Use of recombinant complement regulatory proteins to enhance killing of *Plasmodium falciparum*

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by

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled "Use of recombinant complement regulatory proteins to enhance killing of *Plasmodium falciparum*" is based on work conducted by the author at the University of Leicester mainly during the period between January 2015 and August 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: Mohenned Alsaadawi Date: 30/08/2018

Abstract

Use of recombinant complement regulatory proteins to enhance killing of *Plasmodium falciparum*

Mohenned Alsaadawi

Plasmodium falciparum, the causative agent of malaria, evades complement mediated killing by recruiting host complement regulatory proteins. All of the known complement evasion mechanisms act on the alternative pathway C3 convertase. Asexual blood stages of *Plasmodium falciparum* can sequester factor H, which helps to destabilise and inactivate the C3 convertase. The action of factor H could be reduced by several fluid-phase complement regulators, including properdin and factor H-related protein 1 (CFHR1), which are competitive inhibitors of factor H and enhance complement activation.

The aim of this work was to explore the possibility of enhancing the complement-mediated killing of *P. falciparum* by reducing the efficacy of factor H bound to the parasite surface, either by interfering with its binding to the parasite or its ability to inactivate the C3 convertase. Recombinant properdin and CFHR1 were used as model inhibitors of factor H. Recombinant properdin is particularly effective at augmenting complement activation, as it forms very large oligomers that are much more active than native serum properdin.

Recombinant human, mouse and chimeric properdin (mouse properdin humanised at one or both termini) were expressed in mammalian cells. CFHR1 was expressed in a bacterial expression system, together with a C-terminal fragment of CFHR1 (SCR3-5), which retains the factor H inhibitory activity, but lacks a C5 convertase inhibition activity that resides in the N-terminus of the protein.

The recombinant proteins were purified to near homogeneity and shown to effectively increase C3b deposition on zymosan, a model activator of the alternative pathway. Moreover, the proteins could enhance formation of the membrane attack complex (MAC) via the terminal pathway, leading to haemolysis of erythrocytes. Likewise, serum supplemented with the recombinant proteins had an enhanced ability to opsonize *P. falciparum* schizonts with C3b. The effect of the proteins on live parasites was tested using an *ex vivo* model of parasitaemia in infected erythrocytes. When the

infected erythrocyte cultures were supplemented with rCFHR1, parasitaemia was significantly reduced. Controls using heat-inactivated serum showed that this effect was complement dependent. Immunofluorescence microscopy showed increased C3b and C5b-9 deposition on infected RBC when the recombinant proteins were added to the cultures.

In conclusion, the results indicate that blocking the activity of factor H in serum, or preventing its attachment to *P. falciparum*, is sufficient to reduce the infectivity of the parasite in blood, opening up the possibility of new therapies that exploit this mechanism.

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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
CFHR1, 2, 3, 4, 5	complement factor H related protein 1, 2, 3, 4, 5
CFP	complement factor P (properdin)
CHO	Chinese Hamster Ovary
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
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HIS	heat inactivated normal human serum
HRP	Horse Radish Peroxidase
iC3b	inactive C3b
iRBCs	infected red blood cells
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl Thiogalactoside
kDa	Kilodalton
LB	Luria-Bertani
LA	Lectin pathway
MAC	Membrane Attack Complex
MASP	MBL-Associated Serine Protease
MBL	Mannan Binding Lectin
MEM	Minimum Essential Medium
M	molar
MSP1	Malaria Merozoite Surface Protein 1
µg	Microgram
µL	Microliter
mL	Milliliter
mM	Millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
NHS	Normal Human Serum
niRBCs	non-infected red blood cells
nM	Nanomolar
OD	Optical Density

PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PfGAP50	*P. falciparum* Glideosome Associated Protein-50
PfRh4	*P. falciparum* Reticulocyte binding homologue 4
P_n	high-order oligomers properdin
PNACL	the Protein Nucleic Acid Chemistry Laboratory University of Leicester
RBCs	red blood cells
RCA	Regulation of Complement Activation
rCFHR1	recombinant complement factor H related protein 1
rHMMP	recombinant mouse properdin humanised at N terminus
rHMHP	recombinant mouse properdin humanised at both termini
rHP	recombinant human properdin
rMMHP	recombinant mouse properdin humanised at C terminus
rMP	recombinant mouse properdin
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium
rSCR 3-5	recombinant C terminal of CFHR1
SCRs	short consensus repeats
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

TSRs	Thrombospondin type I Repeats
w/v	weight/volume
WT	Wild Type
Xgal	5-bromo-4-chloro-3-indolyl-beta D-galactopyranoside

1 Chapter One

General Introduction

1.1 Complement system

There are two types of immune response: innate and adaptive immunity. Innate immunity represents the first defence line in the body (Hoffmann *et al*, 1999), while the adaptive response is a specific response, which involves antibodies and cell mediated immunity, with immunological memory (Chaplin, 2010). Complement is traditionally considered to be a part of the innate immune system, although it is also an important effector mechanism of antibody driven immunity and thus cross-links the innate and adaptive systems (Janeway *et al*, 2001).

1.1.1 History of Complement

Complement proteins were identified in blood and body fluids more than 100 years ago. Nuttal (1888) showed that when fresh plasma was heated to 55^0C, its anti-bacterial role was abolished (Alper, 1998). Ehrlich and Morgenroth suggested that bacteria were killed mainly by two components in the blood: a heat stable component called antibody and a heat labile component, which is complement (Figueroa and Densen, 1991). During the 1920s and 1930s, 4 proteins of complement and their lytic mechanism were observed (Gordon, Whitehead and Wormall, 1926; Whitehead, Gordon and Wormall, 1925) and then purified and characterized in 1941 (Pillemer *et al*, 1941).

In the 1950s and 1960s, the immune role of complement was described. Nine proteins of complement were separated from the serum of Guinea pigs and the alternative complement pathway was investigated (Nelson and Nelson, 1959). Nelson and his colleagues discovered the complement components, then later found out their sequence of action and this proteins cascade was later known as the classical pathway (Nelson *et al*, 1966; Nelson and Biro, 1968). During the 1970s and 1980s, the isolation and sequencing of genes encoding the complement proteins were achieved (Campbell *et al*, 1988). Another advance occurred when a third pathway, the lectin pathway was found (Ikeda *et al*, 1987) and complement receptors were characterized (Hourcade, Holers and Atkinson, 1989). Different complement-mediated activities against pathogens can clear the cellular debris and eliminate the pathogenic agents thus controlling the homeostasis (Zipfel and Skerka, 2009). Another important discover was polymorphism in

complement proteins such as factor H, I, and B, which can have different functions according to their genetic variants (Day *et al*, 1988; Clark *et al*, 2006; Córdoba, Rodríguez and De Jorge, 2008; Nilsson *et al*, 2011), an example is the polymorphism of factor H which could release the risk of age-related macular degeneration (AMD) (Clark *et al*, 2006).

1.1.2 The complement pathways

Complement proteins are found as circulating proteins in inactive forms (Beltrame *et al*, 2014). These proteins cleave or interact with other proteins before being activated (Carroll and Sim, 2011). The complement system uses several different mechanisms to protect against pathogenic organisms. Complement proteins such as C3b and C4b can opsonise the activating particles, marking them for phagocytosis by, e.g. macrophages. Activation of the terminal pathway results in the assembly of the membrane attack complex (MAC), which can lyse activating cells (Rus, Cudrici and Niculescu, 2005). The complement system could also contribute in producing proinflammatory molecules like C3a. In addition, it can organise the innate and adaptive immune system (Dunkelberger and Song, 2010). There are three pathways of complement: the classical, alternative and lectin pathways. The three pathways converge with the formation of the C3 and C5 convertases. From this point on, the terminal pathway leads to the formation of the membrane attack complex (MAC) (Carroll and Sim, 2011) (Figure 1-1).

1.1.2.1 The classical pathway (CP)

The recognition molecule, C1q, binds mainly to immune complexes; antigen-IgG or IgM complexes (Kang *et al*, 2009). Serine protease zymogen, C1r, can spontaneously activate when C1q binds to the target surface and subsequently it activates another serine protease proenzyme, C1s, and cleaves it to form C1 serine protease (Dodds *et al*, 1978). This enzyme cleaves C2 and C4, thus forming the C3 convertase, C4b2a (Figure 1-1). After that C3 is cleaved by the C3 convertase into C3a and C3b. The later can bind to C4b2a to form the C5 convertase, C4b3b2a (Figure 1-1). In addition, the deposited C3b on the target surface can activate the alternative pathway as will be explained later.

1.1.2.2 The Lectin pathway (LP)

The lectin pathway is activated when certain structures, like carbohydrates in microorganisms, bind with the lectin pathway recognition complexes. These recognition complexes comprise a lectin such as mannose binding lectin (MBL), Ficolins and collectin 11 (Arlaud *et al*, 2007) and zymogen serine proteases known as MBL-associated serine proteases (MASP1, 2 and 3). Two other proteases are associated with MBL and Ficolins. These are called MAP19 and MAP1, which are alternative splice products of the MASP2 and MASP1/3 genes, respectively (Schwaeble *et al*, 2002; Skjoedt *et al*, 2011). MASP-2 auto-activates when the recognition complexes bind to the pathogen surface (Chen and Wallis, 2004). C4 and C2 can be cleaved by the activated MASP-2 to form the same C3 and C5 convertases produced by the CP (Thiel *et al*, 1997). In addition, MASP1 cleaves MASP2 or C2 bound to C4b and by this way, it contributes in activation the lectin pathway (Schwaeble *et al*, 2011), while MASP3 may play a role in activation of Factor D (FD) (Iwaki *et al*, 2011) (Figure 1-1).

1.1.2.3 The alternative pathway (AP)

The alternative pathway is unlike the classical or lectin pathways which are initiated by a specific pattern recognition molecule such as C1q or MBL/Ficolins. This pathway starts spontaneously when native C3 in plasma is hydrolysed by water. C3(H2O) can bind to factor B (FB)which is subsequently cleaved into FBa and FBb by factor D (FD) (Carroll and Sim, 2011). The resulted product, C3(H2O)Bb is an alternative pathway C3 convertase which cleaves C3 into C3a and C3b. The later can bind more FB to form C3bB.The C3b bound FB can be cleaved by FD to form the alternative pathway C3 convertase; C3bBb which can cleave more C3 (Carroll and Sim, 2011).

The instability of this C3 convertase, which has a 90 second half-life, could be reduced by properdin activity (Medicus, Gotze and Muller-Eberhard, 1976; Pangburn, 1989). Properdin can bind to the alternative pathway convertase and form a quite stable complex; C3bBb-P (Bhakdi and Tranum-Jensen, 1991).

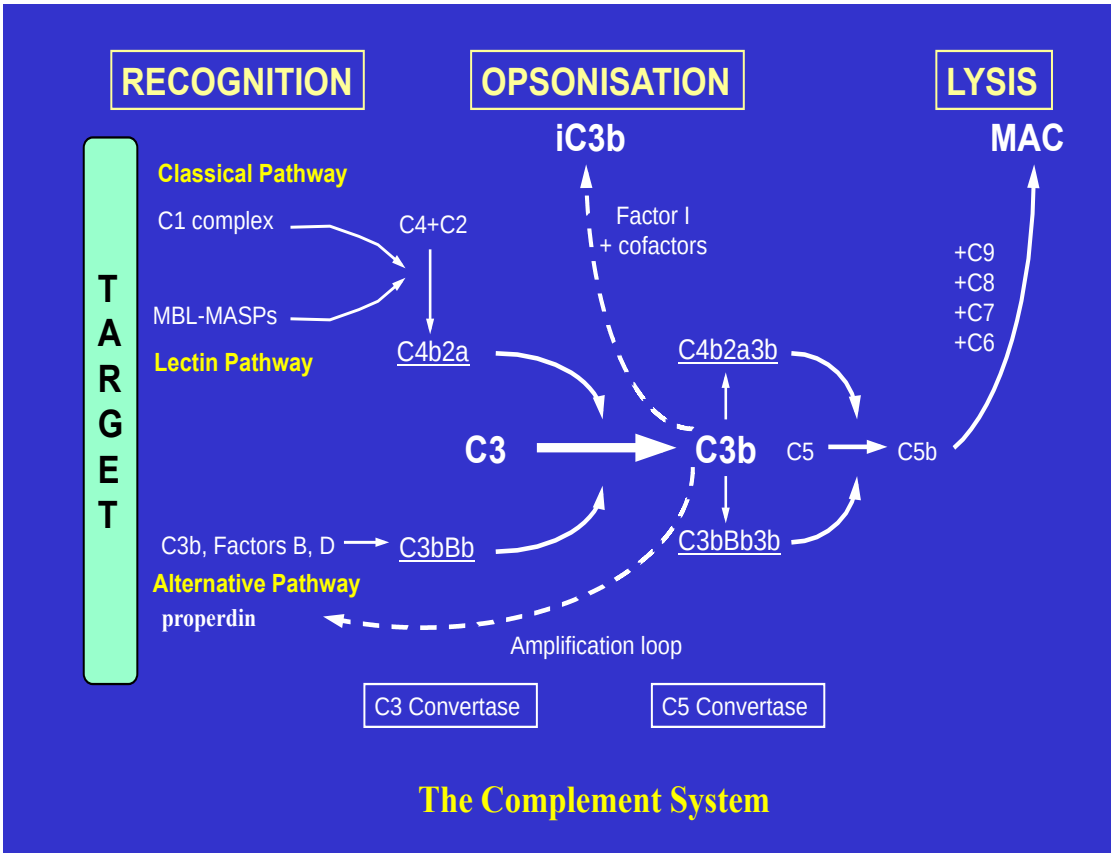

Figure 1-1: Schematic overview of complement pathways activation cascade
During classical pathway activation, C1r cleaves C1s. Activated C1s cleaves C2 and C4 to form the C3 convertase, C4b2a, which cleaves C3 into C3a and C3b. The latter can bind to C4b2a to form the C5 convertase, C4b3b2a. In the lectin pathway, MBL and Ficolins bind to zymogen serine proteases called MBL-associated serine proteases (MASP-1, -2 and -3). C2 and C4 can be cleaved by activated MASP-2 to form the C3 convertase and then C5 convertase as in the classical pathway. During the alternative pathway, FB binds to hydrolyzed C3(H_2O) and is then cleaved by FD resulting in C3(H_2O)Bb, the alternative pathway C3 convertase which in turn cleaves C3 into C3a and C3b. The latter binds to activator surface and can make a new complex with FB. The bound FB in C3bB can be cleaved by FD to form C3bBb which is the alternative pathway C3 convertase. C3bBb is an unstable complex and can be stabilised by properdin and thereby can cleave more C3 to work as an amplifier factor. The terminal pathway starts when C5 associates with C4bC2aC3b on the foreign surfaces, then binds to C6 and 7 resulting in C5b-6-7 complex which acts as a receptor for C8. C9 binds to C5-8 complex to form membrane attack complex (MAC) which creates a pore in plasma membrane and ruptures foreign cells. C3a, C4a and C5a are involved in inflammation and opsonisation. Figure supplied by Robert B. Sim, Oxford University.

* Opsonisation is the process by which specific proteins can cover foreign cells to be clearer for phagocytic cells (Owens III and Peppas, 2006).

** Phagocytosis is the process by which foreign cells are engulfed and destructed by macrophages. It comes after opsonisation (Ambrose, 2006).

1.1.3 Complement 3 (C3)

Mainly, the alternative pathway is contingent on C3 molecules. The average plasma concentration of C3 is about 0.8 g/L which produces every 2 days (Charlesworth *et al*, 1974). It is produced mainly by hepatocytes (de Bruijn and Fey, 1985). The activation of C3 occurs spontaneously through the slowly hydrolysis process to form C3(H2O) in which the internal thioester bond between side chains of cysteine 988 and glutamine 991 could be hydrolysed (Meri, 2016). Similarly to C3b, factor B can bind to C3(H_2O) which cleaved by factor D to form C3(H_2O)Bb and release Ba. C3(H_2O)Bb cleaves C3 into C3b and C3a (Pangburn and Muller-Eberhard, 1980). C3 is critical for all three pathways, in that it is the point at which they converge (Law and Dodds, 1997). The high concentration in the plasma makes C3 the main opsonin of the complement system.

The usefulness of C3 as an opsonin is increased by its internal thioester bond that allows it to form very stable covalent bonds with the activating particle. The α-chain of C3 can be autocatalytically cleaved, which exposes the thioester bond to attack by nucleophiles (Hostetter *et al*, 1982). The majority of activated C3 molecules are hydrolysed to (H_2O)C3b in the fluid phase (Law and Dodds, 1997). However, if C3 is activated in close proximity to a cell surface, the exposed acyl group (Law, Lichtenberg and Levine, 1979) binds covalently with hydroxyl groups on the cell surface (Law and Dodds, 1997). By this means, C3 can react to a wide variety of surfaces.

1.1.4 Complement regulatory proteins

The complement pathways are regulated by many proteins such as properdin, factor H and factor H related proteins.

1.1.4.1 Properdin

Properdin or complement factor P is a serum glycoprotein with a serum concentration of 10-25 µg/ml (Xu *et al*, 2008). It is produced by monocytes, T cells, mast cells and granulocytes. Unlike other complement components, properdin is not synthesised by hepatocytes (Whaley, 1980; Schwaeble *et al*, 1993; Stover *et al*, 2008). Properdin is encoded by the *CFP* gene on the X chromosome, location Xp11.4 (Coleman *et al*, 1991). It physiologically regulates complement activation via the alternative pathway by stabilising the C3 convertase (Farries, Lachmann and Harrison, 1988). Properdin is a

rod shape glycoprotein that is formed by connecting several monomers (53kDa each) from head to tail (Smith *et al*, 1984; Pangburn, 1989). Serum properdin contains dimers, trimers and tetramers. The monomers are composed of seven Thrombospondin type I Repeats (TSRs) (Figure 1-2). TSR4 and TSR5 are responsible for stabilisation of the C3 convertase (Higgins *et al*, 1995).

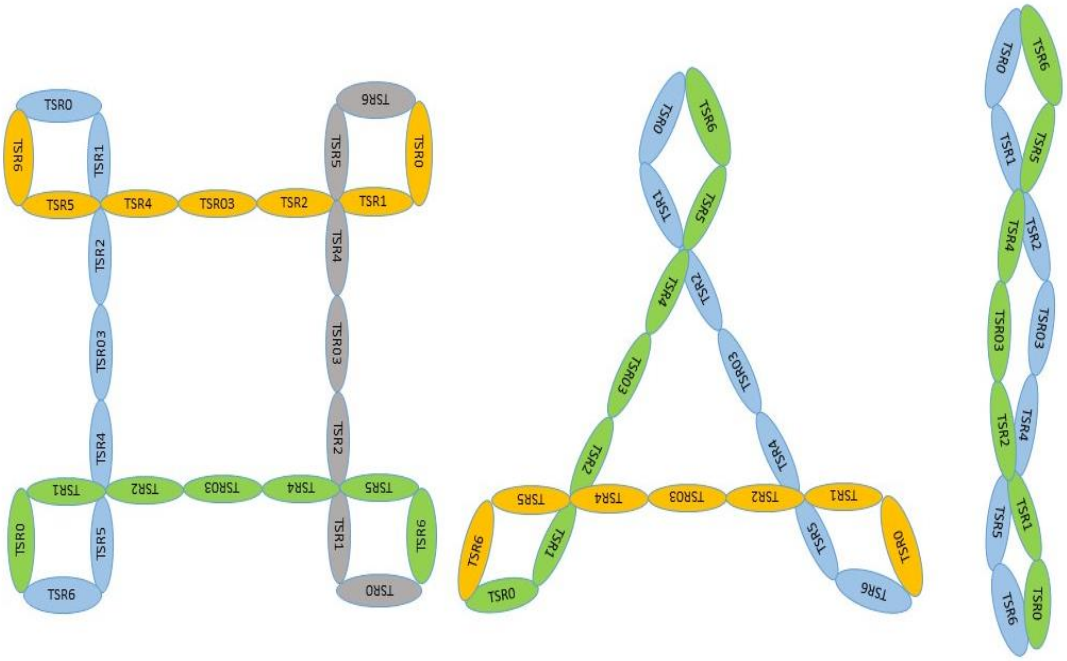

Figure 1-2: Schematic representation of dimer, trimer and tetramer properdin showing head-to-tail binding of monomeric properdin
Properdin is formed by connecting monomers (53 kDa each) from head to tail. Each monomer consists of seven TSR domains numbered from TSR0-6. Serum properdin usually contains cyclic dimers, trimers and tetramers in a ratio 1:2:1. The vertices of these cyclic forms may compose of four TSR domains; two from each of two different monomers forming head to tail structure. (Alcorlo *et al*, 2013) suggested a model with vertices formed by TSR0-1 from one monomer and TSR5-6 from the other.

1.1.4.1.1 Role of Properdin

Properdin can facilitate alternative pathway activation by stabilising the C3 convertase (Hourcade, 2006; Ferreira, Cortes and Pangburn, 2010a). The alternative pathway C3 convertase, C3bBb is responsible for amplification of this pathway by activating more C3 and generating the C5 convertase, C3bBbC3b (Figure 1-1). The half-life of C3bBb is about 90 seconds (Medicus, Gotze and Muller-Eberhard, 1976; Pangburn, 1989). Properdin binds to this complex, increasing the half-life by forming a relatively stable complex, C3bBb-properdin (C3bBbP) (Fearon and Austen, 1975). Therefore, properdin increases the amplification role of the alternative pathway by binding more C3b to form more stable complexes. The binding ability of properdin to C3bBb depends on the size of the polymeric form of properdin. Recombinant human properdin produced and purified *in vitro*, can aggregate to form highly polymerised structures (Farries and Atkinson, 1989; Ali *et al*, 2014). This artificial product (P_n) stabilises C3bBb 5-10 fold more effectively than native properdin. In addition, it could increase the half-life of C3bBb thus increasing the amplification loop of the alternative pathway (Ferreira, Cortes and Pangburn, 2010a). However, the expression of a highly and continuously activated recombinant murine properdin is difficult; recombinant murine properdin is usually less active than native murine properdin (Lynch and Ali, personal communication).

1.1.4.2 Factor H

Factor H is a complement regulatory plasma glycoprotein (Zipfel and Skerka, 2009). It can stop C3 turnover and thus down regulate the alternative pathway (Carroll and Sim, 2011). Factor H is found at a concentration of 150–750 µg/ml in serum (Tan *et al*, 2010). The factor H gene is located on human chromosome 1q32, on the regulation of complement activation (RCA) cluster. This cluster also contains factor H family genes, including factor H related proteins (de Córdoba, Díaz-Guillén and Heine-Suñer, 1999).

1.1.4.2.1 Composition of factor H

The size of factor H is 155 kDa (Sim and DiScipio, 1982). Factor H has two forms, monomers and some dimers, being seen in the circulating system (Perkins *et al*, 2012). Each monomer contains 20 Short Consensus Repeats (SCRs), sometimes called

Complement Control Protein (CCP) domains, each of which has 60 amino acids (Ripoche *et al*, 1988). The main factor H binding sites to C3b are SCR19 and SCR20 (Hannan *et al*, 2016; Ripoche *et al*, 1988). The complement activity mediated by factor H that is located within SCR1-4 (Wu *et al*, 2009). SCR7 and SCR20 of factor H can bind to negatively charged clusters like heparin (Kang *et al*, 2009). In addition, factor H could bind to bacterial and parasitic surfaces and sometimes used by other pathogens to evade complement activity as will be explained in section 1.1.5.4 (Carroll and Sim, 2011).

1.1.4.2.2 Factor H function

Factor H can bind C3b and inhibit the binding of factor B to C3b, thus inhibiting the alternative pathway C3 convertase. This leads to inhibition of the alternative pathway activity by destabilising C3bBb (Conrad, Carlo and Ruddy, 1978). In addition, factor H can mediate the conversion of C3b to an inactive product of C3b (iC3b) by acting as a cofactor for factor I (Whaley and Ruddy, 1976). 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, allowing continuation of the C3 convertase activity (Farries, Lachmann and Harrison, 1988; Bhakdi and Tranum-Jensen, 1991).

1.1.4.3 Complement Factor H Related Proteins

The Complement Factor H-Related Proteins are 5 proteins found in plasma (CFHR1, CFHR2, CFHR3, CFHR4 and CFHR5) that can bind to C3b and modify the activity of factor H as they can share sequence similarities (Skerka *et al*, 2013). They are encoded by genes located on a distinct segment on human chromosome 1q32 within the RCA (Regulation of Complement Activation) gene cluster (Male *et al*, 2000). These proteins could be classified into two groups regarding their ability to be dimerized. Group I include CFHR1, CFHR2, CFHR5 which can form homodimers and heterodimers (heterodimers can be done either between CFHR1 or CFHR2 with CFHR5) as they have dimer forming amino acid motif at the N terminal part. These dimers are linked by the N terminal part. While group II (CFHr3 and CFHR4) cannot form dimers and lack of terminal dimer forming amino acid motif (Skerka *et al*, 2013).

1.1.4.3.1 Group I complement factor H proteins

1.1.4.3.1.1 CFHR1

Composition: CFHR1 contains five Short Consensus Repeat domains (SCRs) (Skerka, Horstmann and Zipfel, 1991) numbered from 1 to 5 (Figure 1-4). The two N-terminal domains (SCR1 and 2) of CFHR1 are responsible for formation of homodimers by connecting the N-terminal part (Figure 1-3) and sometimes can form heterodimers with CFHR2 and CFHR5 (Goicoechea de Jorge *et al*, 2013). They have 34% and 42% similarity to SCR6 and SCR7 of factor H, respectively (Skerka *et al*, 2013). The other SCRs domains at the C-terminal of CFHR1 are mostly identical to SCRs 18–20 of factor H (Figure 1-4).

Function: Two functions are performed by CFHR1. Firstly, the N-terminal domains (SCR 1–2) regulate the terminal pathway by inhibiting MAC assembly (Heinen *et al*, 2009). Secondly, CFHR1, unlike factor H, lacks the cofactor activity which can mediate the cleavage of C3b by factor I and also lacks the dissociation activity of C3b bound factor B in the C3 convertase (Timmann, Leippe and Horstmann, 1991). Thus, CFHR1 can compete for factor H-C3b binding, promoting the alternative pathway activation. This function is likely to be completed by the C-terminal (SCR3, 4, 5) of CFHR1 (Fritsche *et al*, 2010; de Jorge *et al*, 2012).

Figure 1-3: Schematic structure of CFHR1 dimers
CFHR1 consists of five SCR domains numbered from one to five. Dimers can be formed by connecting SCR1 and 2 in one monomer to that in the other. This shape was modified from (Hannan *et al*, 2016).

1.1.4.3.1.2 CFHR2

Composition: It has four SCRs (Skerka *et al*, 1992) which share some similarities to SCRs of factor H as follows: SCR1 of CFHR2 shows 41% similarity to SCR6 of factor H, SCR2 of CFHR2 is 34% similar to SCR7 of factor H and SCR3 of CFHR2 is 89% similar to SCR19 of factor H (89%) and SCR4 of CFHR2 to SCR20 (61%) of factor H (Figure 1-4). It could form dimers in both forms; homo and hetero with CFHR1 only (Skerka *et al*, 2013).

Function: In plasma, CFHR2 concentration is about 50 µg/ml (Skerka *et al*, 2013). It could abolish the alternative pathway activation by reducing C3b deposition thus the amplification loop is no longer being active (Eberhardt *et al*, 2011). It lacks the competitive inhibitory function of factor H on C3b molecule as its C3b binding affinity is lower than factor H (Goicoechea de Jorge *et al*, 2013).

1.1.4.3.1.3 CFHR5

Composition: It has nine SCRs (Skerka *et al*, 2013) in which the SCR3-7 share 46%, 75%, 57%, 48%, and 71% similarity to SCRs 10–14 of factor H. The C terminal part of CFHR5 shows 64% and 41% to SCR19 and 20 of factor H respectively (Figure 1-4). It can form dimers in both forms; homo and hetero, with CFHR1 only (Goicoechea de Jorge *et al*, 2013).

Function: The main function of CFHR5 is related to self and non-self-discrimination. It can bind to C3b and inactivate C3b convertase on self-structures then protect them from the complement activation (McRae *et al*, 2005). It can also play a role as a cofactor for factor I and decay acceleration activity at high concentration (Skerka *et al*, 2013). CFHR5 can compete factor H on its C3b binding site (Goicoechea de Jorge *et al*, 2013).

1.1.4.3.2 Group II complement factor H proteins

1.1.4.3.2.1 CFHR3

Composition: It has five SCRs (Skerka *et al*, 1993) in which the first three SCRs share some similarities to SCR6, SCR7 and SCR8 of factor H (91%, 85% and 62%) respectively while SCR4 of CFHR2 shows 64% similarity to SCR19 of factor H and SCR2 of CFHR2 is 37% similar to SCR20 of factor H (Figure 1-4).

Function: In plasma, CFHR3 concentration is about 70-100 µg/ml (Fritsche *et al*, 2010).CFHR3 can augment factor H activity and also acts as cofactor for factor I (Hellwage *et al*, 1999). CFHR3 can compete with factor H on its C3b binding site (Fritsche *et al*, 2010).

1.1.4.3.2.2 CFHR4

Composition: It has nine SCRs (Skerka *et al*, 1997) and seems to have two symmetrical parts as SCR1-4 are highly similar to SCR5-8 (Figure 1-4). SCR1-3 share 71% similarity to SCR6 of factor H. SCR8 and 9 of CFHR4 show 62% and 68% similarity to SCR19 and 20 of factor H respectively (Skerka *et al*, 1997).

Function: As for CFHR3, CFHR4 can augment factor H activity and also acts as cofactor for factor I (Hellwage *et al*, 1999). CFHR5 may play a role in self and non-self-

discrimination as it can bind to the central part of C3b on self surfaces thus preventing the complement attack (Hellwage *et al*, 1999; Mihlan *et al*, 2009).

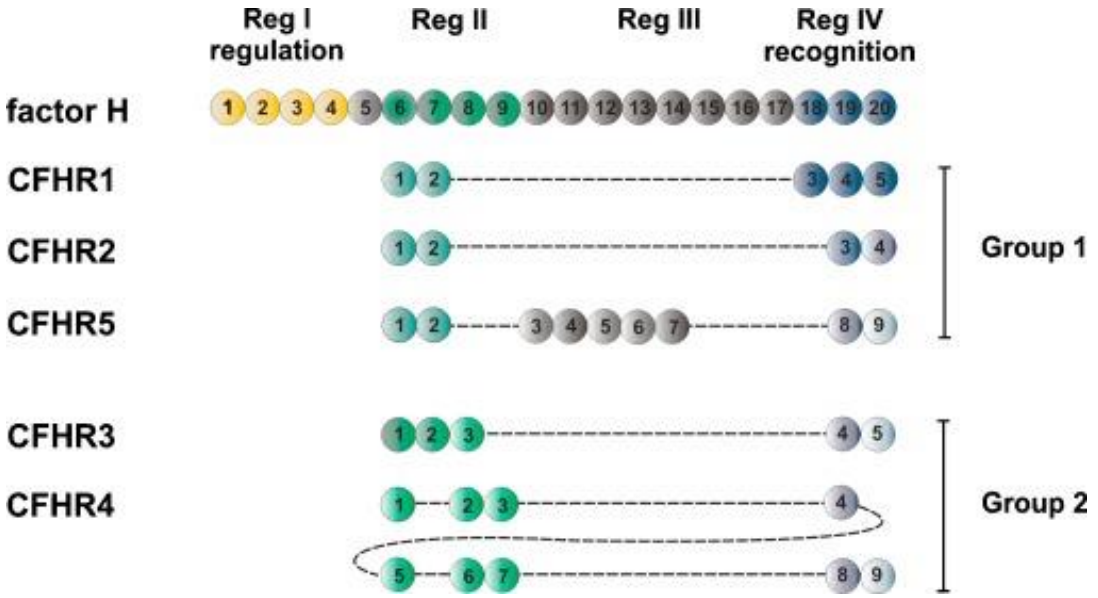

Figure 1-4: Schematic representation of CFHR proteins and factor H structure
Factor H related proteins are encoded by the genes of the factor H family which contain SCR domains with variable similarities to these of factor H. These proteins do not have Reg I as in factor H which is represented by SCR1-4. There are three regions in factor H related proteins which share some similarities with factor H and these are the N-terminal SCR domains of CFHR proteins which show some similarities to SCRs 6–9 (Reg II), SCRs 10–14 (Reg III) of factor H, and the C-terminal parts of both factor H related proteins and factor H SCRs 18–20 (Reg IV). The color intensity relates to the similarity with factor H domains. This figure was taken from (Skerka *et al*, 2013).

1.1.4.4 Complement receptor 1 (CR1):

CR1 can prevent further complement activation and it is found on the host cell surfaces (Zipfel and Skerka 2009). CR1 is encoded by the CR1 gene on chromosome 1 (Rey-Campos *et al.* 1988). The unction of CR1 is accelerating the decay of the alternative pathway convertase (C3bBb) through binding to C3b (Iida and Nussenzweig 1984). It can also play a cofactor role for the factor I-mediated inactivation of C3b (Lesher *et al.*

2013). Many cells can exhibit CR1 on their surfaces such as erythrocytes, white blood cells and visceral epithelial cells (Podocytes) (Gelfand *et al.* 1975 and Fearon, 1980).

1.1.4.5 Factor I

Complement factor I is one of complement regulator protein. It is a serine protease and found in plasma as a zymogen (Roversi *et al*, 2011). It consists of two polypeptide chains linked by a single disulfide bond (Fearon, 1977). Factor I can inactivate C3b by cleaving it into iC3b in presence of factor H which can work as a cofactor (Roversi *et al*, 2011).

1.1.5 Distinguishing of self and non-self-targets

It is important that the immune system can discriminate between non-self and self surfaces. This process can be done by default, which means opsonising or phagocytosing any structures not recognized as self-structures (Meri, 2016). Pathogens can modify some mechanisms in the immune system, disturbing the self and non-self-discrimination process. Understanding these modifications can give some insights about how the microorganisms can avoid complement activation thus developing new ways or vaccines to enhance immune killing of pathogenic microbes (Serruto *et al*, 2010).

1.1.5.1 Role of the alternative pathway in distinguishing self- and non-self surfaces

If the target surfaces can induce complement activation, C3 can be activated to C3b and support the alternative pathway through the amplification loop. C3b deposits on the target structures in a random way and the C3b convertase can be formed by binding factor B to C3b, which leads to activation of more C3b molecules. These active molecules tend to assemble, forming clusters of dimers that deposit on the target surfaces (Meri, 2016). However, when the surfaces do not activate the complement components, C3 activation to C3b is stopped by binding of factor H and I and to form iC3b (Meri, 2016). More C3b-factor H binding could be enough to overcome factor B-C3b binding (Jokiranta *et al*, 2000).

Many domains in factor H show inhibition of C3b, and restriction of alternative pathway activity. Factor H can bind to more than one molecules of C3b (Meri, 2016) through different binding sites located in different SCR domains on factor H including SCR19

and SCR20 (Hannan *et al*, 2016), SCR1-4 (Wu *et al*, 2009) and SCR6-10 (Sharma and Pangburn, 1996). If the C3b density reaches a certain threshold value, the binding affinity of C3b molecules to factor H can increase, but this is not true for factor B (Koistinen, 1991). The role of protecting self-cells from complement attack is not restricted to factor H only, as there are different protein molecules on the self surfaces, such as CR1, which can perform the same function (Hourcade *et al*, 2000).

1.1.5.2 The mechanism of the discrimination includes the binding of factor H to C3b-surfaces

The evasion method of complement killing by human and sheep red blood cells provided evidence of how self-surfaces use sialic acid to avoid complement attack (Pangburn and Muller-Eberhard, 1978; Kazatchkine, Fearon and Austen, 1979). The role of factor H during the alternative pathway activity can distinguish between self and non-self surfaces (Meri and Pangburn, 1990) (Figure 1-5). Polyanions such as sialic acids, glycosaminoglycans (e.g., heparan sulfate) and proteoglycans could be specifically recognized by factor H (Meri and Pangburn, 1994). The C terminal domain of factor H can be responsible for the self-discrimination function (Kuhn, Skerka and Zipfel, 1995).

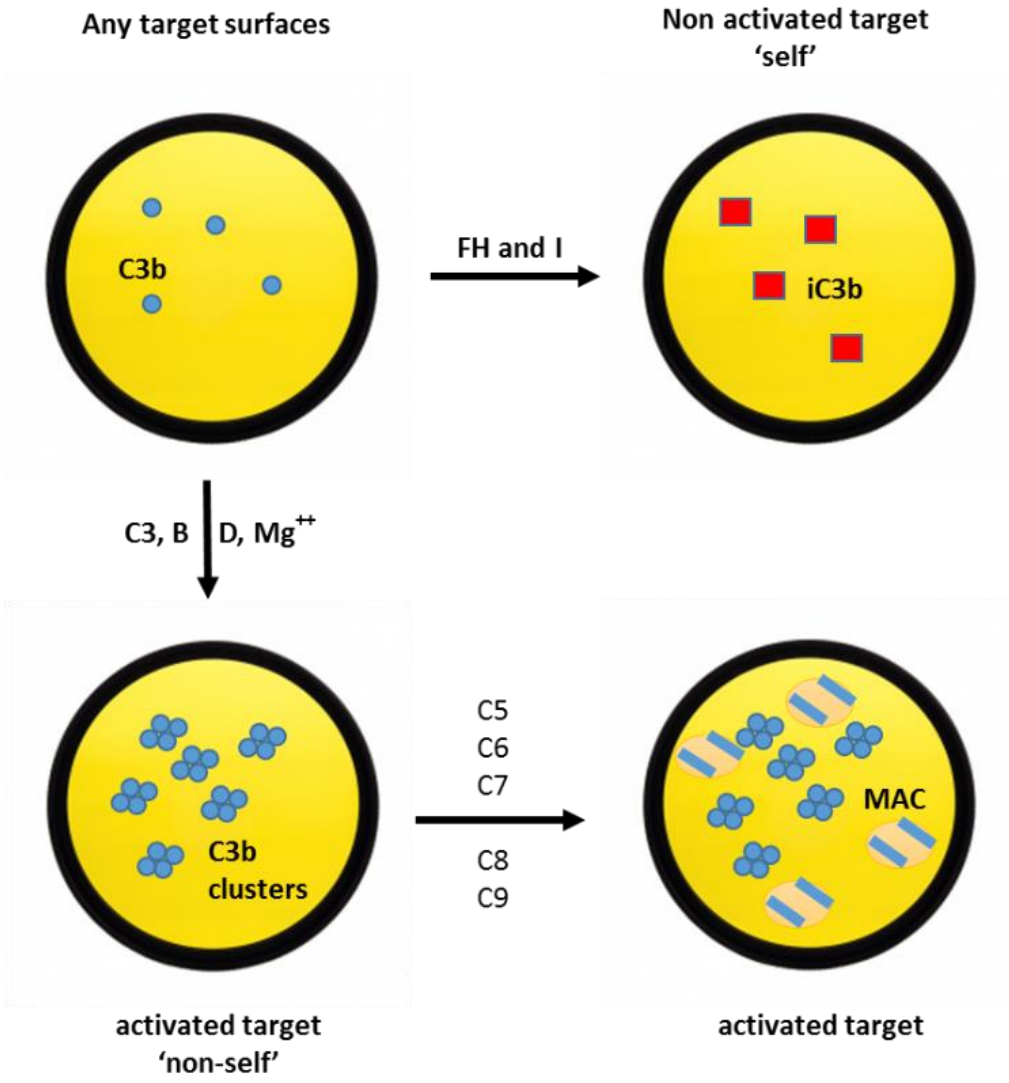

Figure 1-5: Schematic representation of downregulation of the alternative pathway of complement by FH

Firstly, C3b can deposit randomly and individually on any target surfaces. In case of self-targets, C3b activation is not supported and factor H binds to C3b leading to inactive C3b and forming iC3b in the presence of factor I. However, factor B can bind to C3b and then can be cleaved by factor D forming C3bBb convertase which can activate further C3 molecules to form C3b that can assemble as clusters when non-self surfaces do not bind to factor H. After that, the alternative pathway could be amplified by generating new alternative pathway convertase. The further C3b deposited molecules can opsonize the surfaces and the terminal pathway complex (MAC) can be formed.

1.1.5.3 Role of factor H-related proteins in the discrimination of self and non-self

Factor H-related proteins structurally differ in the length and number of SCR domains and functional activities. Their plasma concentrations can be varied but generally it is relatively low between 50-75 μg/ml (Meri, 2016). The concentrations could raise during inflammatory processes (Närkiö-Mäkelä *et al*, 2001). The main homology between factor H related proteins and factor H is the similarity in the last two C terminal domains of factor H (Zipfel *et al*, 2002). This may indicate that these proteins could be involved in discriminating between self and non-self surfaces, as this function resides mainly in the C terminal part of factor H as explained above. However, these proteins can inhibit factor H activity by competing with the binding sites located on the C terminal part of factor H. This could lead to enhancement, but not inhibition, of the complement activation (Meri, 2016). Therefore, factor H related proteins have the ability to fine-tune the inhibitory function of factor H. Factor H related proteins that make dimers can increase the fine-tune function (Goicoechea de Jorge *et al*, 2013). This could be important in case of enhancing the complement activation during some pathogenic infections that express proteins, can bind and induce the inhibitory function of factor H (Caesar *et al*, 2014) as will explain later.

1.1.5.4 Resistance of microbes to complement attack

Many pathogens can escape from the complement killing by sequestering SCR domains of factor H although the complement system has high antimicrobial activity. These microbes can avoid opsonophagocytosis via specific mechanisms include expressing some proteins. For example, *Borrelia burgdorferi* spirochetes, the etiological cause of the Lyme disease, can express specific proteins on their outer membrane that can bind to factor H in a way can inhibit the alternative pathway (Alitalo *et al*, 2002). *Neisseria meningitidis* and *Streptococcus pneumoniae* can also recruit factor H by expressing proteins, which mimic the carbohydrate motif to which factor H binds on self-targets (Dave *et al*, 2004; McNeil *et al*, 2013). However, Ali et al. (2014) succeeded in protecting mice from *N. menigitidis* by injecting the experimental animals with low doses of a recombinant form of properdin (Pn). This may be because recombinant properdin can augment the alternative pathway 5-10-fold more than native properdin thus overcoming the inhibitory role of factor H recruited by the pathogen.

Midgut cells of Anopheles mosquitoes have specific receptors to capture factor H when the Anopheles take a human blood meal. By this mechanism, sexual blood stages of *Plasmodium falciparum*, the causative agent of malaria, can avoid complement attack as the captured factor H can induce the deactivation of C3b thus inhibiting the complement activity (Khattab *et al*, 2015). In addition, 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).

1.2 *Plasmodium falciparum (P. falciparum)*:

Plasmodium is the causative agent of malaria which is a mosquito-borne disease, transmitted by a female of Anopheles mosquitoes to infect a wide variety of intermediate vertebrate hosts such as birds and primates, including humans (Boddey and Cowman, 2013). There are five species of Plasmodium that infect human beings namely *P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi*. The most severe malaria is caused by *P. falciparum* (Boddey and Cowman, 2013).

