by Plassmeyer *et al*. (2009) can identify unfixed *P. falciparum* sporozoites and inhibit sporozoite invasion of HepG2 cells *in vitro.*

I raised mAbs against recombinant TSP of CS. The purified antibodies were checked by western blotting and they clearly bound to CS proteins (Figure 5-5 and Figure 5-7). Monoclonal anti-CS antibodies were tested for their ability to restore complement activation in serum that had been inhibited with recombinant CS proteins. The results showed augmentation of complement activation via the LP when the antibodies were added to CS and mixed with normal human serum. This result may be because the binding of monoclonal anti-CS antibody to CS proteins allowed to properdin work normally to keep the C3 convertase stable, thus increasing lectin pathway activation (Figure 5-8). For the alternative pathway the reactivation by monoclonal anti-CS antibody was less obvious (Figure 5-9), which could be because the inhibition of the alternative pathway is less than that of the lectin pathway The results suggest that the vaccine based on CS (RTS, from GSK) might derive some of its efficacy from the fact that it neutralises CS, rendering the parasite susceptible to complement attack.

6.5 Future work

To date, I have shown that TRAP and CS proteins inhibit complement activity *in vitro*, using model activators of the lectin and alternative pathways. An obvious extension of this work would be to show whether the proteins also inhibit complement attack on live sporozoites.

There is a new technique developed by Soulard *et al*. (2015) that use humanised mice as an intermediate host of the parasite. The complete live cycle of *P.falciparum* can be maintained in these mice. This would be an ideal system to test the efficacy of anti-CS antibody against malarial infection in a whole organism.

Although anti-TRAP and anti-CS might be used as passive vaccines to treat malaria, this is a very expensive approach to therapy, and unrealistic for use in the developing world. A more promising approach, based on the results presented here and the results obtained with RTS,S, might be to optimise an active vaccine based on CS, or to investigate the possibility of small molecule inhibitors of CS for use as anti-malarial drugs. Either of these two approaches should be much cheaper than immunotherapy using anti-malarial mAb.

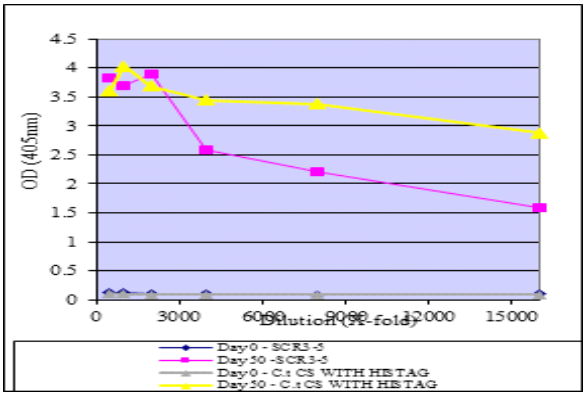
7 Chapter seven

Appendices

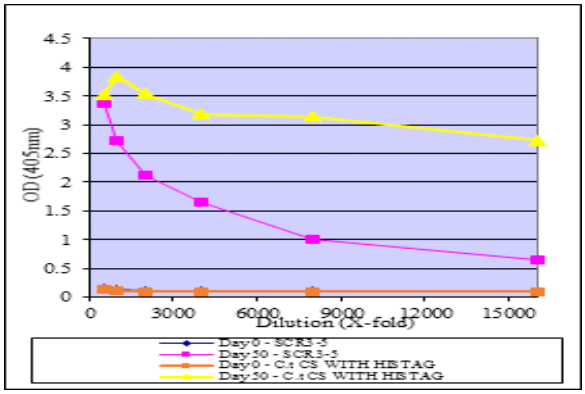
7.1 Determination of antibody titer in mice immunized with the C-terminal fragment of CS

Four Balb/C mice were immunized with native C-terminal CS protein (without the 6x His-tag). A test bleed was carried out at day 0 and at day 50 (when the mice were culled and the spleens extracted for hybridoma production. Sera were tested for binding to the antigen Ct.CS (with the His-tag in this case) and to a control antigen (SCR3-5 of complement factor H-related protein 1, which also contained a His-tag. Two mice (M59065 and M59073), both of which had a high titer of antibody against CS protein, together with low titers against the control protein, were selected for monoclonal antibody production.

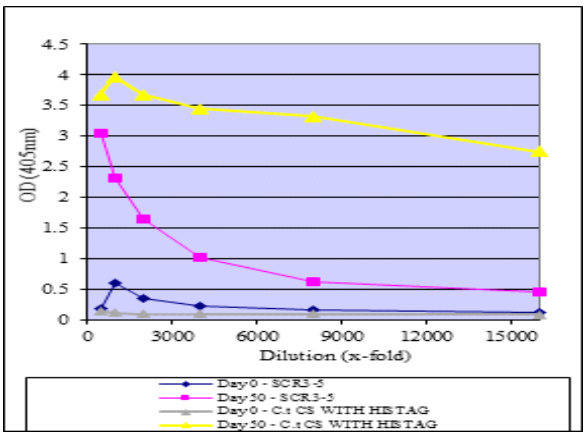
M58907



M59037



M59065



M59073

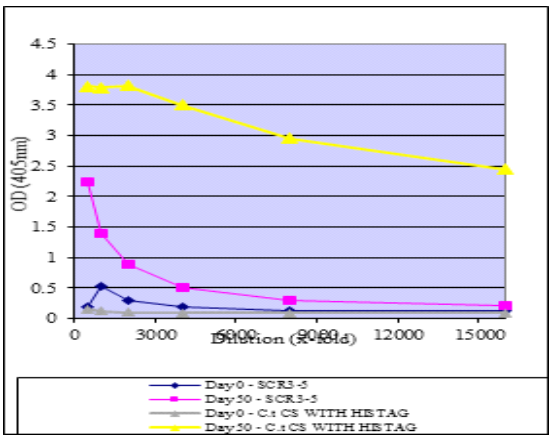


Figure 7-1: Mice immunization to produce mAbs to C-terminus CS protein

Four mice were immunized with native C-terminal CS protein. Bleeds were done on day zero and day 50 then test it on ELISA plates after coated with negative control and antigen (both have His-tag) to check the binding. The results for M59065 and M 59073 showed the binding titres with antigen (C-terminal CS) higher than the negative control (SCR3-5) at day 50. Thus the sera has little or no cross reaction with the His-tag.

7.2 Immunization protocol to select positive clone of monoclonal anti-CS antibody

	Formulaire	Numéro :	F-05/C-HYB-01
	Report	Révision :	01

Eurogentec
Experience true partnership

REPORTING MONOCLONAL ANTIBODY DEVELOPMENT

Customer	Dr. LYNCH	Date :	28/05/2018
Contract	EGT 988		

Order information

Immunisation programme	**Standard**		
Antigen name	*C.t NO HISTAG*		
Carrier	None		
Antigen formulation	In solution		
Total amount	mg	Buffer Concentration	400µg/mL; 770µg/mL 200µg/mL, 258µg/mL
Additional services	n.s.		

n.s. = not specified

Protocol

> **PHASE 1** Immunisation of 4 mice

> **PHASE 2** Fusion/hybridoma production

Myeloma
Immortality Property
Hypoxanthine Phosphoribosyl
Transferase (HPRT)
negative, Ig negative

Spleen cell + Myeloma cell Sp2/OAg 14

> **PHASE 3** Screening for positive hybridomas

Lymphocytes die off on their own over time

Only hybridomas survive
Mixture of immortal antibody producing cells.
Each cell potentially produces a different monoclonal Ab.
Screen wells for positive hybridomas (ELISA).

