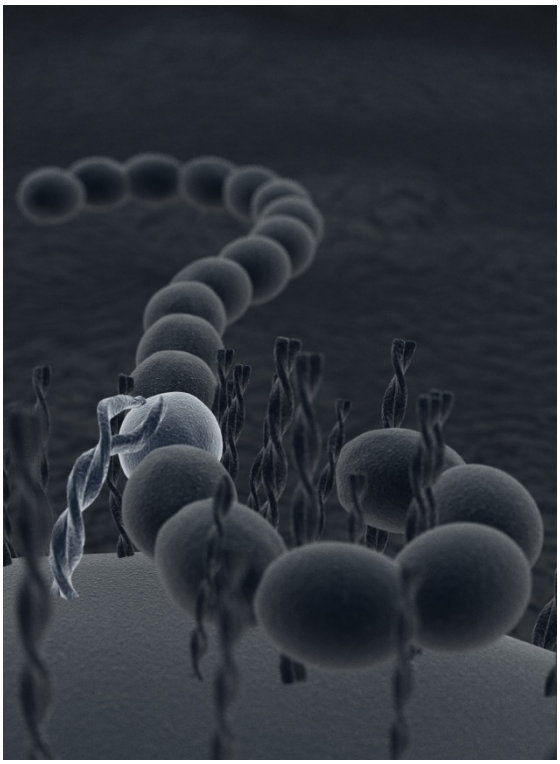
Role of the Soluble Complement Regulator Factor H in Microbial Survival and Host Infection Susceptibility



Karita Haapasalo-Tuomainen
Helsinki 2012

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”Complement factor H attaching on to the surface of *S. pyogenes* via M protein”
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Karita Haapasalo-Tuomainen
2012

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Dedicated to the memory of my mother, Ritva Sinikka Haapasalo

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ORIGINAL PUBLICATIONS

This thesis is based on the following original articles:

I. **Haapasalo K**, Meri T, Jokiranta TS. 2009. *Loa loa* Microfilariae evade complement attack *in vivo* by acquiring regulatory proteins from host plasma. Infect Immun. 77(9):3886-93.

II. **Haapasalo K**, Jarva H, Siljander T, Tewodros W, Vuopio-Varkila J, Jokiranta TS. 2008. Complement factor H allotype 402H is associated with increased C3b opsonization and phagocytosis of *Streptococcus pyogenes*. Mol Microbiol. 70(3):583-94. Corrigendum in Mol Microbiol. 2012. 83(6):1285-86.

III. **Haapasalo K**, Vuopio J, Syrjänen J, Suvilehto J, Massinen S, Karppelin M, Järvelä J, Meri S, Kere J and Jokiranta TS. 2012. Acquisition of complement factor H is important for pathogenesis of *Streptococcus pyogenes* infections: Evidence from bacterial *in vitro* survival and human genetic association. J Immunol. 188:426-35.

ABBREVIATIONS

AGN = acute glomerulonephritis
AMD = age-related macular degeneration
AP = alternative pathway
C1inh = C1-inhibitor
C3aR = C3a receptor
C4BP = C4b-binding protein
C5aR = C5a receptor
C5L2 = C5a-like receptor
CFH = complement factor H
CFH5-7 = a peptide with complement factor H domains 5 to 7
CFHL-1 = complement factor H-like protein 1
CFHR = complement factor H-related protein
CFU = colony-forming unit
CP = classical pathway
CPN = carboxypeptidase N
CR = complement receptor
CRIg = complement receptor of the Immunoglobulin family
CRP = C-reactive protein
DAF = decay accelerating factor, CD55
EDTA = ethylene diamine tetraacetic acid
EGTA = ethylene glycol tetraacetic acid
EIA = enzyme immunoassay
ELISA = enzyme-linked immunosorbent assay
GPI anchor = glycosylphosphatidylinositol anchor
GVB = veronal-buffered saline with gelatin
HRP = horseradish peroxidase
HVR = hypervariable region
iC3b = inactivated C3b
LHR = long homologous repeat
LP = lectin pathway
MAC = membrane attack complex
MASP = MBL-associated serine protease
MBL = mannan-binding lectin
MCP = membrane cofactor protein, CD46
NHS = normal human serum
OF = opacity factor
OPD = o-phenylenediamine
PBS = phosphate-buffered saline
PEG = polyethylene glycol

Por = porin
RCA = regulators of complement activation
SC5b-9 = soluble C5b-9
Scl = streptococcal collagen-like protein
SCR = short consensus repeat
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIC = streptococcal inhibitor of complement
SLO = streptolysin O
Spe = streptococcal pyrogenic exotoxin
STP region = serine/threonine/proline- rich region
TP = terminal pathway
VBS = veronal-buffered saline

ABSTRACT

The key player in human innate immune response is the complement system that attacks microbes and foreign particles invading human body. Complement cascade can be activated through three pathways, the classical, alternative, and lectin pathways. The classical and lectin pathways are activated by recognition of nonself structures while the alternative pathway is constantly in a slightly active stage and is triggered on surfaces that are missing self signals. Surface-deposited complement components act as opsonins for phagocytes while chemotactic and anaphylatoxic components released upon complement activation induce phagocyte recruitment, phagocytosis and the inflammatory response. The components of the late stage of the activation cascade form lytic transmembrane complexes on the surface of the target.

Complement activation is regulated by host surface bound as well as fluid phase molecules that act on several steps during the cascade. Factor H (CFH) is an important fluid phase regulator. It is an elongated protein consisting of 20 domains where the four most amino-terminal domains are responsible for the regulatory activities. A common polymorphism Y402H in CFH that is located in the domain seven has been shown to be associated with age-related macular degeneration (AMD). Several pathogenic microbes capture CFH on their surface from host plasma inhibiting the function of the complement system on the microbial surface. The surface exposed M protein of the pathogenic bacterium, *Streptococcus pyogenes*, binds CFH via the domain seven. The highly variable region of the protein confers to serotype specificity and on the basis of the gene region coding for the hypervariable part of M protein the bacteria can be divided into over 150 different *emm* types.

The aims of this thesis were to analyze the effects of CFH binding on microbial complement evasion *in vivo* and *in vitro* and to analyze how genetic variation in CFH affects *S. pyogenes* survival and host infection susceptibility. It was shown that *Loa loa* microfilariae isolated from patient blood carried host CFH and C4BP on themselves. The complement components deposited on microfilariae surface *in vivo* and *in vitro* were inactive indicating that the microfilariae evade complement attack using surface bound CFH and C4BP.

Most of the *S. pyogenes* strains studied were found to bind CFH and a recombinant CFH fragment consisting of domains 5-7 (CFH5-7) *in vitro*. Binding of CFH and CFH5-7 to 38 studied *S. pyogenes* strains was variable and the binding affinity showed significant inverse relationship with formation of complement activation markers in serum. When a CFH5-7 fragment lacking the cofactor active domains

was incubated with the CFH binding strains in an *in vitro* survival model the multiplication of the bacteria was significantly decreased showing that CFH binding via the domain seven is crucial for survival of *S. pyogenes*.

CFH was isolated from human plasma obtained from donors that were genotyped homozygous for the Y402H polymorphism. The two allotypes bound differentially to the surface of the studied *S. pyogenes* strains and the diminished binding of the allotype CFH(402H) resulted in a significant decrease in multiplication of the bacteria in an *in vitro* survival assay. On the basis of these results it seems that the residue 402Y is crucial in *S. pyogenes*-CFH interaction and that the Y402H polymorphism affects opsonization and phagocytosis of the bacterium.

The Y402H (C1277T) genotype was analyzed from patients with history of erysipelas or recurrent tonsillitis (n=487) and from 455 control subjects. Comparison of the allele and genotype frequencies between the patients and control subjects suggests that the polymorphism is associated with susceptibility to streptococcal infections.

In conclusion, the results show that complement evasion by binding of complement regulators occurs both *in vitro* and *in vivo* and that within the *S. pyogenes* species CFH binding via the domain seven is a common way to evade complement attack and to enhance microbial survival in the host. The ability to capture host CFH varies within different *S. pyogenes* strains as well as within different isolates and it is likely that the affinity of CFH binding determines the ability of bacteria to evade the complement attack. It also seems that the CFH Y402H polymorphism affects *S. pyogenes* survival *in vitro* and this finding probably also has clinical relevance since the studied polymorphism suggests an association with host susceptibility to streptococcal infections erysipelas and recurrent tonsillitis.

TIIVISTELMÄ

Komplementtijärjestelmä on keskeinen luontaiseen immuniteettiin kuuluva tekijä, joka toimii ensimmäisenä puolustuksena isäntään tunkeutuvia mikrobeja vastaan. Se voi aktivoitua sekä tunnistamalla vieraita rakenteita klassisen- tai lektiinitien kautta että ilman mitään spesifiä tunnistuskohdetta oikotieaktivaation kautta. Komplementtiaktivaation käynnistyttyä vieraan kohteen pinnalla, aktivaatio etenee peräkkäisten reaktioiden sarjana. Reaktiosarjan aikana pilkkoutuneista proteiinin paloista osa vapautuu plasmaan toimien houkuttimina ja aktivoijina luonnollisen immuniteetin puolustussoluille. Tietyillä kohteen pinnalle kiinnittyneillä proteiinien osilla on puolestaan kyky opsonoida eli kuorruttaa kohde, jotta fagosyytit voisivat tunnistaa sen. Aktivaatiokaskadin edetessä loppuun kohteen pinnalle voi muodostua solukalvon läpäiseviä komplekseja.

Komplementin oikotie aktivoituu herkästi minkä tahansa kohteen pinnalla ja voi olla tuhoisa elimistön omillekin soluille ellei sitä säädellä tehokkaasti. Elimistömme omia rakenteita suojataan komplementin säätelijäproteiineilla, joita löytyy sekä plasmasta liukoisena että omien solujen solukalvoihin kiinnittyneinä. Faktori H (CFH) on plasmassa liukoisena esiintyvä pitkänomainen komplementin oikotieaktivaatiota säätelevä proteiini, johon kuuluu 20 samankaltaista osaa eli domeenia. Neljä ensimmäistä amino-terminaalista domeenia ovat vastuussa proteiinin säätelyaktiivisuudesta kun taas domeenissa seitsemän sijaitse CFH:n eräs polymorfia Y402H, joka liittyy silmänpohjan ikärappeuman syntyyn.

Useat mikrobit, niin bakteerit, virukset, hiivat kuin vaikkapa verenkierrossa elävät matojen toukkamuodotkin, väistävät komplementin tuhovaikutusta kaappaamalla pintaansa CFH:ta. M proteiini on A-streptokokkibakteerin (*Streptococcus pyogenes*) tärkeä virulenssitekijä, joka tarttuu CFH:n seitsemänteen domeeniin. Sen proteiinirakenteessa esiintyy runsaasti vaihtelua eri *S. pyogenes* serotyyppien kesken ja sen hypervariaabelialueen geenisekvenssin perusteella bakteerit voidaan edelleen jakaa yli 150 *emm*-tyyppiin.