1.2.1 Life cycle

Life cycle of *P. falciparum* is complex and includes two hosts, the female of Anopheles mosquitoes and humans. Inside the human body, the life cycle involves two stages, the exoerythrocytic (liver stage) and erythrocytic (blood) stage. Forms of the parasite in the human body are divided into asexual or sexual forms. Asexual forms include sporozoites and merozoites in hepatocytes, and merozoites, ring form, trophozoites and schizonts in erythrocytes (Scherf, Lopez-Rubio and Riviere, 2008). While sexual forms include gametocytes in the blood stream (Figure 1-6).

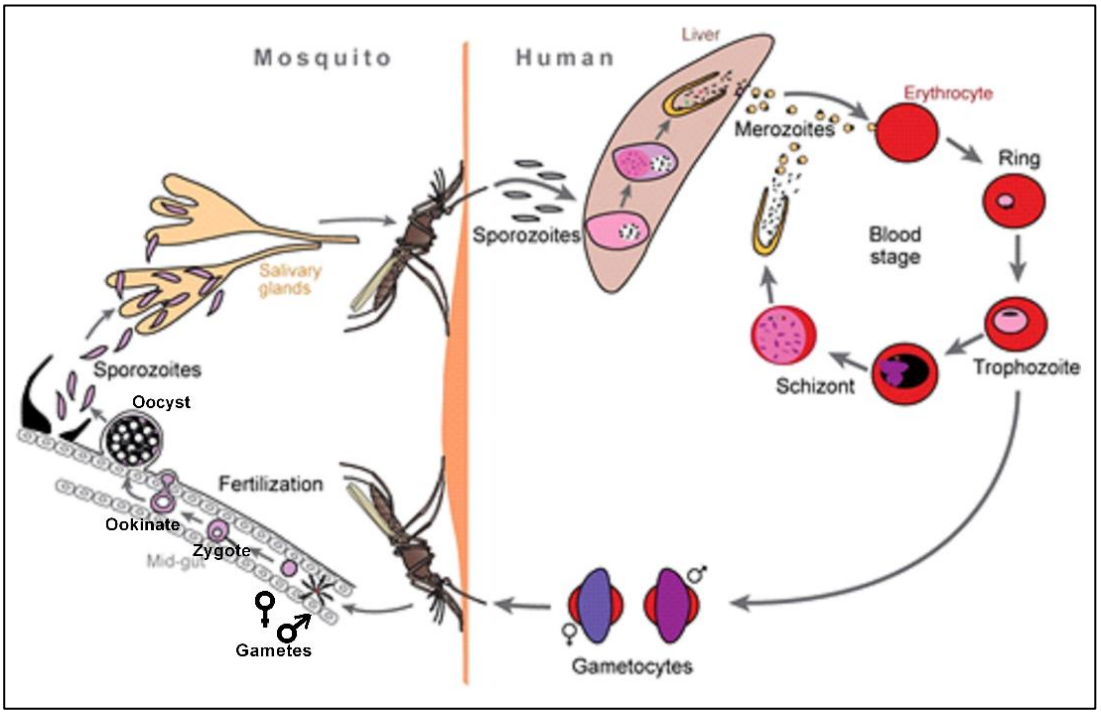

Figure 1-6: Schematic overview of the Life cycle of *P. falciparum*.
The life cycle involves two stages, the exoerythrocytic or liver stage and the erythrocyte or blood stage. The exoerythrocytic stage starts when *P. falciparum* sporozoites are injected into the human bloodstream by female Anopheles mosquitoes. Sporozoites migrate to the liver and grow inside hepatocytes and develop into hepatic merozoites. They can destroy hepatocytes and invade erythrocytes via the bloodstream to start the erythrocyte stage. After that, blood merozoites develop inside erythrocytes through ring, trophozoites and schizont forms. Each schizont divides into16-32 daughter merozoites. The new daughter merozoites, released from the infected erythrocyte, can infect new erythrocytes and form new asexual forms. Some trophozoites develop to sexual forms of Plasmodium, male and female gametocytes. These forms pass into Anopheles gut during mosquito feeding on infected human blood and develop into male and female gametes. Male gametes fuse with female gametes to produce zygotes which then develop into ookinates. Ookinates can penetrate gut epithelial layers and develop into oocysts on the basal lamina. The oocyst contains thousands of sporozoites and when it ruptures, sporozoites migrate to salivary glands of mosquito ready to infect a new human host during blood feeding. The figure was taken from (Scherf, Lopez-Rubio and Riviere, 2008).

1.2.2 Pathogenesis of *P. falciparum* malaria

The clinical symptoms of *P. falciparum* malaria are observed during the asexual blood forms (Cowman and Crabb, 2006). These forms express specialized proteins for adherence to certain receptors on host cells (Malpede and Tolia, 2014). There are three roles of parasite adhesion to erythrocytes; traversal of host cellular membranes, invasion of red blood cells and protection from the human immune clearance (Malpede and Tolia, 2014). Firstly, *Plasmodium* can traverse erythrocyte membrane by interrupting the membrane, and invading the blood cell by gliding in intracellular fluid (Mota *et al*, 2001). The second role is the invasion by invagination of the erythrocytic membrane to form the parasitophorous vacuole in which Plasmodium develops internally (Baum *et al*, 2008; Cowman, Berry and Baum, 2012). Finally, cytoadherence and rosetting of infected red blood cells is mediated by parasite adhesion proteins, providing protection from the host immune response by avoidance of clearance by the spleen (Rowe *et al*, 2009). *P. falciparum* expresses several types of surface proteins 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).

1.2.3 *P. falciparum* and complement activation:

All the known and probable complement evasion mechanisms used by *P. falciparum* act on the alternative pathway C3 convertase (C3bBb) (Rowe *et al*, 2000; Simon *et al*, 2013; Malpede and Tolia, 2014). C3bBb is responsible for producing C3b which has a significant role in pathogen opsonisation and downstream complement-mediated killing since it contains a reactive thioester bond that binds to any membrane or bacterial surfaces (Gros, Milder and Janssen, 2008).

The blood stages of *P. falciparum* produce *P. falciparum* Glideosome Associated Protein-50 (PfGAP50) (Simon *et al*, 2013) and Pf92 (Kennedy *et al*, 2016), which can bind to factor H promoting the breakdown of C3bBb, thus limiting the complement activation. In addition, Plasmodium merozoites can express a CR1 binding protein, *P. falciparum* Reticulocyte binding homologue 4 (PfRh4) (Stubbs *et al*, 2005; Gaur *et al*, 2006; Tham *et al*, 2009), which binds to complement receptor 1 (CR1) in a manner that still allows CR1 to inhibit C3bBb as CR1 can accelerate the decay of the alternative

pathway convertase (C3bBb) by binding to C3b (Iida and Nussenzweig, 1981; Tham *et al*, 2009).

1.3 Hypotheses

All the known and potential mechanisms that Plasmodium uses to evade complement attack act on C3b and the alternative pathway C3 convertase (C3bBb). The *P. falciparum* surface proteins; PfGAP50 and Pf92, bind and activate factor H. Factor H acts as cofactor for factor I, which cleaves and inactivates C3b (and C4b); factor H also inhibits C3bBb and thus down-regulating complement activation.

Properdin binds to and stabilises C3bBb, promoting ongoing C3 convertase activity. The low concentration of properdin, found in serum, limits the rate of alternative pathway activation. Complement activation can be increased by supplementing serum with properdin (Ali *et al*, 2014). Purified recombinant properdin is especially useful in this respect, because it forms large, high-order oligomers (P_n) which are 5-10 fold more active than the dimers, trimers and tetramers found in native serum properdin (Ferreira, Cortes and Pangburn, 2010a).

Properdin could antagonise the inhibitory role of factor H on the alternative pathway (Schwaeble *et al*, 1987a). It has previously been shown that P_n is protective in mouse models of pneumococcal and meningococcal infection (Ali *et al*, 2014). These microorganisms showed recruitment of factor H in order to eliminate the killing effect by the complement system (Dave *et al*, 2004; McNeil *et al*, 2013). It is therefore worth researching whether highly active recombinant properdin could interfere with the recruitment of factor H by the asexual blood stages of *P. falciparum*.

In theory, the protective properties of P_n could be investigated in mouse models of Plasmodium infection, however, the biological activity of properdin is species-specific. Producing highly active recombinant mouse properdin is difficult as recombinant mouse properdin did not show any kind of augmentation to C3b deposition via the alternative pathway (Ali *et al*, 2014). Expressing active chimeric properdin by changing one or both termini to the corresponding human part could be useful in terms of producing an active form of properdin, since it is already known that recombinant human properdin forms higher oligomers, and it is the termini that are responsible for polymerization, while the centre section is responsible for the species-specific biological activity. An alternative

to producing chimeric properdin might be to investigate the properties of human P_n in a humanised mouse. (Soulard *et al*, 2015) succeeded in observing all human malarial stages using humanised mouse. In the future, this gives an opportunity to see the role of active recombinant chimeric properdin on *Plasmodium falciparum* blood stages *in vivo*.

CFHR1 is a soluble competitive inhibitor of factor H that lacks factor I cofactor activity and the C3bBb decay accelerating activity associated with factor H. It does inhibit MAC assembly and hence terminal pathway activation, but this activity resides in the N-terminus of the protein (SCR1 and 2). The N-terminus of CFHR1 is not involved in C3bBb binding and not required for competitive inhibition of factor H. Therefore, CFHR1, or a polypeptide omitting the N-terminus of CFHR1 has the potential to augment alternative pathway activation.

The hypothesis, on which my PhD project is based, is that human and chimeric properdin could enhance the complement alternative pathway activation during *P. falciparum* infections by antagonising factor H activity. In addition, augmentation of complement activation by human recombinant CFHR1 and SCR3-5 would show in principle that by blocking the activity of factor H could render *P. falciparum* more susceptible to complement in blood.

1.4 Aims and Objectives:

The project aims to use a highly polymerised and active, recombinant human properdin, humanised mouse properdin and human recombinant CFHR1 to enhance the complement activity on asexual stages of *P. falciparum* and reduce recruiting factor H by the parasite. Chimeric mouse properdin, humanised at its termini is expected to polymerise better than recombinant murine properdin and can be used in assays to determine the relative importance of the individual pathways. This will be achieved with the following objectives:

1- Molecular biology to produce, amplify and characterise DNA of chimeric properdin and CFHR1 (PCR, Gel Electrophoresis, DNA purification, cloning, transformation and sequencing). Chimeric properdin DNAs were produced by replacing the N and/or C termini of murine properdin cDNA with that of human properdin cDNA, using

specific primers and molecular methods (human and murine properdin cDNA were kindly provided by Dr Nicholas Lynch).

2- Expression of chimeric recombinant properdin in Chinese Hamster Ovary cells (CHO-K1) and CFHR1 in both mammalian and bacterial expression systems.
3- Expression of recombinant human and murine properdin that had been previously transfected into CHO-K1 cells (kindly provided by Professor Wilhelm Schwaeble)
4- Recombinant protein purification (Nickel affinity columns and dialysis techniques)
5- Recombinant protein characterisation (SDS-PAGE and Western blotting)
6- Assaying recombinant protein activity by using complement assays (ELISA-based and haemolytic assays).
7- Testing the role of complement activation induced by the purified recombinant proteins on formalin-fixed and living *P. falciparum* using an ex vivo model of RBC infection and immunofluorescence microscopy.

2 Chapter two

Materials and Methods

2.1 Materials

2.1.1 Chemical materials

Chemical materials	Supplier
1kb plus DNA ladder	Invitrogen
2-mercaptoethanol	Sigma-Aldrich
3-(N-morpholino) propanesulfonic acid (MOPS)	Sigma-Aldrich
4-(2-aminoethyl)-benzene-sulfonyl fluoride (Pefabloc)	Sigma-Aldrich
36.5% formaldehyde solution	Sigma-Aldrich
5-bromo-4-chloro-3-indolyl-beta (Xgal)	Sigma-Aldrich
6X DNA loading dye	Thermo scientific
Acetic acid glacial	Fisher scientific
Acrylamide bis	Sigma-Aldrich
Agarose, electrophoresis grade	Melford
Albumax II	Gibco
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
Dithiothreitol (DDT)	Sigma-Aldrich
Ethanol	University of Leicester, Chemistry Department
Ethidium bromide	Sigma-Aldrich

Ethylene glycol-bis-N,N,Ń,Ń-tetraacetic acid (EGTA)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal bovine serum	Invitrogen
Fuji X-ray film	Fisher Scientific
Gelatin	Serva
Gentamicin	Gibco
Glycerol	Fisher scientific
Glycinee	Fisher scientific
Heparin sodium salt	Sigma-Aldrich
HEPES	Sigma-Aldrich
High fidelity polymerase	New England Biolabs
HisGravi Trap column	GE Healthcare
Hygromycin B	Invitrogen
Hypoxanthine	Sigma-Aldrich
IGEPAL (NP40)	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Imidazole	Sigma-Aldrich
Lipofectamine™ LTX reagent	Invitrogen
Luminata crescendo Western HRP substrate	Millipore
Magnesium sulfate	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 anhydrous	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 bicarbonate	Sigma-Aldrich
Sodium carbonate anhydrous	Fisher scientific
Sodium chloride	Fisher scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium hydrogen carbonate	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
Tryptone	BDH laboratory
Tween-20	Sigma-Aldrich
Urea	VWR international LTD
Zymosan	Sigma-Aldrich

2.1.2 Complement proteins

Complement proteins	Supplier
Factor P depleted serum	Complement technology
Human C3b	Complement technology
Human FH	Complement technology
Human MASP-3	Provided by Dr Sadam Yaseen, Post-doctoral research associate

2.1.3 Antibodies

Antibody	Supplier
FITC Polyclonal rabbit anti human C3c antibody	Dako
Goat anti mouse Alexafluor 546 red colour	Fisher Scientific
Goat anti rabbit Alexafluor 488 green colour	Thermo Fisher Scientific
Goat anti-rabbit IgG alkaline phosphatase conjugated antibody	Sigma-Aldrich
Mouse anti-polyhistidine antibody	Sigma-Aldrich
Monoclonal mouse anti-polyhistidine	Sigma-Aldrich
Monoclonal mouse anti-polyhistidine conjugated with HRP (Horse Radish Peroxidase)	Sigma-Aldrich
Polyclonal mouse anti human C5b-9	Santa Cruz Biotechnology
Polyclonal mouse MSP1 anti asexual blood stages of plasmodium antibody	Santa Cruz Biotechnology
Rabbit anti-human C3c	Dako
Rabbit polyclonal anti-mouse alkaline-phosphatase conjugated	Sigma-Aldrich

2.1.4 Kits

Kits	Supplier
*BamH*I restriction enzyme	New England Biolabs
*Hind*III restriction enzyme	New England Biolabs
pGEM-T easy vector	Promega
Pierce™ Coomassie Plus (Bradford) Assay Kit	Thermo Fisher Scientific
pRSET B	Thermo Fisher Scientific
pSecTag 2/ Hygro A	Thermo Fisher Scientific
QIAquick gel extraction kit	Qiagen
Wizard plus SV minipreps DNA purification kit	Promega
*Xho*I restriction enzyme	New England Biolabs

2.1.5 Blood and sera

	Supplier
Factor P-depleted serum	CompTech
Pooled NHS	SERALAB
Rabbit blood	ENVIGO

2.1.6 Media

	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 Polymerase Chain Reaction (PCR)

Human and mouse liver cDNAs were kindly provided by Dr Nicholas Lynch / University of Leicester, and used as a template to amplify human and mouse properdin with Phusion high fidelity DNA polymerase (Thermo Scientific) and specific primers designed based on a nucleotide sequence of Genbank Accession Number: NM_002621 for human properdin, NM_008823 for mouse properdin and NM_002113 for the full length and SCR3-5 coding sequences of CFHR1.

2.2.1.2 PCR amplification of mouse and human properdin

Human and mouse properdin consist of seven TSR domains, numbered TSR0-TSR6. In the first PCR (Table 2-1), the coding sequences of human properdin TSR0 and the mouse properdin TSR1-TSR6 were PCR amplified using sets of primers (Table 2-2) and specific PCR steps (Table 2-4). The products of the first PCR were ligated in the second PCR step to generate the N-terminus humanised mouse properdin construct.

Human properdin TSR6 coding sequence and mouse properdin TSR0-TSR5 coding sequence were PCR amplified, human TSR6 product was ligated to mouse TSR0-TSR5 to generate the C-terminus humanised mouse properdin construct.

To generate both termini humanised mouse properdin, mouse coding sequence of TSR1-TSR5 was amplified and the product was then ligated to human TSR0 and TSR6 coding sequences. The PCR products were run in 1% agarose gel, prepared as explained in Table 2-5, for 45 minutes at 120 V.

2.2.1.3 PCR amplification of CFHR1

Specific primers (Table 2-3) were used to amplify the two forms of CFHR1 coding sequences using a PCR mixture and program explained in tables 2-1 and 2-4 respectively. A *Bam*HI restriction site was added to the forward primers, while an *Xho*I restriction site was added to CFHR1_C_Xho. The PCR products were separated on 1% agarose gels.

Table 2-1: Preparation of the PCR mixture

Material	Amount	Final concentration
dH_2O	14.75 µl*	
5X Phusion buffer	5.0 µl	1X
10 mM dNTP Mix	0.5 µl	200 µM**
Forward primer	1.5 µl	0.2 µM
Reverse primer	1.5 µl	0.2 µM
Template DNA	1.5 µl	100 ng***
Phusion DNA polymerase	0.25 µl	1.2 Unit
Total	25.0 µl	

* µl= microliter, ** µM= micromolar, *** ng= nanogram

Table 2-2: The set of primers used in PCR amplification for properdin constructs

Primer name	Sequence 5' --> 3'
jP_TSP6_F	GAA AGG ATC ATG GTC AGA GTG GAG TAC
jP_TSP6_R	GTA CTC CAC TCT GAC CAT GAT CCT TTC
jP_TSP1_F	TCC CAG CTG CGA CAC CGG CGC TGT GTG
jP_TSP1_R	CAC ACA GCG CCG GTG TCG CAG CTG GGA
hP_Hind_F	CT AAG CTT GAC CCC GTG CTC TGC TTC ACC CAG TA
mP_Hind_F	CT AAG CTT GAC CCT GTG CTC TGC TTC ACC CAG TA
mP_F	AAG CTT ATG CCT GCT GAA ATG CAA GCC C
mP_R	CTC GAG AGT AGG GTT TCT TCT CTT CTG GGT CTT T
hP_F	AAG CTT ATG ATC ACA GAG GGA GCG CAG
hP_R	CTC GAG AGT AGA GTT CCT CTT CCT CAG GGT CTT TGC A

The underlined sequences indicate the restriction sites for enzymes which are *Hind*III (underline) and *Xho*I (double underline).

Table 2-3: The set of primers used in PCR amplification for CFHR1 constructs

CFHR1_N_*Bam*HI	GGATCCAACATTTTGTGATTTTCCAAA (Full length-Forward)
CFHR1_SCR3_*Bam*HI	GGATCCCTGTGTGAATCCGCCCACA (C terminus-Forward)
CFHR1_C_*Xho*I	CTCGAGATCTTTTTGCACAAGTTGGATAC (Reverse)

The underlined indicate the restriction enzymes which are *Bam*HI (underline) and *Xho*I (double underline).

Table 2-4: PCR cycling program

15 cycles

Stage	**Temperature (**˚C**)**	**Time (**second**)**
Initial denaturation	98	90
Denaturation	98	15
Annealing	75	30
Elongation	72	40

25 cycles

Stage	**Temperature (**˚C**)**	**Time**
Denaturation	98	15 sec
Annealing	65	30 sec
elongation	72	40 sec
Final elongation	72	5 minute
Cooling down	4	∞

Table 2-5: Preparation of agarose gel electrophoresis

Material	**Composition**
Agarose gel	1% of agarose in TAE buffer then ethidium bromide was added at final concentration of 0.5 µg/ml
TAE buffer consists of	1.0 mM acetic acid glacial, 2.0 M Tris-HCl, 50mM EDTA-Na_2

2.2.1.4 Purification of DNA from agarose gel

The QIAquick gel extraction kit (Promega, Cat. No. 28706) was used to purify DNA bands from agarose gels. The desired bands in agarose gels were excised by using a clean and sharp scalpel and gel containing DNA band was dissolved in 3 volumes of QG buffer and incubated at 50˚C for 10 minutes with shaking. 100-200µl of Isopropanol were added to the dissolved gel and mixed gently, and the mixture was transferred to the QIAquick spin column and centrifuged for 1 minute. 500µl of QG buffer were added to the column and spun down for 1 minute and after discarding the supernatant, 750µl of washing buffer (PE) were added to the column and centrifuged for 1 minute. The flow-through was discarded and another centrifugation step was done for 1 minute and the spin column was transferred to a 1.5 ml Eppendorf tube. To elute the DNA, 50µl of nuclease free water were added to the column and stored at -20˚C.

2.2.1.5 Molecular cloning

2.2.1.5.1 A tailing of PCR products

A 3´ terminal A overhang was added to the purified PCR products by using Taq polymerase and dATP. The reaction was run at 70˚C for 25 minutes. The following protocol was used:

Table 2-6: A tailing protocol

Material	Amount
Gel extracted DNA	3 µg
100 mM dATP	0.1 µM
10X taq PCR buffer	1X
Taq polymerase	1.2 Unit

2.2.1.5.2 Cloning of PCR products into pGEM-T easy vector:

The A tailed PCR products were ligated into pGEM-T easy vector (Promega Cat. No. A1360) using T4 DNA ligase (Table 2-7). The mixture was incubated for 2 hours at room temperature or overnight at 4˚C.

Table 2-7: Protocol used for cloning of PCR product into pGEM-T easy vector

Material	Final concentration
2X rapid ligation buffer	1X
pGEM-T easy vector	50 ng
A-tailed PCR product	10 ng
T4 DNA ligase	3 Units
Deionized water to a final volume of	10 µl

2.2.1.5.3 Preparation of chemically competent cells

The frozen stock of Top 10 *Escherichia coli* was thawed on an ice bath and streaked onto Luria-Bertani (LB) agar plates, which were prepared as shown in table 2-7. The plates were incubated for 16 hours at 37˚C. After that, a single colony of growing *E. coli* was harvested and cultured in 5 ml LB broth medium (Table 2-8) containing 20mM $MgSO_4$ and incubated at 37˚C for overnight. After that, 100 ml of LB broth were inoculated with 1 ml of the overnight culture and incubated at 37˚C until OD550nm reached 0.7-0.8. The culture was spun down for 10 minutes at 3000 rpm (revolutions per minute) and 30 ml of Tfb I (Table 2-9) were added to the pellets and incubated in an ice bath for 30 minutes. After 10 minutes of centrifugation at 4˚C, the pellets were resuspended by adding 4 ml of Tfb II (Table 2-9) and aliquoted into small Eppendorf tubes for a single use and stored at -80˚C.

Table 2-8: Media used for bacterial culture

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

* w/v is weight/volume percentage

Table 2-9: Buffers used for competent cell preparation

Buffer	Composition
Transformation buffer (Tfb) I, pH 7.4	3 mM $C_2H_3KO_2$, 50mM $MnCl_2$, 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)

2.2.1.5.4 Transforming chemically competent cells

The heat shock technique was used to transfer the plasmid DNA into chemically competent TOP10F' cells. 40 µl of 0.1 M IPTG and 40 µl of 20 mg/ml Xgal, which were prepared as explained in Table 2-10, were placed onto the entire surface of LB agar plates containing ampicillin at a final concentration of 100 µg/ml. The heat shock method was performed by thawing the frozen TOP10 *E. coli* on ice for 5 minutes, then 2 µl of the ligated chimeric properdin with pGEM-T easy vector were mixed with 50 µl of TOP10 cells in a sterile Eppendorf tube and incubated on ice for 20 minutes. The ice-incubated mixture was heated in a water bath at 42˚C for 4 minutes. After that, cells were immediately transferred into ice for 2 minutes. The transformed cells were incubated with 450 µl of LB broth at 37˚C in a shaking incubator for 1.5 hours. Two volumes, 100 µl and 200 µl, of this culture were cultured on LB ampicillin agar plates at 37˚C overnight.

Table 2-10: Protocol used to prepare IPTG and Xgal stock solutions

Buffer	Composition
IPTG	0.1 M isopropyl thiogalactoside, (Isopropyl beta-D-thiogalactopyranoside) in distilled water
Xgal	20 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside dissolved in DMSO

2.2.1.5.5 Isolation and purification of plasmid DNA

From the overnight culture, a single colony was harvested and inoculated into 2ml of LB broth medium containing ampicillin at a final concentration of 100µg/ml and left overnight at 37˚C in a shaking incubator. The Wizard plus SV minipreps system (Promega) was used to purify plasmid DNA. The overnight culture was centrifuged at 4200rpm for 7 minutes. The supernatant was discarded and 250µl of cell re-suspension solution was added to the pellet and mixed by vortexing. The mixture was transferred into a sterile Eppendorf tube and 250µl of cell lysis solution were added and mixed gently by inversion. The tubes were incubated at room temperature for 2 minutes. After that, 10µl of alkaline protease solution were added to the tube and incubated for 4 minutes at room temperature. 350 µl of neutralization solution were subsequently added to the tube and centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred to a spin column and centrifuged at 13000 rpm for 1 minute. 750 µl of column wash solution were added and the tube was centrifuged for a minute. The washing step was repeated with 250 µl of the column wash solution. Finally, 50 µl of nuclease free water were added to elute the plasmid DNA.

2.2.1.5.6 Restriction digestion

The DNA construct was digested from pGEM-T easy by using *Hind*III, *Bam*HI and *Xho*I restriction enzymes as in the following protocol:

Table 2-11: Protocol used for DNA digestion from pGEM-T easy vector

Material	**Final concentration**
DNA sample	0.1 µg
*Hind*III or *Bam*HI restriction enzyme	5 Units
*Xho*I restriction enzyme	5 Units
10X Restriction buffer 2	1X
10X BSA	1X
Deionized water to a final volume of	10 µl

The mixture was incubated at 37˚C for 2 hours or at 4˚C overnight. The digested products were run on a 1% agarose gel to detect the desired DNA.

2.2.1.5.7 Sub-cloning of purified DNA into expression vector pSecTag2/ Hygro B or pSecTag2/ Hygro A (the mammalian vectors) or pRSET B (the bacterial vector)

The digested DNA constructs of properdin were purified from the agarose gel and ligated into pSecTag2/Hygro B (has 6 histidine at C-terminus). Purified CFHR1 constructs were ligated to pRSET B (has 6 histidine at N-terminus) or pSecTag2/ Hygro A (has 6 histidine at C-terminus) (see Appendices 7). After digesting the vector with the same restriction enzymes (*Hind*III, *Bam*HI and *Xho*I) as explained in the following protocol.

Table 2-12: Protocol used for sub-cloning of DNA product into pSecTag 2/ Hygro B or pRSET B vector

Material	Final concentration
pSecTag 2/ Hygro A or B or pRSET B	50 ng
10X ligase Buffer	1X
Purified DNA	10 ng
T4 DNA ligase	3 Units
Deionized water to a final volume of	10 µl

The ligated product was transformed into competent cells (TOP10 F' *E. coli*). The plasmid DNA was purified, and successful cloning was confirmed by restriction digestion and the construct in the vector was sequenced (Protein & Nucleic Acid Chemistry Laboratory in Hodgkin Building at University of Leicester) to make sure that no mutation had taken place during cloning steps.

2.2.2 Protein methods

2.2.2.1 Protein expression in mammalian Chinese Hamster Ovary (CHO)-K1 cells

2.2.2.1.1 Cultivation of CHO-K1 cells

CHO-K1 cells were used to express recombinant chimeric properdin as they allow for properdin glycosylation which can increase protein folding (Higgins *et al*, 1995). Ham's F-12 Nutrient Mix, GlutaMax (Gibco) culture medium was used to cultivate CHO-K1 cells, supplemented with 10% fetal bovine serum (Gibco) in the presence of 5 ml of 100 U penicillin/0.1 mg/ml streptomycin (Sigma-Aldrich) (Table 2-13).

A frozen stock of CHO-K1 cells was recovered from liquid nitrogen and thawed at 37˚C in a water bath and the cells were diluted with growth medium and centrifuged at 1200 rpm for 10 minutes to get rid of freezing medium. The supernatant was discarded, and the pellet was resuspended in growth medium and added to a small flask and incubated at 37˚C in 5% CO_2.

Table 2-13: Preparation of serum CHO-K1 cell line medium

Medium	Composition
Serum CHO-K1 medium	500 ml Ham's F-12 Nutrient Mix, GlutaMax 10% FBS (v/v), 5 ml 100U penicillin / 0.1 mg/ml streptomycin

2.2.2.1.2 Transfecting of CHO-K1 cells with properdin constructs

Three different properdin constructs ligated with pSecTag2/Hygro B vector were transfected into CHO-K1 cells:

1- Mouse properdin humanised at N-terminus DNA construct (HMMP)

2- Mouse properdin humanised at C-terminus DNA construct (MMHP)

3- Mouse properdin humanised at both termini DNA construct (HMHP)

When CHO-K1 cells were nearly 80% confluent, they were sub-cultured in a 6-well plate and left in an incubator at 37˚C and 5% CO_2 until 50-80% confluent.

Lipofectamine™ LTX reagent (Invitrogen) was used to transfect CHO-K1 cells with the plasmid constructs. 1µg of DNA was diluted with 100 µL of Opti-MEM I reduced serum medium without serum (Invitrogen) followed by addition of 3µL of Lipofectamine™ LTX. The mixture was incubated at room temperature for 25 minutes to form DNA-Lipofectamine™ LTX mixture. The old growth medium of 6-well plate cells was replaced with 1.5ml of new medium and 100µL of DNA-Lipofectamine™ LTX mixture was added to each well with gentle mixing. The plate was incubated at 37˚C in an incubator with 5% CO_2. After 24-48 hours, the medium was replaced with new growth medium containing 300µg/ml hygromycin B (Invitrogen). The cultured cells were washed thrice with phosphate buffered saline (PBS) (Table 2-14) and harvested by adding 100 µl of trypsin-EDTA (Sigma-Aldrich) to each well of the 6-well plate and the plate was incubated at 37˚C for 1 minute. 1ml of growth medium containing 300 µg/ml hygromycin B was added to the cells and cells were re-suspended and transferred to 24-well plates and incubated at 37˚C under 5% CO_2.

At 80-90% cellular confluence, cells were washed thrice with PBS and serum free medium (CHO-S-SFM II) (Gibco) containing 100 Units penicillin, 0.1 mg/ml of streptomycin and 300µg/ml hygromycin B (Table 2-15). This was added to the 24-well plate and the plate was incubated at 37˚C. Two days later, the supernatant was collected in Eppendorf tubes and used to detect recombinant protein expression.

Table 2-14: Preparation of phosphate buffered saline

Buffer	**Composition**
PBS, pH 7.4	1.8 mM KH_2PO_4, 10.1 mM NaH_2PO_4, 136.9 mM NaCl, 2.7 mM KCl

Table 2-15: Preparation of serum free CHO-K1 cell line medium

Medium	**Composition**
Serum free CHO-K1 medium	500 ml CHO-S-SFM II, 5 ml 100 U penicillin / 0.1 mg/ml streptomycin, 1.5 ml of 100 mg/ml hygromycin B

2.2.2.1.3 Protein defining methods in mammalian cells

2.2.2.1.3.1 Dot blot

A dot blot is a simple technique used to detect recombinant protein expression. The supernatant from 24 wells plate was collected and centrifuged for 5 minutes at 13000rpm to remove cell debris. 70µl of the supernatant were spotted onto nitrocellulose membrane (Bio-Rad) and allowed to dry for 5 minutes at room temperature. 5% skimmed milk (Sigma-Aldrich) in PBS was added to block the membrane for 1 hour at room temperature with shaking. Monoclonal mouse anti-polyhistidine HRP (Horseradish Peroxidase) conjugated antibodies (Sigma-Aldrich) were prepared at a ratio of 1:10000 in blocking buffer and added to the membrane which was incubated at room temperature for 1 hour with shaking (see section 3.2.2 and 4.2.1). After that, the membrane was washed thrice with PBS containing 0.05% Tween-20 and the substrate, luminata crescendo Western HRP substrate (Millipore), was transferred onto the entire membrane surface and incubated for few minutes before exposing to Fuji X-ray film (Fisher Scientific) for 1-2 minutes.

2.2.2.1.4 Freezing down CHO-K1 properdin cell line

Positive clones in dot blot were frozen in liquid phase nitrogen. Firstly, cryoprotectant was prepared by adding 10 ml of Dimethyl Sulfoxide (DMSO) to 90 ml of fetal bovine serum and stored at 4C˚ and used within 7 days. A medium cell culture flask with a high cell confluence (>85%) was harvested by using Trypsin-EDTA. Cells were resuspended in 5-6 ml of growth media and then transferred to a centrifuge tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 4ml of cryoprotectant and aliquoted into cryotubes.

2.2.2.1.5 Protein purification

2.2.2.1.5.1 Purification of recombinant properdin

Positive clones were cultured in a medium flask and once the cells were confluent, they were transferred into large triple-layer (400cm^2) flasks and incubated at 37˚C in a CO_2 incubator. At 80-90% of cell confluence, the cells were washed with PBS three times and serum-free medium (Table 2-15) was added to the flasks and incubated at

37˚C for two days. After that, the medium was collected and spun down at 4000 rpm for 10 minutes to remove the cell debris. Binding buffer (Table 2-16) was added to the supernatant at a ratio of 1:1 and the pH was adjusted to 7.4. The solution was passed through the HisGravi Trap column (GE Healthcare) overnight at 4˚C. The column was washed with 10 ml binding buffer (Table 2-16) and the recombinant protein was eluted with 10 ml elution buffer (Table 2-17). The eluted samples were run in SDS-PAGE and confirmed by Western blotting.

To eliminate imidazole from the elution buffer, protein dialysis was performed by using Snake skin membrane 10kDa (Thermo Scientific). The membrane was washed with distilled water, and TBS (Tris Buffered Saline) (Table 2-18). Protein fractions were loaded to the dialysis bag and immersed into 1X TBS buffer and incubated overnight at 4˚C with stirring. Finally, the dialyzed properdin was concentrated by using a concentrator filter tube 10 kDa (Millipore).

Table 2-16: Buffer used for Ni^{2+} affinity chromatography

Buffer	**Composition**
Binding buffer, pH 7.4	500 mM NaCl, 20 mM NaH_2PO_4, 20 mM imidazole

Table 2-17: Buffer used for eluting the properdin protein

Buffer	**Composition**
Elution buffer 4, pH 7.4	500 mM NaCL, 20 mM NaH_2PO_4 and 500 mM Imidazole

2.2.2.2 Protein Expression in a bacterial system

The CFHR1/pRSETB constructs were transformed into *E. coli* BL21(DE3) pLysS and plated on LB agar containing 50 µg/ml ampicillin and 35µg/ml chloramphenicol. Single colonies were picked and incubated overnight at 37^0C in 10ml LB containing the same antibiotics. The next day 100-500 ml of LB were inoculated with the overnight culture and grown until the OD_{550} reached ~0.6. Protein expression was induced by adding 1 mM IPTG. After 5hours, bacterial cells were harvested by

centrifugation at 8000rpm and lysed mechanically using sonication in PBS containing 1 mM Pefabloc and 1 mM DDT. After that, lysates were centrifuged, and the supernatants and pellets stored at -20°C.

2.2.2.2.1 Refolding protocol:

This protocol was modified from Thomson *et al* (2012). Bacterial pellets were re-suspended in 25ml of PBS containing 0.5% Tween20 and incubated with rotation at 4^0C for 2 hours then centrifuged at 21000 rpm, for 30 minutes at 4^0C. The inclusion body pellets were solubilized in 50 mM Tris-HCl, pH 8, 8 M urea, 10 mM DTT on a rotating wheel at 4^0C for 2 hours, after which the solution was centrifuged at 21000 rpm at 4^0C for 20 minutes. The supernatant was then dialysed overnight against 2L of buffer 1 (50 mM TrisCl, pH 8, 6 M urea) then dialyzed again overnight at 4^0C against 2 litres of dialysis buffer 2 (50 mM Tris, pH 8, 2 M urea, 0.4 M Arginine, 3 mM Reduced Glutathione, 0.9 mM Oxidized Glutathione). The following day, the dialysis buffer was diluted 50% with water and dialysis continued overnight at 4^0C. Any insoluble material was centrifuged out (18000 rpm at 4°C for 20 minutes) and finally the sample was dialyzed against 1 litre of dialysis buffer 3 (50 mM Tris, pH 8, 250 mM NaCl, 0.1 M Arginine, 3 mM Reduced Glutathione, 0.9 mM Oxidized Glutathione) overnight at 4^0C to remove the remaining urea. The final dialyzed protein solution was clarified by centrifugation at 21000 rpm at 4^0C for 20 minutes and the supernatant was used in protein purification.

2.2.2.2.2 CFHR1 Purification

The supernatant was loaded onto and purified using a His GraviTrap column (GE Healthcare) equilibrated in equilibration buffer (50 mM Tris, pH 8, 500 mM NaCl). The column was washed with an excess of wash buffer (50 mM Tris, pH 8, 1 M NaCl, 0.1% Triton X-100) followed by a wash with equilibration buffer to remove the Triton X-100. The CFHR1 was eluted in 10 ml elution buffer (350 mM imidazole plus 50 mM Tris, pH 8). Eluted protein was pooled and dialyzed against 2 litres of TBS, pH 7.4, overnight at 4^0C. Protein that precipitated out during the overnight low salt dialysis was removed by centrifugation before proceeding. The purified CFHR1 was checked on Coomassie stained SDS-PAGE gels, western blotted and sequenced at the Biochemistry Department at the University of Leicester (PNACL). The concentration of the recombinant protein was determined by Bradford assay.

2.2.2.2.3 Bradford Assay:

This is done using the Pierce™ Coomassie (Bradford) Protein Assay Kit from Thermo Fisher Scientific ™. Briefly, 5µl of each sample and the standard (different known concentrations of Bovine Serum Albumin) were added an ELISA microplate. Then 250 µl of Coomassie Protein Assay Reagent were added to each well. The results were read after 10 minutes using Bio-Rad microtiter plate reader at 595 nm wavelength.

2.2.2.3 Size-exclusion chromatography (SEC) analysis:

After protein purification, dialysis and protein concentration, the purified proteins were passed through a Superose 6 increase 10/300 GL gel filtration column by Fast Protein Liquid Chromatography (FPLC; GE Healthcare). The principle of gel filtration column depends on the porous beads that are able to separate molecules depending upon their sizes as large molecules pass faster than smaller molecules via the beads through the column (Laurent and Laurent, 1964). 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 FPLC is computerised to measure eluted proteins via sensor. Measured protein is expressed for each fraction using Milli-absorbance Unit (mAU) that is plotted against, time, elution volume and fraction number to illustrate protein concentration. The Superose 6 increase 10/300 GL column was equilibrated with filtered 50 mM Tris-HCl and 145 mM NaCl buffer. Then it was calibrated using the Gel Filtration Markers Kit for Protein Molecular Weights 29,000-700,000 Da from (Sigma Aldrich), in which different proteins are mixed together, then 500 µl of this mixture was loaded into the column using 0.5 ml syringe (HAMILTON Microliter Syringe). The elution curves for the markers were plotted and the data was saved as an Excel sheet then plotted to GraphPad Prism to show the curves of these proteins in order to use them as standard curves showing specific sizes.

Gel filtration is also used for recombinant protein size analysis. After protein dialysis, 0.5 ml of the total volume of the purified recombinant proteins was loaded into the column. The sample was eluted with TBS, with continuous monitoring of the OD280nm, and 1 ml fractions were collected. Fractions containing protein were analysed by SDS-PAGE and Western blotting.

2.2.2.4 Protein defining methods in both mammalian and bacterial expression system

2.2.2.4.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To study the features of recombinant properdin and CFHR1, SDS-PAGE was used under reducing conditions. The reducing conditions were performed by adding 20 µl of 0.1 mg/ml recombinant proteins to 5 µl of SDS protein-loading buffer containing 5% of 2-mercaptoethanol (Table 2-18) and boiling at 95˚C for 10 minutes.

10% SDS polyacrylamide gels were prepared as described by Brunelle and Green (2014) (Table 2-19), and the samples were loaded into the gel together with a molecular weight marker, PageRuler, (Thermo Scientific). Samples were electrophoresed using 1X SDS running buffer (Table 2-18) at 150 V for 1 hour. The gel was fixed by fixing buffer (Table 2-18) and stained with Coomassie Blue for 30 minutes. Then the gel was de-stained by de-staining solution (Table 2-18) until the protein bands became clear.

Table 2-18: 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, 1mg 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-Buffer Saline (TBS), pH 7.4	10 mM Tris-HCl, 140 mM NaCl
Tris/glycine/SDS running buffer, pH 8.3	25 mM Trizma base, 192 mM glycinee, 0.1% SDS (w/v)

Table 2-19: Protocol used to prepare SDS-PAGE gel

Resolving Gel	**Composition**	**Amount (ml)**
	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

Stacking Gel	**Component**	**Amount (ml)**
	30% acrylamide mix	0.623 ml
	Tris–HCl, pH 6.8 1 M	0.63 ml
	10% SDS	0.05 ml
	10% APS	0.05
	TEMED	0.01
	Water	3.697 ml

2.2.2.4.2 Western blotting

Western blotting was performed in order to identify the recombinant proteins and size according to (Mahmood and Yang, 2012). The recombinant proteins, run on the SDS-PAGE gel, were transferred onto nitrocellulose membrane in transfer buffer (Table 2-20) at 300 mA for 1.5 hour. Blocking buffer (5% skimmed milk in 1X TBS) was used to block the membrane for 60 minutes with shaking. After that, monoclonal

mouse anti polyhistidine HRP conjugated antibodies (Sigma-Aldrich), diluted 1:5000 in blocking buffer (5% skimmed milk in TBS), were added to the membrane and incubated at room temperature with shaking for another one hour. The membrane was washed in washing buffer (PBS containing 0.05% Tween-20) three times. A further washing step for the membrane was completed before adding Luminata Crescendo Western HRP substrate. The membrane was visualised using ChemiDoc™ Touch Imaging System.

Table 2-20: Protocol used to prepare Western blotting transfer buffers

Buffer	Composition
Tris/glycine transfer buffer, pH 8.3	25 mM Trizma base, 192 mM glycine, 20% methanol (v/v)

2.2.2.5 Protein functional assays

2.2.2.5.1 Complement assays

2.2.2.5.1.1 Preparation of human and mouse sera for functional complement assay

Pooled human serum was supplied by SERALAB. In addition, some sera were obtained from healthy individuals. Here, human blood was obtained, after signing informed consent form, in sterile tubes and left at room temperature for 15-30 minutes for clotting. Each tube was centrifuged at 3000rpm for 10 minutes at 4˚C. Serum was collected, aliquoted and stored at -80˚C. The mouse serum was prepared as described previously for human serum, but the clotting was done on ice for 3 hours.