Hypoxanthine Aminopterin Thymidine (HAT) treatment kills off excess myeloma cells

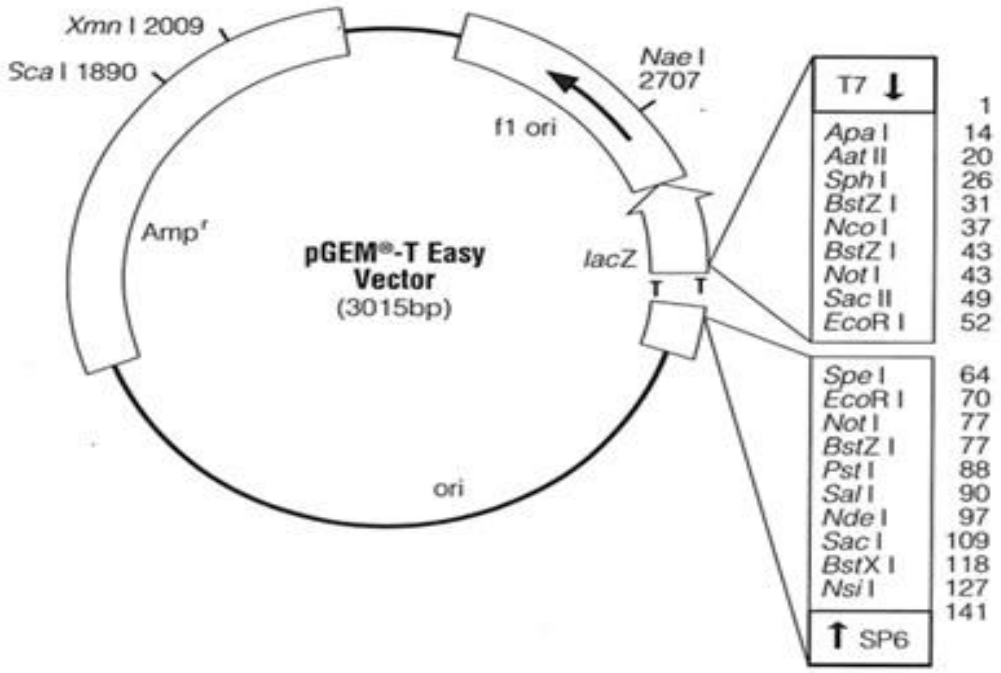
> **PHASE 4** Cloning and isotyping of positive hybridomas

Cloning by serial dilution to get one cell type per well. Test each well to find desired positive clones and then isotype.

7.3 Vectors

There are three types of vectors that used in this project: PGME T easy, pRSET and PSecTag 2/Hygro vector.

PGME T easy vector map



pGEM-T Easy Vector Sequence reference points:

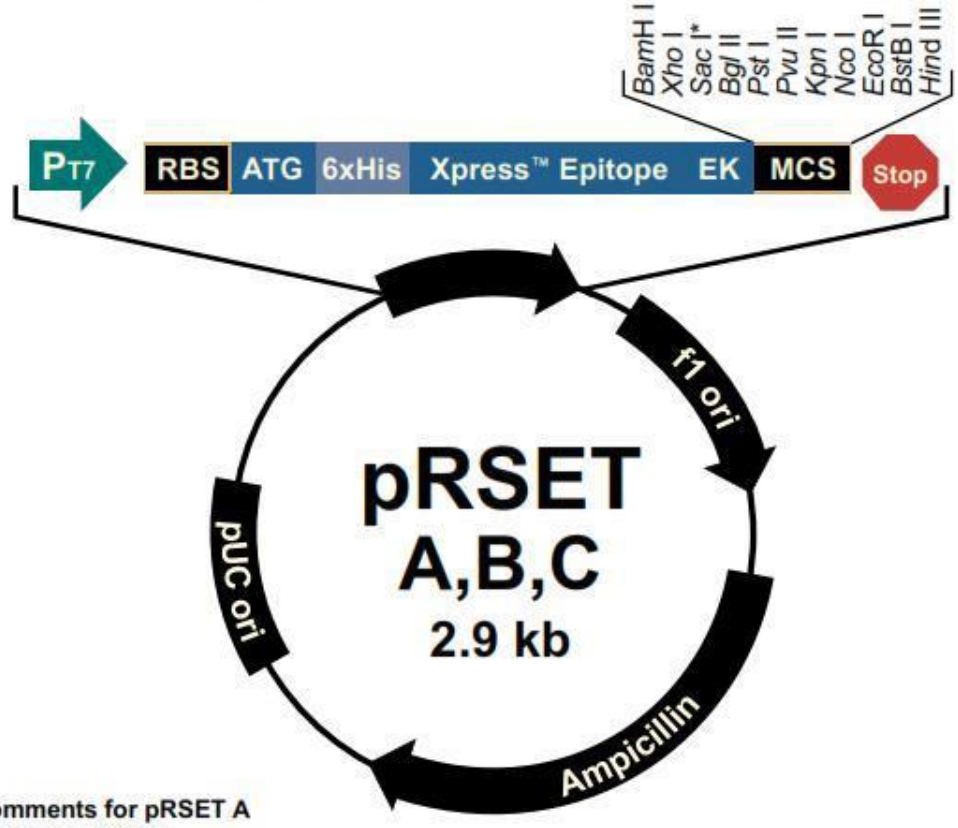
T7 RNA Polymerase transcription initiation site	1
Multiple cloning region	10-128
SP6 RNA Polymerase promoter (-17 to +13)	139-158
SP6 RNA Polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176-197
lacZ start codon	180
lac operator	200-216
β-lactamase coding region	1337-2197
phage f1 region	2380-2835
lac operon sequences	2836-2996, 166-395
pUC/M13 Forward sequencing Primer binding site	2949-2972
T7 RNA Polymerase promoter (-17 - +13)	2999-3

pRSET map

Map of pRSET A, B, and C

pRSET A, B, and C

The map below shows the features of pRSET A, B, and C. **The complete sequence of the vector is available for downloading from our Web site at www.invitrogen.com or from Technical Service (see page 18).**

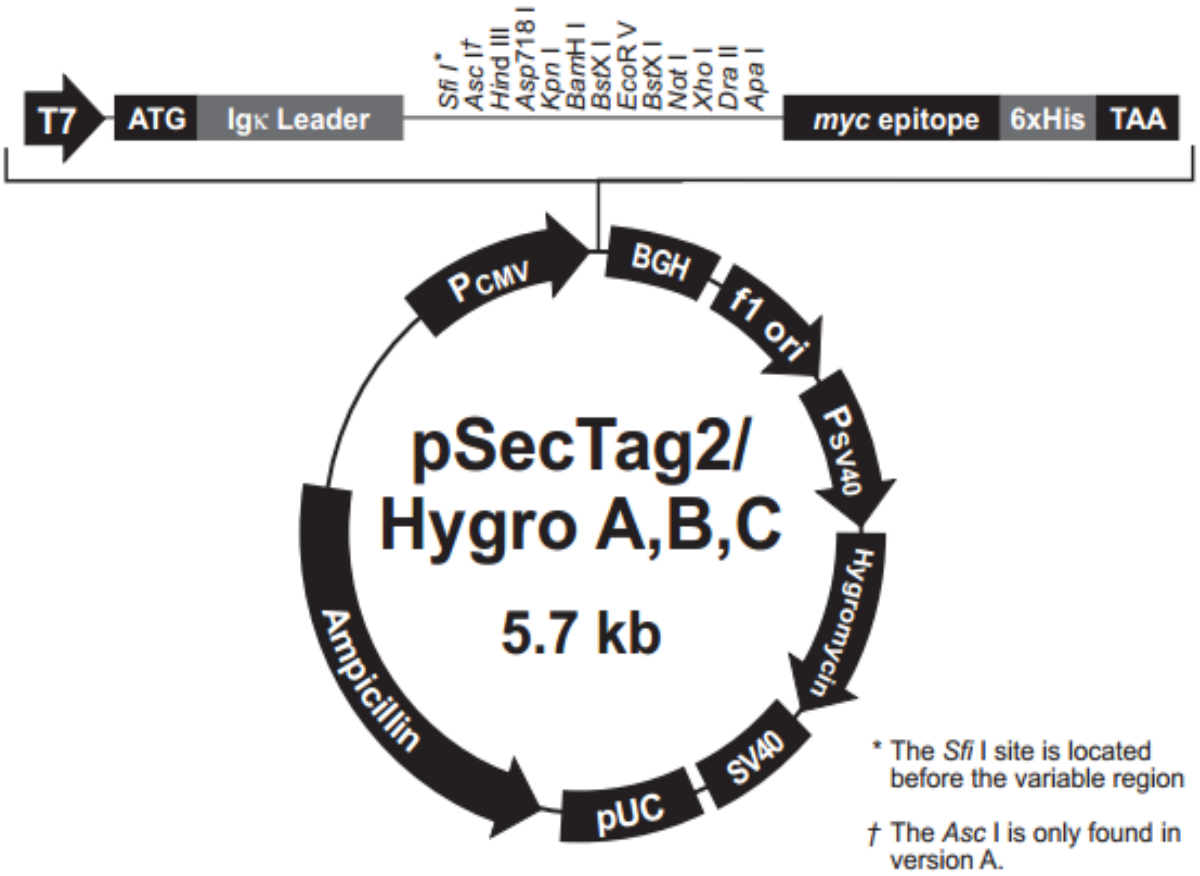


*Version C does not contain *Sac* I

Comments for pRSET A
2897 nucleotides

T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 916-2852 (C)