Tämän väitöskirjatyön tavoitteina oli selvittää kuinka CFH:n sitoutuminen mikrobin pinnalle edistää mikrobin komplementtijärjestelmän väistöä *in vivo* ja *in vitro*, ja vaikuttaako CFH:n geneettinen vaihtelu ihmispopulaatiossa yksilön infektioalttiuteen.

Tutkimuksen tulokset osoittavat, että potilaan verestä eristetyt *Loa loa* sukkulamadon toukkamuodot eli mikrofilariat sitovat pinnalleen CFH ja C4BP säätelijäproteiineja *in vivo* ja *in vitro*. Lisäksi mikrobien pinnalla havaitut komplementin komponentit olivat pääosin inaktivoituja ilmeisesti näiden pinnalle

sitoutuneiden sääteilijäproteiinien toimesta. Löydökset osoittavat, että mikrobi voi sitoa näitä komplementin säätelijöitä ihmisessä *in vivo* ja viittaavat siihen, että säätelijäproteiinien läsnäolo *Loa loa* mikrofilarioiden pinnalla estää komplementin tuhovaikutusta mikrobia vastaan.

Suurin osa tutkittavista *S. pyogenes* kannoista sitoi pinnalleen CFH:ta ja tämän rekombinanttifragmenttia, joka sisälsi CFH:n domeenit 5-7 (CFH5-7). Kun eri *S. pyogenes* kantojen aiheuttamaa komplementtiaktiivisuutta mitattiin seerumissa komplementtiaktivaatiota osoittavien markkerien avulla havaittiin, että kantojen aiheuttama komplementtiaktivaatio korreloi käänteisesti kantojen kykyyn sitoa CFH:ta. CFH5-7 fragmentin osoitettiin myös kilpailevan sitoutumispaikasta *S. pyogenes* bakteerin pinnalla kokopitkän CFH:n kanssa, ja kun viisi tutkittua *S. pyogenes* bakteerikantaa altistettiin CFH5-7 fragmentille, niiden lisääntyminen kokoveressä heikkeni merkitsevästi.

Kokopitkät CFH proteiinit, jotka eristettiin luovuttajien plasmasta, genotyypattiin homotsygoottisiksi Y402H polymorfian suhteen. Sekä CFH5-7 fragmentin että kokopitkän CFH:n sitoutuminen tutkittujen *S. pyogenes* kantojen pinnalle oli heikompaa 402H proteiinilla kuin 402Y proteiinilla. *Ex vivo* mallissa bakteeri selviytyi heikommin allotyypin CFH(402H) suhteen homotsygoottiseksi osoitettujen luovuttajien veressä, johtuen ilmeisimmin CFH:n heikommasta sitoutumisesta bakteerin pinnalle.

Kun verrattiin Y402H genotyyppi ja alleelifrekvenssejä ruusu- tai tonsillitti potilasaineistojen (n=487) ja kontrolliaineiston välillä havaittiin, että Y402H polymorfia voi mahdollisesti liittyä alttiuteen sairastua näihin *S. pyogenes* bakteerin aiheuttamiin infektioihin.

Tämä väitöskirjatutkimus osoittaa, että mikrobit voivat sitoa pinnalleen komplementin säätelijäproteiineja *in vitro* ja *in vivo* ja, että CFH:n sitominen on *S. pyogenes* bakteerille oleellista komplementtivälitteisen tuhoutumisen välttämiseksi. CFH:n Y402H polymorfiasta johtuvat vaihtelut CFH:n sitoutumisvoimakkuuksissa *S. pyogenes* bakteerin pinnalle vaikuttavat ratkaisevasti siihen, kuinka mikrobi pystyy välttämään komplementin tuhovaikutuksen ja tätä kautta selviämään veressä. Tämä selittää saadut tulokset CFH:n Y402H polymorfian assosioitumisesta tiettyihin *S. pyogenes* infektioihin. Koska CFH:n sitominen on *S. pyogenes* bakteerin virulenssille tärkeää, voidaan CFH:n sitoutumista estävää CFH5-7 fragmenttia kenties käyttää *S. pyogenes* infektioiden hoidossa.

1 REVIEW OF LITERATURE

With time the immune system has developed to an organized, powerful, and sensitive system that enables us to cope with foreign materials invading our body. Immunity is divided into two classes called acquired and innate immunity. Antibodies play a major role in the specific acquired immunity while activation of complement components occurs without previous antigenic stimulus. Complement system was described in 1889 when Hans Buchner demonstrated that a heat labile substance in blood serum was able to kill bacteria thus complementing the action of antibodies. Due to the "complementing" function, ten years later the system was named "complement" by Paul Ehrlich (Morgan, 1999). Within next 50 years it was generally belived that complement requires antibodies for activation. In 1954, however, Louis Pillemer demonstrated that the complement system can be activated independently from antibodies and by this way plays a central role in innate immunity (Pillemer et al., 1954). The evolutionary origin of the antibody-independent complement activation system, later named the alternative pathway (AP), is much more ancient than that of the classical antibody-dependent system (classical pathway, CP). It is probable that components of AP first appeared to the superphylum Deuterostomia early in the evolutionary history. Within evolution several duplications in the complement genes took place and the innate immunity developed to a more complex system coincidentally with the adaptive immunity. In addition to the AP the third complement activation system, lectin pathway (LP), seems to have an ancient origin (Krushkal et al., 1998; Nonaka, 2001).

1.1 The complement system

Function of the complement system is based on recognition of targets leading to organized interactions between complement proteins that either promote or down regulate the cascade. The three activation cascades consist of several soluble proteins, and are regulated by at least five surface bound and 10 soluble regulators. In addition, there are surface bound receptors or proteins that, outside the complement system, mediate the complement-initiated immune response. The complement system has various roles in both innate and acquired immunity and also in cleaning immune complexes from body. These functions are mediated by the molecules generated during the activation cascades. Human deficiencies of complement components have emphasized the importance of the complement system in immunity (Figueroa and Densen, 1991).

Defense against infections. The complement system acts directly on invading microbes by causing rapid destruction of some of the targets and generating covalently target-bound opsonizing molecules that enhance phagocytosis of the microbe by phagocytes. Also chemotactic factors and anaphylatoxins are generated at different stages of the activation cascades thus promoting the innate immune response.

Function in clearance. Complement has an important role in removal of immune complexes and debris from the body. Circulating debris and immune complexes are attacked by the complement system followed by capture of the complexes by erythrocytes and transportation to be destroyed by macrophages in spleen or liver. Complement acts also in the clearance of necrotic and apoptotic cells (Gasque, 2004).

Linking the innate and adaptive immunity. Certain cleavage products of the complement activation cascades are recognized by specific complement receptors. Complement receptors expressed on lymphocytes have an important role in activation of these cells thus linking the innate and adaptive immunity (see chapter 1.1.4).

1.1.1 Recognition

In order to function properly the complement system must be activated through specific recognition, one of the key features of the innate immunity. The complement system has three strategies to distinguish self from nonself structures. These are recognition of “microbial nonself”, recognition of “missing self” and recognition of “altered self”(Medzhitov and Janeway, 2002).

Microbial nonself. In general innate immunity can recognize certain conserved molecular patterns appearing commonly on micro-organisms. These are called pathogen associated molecular patterns (PAMPs) as reviewed here (Medzhitov and Janeway, 1997; Mogensen, 2009). Microbial nonself recognition by complement system is mediated via complement components such as C1q, mannan-binding lectin (MBL), and ficolins. C1q recognizes antigen-bound antibodies, MBL mannose and N-acetylglucosamine, and ficolins N-acetylglucosamine residues on microbial surfaces (Matsushita and Fujita, 1992; Matsushita, 2009).

Missing self. Self surfaces have membrane bound complement regulatory proteins that act as markers of normal self. During the last few years it has become more

and more evident that also one plasma protein, factor H (CFH), that acts as a complement regulator of AP, has an important role in differentiating self cells from nonself cells. CFH recognizes the self cells partially by sialic acids and glycosaminoglycans on self surfaces (Fearon, 1978; Kazatchkine et al., 1979b; Pangburn, 2002; Kajander et al., 2011). Particles without these markers are missing self factors and are therefore immediately considered as targets for the complement attack.

Altered self. Complement plays an important role in the safe disposal of apoptotic and necrotic cells. It has been suggested that complement mediated phagocytosis of apoptotic cells requires two signals. Nucleic acids exposed by apoptotic cells serve as self signals for complement component C1q while the loss of surface complement regulators act as missing self signals. This in turn induces complement activation and opsonization on the target leading to phagocytosis of the cell (Elward et al., 2005). Moreover, apoptotic cells acquire fluid phase complement regulators that protect the cells against excessive complement activation and inflammation (Trouw et al., 2007).

1.1.2 Activation

When recognition of the target through the three basic signals, microbial nonself, missing self, or altered self, has occurred the complement can be activated. The activation is mediated through three pathways, CP, AP (Figure 1), and LP. When activated, all of these pathways generate an active enzyme, C5-convertase, that initiates the terminal pathway (TP) and formation of membrane attack complex (MAC).

Classical pathway (CP). Activation of CP is initiated when C1q, a component of the C1-complex, recognizes its ligand attached to the target. The most well-known activating ligands of C1q are antigen-bound antibodies IgG1, IgG3 and polymerized IgM (Bindon et al., 1988; Collins et al., 2002). At least two IgG molecules are required for triggering the activation so that C1q is bound to the Fc parts of the antibodies (Hughes-Jones et al., 1984). This results in conformational change in C1-complex (Brier et al., 2010) ensuring that a fluid phase antibody does not cause unnecessary complement activation. Molecules other than immunoglobulins, including lipid A region of bacterial lipopolysaccharide (Morrison and Kline, 1977) and components of damaged cells such as DNA, (Jiang et al., 1992a), C-reactive protein (CRP), (Jiang et al., 1992b) serum amyloid P component (SAP)(Ying et al., 1993), and pentraxin 3 (PTX3) can activate the classical pathway in an antibody-independent manner (Deban et al., 2010; Deban et al., 2011).