2.2.2.5.1.2 Enzyme Linked Immuno-Sorbent Assay (ELISA)

2.2.2.5.1.2.1 The alternative pathway activation assay

Normal human serum (NHS) and wild type mouse serum were used to test the activity of alternative activation pathway of complement following the addition of recombinant chimeric, human and mouse properdin on zymosan. Microtiter ELISA plates (Nunc MaxiSorp 96 well plate) were coated with 100µl coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3 pH 9.6) containing 10µg/ml of zymosan and incubated at 4˚C overnight. The protein binding sites were blocked with 250µl of 1% BSA in 1X TBS for 2 hours at room temperature. The plate was washed thrice with 1X TBS containing 0.05% Tween-20. Different concentrations of recombinant properdin and CFHR1 were added to serially diluted normal human serum, mouse serum or factor P depleted serum (Complement Technology) in alternative pathway buffer; GVB/Mg^{2+}/EGTA (GVB: Gelatin veronal buffer) (see section 2.2.2.5.1.2.2) which allows alternative pathway activation and prevents the activation of the classical and lectin pathways. 100µl of diluted sera with properdin or CFHR1 were added to plate wells and incubated at 37˚C for 1 hour. The plate was washed and 100µl of polyclonal rabbit anti-human C3c antibody (Dako) diluted 1:5000 in washing buffer were added and incubated at 37˚C for 1 hour (C3c is a fragment of C3b). After that, the plate was washed and 100µl of alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) diluted 1:5000 in washing buffer were added and incubated at room temperature for 1 hour. Finally, the plate was washed and filled with 100µl/well of colorimetric substrate solution (Sigma Fast p-Nitrophenyl phosphate tablet). After 5 minutes, the absorbance of reaction product was measured by BioRad microtiter plate reader at 415 nm wavelength.

2.2.2.5.1.2.2 Preparing alternative and lectin pathway buffers

To prepare complement pathways buffer, BBS buffer (Barbital Buffer Saline) was prepared (BBS) by mixing 145mM of NaCl+4mM of Barbituric acid+ 1mM Na Barbiturate, pH 7.3-7.4, with 500ml with DW. After that the mixture was filtered. Alternative pathway buffer was prepared by mixing 1X BBS+5mM of $MgCl_2$+8mM EGTA+ 0.1% Gelatin. Lectin pathway buffer was prepared by adding 1X BBS to 1mM

of $MgCl_2$ and 2mM $CaCl_2$+ 0.1% Gelatin. Both buffers were filtered and stored at 4 °C.

2.2.2.5.1.2.3 Binding assay to heparin, schizont and C3b

Heparin (25 µg/mL) in PBS or isolated schizonts (1 mg/ml) (see section 2.2.2.5.2.4 and 2.2.2.5.2.5) or C3b (1 mg/ml) were used to coat microtiter ELISA 96-well plates in single concentration of 10µg/ml. The stock concentration of isolated schizonts was checked using Bradford assay. After overnight incubation, the plates were blocked with 250 µl of 1% (v/v) Bovine Serum Albumin (BSA) in Tris-buffered saline (TBS) for 2 hours at room temperature. Uncoated wells were just blocked with BSA were used for negative controls. Then the plates were washed three times with 250µl of washing buffer (TBS and Tween 20 0.05%). Next, serial dilutions of recombinant human Properdin and mouse properdin humanised at N terminus in TBS were added to schizonts and C3b coated plate while the dilutions of full length CFHR1 and SCR3-5 were tested on heparin, schizonts and C3b coated plates. All of which were applied into the coated materials in the microtitration plate in duplicate. After 1hour incubation at 37°C, wells were washed three times using washing buffer. Then, 100µl of diluted mouse polyclonal anti-polyhistidine (Sigma-Aldrich) 1:10000 in washing buffer were added as primary antibodies. Next, plates were incubated for 1hour at 37°C followed by the three times of washing step. After that, and in order to detect recombinant protein binding, 100 µl of alkaline phosphatase conjugated rabbit polyclonal anti-mouse IgG (Sigma-Aldrich; diluted 1:5000) in washing buffer were added as secondary antibodies, respectively. Next, plates were incubated for 1hour at room temperature followed by a last washing step three times. Following washing, the tablets of alkaline phosphatase substrate (Fast p-Nitrophenyl Phosphate tablet sets, Sigma-Aldrich) were prepared in 20ml distilled water. Then, 100 µl of the prepared substrate were added into each well to determine the presence of the Alkaline-phosphatase conjugate by which developing the chromogenic substrate to a yellow colour as evidence for binding. Lastly, plates were incubated at room temperature away from direct light until the reaction between substrate and alkaline phosphatase reached the optimum desired colour which then was measured at wavelength 415 nm using Bio-Rad iMark microplate reader. All ELISA assays were repeated three times using fresh buffers for each time.

2.2.2.5.1.3 Haemolysis assays

2.2.2.5.1.3.1 Day 1. Preparation of Rabbit RBC

Rabbit blood was ordered from ENVIGO. 2ml of whole blood were spun down for 1-2 minutes at 3200 rpm in a refrigerated centrifuge at 4˚C then plasma and buffy coat were aspirated off. The RBC pellet was washed 3x by re-suspending in 2ml ice-cold lectin pathway or alternative pathway buffer and repeating the centrifugation step and after third wash, cells were re-suspended in 4ml of alternative and lectin pathway buffer. For lectin pathway experiment, a 2 ml aliquot of the RBC was set aside as uncoated controls. To the remaining 2 ml, 2 ml $CrCl_3$ (0.5 mg/ml in lectin pathway buffer) and 2 ml mannan (100 µg/ml in lectin pathway buffer) were added then incubated with gentle mixing at room temperature for 5 minutes. The reaction was stopped by adding 7.5 ml lectin pathway buffer. After that, cells were spun down as above and re-suspended in 2 ml lectin pathway buffer and washed a further two times as above and stored at 4˚C.

2.2.2.5.1.3.2 Day 2. Haemolysis assay

The concentration of RBC was determined to be used in assay: for each type of RBC, a series of 100 µl dilutions in lectin pathway or alternative pathway buffer was set up in a round-bottomed plate (5x, 10x, 15x, and 20x). Then 100 µl of Nano pure water were added to lyse cells and the plate was spun down for 5 minutes at max speed. After that, 100 µl of the fluid-phase were sampled from each well and OD at 540 nm was recorded. The dilution that gives an OD 540 for maximum lysis of approximately 0.7-0.9 was used for the future experiments. (An OD 540 of 0.9 corresponding to approximately 10^8 cells/ml) was chosen. Dilutions of test sera were prepared in ice-cold lectin pathway or alternative pathway buffer. Then 100 µl of each serum dilution were pipetted into a round-bottomed plate. After that, 100 µl of appropriately diluted RBCs preparation were added and incubated at room temperature for circa 1 hour, observing for lysis. Plate was spun down at 1200 rpm for 5 minutes then 100µl of the fluid-phase were sampled and OD at 540 nm was recorded.

2.2.2.5.2 Parasitic assays

2.2.2.5.2.1 Culture of *P. falciparum*

P. falciparum asexual blood stages of strain 3D7 were cultured in RPMI-HEPES culture medium, supplemented with 50 μg ml hypoxanthine (Sigma Aldrich), 10 μg/ml gentamicin (Gibco) and O^+ human RBC at 5% haematocrit. The maintenance medium contained 0.5% Albumax II (Gibco); for experiments the Albumax II was replaced with 10% v/v pooled heat-inactivated human O^+ serum (HIS, inactivated for 1 h at 55°C), or pooled O^+ NHS (as a source of complement) (Trager and Jensen, 1977), (Ifediba and Vanderberg, 1981). All cultures were maintained at 37°C in an atmosphere of 1% O_2, 5% CO_2 and 90% N_2.

2.2.2.5.2.2 Synchronizing the asexual blood stages

Parasite culture with 5% Parasitaemia (mainly ring stages) was centrifuged 1800 rpm for 3 minutes at room temperature; the supernatant was discarded, and the pellet was resuspended in 5% sorbitol in dH_2O five times and incubated for 20 minutes at room temperature (Lambros and Vanderberg, 1979). Sorbitol kills other plasmodium stages but not ring stage. Then the cultures were washed thrice with cell culture medium to remove the sorbitol. Finally, the pellets were resuspended in new culture media contained heat inactivated serum with 4% haematocrit and further cultivated.

2.2.2.5.2.3 Schizont infected RBCs (iRBCs) preparation

Schizont iRBCs (iRBC) were purified using Percoll gradient centrifugation as described (Radfar *et al*, 2009) where Percoll 36% was prepared and added slowly to iRBCs in 1:1 proportion. Without mixing, the mixture was spun down at room temperature, 1100 rpm for 10 minutes, acceleration 7 and deceleration 6. Three layers were separated, iRBCs were floated on the top, Percoll layer in the middle while other parasitic stages and non-infected RBCs were down in the bottom. iRBCs were collected and resuspended in new culture media then spun down at 2500 rpm at room temperature for 5 minutes then the supernatant was discarded and resuspended again in new culture media.

2.2.2.5.2.4 Isolating schizonts from iRBCs by Saponin treatment

After schizont iRBCs had been prepared, some of them were saponin-treated in order to separate the schizonts from the RBC as saponin can lyse the RBC membrane. 0.05% of saponin was added to iRBCs and incubated on ice for 1 minute then centrifuged and resuspended in media. After that, they were spun down at 3000 rpm at room temperature for 5 minutes to get rid of remaining saponin. The supernatant was discarded, and pellets were fixed with 4% paraformaldehyde and 0.1% of glutaraldehyde for 10 minutes on ice, then were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS).

2.2.2.5.2.5 Quantification the amount of isolated schizonts

Isolated schizonts were resuspended in 1 ml of PBS. 100 µl of the mixture were centrifuged and the supernatant was discarded. After that the lysis buffer was prepared by mixing 100mM NaCl, 1mM DDT, 1mM EDTA and 1% NP40 (IGEPAL). 900 µl of lysis buffer were added to the parasite pellets. The supernatant was again centrifuged and collected. The amount of protein was quantified using Bradford assay. The total amount of protein in 100 µl of parasite was calculated.

2.2.2.5.2.6 Growth inhibition assay:

2.2.2.5.2.6.1 Growth inhibition assay by adding rCFHR1 for 24 hours incubation

An assay that measures the invasion of erythrocytes by asexual blood stages of *P. falciparum* was used to determine the effect of recombinant CFHR1 on complement-mediated parasite killing. In this assay, fresh uninfected RBCs were mixed with starting culture of infected RBCs and cultured for 48 hours to allow new invasion of RBCs. The number of infected cells at the beginning of the experiment is adjusted to 1% by varying the volume of the infected starting culture, and the number at the end of the experiment was determined by microscopy.

Suspensions of infected and non-infected RBCs in RPMI 1640 were prepared separately; so that the haematocrit was 5% (i.e. the suspensions contained 4% v/v packed RBCs). The infected and uninfected suspensions were mixed in a ratio calculated to give 1% parasitaemia. Eighty microliter aliquots of the mixture were

added to the wells of a 96 well plate. NHS as a source of complement (or heated-inactivated NHS for negative controls) was added to a final concentration of 10%. Recombinant CFHR1 with 50was added and the final volume adjusted to 100µl per well. The wells with HIS and NHS were received DPBS in the same amount of rCFHR1. The suspensions were incubated 48 hours at 37°C in 5% CO_2 and 1% O_2. At the end of the experiment light microscopy was used to determine the number of infected RBC.

2.2.2.5.2.6.2 Growth assays for 3 generations of asexual blood stages

An assay that measures the invasion of erythrocytes by asexual blood stages of *P. falciparum* was used to determine the effect of recombinant rHP, rHMMP, rCFHR1 and rSCR3-5 on complement-mediated parasite killing. In this assay, fresh uninfected RBCs were mixed with a 0.3% starting culture of infected RBC and cultured for 48 hours or 96 hours to allow new invasion of RBCs. The number of infected cells at the beginning of the experiment was adjusted to 0.3% by varying the volume of the infected starting culture, and the number at the end of the experiment was determined by microscopy.

Suspensions of infected and non-infected RBCs in RPMI 1640 were prepared separately; so that the haemotacrit was 5%. The infected and uninfected suspensions were mixed in a ratio calculated to give 0.3% parasitaemia. Eighty microliters aliquots of the mixture were added to the wells of a 96 well plate. NHS as a source of complement (or heated-inactivated NHS for negative controls) was added to a final concentration of 10%. Recombinant proteins were added, and the final volume adjusted to 100µl per well. The wells with HIS and NHS were received DPBS in the same amount of recombinant proteins. The suspensions were incubated for 48 hours or 96 hours at 37°C in 5% CO_2 and 1% O_2. NHS or HIS plus/minus the recombinant proteins were replaced once per 12 hours. Three generations were exposed to these supplementations. Parasitaemia was calculated twice; after 48 hours and 96 hours. At the end of the experiment, the light microscopy was used to determine the number of infected RBC by smearing the samples on glass slides then staining them by Giemsa stain and finally counting the number of RBCs containing blue dots. The experiments were done in triplicate; data analysis was performed using Microsoft Excel 2013.

2.2.2.5.2.7 C3b deposition on asexual blood parasite

iRBCs or isolated schizonts of *P. falciparum* of strains 3D7 were incubated with 10% NHS supplemented with fixed concentration of recombinant proteins (rHP, rHMMP, rCFHR1 and rSCR3-5) diluted in RPMI-HEPES culture medium for 1 hour at 37°C. After that, the cells were washed 3 times with PBS then air-dried on Teflon slides and fixed in 4% paraformaldehyde/PBS in suspension for 10 minutes at room temperature. After that, these slides were blocked with 5% BSA diluted in PBS for 2 hours at room temperature. Primary antibodies (rabbit anti human C3c antibody and polyclonal rabbit or mouse anti-MSP1 antibody) were diluted with PBS and added to slides which were then incubated at 37°C for 2 hours. For negative controls, iRBCs or non-infected red blood cells (niRBCs) were incubated with NHS without adding primary antibodies (Abs) or with primary antibodies without adding NHS, then for visualization, the secondary antibodies were added to both controls. Binding of primary antibody was visualized using Alexa Fluor 488 conjugated goat anti-mouse or Alexa Fluor 546 goat anti rabbit secondary antibody depending on the primary antibody used. The asexual blood stages were routinely double-labelled with polyclonal rabbit anti-MSP1 antibody in combination with Alexa Fluor 594 conjugated goat anti-rabbit secondary antibody for 2 hours at 37°C. Images were acquired with a ZEISS microscope (Axiovert 200M) using software (Fiji Is Just (ImageJ)).

2.2.2.5.2.8 Immunofluorescence (IF):

2.2.2.5.2.8.1 Principle:

To detect and evaluate complement deposition and activation on Plasmodium asexual stages, immunofluorescent microscopy was done using anti C3b (FITC) and anti C5b-9 as primary antibodies. Two types of secondary antibodies were used to detect primary antibody binding; goat anti mouse Alexafluor 546 red colour and goat anti rabbit Alexafluor 488 green colour. However, FITC antibodies do not need any secondary antibodies as they were directly detected from there green fluorescence of fluorescein isothiocyanate (FITC). The fluorophore allows visualising the distribution of the targeted antigens in the tissue under a fluorescent microscope.

2.2.2.5.2.8.2 Technique

Smears of schizont infected RBCs treated with serum with or without recombinant proteins were made on previously labelled, polystyrene slides and dried then fixed using 4% paraformaldehyde for 10 minutes at room temperature. After drying, the slides were frozen until doing the experiment. The frozen sections were thawed at room temperature for 10 minutes. Then, the sections were washed with TBS and 0.05% Tween 20 (3 times, 3 minutes each) to remove the fixative. After that, the sections were blocked with 5% BSA to block non-specific binding sites and incubated for 30 minutes at room temperature. 40µl of the primary antibody solution (Table 2-21) were added to each section, then the sections were covered with plastic coverslips and incubated at 37°C for 1hour. The sections were washed with TBS-Tween 20 (3 times, 3 minutes). 40 µl of the secondary antibody solution (Table 2-21) were added to each section, and the sections were covered with plastic coverslips and incubated at room temperature for 1h. Later, the RBC smears were washed with TBS-Tween 20 (3 times, 3 minutes). Fluoroshield Mounting Medium with DAPI (It binds to the DNA to passes through the cell membrane) (Absorption wave length 358 nm) was added to each section, and then glass coverslips were added. Images were acquired with a ZEISS microscope (Axiovert 200M) using software (Fiji Is Just (ImageJ)).

Table 2-21: Dilution of primary and secondary antibodies

Targets	Primary antibody	Dilution	Secondary antibody	Dilution
C3 fragments (C3c, C3b and C3)	FITC Polyclonal rabbit anti human C3c antibody (Dako)	1:100 with 1xTBS	-	-
Asexual blood stages of *P. falciparum* strain 3D7	polyclonal mouse MSP1 anti asexual blood stages of plasmodium antibody (Santa Cruz Biotechnology)	1:100 with 1xTBS	goat anti mouse Alexafluor 546 red colour (Thermo Fisher Scientific)	1:100 with 1xPBS
C5b-9	Polyclonal mouse anti human C5b-9 (Santa Cruz Biotechnology)	1:100 with 1xTBS	goat anti rabbit Alexafluor 488 green colour (Thermo Fisher Scientific)	1:100 with 1xPBS

*Absorption wave length FITC (494-518nm).

2.2.3 Statistical analysis

All values of ELISA experiments were arranged and tabulated in Microsoft Excel program. Results were expressed as mean ± S.E.M. Statistical analysis was performed (t-test, one-way or two-way ANOVA) in GraphPad Prism version 6, P-value <0.05 was considered as significant. The experiments were set up independently, but some reagents were technical replicates.

3 Chapter three Expression of a Highly Polymerised and Active, Recombinant Human and Humanised Mouse Properdin

3.1 Introduction

Properdin is found as a glycoprotein in the serum with a concentration of 10-25 µg/ml (Xu *et al*, 2008). Different cells can produce properdin such as monocytes, T cells, mast cells and granulocytes. However, hepatocytes cannot produce properdin (Whaley, 1980; Schwaeble *et al*, 1993; Stover *et al*, 2008). Properdin is encoded by the CFP gene on the X chromosome, location Xp11.4 (Coleman *et al*, 1991). It plays an important role in regulating the complement activation via the alternative pathway by stabilising the C3 convertase (Pillemer *et al*, 1954; Farries, Lachmann and Harrison, 1988). It has a rod shape which is formed by connecting several monomers (53 kDa each) from a head to tail (Smith *et al*, 1984; Pangburn, 1989b). Serum properdin has different oligomeric forms; dimers, trimers and tetramers. The monomers are composed of seven Thrombospondin type I Repeats (TSRs) (Figure 1-2).

3.1.1 Role of Properdin

Properdin can augment the alternative pathway by stabilising the C3 convertase (Hourcade, 2006; Ferreira, Cortes and Pangburn, 2010a) . The alternative pathway C3 convertase, C3bBb could work as amplifier of the alternative by cleaving more C3 (Figure 1-1). The half-life of the alternative pathway C3 convertase is about 90 seconds (Medicus, Gotze and Muller-Eberhard, 1976; Pangburn and Muller-Eberhard, 1986). Properdin can increase the half-life of C3bBb by binding to this complex and forming a relatively stable complex, C3bBb properdin (C3bBbP) (Fearon and Austen, 1975). The stabilisation role is related to TSR4 and TSR5 (Higgins *et al*, 1995). In that way properdin could increase the amplification role of the alternative pathway by binding more C3b to form more stable complexes. The polymeric form of properdin could play an important role in the binding ability to C3bBb. Highly polymerised structures of recombinant human properdin could be produced and purified *in vitro* (Farries and Atkinson, 1989; Ali *et al*, 2014). This artificial product (P_n) stabilises C3bBb 5-10 folds more effectively than native properdin (Ferreira *et al.* 2010). However, the expression of a highly oligomerized and continuously activated recombinant mouse properdin is difficult; recombinant mouse properdin is usually less active than native mouse properdin (Lynch and Ali, personal communication).

3.1.2 Aims

This part of my thesis aims to express a highly active and polymerised, recombinant human and humanised mouse properdin that might be used to reduce parasitaemia for malaria infections as Ali *et al.* (2014) cited that recombinant human properdin can protect mouse from *S. pneumoniae*. This can be done by investigating whether recombinant properdin; a factor stabilising the C3bBbP complex, might be useful against asexual blood stages of *P. falciparum*. Chimeric mouse properdin, humanised at its termini is expected to polymerise better than recombinant murine properdin (since recombinant mouse properdin is less active than native mouse properdin) and can be used in assays that require human or murine sera to determine the relative importance of the individual pathways.

3.2 Results

3.2.1 Construction of humanised mouse properdin cDNA

Properdin contains seven repeat motifs called Thrombospondin I domains (TSRs) which are numbered from 0 to 6. The functional activity of these TSR is different. TSR0 and TSR6 are involved in the oligomerization of properdin (Sun, Reid and Perkins, 2004). TSR4 and TSR5 are responsible for C3 convertase stabilization (Higgins, Wiedemann *et al*. 1995), whilst TSR2 and TSR3 may play a role in facilitating the function of other domains (Sun, Reid and Perkins, 2004).

This part of the project aims to produce human and humanised mouse properdin in eukaryotic expression systems. Therefore, producing full length chimeric properdin without mutation is critical as this can be the most important part which can ensure getting correct amino acid sequencing of properdin which has an important role in protein activity.

Human Properdin cDNA contains an open reading frame of 1785 base pairs (bp) which encode 469 amino acid residues (Figure 3-1), while murine properdin cDNA is encoded by 1636 bp, which yields 464 amino acids (Figure 3-2). Properdin contains seven repeat motifs called Thrombospondin I domains (TSRs) which are numbered from 0 to 6. The coding sequences of human TSP0 plus signal peptides are 228 bp which code for 76 amino acid (Figure 3-1), while the coding sequences for mouse TSP0 plus signal peptides

are 216 bp which code to 72 amino acids (Figure 3-2). The coding sequences of human TSP6 are 252 bp, which codes for 84 amino acids, whilst the coding sequences of TSP6 for mouse properdin are 276 bp that code to 92 amino acids (Figure 3-1 and Figure 3-2).

Figure 3-1: amino acid sequences of human properdin and its TSPs, all derived from NM_002621. Reference amino acid sequences of human properdin (HP) (derived from NM_002621), arranged by colors as a following: signal peptide grey, TSR0 red, TSR1 green, TSR2 blue, TSR3 yellow, TSR4 black, TSR5 brown, TSR6 purple.

Figure 3-2: Amino acid sequences of mouse properdin and its TSPs, all derived from NM_008823. Reference amino acid sequences of mouse properdin (MP) (derived from NM_008823), arranged by colors as followings: signal peptide grey, TSR0 red, TSR1 green, TSR2 blue, TSR3 yellow, TSR4 black, TSR5 brown, TSR6 purple.

Dr Nicholas Lynch (University of Leicester, UK) kindly provided the template cDNA of human and mouse properdin. The template was used with different sets of primers to generate the chimeric properdin (see Table 2-2 and see section 2.2.1.2). The coding sequences (CDS) of human properdin TSR0 and TSR6 and mouse properdin TSR1-TSR5 were PCR amplified using Phusion DNA polymerase (Figure 3-3 A, B, and C). In another PCR experiment, mouse TSR0-TSR5 and TSR1-TSR6 were amplified (Figure 3-3 D, E, and F). All PCR products were visualised on a 1% agarose gel and were confirmed to be the correct size.

Human properdin TSR6 coding sequence and mouse properdin TSR0-TSR5 coding sequence were PCR ligated to generate C-terminus humanised mouse properdin (MMHP) construct (Figure 3-4 A and D). In a second PCR step, the human TSR0 was ligated to

mouse TSR1-TSR6 to generate N-terminus humanised mouse properdin (HMMP) construct (Figure 3-4 B and D). To generate mouse properdin humanised at both termini (HMHP) construct, human TSR0 and TSR1 coding sequences were ligated to mouse coding sequence of TSR1-TSR5 (Figure 3-4 C and D). The PCR products were analysed in 1% agarose gel.

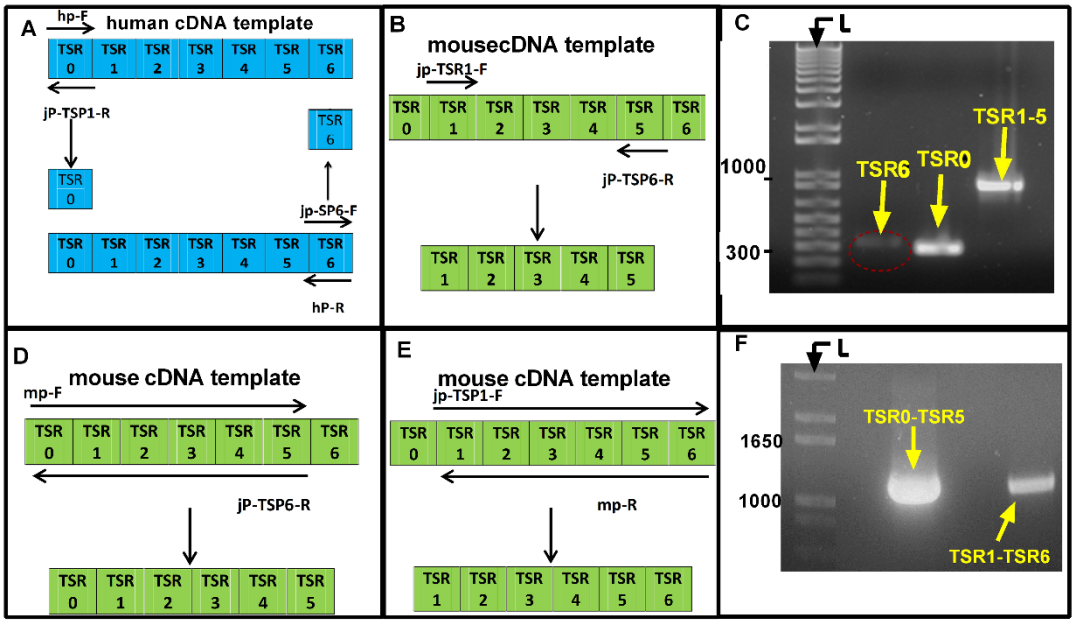

Figure 3-3: Schematic representation and agarose gel electrophoresis of PCR amplified human and mouse properdin TSRs.
Different sets of primers were used to generate human TSR0 and TSR6 (A and C), mouse TSR1-TSR5 (B and C), mouse TSR0-TSR5 (D and F) and TSR1-TSR6 (E and F). The products were analyzed on agarose gel. The coding sequences of human TSR0 and TSR6 show a band of approximately 300bp (C) while mouse TSR1-TSR5 coding sequences show a band of around 850bp (C) while mouse TSR0-TSR5 and TSR1-TSR6 CDS show bands of around 1100bp (E). jp=joining primer, hp=human properdin, mp=mouse properdin, F= forward primer, R= reverse primer, L= 1kb DNA plus ladder.

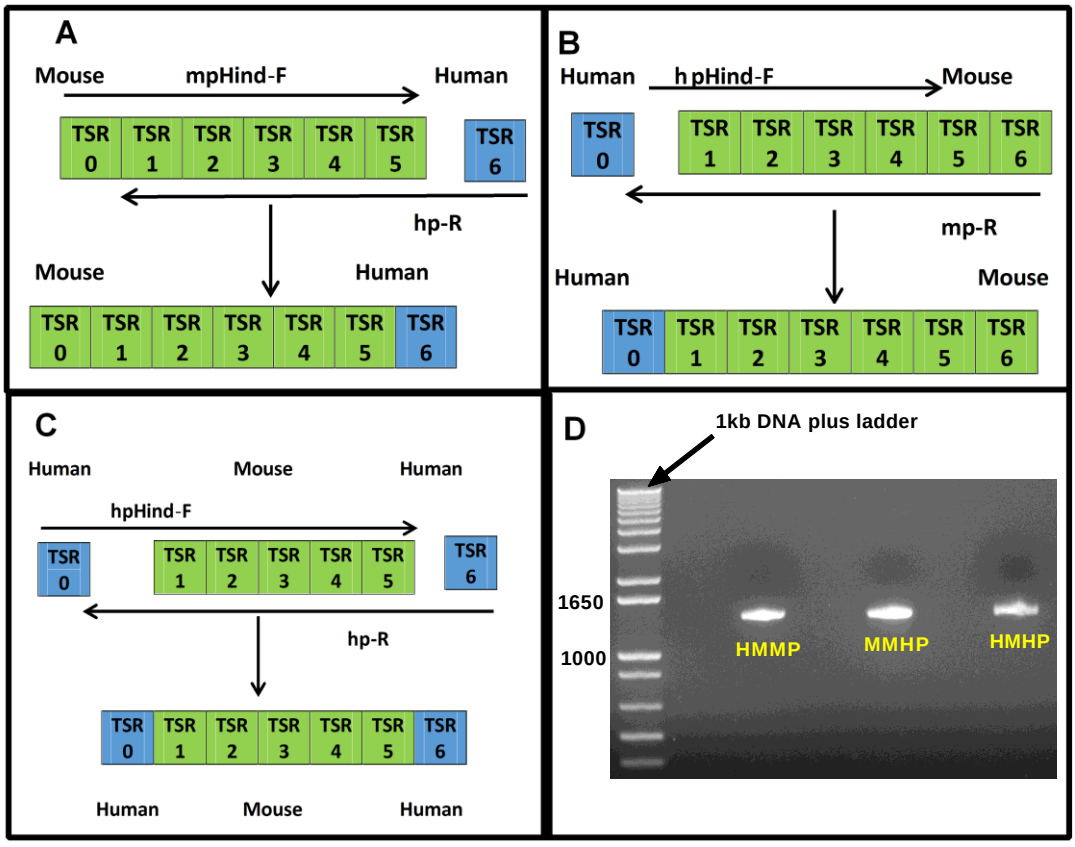

Figure 3-4: Schematic representation and agarose gel electrophoresis of chimeric mouse properdin. The gel purified human TSR6 coding sequence was used as a template beside mouse TSR0-TSR5 generating the coding sequence for MMHP (A and D). Human properdin TSR0 coding sequence was ligated to mouse properdin TSR1-TSR6 coding sequences generating the coding sequence for HMMP (B and D). TSR0 and TSR6 coding sequences of human properdin were ligated to mouse properdin TSR1-TSR5 coding sequences generating the coding sequence for HMHP (C and D). The three different chimeric constructs show a single band of approximately 1400bp (D).

3.2.2 Cloning the properdin DNA into pGEM-T easy and pSecTag2/Hygro B vectors

pGEM-T easy vector was used to facilitate easy cloning of the PCR products (Mezei *et al* 1994) (see sections 2.2.1.5.2). It contains 3018 base pairs and has a single thymidine residue at both ends. A tailing was done using Taq polymerase to add adenine nucleotides to the PCR product to make them complementary to the T-overhangs in pGEM-Teasy. The products were then ligated into the vector (see section 2.2.1.5.1). After that the DNA constructs were transfected and expressed in *E.coli* TOP10 competent cells. These cells were plated on LB ampicillin agar plates (see section 2.2.1.5.4). The colors of the resulting colonies indicate whether ligation was successful or not. White colonies indicate correct ligation, while cells containing only vector appear bluish. This happened because of the α-complementation of the β-galactosidase gene, in which β-galactosidase was inactivated by deleting certain sequences (α peptide). An inactive mutant β-galactosidase was rescued by inserting α peptide to a vector. This activated β-galactosidase show blue colonies in presence of X-gal when only a vector was successfully transfected. DNA recombinant ligated to a vector disrupts the α peptide and the α-complementation process, and shows white colonies (Langley, Villarejo *et al* 1975; Messing, Gronenborn *et al* 1977; Langley, Villarejo *et al* 1975, Vieira, Messing 1982).

The pGEM T easy constructs were amplified by growing selected white colonies in ampicillin supplemented LB broth. These constructs were extracted using Wizard plus SV Minipreps (Promega) (see section 2.2.1.5.5). The successful ligation was checked by digesting with *Xho*I and *Hind*III, sites for which had been engineered into the PCR primers (see section 2.2.1.5.6). When the digested samples were run on a 1% agarose gel, two bands, 3000 bp for pGEM T easy and 1400 bp for properdin were seen, showing that the ligation had succeeded (Figure 3-5 A and B).

After running on agarose gel, properdin construct bands were excised and extracted by using a gel extraction kit (see section 2.2.1.4) and then sub-cloned into a mammalian expression vector; pSectag2/hygroB, in frame with the C terminal 6x histidine tag. This tag can be used for purification purposes. The successful ligation was checked by digesting some properdin- pSectag2/hygroB plasmids using *Xho*I and *Hind*III restriction enzymes and then run on agarose gel, as previously done with pGEM T easy (Figure 3-5 C and D). The chimeric properdin constructs show bands of approximately 1400bp while pSecTag

2/Hygro B (Figure 3-5 C and D) show bands of about 5000bp. Before transfecting mammalian cells, the DNA sequence of all constructs were determined in the DNA sequence unit (PNACL) at the University of Leicester, to be sure there is no mutation during PCR amplification or UV- light exposure. The sequencing results of the constructs show that the cloning was done without mutation (Figure 3-6).

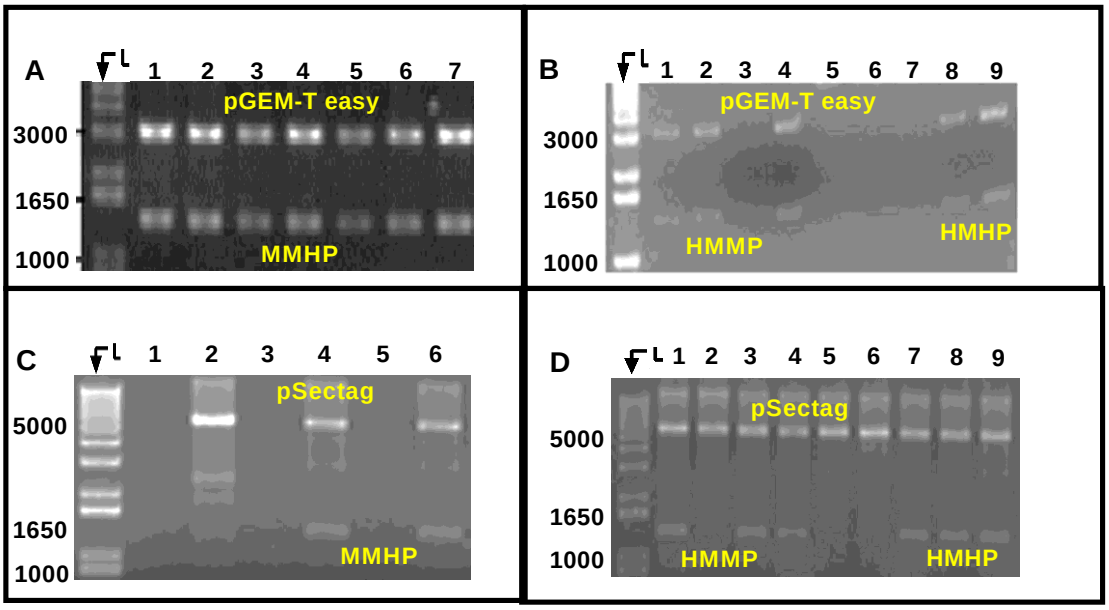

Figure 3-5: Cloning of chimeric properdin DNA into pGEM-T easy and pSecTag2/Hygro B vectors. Agarose gel analysis of plasmid minipreps of different colonies (numbered starting from 1) of MMHP (A), HMMP (lanes 1, 2 and 4) and HMHP (lanes 1, 3 and 4) (B) of humanised mouse properdin constructs into pGEM T easy and pSecTag2/Hygro B (HMMP in panel C and HMMP (lanes 1, 3 and 4) and HMHP (lanes 7, 8 and 9) in panel D) (3018bp and 5000 pb) digested with Xho*I* and Hind*III*. Chimeric mouse properdin DNA show a band of 1400bp. The DNA constructs were gel purified and sub-cloned into pSecTag 2/Hygro B (5000bp), L= 1kb DNA plus ladder.

CLUSTAL O(1.2.1) multiple sequence alignment

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HP NP_002621      MITEGAQAPRLLLPPLLLLLLTLPATGSDPVLCFTQYEESSGKCKGLLGGGVSVEDCCLNT
HMMP construct    ------------------------DPVLCFTQYEESSGKCKGLLGGGVSVEDCCLNT
HMHP construct    ------------------------DPVLCFTQYEESSGKCKGLLGGGVSVEDCCLNT
MMHP construct    -------------------------DPVLCFTQYEESSGRCKGLLGRDIRVEDCCLNA
MP NP_032849      -MPAEMQAPQWL---LLLLVILPATGSDPVLCFTQYEESSGRCKGLLGRDIRVEDCCLNA

HP NP_002621      AFAYQKRSGGLCQPCRSPRWSLWSTWAPCSVTCSEGSQLRYRRCVGWNGQCSGKVAPGTL
HMMP construct    AFAYQKRSGGLCQPCRSPRWSLWSTWAPCSVTCSEGSQLRHRRCVGRGGQCSENVAPGTL
HMHP construct    AFAYQKRSGGLCQPCRSPRWSLWSTWAPCSVTCSEGSQLRHRRCVGRGGQCSENVAPGTL
MMHP construct    AYAFQEHDGGLCQACRSPQWSAWSLWGPCSVTCSEGSQLRHRRCVGRGGQCSENVAPGTL
MP NP_032849      AYAFQEHDGGLCQACRSPQWSAWSLWGPCSVTCSEGSQLRHRRCVGRGGQCSENVAPGTL

HP NP_002621      EWQLQACEDQQCCPEMGGWSGWGPWEPCSVTCSKGTRTRRRACNHPAPKCGGHCPGQAQE
HMMP construct    EWQLQACEDQPCCPEMGGWSEWGPWGPCSVTCSKGTQIRQRVCDNPAPKCGGHCPGEAQQ
HMHP construct    EWQLQACEDQPCCPEMGGWSEWGPWGPCSVTCSKGTQIRQRVCDNPAPKCGGHCPGEAQQ
MMHP construct    EWQLQACEDQPCCPEMGGWSEWGPWGPCSVTCSKGTQIRQRVCDNPAPKCGGHCPGEAQQ
MP NP_032849      EWQLQACEDQPCCPEMGGWSEWGPWGPCSVTCSKGTQIRQRVCDNPAPKCGGHCPGEAQQ

HP NP_002621      SEACDTQQVCPTHGAWATWGPWTPCSASCHGGPHEPKETRSRKCSAPEPSQKPPGKPCPG
HMMP construct    SQACDTQKTCPTHGAWASWGPWSPCSGSCLGGAQEPKETRSRSCSAPAPSHQPPGKPCSG
HMHP construct    SQACDTQKTCPTHGAWASWGPWSPRSGSCLGGAQEPKETRSRSCSAPAPSHQPPGKPCSG
MMHP construct    SQACDTQKTCPTHGAWASWGPWSPCSGSCLGGAQEPKETRSRSCSAPAPSHQPPGKPCSG
MP NP_032849      SQACDTQKTCPTHGAWASWGPWSPCSGSCLGGAQEPKETRSRSCSAPAPSHQPPGKPCSG

HP NP_002621      LAYEQRRCTGLPPCPVAGGWGPWGPVSPCPVTCGLGQTMEQRTCNHPVPQHGGPFCAGDA
HMMP construct    PAYEHNKACSGLPCPVAGGWGPWSPLSPCSVTCGLGQTLEQRTCDHPAPRHGGPFCAGDA
HMHP construct    PAYEHKACSGLPPCPVAGGWGPWSPLSPCSVTCGLGQTLEQRTCDHPAPRHGGPFCAGDA
CHMP construct    PAYEHKACSGLPPCPVAGGWGPWSPLSPCSVTCGLGQTLEQRTCDHPAPRHGGPFCAGDA
MP NP_032849      PAYEHKACSGLPPCPVAGGWGPWSPLSPCSVTCGLGQTLEQRTCDHPAPRHGGPFCAGDA

HP NP_002621      TRTHICNTAVPCPVDGEWDSWGEWSPCIRRNMKSISCQEIPGQQSRGRTCRGRKFDGHRC
HMMP construct    TRNQMCNKAVPCPVNGEWEAWGKWSDCSRLRM-SINCEGTPGQQSRSRSCGGRKFNGKPC
HMHP construct    TRNQMCNKAVPCPVNGEWEAWGKWSDCSRLRM-SINCEGTPGQQSRSRSCGGRKFNGKPC
MMHP construct    TRNQMCNKAVPCPVNGEWEAWGKWSDCSRLRM-SINCEGTPGQQSRSRSCGGRKFNGKPC
MP NP_032849      TRNQMCNKAVPCPVNGEWEAWGKWSDCSRLRM-SINCEGTPGQQSRSRSCGGRKFNGKPC

HP NP_002621      AGQQQDIRHCYSIQHCPLKGSWSEWSTWGLCMPPCGPNPTRARQRLCTPLLPKYPPTVSM
HMMP construct    AGKLQDIRHCYNIHNCIMKGSWSQWSTWSLCTPPCSPNATRVRQRLCTPLLPKYPPTVSM
HMHP construct    AGKLQDIRHCYNIHNCIMKGSWSEWSTWGLCMPPCGPNPTRARQRLCTPLLPKYPPTVSM
MMHP construct    AGKLQDIRHCYNIHNCIMKGSWSEWSTWGLCMPPCGPNPTRARQRLCTPLLPKYPPTVSM
MP NP_032849      AGKLQDIRHCYNIHNCIMKGSWSQWSTWSLCTPPCSPNATRVRQRLCTPLLPKYPPTVSM