PSecTag 2/Hygro vector



Comments for pSecTag2/Hygro A
5745 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Murine Ig kappa-chain V-J2-C signal peptide: bases 905-967
Multiple cloning site: bases 970-1081
c-myc epitope: bases 1082-1111
Polyhistidine tag: bases 1127-1144
BGH reverse priming site: bases 1167-1184
BGH polyadenylation site: bases 1166-1380
f1 origin: bases 1443-1856
SV40 promoter and origin: bases 1924-2245
Hygromycin B phosphotransferase ORF (Hyg^R): bases 2263-3288
SV40 polyadenylation site: bases 3418-3547
pUC origin: bases 3931-4604
β-lactamase ORF (Amp^R): bases 4749-5609

8 Chapter eight

References

Bibliography

Adam, C., Geniteau, M., Gougerot-Pocidalo, M., Verroust, P., Lebras, J., Gibert, C. and Morel-Maroger, L. (1981) 'Cryoglobulins, circulating immune complexes, and complement activation in cerebral malaria', *Infection and immunity,* 31(2), pp. 530-535.

Ahearn, J.M. and Fearon, D.T. (1989) 'Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21)', in Anonymous *Advances in immunology*. Elsevier, pp. 183-219.

Ajees, A.A., Gunasekaran, K., Volanakis, J.E., Narayana, S.V., Kotwal, G.J. and Murthy, H.K. (2006) 'The structure of complement C3b provides insights into complement activation and regulation', *Nature,* 444(7116), pp. 221.

Ali, Y.M., Lynch, N.J., Haleem, K.S., Fujita, T., Endo, Y., Hansen, S., Holmskov, U., Takahashi, K., Stahl, G.L. and Dudler, T. (2012) 'The lectin pathway of complement activation is a critical component of the innate immune response to pneumococcal infection', *PLoS pathogens,* 8(7), pp. e1002793.

Alitalo, A., Meri, T., Lankinen, H., Seppala, I., Lahdenne, P., Hefty, P.S., Akins, D. and Meri, S. (2002) 'Complement inhibitor factor H binding to Lyme disease spirochetes is mediated by inducible expression of multiple plasmid-encoded outer surface protein E paralogs', *Journal of immunology (Baltimore, Md.: 1950),* 169(7), pp. 3847-3853.

Alonso, P.L., Sacarlal, J., Aponte, J.J., Leach, A., Macete, E., Aide, P., Sigauque, B., Milman, J., Mandomando, I. and Bassat, Q. (2005) 'Duration of protection with RTS, S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial', *The Lancet,* 366(9502), pp. 2012-2018.

Alonso, P.L., Sacarlal, J., Aponte, J.J., Leach, A., Macete, E., Milman, J., Mandomando, I., Spiessens, B., Guinovart, C. and Espasa, M. (2004) 'Efficacy of the RTS, S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial', *The Lancet,* 364(9443), pp. 1411-1420.

Anstey, N.M., Russell, B., Yeo, T.W. and Price, R.N. (2009) 'The pathophysiology of vivax malaria', *Trends in parasitology,* 25(5), pp. 220-227.

Aponte, J.J., Aide, P., Renom, M., Mandomando, I., Bassat, Q., Sacarlal, J., Manaca, M.N., Lafuente, S., Barbosa, A. and Leach, A. (2007) 'Safety of the RTS, S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial', *The Lancet,* 370(9598), pp. 1543-1551.

Ariki, S., Takahara, S., Shibata, T., Fukuoka, T., Ozaki, A., Endo, Y., Fujita, T., Koshiba, T. and Kawabata, S. (2008) 'Factor C acts as a lipopolysaccharide-

responsive C3 convertase in horseshoe crab complement activation', *Journal of immunology (Baltimore, Md.: 1950),* 181(11), pp. 7994-8001.

Atkinson, J.P., Glew, R.H., Neva, F.A. and Frank, M.M. (1975) 'Serum complement and immunity in experimental simian malaria. II. Preferential activation of early components and failure of depletion of late components to inhibit protective immunity', *Journal of infectious diseases,* 131(1), pp. 26-33.

Bauza, K., Malinauskas, T., Pfander, C., Anar, B., Jones, E.Y., Billker, O., Hill, A.V. and Reyes-Sandoval, A. (2014) 'Efficacy of a Plasmodium vivax malaria vaccine using ChAd63 and modified vaccinia Ankara expressing thrombospondin-related anonymous protein as assessed with transgenic Plasmodium berghei parasites', *Infection and immunity,* 82(3), pp. 1277-1286.

Beare, N.A., Taylor, T.E., Harding, S.P., Lewallen, S. and Molyneux, M.E. (2006) 'Malarial retinopathy: a newly established diagnostic sign in severe malaria', *The American Journal of Tropical Medicine and Hygiene,* 75(5), pp. 790-797.

Behet, M.C., Foquet, L., van Gemert, G., Bijker, E.M., Meuleman, P., Leroux-Roels, G., Hermsen, C.C., Scholzen, A. and Sauerwein, R.W. (2014) 'Sporozoite immunization of human volunteers under chemoprophylaxis induces functional antibodies against pre-erythrocytic stages of Plasmodium falciparum', *Malaria journal,* 13(1), pp. 136.

Behet, M.C., Kurtovic, L., van Gemert, G.J., Haukes, C.M., Siebelink-Stoter, R., Graumans, W., van de Vegte-Bolmer, M.G., Scholzen, A., Langereis, J.D., Diavatopoulos, D.A., Beeson, J.G. and Sauerwein, R.W. (2018) 'The complement system contributes to functional antibody-mediated responses induced by immunization with Plasmodium falciparum malaria sporozoites', *Infection and immunity,* .

Bermúdez, A., Vanegas, M. and Patarroyo, M.E. (2008) 'Structural and immunological analysis of circumsporozoite protein peptides: a further step in the identification of potential components of a minimal subunit-based, chemically synthesised antimalarial vaccine', *Vaccine,* 26(52), pp. 6908-6918.

Bhakdi, S. and Tranum-Jensen, J. (1991) 'Complement lysis: a hole is a hole', *Immunology today,* 12(9), pp. 318-320.

Bijker, E.M., Teirlinck, A.C., Schats, R., van Gemert, G., van de Vegte-Bolmer, M., van Lieshout, L., IntHout, J., Hermsen, C.C., Scholzen, A. and Visser, L.G. (2014) 'Cytotoxic markers associate with protection against malaria in human volunteers immunized with Plasmodium falciparum sporozoites', *The Journal of infectious diseases,* 210(10), pp. 1605-1615.

Biryukov, S. and Stoute, J.A. (2014) 'Complement activation in malaria: friend or foe?', *Trends in molecular medicine,* 20(5), pp. 293-301.

Blom, A.M., Villoutreix, B.O. and Dahlbäck, B. (2004) 'Complement inhibitor C4b-binding protein—friend or foe in the innate immune system?', *Molecular immunology,* 40(18), pp. 1333-1346.

Boddey, J.A. and Cowman, A.F. (2013) 'Plasmodium nesting: remaking the erythrocyte from the inside out', *Annual Review of Microbiology,* 67, pp. 243-269.

Bongfen, S.E., Ntsama, P.M., Offner, S., Smith, T., Felger, I., Tanner, M., Alonso, P., Nebie, I., Romero, J.F. and Silvie, O. (2009) 'The N-terminal domain of Plasmodium falciparum circumsporozoite protein represents a target of protective immunity', *Vaccine,* 27(2), pp. 328-335.

Bousema, T. and Drakeley, C. (2011) 'Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination', *Clinical microbiology reviews,* 24(2), pp. 377-410.