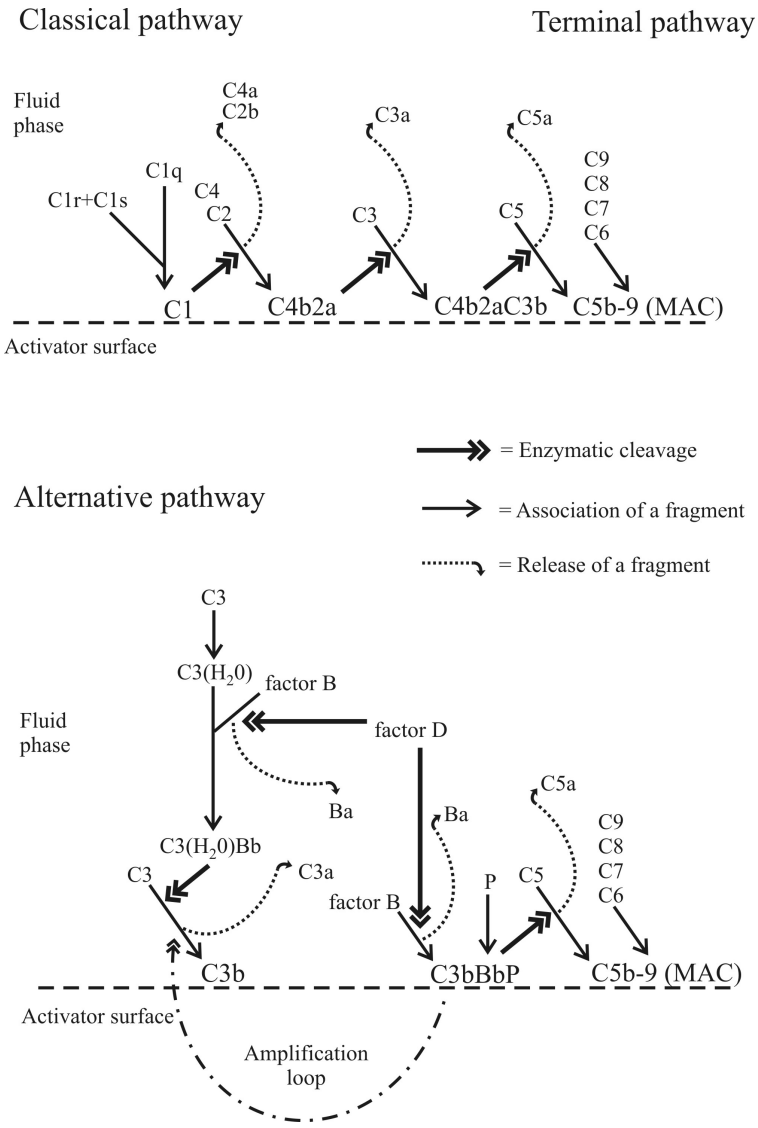

Figure 1. Classical and alternative pathways of complement activation. Complement proteins interact with each other in a sequence. When activated, both pathways lead to activation of the terminal pathway and formation of the membrane attack complex (MAC). The alternative pathway amplification loop is marked as a dashed curve.

The C1 complex is a large multimolecular complex (approximately 750 kDa) composed of a single C1q molecule, two molecules of C1r, and two molecules of C1s. C1s and C1r are linked noncovalently to the complex in a Ca^{2+} dependent manner (Lepow et al., 1963; Ziccardi and Cooper, 1977; Bally et al., 2009). They are both serine proteases having a molecular weight approximately 93 kDa (Sim et al., 1977). C1q is composed of six identical subunits that are bound together noncovalently resembling a bunch of tulips. Each of these subunits can carry the antibody binding site within the globular head domain (Calcott and Müller-Eberhard, 1972; Gadjeva et al., 2008). When C1-complex is bound to the antibodies the C1r subcomponents in the complex are autocatalytically activated thus activating the C1s subcomponents by cleavage (Naff and Ratnoff, 1968; Dodds et al., 1978; Budayova-Spano et al., 2002). Complement components C4 and C2 are both cleaved by C1s (Müller-Eberhard et al., 1967; Matsumoto et al., 1989).

C4 is composed of three chains, α (93 kDa), β (78 kDa) and γ (44 kDa). These are linked together via three disulphide-bonds and noncovalent interactions. In fluid phase C4 is cleaved by C1s to C4b (198 kDa) and C4a (9 kDa) (Schreiber and Muller-Eberhard, 1974). Upon activation C4b undergoes a conformational change exposing a thioester group that can form covalent amide- or ester bond with an amine or hydroxyl group nearby, presumably on the activation surface (Isenman and Kells, 1982; Dodds et al., 1996). Membrane-bound C4b then binds C2 in the presence of Mg^{2+} ions, exposing the C2 component for cleavage by C1s thus resulting in formation of a C4b2a complex, called CP C3-convertase (Müller-Eberhard et al., 1967; Nagasawa and Stroud, 1977). The C3-convertase is an active enzyme where the C2a component promotes the CP activation by cleaving the central complement component, C3 (Cooper, 1975).

The component C2 is an approximately 102 kDa single chain plasma protein and the two subunits cleaved by C1s have molecular weights 74 kDa (C2a) and 34 kDa (C2b) (Kerr and Porter, 1978). The role of C2b that is released upon activation of CP is not known but at least the C2b part has an important role in attachment of the intact C2 to C4b (Nagasawa and Stroud, 1977). The component C2 has an analogous function to factor B in AP (more detailed description in AP section) and these proteins also resemble each other in structure (Pryzdial and Isenman, 1987; Ponnuraj et al., 2004; Milder et al., 2006).

Lectin pathway (LP). The first component of the LP was described when MBL, a serum lectin, was found to initiate the CP in an antibody independent way (Ikeda et al., 1987). MBL binds mannose and N-acetylglucosamine residues that are abundant in bacterial cell walls. The initiation of LP activation requires association of two C1r- and C1s-like mannose-associated serine proteases MASP-1 and MASP-2 with MBL. Complement components C4 and C2 are both cleaved by MASP-2 leading to propagation of the complement activation similarly to CP (Matsushita et

al., 1992; Sato et al., 1994; Thiel et al., 1997). It has also been shown that mouse MASP-1 and probably also MASP-3 both cleave and activate pro-factor D to factor D indicating their importance in AP activation (Takahashi et al., 2010). MBL and C1q are structurally related but, unlike C1q, MBL possesses sugar-binding lectin domains (Turner, 1996). In addition to MBL, LP can be activated by relatively recently described M-, L-, and H-ficolins or 1-, 2- and 3-ficolins, respectively. These all act as pattern recognition molecules that recognize carbohydrates on microbial surfaces such as N-acetylglucosamine. Similarly to MBL, ficolins are associated with MASPs at the early stage of complement activation cascade as reviewed recently (Matsushita, 2009).

Alternative pathway (AP). C3 is the key component of all three pathways since all major effector functions of all complement pathways are mediated through activation of this molecule. It is a large 185 kDa molecule composed of two chains, α and β (Tack et al., 1979b). AP is activated spontaneously since C3 is continuously hydrolyzed at a low rate in human plasma to form a metastable C3(H_20) (Pangburn et al., 1981). Within a short period of time (milliseconds) C3(H_20) is able to bind factor B in a Mg^{2+}-dependent manner exposing it to cleavage by factor D thus forming a C3(H_20)Bb complex, the initial C3 convertase, in fluid phase. This complex cleaves fluid phase C3 to C3a and C3b and the freshly formed C3b can then attach onto any surfaces within a short time (Fearon and Austen, 1975a; Pangburn and Müller-Eberhard, 1980; Fishelson et al., 1984). The smaller cleavage fragment, C3a (9 kDa) of C3, is released and acts as an anaphylatoxin and chemotactic factor as reviewed earlier (Hugli, 1975). This spontaneous complement activation in fluid phase, characteristic only for AP, is constantly targeting all surfaces lacking sufficient down-regulators.

Factor D is a 26 kDa serine protease. It can cleave the single chain factor B only when it is bound to C3(H_2O) or C3b (Lesavre and Muller-Eberhard, 1978) since the conformational change in factor B during the complex formation, exposes factor B for activation by factor D (Janssen et al., 2009; Hourcade and Mitchell, 2011). When cleaved, Bb releases a fragment Ba (30 kDa) while Bb (60 kDa) acts as the serine protease in the formed C3-convertase, C3bBb (Medicus et al., 1976b; Lesavre et al., 1979).

The cleaved C3b exposes a thioester group that can bind covalently to a target surface similarly to C4b in CP. At this stage the interaction between C3b and either factor B or CFH (see section 1.1.3.1) forms the molecular basis of the nonself *vs.* self discrimination of AP (Pangburn and Müller-Eberhard, 1978; Kazatchkine et al., 1979a; Meri and Pangburn, 1990). Upon activation the C3 convertase, C3bBb, is formed similarly to the fluid phase C3(H_20)Bb but unlike C3(H_20)Bb the C3bBb complex can furthermore be stabilized by properdin (see section 1.1.3.1) (Medicus et al., 1976a). The amplification of AP occurs at this stage when C3 convertase

activates more fluid phase C3 by cleavage to C3b (Müller-Eberhard and Götze, 1972). This amplification step causes effective C3b opsonization of the target (Shin et al., 1969).

Terminal pathway (TP). Initiation of the TP and formation of MAC is caused by the surface bound C5 convertases (C4b2aC3b or C3bBbC3b) that cleave fluid phase C5 (Muller-Eberhard, 1986).

C5 is structurally related to C3 and C4 components consisting of two-chains, α (~140 kDa) and β (~80 kDa). These chains are linked together with disulphide bonds (Nilsson et al., 1975). C5 does not contain a thioester group that becomes exposed when C5 is converted to C5b. Therefore, C5b cannot bind covalently to surfaces (DiScipio, 1981). To get activated C5 can bind to surface-associated C5 convertases (C4b2aC3b or C3bBbC3b) exposing itself for cleavage by C2a or Bb (Medicus et al., 1976b; Vogt et al., 1978). The C5a fragment cleaved from the α-chain of C5 is an important anaphylatoxin while the C5b fragment remains attached to C3b in the complex (Tack et al., 1979a). The next two single chain proteins C6 (~104 kDa) and C7 (~92 kDa) bind to C5b in sequence (Discipio and Gagnon, 1982; DiScipio, 1992a) where the attachment of C7 to C6 causes a conformational change in the C5b67 complex releasing it from the convertase into the fluid phase. Within a short period of time the C5b67 complex is able to associate tightly into the target membrane (Podack et al., 1978a). Majority of the complexes do not reach the membrane in time and will be inactivated in fluid phase by S-protein (Podack et al., 1978b) or by C8 described next.

Component C8 is a molecule having similarities with C6 and C7. It is composed of three chains, α (64 kDa), β (64 kDa), and γ (22 kDa). The subunit α is disulphide bonded to γ while this dimer is then noncovalently associated with β. C8 α subunit of C8 is the first complement protein penetrating the cell membrane (Steckel et al., 1980; Hadders et al., 2007). C8 has a dual role in the complement cascade, inhibitory and promoting. Binding of C8 to C5b67 in fluid phase limits attachment of the complex into the membrane while being required for the MAC formation on the surface (Nemerow et al., 1979). Binding of C8 to the surface-attached C5b67 attaches the complex more deeply into the membrane and enables binding of the last complement component, C9, to C8 (Kolb and Müller-Eberhard, 1974). C9 is a single chain protein (69 kDa) homologous with C6, C7, and C8. While attached to C8 C9 is unfolded exposing binding sites for additional C9 components (up to 12-16 molecules) that together form a transmembrane pore (Podack et al., 1982).

1.1.3 Regulation

In the activation cascade C3b must reach the target within a short period of time to avoid hydrolysis and rapid degradation. Furthermore, the formed C3 convertases decay rapidly in the absence of properdin. In the TP the C5b-7 complexes that do not attach to a surface bind C8 in the fluid phase and thereby become inactivated. However, this non-specific regulation is not sufficient to control complement activation because of the efficient amplification steps of the system. Upon activation the complement system has a tendency to propagate in an explosive manner. Especially the low grade constant activation of AP in plasma can lead to amplification very easily and become harmful even for the host as exemplified by several disease processes (see section 1.1.3.1). Without specific regulation (Figure 2) complement activation would also consume the components that are needed for activation leading to acquired (secondary) complement deficiency and thereby complement inactivity.