HP NP_002621      VEGQGEKNVTFWGRPLPRCEELQGQKLVVEEKRPCLHVPACKDPEEEEL--
HMMP construct    VEGQGEKNVTFWGTPRPLCEALQGQKLVVEEKRSCLHVPVCKDPEEKKPYSR
HMHP construct    VEGQGEKNVTFWGRPLPRCEELQGQKLVVEEKRPCLRVPACKDPEEEELYSR
MMHP construct    VEGQGEKNVTFWGRPLPRCEELQGQKLVVEEKRPCLRVPACKDPEEEELYSR
MP NP_032849      VEGQGEKNVTFWGTPRPLCEALQGQKLVVEEKRSCLHVPVCKDPEEKKP--
```

Figure 3-6: Alignment of amino acid sequences of recombinant properdin.
Reference amino acid sequences of human properdin (HP) (top line; derived from NP_002612), and mouse properdin (MP) (bottom line, derived from NP_032849), aligned with the cDNA-derived amino acid sequences determined for chimeric properdin HMHP, MMHP and HMMP. Human sequence shown in orange, mouse in blue. CLUSTAL O (1.2.1) program was used to align the multiple sequences.

3.2.3 Expression of humanised mouse properdin

Chimeric properdin was expressed in CHO-K1 mammalian cells. Cells were transfected with DNA plasmid using Lipofectamine™ reagent (see section 2.2.2.1.2). Hygromycin-B, a selective antibiotic was added to F12 nutrient media, culture media of CHO-K1 cells, with a concentration of 300µg/ml to prevent growth of non-transfected cells. In addition, human and mouse properdin, already transfected in CHO-K1 cells and kindly provided by Professor Wilhelm Schwaeble, were also expressed. Three weeks later, after changing hygromycin-B containing growth media each 5 days, the tissue culture flasks become confluent with protein expressing cells. The supernatant was collected, filtered to remove debris and dead cells, and dotted onto nitrocellulose membrane and probed with HRP conjugated monoclonal mouse anti-polyhistidine antibodies to identify the positive clones that express recombinant proteins (Figure 3-7).

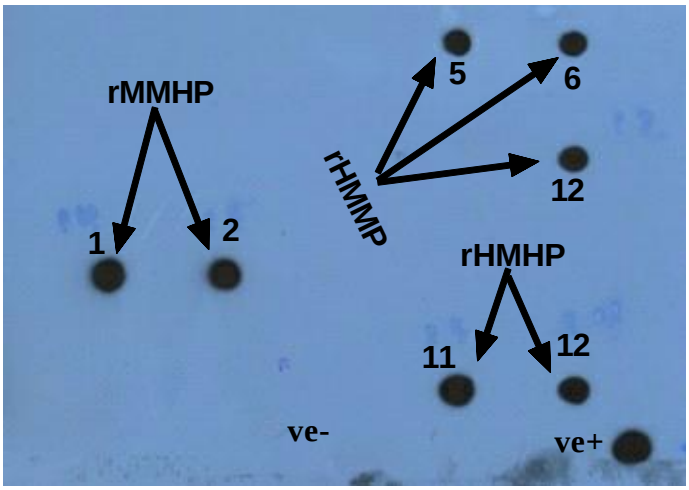


Figure 3-7: Dot blot assay of supernatant of different clones for expression of recombinant chimeric properdin.
Screening of selected clones for expression of recombinant mouse properdin humanised at the C terminus (rMMHP), at the N terminus (rHMMP) and at both termini (rHMHP). The protein expression was visualized using HRP conjugated monoclonal mouse anti-polyhistidine antibodies. Recombinant mouse MASP-3 with histidine linker, kindly provided by Dr. Sadam Yaseen who was a post-doctoral research associate in our laboratory, was used as a positive control (+ve). Supernatant from non-transfected CHO-K1 cells was used as a negative control (-ve). The positive clones are indicated.

3.2.4 Purification of recombinant properdin

Triple layer flasks were cultivated with the positive clones in a dot blot test only, and the supernatant was collected and purified through a His GraviTrap column (see section 2.2.2.1.5.1). These are chelating columns, pre-loaded with Ni^{2+}, that have a high affinity for the 6X histidine tag. The protein is eluted with imidazole, which competes with the binding of 6x histidine tag. 350 mM of imidazole was used to elute the protein. The protein concentration was checked twice, once at end of the purification and the other one when the protein was frozen at -80°C, as there is a chance that aggregated properdin (Pn) formed during freeze–thaw cycles precipitates (Ferreira, Cortes *et al* 2010). A Bradford assay was used to check protein concentration (see section 2.2.2.2.3).

3.2.5 Characterization of recombinant properdin

The recombinant properdin was then tested by SDS-PAGE under reducing conditions (see section 2.2.2.4.1). The chimeric recombinant properdin ran at the anticipated size of approximately 55 kDa (Figure 3-8). To confirm the presence of the desired recombinant properdin, Western blotting was performed (see section 2.2.2.4.2) using HRP conjugated monoclonal mouse anti polyhistidine antibodies. Recombinant SCR3-5 of CFHR1 (I produced as a part of the thesis) with histidine tag was used as a positive control (+ve) and supernatant medium from non- transfected CHO-K1 cells was used as a negative control (-ve) (Figure 3-9). The chimeric properdin bands were visualised clearly at 55 kDa.

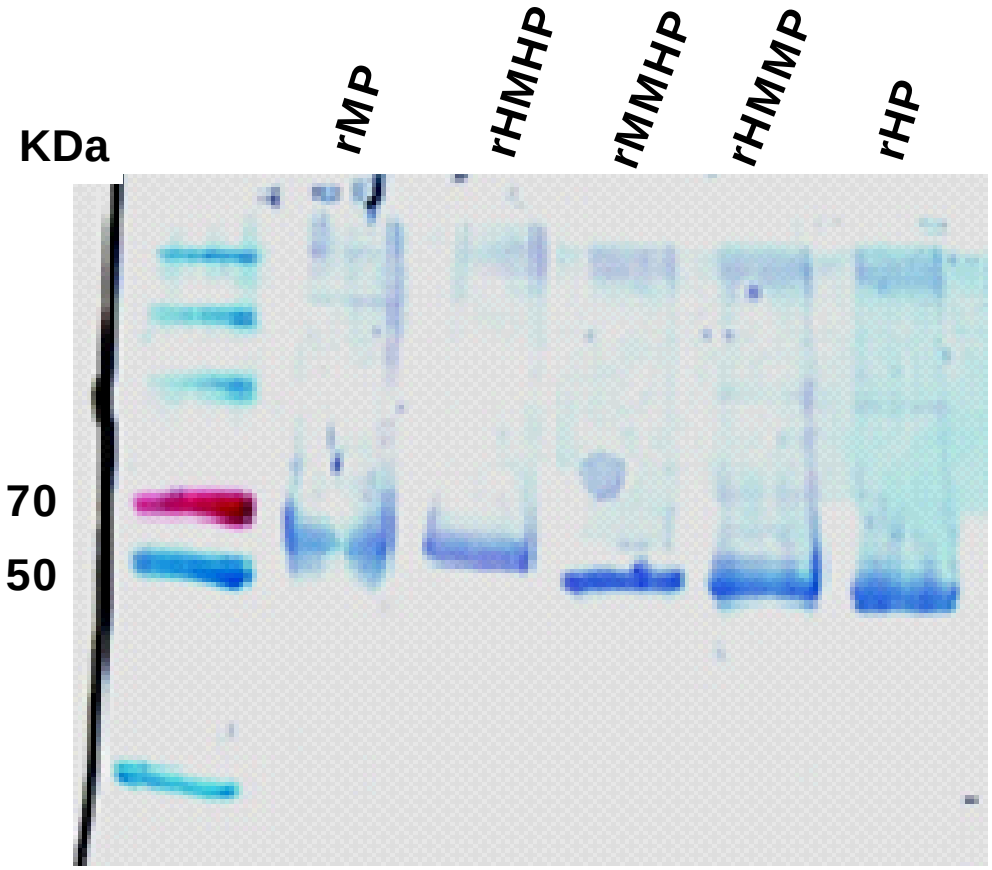


Figure 3-8: SDS-PAGE analysis of different forms of recombinant properdin
20µg of the recombinant proteins were separated in 10% polyacrylamide gel under reducing conditions and stained with Coomassie Blue. rHMHP, rMMHP and rHMMP show single bands of approximately 55 kDa while the purified recombinant mouse (rMP) (lane 2) and human (rHP) properdin also show single bands of approximately 55 kDa.

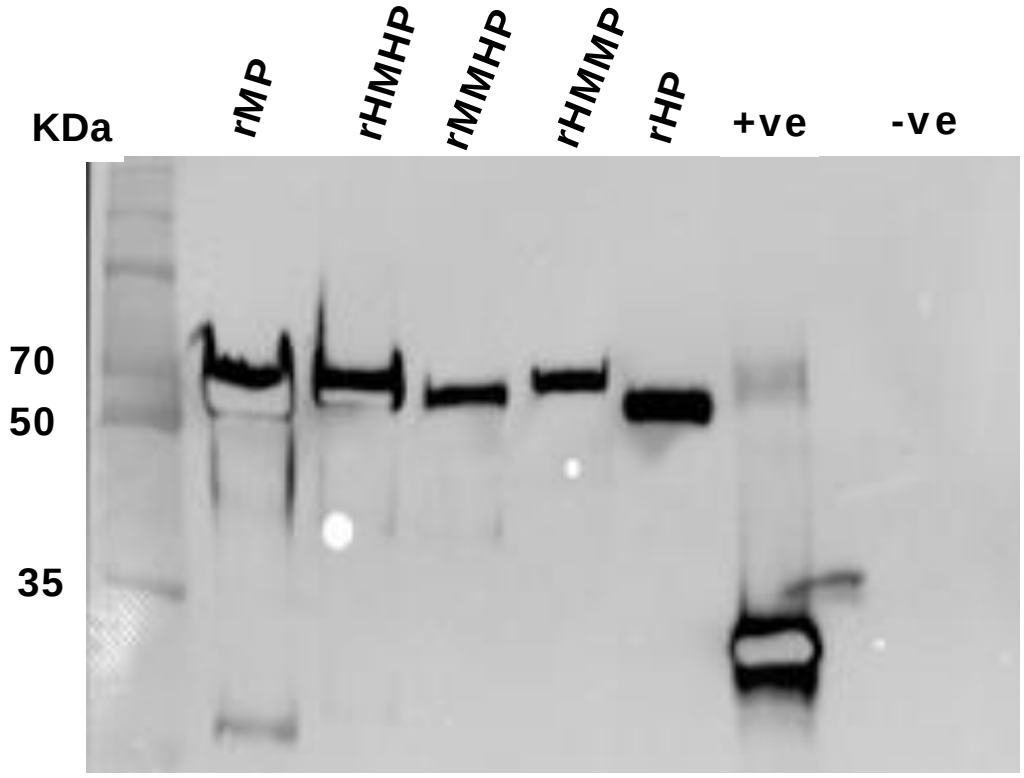


Figure 3-9: Western blotting analysis of different forms of recombinant humanised properdin
Recombinant properdin (1µg) was separated in SDS polyacrylamide gel and proteins were electrophoretically transferred onto a nitrocellulose membrane. The blot was probed with HRP conjugated monoclonal mouse anti-polyhistidine antibodies. rHMHP, rMMHP and rHMMP show single bands of approximately 55 kDa whilst rMP and rHP properdin also show single bands of approximately 55 kDa. Recombinant SCR3-5 of CFHR1 with histidine tag was used as a positive control (+ve) and supernatant medium from non- transfected CHO-K1 cells was used as a negative control (-ve).

3.2.6 Protein sequences:

Protein sequences were checked by TOF mass spectrometry of single bands excised from SDS-PAGE gels at the University of Leicester proteomic unit (PNACL). The peptides identified all agreed with the anticipated amino acid sequences.

3.2.7 Analysis of human, mouse and chimeric recombinant properdin using gel filtration Fast Protein Liquid Chromatography (FPLC)

Gel filtration was performed to determine the oligomerisation state of the recombinant proteins, using a GE healthcare Fast Protein Liquid Chromatography (FPLC) system, fitted with a Superose increase 6 10/300 column. This technique depends on separation

proteins by the size (see section 2.2.2.3). Thus, the largest particles pass through the column faster than that are smaller size. This column was calibrated first using Gel Filtration Markers Kit for Protein Molecular Weights 29,000-700,000 Da from Sigma, (Figure 3-10).

When recombinant properdin was applied to the column, multiple peaks appeared at different elution amounts. Pn appeared to elute firstly between 12-14 ml which looks to be aggregated higher-order polymers, then other forms of properdin; tetramers (P4), trimers (P3) and dimers (P2), were eluted subsequently between 14-16 ml, 16-17 ml and 18-20 ml respectively (Figure 3-11). It has been successful in producing predominantly P3, P4 and bigger forms while P1 was noticeable by its absence. However, other forms of chimeric and murine properdin do not show correct oligomerization state as Pn did not appear clearly (Figure 3-12). Fractions were collected from each gel filtration run, concentrated then assayed for their ability to augment alternative pathway activation on zymosan.

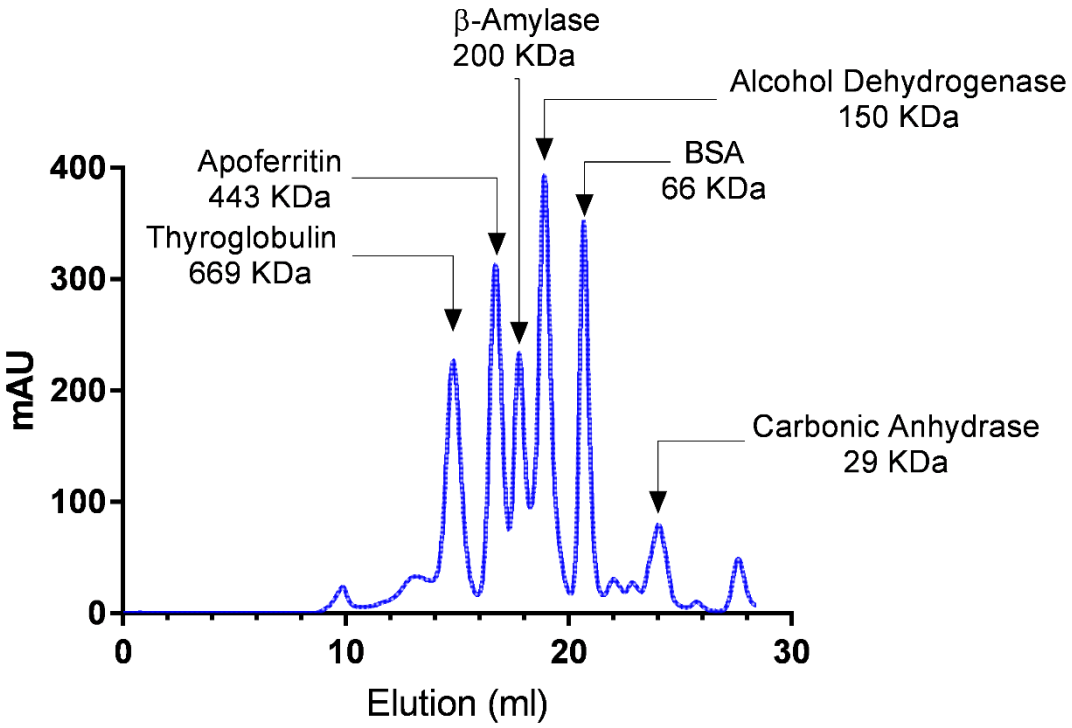


Figure 3-10: Elution of molecular weight markers used to calibrate a Superose 6 Incease 10/300 column.

0.5 ml of a mixture of marker proteins (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), were loaded on a Superose 6 increase 10/300 GE healthcare column which was eluted with 3 column volumes of TBS. Marker proteins were eluted in multiple peaks between 14 and 20 ml. The molecular weights of standard proteins are marked by arrows.

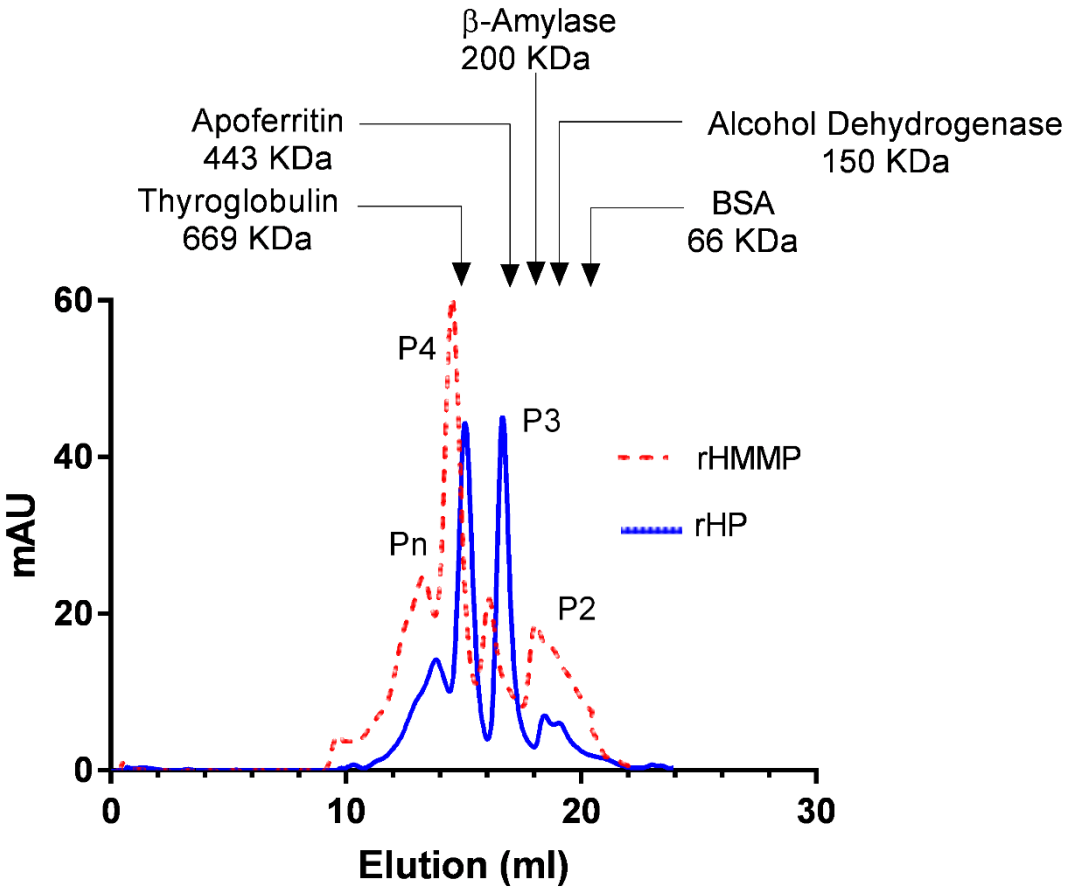


Figure 3-11: Separation of different oligomeric forms of both rHP and rHMMP.
0.5 ml of rHP or rHMMP were loaded on Superose 6 increase 10/300 GE healthcare column which was then eluted with one column volume of TBS. Recombinant properdin was eluted in multiple peaks between 14 and 20 ml. The oligomeric form (Pn) comes out first from the column, as it is an aggregate of properdin, then other forms; P4, P3, P2 and P1 come later respectively. The elution volumes and molecular weights of standard proteins are marked by arrows.

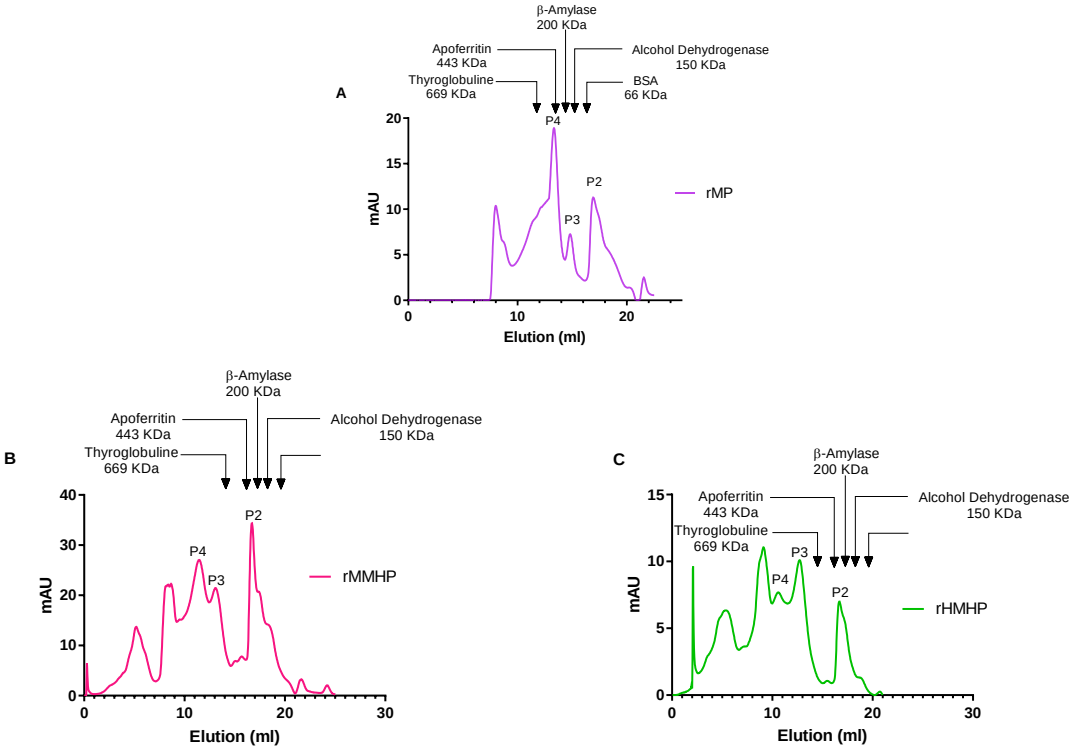


Figure 3-12: Separation of different oligomeric forms of both rMP, rMMHP and rHMHP.
0.5 ml of rMP (A), rMMHP (B) or rHMHP (C) were loaded on superpose 6 increase 10/300 GE healthcare column which was washed thrice column volume with TBS. recombinant properdin were eluted in multiple peaks. The elution volumes and molecular weights of standard proteins are marked by arrows.

3.2.8 Complement assays

3.2.8.1 Complement assays done on ELISA microplates

3.2.8.1.1 Role of previously transfected rHP and rMP in C3b deposition

As mentioned before, properdin has the ability to stabilize the C3b and the C5 convertase, (C3bBb and $(C3b)_nBb$), respectively which increases the half-life of the C3b convertase and then augments the alternative pathway through an amplification loop (Schwaeble, Reid 1999). Ali *et al*. (2014) reported that recombinant properdin can increase the alternative pathway activation and C3b deposition in NHS without calcium. To check human and mouse properdin activity, rHP and rMP (previously transfected to CHO-K1 cells by Professor Wilhelm Schwaeble group) were expressed and purified then tested on NHS and WT mouse serum.

To check the effect of these recombinant proteins on complement activity, a fixed concentration of rHP and rMP (10 µg/ml) was added to serial dilutions of NHS and WT mouse serum respectively in GVB/Mg^{2+}/EGTA buffer. The mixtures were incubated on zymosan coated microtiter plates. Polyclonal rabbit anti-human C3c was used to determine C3b deposition. The exogenous properdin augmented alternative pathway activation through increasing C3b deposition on zymosan (Figure 3-13). This experiment shows that rHP added to NHS activates more C3b deposition on zymosan compared to NHS without protein addition. AP_{50} (the serum concentration required for half maximal activity) values were shifted by adding rHP from 3.5% for NHS to 1.6% for NHS supplemented with rHP. However, rMP added to WT mouse serum does not show any shifting for AP_{50}. The t test between NHS only vs rHP+NHS showed significantly differences. However, no significant differences between WT mouse serum only vs WT mouse serum + rMP. It could be said, recombinant mouse properdin is usually less active than native mouse properdin while rHP is more active than the native human properdin.

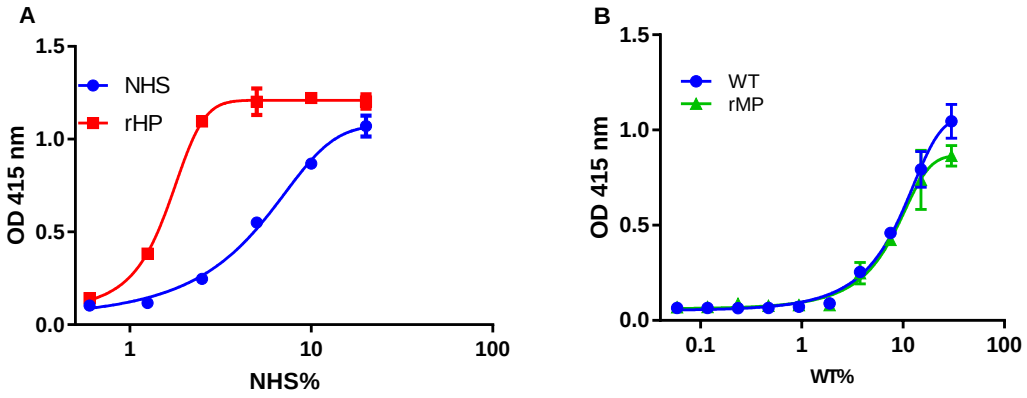


Figure 3-13: comparison between the alternative pathway induced by rHP and rMP in NHS and WT mouse serum

Fixed concentration (10 µg/ml) of rHP (A) and rMP (B) expressed in CHO-K1 was added to serial dilutions of NHS (A) and WT mouse serum (B) on zymosan coated plate. C3b deposition was determined using anti-human C3c. The differences were checked by t test- one per row between NHS only vs rHP+NHS and between WT mouse serum only and WT mouse serum + rMP, Significant values=P<0.05. Data are the means ± SEM from three independent experiments carried out.

3.2.8.1.2 Augmentation of the alternative pathway activity by adding recombinant chimeric properdin

Based on the positive role of properdin in regulating the complement alternative pathway, the purified recombinant proteins were tested for their ability to augment activation of this pathway. Different concentrations of recombinant properdin were added to serially diluted normal human serum starting at maximum serum concentration 20% in GVB/Mg^{2+}/EGTA buffer. Recombinant murine Properdin humanised at the N terminus (rHMMP) could augment the alternative pathway. rHMMP was more effective in enhancing C3b deposition on zymosan coated plate (Figure 3-14 E) than the other chimeric properdin (Figure 3-14 A and C). rHMMP could shift down the AP_{50} of NHS from 2.1% to 1.2% (Figure 3-14F). Thus, targeting the N terminus of mouse properdin by replacing TSR0 with the corresponding human domain was more effective than targeting the C terminus, or both ends of mouse properdin.

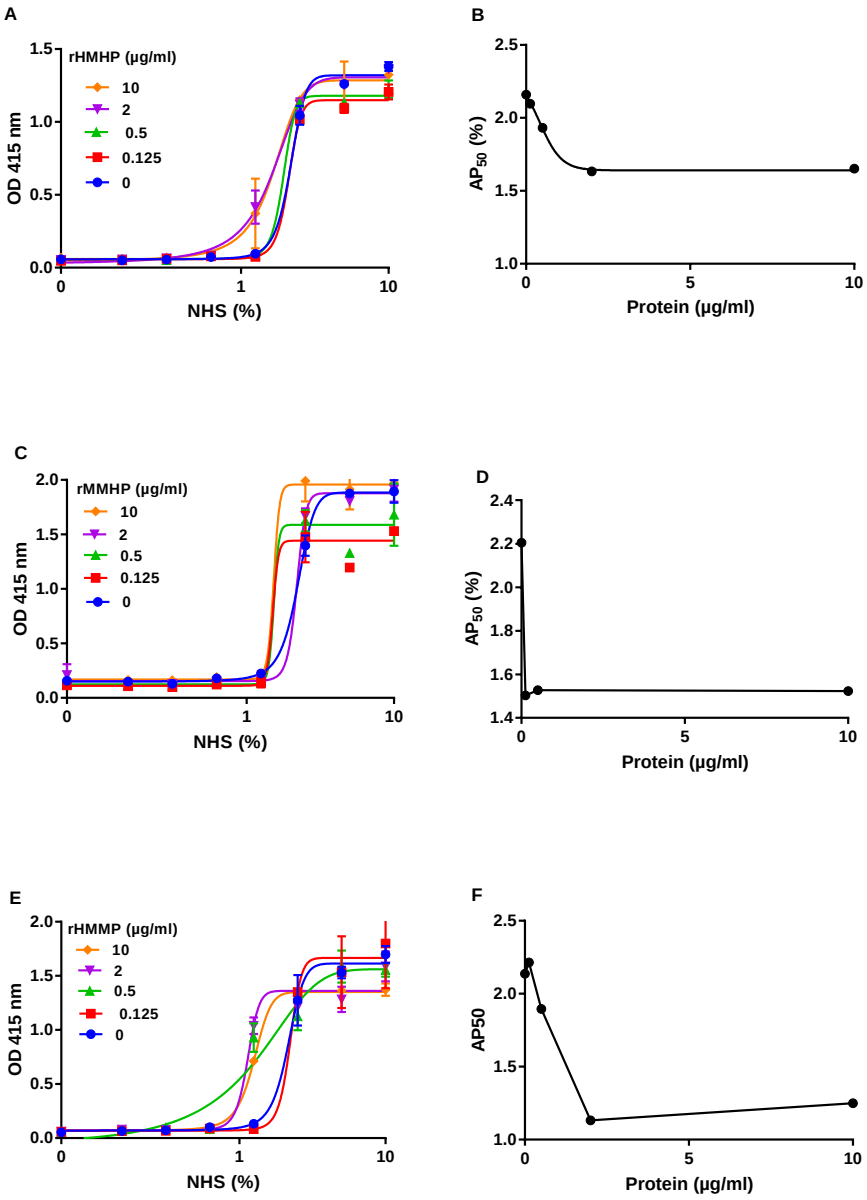


Figure 3-14: Comparison of the alternative pathway of different types of chimeric properdin in NHS (**A, C and E**) Different concentrations of recombinant chimeric proteins were added to serially diluted normal human serum in GVB/Mg^{2+}/EGTA buffer. C3b deposition was determined using anti-human C3c on zymosan coated plate. **(B, D and F):** Sigmoidal curves of rMMHP, rHMHP and rHMMP were fitted to the data and the AP_{50} was calculated. Data are the means ± SEM from three independent experiments carried out.

3.2.8.1.3 Comparison between rHMMP and other chimeric properdin activity in inducing the C3b deposition in WT mouse serum

Recombinant mouse properdin failed to augment the alternative pathway (Figure 3-13) (Ali *et al*, 2014). To explore the role of the chimeric properdin in wild type mouse serum, alternative pathway dependent C3b deposition test was studied in wild type mouse serum supplemented with 10µg/ml final concentration of chimeric rMMHP, rHMHP or rHMMP on zymosan coated plate (Figure 3-15). rHMMP showed a significant increase in the alternative pathway activity compared to the wild type serum alone. rMMHP and rHMHP increased alternative pathway activation but quickly showed decrease at the higher concentrations of serum that could be because rMMHP and rHMHP interferes with the endogenous properdin. AP_{50} was shifted down when these proteins were added to WT mouse serum. AP_{50} was shifted down from 6.3% of WT mouse serum to 2.8 % for rHMMP, 1.7% for rMMHP and 1.8% for rHMHP. Showing that all chimeric properdin could enhanced the alternative pathway in wild type mouse serum.

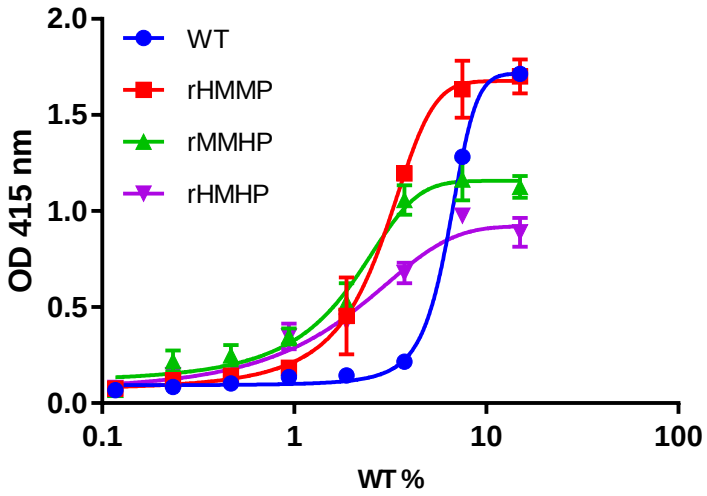


Figure 3-15: comparison between alternative pathway activity of different concentrations of WT mouse serum supplemented with 10 µg/ml of recombinant chimeric properdin.
10 µg/ml of rHMMP, rMMHP and rHMHP was added to different concentrations of wild type mouse serum on zymosan coated plate. C3b deposition was probed with polyclonal rabbit anti-human C3c. This experiment shows that rHMMP is more active than other proteins. AP_{50} was calculated and fitted to the data. Data are the means ± SEM from three independent experiments carried out.

3.2.8.1.4 Comparing the C3b deposition activity between rHMMP and rMP in WT mouse serum

rMP did not show any enhancement to the alternative pathway (Ali *et al*, 2014). It did not augment the C3b deposition in wild type mouse serum (Figure 3-13). To show the role of rHMMP in C3b deposition compared to rMP in WT mouse serum, serial dilutions of WT mouse serum were supplemented with 10 µg/ml of rHMMP and rMP on zymosan coated plates. The activity was checked using polyclonal rabbit anti human C3c (Figure 3-16). It is clear that rHMMP can induce the alternative pathway and add more C3b through amplification loop of this pathway which is significantly different compared to WT mouse serum without adding or with rMP. rHMMP shifted AP_{50} of WT mouse serum from 6.2% to 3.4%. It therefore seems that, targeting the N-terminus of mouse properdin by replacing it with that of human properdin making mouse properdin an active component.

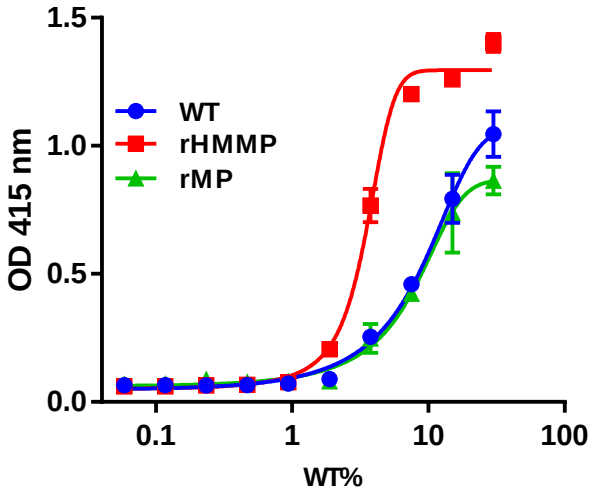


Figure 3-16: comparison between the alternative pathway activities of different concentrations of WT mouse serum against WT mouse serum supplemented with fixed concentration (10 µg/ml) of rHMMP and rMP.
10 µg/ml of rHMMP or rMP was added to different concentrations of wild type mouse serum on zymosan coated plate. C3b deposition was determined using polyclonal rabbit anti-human C3c. This experiment shows that rHMMP is more active compared to rMP. The AP_{50} was calculated and fitted to the data. Data are the means ± SEM from three independent experiments carried out.

3.2.8.1.5 C3b deposition of rHP on zymosan coated plate

As mentioned before, properdin is the only positive regulator that augments the alternative pathway through amplification loop due to its ability to stabilize and increase the half-life of the C3b convertase (C3bBb) then activate more C3 and generating the C5 convertase (C3bBbC3b) (Schwaeble, Reid 1999). Recombinant properdin can restore complement activation and reduce AP_{50} in NHS without adding calcium (Ali *et al.*, 2013). In order to investigate the whole role of recombinant properdin without any doubt about the possibility of interfering with remained native properdin, properdin depleted serum (PDS) (CompTech) was supplemented with both rHP and rHMMP. The alternative pathway can be activated slowly and partially when PDS was used. This can be a good way to show how recombinant properdin can work in terms of C3b deposition thus restoring the alternative pathway.

Different concentrations of properdin-depleted human serum (PDS) were supplemented with varying concentrations of rHP starting at maximum protein concentration of 10µg/ml. The C3b fixation on zymosan coated plate showed a dose-dependent increase with the amount of the recombinant protein (Figure 3-17). The AP_{50} was shifted by adding more rHP compared to PDS only. The AP_{50} of PDS is 5.2% which dropped to 0.23% in the same serum when it was supplemented with 10µg/ml of rHP (Figure 3-13).

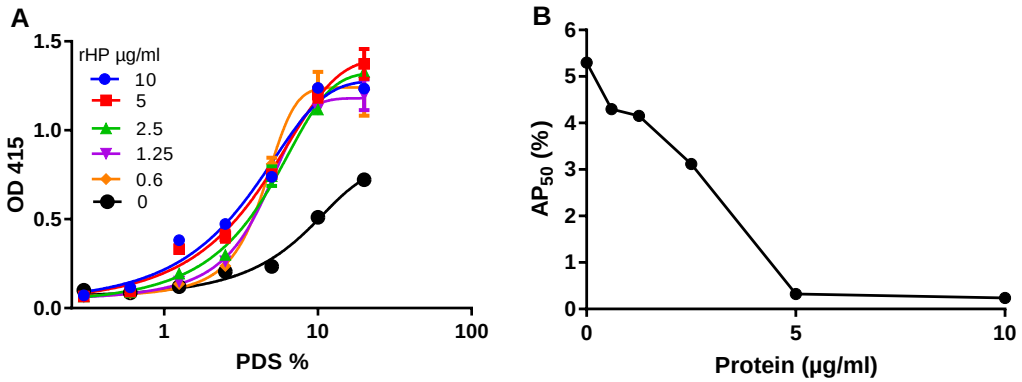


Figure 3-17: C3b deposition induced by different concentrations of rHP in properdin depleted human serum (PDS)
Different concentrations of rHP were added to different concentrations of PDS. After that, the mixture was added to zymosan coated ELISA plate. After incubation for 90 minutes at 37°C, C3b deposition was determined by polyclonal rabbit anti-human C3c. Sigmoidal curves were fitted to the data and AP_{50} was determined. Data are the means ± SEM from three independent experiments carried out.

3.2.8.1.6 Comparison of the alternative pathway activity induced by rHP and rHMMP in properdin depleted serum

Recombinant properdin can restore the alternative pathway activity when the used serum is properdin depleted (Ali *et al*, 2013). The ability of rHP and rHMMP to enhance the alternative pathway activity was tested in properdin depleted serum. To compare the activity of rHP and rHMMP on C3b deposition in DPS, a fixed concentration (10 µg/mL) of rHMMP and rHP was added in GVB/Mg2+/EGTA buffer to different concentrations of PDS on zymosan coated plate. The alternative pathway activity was checked by calculating C3b deposition on the plate. Polyclonal rabbit anti human C3c was used for checking C3b deposited on the plate. One-way ANOVA showed there is no significant increase in C3b fixation induced by rHP compared to rHMMP (Figure 3-18). However, both types of recombinant properdin showed significant increase in C3b deposition compared to DPS only. This result showed that rHMMP augments the alternative pathway in DPS as does rHP. From the results shown in Figure 3-14, Figure 3-15 and Figure 3-18, it is clear that rHMMP could augment the alternative pathway in NHS, wild type mouse serum and DPS.

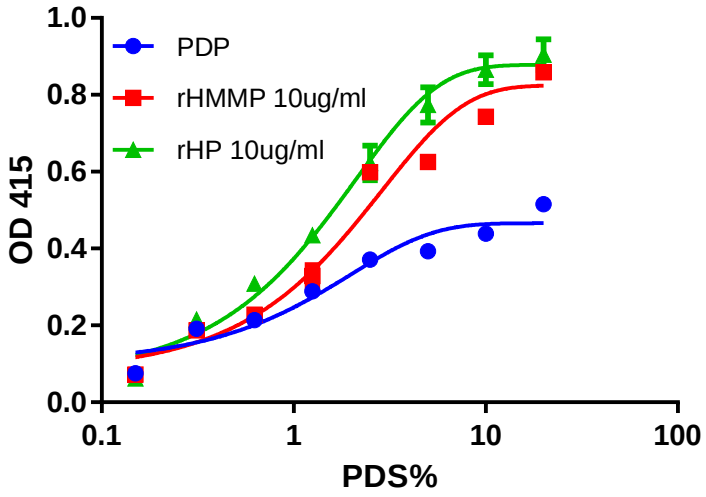


Figure 3-18: A Comparison the alternative pathway of complement activation induced by 10 µg/ml of rHP or rHMMP in PDS
Constant concentration of rHP and rHMMP was added to different concentrations of PDS and diluted in GVB/Mg2+/EGTA buffer. The mixture was added to zymosan coated ELISA plate and C3b deposition was determined. The statistical significance of the amounts of C3 deposition was calculated between the amounts of C3b deposition generated by PDS in presence or absence of rHP or rHMMP by using multiple t test- one per row. One-way ANOVA was used to compare between the activity of rHP and rHMMP. The statistical significance is = ($P<0.05$). Data are the means ± SEM from three independent experiments carried out.

3.2.8.1.7 The activity of different oligomeric forms of recombinant properdin

Properdin could be separated into dimers (P2), trimers (P3), and tetramers (P4) (Smith *et al*, 1984; Pangburn, 1989). The recombinant properdin can aggregate to form high oligomers characterised by highly ability to activate the alternative pathway (Higgins *et al*, 1995; Ferreira, Cortes and Pangburn, 2010). A C3b deposition assay was used to investigate the role of each oligomeric form in alternative pathway activation. Different concentrations of each form were added to a fixed concentration of NHS (2.5% was chosen because this is the concentration at which alternative pathway activity just becomes detectable, i.e. the lower inflection point of the AP_{50} curve, therefore any significant difference could be easily shown when recombinant proteins were added) (Ali *et al*, 2014). The result showed that Pn of both rHP and rHMMP could activate the alternative pathway dependent C3b deposition at very low doses (Figure 3-19), while other forms augment the C3b deposition with higher concentrations. This result confirmed that properdin role in the amplification loop of the alternative pathway mainly belongs to the activity of Pn.

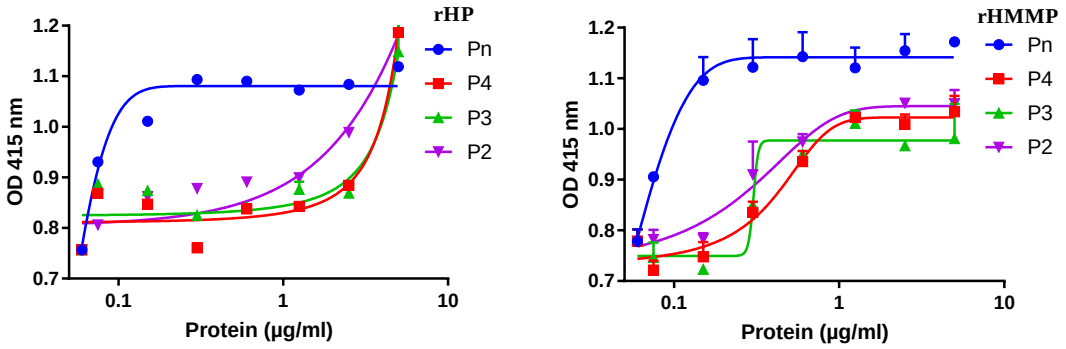


Figure 3-19: determination of C3b deposition induced by different gel filtration elution of properdin
Different concentrations of recombinant human and humanised at N-terminus mouse properdin were added to 2.5% NHS. C3b deposition was determined using anti-human C3c. The highly oligomerized form (Pn) of both proteins added to NHS activates more C3b deposition on zymosan compared to NHS without protein addition or compared to other oligomeric forms of properdin. These differences were statistically significant between NHS vs NHS supplemented with oligomeric forms when they checked by Dunnett's multiple comparisons test, One-way ANOVA. Significant values=$P<0.05$. Data are the means ± SEM from three independent experiments carried out.

3.2.8.1.8 Binding of purified recombinant properdin to C3b:

Properdin stabilises the C3b convertase by binding to C3b in C3bBb, which increases the half-life of the C3 convertase leading to cleavage of more C3 and generating more C3b. This is the amplification loop of the alternative pathway (Fearon and Austen, 1975). To investigate whether recombinant human and chimeric properdin bind to human C3b, an assay was designed, by coating an ELISA microtiter plates with human C3b. This experiment was done as mentioned in section 2.2.2.5.1.2.3. The binding was checked by mouse monoclonal anti polyhistidine. Purified rHP and rHMMP showed clear binding to C3b when they were compared to uncoated wells which also received the same corresponding concentrations of recombinant proteins (Figure 3-20). The half maximum protein concentration which showed half maximum binding (EC_{50}) was counted for both proteins; rHP and rHMMP, which were 3 and 1 µg/ml respectively. This could explain that the C3b binding ability of rHMMP is more than that of rHP. Generally, both rHP and rHMMP could bind to C3b efficiently.

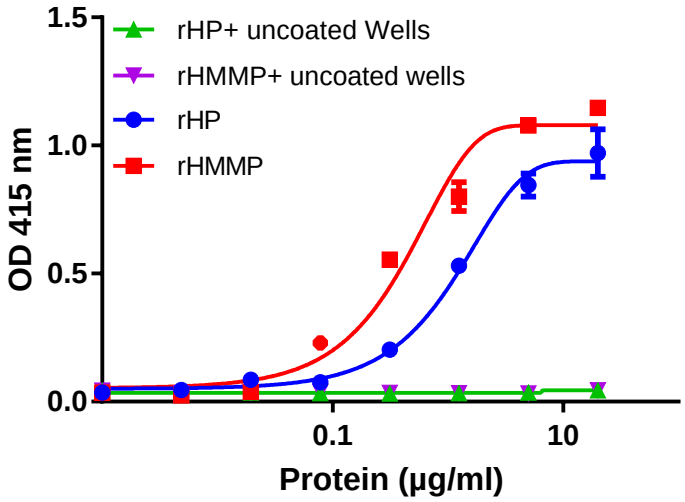


Figure 3-20: Binding of rHP and rHMMP to C3b.
Different serial dilutions of rHP and rHMMP were added to C3b coated plates after blocking the plate with 2.5% BSA. The binding was checked by monoclonal mouse anti poly histidine antibodies. The statistical significance of the C3b binding was calculated between the C3b coated wells and non-coated wells in presence of rHP or rHMMP by using Sidak's multiple comparisons test, one-way ANOVA test. The statistical significance is = ($P<0.05$). Data are the means ± SEM from three independent experiments carried out.

3.2.8.2 Complement assays based on haemolysis tests

The above experiments showed that recombinant properdin could increase C3b deposition and activation of the alternative pathway. To show its role in the terminal pathway, the haemolysis assay was applied to check the complement activity of serum on lysis of different individual RBCs (Sakai 1992). Supplementing serum with different concentrations of properdin and adding to rabbit RBCs can be used to show the role of recombinant properdin on the terminal pathway and MAC production. Rabbit RBCs were chosen because they are less fragile thus, they do not spontaneously lyse without complement activation (Saeed 2016). In addition, these RBCs are susceptible to complement lysis by human or mouse sera because the RBC surface complement regulators or other cell membrane structures do not interfere with fluid phase regulators of serum complement activation preventing RBC opsonisation and MAC lysis (Saeed 2016). Before starting, the sera and recombinant proteins should not be exposure to heat for a long time and all steps should be done on ice as this could decrease the complement or protein activity (Saeed 2016). To activate the alternative pathway, no recognition molecules were needed as it is spontaneously started. Therefore, the RBCs were not sensitised as it is necessary for classical or lectin assays (Lange, Magnadóttir 2003).