Bouwman, L.H., Roep, B.O. and Roos, A. (2006) 'Mannose-binding lectin: clinical implications for infection, transplantation, and autoimmunity', *Human immunology,* 67(4-5), pp. 247-256.

Boyle, M.J., Reiling, L., Feng, G., Langer, C., Osier, F.H., Aspeling-Jones, H., Cheng, Y.S., Stubbs, J., Tetteh, K.K. and Conway, D.J. (2015) 'Human antibodies fix complement to inhibit Plasmodium falciparum invasion of erythrocytes and are associated with protection against malaria', *Immunity,* 42(3), pp. 580-590.

Carroll, M.C. (2004) 'The complement system in regulation of adaptive immunity', *Nature immunology,* 5(10), pp. 981-986.

Carvalho, G.B.d. and Carvalho, G.B.d. (2011) 'Duffy Blood Group System and the malaria adaptation process in humans', *Revista brasileira de hematologia e hemoterapia,* 33(1), pp. 55-64.

Charlesworth, J.A., Scott, D.M., Pussell, B.A. and Peters, D.K. (1979) 'Metabolism of human beta 1H: studies in man and experimental animals', *Clinical and experimental immunology,* 38(3), pp. 397-404.

Chen, M., Daha, M.R. and Kallenberg, C.G. (2010) 'The complement system in systemic autoimmune disease', *Journal of Autoimmunity,* 34(3), pp. J276-J286.

Cole, D.S. and Morgan, B.P. (2003) 'Beyond lysis: how complement influences cell fate', *Clinical science (London, England : 1979),* 104(5), pp. 455-466.

Collins, W.E. and Jeffery, G.M. (2007) 'Plasmodium malariae: parasite and disease', *Clinical microbiology reviews,* 20(4), pp. 579-592.

Combe, A., Moreira, C., Ackerman, S., Thiberge, S., Templeton, T.J. and Ménard, R. (2009) 'TREP, a novel protein necessary for gliding motility of the malaria sporozoite', *International journal for parasitology,* 39(4), pp. 489-496.

Curtidor, H., García, J., Vanegas, M., Puentes, F., Forero, M. and Patarroyo, M.E. (2008) 'Identification of peptides with high red blood cell and hepatocyte binding activity in the Plasmodium falciparum multi-stage invasion proteins: PfSPATR and MCP-1', *Biochimie,* 90(11), pp. 1750-1759.

Dasari, P., Heber, S.D., Beisele, M., Torzewski, M., Reifenberg, K., Orning, C., Fries, A., Zapf, A.L., Baumeister, S., Lingelbach, K., Udomsangpetch, R., Bhakdi, S.C., Reiss, K. and Bhakdi, S. (2012) 'Digestive vacuole of Plasmodium falciparum released during erythrocyte rupture dually activates complement and coagulation', *Blood,* 119(18), pp. 4301-4310.

Dasari, P., Reiss, K., Lingelbach, K., Baumeister, S., Lucius, R., Udomsangpetch, R., Bhakdi, S.C. and Bhakdi, S. (2011) 'Digestive vacuoles of Plasmodium falciparum are selectively phagocytosed by and impair killing function of polymorphonuclear leukocytes', *Blood,* 118(18), pp. 4946-4956.

Dave, S., Pangburn, M.K., Pruitt, C. and McDaniel, L.S. (2004) 'Interaction of human factor H with PspC of Streptococcus pneumoniae', *Indian Journal of Medical Research,* 119, pp. 66-73.

Davis III, A.E., Mejia, P. and Lu, F. (2008) 'Biological activities of C1 inhibitor', *Molecular immunology,* 45(16), pp. 4057-4063.

Doolan, D.L., Dobano, C. and Baird, J.K. (2009) 'Acquired immunity to malaria', *Clinical microbiology reviews,* 22(1), pp. 13-36, Table of Contents.

Eisen, D.P. and Minchinton, R.M. (2003) 'Impact of mannose-binding lectin on susceptibility to infectious diseases', *Clinical Infectious Diseases,* 37(11), pp. 1496-1505.

Elsworth, B., Matthews, K., Nie, C.Q., Kalanon, M., Charnaud, S.C., Sanders, P.R., Chisholm, S.A., Counihan, N.A., Shaw, P.J. and Pino, P. (2014) 'PTEX is an essential nexus for protein export in malaria parasites', *Nature,* 511(7511), pp. 587-591.

Endo, Y., Matsushita, M. and Fujita, T. (2007) 'Role of ficolin in innate immunity and its molecular basis', *Immunobiology,* 212(4-5), pp. 371-379.

Endo, Y., Takahashi, M. and Fujita, T. (2006) 'Lectin complement system and pattern recognition', *Immunobiology,* 211(4), pp. 283-293.

Farries, T.C. and Atkinson, J.P. (1989) 'Biosynthesis of properdin', *Journal of immunology (Baltimore, Md.: 1950),* 142(3), pp. 842-847.

Farries, T.C., Lachmann, P.J. and Harrison, R.A. (1988) 'Analysis of the interaction between properdin and factor B, components of the alternative-pathway C3 convertase of complement', *The Biochemical journal,* 253(3), pp. 667-675.

Fearon, D.T. and Austen, K.F. (1975a) 'Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase', *The Journal of experimental medicine,* 142(4), pp. 856-863.

Fearon, D.T. and Austen, K.F. (1975b) 'Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase', *The Journal of experimental medicine,* 142(4), pp. 856-863.

Ferreira, V.P., Cortes, C. and Pangburn, M.K. (2010) 'Native polymeric forms of properdin selectively bind to targets and promote activation of the alternative pathway of complement', *Immunobiology,* 215(11), pp. 932-940.

Ferreira, V.P., Pangburn, M.K. and Cortés, C. (2010) 'Complement control protein factor H: the good, the bad, and the inadequate', *Molecular immunology,* 47(13), pp. 2187-2197.

Francis, C. and Kaplan, K. (2006) 'Principles of antithrombotic therapy', *Williams Hematology.Seventh.New York: McGraw-Hill, ,* pp. 283-300.

Frederiksen, P.D., Thiel, S., Larsen, C.B. and Jensenius, J.C. (2005) 'M-ficolin, an innate immune defence molecule, binds patterns of acetyl groups and activates complement', *Scandinavian Journal of Immunology,* 62(5), pp. 462-473.

Frevert, U., Sinnis, P., Cerami, C., Shreffler, W., Takacs, B. and Nussenzweig, V. (1993) 'Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes', *The Journal of experimental medicine,* 177(5), pp. 1287-1298.

Fujita, T. (2002) 'Evolution of the lectin–complement pathway and its role in innate immunity', *Nature Reviews Immunology,* 2(5), pp. 346.

Gadjeva, M., Takahashi, K. and Thiel, S. (2004) 'Mannan-binding lectin—a soluble pattern recognition molecule', *Molecular immunology,* 41(2-3), pp. 113-121.

Gantt, S., Persson, C., Rose, K., Birkett, A.J., Abagyan, R. and Nussenzweig, V. (2000) 'Antibodies against thrombospondin-related anonymous protein do not inhibit Plasmodium sporozoite infectivity in vivo', *Infection and immunity,* 68(6), pp. 3667-3673.

Garred, P., Larsen, F., Seyfarth, J., Fujita, R. and Madsen, H.O. (2006) 'Mannose-binding lectin and its genetic variants', *Genes and immunity,* 7(2), pp. 85.

Garred, P., Nielsen, M.A., Kurtzhals, J.A., Malhotra, R., Madsen, H.O., Goka, B.Q., Akanmori, B.D., Sim, R.B. and Hviid, L. (2003) 'Mannose-binding lectin is a disease modifier in clinical malaria and may function as opsonin for Plasmodium falciparum-infected erythrocytes', *Infection and immunity,* 71(9), pp. 5245-5253.

Ghani, A.C., Sutherland, C.J., Riley, E.M., Drakeley, C.J., Griffin, J.T., Gosling, R.D. and Filipe, J.A. (2009) 'Loss of population levels of immunity to malaria as a result

of exposure-reducing interventions: consequences for interpretation of disease trends', *PloS one,* 4(2), pp. e4383.

Good, M.F. and Doolan, D.L. (1999) 'Immune effector mechanisms in malaria', *Current opinion in immunology,* 11(4), pp. 412-419.