Genes coding several regulators of complement activation (RCA) are located in chromosome 1q32 within a 900 kb segment called RCA gene cluster (Lublin et al., 1987). These regulators are C4b-binding protein (C4BP), complement receptor 1 (CR1) (Rodriguez de Cordoba et al., 1984), CFH/factor H-like protein 1 (CFHL-1) (Rodriguez de Cordoba et al., 1985), decay accelerating factor (DAF) (Lublin et al., 1987) and membrane cofactor protein (MCP) (Lublin et al., 1988) presented in the order of description. CR1, DAF and MCP are membrane-associated regulators while CFH, CFHL-1, and C4BP are fluid-phase regulators. In addition to their close location in genome these proteins share similarities in their structure and function. They all contain four or more short consensus repeat domains (SCR or complement control protein module, CCP) that are arranged in row resembling “beads on a string”. Each SCR is a globular domain containing approximately 60 amino acids. The bead-like conformation is maintained by four cystein residues that form a triple loop structure via two intradomain disulphide bonds that are always linked in a 1-3, 2-4 pattern (Klickstein et al., 1987).

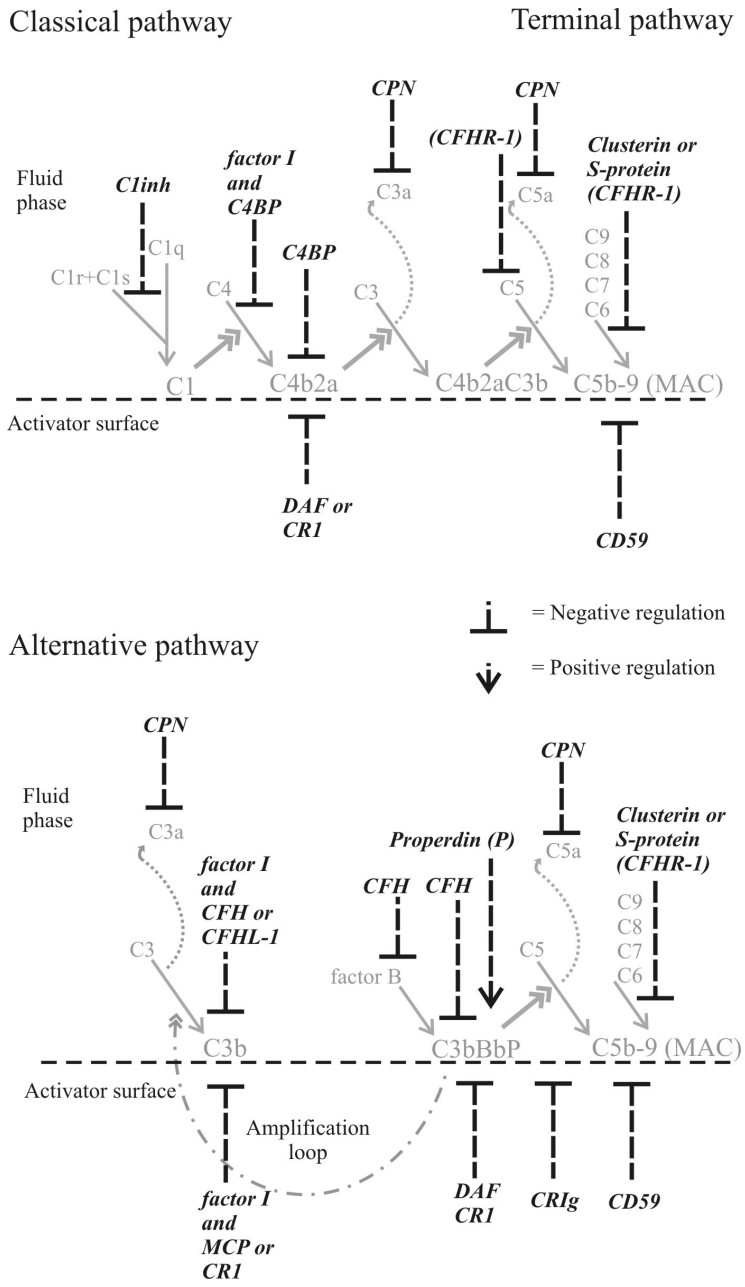

Figure 2. Classical and alternative pathways of complement activation are regulated by soluble and surface bound regulators (marked in italics). Carboxypeptidase N (CPN) acts on C3a and C5a formed by both pathways. The alternative pathway amplification loop is marked as a dashed curve.

1.1.3.1 Soluble complement regulators

C1-inhibitor (C1inh). Upon activation of CP cross-linking of the globular head domains of C1q results in a conformational change in this molecule (Vandenberg and Easterbrook-Smith, 1986) causing autoactivation of C1r and further activation of the serine protease C1s by C1r as explained above. At this stage C1inh is able to regulate the activation by binding to C1r and C1s causing their dissociation from C1q. C1inh also regulates CP activation in fluid phase by acting on C1 that otherwise could be spontaneously autoactivated by non-immune activators at low levels (Ziccardi, 1982). C1inh has an important role in inhibiting various other proteases outside the complement system such as factors in the blood coagulation cascade and kinin-kallikrein system (Schapira et al., 1983; Wuillemin et al., 1995). C1inh is a serine protease inhibitor (serpin) and is consumed by the interaction with the target protease. It is a heavily glycosylated single chain protein with a size of 105 kDa (Harrison, 1983). It has a two-domain structure with the reactive serpin domain in the globular carboxyl-terminus and the glycosylated domain in the tail-like amino-terminus (Odermatt et al., 1981; Bock et al., 1986; Davis et al., 1986). It is the only inhibitor of C1q and a deficiency in C1inh results in hereditary angioedema (HAE), a disease characterized by recurrent acute edema of skin or mucosa. The symptoms in this disease are mainly or totally due to the lack of regulation of kallikrein by C1inh and the generation of the potent vasoactive peptide bradykinin in patient plasma (Pappalardo et al., 2002).

C4b-binding protein (C4BP). After initiation of CP by C1 the next step which is regulated specifically is the C4b stage. This is performed by C4BP that interferes with the assembly of surface bound CP C3-convertase C4b2a by accelerating its natural decay. It also acts as a cofactor for factor I in cleavage of both surface bound and fluid phase C4b to C4c and C4d (Gigli et al., 1979).

C4BP is encoded by a gene in the RCA gene cluster and is mainly expressed in the liver. It is a 540-590 kDa protein consisting of several disulphide bonded subunits (Scharfstein et al., 1978) forming together a spider-like structure (Figure 3) (Dahlbäck et al., 1983) and the most common isoform has seven identical α-chains (70 kDa each) and one β-chain (45 kDa) (Sanchez-Corral et al., 1995). The α-chain is composed of eight SCR-domains (Chung et al., 1985) and the β-chain from three (Hillarp and Dahlbäck, 1990). The chains are carboxyl-terminally linked to a central “core” of the spider-like molecule. The most amino-terminal domains 1 and 2 of the α-chain are likely required for C4b binding and cofactor activities (Blom et al., 2000b) while the β-subunit is required for binding to protein S (Hillarp and Dahlbäck, 1988) probably via domains 1 and 2 (van de Poel et al., 1999a; van de Poel et al., 1999b). Protein S is a vitamin K-dependent molecule of coagulation cascade and its role in the C4BP-protein S -complex is unclear. However, when

bound to C4BP, coagulation-associated functions of protein S remain inactive and it does not affect the regulatory functions of C4BP (Dahlbäck, 1986).

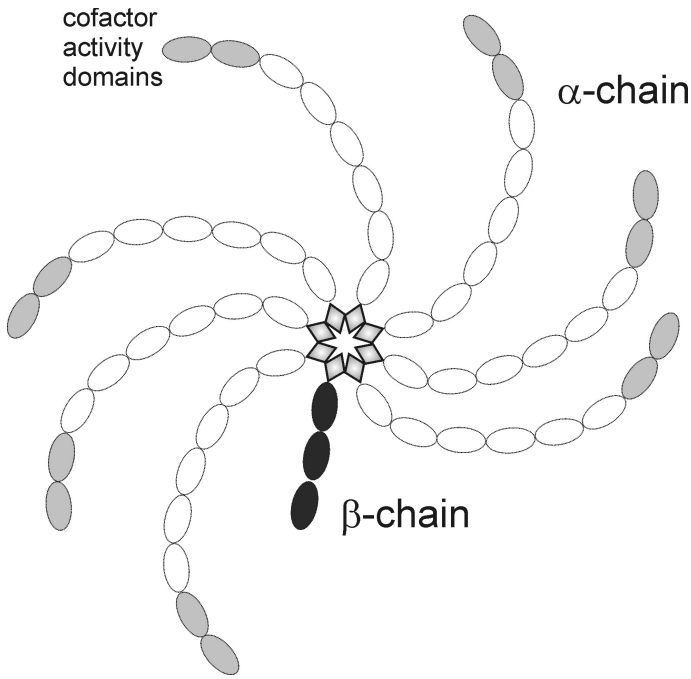

Figure 3. C4BP has a spider-like structure. The seven identical α-chains contain eight domains and the cofactor activity site is located within domains 1 and 2. The β-chain has three domains and binds protein S. Figure modified from Sanchez-Corral et al. 1995 (Sanchez-Corral et al., 1995).

Factor H (CFH). AP can be initiated on any surface where C3b becomes deposited. Whether AP is activated or not is believed to depend on the relative abundance of polyanions on the cell surface, such as glycosaminoglycans or sialic acids. These anionic structures favor binding of CFH rather than factor B to surface bound C3b leading to down-regulation of AP. When bound on the surface CFH regulates AP indirectly by acting as a cofactor for the inactivation of C3b by factor I (Pangburn et al., 1977). It also directly inhibits the formation of C3-convertases by interfering with the binding of factor B to C3b (Pangburn and Müller-Eberhard, 1978) and furthermore increases the pace of the natural decay of already formed AP convertases (Weiler et al., 1976; Whaley and Ruddy, 1976).