To investigate whether haemolysis assays can be initiated through the alternative or lectin pathway, serial dilutions of NHS (with normal amount of MBL) and MBL deficient serum (MBL-) were incubated with rabbit RBCs for an hour under humid conditions (see section 2.2.2.5.1.3). NHS and MBL- showed RBC lysis when GVB/Mg^{2+}/EGTA buffer was used to initiate the alternative pathway (Figure 3-21-A). There is no statistically significant difference in the AH_{50} (the serum concentration required for half maximal haemolysis) (AH_{50}=12.2 and 12.68 respectively) for both kinds of sera.

However, NHS lyses more RBCs when the lectin pathway was activated by adding serial dilutions of these sera in GVB^{++} buffer to mannan coated RBCs compared to MBL- which does not show any difference in the haemolysis (Figure 3-21-B). Here, uncoated RBCs were used as a negative control. RBCs haemolysis was read under OD 540 wavelength. The LH_{50} (the serum concentration required for half maximal haemolysis) was calculated which is 1.5%, confirming that the MAC formation through the lectin pathway is MBL dependant as it is known. Both last results confirmed that haemolysis assays can be done through the alternative and lectin pathway.

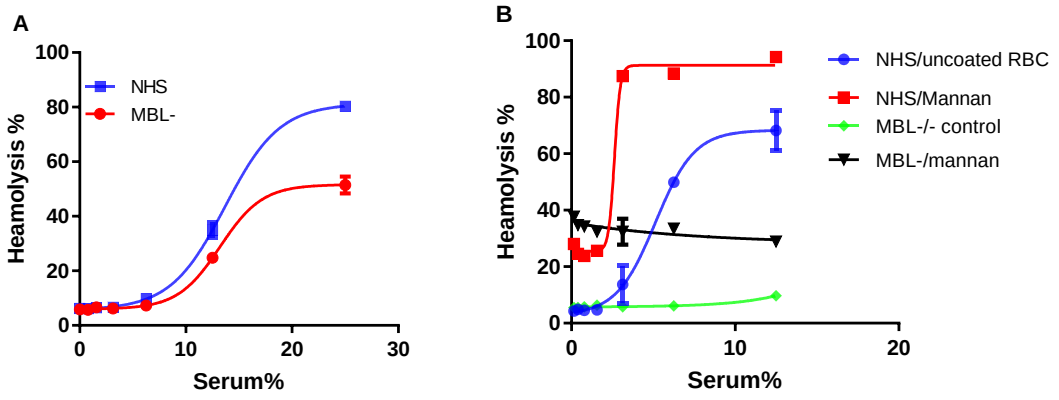


Figure 3-21: Haemolysis assay through the alternative pathway and lectin pathway.
A- NHS and MBL-deficient serum lyse RBCs through the alternative pathway. In a hemolysis assay, different concentrations of NHS and MBL-deficient serum in GVB/Mg2+/EGTA buffer lyse rabbit RBC via the alternative pathway. The AH50 was calculated and fitted to the data. Data are the means ± SEM from three independent experiments carried out.
B- NHS augments RBCs haemolysis through the lectin pathway more than MBL-. In a hemolysis assay, different concentrations of NHS and MBL-deficient serum GVB^{++} buffer lyse rabbit RBC via the lectin pathway. The LH50 was calculated and fitted to the data. Data are the means ± SEM from three independent experiments carried out.

3.2.8.2.1 The role of rHP and rHMMP in terminal pathway activation

In addition to the role of properdin in C3b convertase stabilization, it can do the same for the C5 convertase ((C3b)nBb) (Schwaeble and Reid, 1999). C5 convertase could cleave C5 to C5a and C5b which binds to C6 and 7 thus ending with formation of the MAC. To assess the role of recombinant properdin in the terminal pathway, different concentrations of rHP and rHMMP were added to fixed concentration 6.5% of NHS (6.5% was chosen as it had previously been shown in Figure 3-21-A to be the concentration at which alternative pathway activity just becomes detectable) in GVB/Mg2+/EGTA buffer through the alternative pathway. This mixture was added to U shape microplate and incubated for 1 hour at 37°C under humid conditions and the haemolysis degree (due to the complement activity) was read. RBC lysis was significantly more when rHP was added to NHS compared to rHMMP (Figure 3-22-A). Therefore, the alternative pathway was activated more when rHP was added to NHS compared to rHMMP. Another

experiment was done by using 15% WT mouse serum concentration, which was supplemented by different concentrations of rHP, rHMMP and rMP in GVB/Mg2+/EGTA buffer to initiate the alternative pathway. These amounts were then added to rabbit RBCs and incubated for 1 hour at 37°C under humid conditions to induce RBC haemolysis. 10µg/ml rHMMP shows significantly more lysis than other recombinant properdin (Figure 3-22-B). Here, rHMMP supplemented WT mouse serum lysed more RBCs than the other two kinds of recombinant properdin.

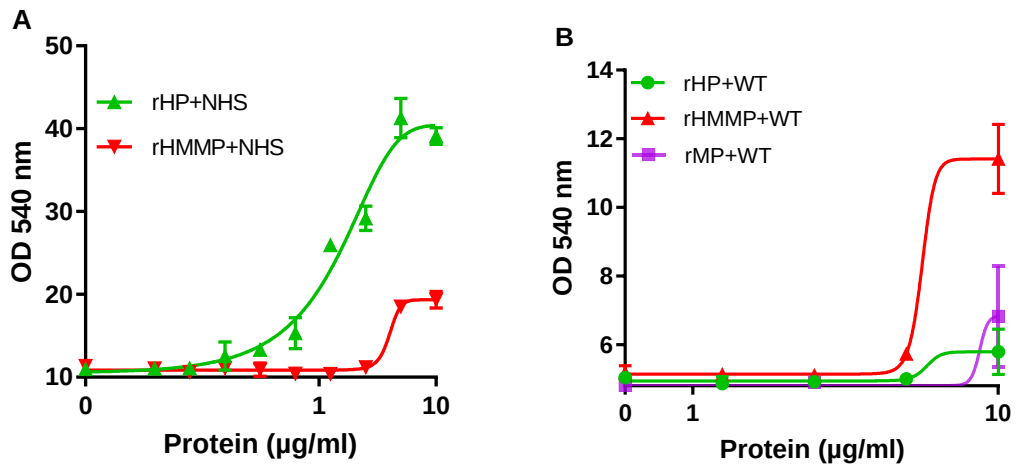


Figure 3-22: Haemolysis assay through the alternative pathway induced by adding rHP and rHMMP to NHS and WT mouse serum.
A: In haemolysis assay, different concentrations of rHP and rHMMP added to 6.5% NHS in GVB/Mg2+/EGTA buffer lyse rabbit RBC via the alternative pathway. The differences between addition of rHP and rHMMP were significant using multiple t test- one per row. Data are the means ± SEM from three independent experiments carried out.
B: In a haemolysis assay, different concentrations of rHP and rHMMP added to 15% WT mouse serum in GVB/Mg2+/EGTA buffer lyse rabbit RBC via the alternative pathway. Tukey's multiple comparisons test, Two way ANOVA test showed that there are significant differences between adding of rHMMP vs rHP or rMP in maximum concentration only. Data are the means ± SEM from three independent experiments carried out.

3.2.9 Summary

Different sets of primers were used to generate mouse properdin humanised at C, N and both termini. These DNA constructs were size confirmed and visualised on 1% agarose. Three chimeric properdin were cloned successfully to mammalian expression vector, pSectag2/hygroB. Recombinant human, mouse and chimeric properdin (mouse properdin humanised at one or both termini) were expressed in mammalian cells. The recombinant proteins were purified to near homogeneity. Gel filtration of the recombinant properdin showed that only rHP and rHMMP were well oligomerized. rHP and rHMMP effectively bind to C3b and enhanced the C3b deposition on zymosan. The oligomeric forms of properdin were tested on zymosan to check their activity. The higher oligomer; Pn showed higher augmentation to the C3b deposition than occurred with other form. In addition, rHP and rHMMP could augment the terminal pathway and increase the formation of the membrane attack complex (MAC) via the terminal pathway, leading to haemolysis of erythrocytes. Therefore, these proteins could be ideal products in overcoming the complement inhibitory roles by asexual blood stages of *P. falciparum*. In the next experiments, rHP and rHMMP were used in assays targeting inhibition of asexual blood stages of *P. falciparum.*

4

Chapter four

Expression of Active CFHR1; Full Length and SCR 3-5

4.1 Introduction

The complement pathways converge with the formation of a C3 convertase (Carroll and Sim, 2011). The Complement Factor H-Related Proteins are five proteins found in plasma (CFHR1, CFHR2, CFHR3, CFHR4 and CFHR5) that can bind to C3b and modify the activity of factor H (Skerka *et al*, 2013). CFHR1 contains five Short Consensus Repeat domains (SCRs) (Skerka, Horstmann and Zipfel, 1991) numbered from 1 to 5 (Figure 1-4). CFHR1 could form dimers in plasma. This function is restricted to the N-terminus (SCR1 and SCR2) (Goicoechea de Jorge *et al*, 2013). The similarities between SCR1 and SCR2 of CFHR1 and SCR6 and SCR7 of factor H is 34% and 42% respectively (Skerka *et al*, 2013). The three C-terminal SCR domains of CFHR1 are mostly identical to SCR19 and SCR20 of factor H (Figure 1-4).

4.1.1 The role of CFHR1

CFHR1 is a soluble competitive inhibitor of factor H that lacks factor I cofactor activity and the C3bBb decay accelerating activity associated with factor H (Timmann, Leippe and Horstmann, 1991). The N-terminus of CFHR1 (SCR 1 and 2) is not involved in C3bBb binding and not required for competitive inhibition of factor H (Fritsche *et al*, 2010a). Therefore, CFHR1 or a polypeptide omitting the N-terminus of CFHR1 has the potential to augment alternative pathway activation by inhibiting factor H (Fritsche *et al*, 2010b; De Jorge *et al*, 2012).

4.1.2 Aims and Objectives:

The aim of this part of my project was to use recombinant CFHR1 or SCR3-5 as an alternative pathway enhancer by inhibiting factor H. This was achieved by the following steps: molecular biology to produce, amplify and define DNA of CFHR1; expression of CFHR1 in both bacterial and mammalian systems; recombinant protein purification; protein characterization and finally checking recombinant protein activity by using complement assays.

4.2 Results

CFHR1 consists of five SCR domains numbered 1 to 5. These domains share some similarities with those of factor H. Therefore, CFHR1 can act as a competitive inhibitor

of factor H and interfere with its activity in down-regulating the alternative pathway (Skerka *et al*, 2013). However, SCR1 and SCR2 of CFHR1 are thought to inhibit the C5 convertase (Heinen *et al*, 2009), so a polypeptide omitting these domains is expected to be a better enhancer of overall complement activation than full-length CFHR1. Therefore full length CFHR1 and SCR3-5 (excluding N terminus) were expressed and checked for their complement activity. Human CFHR1 cDNA contains an open reading frame of 900 base pairs (bp), which encodes 330 amino acids residues. SCR3-5 (208 residues) are encoded by the last 550 bp, (Figure 4-1).

Figure 4-1: Amino acid sequences of human CFHR1 and its SCR domains, all derived from NM_008823.
Reference amino acid sequences of human CFHR1 (derived from NM_002113), arranged by colors as followings: signal peptide grey, SCR1 red, SCR2 green, SCR3 blue, SCR4 yellow, SCR5 purple.

Dr Lynch (University of Leicester, UK), kindly provided the template of cDNA of human CFHR1. Different sets of primers were used to generate full length and SCR3-5 of human CFHR1 (see section 2.2.1.3) using Phusion DNA polymerase. Primers were designed to amplify full length CHFR1 and SCR3-5, with *BamH*I and *Xho*I restriction sites arranged to allow in-frame cloning into the expression vectors (Table 2-3). The PCR products obtained were nearly 900bp and 550bp for the full length and SCR3-5 fragments respectively, as anticipated from the published cDNA sequence (Figure 4-2).

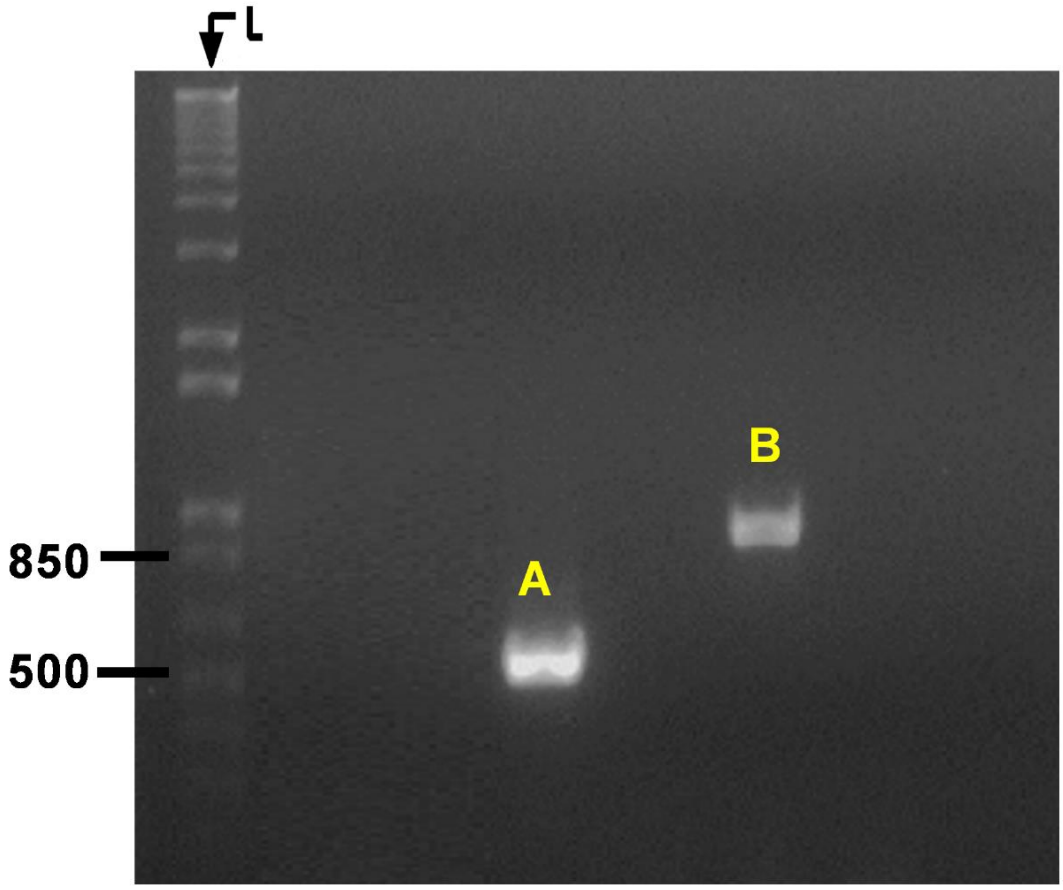


Figure 4-2: Agarose gel electrophoresis of PCR amplified full length and SCR3-5 of human CFHR1. Different sets of primers were used to generate rCFHR1 and rSCR3-5. The products were analysed on the agarose gel. rSCR3-5 CDS show a band of around 550bp (A), while full length CDS show a band of approximately 900bp (B), L= 1kb DNA plus ladder.

4.2.1 Cloning CFHR1 DNA into pGEM-T easy, pRSET/B and pSecTag2/HygroA vectors

pGEM-T easy was used to facilitate cloning of the CFHR1 and SCR3-5 constructs.(Mezei *et al*, 1994) (see section 2.2.1.5.2). pGEM T easy has a single thymidine at both ends, which are complementary to the single nucleotide adenosine overhangs normally added to the end of PCR products by Taq polymerase. Since Phusion DNA polymerase does not

add a single nucleotide extension to the end of PCR products, adenine nucleotides were added by briefly incubating the products with dATP and Taq polymerase (see section 2.2.1.5.1). As explained in chapter 3, the ligated plasmids were transfected into *E.coli* TOP10 competent cells which were used for DNA construct amplification and maintenance (see section 2.2.1.5.4). These cells were plated on LB ampicillin agar plates. Different colored colonies appeared, indicating whether transfection was correct or not. White colonies indicate correct ligation as DNA-vector recombinant containing cells produce white colonies in presence of X-gal, while cells that are not successfully transfected with recombinant DNA (for example only vector) appear bluish (Langley *et al*, 1975; Messing *et al*, 1977).

White colonies were grown in ampicillin supplemented LB broth to get more pGEM-T easy ligated CFHR1 and SCR3-5 constructs. The plasmids were extracted using Wizard plus SV Minipreps (Promega) (see section 2.2.1.5.5). For confirmation, constructs were digested with *BamH*I and *Xho*I restriction enzymes, which cut sites at the ends of CFHR1 and SCR3-5 (see section 2.2.1.5.6). Running the digested samples on a 1% agarose gel showed that the ligation was successful. The obtained bands were 3000 bp for pGEM T easy, 900 bp for CFHR1 or 550 bp for SCR3-5 (Figure 4-3 A and B)

As explained before (see section 2.2.1.4), digested CFHR1-PGEM T easy plasmid was run on agarose gel for purification purposes. CFHR1 and SCR3-5 construct bands were excised by using new sterile small blade without exposing to UV-light for a long time to prevent mutation. A gel extraction kit was used to extract the constructs (see section 2.2.1.4).

Two expression systems were used to express rCFHR1 and rSCR3-5; bacterial and mammalian. The extracted constructs were sub-cloned into a mammalian expression vector; pSectag2/hygroA, which adds a C-terminal histidine tag. These constructs were also sub-cloned into a bacterial expression system; pRSET/B, which adds a histidine tag at the beginning of the constructs. The purification of recombinant proteins depends mainly on these tags. After ligation and transformation into TOP10F', plasmid midipreps were prepared and digested using *BamH*I and *Xho*I restriction enzymes to check success of the ligation.

The resulting products were run on a 1% agarose gel. CFHR1 and SCR3-5 constructs show bands of approximately 900bp and 550bp respectively and pSecTag 2/Hygro A about 5000bp (Figure 4-3 C and D) or pRSET/B around 3000bp (Figure 4-3 E and F).

DNA sequencing for all CFHR1 and SCR3-5 constructs was done in the DNA sequence unit (PNACL) at the University of Leicester to check if there were any mutations through the PCR process or by UV- light exposure. The sequencing results of the constructs show that the cloning was done without mutation (Figure 4-4).

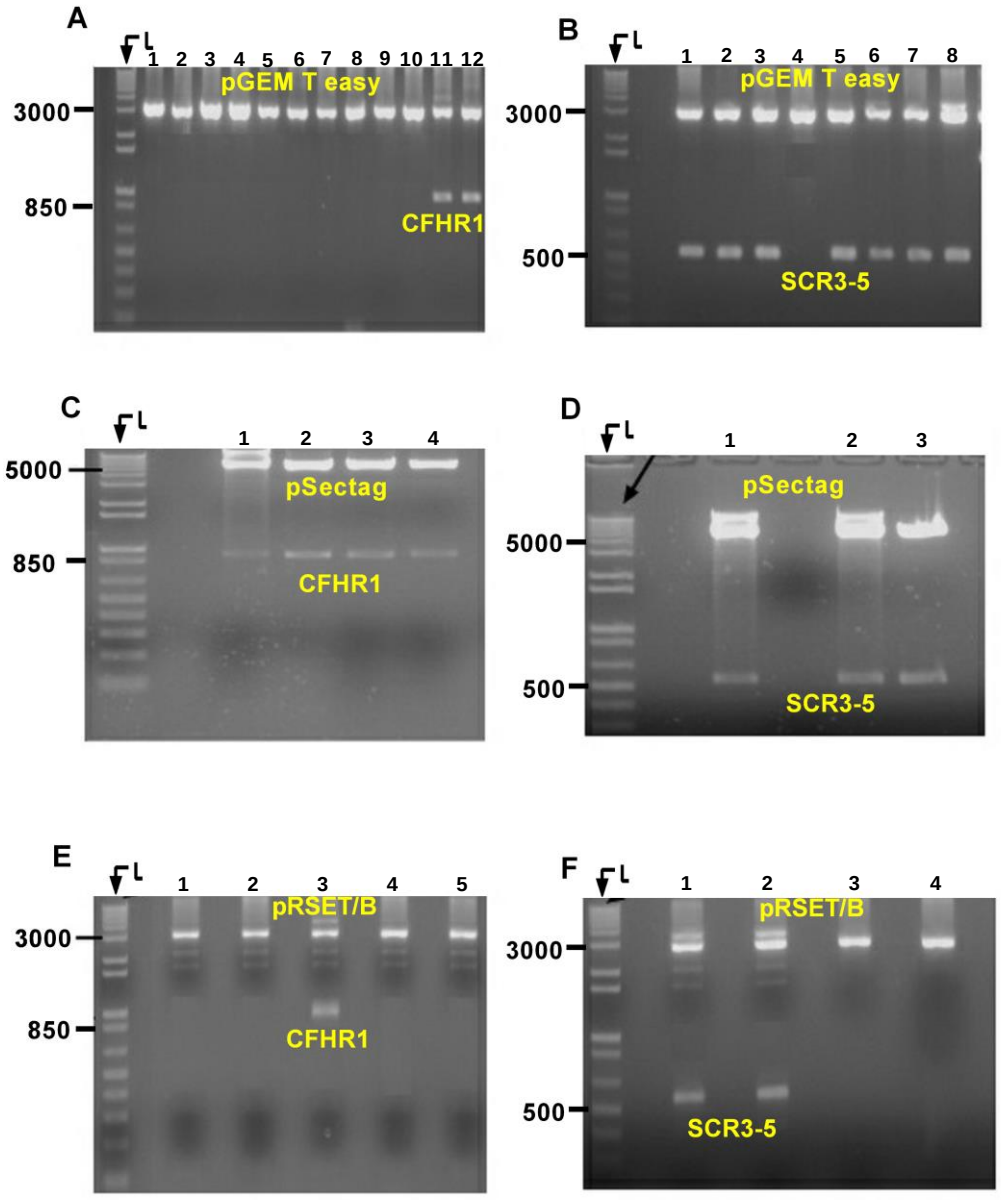


Figure 4-3 Cloning of CFHR1 and SCR3-5 DNA into pGEM-T easy pRSET/B and pSecTag/Hygro A vectors.
Different colonies of Agarose gel analysis of cloning of CFHR1 (A) and SCR3-5 (B) of human CFHR1 constructs into pGEM T easy vector (3018bp). CFHR1 and SCR3-5 CDS show bands of approximately 900bp and 550bp respectively. The DNA constructs were then gel purified and sub-cloned into pSecTag 2/Hygro A (5700bp) (C and D) and pRSET/B (3000bp) (E and F), L= 1kb DNA plus ladder.

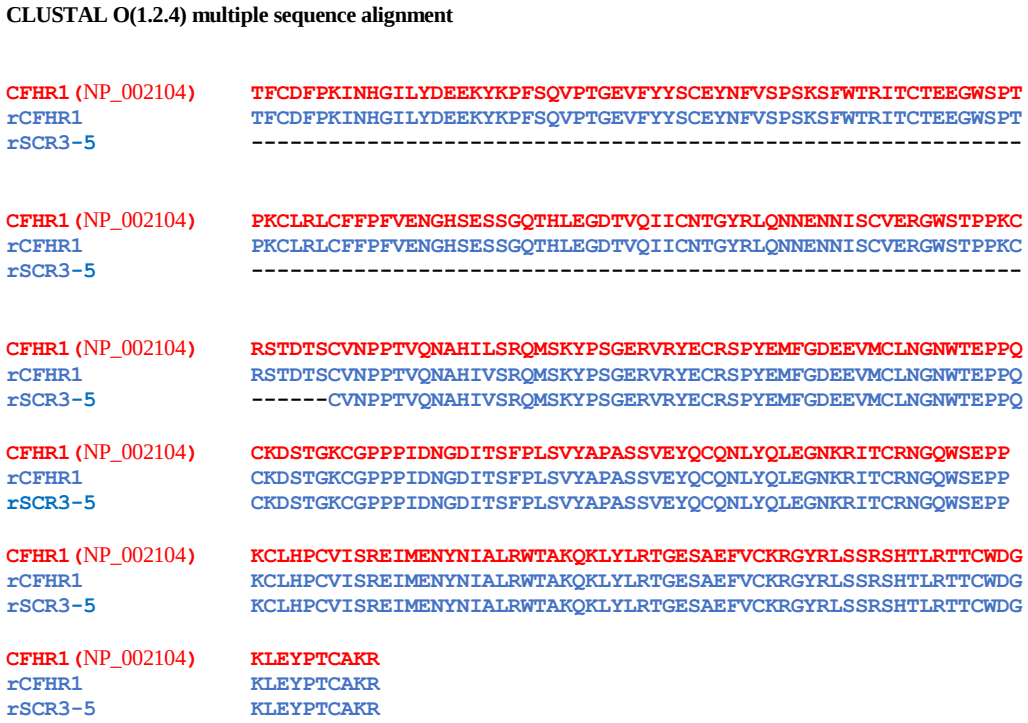


Figure 4-4: Alignment of amino acid sequences of recombinant CFHR1.
The cDNA-derived amino acid sequences determined for full length (blue) and C terminus (blue) were aligned with the reference amino acid sequence of human CFHR1 (red, top line; derived from NP_002104). CLUSTAL O (1.2.1) program was used to align the multiple sequences.

4.2.2 Expression of human CFHR1

pRSET/B ligated CFHR1 constructs were expressed in bacteria (One Shot® BL21(DE3)pLysS chemically competent *E. coli*). Ampicillin and chloramphenicol were used as selective antibiotics and added to LB agar; the culture media for BL21 cells, to prevent growth of non-transfected cells, with a concentration of 50µg/ml and 35µg/ml respectively (see section 2.2.2.2).

Some bacterial clones were selected to check protein expression by dot blot. These clones were grown up in 10 ml of LB broth containing ampicillin and chloramphenicol. When the OD reached 0.6-0.8, 1 mM of IPTG was added to induce expression. After 3-5 hours, bacterial culture was collected and subjected to mechanical destruction (sonication) (see section 2.2.2.1.3.1). After centrifugation, the supernatant and pellets were blotted onto nitrocellulose membrane and then probed with anti-polyhistidine antibodies (Figure 4-5).

No positive clones were shown from the supernatant while the pellets showed varied protein expression. The positive clone was selected for the next protein expression step.

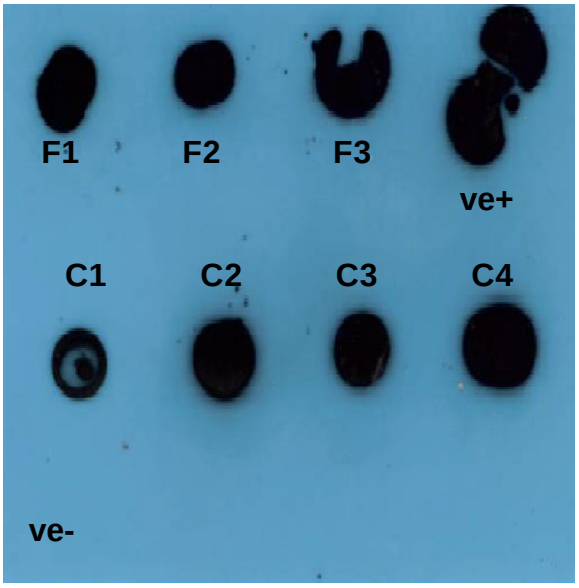


Figure 4-5: Dot blot assay of different clones for expression of recombinant rCFHR1 and rSCR3-5
Screening a number of selected bacterial clones for expression of recombinant rCFHR1 (F) and rSCR3-5 (C). The protein expression was visualized using HRP conjugated monoclonal mouse anti-polyhistidine antibodies. Recombinant N-terminus humanised mouse properdin with histidine linker was used as a positive control (+ve). Pellets from non-transfected BL21(DE3)pLysS cells was used as a negative control (-ve). The positive clones are indicated.

4.2.3 Refolding the recombinant proteins

rCFHR1 and rSCR3-5 when expressed in BL21(DE3)pLysS were insoluble as inclusion bodies. To purify recombinant proteins from inclusion bodies, a refolding protocol was needed, in which bacterial pellets were washed and solubilised then refolded by dialysis (see section 2.2.2.2.1). This protocol was modified from Thomson *et al* (2012).

4.2.4 Purification:

rCFHR1 and rSCR3-5 were purified using His GraviTrap columns (GE Healthcare) (see section 2.2.2.2.2). 350 mM of imidazole was added to the column to elute the proteins from the His GraviTrap column at near homogeneous purity. rCFHR1 and rSCR3-5 were

analysed using SDS-PAGE 10% under reducing conditions and Coomassie stained (Figure 4-6) (see section 2.2.2.4.1). To confirm the results, purified histidine tagged proteins were visualised by Western blotting using monoclonal mouse anti polyhistidine (Figure 4-7) (see section 2.2.2.4.2). The expected bands of approximately 43kDa and 29kDa respectively, were seen.

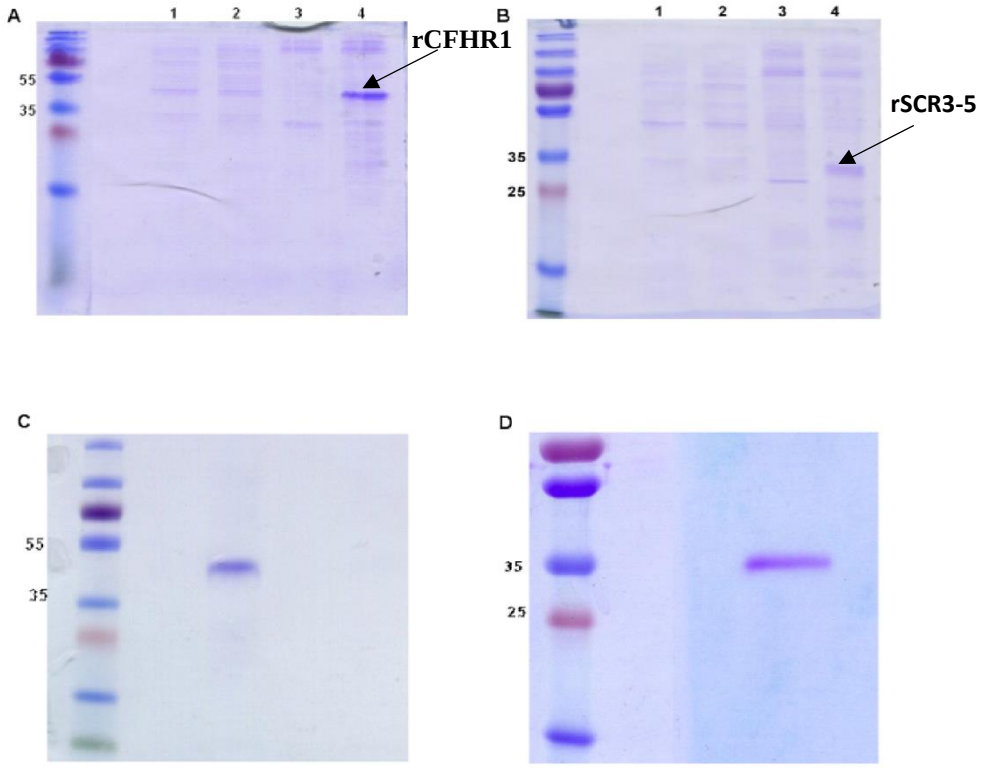


Figure 4-6: SDS-Polyacrylamide gel electrophoresis of rCFHR1 and rSCR3-5 expressed in BL21 (DE3) pLysS.
20µg of the recombinant proteins was separated in 10% polyacrylamide gel under reducing conditions and stained with Coomassie Blue. **A**: Full length of recombinant CFHR1 expressed in *E. coli* is insoluble. After expression, cells were lysed and separated into lysates and insoluble pallets as followings; 1- Supernatant of non-transfected BL21 as a negative control. 2- Supernatant of BL21 expressing rCFHR1. 3- Pellets of non-transfected BL21 as a negative control. 4- Pellets of BL21 expressing rCFHR1 which shows that rCFHR1 band is approximately 43 kDa. **B**: SCR3-5 of recombinant CFHR1 expressed in *E. coli* is insoluble also. As with rCFHR1, after expression, *E. coli* cells were lysed and separated into lysates and insoluble pallets as following; 1- Supernatant of non-transfected BL21 as a negative control. 2- Supernatant of BL21 expressing rSCR3-5. 3- Pellets of non-transfected BL21 as a negative control. 4- Pellets of BL21 expressing rSCR3-5, which shows that rSCR3-5 band is approximately 28 kDa. **C and D**: SDS gel of purified rCFHR1 and rSCR3-5: Purified rCFHR1 (C) or rSCR3-5 after Nickel column purification was run on SDS-PAGE and show single bands of approximately 43kDa and 28KDa respectively.

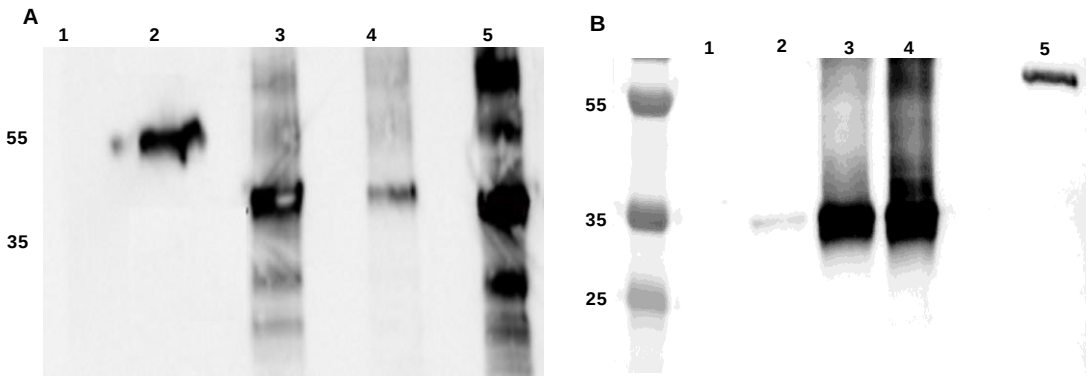


Figure 4-7: Western blotting analysis of rCFHR1 and rSCR3-5.
1µg of Nickel column purified rCFHR1 (A) and rSCR3-5 (B) were separated on SDS polyacrylamide gel then transferred onto nitrocellulose membrane. The blot was probed with HRP conjugated monoclonal mouse anti-polyhistidine antibodies. These samples were transferred as a following**: A**: (1) Non-transfected BL21 as a negative control. (2) Recombinant human properdin, kindly provided by Dr. Sadam Yaseen, as a positive control. (3) Pure protein. (4) Washing. (5) Flow through. **B**: (1) Non-transfected BL21 as a negative control. (2) Washing. (3) Pure protein. (4) Flow through. (5) Recombinant human properdin, kindly provided by Dr. Sadam Yaseen, as a positive control. Western blotting of pure recombinant proteins shows single bands of rCFHR1 and rSCR3-5 at approximately 43KDa and 29 KDa respectively.

4.2.5 Binding assays

4.2.5.1 Binding of rCFHR1 and rSCR3-5 to heparin

Heparin is a surface structure of host cells (Meri et al, 2013), and its ability to bind factor H might help to protect the cells from complement attack. The heparin binding capacity of rCFHR1 and rSCR3-5 were tested as a quick and easy indicator of whether the protein was correctly folded. Based on that serial dilutions of rCFHR1 and rSCR3-5 in TBS buffer were added to heparin coated plates after blocking the plate, protein binding was detected using monoclonal mouse anti polyhistidine conjugated with HRP (Figure 4-8). These proteins showed dose-dependent binding to heparin which indicates that the proteins are probably correctly refolded. The EC_{50} was 0.17 µM and 0.11 µM for rCFHR1 and rSCR3-5 respectively. It was concluded that rCFHR1 and rSCR3-5 were efficiently bound to heparin and correctly refolded.

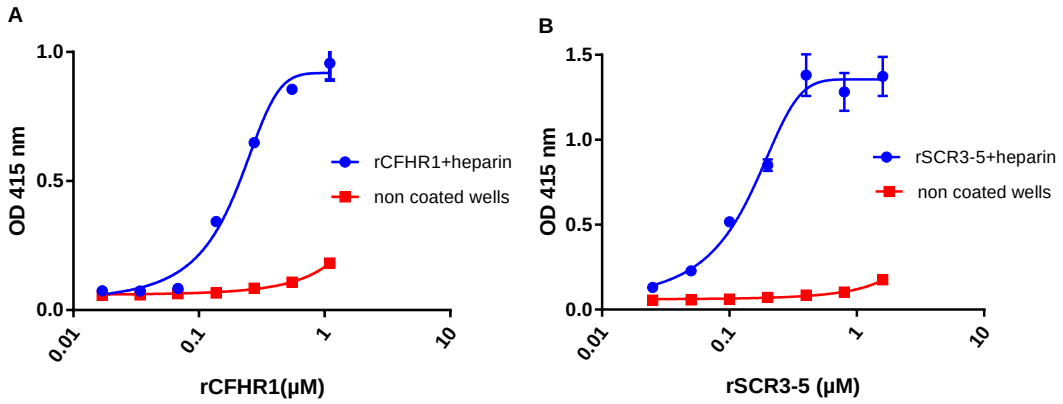


Figure 4-8: Binding of rCFHR1 and rSCR3-5 to heparin.
Different serial dilutions of rCFHR1 (**A**) and rSCCR3-5 (**B**) were added to heparin coated plates (25 µg/ml in PBS) after blocking the plate with 1% BSA. The binding was checked by monoclonal mouse anti poly histidine antibodies. Heparin binding was statistically different when multiple t test- one per row was used between rCFHR1 or rSCR3-5 vs non coated wells. Data are the means ± SEM from three independent experiments carried out.

4.2.5.2 Factor H inhibitory effect of rCFHR1 or rSCR3-5 to heparin

Factor H can bind to heparin in two places namely SCR7 and 20 (Perkins, Fung and Khan, 2014). SCR5 of CFHR1 is 100% similar to SCR20 of factor H and SCR2 of CFHR1 is 41% similar to SCR7 of factor H (Martínez-Barricarte *et al*, 2012). A competition ELISA assay was used to investigate whether factor H can inhibit the binding between rCFHR1 or rSCR3-5 and heparin. A constant concentration of recombinant full length CFHR1 or SCR3-5 (0.5 µM) was mixed with serial dilutions of factor H and the mixtures incubated on a heparin-coated microtiter plate. The binding was determined using monoclonal mouse anti-poly histidine antibodies. Factor H inhibited the binding of both recombinant proteins to heparin binding in a dose-dependent manner (Figure 4-9). This inhibition may be because there is competition between heparin binding sites in both factor H and recombinant proteins. The inhibition was partial as factor H might not block all CFHR1 or SCR3-5-heparin binding sites.

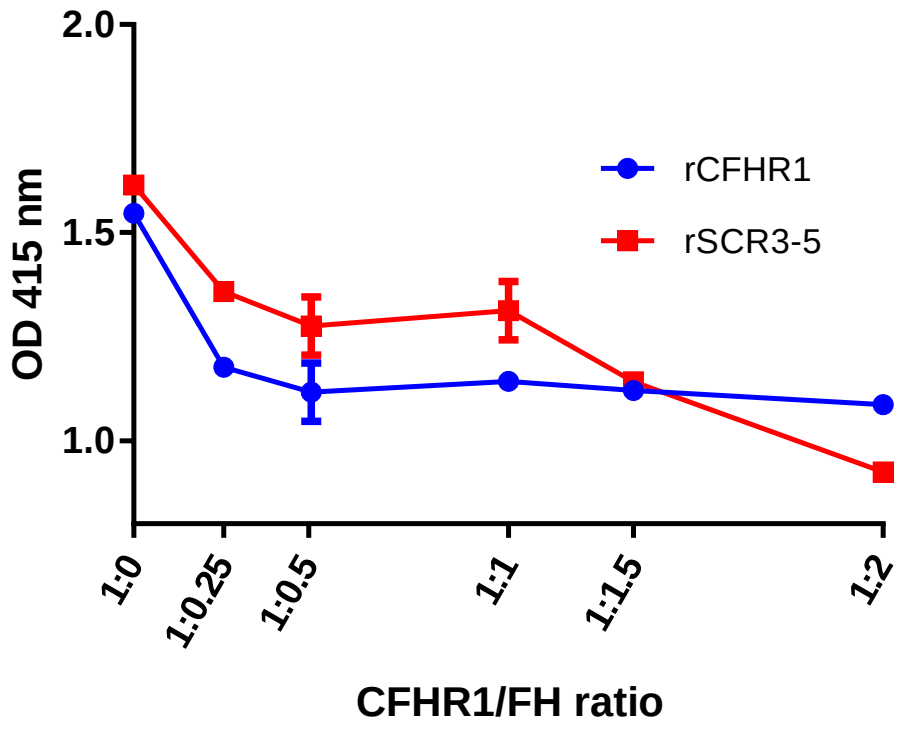


Figure 4-9: Competition between factor H to rCFHR1 and rSCR3-5 on heparin binding sites
Different serial dilutions of factor H were added to 0.5 µM of rCFHR1 or rSCR3-5 on a heparin coated plate (25 µg/mL in PBS) after blocking the plate with 1% BSA. The binding was checked by monoclonal mouse anti poly histidine antibodies. Statistically significant difference (One sample t test, $P<0.05$) was seen between the wells received only rCFHR1 or rSCR3-5 vs the wells received different factor H concentrations. Data are the means ± SEM from three independent experiments carried out.

4.2.5.3 Binding of purified rCFHR1 and rSCR3-5 to C3b:

CFHR1 can bind to C3b and act as a competitive inhibitor of factor H on its binding sites on C3b molecules (Hannan *et al*, 2016). To investigate whether rCFHR1 and rSCR3-5 could bind to human C3b, an assay was designed, by coating an ELISA microtiter plates with human C3b. This experiment was done as mentioned in section 2.2.2.5.1.2.3. The binding of rCFHR1 and rSCR3-5 was determined using mouse monoclonal anti polyhistidine. Both recombinant proteins showed clear binding to C3b when they were compared to uncoated wells (that also received the same corresponding concentrations of recombinant proteins) (Figure 4-10).

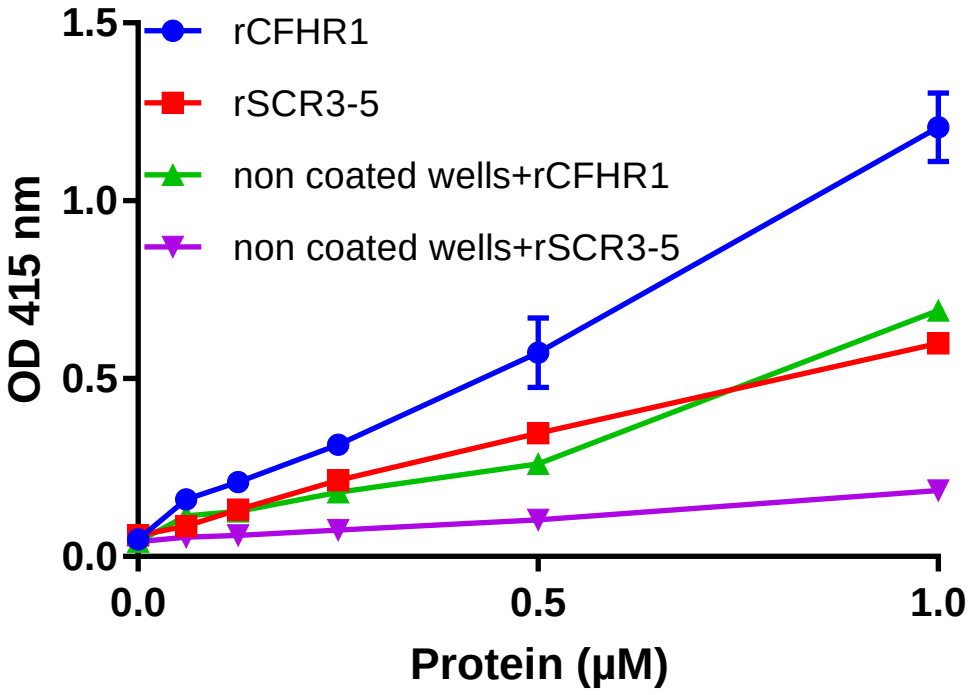


Figure 4-10: Binding of rCFHR1 and rSCR3-5 to C3b.
Different serial dilutions of rCFHR1 and rSCR3-5 were added to C3b coated plates (25µg/ml in PBS) after blocking the plate with 2.5% BSA. The binding was checked by using monoclonal mouse anti poly histidine antibodies. The binding of both kinds of recombinant CFHR1 on C3b is significantly distinguishable from C3b uncoated wells which is considered as a negative control. The significant P value was statistically calculated by using Sidak's multiple comparisons test, One Way ANOVA between C3b coated and non coated wells, Significant values=P<0.05. Data are the means ± SEM from three independent experiments carried out.