Goto, H. and Sanchez, M.C.A. (2013) 'Does the complement system work for or against the host during parasite infections', *International trends in immunity,* 1(2), pp. 11-23.

Goundis, D. and Reid, K.B. (1988) 'Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs', *Nature,* 335(6185), pp. 82.

Guyatt, H.L., Corlett, S.K., Robinson, T.P., Ochola, S.A. and Snow, R.W. (2002) 'Malaria prevention in highland Kenya: indoor residual house-spraying vs. insecticide-treated bednets', *Tropical Medicine & International Health,* 7(4), pp. 298-303.

Guyton, A. and Hall, J. (2006) 'Texbookof medical physiology Elsevier Saunders', .

Hanahan, D. (1983) 'Studies on transformation of Escherichia coli with plasmids', *Journal of Molecular Biology,* 166(4), pp. 557-580.

Hemingway, J. and Ranson, H. (2000) 'Insecticide resistance in insect vectors of human disease', *Annual Review of Entomology,* 45(1), pp. 371-391.

Henriksen, M.L., Brandt, J., Andrieu, J.P., Nielsen, C., Jensen, P.H., Holmskov, U., Jorgensen, T.J., Palarasah, Y., Thielens, N.M. and Hansen, S. (2013) 'Heteromeric complexes of native collectin kidney 1 and collectin liver 1 are found in the circulation with MASPs and activate the complement system', *Journal of immunology (Baltimore, Md.: 1950),* 191(12), pp. 6117-6127.

Herrington, D.A., Clyde, D.F., Losonsky, G., Cortesia, M., Murphy, J.R., Davis, J., Baqar, S., Felix, A.M., Heimer, E.P. and Gillessen, D. (1987) 'Safety and immunogenicity in man of a synthetic peptide malaria vaccine against Plasmodium falciparum sporozoites', *Nature,* 328(6127), pp. 257.

Hill, D.L., Eriksson, E.M., Suen, Connie SN Li Wai, Chiu, C.Y., Ryg-Cornejo, V., Robinson, L.J., Siba, P.M., Mueller, I., Hansen, D.S. and Schofield, L. (2013) 'Opsonising antibodies to P. falciparum merozoites associated with immunity to clinical malaria', *PLoS One,* 8(9), pp. e74627.

Hodgson, S.H., Ewer, K.J., Bliss, C.M., Edwards, N.J., Rampling, T., Anagnostou, N.A., de Barra, E., Havelock, T., Bowyer, G. and Poulton, I.D. (2014) 'Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naive individuals', *The Journal of infectious diseases,* 211(7), pp. 1076-1086.

Holmberg, V., Schuster, F., Dietz, E., Visconti, J.C.S., Anemana, S.D., Bienzle, U. and Mockenhaupt, F.P. (2008) 'Mannose-binding lectin variant associated with severe malaria in young African children', *Microbes and Infection,* 10(4), pp. 342-348.

Holmskov, U., Malhotra, R., Sim, R.B. and Jensenius, J.C. (1994) 'Collectins: collagenous C-type lectins of the innate immune defense system', *Immunology today*, 15(2), pp. 67-74.

Holt, G.D., Krivan, H.C., Gasic, G.J. and Ginsburg, V. (1989) 'Antistasin, an inhibitor of coagulation and metastasis, binds to sulfatide (Gal(3-SO4) beta 1-1Cer) and has a sequence homology with other proteins that bind sulfated glycoconjugates', *The Journal of biological chemistry*, 264(21), pp. 12138-12140.

Hourcade, D.E. (2006) 'The role of properdin in the assembly of the alternative pathway C3 convertases of complement', *The Journal of biological chemistry*, 281(4), pp. 2128-2132.

Jurianz, K., Ziegler, S., Garcia-Schüler, H., Kraus, S., Bohana-Kashtan, O., Fishelson, Z. and Kirschfink, M. (1999) 'Complement resistance of tumor cells: basal and induced mechanisms', *Molecular immunology,* 36(13-14), pp. 929-939.

Kang, Y., Tan, L.A., Carroll, M.V., Gentle, M.E. and Sim, R.B. (2009) 'Target pattern recognition by complement proteins of the classical and alternative pathways', in Anonymous *Target Pattern Recognition in Innate Immunity*. Springer, pp. 117-128.

Keizer, R.J., Huitema, A.D., Schellens, J.H. and Beijnen, J.H. (2010) 'Clinical pharmacokinetics of therapeutic monoclonal antibodies', *Clinical pharmacokinetics,* 49(8), pp. 493-507.

Kennedy, A.T., Schmidt, C.Q., Thompson, J.K., Weiss, G.E., Taechalertpaisarn, T., Gilson, P.R., Barlow, P.N., Crabb, B.S., Cowman, A.F. and Tham, W.H. (2016) 'Recruitment of Factor H as a Novel Complement Evasion Strategy for Blood-Stage Plasmodium falciparum Infection', *Journal of immunology (Baltimore, Md.: 1950),* 196(3), pp. 1239-1248.

Kinoshita, T. (1991) 'Biology of complement: the overture', *Immunology today*, 12(9), pp. 291-295.

Klabunde, J., Uhlemann, A., Tebo, A.E., Kimmel, J., Schwarz, R.T., Kremsner, P.G. and Kun, J.F. (2002) 'Recognition of Plasmodium falciparum proteins by mannan-binding lectin, a component of the human innate immune system', *Parasitology research,* 88(2), pp. 113-117.

Kruger, N.J. (2009) 'The Bradford method for protein quantitation', in Anonymous *The protein protocols handbook.* Springer, pp. 17-24.

Kumar, K.A., Sano, G., Boscardin, S., Nussenzweig, R.S., Nussenzweig, M.C., Zavala, F. and Nussenzweig, V. (2006) 'The circumsporozoite protein is an

immunodominant protective antigen in irradiated sporozoites', *Nature,* 444(7121), pp. 937.

Kurtovic, L., Behet, M.C., Feng, G., Reiling, L., Chelimo, K., Dent, A.E., Mueller, I., Kazura, J.W., Sauerwein, R.W. and Fowkes, F.J. (2018) 'Human antibodies activate complement against Plasmodium falciparum sporozoites, and are associated with protection against malaria in children', *BMC medicine,* 16(1), pp. 61.

Lawler, J. and Hynes, R.O. (1986) 'The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins', *The Journal of cell biology,* 103(5), pp. 1635-1648.

Lipscombe, R., Sumiya, M., Hill, A., Lau, Y., Levinsky, R., Summerfield, J. and Turner, M. (1992) 'High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene', *Human molecular genetics,* 1(9), pp. 709-715.

Liu, Y., Endo, Y., Iwaki, D., Nakata, M., Matsushita, M., Wada, I., Inoue, K., Munakata, M. and Fujita, T. (2005) 'Human M-ficolin is a secretory protein that activates the lectin complement pathway', *Journal of immunology (Baltimore, Md.: 1950),* 175(5), pp. 3150-3156.

Luz, P.R., Boldt, A.B., Grisbach, C., Kun, J.F., Velavan, T.P. and Messias-Reason, I.J. (2013) 'Association of L-ficolin levels and FCN2 genotypes with chronic Chagas disease', *PloS one,* 8(4), pp. e60237.

Madsen, H.O., Satz, M.L., Hogh, B., Svejgaard, A. and Garred, P. (1998) 'Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America', *Journal of immunology (Baltimore, Md.: 1950),* 161(6), pp. 3169-3175.

Malpede, B.M. and Tolia, N.H. (2014) 'Malaria adhesins: structure and function', *Cellular microbiology,* 16(5), pp. 621-631.

Markiewski, M.M., DeAngelis, R.A., Strey, C.W., Foukas, P.G., Gerard, C., Gerard, N., Wetsel, R.A. and Lambris, J.D. (2009) 'The regulation of liver cell survival by complement', *Journal of immunology (Baltimore, Md.: 1950),* 182(9), pp. 5412-5418.

Martens, P. and Hall, L. (2000) 'Malaria on the move: human population movement and malaria transmission', *Emerging infectious diseases,* 6(2), pp. 103-109.

Marx, U., Embleton, M.J., Fischer, R., Gruber, F.P., Hansson, U., Heuer, J., De Leeuw, W.A., Logtenberg, T., Merz, W. and Portetelle, D. (1997) 'Monoclonal antibody production', *ATLA-NOTTINGHAM-,* 25, pp. 121-138.