CFH is an elongated, approximately 155 kDa single-chain glycoprotein (Sim and DiScipio, 1982) consisting of 20 SCR domains (Ripoche et al., 1988) (Figure 4). The cofactor and decay acceleration activities of CFH are located within the first four amino-terminal domains (Gordon et al., 1995; Kühn et al., 1995; Kühn et al., 1996) while the main surface recognition site is within domains 19-20 (Pangburn, 2002; Jokiranta et al., 2005; Ferreira et al., 2006). CFH interacts with C3b via both domains 1-4 and 19-20 while an additional, probably less important, C3b interaction sites are found within domains 8-18 (Jokiranta et al., 1996; Sharma and Pangburn, 1996; Jokiranta et al., 2000; Jokiranta et al., 2005; Wu et al., 2009). The dual interaction of CFH via domains 19-20 with C3b and nonactivator surface glycosaminoglycans or sialic acids explains the molecular basis between self and nonself discrimination by CFH (Kajander et al., 2011). CFH interacts with heparin via domains seven (Blackmore et al., 1996; Sharma and Pangburn, 1996) and 20 (Blackmore et al., 1998b), and possibly weakly through domain 9 (Ormsby et al., 2006). In addition, it has been suggested, although not confirmed, that CFH binds polyanions/sialic acids via domains 13 (Pangburn et al., 1991). Moreover, CFH interacts with neisserial sialic acids via domains 16-20 (Ram et al., 1998b).

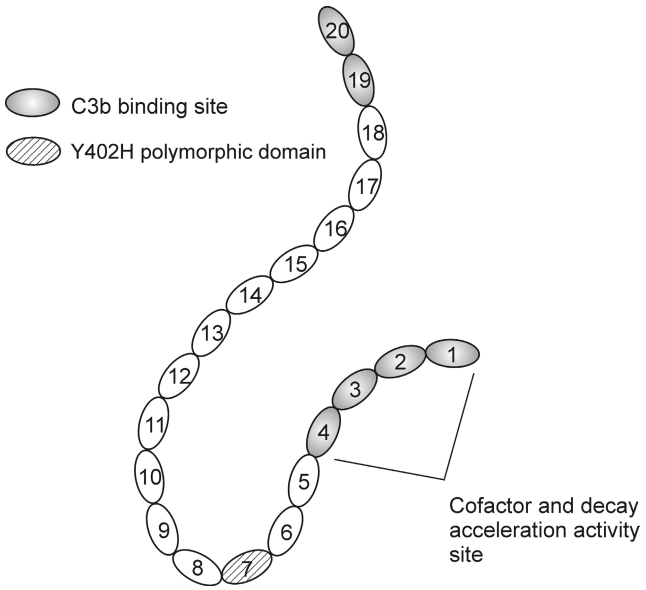

Figure 4. CFH has a elongated structure with 20 domains. The major C3b-binding sites (marked with grey color) are located in domains 19-20 and 1-4 where the latter is the cofactor activity site. CFH polymorphism Y402H is located within domain seven (marked with stripes). CFH is presented as a flexible molecule according to electron microscopy studies (DiScipio, 1992b).

Several mutations in CFH have been reported in renal diseases. Those located in the carboxyl-terminus are associated with atypical hemolytic-uremic syndrome (aHUS) (Warwicker et al., 1998; Richards et al., 2001) while at least one mutation in the amino-terminus is associated with dense deposit disease (DDD), earlier called membranoproliferative glomerulonephritis type II (MPGN) (Licht et al., 2006). In addition to mutations, a large number of CFH polymorphisms have been identified (Rodriguez de Cordoba et al., 2004). Some polymorphisms have been found to be associated with aHUS (Caprioli et al., 2003) while one polymorphism in the domain seven is strongly associated with AMD, the most common cause of visual loss of the elderly in industrialized countries (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). The Y402H polymorphism is located in domain seven close to or within the sites that have been shown to bind heparin, CRP, and *Streptococcus pyogenes* M protein (Giannakis et al., 2003). Further studies have shown that the CFH(402H) allotype shows impaired binding to CRP partially explaining the inflammation in macula of individuals with AMD (Laine et al., 2007). The Y402H polymorphism has also been shown to affect CFH binding to heparin (Clark et al., 2006) and a crystal structure of the CFH6-8 region containing the polymorphic amino acid suggests that the 402Y residue is directly involved in binding of CFH on surface glycosaminoglycans (Prosser et al., 2007). Additionally the Y402H polymorphism affects CFH binding to necrotic cells (Sjöberg et al., 2007) indicating that the alteration of CFH interaction with these structures could potentially lead to increased or reduced complement activation at the location and thereby affect the disease progression. However, it is still unclear why these polymorphisms have been enriched in the human population despite of their association with harmful diseases.

Factor H-like protein 1 (CFHL-1). CFHL-1 is encoded by the same gene as CFH by alternative splicing. It is a 45-50 kDa protein consisting of the first seven amino-terminal domains of CFH followed by four additional amino acids (Ripoche et al., 1988). Similarly to CFH, CFHL-1 has cofactor and decay accelerating activities within domains 1-4 (Kühn et al., 1995; Kühn et al., 1996) but it lacks the surface recognition capacity of full length CFH.

Complement factor H-related proteins 1-5 (CFHR-1, CFHR-2, CFHR-3, CFHR-4A , CFHR-4B, CFHR-5). There are six complement CFH-related proteins (CFHR) that share structural similarities with CFH and are also encoded by genes in the RCA gene cluster. CFHR-1 is composed of five SCR domains and can be found in two different glycosylated forms in plasma, CFHR-1α (37 kDa) and CFHR-1β (42 kDa) (Timmann et al., 1991). Regulatory function for CFHR-1 has been suggested. It probably acts on the TP by binding C5 and C5b6 thus inhibiting the cleavage of C5 and formation of C5b67 complex (Heinen et al., 2009). CFHR-2 consists of four SCR domains (Skerka et al., 1992) while both CFHR-3 and CFHR-4B have five SCR

domains and CFHR-4A and CFHR-5 have nine (Skerka et al., 1997; McRae et al., 2001; Jozsi et al., 2005). At least CFHR-5 has weak cofactor activity for factor I (McRae et al., 2005). CFHR-3 and CFHR-4B both interact with the C3d region of C3b similarly to CFH indicating that they also might have regulatory activity which, however, has not been shown (Hellwage et al., 1999).

Properdin. Stabilization of the formed C3/C5 convertases of the AP is mediated by properdin making it the only positive regulator of complement activation. Stabilizing the convertases decreases the rate of their natural decay (Fearon and Austen, 1975b; Medicus et al., 1976a). According to the early suggestion of Louis Pillemer (Pillemer et al., 1954) properdin can probably also initiate AP activation. Recent studies have shown that it directly (in the absence of C3b) binds on surfaces of *Neisseria gonorrhoeae* (Spitzer et al., 2007) and *Chlamydia pneumoniae* (Cortes et al., 2011) indicating its role as a pattern recognition molecule. The gene encoding properdin is located in X-chromosome (Goundis et al., 1989). Properdin exists in plasma as oligomers of two to four monomers linked together in a cyclic fashion (Figure 5) (Smith et al., 1984). Each 52 kDa monomer consists of six thrombospondin repeat-domains with conserved residues that are also found in thrombospondin and complement components C6, C7, C8, and C9 (Nolan et al., 1991).

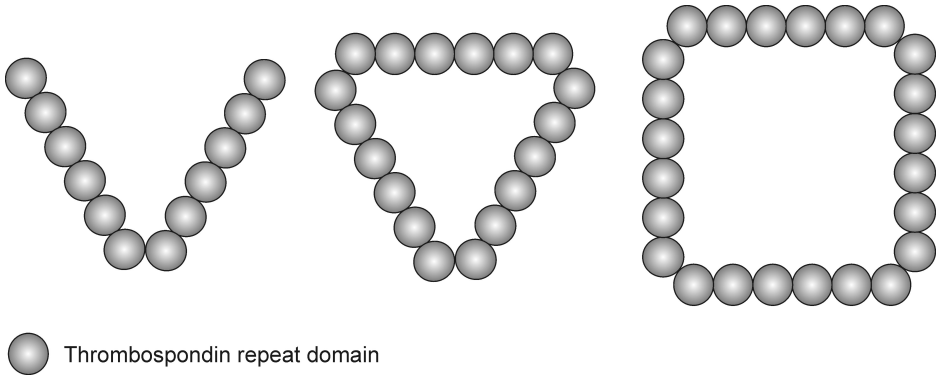

Figure 5. Properdin oligomers. Each properdin monomer has six thrombospondin repeat-domains. The monomers are arranged as cyclic dimers, trimers and tetramers. Figure modified from (Smith et al., 1984).

S-protein (vitronectin). S-protein regulates the complement activation by binding fluid phase C5b-7 thereby preventing the association of the unstable hydrophobic membrane-binding site of the complex onto the membrane. It has also been

suggested that S-protein interferes with C9 polymerization within the membrane-associated C5b-9 complex (Podack et al., 1984). The name vitronectin comes from its property to mediate cell attachment to tissues similarly to fibronectin (Hayman et al., 1983). The name S-protein (S for site specific) comes from the observation that it regulates the C system competing for the membrane-binding sites with terminal activation complexes (Podack et al., 1978b). S-protein also binds heparin suggesting that it interferes with the anticoagulation system (Preissner and Muller-Berghaus, 1987). In plasma, S-protein exists at least in two forms, a single chain molecule (84 kDa) and a two chain molecule where the subunits (69 kDa and 15 kDa) are linked together with disulfide bonds. It probably also forms dimers and higher molecular weight aggregates in plasma (Podack and Muller-Eberhard, 1979).

Clusterin (SP-40,40). Similarly to S-protein clusterin binds fluid phase C5b-7 making it incapable to associate with membranes (Murphy et al., 1989). Of the isolated late complement components, clusterin associates with C7, C8, and C9. The binding of C9 to clusterin suggests that, in addition to membrane association, clusterin also inhibits the C9-polymerization and thereby formation of the transmembrane pore (Tschopp et al., 1993). The name clusterin comes from the property of the protein to stimulate cell adhesion and aggregation (Blaschuk et al., 1983). It is a heterodimeric serum glycoprotein composed of α- and β-chains both having a molecular weight of approximately 40 kDa. The heavily glycosylated chains are linked together by disulphide bonds (Murphy et al., 1988).

Factor I (C3b inactivator). Factor I regulates the complement system by inactivating C4b and C3b molecules generated upon activation. However, factor I cannot function without a cofactor. In the presence of a cofactor, either C4BP (Fujita et al., 1978), CR1 (Iida and Nussenzweig, 1981), or MCP (Seya et al., 1986), factor I cleaves the α-chain of C4b at two sites leaving the surface bound C4d (47 kDa α^2 chain) attached on the membrane and releasing the large C4c (consisting of chains α^3, α^4, β, and γ) fragment in the fluid phase.

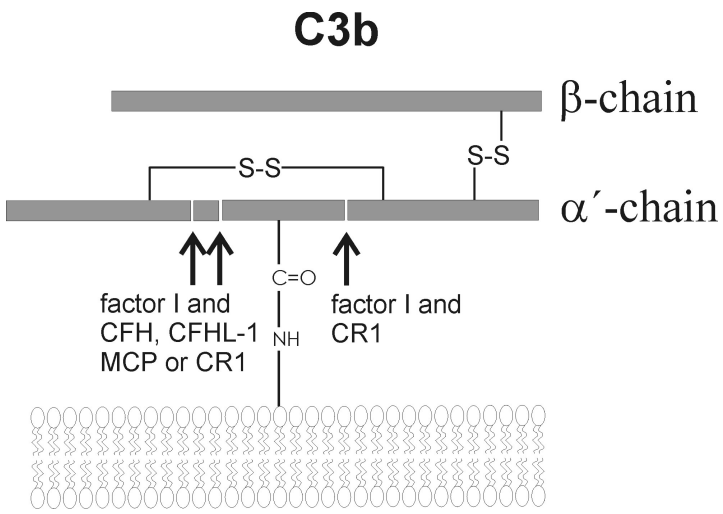

Figure 6. Inactivation of C3b by factor I and the cofactors. The α-chain is cleaved by factor I in two steps. In the first step two first cleavages require the cofactor activities of CFH, MCP or CR1 while in the second only CR1 can act as a cofactor.