4.2.5.4 Competitive inhibitory effect between factor H to rCFHR1 or rSCR3-5 on C3b

The main C3b binding sites in factor H are SCR19 and 20 (Hannan *et al*, 2016), which is also required for regulating the complement activity mediated by factor H that is located within SCR1-4 (Wu *et al*, 2009) and SCR6-10 (Sharma and Pangburn, 1996). SCR4 and SCR5 of CFHR1 show high similarity to the C-terminus of factor H SCR19 and 20 while SCR1-2 of CFHR1 show less similarity to SCR6 and SCR7 of factor H (Skerka *et al*, 2013). The similarities between SCR domains of CFHR1 and factor H could lead to competition between these complement proteins for C3b binding. A competition assay was devised to determine whether factor H could inhibit the binding of rCFHR1 and rSCR3-5 to C3b. Serial dilutions of factor H were added to 0.5µM recombinant proteins

in different ratios, and incubated ELISA plates were coated with 10 µg/mL of C3b. Protein binding was checked using monoclonal mouse anti-polyhistidine antibodies. Factor H inhibited the binding of both recombinant proteins to C3b (Figure 4-9). Increasing amounts of factor H affected rCFHR1 and rSCR3-5 binding in a dose-dependent manner.

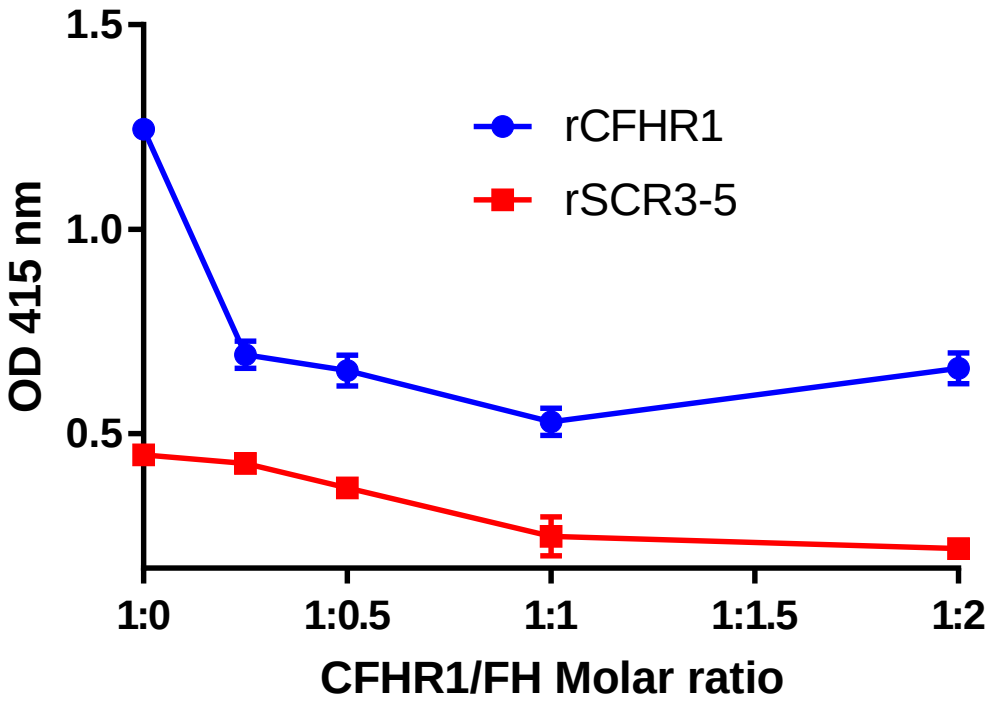


Figure 4-11: Competition between factor H to rCFHR1 and rSCR3-5 on C3b binding sites
Different serial dilutions of factor H were added to 0.5 µM of rCFHR1 and rSCR3-5 on a C3b coated plate (10 µg/mL in PBS) after blocking the plate with 1% BSA. The binding was checked by adding monoclonal mouse anti poly histidine antibodies. Statistically significant differences were found between the wells receiving rCFHR1 or rSCR3-5 only vs the wells receiving both recombinant proteins plus different concentrations of factor H using one sample t test, $P<0.05$. Data are the means ± SEM from three independent experiments carried out.

4.2.6 Complement assays induced on ELISA plate

4.2.6.1 Complement alternative pathway activation by rCFHR1 and rSCR3-5

CFHR1 can increase C3b deposition because it acts as a competitive inhibitor of factor H on its binding sites on C3b molecules and also because it lacks factor I cofactor activity and the C3bBb decay accelerating activity associated with factor H (Hannan *et al*, 2016).

Therefore, rCFHR1 and rSCR3-5 were tested for their ability to augment activation of the alternative pathway. These recombinant human proteins increase the alternative pathway driven C3b deposition (Figure 4-12 A and C), reducing the AP_{50} from 4% to approximately 1.2% in the case of rCFHR1 and from 4% to 2.2% when rSCR3-5 was used (Figure 4-12 B and D).

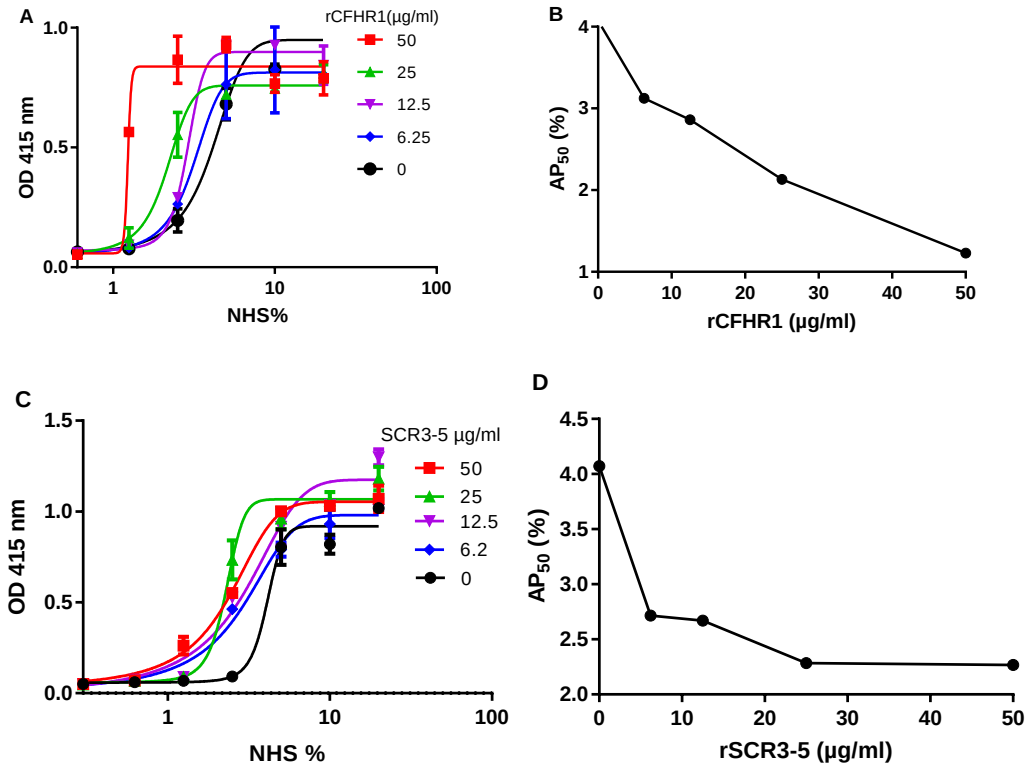


Figure 4-12: The alternative pathway of complement activation in normal human serum supplemented with different concentrations of rCFHR1 and rSCR3-5.
A and C: Different concentrations of rCFHR1 (**A**) and rSCR3-5 (**C**) were added to serially diluted NHS in GVB/Mg2+/EGTA buffer. Then these mixtures were added to a zymosan coated plate. C3b deposition was determined using polyclonal rabbit anti-human C3c. **B and D:** Sigmoidal curves were fitted to the data and the AP_{50} was calculated. Data are the means ± SEM from three independent experiments carried out.

4.2.7 Haemolysis assays demonstrate the role of rCFHR1 and rSCR3-5 in terminal pathway activation.

As can be seen in Figure 4-12, rCFHR1 and rSCR3-5 can augment C3b fixation and increase C3b deposition, leading to activation of the amplification loop of the alternative pathway. Whether this results in activation of the terminal complement pathway and production of MAC can be determined using haemolysis assays, which measure the ability of serum to lyse heterologous RBCs (Sakai, 1992). To check the effect of rCFHR1 and rSCR3-5 on complete complement activity, haemolysis assays were done using rabbit RBCs. Serum was supplemented with different concentrations of rCFHR1 and rSCR3-5 and added to rabbit blood cells to show the role of these proteins on the terminal pathway.

4.2.7.1 Role of rCFHR1 and rSCR3-5 in rabbit RBC haemolysis through the alternative pathway

CFHR1 could inhibit C5 convertase thus the MAC production (Heinen *et al*, 2009). To show the role of recombinant types of CFHR1 on terminal pathway activation, serial dilutions of rCFHR1 and rSCR3-5 were added to a fixed concentration of 6.5% NHS in GVB/Mg2+/EGTA buffer to initiate RBCs lysis through the alternative pathway. The experiment was done under humid conditions and incubated for 1 hour at 37°C and the haemolysis degree was read. Surprisingly, both recombinant proteins, rCFHR1 and rSCR3-5, significantly activated the terminal pathway and induced RBC lysis (Figure 4-13). However, there are no statistical differences between rCFHR1 vs rSCR5. It could be said that even if native CFHR1 inhibits the terminal pathway, both rCFHR1 and rSCR3-5 enhance MAC formation.

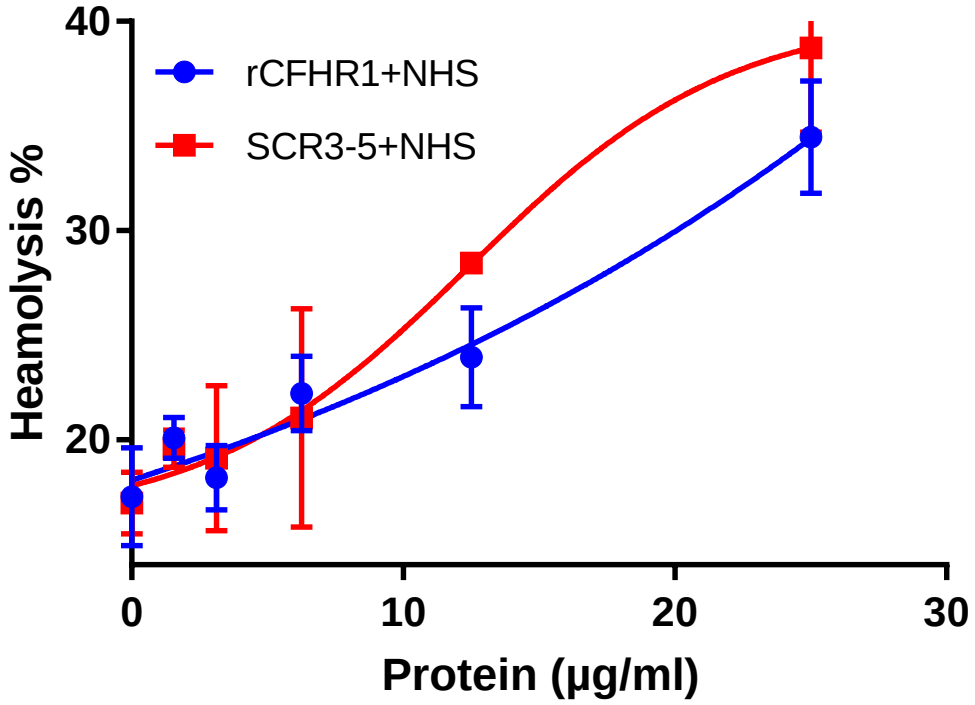


Figure 4-13: Haemolysis assay induced by adding rCFHR1 or rSCR3-5 through the alternative pathway.
In a hemolysis assay, with different concentrations of rCFHR1 or SCR3-5 added to 6.5% normal human serum using the alternative pathway initiating rabbit erythrocytes, the addition of recombinant proteins stimulates more the RBC lysis. The haemolysis caused by adding NHS only was statistically different from rCFHR1 or rSCR3-5 using one sample t test, $P<0.05$. However, multiple t test- one per row showed that there is no significant differences between adding rCFHR1 vs adding rSCR3-5. Data are the means ± SEM from three independent experiments carried out.

4.2.7.2 Role of rCFHR1 and rSCR3-5 in haemolysis of rabbit RBC initiated through the lectin pathway

To see whether these proteins could augment the terminal pathway when the lectin pathway was activated, another experiment was done by initiating RBC lysis through the lectin pathway. RBC were coated with mannan to drive the lectin pathway (Suankratay *et al*, 1998). Serial dilutions of rCFHR1and rSCR3-5 were mixed with NHS (MBL sufficient serum) to a final serum concentration of 1.5% in GVB^{++} buffer (the LH_{50} was calculated in section 3.2.8.2). Then the mixtures were added to rabbit RBCs and incubated for 1 hour at 37°C under humid conditions to induce RBC lysis (Figure 4-14). The recombinant proteins had no effect whatsoever on uncoated RBCs, since they do not induce lectin pathway activation and the low serum concentration used does not permit

alternative pathway activation. rCFHR1 and rSCR3-5 both induced haemolysis of mannan-coated RBCs. rSCR3-5 appeared to lyse the RBC more completely than rCFHR1. At the highest concentration, rSCR3-5 gave 38% lysis, rCFHR1, only 26%. Two-way ANOVA showed significantly higher lysis with SCR3-5 at two concentrations; 50 µg/ml and 25 µg/ml. LH_{50} for rSCR3-5 is slightly higher than that for rCFHR1. These results are slightly surprising, given that rCFHR1 is thought to act mainly on C3bBb. There are two possible interpretations for that; first, the amplification loop of the alternative pathway is active at low serum concentrations, even though initiation is effectively blocked, and the effect of rCFHR1 is attributable to its known activity on the alternative pathway C3 convertase, or; second, that CFHR1 has a previously undescribed effect on the lectin pathway. The near identical LH_{50} values suggest that rCFHR1 and rSCR3-5 use the same mechanism to augment complement activation, while the lower absolute haemolysis observed with rCFHR1 might result from its reported ability to inhibit the C5 convertase.

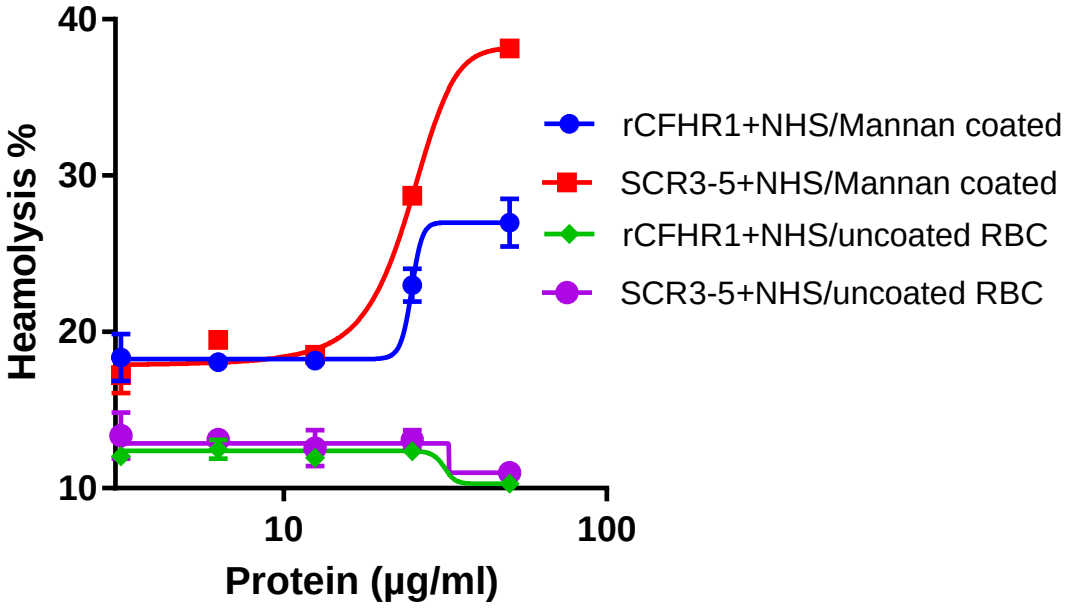


Figure 4-14: Haemolysis assay induced by adding rCFHR1 and rSCR3-5 through the lectin pathway
In a haemolysis assay, different concentrations of rCFHR1 and SCR3-5 were added to normal human serum (end concentration = 1.5%) in GVB^{++}. The mixtures were used to lyse mannan-coated rabbit RBC, which activate the lectin pathway via MBL. Uncoated RBC were used as a negative control. One-way ANOVA (with Sidak's multiple comparisons test) showed that the differences between the coated and non-coated RBCs were significant in both cases ($P<0.05$). The differences between the haemolysis induced by adding rCFHR1 and rSCR3-5 was tested using Two-way ANOVA (Sidak's multiple comparisons test). Data are means ±SEM of duplicates and are representative of three experiments.

4.2.8 Summary

CFHR1 and SCR3-5 constructs were produced and successfully cloned into the bacterial expression vector, pRSETB, then transfected into BL21(DE3)pLysS chemically competent *E. coli*. The proteins were insoluble and expressed in inclusion bodies. Therefore, they were solubilised and refolded before purification by nickel affinity chromatography. The refolded proteins bound to heparin, a known ligand for CFHR1 (and FH), indicating that they were active and correctly folded. Another result showed that factor H can inhibit the binding of both recombinant proteins to heparin. The proteins were used in complement assays to determine their activity and roles in complement pathway activation. Both recombinant proteins can enhance C3b deposition on zymosan-coated plate, as they act as competitive inhibitors of factor H. Thus, CFHR1 can enhance the alternative pathway activation by inhibiting factor H. In addition, these recombinant CFHR1 forms significantly activate the terminal pathway and induced RBC lysis by MAC activity. In conclusion, these proteins can compete with factor H and show augmentation for the alternative pathway and are ready to use in the next *P. falciparum* asexual blood stages inhibition assays.

5

Chapter five

The Role of Recombinant Properdin and CFHR1 on Plasmodium falciparum Asexual Blood Stages

5.1 Introduction

Rosa *et al* (2016) used immunofluorescence microscopy and western blotting to demonstrate that factor H and CFHR1 can bind to asexual blood stages. Asexual blood stages of *P. falciparum* express the *P. falciparum* surface proteins PfGAP50 and Pf92, which can bind factor H in a way that can induce the cofactor activity of factor H (Kennedy *et al*, 2016), thus inhibiting the alternative pathway.

In addition, Pn (the highly oligomerized form of recombinant human properdin) can protect mice from *Neisseria meningitidis* through augmenting the alternative pathway by stabilising C3bBb (Ali *et al*, 2014). Recombinant properdin could overcome the ability of *N. meningitidis* to down regulate the alternative pathway by recruitment of factor H. This role could mainly be because Pn can be 5-10 folds more active than native serum properdin (Ali *et al*, 2014). Two forms of properdin were expressed and then functionally checked on the complement activation. These tests showed that ability of recombinant proteins to restore the alternative and the terminal pathways. In this chapter, these proteins were used to augment the complement activation on asexual blood stages of *P. falciparum*.

CFHR1 could inhibit factor H-C3b binding. This inhibition relate to the similarities between the C-terminal parts of CFHR1 and factor H (Skerka, Horstmann and Zipfel, 1991). CFHR1 is not associated with the factor I cofactor activity and the C3bBb decay accelerating activity associated with factor H (Timmann, Leippe and Horstmann, 1991). However, SCRs 1 and 2 of CFHR1 are not involved in C3bBb binding and not required for competitive inhibition of factor H binding to the cell surface (Fritsche *et al*, 2010). It was shown that C3b and C5b-9 deposition can be increased by supplementing serum with recombinant full length or recombinant SCR3-5 of CFHR1 (Figure 4-12, Figure 4-13 and Figure 4-14). CFHR1 or a polypeptide omitting N-terminus of CFHR1 has the potential to augment alternative pathway activation and inhibit factor H which can be activated during *P. falciparum* infections.

The hypothesis, on which this part is based, is that these recombinant complement regulatory proteins (human, chimeric properdin, human CFHR1 and SCR3-5) could augment the complement activation on asexual blood stages of *P. falciparum* because they directly counteract all the proven and likely mechanisms that the organism uses to

evade complement activation. The experiments could be classified into two groups; *in vitro* and *ex vivo*. *In vitro* experiments involve binding and C3b deposition while *ex vivo* experiments include growth assays, C3b and C5b-9 deposition on live asexual blood stages.

5.2 Results

5.2.1 *In vitro* experiments:

The last *in vitro* experiments described in chapter 3 showed that recombinant properdin could bind to C3b (Figure 3-20) and enhance the alternative (Figure 3-13 and Figure 3-14) and terminal pathways activation (Figure 3-22). In chapter 4, I showed that rCFHR1 and rSCR3-5 can bind to C3b (Figure 4-10) and augment the complement pathways (Figure 4-12 and Figure 4-13). The next step was to test the activity of these recombinant proteins on asexual blood stages of *P. falciparum*. Different kinds of *in vitro* experiments were done including binding and C3b deposition assays.

5.2.1.1 Direct binding of recombinant proteins to schizonts

Various researchers have suggested that properdin can augment complement activation by binding directly to pathogens (Spitzer *et al*, 2007). However, this mechanism is disputed by other researchers, who reported that no direct binding between properdin and the microorganisms is needed to augment complement activation (Moticka, 2015). Asexual blood stages of *P. falciparum* use factor H to evade complement killing and down regulate the alternative pathway (Rosa *et al*, 2016). Kennedy *et al.* (2016) mentioned that serum CFHR1 can bind to merozoites. To check whether rHP, rHMMP, rCFHR1 and rSCR3-5 were bound directly to isolated schizonts, different concentrations of these proteins were added to a plate coated with schizonts. After blocking the plate with 1% of BSA, the binding was probed with monoclonal mouse anti polyhistidine conjugated with HRP. rHP and rHMMP do not show any kind of binding to schizonts (Figure 5-1). rCFHR1 and SCR3-5 showed strong binding to schizonts (Figure 5-2). The EC_{50} was 0.97 µM and 1.5 µM for rCFHR1 and rSCR3-5 respectively.

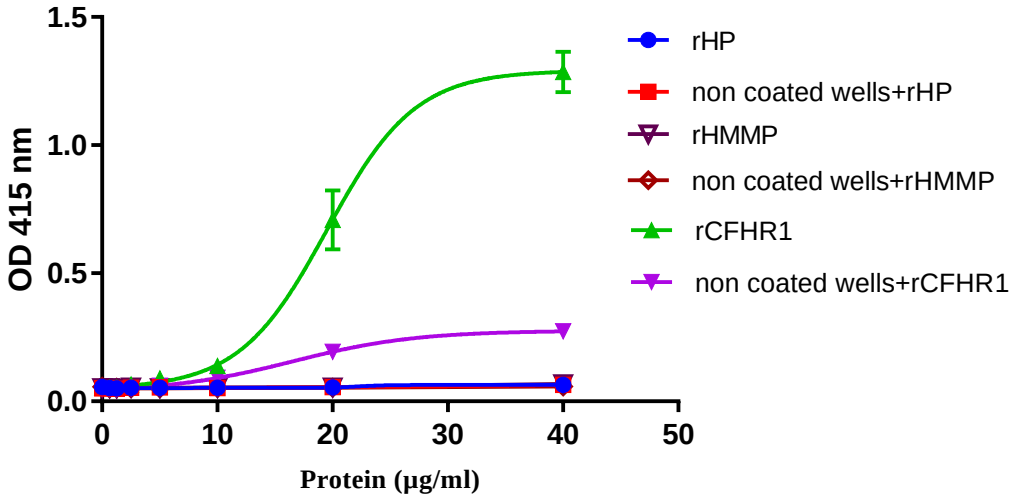


Figure 5-1: Binding of properdin to schizonts
Binding of recombinant properdin was checked by adding different concentrations of rHP and rHMMP were to schizont coated plate in GVB/Mg^{2+}/EGTA buffer after blocking the plate with 1% BSA. rCFHR1 was used as a positive control. The binding was detected by monoclonal mouse anti polyhistidine. No binding between both types of properdin and schizonts was observed. Data are the means ± SEM from three independent experiments carried out.

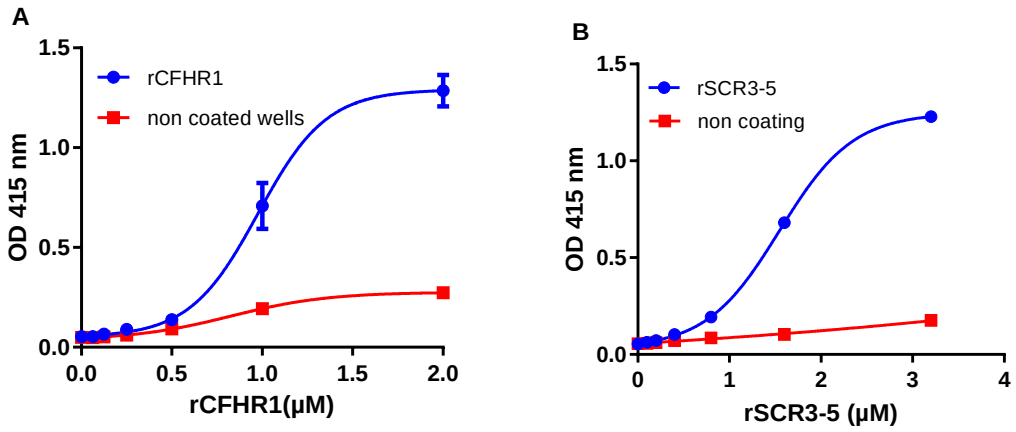


Figure 5-2: Binding of rCFHR1 and rSCR3-5 to schizonts of P. falciparum.
Serial dilutions of rCFHR1 (**A**) and rSCR3-5 (**B**) were added to schizont coated plates after blocking the plate with 1% BSA. The binding was checked by monoclonal mouse anti poly histidine antibodies. Statistical significance difference was checked using multiple t test – one per row ($P<0.05$) between non schizont coated wells vs coated wells. Data are the means ± SEM from three independent experiments carried out.

5.2.1.2 Competition between factor H and rCFHR1 or rSCR3-5 for schizont binding

We found that full length and SCR3-5 of rCFHR1 can bind efficiently to schizonts (see section 5.2.1.1). It has previously been shown that rCFHR1 and rSCR3-5 compete with

factor H for heparin and C3b binding (Figure 4-9 and Figure 4-11). The similarities between SCRs of CFHR1 and factor H could cause a competitive inhibition on schizont binding. In order to look at the competition of CFHR1 and factor H on binding to schizonts, different concentrations of factor H were added to 0.5 µM rCFHR1 or rSCR3-5. This mixture then was added to schizont coated plate. The binding of recombinant proteins was checked using monoclonal mouse anti-polyhistidine. The different concentrations of factor H compete with fixed concentration of rCFHR1 and rSCR3-5 on binding to schizonts (Figure 5-3). Factor H showed statistically significant differences ($p<0.05$) in the ability to compete with the two proteins. Factor H inhibits binding of rCFHR1 to schizonts more than rSCR3-5.

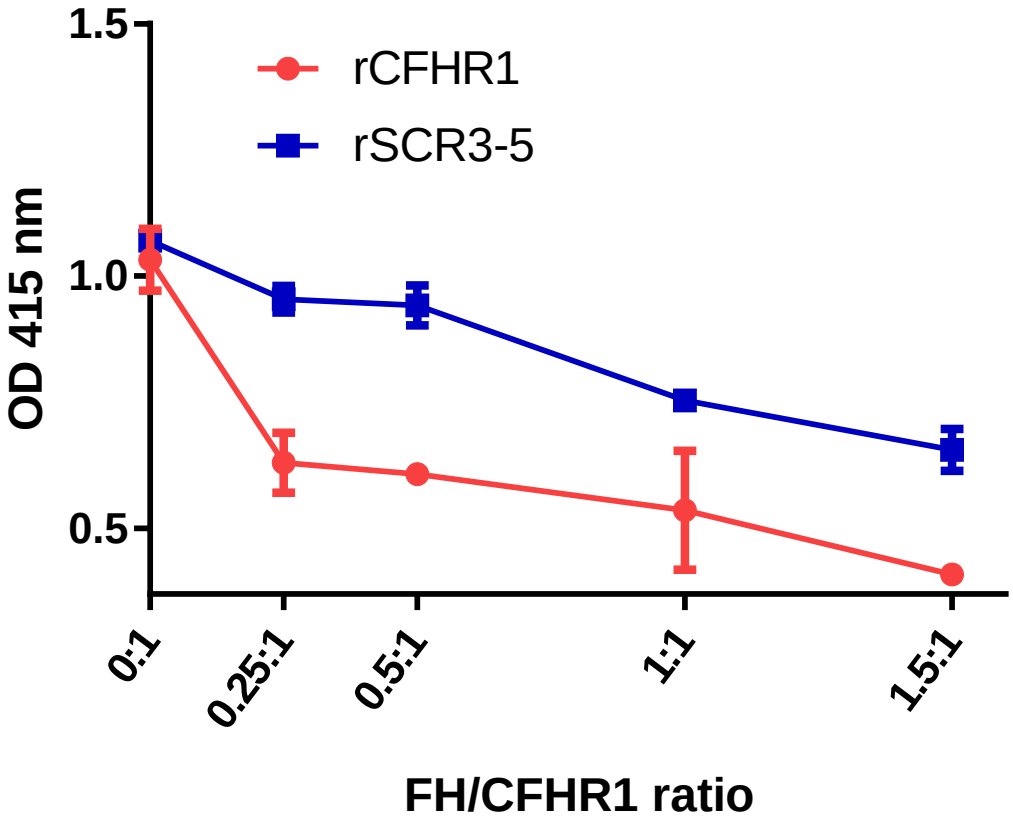


Figure 5-3: Competition of factor H to the binding of rCFHR1 and rSCR3-5 to schizonts
Different serial dilutions of factor H were added to 0.5 µM rCFHR1 or 0.7 µM rSCR3-5 on a schizont coated plate after blocking the plate with 1% BSA. The binding of the recombinant protein was checked using monoclonal mouse anti poly histidine antibodies. Statistical significant differences (one sample t test, $P<0.05$) between factor H only added wells vs rCFHR1 or rSCR3-5 added wells were statistically different. Data are the means ± SEM from three independent experiments carried out.

5.2.1.3 C3b deposition on schizonts

The complement alternative pathway starts when C3 in plasma is hydrolysed by water. Complement activation on blood stages of *P. falciparum* starts when merozoites rupture RBCs and invade new RBCs (Dasari *et al*, 2012). To show the complement activity on schizonts of *P. falciparum*, serial dilutions of NHS in CVB/EGTA/Mg^{2+}, starting at a maximum of 20% were added to microtiter plates half-coated with zymosan, half-coated with schizont of *P. falciparum*. C3b deposition was determined using anti-human C3c. NHS can induce AP-dependent C3b deposition on schizonts with the same efficiency as on zymosan (Figure 5-4).

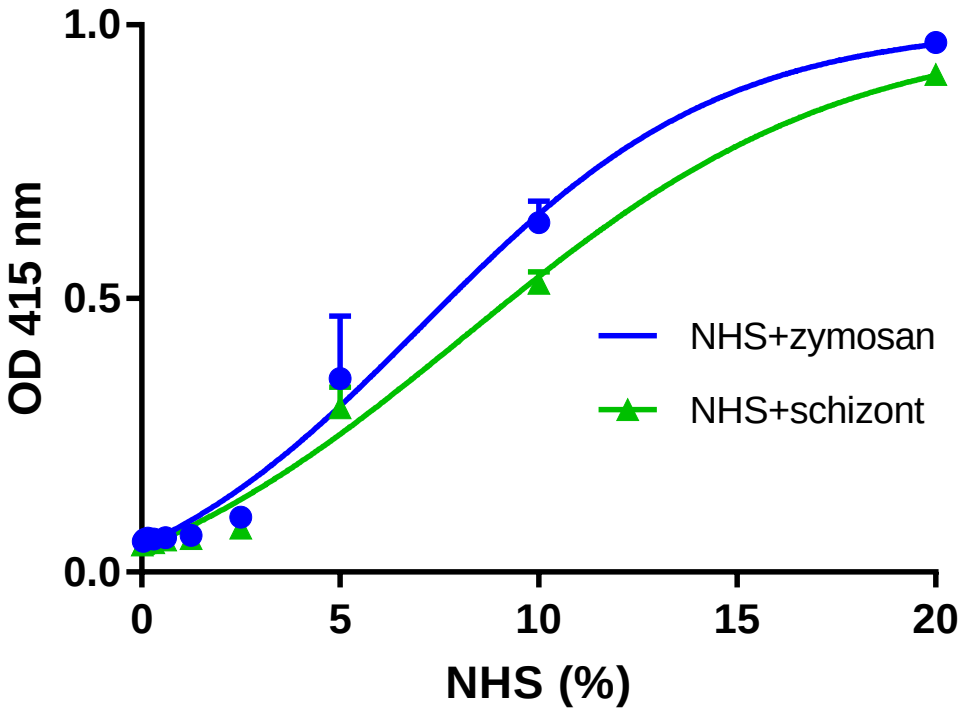


Figure 5-4: C3b deposition on zymosan and schizonts.
Serial dilutions of NHS at maximum of 20% were added to a microplate, half coated with zymosan and half –coated with schizont of *P. falciparum*. C3b deposition was determined using anti-human C3c. NHS induces C3b deposition on schizonts as the same efficiency as on zymosan. Statistically significant difference (one sample t test, $P<0.05$) was observed between the different NHS concentrations on zymosan or schizont coated plate. Data are the means ± SEM from three independent experiments carried out.

5.2.1.4 The role of recombinant proteins in augmenting alternative pathway activity on schizonts

Based on the experiments previously done regarding the role of recombinant rHP, rHMMP, rCFHR1 and rSCR3-5 in augmenting the complement alternative and terminal pathways, the purified recombinant proteins were tested for their ability to augment the alternative pathway on schizonts.

5.2.1.4.1 Role of rHP and rHMMP in augmenting the C3b deposition on Schizonts

Properdin can augment C3b deposition by increasing the C3b convertase half-life thus enhancing the amplification loop of the alternative pathway (Ferreira, Cortes and Pangburn, 2010). Recombinant properdin could enhance the bactericidal effect by increasing the alternative pathway activity on *N. meningitidis* coated plates (Ali *et al*, 2014). Our previous experiments in Chapter 3 showed that rHP and rHMMP could fix more C3b on zymosan coated plate (Figure 3-13 and Figure 3-14- E and F). Two experiments were done to explore the recombinant properdin role on alternative pathway activity. In the first experiment (Figure 5-5) different concentrations of rHMMP were added to serial dilutions of NHS starting at maximum serum concentration 20% in GVB/Mg^{2+}/EGTA buffer. This experiment showed that rHMMP could augment the C3b deposition on schizont coated plates (Figure 5-5 A), and the AP_{50} of NHS was shifted down from 3.6% to 1.5% (Figure 5-5 B). C3b deposition was statistically different in rHMMP vs NHS.

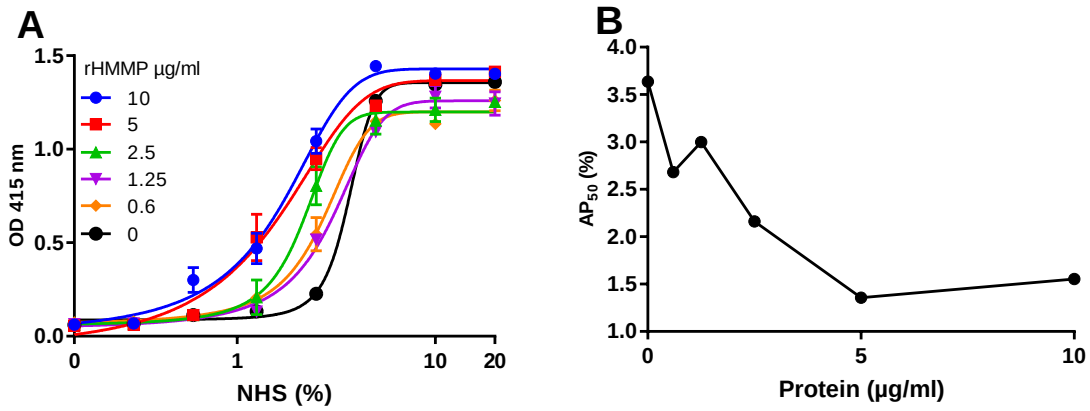


Figure 5-5: C3b deposition induced by adding rHMMP in NHS on schizont coated plate:
A: Different concentrations of recombinant proteins were added to serially diluted NHS in alternative pathway buffer. C3b deposition was determined using anti-human C3c. **B**: Sigmoidal curves of rHMMP were fitted to the data and the AP_{50} was calculated. The AP_{50} was shifted down from 3.6% to 1.5% serum concentration. Data are the means ± SEM from three independent experiments carried out.

The second experiment was done to show the role of rHP on the alternative pathway activity through C3b deposition on schizont coated plate. A fixed concentration of NHS (2.5%) was supplemented with different concentrations of the recombinant protein starting at maximum of 10 µg/ml then they were added to a schizont coated plate in GVB/Mg2+/EGTA buffer. The plate was probed with polyclonal rabbit anti human C3c. All protein concentrations tested showed significant activation compared to NHS without adding proteins (Figure 5-6). These results indicate that rHP is as effective as rHMMP in activating C3b deposition and thus augmenting alternative pathway activation on schizonts of *P. falciparum.*

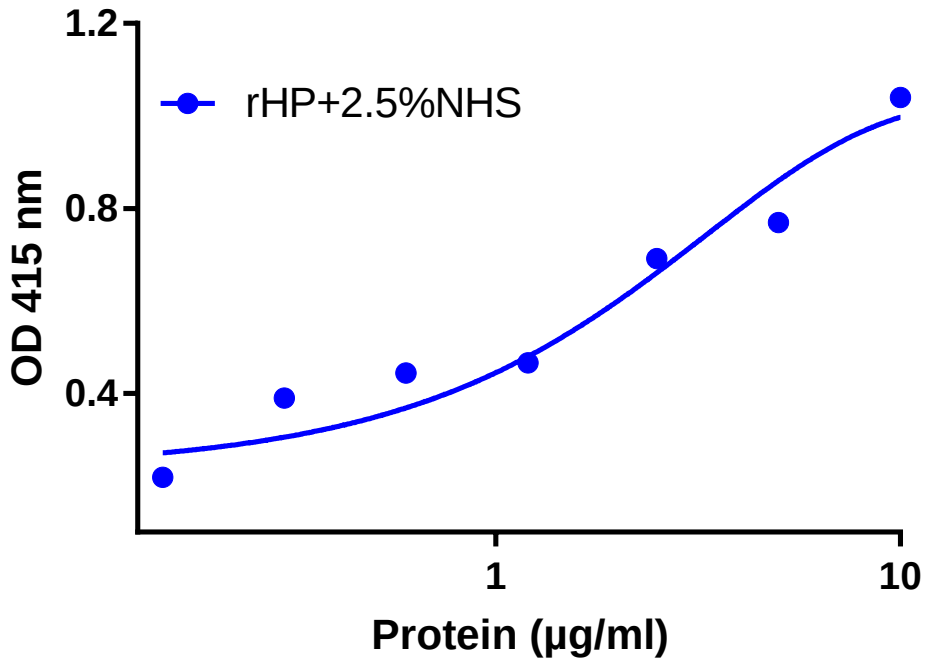


Figure 5-6: Augmentation of alternative pathway-dependent C3b deposition on schizonts by supplementing NHS with different concentrations of rHP.
Different concentrations of rHP were added to 2.5% NHS in alternative pathway buffer and incubated on schizont coated plates. C3b deposition was determined using anti-human C3c. The differences were checked by using one sample t test between the wells supplemented with NHS only vs with these supplemented with NHS plus rHP ($P<0.05$). Data are the means ± SEM from three independent experiments carried out.

5.2.1.4.2 C3b fixation on *P. falciparum* schizonts when rCFHR1 and rSCR3-5 were added

CFHR1 competes with factor H on C3b by mimicking of SCR19 and 20 of factor H, which are 97% to 100% similar to SCR4 and 5 of CFHR1, leading to inhibition of factor H activity on C3b (Skerka, Horstmann and Zipfel, 1991). Both rCFHR1 and rSCR3-5 previously showed augmentation the alternative pathway by increasing the C3b deposition on zymosan coated plate (Figure 4-12). Based on that, the role of rCFHR1 and rSCR3-5 in enhancing the alternative pathway on schizonts was tested. Through these experiments, different serial dilutions of rCFHR1 and rSCR3-5 were added to 2.5% NHS in GVB/Mg2+/EGTA on schizonts. Then C3b deposition was determined using polyclonal rabbit anti human C3c. In both experiments, both proteins showed significant augmentation of the alternative pathway activation (Figure 5-7).

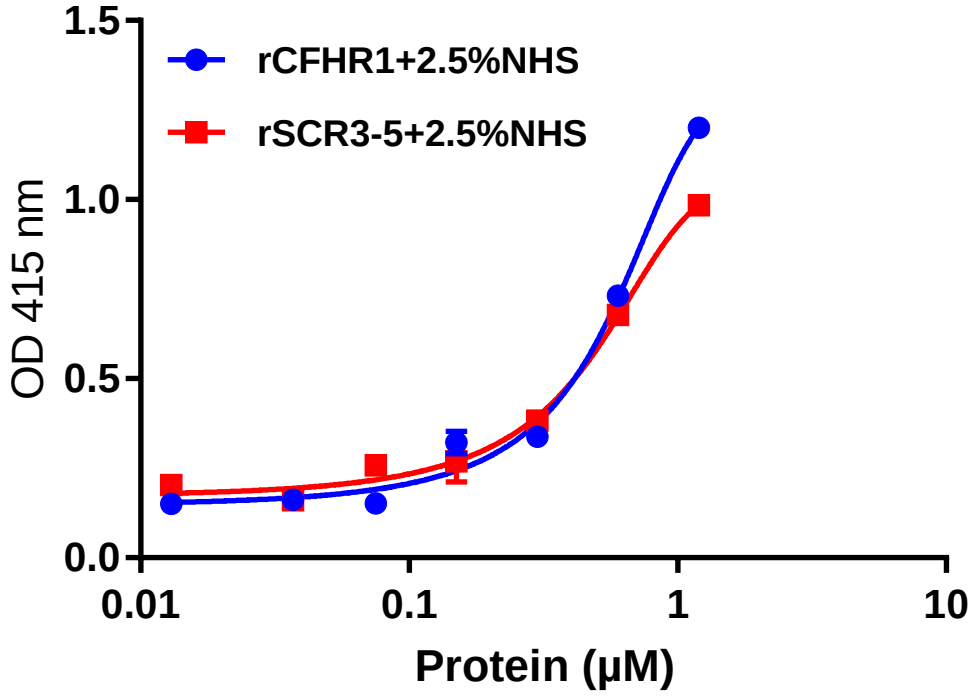


Figure 5-7: Recombinant human CFHR1 full length and SCR 3-5 augment alternative pathway activation in normal human serum on schizonts.
Different concentrations of recombinant human rCFHR1 and rSCR3-5 were added to 2.5% NHS in alternative pathway buffer on schizont coated plates. C3b deposition was determined using anti-human C3c. Statistically significant difference (one sample t test, $P<0.05$) was observed between the wells supplemented with NHS only vs with these supplemented with NHS plus rCFHR1 or rSCR3-5. Data are the means ± SEM from three independent experiments carried out.

5.2.2 *Ex vivo* experiments (*P. falciparum* assays)

In vitro experiments showed that schizonts can induce the complement alternative pathway by increasing C3b deposition on the plate (Figure 5-4). This activity was much stronger when NHS was supplemented with the recombinant properdin or CFHR1 (Figure 5-6 and Figure 5-7). Doing *in vivo* experiments on *P. falciparum* tends to be very difficult, therefore another way is to test the complement regulators by doing *ex vivo* experiments. *P falciparum* blood stages can be cultured in medium containing human RBCs. The asexual blood stages reproduce in this system, infecting, rupturing and re-infecting new erythrocytes. The cycle (generation time) takes about 48 hours (see section 2.2.2.5.2.1). Each cycle starts with the ring stage then trophozoite after that schizonts and finally merozoites which again transformed to ring stage at the beginning of second