Matsushita, M., Endo, Y., Taira, S., Sato, Y., Fujita, T., Ichikawa, N., Nakata, M. and Mizuochi, T. (1996) 'A novel human serum lectin with collagen- and fibrinogen-

like domains that functions as an opsonin', *The Journal of biological chemistry*, 271(5), pp. 2448-2454.

Matuschewski, K., Nunes, A.C., Nussenzweig, V. and Menard, R. (2002) 'Plasmodium sporozoite invasion into insect and mammalian cells is directed by the same dual binding system', *The EMBO journal*, 21(7), pp. 1597-1606.

McCormick, C.J., Tuckwell, D.S., Crisanti, A., Humphries, M.J. and Hollingdale, M.R. (1999) 'Identification of heparin as a ligand for the A-domain of Plasmodium falciparum thrombospondin-related adhesion protein', *Molecular and biochemical parasitology*, 100(1), pp. 111-124.

McNeil, L.K., Zagursky, R.J., Lin, S.L., Murphy, E., Zlotnick, G.W., Hoiseth, S.K., Jansen, K.U. and Anderson, A.S. (2013) 'Role of factor H binding protein in Neisseria meningitidis virulence and its potential as a vaccine candidate to broadly protect against meningococcal disease', *Microbiology and molecular biology reviews : MMBR*, 77(2), pp. 234-252.

Medicus, R.G., Gotze, O. and Muller-Eberhard, H.J. (1976) 'Alternative pathway of complement: recruitment of precursor properdin by the labile C3/C5 convertase and the potentiation of the pathway', *The Journal of experimental medicine*, 144(4), pp. 1076-1093.

Medof, M.E., Iida, K., Mold, C. and Nussenzweig, V. (1982) 'Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes', *The Journal of experimental medicine*, 156(6), pp. 1739-1754.

Medof, M.E. and Nussenzweig, V. (1984) 'Control of the function of substrate-bound C4b-C3b by the complement receptor Cr1', *The Journal of experimental medicine*, 159(6), pp. 1669-1685.

Ménard, R. (2000) 'The journey of the malaria sporozoite through its hosts: two parasite proteins leadthe way', *Microbes and Infection*, 2(6), pp. 633-642.

Mendis, K.N., Naotunne, T.d.S., Karunaweera, N.D., Del Giudice, G., Grau, G.E. and Carter, R. (1990) 'Anti-parasite effects of cytokines in malaria', *Immunology letters*, 25(1-3), pp. 217-220.

Meri, T., Amdahl, H., Lehtinen, M., Hyvärinen, S., McDowell, J., Bhattacharjee, A., Meri, S., Marconi, R., Goldman, A. and Jokiranta, T. (2013) 'Microbes bind complement inhibitor factor H via a common site', *PLoS pathogens*, 9(4), pp. e1003308.

Miller, L.H., Baruch, D.I., Marsh, K. and Doumbo, O.K. (2002) 'The pathogenic basis of malaria', *Nature*, 415(6872), pp. 673-679.

Morahan, B.J., Wang, L. and Coppel, R.L. (2009) 'No TRAP, no invasion', *Trends in parasitology*, 25(2), pp. 77-84.

Mordmüller, B., Surat, G., Lagler, H., Chakravarty, S., Ishizuka, A.S., Lalremruata, A., Gmeiner, M., Campo, J.J., Esen, M. and Ruben, A.J. (2017) 'Sterile protection against human malaria by chemoattenuated PfSPZ vaccine', *Nature,* 542(7642), pp. 445.

Naik, A., Sharma, S. & Quigg, R.J. (2013) 'Complement regulation in renal disease models', *Seminars in nephrology*Elsevier, pp. 575.

Naitza, S., Spano, F., Robson, K. and Crisanti, A. (1998) 'The thrombospondin-related protein family of apicomplexan parasites: the gears of the cell invasion machinery', *Parasitology today,* 14(12), pp. 479-484.

Nardin, E.H., Oliveira, G.A., Calvo-Calle, J.M., Castro, Z.R., Nussenzweig, R.S., Schmeckpeper, B., Hall, B.F., Diggs, C., Bodison, S. and Edelman, R. (2000) 'Synthetic malaria peptide vaccine elicits high levels of antibodies in vaccinees of defined HLA genotypes', *The Journal of infectious diseases,* 182(5), pp. 1486-1496.

Nardin, E.H., Calvo-Calle, J.M., Oliveira, G.A., Nussenzweig, R.S., Schneider, M., Tiercy, J.M., Loutan, L., Hochstrasser, D. and Rose, K. (2001) 'A totally synthetic polyoxime malaria vaccine containing Plasmodium falciparum B cell and universal T cell epitopes elicits immune responses in volunteers of diverse HLA types', *Journal of immunology (Baltimore, Md.: 1950),* 166(1), pp. 481-489.

Nilsson, S.C., Sim, R.B., Lea, S.M., Fremeaux-Bacchi, V. and Blom, A.M. (2011) 'Complement factor I in health and disease', *Molecular immunology,* 48(14), pp. 1611-1620.

Nussenzweig, V. and Nussenzweig, R.S. (1985) 'Circumsporozoite proteins of malaria parasites', *Cell,* 42(2), pp. 401-403.

Pangburn, M. and Muller-Eberhard, H. (1986) 'The C3 convertase of the alternative pathway of human complement. Enzymic properties of the bimolecular proteinase.', *Biochem.J,* 235, pp. 723-730.

Pangburn, M.K., Schreiber, R.D. and Muller-Eberhard, H.J. (1981) 'Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3', *The Journal of experimental medicine,* 154(3), pp. 856-867.

Peitsch, M.C. and Tschopp, J. (1991) 'Assembly of macromolecular pores by immune defense systems', *Current opinion in cell biology,* 3(4), pp. 710-716.

Peterson, D.S., Gao, Y., Asokan, K. and Gaertig, J. (2002) 'The circumsporozoite protein of Plasmodium falciparum is expressed and localized to the cell surface in the free-living ciliate Tetrahymena thermophila', *Molecular and biochemical parasitology,* 122(2), pp. 119-126.

Plassmeyer, M.L., Reiter, K., Shimp, R.L.,Jr, Kotova, S., Smith, P.D., Hurt, D.E., House, B., Zou, X., Zhang, Y., Hickman, M., Uchime, O., Herrera, R., Nguyen, V.,

Glen, J., Lebowitz, J., Jin, A.J., Miller, L.H., MacDonald, N.J., Wu, Y. and Narum, D.L. (2009a) 'Structure of the Plasmodium falciparum circumsporozoite protein, a leading malaria vaccine candidate', *The Journal of biological chemistry,* 284(39), pp. 26951-26963.

Plassmeyer, M.L., Reiter, K., Shimp, R.L.,Jr, Kotova, S., Smith, P.D., Hurt, D.E., House, B., Zou, X., Zhang, Y., Hickman, M., Uchime, O., Herrera, R., Nguyen, V., Glen, J., Lebowitz, J., Jin, A.J., Miller, L.H., MacDonald, N.J., Wu, Y. and Narum, D.L. (2009b) 'Structure of the Plasmodium falciparum circumsporozoite protein, a leading malaria vaccine candidate', *The Journal of biological chemistry,* 284(39), pp. 26951-26963.

Podack, E.R., Preissner, K.T. and Muller-Eberhard, H.J. (1984) 'Inhibition of C9 polymerization within the SC5b-9 complex of complement by S-protein', *Acta pathologica, microbiologica, et immunologica Scandinavica.Supplement,* 284, pp. 89-96.

Pombo, D.J., Lawrence, G., Hirunpetcharat, C., Rzepczyk, C., Bryden, M., Cloonan, N., Anderson, K., Mahakunkijcharoen, Y., Martin, L.B. and Wilson, D. (2002) 'Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum', *The Lancet,* 360(9333), pp. 610-617.

Ramasamy, R., Ramasamy, M. and Yasawardena, S. (2001) 'Antibodies and Plasmodium falciparum merozoites', *Trends in parasitology,* 17(4), pp. 194-197.

Ramos, T., Bullard, D.C. and Barnum, S. (2012) 'Deletion of the complement phagocytic receptors CR3 and CR4 does not alter susceptibility to experimental cerebral malaria', *Parasite immunology,* 34(11), pp. 547-550.