Unlike the cleavage of C4b the cleavage of C3b by factor I occurs in two steps. The first two cleavages of C3b splits the α´-chain into two fragments (68 kDa and 43 kDa) giving rise to an inactive iC3b molecule while releasing a small C3f fragment (Figure 6) (Harrison and Lachmann, 1980). The generated iC3b does not bind factor B and therefore cannot promote further AP activation (Ross et al., 1983). The first two cleavages requires the presence of cofactors CFH (Pangburn et al., 1977), MCP (Seya et al., 1986) or CR1 (Fearon, 1979). Factor I can further cleave the iC3b molecule releasing C3c in the fluid phase and leaving the C3dg attached on the membrane. Only CR1 has cofactor activity for this third cleavage under physiological conditions (Ross et al., 1982). C3b and its cleavage fragments iC3b are both opsonic fragments. Therefore the cleavage of iC3b to C3dg by CR1 on host cell surfaces is crucial for protection against phagocytosis (van Lookeren Campagne et al., 2007).

Factor I is approximately a 90 kDa enzyme consisting of a heavy (50 kDa) and a light chain (38 kDa) linked together by a disulphide bond. The serine protease activity is located in the light domain while the C3b-binding site is in the heavy domain (Goldberger et al., 1987; DiScipio, 1992b; Roversi et al., 2011).

Carboxypeptidase N (CPN). When activated, the complement proteases cleave components C3, C4 and C5 releasing small polypeptides to the fluid phase. The released C5a and C3a are both powerful anaphylatoxins that trigger the inflammatory response, C5a being clearly more efficient. Although these polypeptides share similarities in structure and function, only C5a is able to promote chemotaxis (Fernandez and Hugli, 1978). To inhibit an excessive inflammatory response, these mediators are quickly and efficiently inactivated by an anaphylatoxin inactivator, CPN, that removes an arginine residue from the mediators thus forming the molecules C5aDes-Arg or C3aDes-Arg (Bokisch and Müller-Eberhard, 1970). These DesArg forms have reduced or no anaphylatoxic or chemotaxic activity because of reduced binding to C3aR or C5aR (see section 1.1.4).

1.1.3.2 Membrane associated regulators

Complement receptor 1 (CR1, CD35). This 205 kDa molecule was first isolated from erythrocyte membranes. It has an important role in complement regulation on cell surfaces and in the clearance of fluid phase immunocomplexes. In addition to the cofactor activities in cleavage of C4b and C3b (see above) it also has decay accelerating activity on the convertases C3bBb (Fearon, 1979), C4b2a and C4b2aC3b (Iida and Nussenzweig, 1981). Processing of immune complexes by CR1 is a dynamic event where both CR1 mediated cofactor activity and ligand recruitment plays an important role. CR1 is a receptor for C3 and C4 cleavage fragments C3b, iC3b and C4b (Ross et al., 1983). It effectively attracts C3b opsonized fluid phase immune complexes to the surface of erythrocytes but easily releases them when C3b is inactivated to iC3b by factor I. Thereby the CR1 molecule becomes unoccupied and can rapidly rebind to adjacent C3b molecule causing a dynamic “binding and release” event (Medof et al., 1982a; Medof et al., 1982b; Medof et al., 1982c). As a result the opsonized immune complexes are easily delivered to tissue macrophages for phagocytosis. CR1 is also a cellular receptor for C1q (Klickstein et al., 1997) and MBL (Ghiran et al., 2000).

The gene for CR1 is located in the RCA gene cluster. CR1 is a polymorphic membrane associated protein where the most common form, type F (Wong et al., 1983), is composed of four repeating units (long homologous repeats, LHR) consisting of seven SCRs each, two additional carboxyl-terminal SCRs (total of 30 SCRs) followed by transmembrane and cytoplasmic regions (Figure 7) (Klickstein et al., 1987). CR1 has distinct binding sites for C3b and C4b. The two amino-terminal SCRs in LHR-A bind C4b while the two amino-terminal domains in LHR-B and LHR-C bind C3b (Klickstein et al., 1988).

Complement receptor of the Immunoglobulin family (CRIg). CRIg is a complement receptor expressed on tissue macrophages. It mediates opsonophagocytosis by binding C3b and iC3b on opsonized particles (Helmy et al., 2006). When bound to C3b CRIg regulates AP activation by inhibiting the interaction of C3 and C5 with AP convertases (Wiesmann et al., 2006).

Decay accelerating factor (DAF or CD55). As the name indicates DAF is able to regulate the AP and CP by accelerating the decay of C3-convertases. It associates with C4b and C3b on the membrane and dissociates Bb and C2a from the convertases (Fujita et al., 1987). Unlike CFH, C4b, MCP, and CR1 it has no cofactor activity. DAF is a 70 kDa membrane protein composed of four SCR domains and a "spacer" serine/threonine/proline (STP)-rich region (Figure 7). It is anchored on the cell membrane via a glycosylphosphatidylinositol (GPI) anchor (Medof et al., 1987). For the decay accelerating function on C4b2a the domains SCR2-3 are essential while in the case of C3bBb the domains SCR2-4 are needed (Brodbeck et al., 1996). Also the STP region is essential for regulation in both pathways (Coyne et al., 1992).

Membrane cofactor protein (MCP or CD46). MCP was originally named gp45-70. This 50-85 kDa heavily glycosylated membrane protein has cofactor activity for the cleavages of C4b and the first cleavage of C3b by factor I but has no decay accelerating activity on the C3-convertases (Seya et al., 1986). MCP is widely expressed on cells but absent from human erythrocytes (Seya et al., 1988). MCP has structural similarity with DAF having four SCR domains and a heavily glycosylated STP region but unlike DAF, MCP has transmembrane and cytoplasmic domains (Figure 7) (Lublin et al., 1988). Alternative splicing of the gene encoding MCP results in isoforms that differ from each other in the composition of STP and cytoplasmic tail regions. Several isoforms can be expressed in the same cell (Post et al., 1991). SCR domains 2-4 of MCP are important for the regulatory activity while the domains 3 and 4 are essential for both ligand binding and cofactor activity (Adams et al., 1991).

CD59 (protectin). At the final step of TP, multiple C9 molecules incorporate into the target membrane forming a channel that causes cell lysis. CD59 binds tightly to the membrane associated C5b-8 complex preventing the unfolding of C9 (Meri et al., 1990) and thereby C9-incorporation to the membrane (Rollins and Sims, 1990). CD59 is widely and abundantly expressed by blood cells and several other cell types (Meri et al., 1991). This 18-20 kDa single chain protein has five intra-chain disulphide bonds, it is heavily glycosylated and has a GPI anchor (Davies et al., 1989; Sugita et al., 1993; Jokiranta et al., 1995).

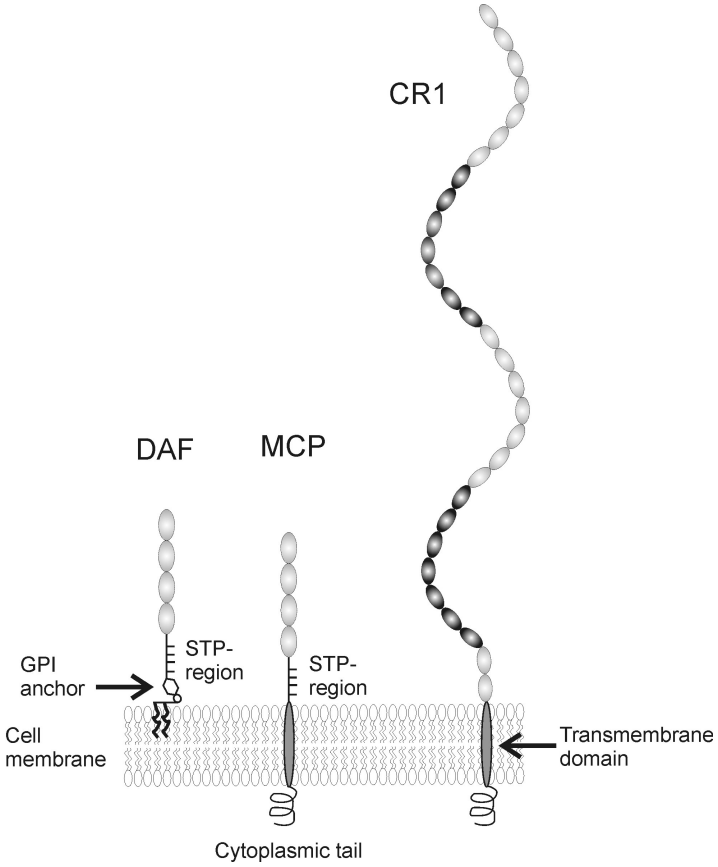

Figure 7. Cell surface regulators DAF, MCP, and CR1. DAF and MCP are composed of four SCR domains and a glycosylated STP rich spacer between the domains and the membrane. CR1 is composed of repeating seven domain units and transmembrane and cytoplasmic regions. Also MCP is anchored on the cell membrane via a transmembrane domain while DAF has a GPI anchor.

1.1.4 Complement receptors

The function of most complement receptors is to mediate a signal for activation of the cell upon binding to a complement cleavage product. Two receptors, CR1 and CRIg (described above), have also complement regulatory activity.

Complement receptor 2 (CR2, CD21). CR2 has an important role in linking innate and adaptive immunity by mediating activation of B-cells. Cleavage of C3b to iC3b and further to C3dg by factor I exposes a CR2-binding site on the components

(Ross et al., 1983; van den Elsen and Isenman, 2011). The role of CR2 in B-cell activation is based on covalent attachment of C3dg to an antigen recognized by B-cell receptor (surface immunoglobulin, sIgM). Crosslinking of sIgM and the B-cell surface complex consisting of CR2, CD19 and CD81 (TAPA-1) significantly lowers the threshold required for B-cell activation as reviewed earlier (Carter et al., 1988; Fearon and Carter, 1995).

C3d, or actually C3dg, has been shown to act as molecular adjuvant *in vivo* in a mouse model increasing the immunogenicity of the antigen 1000 to 10,000 fold (Dempsey et al., 1996). The gene encoding CR2 is located in RCA gene cluster (Weis et al., 1987) and the expressed protein is composed of 15 or 16 SCR domains, a transmembrane domain, and a cytoplasmic tail (Weis et al., 1988). CR2 is sometimes referred as EBV receptor because Epstein -Barr virus uses it for entry into host B-cells (Fingeroth et al., 1984).