generation. This model system of the blood stages of infection enables several different kinds of experiments: *P. falciparum* growth assays can be done by starting with a known level of infection (parasitaemia) and sampling at different time points to determine growth, and; Immunofluorescence microscopy can be used to detect parasite and erythrocyte components, and surface-bound exogenous components, e.g. complement components from serum.

5.2.2.1 Growth assay

Asexual blood parasites express surface proteins which can play an important role in decreasing complement activity. PfGAP50 and Pf92 (asexual blood stages surface proteins) can bind to factor H in a way can induce the cofactor activity of factor H and down regulate the alternative pathway (Simon *et al*, 2013; Kennedy *et al*, 2016). Recombinant properdin can enhance complement alternative pathway activity on schizonts (Figure 5-6) and counteract the factor H activity. CFHR1 (both recombinant forms) can also augment the alternative pathway and act as a competitive inhibitor for binding of factor H and schizonts (Figure 5-7 and Figure 5-3).

5.2.2.1.1 Initial experiment using infected RBC culture supplemented with NHS and rCFHR1

The first *ex vivo* experiments tested the effect of adding rCFHR1 to parasitic culture for two generations. *P. falciparum* asexual blood stages of strain 3D7 were cultured in RPMI-HEPES culture medium supplemented with 50µg/ml hypoxanthine and 10µg/ml gentamicin (see section 2.2.2.5.2.6.1). At the start of the experiment the haematocrit was adjusted to 5% and the parasitaemia to 1% (by mixing fresh RBC with infected RBC with a known level of parasitaemia). Samples of the starting culture were incubated with 10% NHS, heat-inactivated NHS (HIS), or NHS + 50µg/ml rCFHR1 for 48 hours; two parasitic stage generations. To determine parasitaemia, 10µl of infected RBCs culture was spread on glass slides and stained with Giemsa. The asexual blood stages of *P. falciparum* appeared as blue spots inside or outside RBCs (Figure 5-8). Counting the dots inside RBCs gives a measure of parasite infected RBCs (parasitaemia). In this initial experiment, the highest level of parasitaemia (2.5%) was observed in HIS (which has reduced complement activity), falling to 2% in cultures containing NHS (presumably due to complement-mediated attack on the parasite). Adding rCFHR1 to NHS reduced parasitaemia even further, to 1.7%. (Figure 5-9). However the differences between HIS

and NHS or NHS supplemented with rCFHR1 failed to reach statistical significance in this experiment.

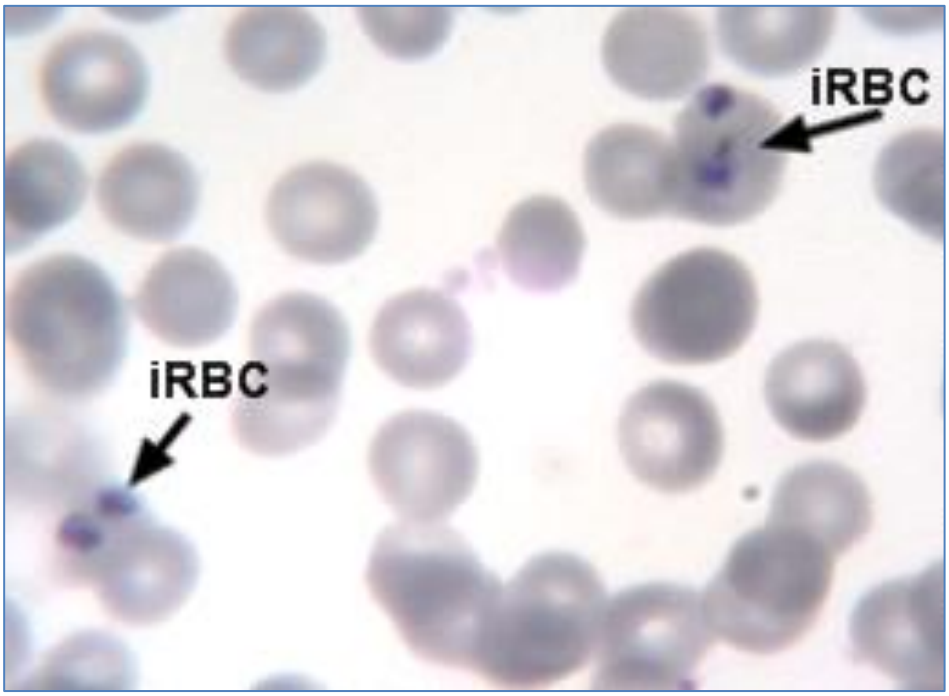


Figure 5-8: infected RBCs stained with Giemsa.
80µl of supernatant from each well of the 96-tissue culture plate was discarded and 10µl of the remainder after mixing was spread on a clean slide and left until drying. Then the slide was put in methanol for 1 minutes then left for drying. After that, it was put in Giemsa stain for 10-15minutes. Finally, it was washed with water and examined under the microscope (100X). Infected RBCs (iRBC) were orange and contained blue spots representing the parasite stages.

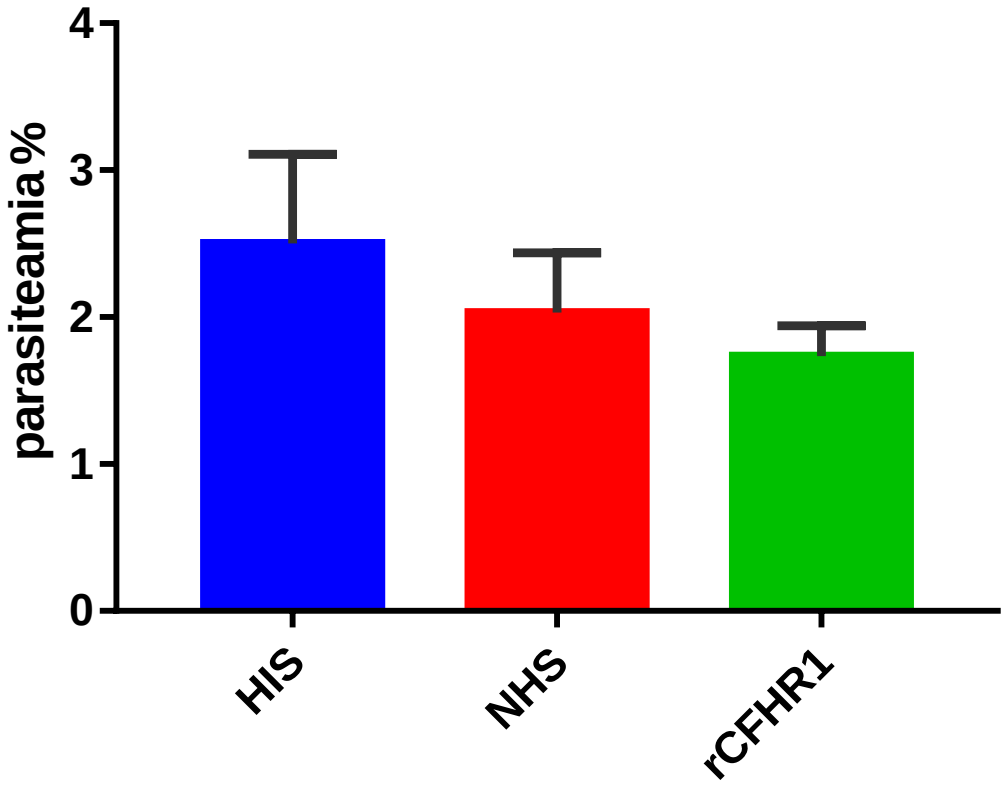


Figure 5-9: Parasitaemia in rCFHR1 containing culture.
360 µl of 1% parasitaemia starting culture was added to 40 µl of each heat inactivated normal human serum (HIS) (10%), NHS (10%) and 50µg/ml of CFHR1 plus NHS (10%). Then 100 µl of each was transferred in triplicates into a 96 well U shape tissue culture plate. It was incubated in 5% CO2, 1% O2 at 37°C for 48 hours. The parasitaemia was checked by randomly counting number of the infected RBCs in 10 different fields of each Giemsa stained slides for each well under the microscope (100x) using the following equation:

$$Parasitaemia = \frac{infected\ RBC}{total\ RBC\ No.}\ x\ 100$$

Statistically significant difference was not seen between HIS and NHS or NHS supplemented with rCFHR1 One-way ANOVA, Sidak's multiple comparisons test.

5.2.2.1.2 Growth assay using infected RBC culture supplemented with different types of recombinant proteins

Based on the above, a second parasite growth assay was done using NHS supplemented with different complement inducers; recombinant properdin and CFHR1, to try to compensate for the deficiency of the alternative pathway due to the parasite activity. The experiments were done for three asexual blood parasitic stages generations changing the media and the supplements twice a day to ensure a continuous supply of recombinant proteins and complement. Infected RBC cultures mostly at the late ring stage were

supplemented with 5 µg/ml of rHP or rHMMP and 25 µg/ml of rCFHR1 or rSCR3-5. The mixture was incubated for 96 hours at 36°C 5% CO_2 and 1% O_2. NHS or HIS plus/minus the recombinant proteins were replaced once per 12 hours. Three generations were exposed to these supplementations.

Parasitaemia was calculated twice; after 48 hours and 96 hours. The results showed that there is no significant effect of adding recombinant properdin to NHS or HIS. When rCFHR1 or rSCR3-5 were added to NHS there was a significant decrease in parasitaemia compared to NHS without additional protein (Figure 5-10 A and Figure 5-10 B). Another result could be seen in this experiment, which is inhibition of parasitic growth when rCFHR1 and rSCR3-5 were added to HIS (Figure 5-10 B), suggesting that rCFHR1 and rSCR3-5 have complement-dependent and complement-independent effects on parasitaemia.

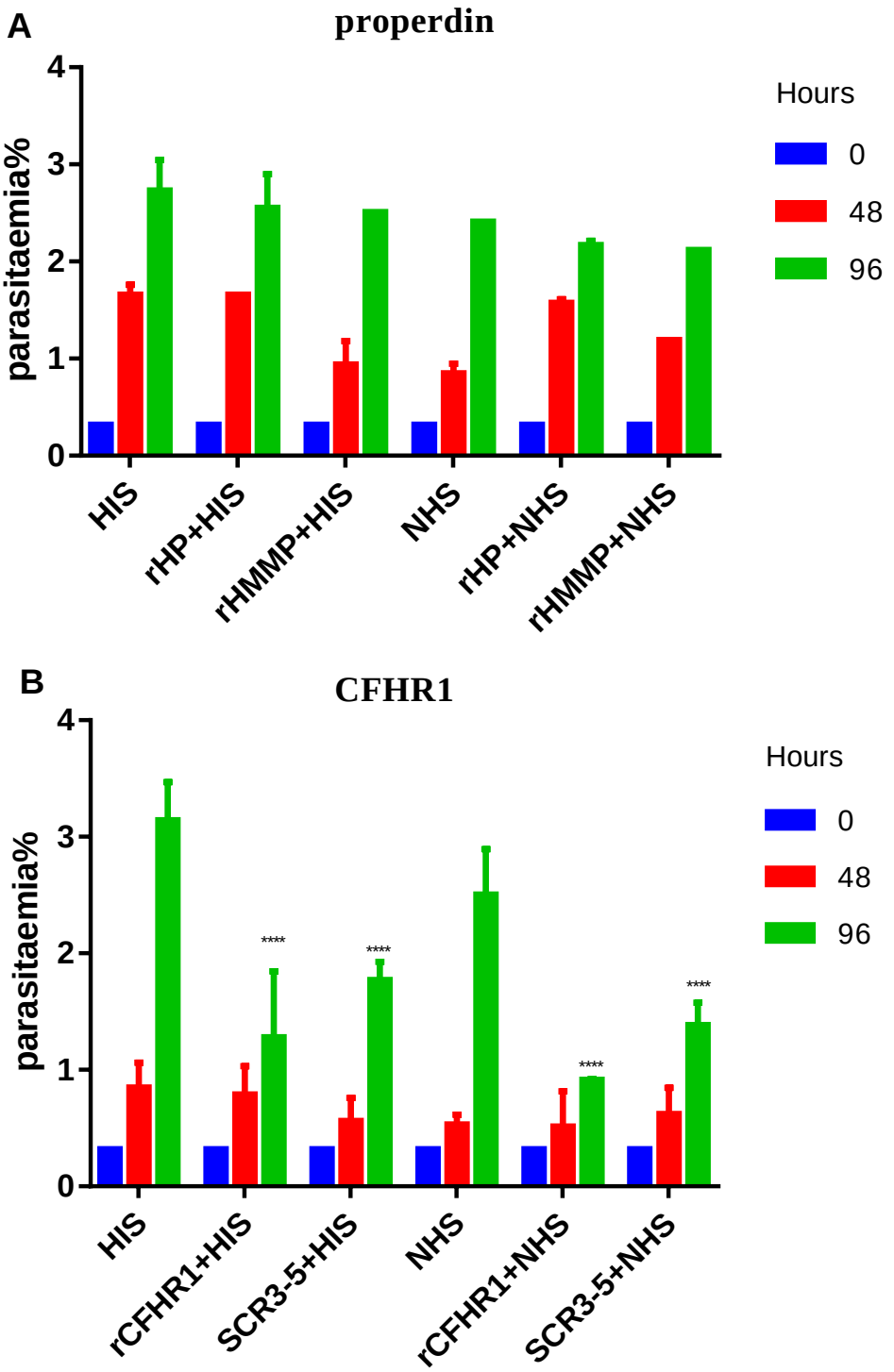


Figure 5-10: Parasitaemia in asexual blood stages infected RBC culture supplemented by recombinant proteins.

Start culture media of 0.3% parasitaemia was supplemented with either HIS (10%) or NHS (10%) plus/minus 5 µg\ml of rHP or rHMMP (**A**), or 25 µg\ml of rCFHR1 or rSCR3-5 (**B**) while PBS was added to HIS and NHS instead of proteins as a control. Then 100 µl of each was transferred in triplicate into a 96 well U shape tissue culture plate. The plate was incubated in 5% CO_2, 1% O_2 at 36^0C for 96 hours. The parasitaemia was checked twice; after 48 hours and 96 hours by blindly counting number of the infected RBCs as in the last experiment. The statistical significance is indicated (**** $p<0.0001$; two-way ANOVA with Dunnett's correction for multiple comparisons). The experiment was performed in triplicate (mean ± SE); the data are representative for one of two independent experiments.

5.2.2.2 Immunofluorescent assay

C3b can deposit on the surface of schizonts (Figure 5-4). This activation could progress to the end of the terminal pathway (Rosa *et al*, 2016). Here, we used indirect immunofluorescence assays to examine the deposition of complement components on the surfaces of schizonts and iRBCs, in the presence and absence of recombinant CFHR1 and properdin.

5.2.2.2.1 Antibody optimization

Rabbit anti-human C3c FITC was used to detect C3b deposition, in combination with mouse anti-human merozoite surface 1 (MSP1) to show co-localization to the plasmodium asexual blood stage surface. To for check terminal pathway activity, mouse anti-human C5b-9, an antibody that recognises a neoepitope on assembled C5b-9, was used in conjunction with rabbit anti-human MSP1. To check which concentration of antibody to use, isolated schizonts were incubated with 20% serum for 1 hour at 37°C. The samples were spread on slides and fixed with methanol for 10 minutes. After blocking the slides with 5% BSA, different concentrations: 1/100, 1/50, and 1/10 of primary antibodies (rabbit anti-human C3c and mouse anti-human MSP1) (Figure 5-11 A) or mouse anti human C5b-9 and rabbit anti human MSP1 (Figure 5-11 B) were added to the slides. Visualization was done by using fluoroconjugate goat anti-mouse and goat anti-rabbit in 1/100 concentration. For all primary antibodies, a 1/100 concentration was chosen for use in subsequent experiments, as it gave sufficient signal with low background staining.

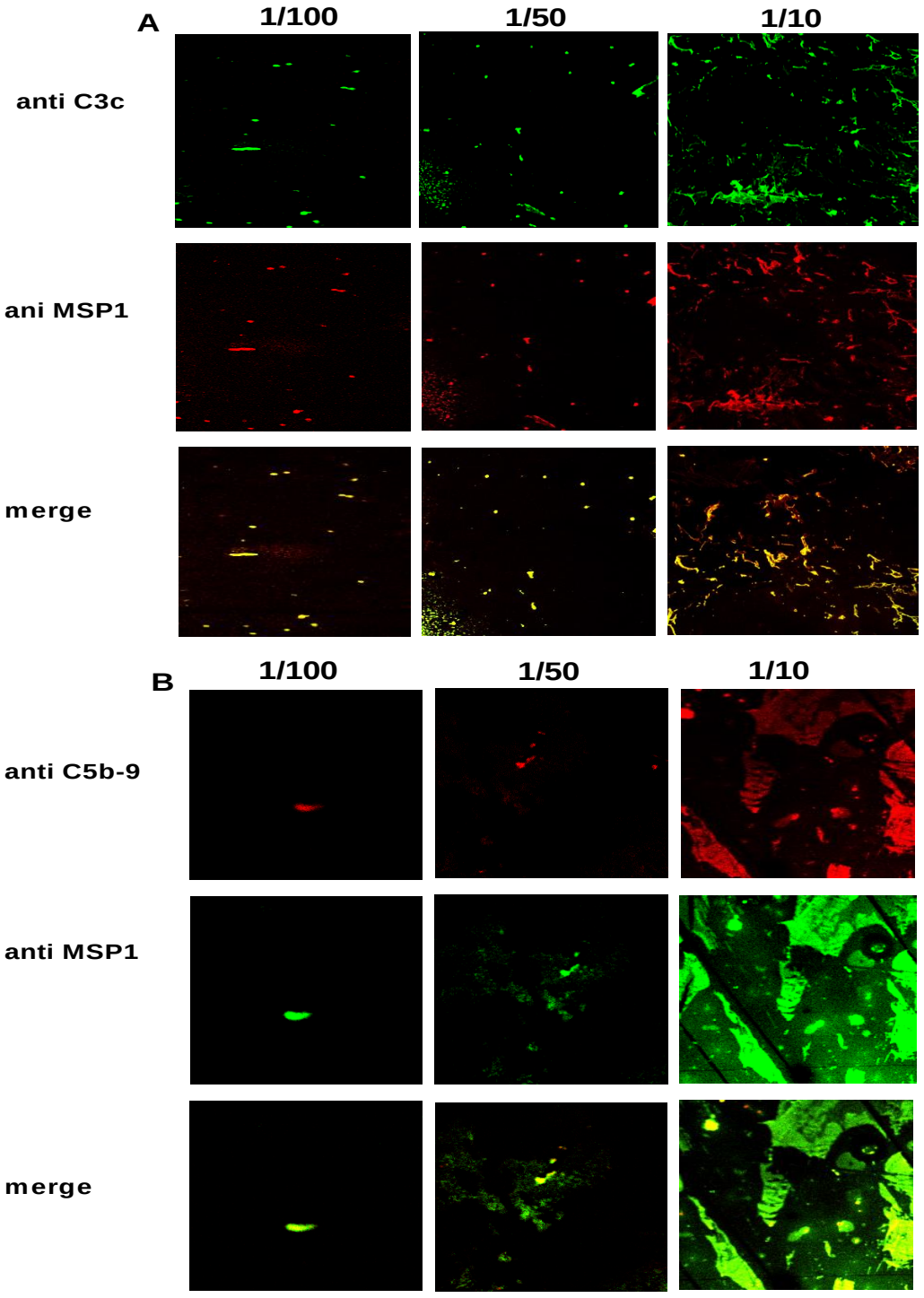


Figure 5-11: Antibody optimization
Isolated schizonts were incubated with 20% serum for 1 hour at 37°C, 5% CO_2 1% O_2. Slides were made by making smears and fixing with methanol for 10 minutes. After blocking with 5% BSA for 2 hours, the slides were probed using different concentrations (1/100, 1/50 and 1/10) of rabbit anti human C3c and mouse anti human MSP1 (**A**) or mouse anti human C5b-9 and rabbit anti human MSP1 (**B**) for an hour at 37°C. Visualization was done by adding goat anti-mouse Alexafluor 546 red colour and goat anti-rabbit Alexafluor 488 green colour in 1/100 concentration. Two colours appeared, green represents C3b and red for asexual blood stages (**A**). While in panel **B,** red represents C5b-9 and green for asexual blood stages. C3b and C5b-9 were deposited on schizont. The intensity of antibody binding was different depending on primary antibody concentration.

5.2.2.2.2 Setting different kinds of controls

Different types of controls were used in order to check if the primary and secondary antibodies were specific and also to compare between the results with positive and negative controls. Non-infected RBCs were incubated with 10% pooled NHS at 37°C for an hour then a drop of this mixture was smeared on a glass slide. After that this slide was incubated with rabbit anti human C3c and mouse anti human MSP1 or mouse anti human C5b-9 and rabbit anti-human MSP1. Visualization was done by using goat anti-mouse and goat anti-rabbit in 1/100 concentration (Figure 5-12-1). This control was done to check if the primary antibodies make any kind of cross reaction with non-infected RBCs. In a second control, schizont infected RBCs were incubated with 10% NHS. No primary antibodies were used in this control and the visualization was done as in the above (Figure 5-12-2). This control was done to show that the secondary antibodies do not make any kind of cross reaction with infected RBCs. For the other controls, schizont infected RBCs were not incubated with NHS. After smearing these slides, some of them were incubated with rabbit anti-human C3c and mouse anti-human MSP1 (Figure 5-12-3) while others were incubated with mouse anti-human C5b-9 and rabbit anti-human MSP1 (Figure 5-12-4). These controls showed that there is no binding between iRBCs and anti C3b or anti C5b-9.

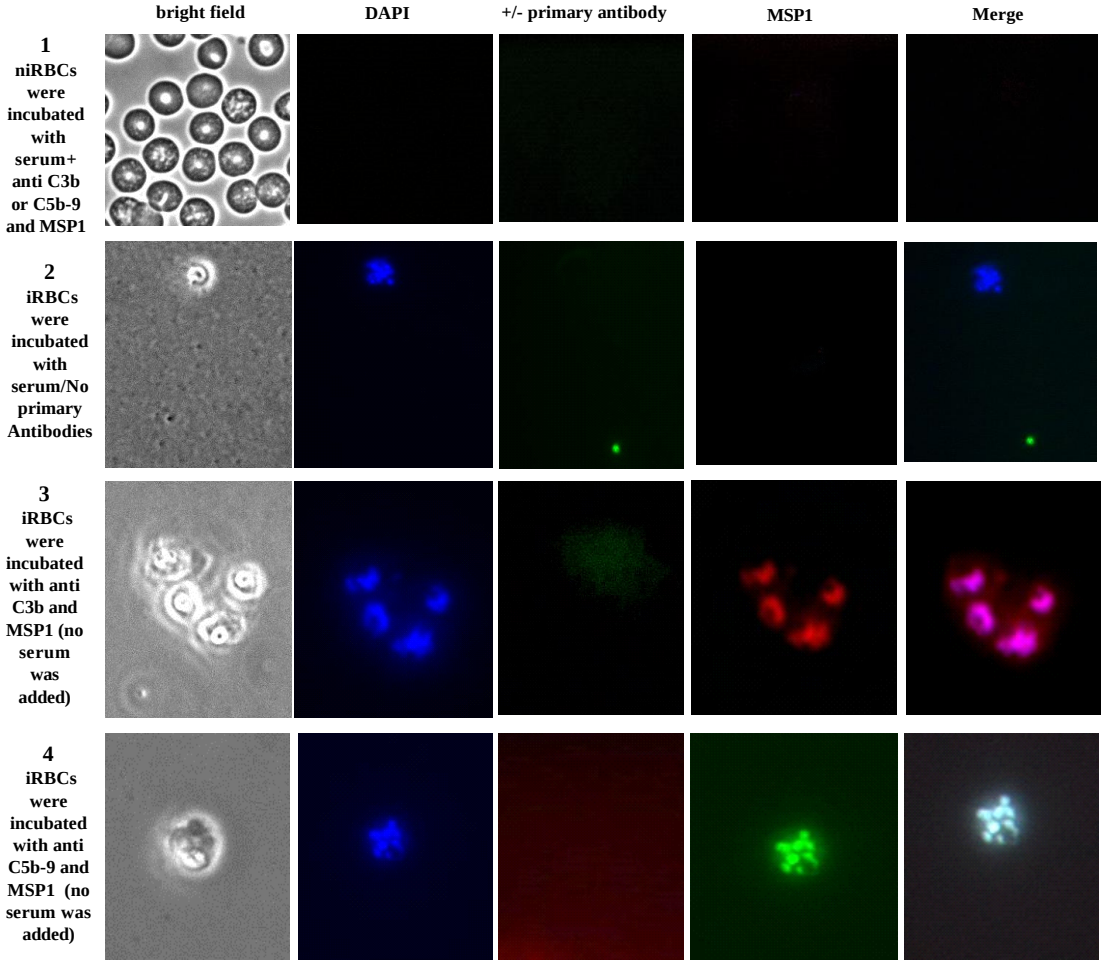
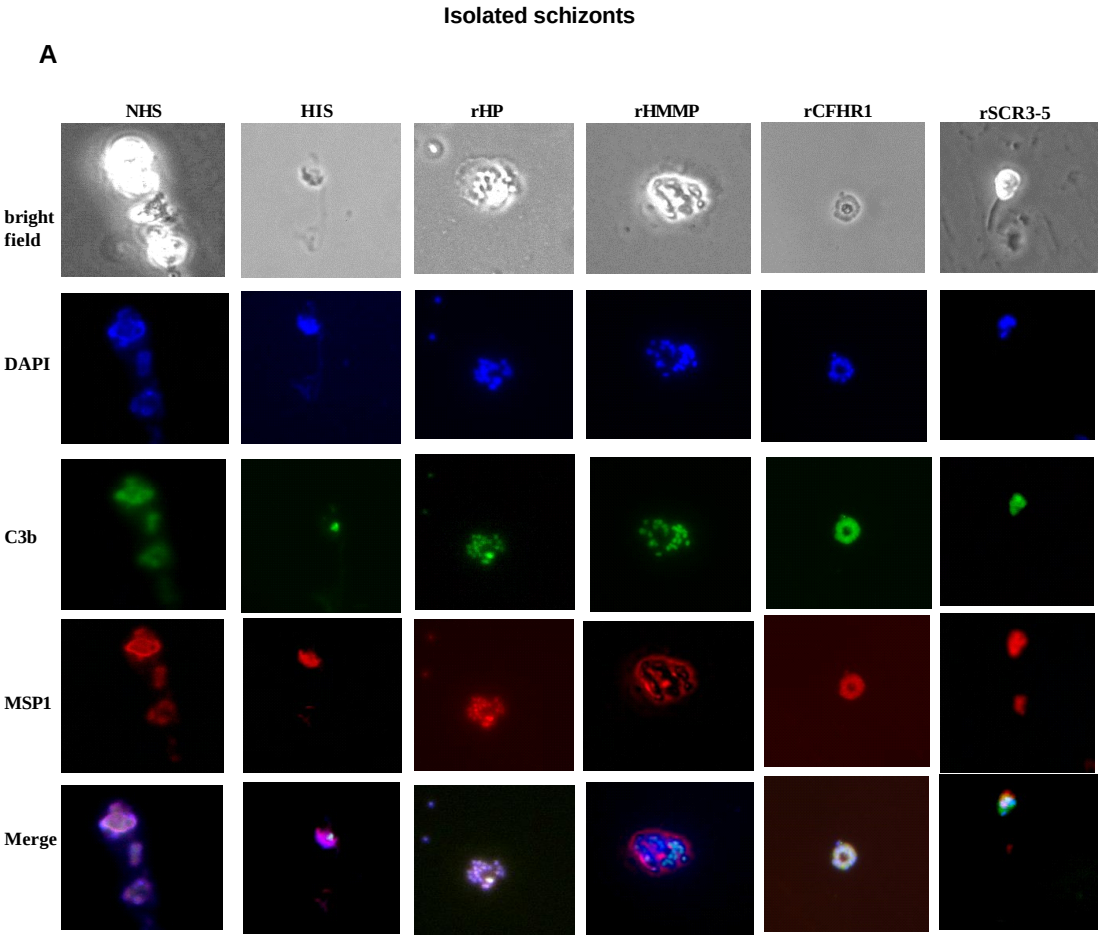


Figure 5-12: Different sets of controls
(1) Non-infected RBCs were incubated with 10% serum. Rabbit anti human C3c (3) and mouse anti human MSP1 (4) in 1/100 concentration were added as primary antibodies. Visualization was done by using goat anti mouse and goat anti rabbit in 1/100 concentration.
(2) Schizont infected RBCs were incubated with 10% serum. No primary antibodies were added. Visualization was done by using goat anti mouse and goat anti rabbit in 1/100 concentration.
(3 and 4) Schizont infected RBCs were incubated with both primary antibodies (no serum); rabbit anti human C3c (3) and mouse anti human MSP1 (4) in 1/100 concentration. Visualization was done by using goat anti mouse and goat anti rabbit in 1/100 concentration.

5.2.2.2.2.1 C3b and C5b-9 deposition on iRBCs or isolated schizonts

iRBCs or isolated schizonts were incubated with NHS, HIS, NHS+ rHP, NHS+ rHMMP, NHS+rCFHR1, or NHS+rSCR3-5. C3b deposition was demonstrated by immune-labelling with FITC polyclonal rabbit anti-human C3c antibody (green). Asexual blood stages of *Plasmodium falciparum* strain 3D7 were detected by polyclonal mouse MSP1

(Santa Cruz Biotechnology) then visualized by goat anti-mouse Alexafluor 546 (red). Another experiment for C5b-9 was done using polyclonal mouse anti-human C5b-9 (Santa Cruz Biotechnology) then visualized by goat anti-rabbit Alexafluor 488 (green). While the parasitic stages were checked by using rabbit MSP19 anti asexual blood stages of *P. falciparum* then visualised by using goat anti-rabbit Alexafluor 488 (green). Different sets of controls were set up to ensure the experiments correctly worked (Figure 5-12). The experiments showed that rHP, rHMMP, rCFHR1 and rSCR3-5 induced the C3b and C5b-9 deposition on isolated schizonts as well as on schizont iRBCs (Figure 5-13 and Figure 5-14). The results were independently repeated three times. However, adding HIS did not show C3b or C5b-9 deposition, showing that deposition is dependent upon complement activation (Kennedy *et al*, 2016). These experiments were done thrice independently.


Isolated schizonts
A
NHS
HIS
rHP
rHMMP
rCFHR1
rSCR3-5
bright field
DAPI
C3b
MSP1
Merge

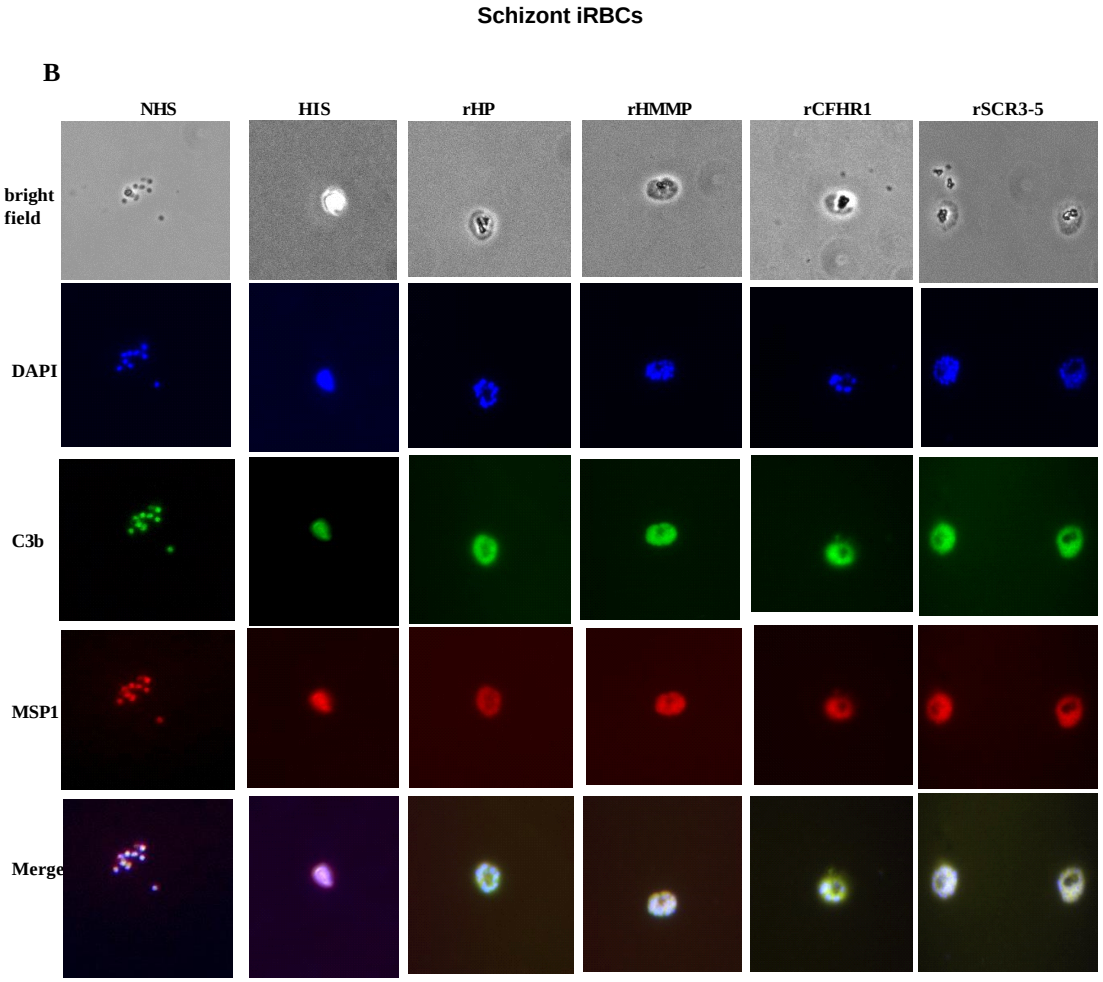
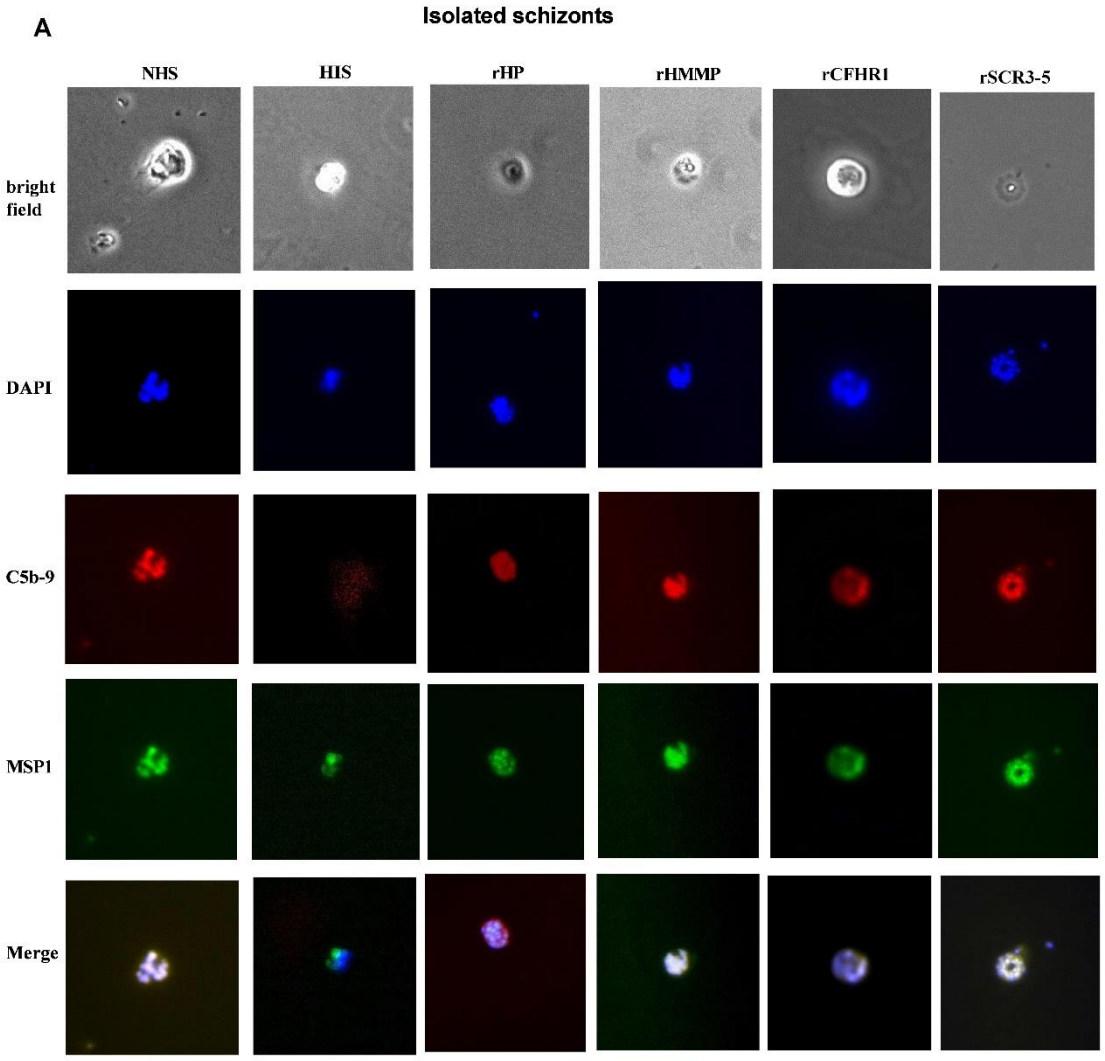


Figure 5-13: C3b deposition on the surfaces of asexual blood stages cultures supplemented with NHS and recombinant properdin.
iRBCs (**A**) or isolated schizont (**B**) were incubated with NHS+ buffer, HIS+ buffer, NHS+ rHP, NHS+ rHMMP, NHS+rCFHR1 and NHS+rSCR3-5 for 1 hour at 36°C, 5% CO_2 and 1% O_2. **C3b** deposition was demonstrated by immunolabelling with primary antibodies (FITC Polyclonal rabbit anti human C3c antibody, green). Asexual blood stages of *P. falciparum* strain 3D7 were detected by polyclonal mouse MSP1 anti asexual blood stages of plasmodium antibody (Santa Cruz Biotechnology) then visualized by goat anti mouse Alexafluor 546 (red).


A
Isolated schizonts
NHS
HIS
rHP
rHMMP
rCFHR1
rSCR3-5
bright field
DAPI
C5b-9
MSP1
Merge

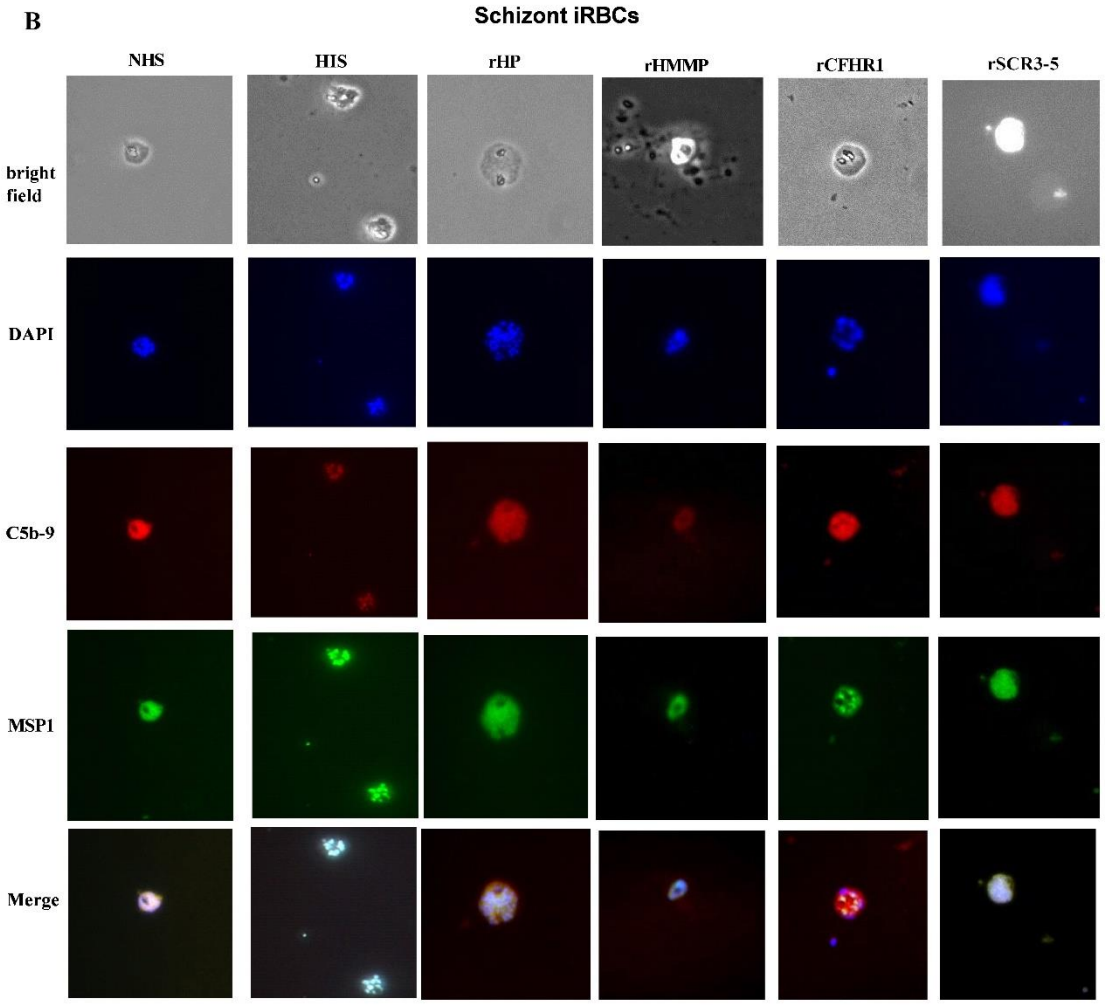


Figure 5-14: C5b-9 deposition on the surfaces of asexual blood stages cultures supplemented with NHS and recombinant properdin.
MAC formation on the surfaces of iRBCs was detected by checking **C5b-9** deposition on both iRBCs (**A**) and isolated schizonts (**B**) which were detected by primary antibodies (Polyclonal mouse anti human C5b-9) (Santa Cruz Biotechnology) then visualized by goat anti mouse Alexafluor 546 (red). Asexual blood stages of Plasmodium falciparum strain 3D7 were detected by polyclonal rabbit MSP19 anti asexual blood stages of Plasmodium antibody (kindly provided by professor Andrew Tobin) then visualized by goat anti rabbit Alexafluor 488 (green).

5.2.3 Summary

rCFHR1 and rSCR3-5 showed significant binding to schizont-coated plates. Factor H could compete with rCFHR1 and rSCR3-5 (which contain structures similar to SCR19 and 20 of factor H) for binding to schizonts. This is consistent with the idea that P. falciparum surface proteins actively recruit factor H to avoid complement activation. rHP and rHMMP did not bind to schizont-coated plates. Schizonts can induce C3b deposition and thus activate the alternative pathway. Moreover, adding rHP, rHMMP, rCFHR1 and rSCR3-5 can fix more C3b on schizont coated plates, thus augmenting alternative pathway activation on schizonts of *P. falciparum*. When growth assays were done, all recombinant proteins could significantly decrease parasitaemia when they were added to NHS. This could be because the activity of recombinant proteins, can overcome the inhibitory effects of the parasite surface proteins, lead to exposure of the parasite to the direct effect of the complement system and deposition of more complement components on asexual blood stages. Inhibition of parasitic growth by adding rCFHR1 and rSCR3-5 was significantly higher than that obtained using properdin supplementation. C3b deposition largely reduced when NHS was heated to 65°C (heat inactivated serum). Parasitic stages can recruit factor H even if HIS was added. This means that asexual blood stages can recruit factor H independently on C3 activity (Kennedy *et al*, 2016). C3b and C5b-9 fixation on iRBCs and isolated schizonts were shown by immunofluorescence assays.

6Chapter six

Discussion

6.1 Brief introduction

The complement system protects the human body from infectious organisms through three pathways: the classical, lectin and alternative pathways (Figure 1-1). All of the pathways converge with the formation of a C3 convertase and, subsequently, a C5 convertase. Surface-bound C3 activation products (chiefly C3b) are the main opsonins of the complement system proteins, which lead to phagocytosis (Carroll and Sim, 2011). Cleavage of C5 starts the terminal pathway and finally leads to the formation of the Membrane Attack Complex (MAC) (Figure 1-1).

Factor H can inhibit the alternative pathway activation through inhibiting the C3 convertase of the alternative pathway by preventing the binding between factor B and C3b (Conrad, Carlo and Ruddy, 1978). Factor H could also inhibit the alternative pathway by acting as a cofactor for factor I, thus it can mediate the conversion of C3b to an inactive product of C3b (iC3b) (Whaley and Ruddy, 1976). Therefore, factor H could accelerate the breakdown of C3bBb and inactivate C3b to iC3b (Skerka and Zipfel, 2008). Many microorganisms such as *P. falciparum* can recruit factor H to escape from the complement attack (Simon *et al*, 2013).

All the known and probable complement evasion mechanisms used by *P. falciparum* act on C3b and the alternative pathway C3 convertase (C3bBb) (Rowe *et al*, 2000; Simon *et al*, 2013; Malpede and Tolia, 2014). Inhibiting the C3bBb during Plasmodium infections leads to decrease C3b deposition (Fearon and Austen, 1975). Asexual blood stages of *P. falciparum* can recruit factor H to evade the complement attack. Meri *et al.* (2013) studied the recruitment of factor H by *Haemophilus influenzae, Bordetella pertussis, Pseudomonas aeruginosa, Streptococcus pneumonia, Candida albicans, Borrelia burgdorferi,* and *Borrelia hermsii*. They found that all pathogens recruited factor H by binding to the same region in SCR20. This binding could augment factor H-C3b binding thus down regulating the complement activation. Our results showed that rCFHR1 or rSCR3-5 could act as competitive inhibitors to factor H by competing to SCR19 and SCR20.

Asexual blood stages of *P. falciparum* can down regulate the alternative pathway by expressing different types of surface proteins such as PfGAP50 and Pf92, which can bind to factor H in a way that can induce factor H activity (Simon *et al*, 2013; Kennedy *et al*,

2016). Binding of PfGAP50 to factor H was investigated. Simon *et al.* (2013) found that recombinant GAP50 could bind to full length and SCR1-7 but not to SCR1-4 of factor H. They mentioned that GAP50 preferred to bind SCR5-7. However, they did not check the binding activity on the SCR19-20 separately. Kennedy *et al*. (2016) reported that Pf92 is the only merozoites surface protein that could interact with factor H. They found that Pf92 could bind only to SCR4-6 of factor H.

Properdin can increase the half-life of C3 convertase by stabilising the C3 convertase (Hourcade, 2006; Ferreira, Cortes and Pangburn, 2010). The alternative pathway C3 convertase, C3bBb is responsible for amplification of the alternative pathway by activating more C3 thus augmenting the alternative pathway activation (Figure 1-1). Properdin can bind to C3bBb forming a relatively more stable complex, C3bBb properdin (C3bBbP) (Fearon and Austen, 1975). Therefore, properdin and factor H have opposite functions as properdin could stabilise the C3bBb complex, allowing ongoing the C3 convertase activity (Farries, Lachmann and Harrison, 1988; Bhakdi and Tranum-Jensen, 1991).

CFHR1 lacks factor I cofactor activity and the C3bBb decay accelerating activity associated with factor H (Timmann, Leippe and Horstmann, 1991). SCRs 1 and 2 of CFHR1 are not involved in C3bBb binding and not required for competitive inhibition of factor H binding to the cell surface (Fritsche *et al*, 2010). CFHR1 or SCR3-5 have the potential to augment alternative pathway activation through inhibiting factor H that can be activated during *P. falciparum* infections.

The binding ability of properdin to C3bBb depends on the size of polymeric forms of properdin. Recombinant human properdin produced and purified *in vitro*, can aggregate to form highly polymerised structures (Farries and Atkinson, 1989a; Ali *et al*, 2014). This artificial product (P_n) can stabilise C3bBb 5-10-fold more effectively than native properdin (Ferreira *et al.* 2010). In addition, it could increase production of C3bBb thus increasing the amplification loop of the alternative pathway (Ferreira *et al.* 2010). However, the expression of a highly and continuously activated recombinant mouse properdin is difficult; recombinant mouse properdin is usually less active than native mouse properdin (Lynch and Ali, personal communication).

6.2 Recombinant properdin experiments

Recombinant human properdin produced and purified *in vitro*, can aggregate to form highly polymerised structures called activated properdin or P_n (Farries and Atkinson, 1989; Ali *et al*, 2014), which can stabilise C3bBb 5-10 fold more effectively than native properdin (Ferreira, Cortes and Pangburn, 2010). Freeze-thaw cycles can further increase properdin oligomerization, yielding a highly oligomerized form (P_n) (Ferreira, Cortes and Pangburn, 2010). Attempts to express highly and continuously active recombinant murine properdin failed (Lynch and Ali, personal communication); recombinant murine properdin is usually less active than native murine properdin, while recombinant human properdin was more active than native human properdin (Ali *et al*, 2014).

Properdin termini play an important role in properdin activity, as the C terminal part (TSR6) and the N terminal part (TSR0) are involved in dimer, trimer and tetramer formation (Sun, Reid and Perkins, 2004). Given that the termini are responsible for the oligomerization of properdin, and that recombinant human properdin forms large oligomers much more readily than murine properdin, we tried replacing the N- and/or C-termini of mouse properdin with those of human properdin to produce humanised mouse properdin. Some properdin cDNA construct bands were not visible as some transfected colonies have received the vector only (ligation the vector with properdin cDNA constructs was failed).

Recombinant, murine and chimeric properdin were successfully expressed and purified. The oligomerization state of properdin was checked by gel filtration. Both rHP and HMMP showed similar peaks on the same column, which are dimers, trimers, tetramers and higher-oligomers (Pn) (Figure 3-11). Therefore, exchanging the N-terminus of mouse properdin with that of human properdin increased oligomerization and produced Pn. However, other forms did not show the oligomerization states correctly (Figure 3-12). At the beginning, some peaks appeared before 10 ml elution. When these elutions were checked in SDS gels, they did not show the properdin bands, which suggests that they could represent contaminants. The properdin curves look to elute higher than marker curves with corresponding molecular weights. This could be because properdin is a rod-shaped molecule which behaves as though it were slightly larger than the globular proteins used for calibration.

C3b deposition experiments on ELISA plates were used to measure alternative pathway activation. To activate the alternative pathway only, without the other complement pathways functioning, sera were diluted in EGTA containing buffer (no calcium was added) (see section 2.2.2.5.1.2.1), which preferentially consumes calcium, which is very necessary to activate the classical and lectin pathways, leaving Mg^{2+}, which is required for the alternative pathway (Lesavre and Muller-Eberhard, 1978). Anti C3c antibodies were used to detect C3b as it can cross react with surface bound C3b.

The role of rHP and rMP in C3b deposition has been also tested. rHP can augment the C3b deposition in NHS and also shifted down the AP_{50} as expected, while rMP did not show any augmentation in C3b deposition compared to WT mouse serum that was not supplemented with rMP (Figure 3-13). This result agrees with Schwaeble and Reid (1999) and Ali et al (2014) who mentioned that properdin has the ability to stabilize the C3b and the C5 convertase (C3bBb and (C3b)nBb), respectively which increase the half-life of the C3b convertase and then augments alternative pathway activation through the amplification loop. In summary, rHP is more active than native HP, while rMP is less active than native mouse properdin.