Rathore, D., Sacci, J.B., de la Vega, P. and McCutchan, T.F. (2002) 'Binding and invasion of liver cells by Plasmodium falciparum sporozoites. Essential involvement of the amino terminus of circumsporozoite protein', *The Journal of biological chemistry,* 277(9), pp. 7092-7098.

Rawal, N., Rajagopalan, R. and Salvi, V.P. (2008) 'Activation of complement component C5: comparison of C5 convertases of the lectin pathway and the classical pathway of complement', *The Journal of biological chemistry,* 283(12), pp. 7853-7863.

Reid, K.B. and Porter, R.R. (1981) 'The proteolytic activation systems of complement', *Annual Review of Biochemistry,* 50(1), pp. 433-464.

Ren, Y., Ding, Q. and Zhang, X. (2014) 'Ficolins and infectious diseases', *Virologica Sinica,* 29(1), pp. 25-32.

Rey-Campos, J., Rubinstein, P. and Rodriguez de Cordoba, S. (1988) 'A physical map of the human regulator of complement activation gene cluster linking the complement genes CR1, CR2, DAF, and C4BP', *The Journal of experimental medicine,* 167(2), pp. 664-669.

Ricklin, D., Hajishengallis, G., Yang, K. and Lambris, J.D. (2010) 'Complement: a key system for immune surveillance and homeostasis', *Nature immunology,* 11(9), pp. 785.

Roberts, L. and Janovy, J. (2005) 'Foundations parasitology. 7th eds', .

Robson, K.J., Hall, J.R., Jennings, M., Harris, T., Marsh, K., Newbold, C., Tate, V.E. and Weatherall, D. (1988) 'A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite', .

Robson, K., Frevert, U., Reckmann, I., Cowan, G., Beier, J., Scragg, I., Takehara, K., Bishop, D., Pradel, G. and Sinden, R. (1995) 'Thrombospondin-related adhesive protein (TRAP) of Plasmodium falciparum: expression during sporozoite ontogeny and binding to human hepatocytes.', *The EMBO journal,* 14(16), pp. 3883-3894.

Robson, K.J., Frevert, U., Reckmann, I., Cowan, G., Beier, J., Scragg, I.G., Takehara, K., Bishop, D.H., Pradel, G. and Sinden, R. (1995) 'Thrombospondin-related adhesive protein (TRAP) of Plasmodium falciparum: expression during sporozoite ontogeny and binding to human hepatocytes', *The EMBO journal,* 14(16), pp. 3883-3894.

Roestenberg, M., McCall, M., Mollnes, T.E., van Deuren, M., Sprong, T., Klasen, I., Hermsen, C.C., Sauerwein, R.W. and van der Ven, A. (2007) 'Complement activation in experimental human malaria infection', *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 101(7), pp. 643-649.

Roggero, M., Filippi, B., Church, P., Hoffman, S., Blum-Tirouvanziam, U., Lopez, J., Esposito, F., Matile, H., Reymond, C. and Fasel, N. (1995) 'Synthesis and immunological characterization of 104-mer and 102-mer peptides corresponding to the N-and C-terminal regions of the Plasmodium falciparum CS protein', *Molecular immunology,* 32(17), pp. 1301-1309.

Rowe, J.A., Claessens, A., Corrigan, R.A. and Arman, M. (2009) 'Adhesion of Plasmodium falciparum-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications', *Expert reviews in molecular medicine,* 11.

Salas, F., Fichmann, J., Lee, G.K., Scott, M.D. and Rosenthal, P.J. (1995) 'Functional expression of falcipain, a Plasmodium falciparum cysteine proteinase, supports its role as a malarial hemoglobinase', *Infection and immunity,* 63(6), pp. 2120-2125.

Scherf, A., Lopez-Rubio, J.J. and Riviere, L. (2008) 'Antigenic variation in Plasmodium falciparum', *Annu.Rev.Microbiol.,* 62, pp. 445-470.

Schwaeble, W.J. and Reid, K.B. (1999) 'Does properdin crosslink the cellular and the humoral immune response?', *Immunology today,* 20(1), pp. 17-21.

Schwaeble, W., Zwirner, J., Schulz, T.F., Linke, R.P., Dierich, M.P. and Weiss, E.H. (1987) 'Human complement factor H: expression of an additional truncated gene

product of 43 kDa in human liver', *European journal of immunology,* 17(10), pp. 1485-1489.

Schwaeble, W.J., Lynch, N.J., Clark, J.E., Marber, M., Samani, N.J., Ali, Y.M., Dudler, T., Parent, B., Lhotta, K., Wallis, R., Farrar, C.A., Sacks, S., Lee, H., Zhang, M., Iwaki, D., Takahashi, M., Fujita, T., Tedford, C.E. and Stover, C.M. (2011) 'Targeting of mannan-binding lectin-associated serine protease-2 confers protection from myocardial and gastrointestinal ischemia/reperfusion injury', *Proceedings of the National Academy of Sciences of the United States of America,* 108(18), pp. 7523-7528.

Selman, L. and Hansen, S. (2012) 'Structure and function of collectin liver 1 (CL-L1) and collectin 11 (CL-11, CL-K1)', *Immunobiology,* 217(9), pp. 851-863.

Serruto, D., Rappuoli, R., Scarselli, M., Gros, P. and Van Strijp, J.A. (2010) 'Molecular mechanisms of complement evasion: learning from staphylococci and meningococci', *Nature reviews Microbiology,* 8(6), pp. 393.

Seya, T., Nakamura, K., Masaki, T., Ichihara-Itoh, C., Matsumoto, M. and Nagasawa, S. (1995) 'Human factor H and C4b-binding protein serve as factor I-cofactors both encompassing inactivation of C3b and C4b', *Molecular immunology,* 32(5), pp. 355-360.

Sharma, P., Bharadwaj, A., Bhasin, V.K., Sailaja, V.N. and Chauhan, V.S. (1996) 'Antibodies to a conserved-motif peptide sequence of the Plasmodium falciparum thrombospondin-related anonymous protein and circumsporozoite protein recognize a 78-kilodalton protein in the asexual blood stages of the parasite and inhibit merozoite invasion in vitro', *Infection and immunity,* 64(6), pp. 2172-2179.

Shin, S.C.J., Vanderberg, J.P. and Terzakis, J.A. (1982) 'Direct infection of hepatocytes by sporozoites of Plasmodium berghei 1', *The Journal of protozoology,* 29(3), pp. 448-454.

Silver, K.L., Higgins, S.J., McDonald, C.R. and Kain, K.C. (2010) 'Complement driven innate immune response to malaria: fuelling severe malarial diseases', *Cellular microbiology,* 12(8), pp. 1036-1045.

Sim, R., Day, A., Moffatt, B. and Fontaine, M. (1993) '[1] Complement factor I and cofactors in control of complement system convertase enzymes', in Anonymous *Methods in enzymology.* Elsevier, pp. 13-35.

Simon, N., Lasonder, E., Scheuermayer, M., Kuehn, A., Tews, S., Fischer, R., Zipfel, P.F., Skerka, C. and Pradel, G. (2013) 'Malaria parasites co-opt human factor H to prevent complement-mediated lysis in the mosquito midgut', *Cell host & microbe,* 13(1), pp. 29-41.

Sinnis, P., Willnow, T.E., Briones, M.R., Herz, J. and Nussenzweig, V. (1996) 'Remnant lipoproteins inhibit malaria sporozoite invasion of hepatocytes', *The Journal of experimental medicine,* 184(3), pp. 945-954.

Skerka, C. and Zipfel, P.F. (2008) 'Complement factor H related proteins in immune diseases', *Vaccine,* 26, pp. I9-I14.

Smith, M.C., Pensky, J. and Naff, G.B. (1982) 'Inhibition of zymosan-induced alternative complement pathway activation by concanavalin A', *Infection and immunity,* 38(3), pp. 1279-1284.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y. and Hay, S.I. (2005) 'The global distribution of clinical episodes of Plasmodium falciparum malaria', *Nature,* 434(7030), pp. 214-217.

Soldati, D., Foth, B.J. and Cowman, A.F. (2004) 'Molecular and functional aspects of parasite invasion', *Trends in parasitology,* 20(12), pp. 567-574.