Complement receptor 3 and 4 (CR3, CD11b/CD18 and *CR4, CD11c/CD18)*. The integrin receptors CR3 and CR4 are both transmembrane heterodimers and expressed on monocytes, macrophages, neutrophils and lymphocytes as reviewed recently (van Lookeren Campagne et al., 2007). The C3b-derived cleavage product iC3b is a ligand for both CR3 (Ross et al., 1983) and CR4 (Micklem and Sim, 1985). These interactions enhance phagocytosis of the target by monocytes and macrophages (Myones et al., 1988).

C3a receptor, C5a receptor, and C5a-like receptor (C3aR, C5aR and C5L2). C3a, C5a, and to lesser extent C5aDesArg, are pro-inflammatory polypeptides generated after the proteolytic cleavage of C3 and C5. These are then captured by specific receptors C3aR, C5aR, and C5L2 on various surfaces causing activation of cells carrying these receptors. These receptors share high sequence homology (Lee et al., 2001) but differ in ligand specificity and function. C3aR is the receptor for C3a but cannot recognize and be activated by C3aDesArg or C5a (Wilken et al., 1999). C5aR binds C5a and more weakly C5aDesArg while C5L2 has equal or even higher affinity for C5aDesArg compared to C5a. Therefore, it has been suggested that, while C5aR has a pro-inflammatory role, C5LR is controlling both pro-inflammatory and regulatory responses (Gerard et al., 1989; Cain and Monk, 2002; Okinaga et al., 2003; Chen et al., 2007; Scola et al., 2007). Anaphylatoxins C3a and C5a can also interact with C3aR and C5aR on T-cells and by this way participate in T-cell activation and thereby in adaptive immunity responses (Strainic et al., 2008).

1.2 Microbes and complement resistance

An invading microbe is a definite target for the innate immune system and will be rapidly eliminated if the microbe does not possess any immune evasion mechanisms. Some decades ago it was noticed that complement, in the absence of antibodies, can be efficient enough to kill gram-negative bacteria by direct lysis (Schreiber et al., 1979) or gram-positive bacteria via complement-mediated opsonophagocytosis (Li and Mudd, 1965). Since complement forms one of the major innate immune defense mechanisms against bacterial pathogens, emergence of virulence factors that specifically inhibit complement have been favored by natural selection. Over the last two decades it has become clear that there are several microbial complement evasion strategies that practically cover each single complement activation step. Several examples show that one microbe can have multiple complement evasion strategies and these in combination contribute to complement resistance.

Interference with complement activation by microbial components. Certain microbial structures such as the capsule of *Escherichia coli* or *Staphylococcus aureus* cannot activate AP in the absence of anti-capsular antibodies (Verbrugh et al., 1982; Bortolussi et al., 1983). Even when antibodies are present *S. aureus*, for example, uses protein A for antimicrobial IgG Fc binding and consequently interferes with IgG opsonization and CP activation (Forsgren and Sjöquist, 1966; Sjöquist and Stålenheim, 1969). Some microbes expose structures similar to complement regulatory proteins and use these to interfere with complement activation. For example Vaccinia virus expresses a secretory protein, Vaccinia virus complement-binding protein (VCP), that is structurally related to C4BP (Kotwal and Moss, 1988). *Trypanosoma cruzi* trypomastigotes (Joiner et al., 1988) and the herpes simplex virus 1 (HSV-1) (Fries et al., 1986) both express a protein with decay accelerating activity on C3bBb. In addition, proteins produced by *S. aureus* have been shown to interfere with formation or function of C3- and/or C5-convertases and thereby C3b deposition, C5a formation and phagocytosis (Rooijakkers et al., 2005; Bestebroer et al., 2010; Jongerius et al., 2010).

Degradation of complement components. Several pathogenic microbes have been shown to secrete enzymes that degrade complement components. C3 is the key component of all activation pathways and for this reason probably the main target for complement component cleaving microbial enzymes. For example, *S. pyogenes* protease SpeB (Terao et al., 2008), certain *Pseudomonas aeruginosa* enzymes (Hong and Ghebrehiwet, 1992; Schmidtchen et al., 2003), and *S. aureus* aureolysin (Laarman et al., 2011) attack C3 while a enterohemorrhagic *E. coli* serine protease EspP degrade both C3 and C5 (Orth et al., 2010) and a surface protease PgtE of

Salmonella enterica cleaves C3b, C4b and C5 (Ramu et al., 2007). Moreover, enzymes expressed by *Aspergillus fumigatus* (Behnsen et al., 2010) and *Candida albicans* (Gropp et al., 2009) have been shown to act on C3 and on intact or cleaved C4 thus causing non-specific degradation of complement components.

The C5a anaphylatoxin and chemotactic factor is the key component enhancing inflammation and chemotaxis towards the invading microbe. Streptococcal bacteria, *S. pyogenes* (Wexler et al., 1985) and *Streptococcus agalactiae* (Bohnsack et al., 1991), both produce C5a-cleaving enzymes. Whether these enzymes also cleave C3a, like a proteinase produced by the protozoa *Entamoeba histolytica* (Reed et al., 1995), is not yet known.

1.2.1 Binding of host complement regulators

Another way to regulate complement activation on microbial surface is the acquisition of host complement regulators. The usage of complement regulatory proteins in immune evasion by several pathogenic microbes is summarized in Tables 1 and 2.

1.2.1.1. Binding of C4BP

Binding of C4BP to C4b down regulates both CP and LP. Several microbes have been shown to use specific ligands for C4BP binding. The studies on the interactions between C4BP and microbial ligands have shown that binding is mediated via the domains within the α-chain of C4BP. From these *E. coli* K1 OmpA binds to domain 3 of the α-chain, (Prasadarao et al., 2002) and *Neisseria gonorrhoeae* porin Por1A to domain 1 (Ram et al., 2001b). Domains two, five, and seven are needed for full binding of *Moraxella catarrhalis* UspA (Nordström et al., 2004). For binding of C4BP to *Neisseria meningitidis* PorA, domains 2 and 3 (Jarva et al., 2005) and for yeast *C. albicans* Pra1, domains four, seven, and eight of the α-chain are needed (Luo et al., 2011). Host C4BP is also bound by YadA and Ail of *Yersinia enterocolitica* (Kirjavainen et al., 2008), *Bordetella pertussis* FHA (Berggård et al., 1997) and *Borrelia recurrentis* CihC (Grosskinsky et al., 2010) but the interacting domains are not yet known. In addition, several *S. pyogenes* serotypes have been shown to bind C4BP (Thern et al., 1995) where the specific binding sites of M4 and M22 proteins have been shown to overlap with the C4b-binding site in domains 1-2 of the C4BP α-chain (Accardo et al., 1996; Blom et al., 2000a).

Table 1. Microbial acquisition of host complement regulatory proteins C4BP and CFH

Protein	Microbe	Ligand	Reference
C4BP	*Bordetella pertussis*	FHA	(Berggård et al., 1997)
	Borrelia burgdorferi s.l.	?	(Pietikäinen et al., 2010)
	Borrelia recurrentis	CihC	(Grosskinsky et al., 2010)
	Candida albicans	Pra1	(Luo et al., 2011)
	Escherichia coli	OmpA	(Prasadarao et al., 2002)
	Moraxella catarrhalis	UspA	(Nordström et al., 2004)
	Neisseria gonorrhoeae	Por1A	(Ram et al., 2001b)
	Neisseria meningitidis	PorA	(Jarva et al., 2005)
	Salmonella enterica	Rck	(Ho et al., 2011)
	Streptococcus pyogenes	M protein	(Thern et al., 1995)
	Yersinia enterocolitica	YadA, Ail	(Kirjavainen et al., 2008)
CFH	*Aspergillus fumigatus**#*	?	(Behnsen et al., 2008)
	Bordetella parapertussis	?	(Amdahl et al., 2011)
	Bordetella pertussis	?	(Amdahl et al., 2011)
	Borrelia burgdorferi s.l.	BbCRASP*	(Kraiczy et al., 2001; Hartmann et al., 2006)
		OspE	(Hellwage et al., 2001)
	Borrelia hermsii	FhbA	(Hovis et al., 2006)
	Candida albicans	Gpm1p*	(Poltermann et al., 2007)
		Pra1*	(Luo et al., 2009)
	Fusobacterium necrophorum	?	(Friberg et al., 2008)
	Haemophilus influenzae	?	(Hallström et al., 2008)
	Leptospira interrogans	LfhA	(Verma et al., 2006)
	Neisseria gonorrhoeae	Por1A*,#	(Ram et al., 1998a)
		Por1B*,#	(Ngampasutadol et al., 2008)
	Neisseria meningitidis	Fhbp	(Schneider et al., 2009)
		NspA	(Lewis et al., 2010)
	Onchocerca volvulus	?	(Meri et al., 2002)
	Pseudomonas aeruginosa	Tuf#	(Kunert et al., 2007)
	Salmonella enterica	Rck	(Ho et al., 2010)
	Streptococcus agalactiae	β protein,	(Areschoug et al., 2002)
		SHT	(Maruvada et al., 2009)
	Streptococcus pneumoniae	Hic	(Jarva et al., 2004)
		PspC	(Hammerschmidt et al., 2007)
	Streptococcus pyogenes	Fba*	(Pandiripally et al., 2002)
		M protein*	(Horstmann et al., 1988)
		Scl#	(Reuter et al., 2010)
	Yersinia enterocolitica	Ail	(Biedzka-Sarek et al., 2008)
		YadA	(China et al., 1993)

*Binds also CFHL-1, # Binds also CFHR-1

Abbreviations: Ail, attachment-invasion locus protein; CihC, complement inhibition via C4bp; BbCRASP, *Borrelia burgdorferi* complement regulator-acquiring surface proteins; Fba, fibronectin-binding protein; FHA, filamentous hemagglutinin; FhbA, factor H-binding protein; Fhbp, factor H-binding protein; Gpm, phosphoglycerate mutase: Hic, factor H-binding inhibitor of complement; LfhA, Leptospira factor H-binding protein; Nsp, neisserial surface protein; Omp, outer membrane protein; Osp, outer surface protein; Por, porin; Pra, pH-regulated antigen; PspC, pneumococcal surface protein; Rck, resistance to complement killing; Scl, streptococcal collagen-like protein; SHT, streptococcal histidine triad; Tuf, elongation factor Tu ; UspA, ubiquitous surface protein A; YadA, *Yersinia* adhesin A

1.2.1.2. Binding of CFH

Alternative pathway evasion is probably the main reason why microbes acquire CFH or CFHL-1 on their surface. In 1988 Horstmann et al. (Horstmann et al., 1988) described for the first time that a microbe selectively binds human complement regulatory protein (CFH) and by this way evades complement mediated opsonophagocytosis. This was done by studying *Streptococcus pyogenes* serotypes M5, M6, M19, M24 and M28 as well as an M protein deletion mutant strain. In the same study the interaction between CFH and M protein was further studied by using a recombinant M6 protein. Thereafter the binding site on CFH for *S. pyogenes* M6 has been localized to CFH domains 6-10 (Sharma and Pangburn, 1997) and later to the domain seven (Blackmore et al., 1998a).