Recombinant murine properdin humanised at the N-terminus (rHMMP) can augment the alternative pathway in NHS or WT mouse serum, as shown by the increase in C3b deposition on zymosan. Targeting the N-terminus of mouse properdin by adding human TSR0 instead of opposite murine domains showed more activity than that targeting the C terminus, or both ends of mouse properdin (Figure 3-14). Targeting TSR0 of inactive mouse properdin may change the oligomerization properties of mouse properdin, as the N-terminal part can be involved in oligomerization (Sun, Reid and Perkins, 2004). Our results suggest that, the N-terminal part (TSR0) is required for oligomerization and stabilization of C3bBb.

Both types of recombinant properdin were added to properdin-depleted serum (PDS) to compare their role in restoring the complement alternative pathway. When PDS was used, rHP and rHMMP can augment the C3b deposition on zymosan (Figure 3-18). Properdin could increase the half-life of the C3b convertase, thus more the C3b convertase can be deposited and alternative amplification loop starts. This antagonises the factor H activity (Schwaeble *et al*, 1987). Our result showed that both kinds of properdin could augment the C3b deposition on human depleted serum.

The biological activity of the oligomeric forms of properdin differs. From the results, Pn of rHP and rHMMP showed more ability to activate the C3b deposition compared to other forms of recombinant properdin (Figure 3-19). The ability of properdin to stabilize the C3b convertase increased with increasing size of the oligomers (Higgins *et al*, 1995). Pn could be a highly active artificial product in augmenting the alternative pathway, while the other forms could activate this pathway when they bind to complement activators (Farries *et al*, 1987; Pangburn, 1989). Ferreira, Cortes and Pangburn (2010) found that the separated P3 can active the C3b deposition 3-fold more effectively than native serum when P3 was bound to zymosan. Our findings showed that the purified Pn of both forms of recombinant properdin, rH and rHMMP, showed high C3b deposition (Figure 3-19).

Purified rHP and rHMMP showed clear binding to C3b (Figure 3-20). C3b fixation increases when properdin binds to the C3b molecule, leading to more C3b convertase formation and thus more C3 cleavage. In this way, properdin can start the amplification loop of the alternative pathway (Fearon and Austen, 1975).

NHS and MBL-/- serum showed no difference in RBC lysis through the alternative pathway activation (Figure 3-21-A). This is expected, as the alternative pathway can be activated spontaneously and independently from MBL concentration or mannan coating (Lange, Magnadóttir 2003). However, NHS lyses more RBCs than MBL deficient serum (MBL-/-) when the sera were incubated with rabbit RBCs previously coated with mannan through the lectin pathway (Figure 3-21-B). This is because MBL (one of the recognition molecules of the lectin pathway) in NHS is required to activate the lectin pathway (Suankratay *et al*, 1998). Our results also demonstrated that mannan coated RBCs were lysed more than uncoated RBCs through the lectin pathway. This result agreed with Suankratay *et al*. (1998), who reported that uncoated RBCs could not be lysed by the lectin pathway, while mannan coated RBCs were lysed as mannan could work as a complement lectin pathway activator.

Haemolysis assays were done to show the effect of recombinant properdin on the whole complement system, which ends with MAC formation. rHMMP and rHP induced the alternative pathway and MAC formation leading to more rabbit RBC haemolysis when both recombinant proteins were added to NHS or WT mouse serum (Figure 3-22 A and B). This could be because of the role of properdin in stabilizing the C5 convertase ((C3b)nBb) (Schwaeble and Reid, 1999). Thereafter, this leads to the cleavage of C5 to

C5a and C5b which binds to C6 and 7, resulting in the activation of the terminal pathway, which ends with formation of the membrane attack complex (MAC) (Figure 1-1). However, rHP was more active in MAC formation in NHS than rHMMP, while the later lysed more RBCs by forming more MAC in WT mouse serum than rHP. This could be a result of the host specificity of properdin, which means human properdin works well in human serum while chimeric properdin which mainly contains mouse domains is more active in mouse serum.

6.3 Recombinant CFHR1 experiments

CFHR1 is unlike factor H, as it lacks the cofactor activity that can mediate the cleavage of C3b by factor I and it also lacks the dissociation activity of factor B bound C3b in the C3 convertase (Timmann, Leippe and Horstmann, 1991). CFHR1 can compete for factor H-C3b binding, promoting alternative pathway activation. The C terminus (SCR3-5) of CFHR1 might be a better complement inhibitor for two reasons. Firstly, the C-terminus (SCR3, 4, 5) of CFHR1 is responsible for the above function (Fritsche *et al*, 2010; de Jorge *et al*, 2012). The other reason is that the N-terminal domains (SCR 1–2) of CFHR1 can inhibit MAC assembly (Heinen *et al*, 2009), which may lead to less complement activation.

rCFHR1 and rSCR3-5 were cloned to two expression vectors in order to express these proteins in bacterial and mammalian systems (Figure 4-3). The bacterial system proved sufficient to get high amounts of active proteins in a short period, so the mammalian system was dropped.

Both recombinant rCFHR1 and SCR3-5 showed dose-dependent binding to heparin and this indicates that the proteins are probably correctly refolded (Figure 4-8). However, the binding affinity of rCFHR1 (EC_{50}=0.17 μM) is less than that of rSCR3-5 (EC_{50}=0.11 μM). This does not agree with Zipfel *et al*. (2002) who mentioned that the full length of CFHR1 could bind to heparin in two SCR domains (SCR2 and 5), which means the heparin binding was restricted to only SCR5 domain of rSCR3-5. The result also explained that the binding affinity of rCFHR1 and rSCR3-5 was more than that of factor H which is K_D=0.5 μM (Perkins, Fung and Khan, 2014). A competition experiment between these two types of CFHR1 and factor H to heparin showed that, increasing amounts of factor H affected rCFHR1 and rSCR3-5 binding in a dose-dependent manner (Figure 4-9). This could be because the competition of SCR2 and SCR5 of rCFHR1 and

rSCR3-5 with SCR7 and SCR20 of factor H on heparin binding sites as these domains are responsible for heparin binding (Józsi *et al*, 2015). SCR5 of both recombinant proteins shares exactly the same structure with SCR20 of factor H, and there is 41% similarity between amino acids of SCR2 of CFHR1 and SCR7 of factor H (Skerka *et al*, 2013).

Both recombinant proteins show clear binding to C3b (Figure 4-10). Hannan *et al*. (2016) mentioned that CFHR1 can bind to C3b and act as a competitive inhibitor of factor H on its binding sites on C3b molecules. The main C3b binding sites in factor H are SCR1-4 (Wu *et al*, 2009), SCR6-10 (Sharma and Pangburn, 1996) and SCR19 and 20 (Hannan *et al*, 2016). SCR4 and 5 of CFHR1 show high similarity to the C-terminus of factor H SCR19 and 20, while SCR1-2 of CFHR1 show less similarity to SCR6-7 of factor H (Skerka *et al*, 2013). Figure 4-10 showed that rCFHR1 and rSCR3-5 have approximately the same binding affinity to C3b (EC_{50}=0.4 μM), indicating that both rCFHR1 and rSCR3-5-C3b binding can mediate augmentation of the alternative pathway. However, factor H show lower binding affinity to C3b (K_D=0.59 μM) (Perkins, Fung and Khan, 2014) compared to rCFHR1 and rSCR3-5. Our results revealed that increasing amounts of factor H affected rCFHR1 and rSCR3-5 binding in a dose-dependent manner and led to inhibition of both recombinant proteins-C3b binding (Figure 4-10). This could be because the competition between SCR4 and 5 of both recombinant types of CFHR1 and SCR19 and 20 of factor H which incorporated in C3b binding sites as they share the same similarities in their structures (Skerka *et al*, 2013).

rCFHR1 and rSCR3-5 could augment C3b deposition and thus enhance the alternative pathway (Figure 4-12). It is known that CFHR1 can induce the alternative pathway because it could competitively inhibit factor H-C3b binding (Hannan *et al*, 2016). This competitive inhibition arises because SCR4 and 5 of CFHR1 share approximately the same amino acid structure as SCR19 and 20 of factor H, which are responsible for C3b binding (Skerka *et al*, 2013). The results also showed that rCFHR1 can activate C3b deposition more than rSCR3-5, since the later reduced the AP_{50} less than rCFHR1, which means that the full-length polypeptide has more affinity to bind to C3b and inhibit its binding to factor H.

Both recombinant proteins, rCFHR1 and rSCR3-5 significantly activated the terminal pathway and induced rabbit RBC lysis by MAC activity through both the alternative and lectin pathways (Figure 4-13 and Figure 4-14). Although CFHR1 inhibits C5 convertase

and so MAC formation (Heinen *et al*, 2009), rCFHR1 and rSCR3-5 activate MAC formation and the terminal pathway through increasing rabbit RBC lysis. This suggests that the high amount of C3b fixed by recombinant proteins can compensate the inhibition of C5 convertase (Figure 4-12). Nonetheless rSCR3-5 did lyse more RBCs than rCFHR1 through the lectin pathway. These differences can be attributed to the lack of the N terminal part of CFHR1 in rSCR3-5, which therefore has less inhibitory activity on the MAC assembly (Heinen *et al*, 2009). Generally, both recombinant proteins can augment the terminal pathway and MAC formation.

6.4 *P. falciparum* experiments

Recombinant proteins showed varied binding ability to asexual blood stages. No binding was seen when rHP and rHMMP were added to schizonts (Figure 5-1). This agreed with Moticka (2015) who mentioned that even though properdin does not show any binding to pathogens, the C3b deposition was enhanced. However, some researchers reported that pathogen-properdin binding was needed to augment the alternative pathway (Spitzer *et al*, 2007). The role of properdin starts with binding to C3bBb complex (Medicus, Gotze and Muller-Eberhard, 1976; Pangburn, 1989). It looks like properdin does not localise to the *P. falciparum* surface in the absence of C3b.

Other recombinant proteins, rCFHR1 and rSCR3-5, showed significant binding to schizonts (Figure 5-2). This agrees with Rosa *et al*. (2016) and Kennedy *et al*. (2016) who demonstrated that asexual blood stages can bind to factor H and CFHR1 using immunofluorescence and Western blotting. Our result could suggest that the schizont binding sites can involve SCR3, 4 and 5 of CFHR1. Although domains SCR4 and SCR5 of CFHR1 are very similar to SCR19 and SCR20 of factor H, Kennedy *et al*. (2016) found that SCR19 and 20 of factor H did not bind to merozoites of *P. falciparum* and this role was restricted to SCR5 and 6 only. Our result also showed that rCFHR1 and SCR3-5 have similar binding affinity to schizonts, suggesting that all the binding sites in full length of CFHR1 are contained in the C-terminus.

The competition between factor H and the recombinant types of CFHR1 for schizont binding was investigated. Factor H can compete with rCFHR1 and rSCR3-5 binding to schizonts in a concentration dependant manner (Figure 5-3). This could be explained by the competition among the similar SCR domains (SCR4 and 5 of CFHR1 and SCR19 and 20 of factor H) (Skerka *et al*, 2013). According to (Kennedy *et al*, 2016), it is not expected

to see any kind of inhibition between factor H and rSCR3-5 on schizont surfaces, as they found that only SCR5 and 6 of factor H bind to merozoites of *P. falciparum*. However, our results showed that the binding between SCR3-5 of CFHR1 and schizont could be inhibited by adding factor H. This suggests new factor H-schizont binding sites in the last two SCRs; SCRs 19 and 20.

Another notable point is that factor H inhibits binding of rCFHR1 to schizonts more than rSCR3-5. This could be because factor H can compete with rCFHR1 on more schizont binding sites compared to rSCR3-5 due to the similarity between SCR1, 4 and 5 of rCFHR1 with SCR6, 19 and 20 of factor H, respectively (Skerka *et al*, 2013).

Schizonts could induce alternative pathway activation, in a dose-dependent and saturable manner (Figure 5-4). This kind of induction is similar to other types of pathogenic microorganisms, which could induce the alternative pathway activation when C3 is hydrolysed by water (Dasari *et al*, 2012). In the case of *P. falciparum*, the C3b deposition can start when the asexual blood stages invade new RBCs (Dasari *et al*, 2012).

Recombinant proteins were tested to see whether they enhance the alternative pathway on schizonts. Both rHP and rHMMP augment alternative pathway activation on isolated schizonts, as evidenced by increased C3b deposition (Figure 5-5 A, B and Figure 5-6). This is probably due to the role of properdin in increasing the half-life of C3b convertase, thus augmenting the alternative pathway by enhancing the amplification loop of this pathway (Fearon and Austen, 1975). The use of recombinant properdin, that can aggregate and oligomerize to a highly active product Pn (Ali *et al*, 2014), is 5-10 times more effective than native properdin (Ferreira, Cortes and Pangburn, 2010).

Regarding recombinant types of CFHR1; rCFHR1 and rSCR3-5 can fix more C3b on schizont coated plates (Figure 5-7). This can be explained by the role of rCFHR1 or SCR3-5 in inhibiting FH-C3b binding thus inhibition the C3bBb decay-accelerating activity associated with factor H (Skerka, Horstmann and Zipfel, 1991).

It is not the first time that regulatory proteins have been used to augment the complement activation against the pathogenic microorganisms. Ali *et al*. (2014) tested the protective role of Pn against *Neisseria meningitidis and Streptococcus pneumoniae* on mice. These bacteria inhibit the alternative pathway by recruitment of factor H (Dave *et al*, 2004; McNeil *et al*, 2013). Experiments showed that *N. meningitidis and S. pneumoniae* infected mice treated with recombinant human properdin were less susceptible to

infection than untreated mice. Therefore, it could be useful to test the expressed recombinant proteins on asexual stages of *P. falciparum*, as these stages recruit factor H to evade the complement attack (Simon *et al*, 2013).

It is very difficult to check the recombinant complement regulatory proteins role on *P. falciparum* stages *in vivo* in humans, because of the host specificity. In order to test the role of these products on live asexual blood stages, an *ex vivo* model of the blood stage was used. *P. falciparum* blood stages were cultured in RBCs in media supplemented with NHS (as a source of complement) or NHS plus recombinant complement control proteins. Growth assays and immunofluorescence microscopy were used to determine the effect of the regulatory proteins on parasitaemia and complement deposition.

In the growth assays, parasitaemia was determined by making blood smears and staining the slides with Giemsa stain. The parasitaemia was calculated by counting the number of RBCs with blue dots inside (Figure 5-8), which represent the infected cells with blood stages of the parasite. This assay was blinded, by hiding the slide name by a third person other than the researcher. Each slide was counted ten times of a hundred small squares, which could ensure covering a statistically considerable area of slides.

The first growth experiment was done using only one recombinant protein, with a single supplementation for 48 hours (Figure 5-9). Non-significant reduction was shown when rCFHR1 was added to parasitic culture supplemented with NHS. Significance may not have been reached because rCFHR1 and NHS was only added at the start of the experiment, which means no continuous supply for serum or rCFHR1. It is important to provide serum or protein each 12 hours to ensure there is continuous complement activity, which mirrors the in vivo situation more exactly (Rosa *et al*, 2016).

The other growth assay experiment was done for 96 hours over three generations of asexual blood stages, with continuous supply of all prepared recombinant proteins. rHP and rHMMP showed no significant decrease in parasitaemia when they were added to NHS compared to NHS without addition (Figure 5-10 A). In Figure 5-10 panel B, rCFHR1 and rSCR3-5 showed significant decrease in parasitaemia when these proteins were added to NHS compared to NHS without addition. This growth inhibition may be related to the competition to the binding between rCFHR1 or rSCR3-5 to factor H on both asexual blood parasites and C3bBb, which leads to reduced recruitment of factor H by the parasite and activation of the complement alternative pathway by stopping decay

accelerating activity associated with factor H (Timmann, Leippe and Horstmann, 1991). This result agrees with (Kennedy *et al*, 2016) who mentioned that merozoites of *P. falciparum* can recruit factor H to evade complement activation. Another issue could be seen in this experiment: the parasitic growth was inhibited even when rCFHR1 and rSCR3-5 were added to HIS compared to HIS without addition. During heating the normal human serum at 65°C, C3b activity can be reduced. This may refer to the fact that the recruitment of factor H by asexual blood parasites is independent of C3 activity (Kennedy *et al*, 2016).

Immunofluorescence was used to study C3b deposition and MAC formation on live asexual blood stages. All samples supplemented with NHS and the recombinant proteins showed C3b and C5b-9 deposition (Figure 5-13 and Figure 5-14). It was expected to see the complement component deposition as it has been previously shown that C3b can deposit on schizonts (Figure 5-5, Figure 5-6 and Figure 5-7). In addition, C3b can trigger complement pathways, which continue with deposition of C5b to C9 forming the MAC. It was seen before that MAC can deposited on asexual blood stages (Rosa *et al*, 2016). However, when HIS was added, no C3b or C5b-9 deposition were seen which could relate to reduction of C3b activity when NHS is heated to 65°C (Kennedy *et al*, 2016). Immunofluorescent assay results confirmed our last findings, which show that rHP, rHMMP, rCFHR1, and rSCR3-5 can induce more C3b and C5b-9 deposition on isolated schizonts and showed that this deposition was also on live schizont iRBCs.

There appear to be two mechanisms to enhance the complement activation on *P. falciparum* at play here; Firstly, CFHR1 may compete factor H off the parasite surface, and secondly, either CFHR1 or properdin can competitively inhibit the interaction between factor H and surface bound C3b (perhaps in the context of C3bBb).

The plasma concentration of CFHR1 dimers is 70 µg/ml, while factor H concentration is 500 µg/ml. The molar ratio of plasma concentrations is 0.3:1. (CFHR1:factor H). Therefore, CFHR1 is not a credible therapeutic agent, as at least 200 µg/ml is needed to interfere with factor H activity. However, targeting factor H, especially with Pn of properdin, can result in systemic complement dysregulation, perhaps even de-complementing the patient, which would be counterproductive.

It might be better to target the *P. falciparum* proteins, using monoclonal antibodies, drugs, vaccination or inhibitory peptides derived from the *P. falciparum* asexual blood stage

proteins. However, production of monoclonal antibodies is very expensive, while expression of peptides and active vaccination are cheaper. Attempts were made to produce new vaccines targeting the whole sporozoites or circumsporite protein of sporozoites (Hill, 2011; Epstein *et al*, 2017).

6.5 Future work

CFHR1 consists of five SCR domains. SCR1-2 represent the N terminal part, while SCR3-5 represent the C terminus (Figure 1-4). Supplementing NHS with the full length of CFHR1 could reduce the parasitaemia and inhibit infecting new RBCs, while rSCR3-5 showed less effect (Figure 5-10 B). It could be concluded that the N terminal part could be involved in inhibiting factor H recruitment. It can be suggested that expressing each SCR domain of CFHR1 could be valuable. These domains could be tested for their role in reducing the parasitaemia. This could give an insight about which domains are exactly included in sequestering asexual blood stages of *P. falciparum* to factor H. Thus, the effective concentrations of the active domains may decline much more than the full length of CFHR1 which then could be suggested as a therapeutic agent.

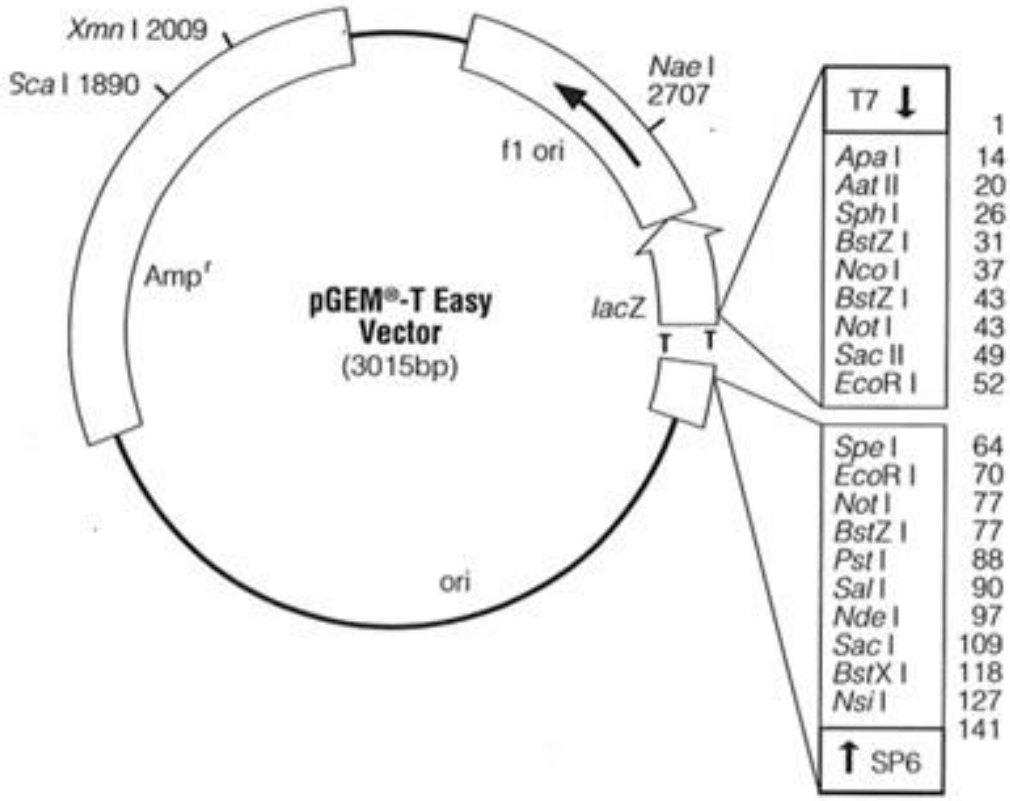
There are different binding sites of asexual blood stages to factor H. Two proteins; PfGAP50 (Simon *et al.* 2013) and Pf92 (Kennedy *et al*, 2016), were reported to be the binding sites. These proteins could be expressed and checked for their activity in factor H binding. Blockage of these sites could be effective in preventing factor H sequestration. It is possible that antibodies directed against these sites could block factor H binding sites, thus stopping the recruitment of factor H. However, producing monoclonal antibodies can be expensive.

Studying *in vivo* models of *P. falciparum* experimentally is very difficult. A new technique was developed by Soulard *et al*. (2015) to use humanised mice as an intermediate host of the parasite. They succeeded in seeing the complete life cycle of human *P. falciparum* in this kind of mice. Doing inhibition experiments using this technique could show more realism regarding use the complement regulatory proteins to inhibit the parasitaemia.

7 Chapter seven

Appendices

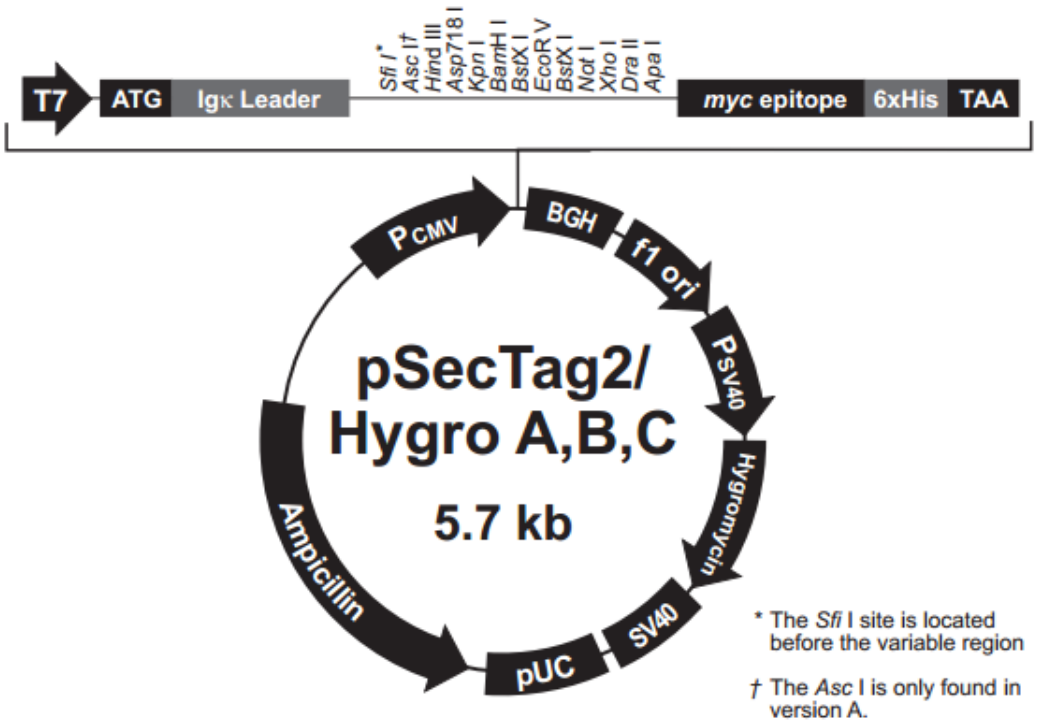
PGEM T easy vector map



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

PsecTag 2/Hygro vector



Comments for pSecTag2/Hygro A
5745 nucleotides

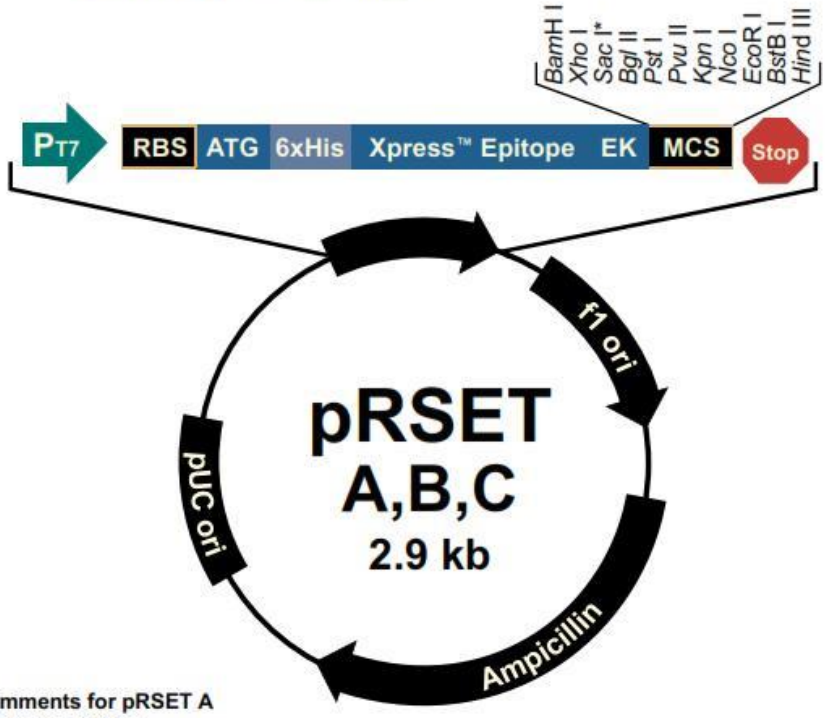
CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Murine Ig kappa-chain V-J2-C signal peptide: bases 905-967
Multiple cloning site: bases 970-1081
c-myc epitope: bases 1082-1111
Polyhistidine tag: bases 1127-1144
BGH reverse priming site: bases 1167-1184
BGH polyadenylation site: bases 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

pRSET B 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)

Contributions

Name	Year	Type and title	Place
3rd Complement UK: Symposium and Training Course	23th to 24th March 2015	attendance	Christ's College St Andrew's Street Cambridge CB2 3BU UK
The 8th annual Postgraduate Conference of University of Leicester	18th-20th April 2016	Presentation: Development of immunotherapeutic to enhance complement-mediated killing of P. falciparum	Leicester, UK
4th Complement UK Annual Conference	24th-25th November 2016	attendance	College Court, Leicester Knighton Rd, Leicester, East Midlands LE2 3UF
Complement for Practitioners: Managing Renal Disease	6th-7th April 2017	attendance	King's College London Guy's Campus Henriette Raphael House
The 9th annual Postgraduate Conference of University of Leicester	27th-29th March 2017	Presentation: Humanised mouse properdin could augment the alternative pathway of wild type mouse and normal human sera on zymosan and *Plasmodium falciparum* merozoites coated plate	Leicester, UK
EMBL Conference: BioMalPar XIII: Biology and Pathology of the Malaria Parasite	29th– 31th May 2017	Poster: CFHR1 Augments Complement Activation on Plasmodium falciparum	EMBL Heidelberg, Germany
10th International Conference on Complement Therapeutics	24th-29th June 2017	Poster: Recombinant human and chimeric properdin augment Complement Activation on Plasmodium falciparum	Aldemar Knossos Royal Village Conference Center Location: Heraklion, Crete, Greece
5th Complement UK Training Course &Symposium	26th & 27th March 2018	Poster: CFHR1 augments opsonisation of Plasmodium falciparum blood stages and reduces parasitaemia	Manchester Meeting Place, Manchester, UK
ROYAL SOCIETY OF BIOLOGY EAST MIDLANDS BRANCH POSTGRADUATE POSTER COMPETITION	21st May 2018	Poster: Inhibition of factor H-recruitment mechanism, used by Plasmodium falciparum blood stages (causative agent of malaria), and reduces parasitaemia	DE Montfort university, UK

8Chapter eight

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