Song, G., Koksal, A.C., Lu, C. and Springer, T.A. (2012) 'Shape change in the receptor for gliding motility in Plasmodium sporozoites', *Proceedings of the National Academy of Sciences of the United States of America,* 109(52), pp. 21420-21425.

Soulard, V., Bosson-Vanga, H., Lorthiois, A., Roucher, C., Franetich, J., Zanghi, G., Bordessoulles, M., Tefit, M., Thellier, M. and Morosan, S. (2015) 'Plasmodium falciparum full life cycle and Plasmodium ovale liver stages in humanized mice', *Nature communications,* 6, pp. 7690.

Stellwagen, E. (1990) '[25] Gel filtration', in Anonymous *Methods in enzymology*. Elsevier, pp. 317-328.

Stevenson, M.M. and Riley, E.M. (2004) 'Innate immunity to malaria', *Nature Reviews Immunology,* 4(3), pp. 169-180.

Stills, H.F. (2012) 'Polyclonal antibody production', in Anonymous *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents.* Elsevier, pp. 259-274.

Stoute, J.A., Odindo, A.O., Owuor, B.O., Mibei, E.K., Opollo, M.O. and Waitumbi, J.N. (2003) 'Loss of red blood cell–complement regulatory proteins and increased levels of circulating immune complexes are associated with severe malarial anemia', *The Journal of infectious diseases,* 187(3), pp. 522-525.

Stoute, J.A., Slaoui, M., Heppner, D.G., Momin, P., Kester, K.E., Desmons, P., Wellde, B.T., Garçon, N., Krzych, U. and Marchand, M. (1997) 'A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria', *New England Journal of Medicine,* 336(2), pp. 86-91.

Sultan, A.A., Thathy, V., Frevert, U., Robson, K.J., Crisanti, A., Nussenzweig, V., Nussenzweig, R.S. and Ménard, R. (1997) 'TRAP is necessary for gliding motility and infectivity of Plasmodium sporozoites', *Cell,* 90(3), pp. 511-522.

Sun, T., Holowka, T., Song, Y., Zierow, S., Leng, L., Chen, Y., Xiong, H., Griffith, J., Nouraie, M., Thuma, P.E., Lolis, E., Janse, C.J., Gordeuk, V.R., Augustijn, K. and Bucala, R. (2012) 'A Plasmodium-encoded cytokine suppresses T-cell immunity

during malaria', *Proceedings of the National Academy of Sciences of the United States of America,* 109(31), pp. E2117-26.

Takahashi, K. (2011) 'Mannose-binding lectin and the balance between immune protection and complication', *Expert review of anti-infective therapy,* 9(12), pp. 1179-1190.

Taylor, P., Botto, M. and Walport, M. (1998a) 'The complement system', *Current Biology,* 8(8), pp. R259-R261.

Taylor, P., Botto, M. and Walport, M. (1998b) 'The complement system', *Current Biology,* 8(8), pp. R259-R261.

Taylor, P.R., Seixas, E., Walport, M.J., Langhorne, J. and Botto, M. (2001) 'Complement contributes to protective immunity against reinfection by Plasmodium chabaudi chabaudi parasites', *Infection and immunity,* 69(6), pp. 3853-3859.

Tero, P., Tommi, K., Juho, K., Kaisa, H., Amit, S. and Perttu, P. (2013) 'Structure of Plasmodium falciparum TRAP (thrombospondin-related anonymous protein) A domain highlights distinct features in apicomplexan von Willebrand factor A homologues', *Biochemical Journal,* 450(3), pp. 469-476.

Tewari, R., Spaccapelo, R., Bistoni, F., Holder, A.A. and Crisanti, A. (2002) 'Function of region I and II adhesive motifs of Plasmodium falciparum circumsporozoite protein in sporozoite motility and infectivity', *The Journal of biological chemistry,* 277(49), pp. 47613-47618.

Thevenon, A.D., Leke, R.G., Suguitan, A.L.,Jr, Zhou, J.A. and Taylor, D.W. (2009) 'Genetic polymorphisms of mannose-binding lectin do not influence placental malaria but are associated with preterm deliveries', *Infection and immunity,* 77(4), pp. 1483-1491.

Thiel, S. and Gadjeva, M. (2009a) 'Humoral pattern recognition molecules: mannan-binding lectin and ficolins', in Anonymous *Target Pattern Recognition in Innate Immunity*. Springer, pp. 58-73.

Thiel, S. and Gadjeva, M. (2009b) 'Humoral pattern recognition molecules: mannan-binding lectin and ficolins', in Anonymous *Target Pattern Recognition in Innate Immunity*. Springer, pp. 58-73.

Thomas, A., Gasque, P., Vaudry, D., Gonzalez, B. and Fontaine, M. (2000) 'Expression of a complete and functional complement system by human neuronal cells in vitro', *International immunology,* 12(7), pp. 1015-1023.

Thompson, J., Cooke, R.E., Moore, S., Anderson, L.F., Janse, C.J. and Waters, A.P. (2004) 'PTRAMP; a conserved Plasmodium thrombospondin-related apical merozoite protein', *Molecular and biochemical parasitology,* 134(2), pp. 225-232.

Thomson, C.A., Olson, M., Jackson, L.M. and Schrader, J.W. (2012) 'A simplified method for the efficient refolding and purification of recombinant human GM-CSF', *PLoS One,* 7(11), pp. e49891.

Tomley, F.M. and Soldati, D.S. (2001) 'Mix and match modules: structure and function of microneme proteins in apicomplexan parasites', *Trends in parasitology,* 17(2), pp. 81-88.

Trampuz, A., Jereb, M., Muzlovic, I. and Prabhu, R.M. (2003) 'Clinical review: Severe malaria', *CRITICAL CARE-LONDON-,* 7(4), pp. 315-323.

Tucker, R.P. (2004) 'The thrombospondin type 1 repeat superfamily', *The international journal of biochemistry & cell biology,* 36(6), pp. 969-974.

Turner, M.W. (1996) 'Mannose-binding lectin: the pluripotent molecule of the innate immune system', *Immunology today,* 17(11), pp. 532-540.

Venkatraman Girija, U., Gingras, A.R., Marshall, J.E., Panchal, R., Sheikh, M.A., Gal, P., Schwaeble, W.J., Mitchell, D.A., Moody, P.C. and Wallis, R. (2013) 'Structural basis of the C1q/C1s interaction and its central role in assembly of the C1 complex of complement activation', *Proceedings of the National Academy of Sciences of the United States of America,* 110(34), pp. 13916-13920.

Walport, M.J. (2001) 'Complement', *New England Journal of Medicine,* 344(14), pp. 1058-1066.

Ward, P.A., Sterzel, R.B., Lucia, H.L., Campbell, G.H. and Jack, R.M. (1981) 'Complement does not facilitate plasmodial infections', *Journal of immunology (Baltimore, Md.: 1950),* 126(5), pp. 1826-1828.

Weekley, C. and Smith, D.S. (2013) 'Malaria: the clinical basics', *Global Health Education Consortium (GHEC),* .

World Health Organization (2015) *Guidelines for the treatment of malaria.* World Health Organization.

Ying, P., Shakibaei, M., Patankar, M.S., Clavijo, P., Beavis, R.C., Clark, G.F. and Frevert, U. (1997) 'The malaria circumsporozoite protein: interaction of the conserved regions I and II-plus with heparin-like oligosaccharides in heparan sulfate', *Experimental parasitology,* 85(2), pp. 168-182.

Zipfel, P.F. and Skerka, C. (2009) 'Complement regulators and inhibitory proteins', *Nature Reviews Immunology,* 9(10), pp. 729-740.

Zipfel, P.F., Würzner, R. and Skerka, C. (2007) 'Complement evasion of pathogens: common strategies are shared by diverse organisms', *Molecular immunology,* 44(16), pp. 3850-3857.

Zundel, S., Cseh, S., Lacroix, M., Dahl, M.R., Matsushita, M., Andrieu, J.P., Schwaeble, W.J., Jensenius, J.C., Fujita, T., Arlaud, G.J. and Thielens, N.M.

(2004) 'Characterization of recombinant mannan-binding lectin-associated serine protease (MASP)-3 suggests an activation mechanism different from that of MASP-1 and MASP-2', *Journal of immunology (Baltimore, Md.: 1950)*, 172(7), pp. 4342-4350.