Most microbes acquire CFH via two regions of this control protein, domains 5-7 and 18-20 (Figure 8). The reason for this specific interaction has not been described but it seems likely that the conserved location for the interaction on CFH might have a functional relevance. Although most of the known CFH-microbe interactions are mediated via two distinct sites in CFH, several *S. pyogenes* and *N. meningitidis* strains have been shown to interact with CFH via the domains 6-7 only. More specifically, *N. meningitidis* has two ligands for CFH binding, Fhbp (Schneider et al., 2009) and NspA (Lewis et al., 2010). Two CFH ligands that have been found from *N. gonorrhoeae* interact at distinct sites of the regulatory protein. Por1A binds CFH via domains 6 and 18-20 while Por1B binds only domains 18-20 (Ram et al., 1998a; Ngampasutadol et al., 2008). Also *Borrelia burgdorferi* uses two ligands for CFH binding, OspE for binding domains 18-20 (Hellwage et al., 2001; Alitalo et al., 2004) and BbCRASPs for 6-7 interaction (Kraiczy et al., 2001; Hartmann et al., 2006). Moreover, two CFH-binding ligands have been found from *Y. enterocolitica* and *Streptococcus pneumoniae* (China et al., 1993; Biedzka-Sarek et al., 2008). On the contrary, *P. aeruginosa* (Kunert et al., 2007) and *C. albicans* (Poltermann et al., 2007; Luo et al., 2009), use a single ligand for interacting with two distinct sites in CFH. Probaly also Rck of *S. enterica* uses two CFH interaction sites and the expression of the ligand in *E. coli* has been shown to mediate complement resistance (Ho et al., 2010)

Interestingly, *S. aureus* Sbi and shiga toxin (Stx2) of enterohemorrhagic *E. coli* bind CFH in a unique way. From these Sbi binds domains 19-20 by not directly interacting with the regulator but by forming a tripartite complex with C3b (Haupt et al., 2008) while Stx2 binds both CFH domains 6-7 and 18-20 in fluid phase (Orth et al., 2009). Moreover, unlike the other CFH binding microbes, *S. pneumoniae* binds CFH via the middle part of the protein using domains 8-11, 12-14 and 19-20 (Jarva et al., 2004; Hammerschmidt et al., 2007).

The ligand interacting with CFH is unknown for *Haemophilus influenzae* (Hallström et al., 2008), *Fusobacterium necrophorum* (Friberg et al., 2008), *A. fumigatus* (Behnsen et al., 2008), *Bordetella pertussis*, *Bordetella parapertussis* (Amdahl et al., 2011) and *Onchocerca volvulus* (Meri et al., 2002). All of these microbes interact at least with CFH domains 6-7 and 18-20. The microfilariae of the nematode *Onchocerca volvulus* have been shown to acquire CFH domains 8-20.

In addition to bacteria, fungi and nematodes, CFH also binds to viral pathogens such as HIV-1 (Pinter et al., 1995; Stoiber et al., 1996) and West Nile virus (Chung et al., 2006). The constantly growing list demonstrating microbial interactions with CFH clearly shows that binding of CFH is a widely used innate immunity evasion mechanism used by a variety of pathogenic microbes.

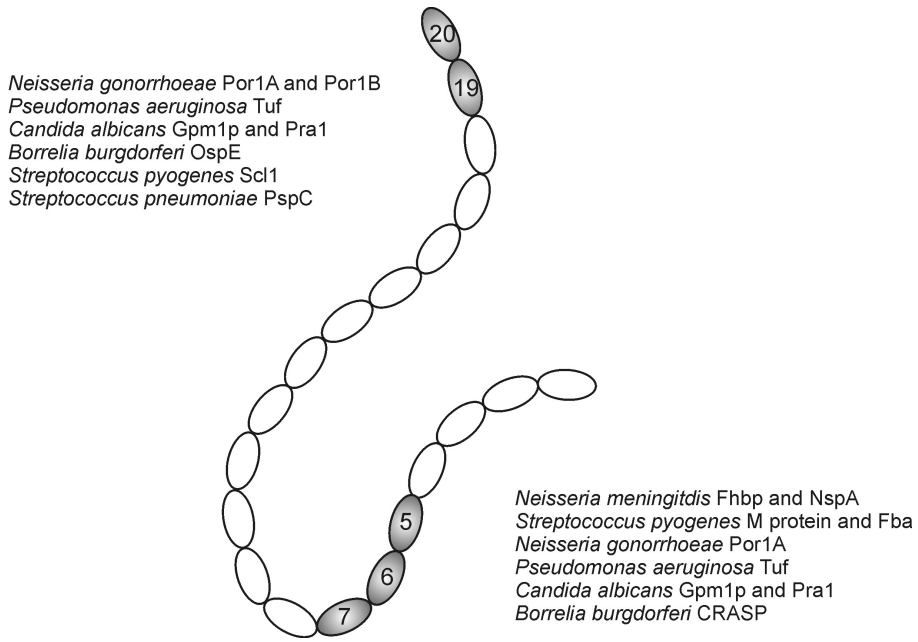

Figure 8. Binding of CFH on microbial ligands. CFH is known to interact with several microbial ligands via domains 19-20 (left panel) and/or 5-7 (right panel).

1.2.1.3. Binding of host complement regulators other than C4BP and CFH

C1inh. Complement evasion via recruitment of C1inh on the surface of at least three bacteria has been shown (Table 2) (Lathem et al., 2004; Marr et al., 2007; Grosskinsky et al., 2010). Whether these interactions could also influence other

host responses such as the kinin-kallikrein or coagulation systems where C1inh is involved is still unclear.

Properdin. As described earlier (Section 1.1.3.1), properdin is a positive regulator of AP increasing the half life of surface bound C3/C5-convertases. In addition recent investigations have suggested that properdin plays an important role as a pattern recognition molecule since it binds directly (in the absence of C3b) on bacterial surfaces (Spitzer et al., 2007; Cortes et al., 2011). Therefore, it is likely that cleavage rather than binding of properdin on microbial surface would be beneficial for the pathogen. It has been suggested that *S. pyogenes* enzyme SpeB could cleave properdin and by this way decrease AP activation and bacterial opsonophagocytosis (Tsao et al., 2006). Whether decreased survival of bacteria in these assays was due to the action of SpeB on properdin or on C3 (shown by (Terao et al., 2008)) or on both remains unclear.

S-protein (vitronectin). In 1987 Chhatwal et al. (Chhatwal et al., 1987) showed that several gram-positive bacteria (*S. pyogenes*, group C streptococci, group G streptococci and *S. aureus*) and *E. coli* bind S-protein and hypothesized that the interaction could mediate binding of the pathogen to host cells. This is probably the main purpose of the phenomenon especially within gram-positive bacteria that are resistant to the lytic effect of MAC because of the thick peptidoglycan cell wall. The binding of S-protein by gram-negative bacteria such as *H. influenzae* (Hallström et al., 2006) and *Moraxella catarrhalis* (Singh et al., 2010) has been described to confer to serum resistance of the bacteria.

Clusterin. Binding of clusterin on surface of *S. aureus* (Partridge et al., 1996) and *S. epidermidis* (Li and Ljungh, 2001) has been described. Clusterin also binds on secreted microbial proteins Dengue virus NS1 protein (Kurosu et al., 2007) and *S. pyogenes* streptococcal inhibitor of complement (SIC) (Åkesson et al., 1996). Whether these interactions have an importance in evasion of complement MAC formation is unlikely at least for the investigated bacteria that are gram-positive. However, it is possible that the cell aggregating action of clusterin could enhance microbial pathogenicity.

Factor I. In order to function as a C3b inactivator, factor I requires either a cell surface cofactor (CR1 or CD46) or a cofactor from the fluid phase (CFH or C4BP). Certain poxviruses (vaccinia-, variola- and monkeypox virus) (Sahu et al., 1998; Rosengard et al., 2002; Liszewski et al., 2006) and one bacterium *S. aureus* (Hair et al., 2008) all express proteins with cofactor activies for factor I.

Receptors. CR1 receptor is a surface regulator having both cofactor and decay accelerating activities. Being very abundant on erythrocyte surfaces intracellular parasites such as *Plasmodium falciparum* (Spadafora et al., 2010) and *Leishmania*

major (Da Silva et al., 1989) interact with this receptor and use it for invasion into the host cells. In addition, DAF and MCP are both used as cellular receptors for viral entry into host cells (Naniche et al., 1993; Ward et al., 1994; Shafren et al., 1997) while *S. pyogenes* probably uses MCP for surface attachment (Okada et al., 1995; Giannakis et al., 2002). In contrast, CD59 has been detected from microbial surfaces indicating that this GPI-anchored protein can be transferred from host cell to the microbial surface (Rautemaa et al., 1998; Rautemaa et al., 2001).

Table 2. Microbial acquisition of host complement regulatory proteins others than CFH and C4BP

Protein	Microbe	Ligand	Reference
C1inh	*Bordetella pertussis*	Vag8	(Marr et al., 2007; Marr et al., 2011)
	Borrelia recurrentis	CihC	(Grosskinsky et al., 2010)
	Escherichia coli	StcE	(Lathem et al., 2004)
S-protein	*Haemophilus influenzae*	Hsf	(Hallström et al., 2006)
	Moraxella catarrhalis	UspA2	(Singh et al., 2010)
	several bacteria	?	(Chhatwal et al., 1987)
Clusterin	*Staphylococcus*	?	(Partridge et al., 1996)
	Streptococcus pyogenes	SIC	(Åkesson et al., 1996)
Factor I	*Staphylococcus aureus*	ClfA	(Hair et al., 2008)
CR1	*Leishmania major*	?	(Da Silva et al., 1989)
	Plasmodium falciparum	PfRh4	(Spadafora et al., 2010; Tham et al., 2010)
CD59	*Escherichia coli*	?	(Rautemaa et al., 1998)
	Helicobacter pylori	?	(Rautemaa et al., 2001)
DAF	Coxsackie A 21 virus	?	(Shafren et al., 1997)
	Echovirus 7	?	(Ward et al., 1994)
MCP	Measles virus	Hemagglutinin	(Naniche et al., 1993; Manchester et al., 2000)
	Streptococcus pyogenes	M protein	(Okada et al., 1995)

Abbreviations: CihC, complement inhibition; ClfA, clumping factor A; Hsf, Haemophilus surface fibrils; PfRh, reticulocyte-binding–like homolog protein; SIC, Streptococcal inhibitor of complement; StcE, secreted protease of C1 esterase inhibitor from EHEC; UspA, ubiquitous surface protein A; Vag, autotransporter encoded by virulence-activated gene 8

1.3 Examples of complement evading microbes

1.3.1 *Loa loa*

The “eye worm” was first described in medical literature in 1770 and 1777 when occasional subconjunctival migration of a filarial worm was reported as reviewed earlier (Grützig and Jennes, 1977). The African eye worm, *Loa loa,* belongs to the phylum Nematoda or roundworms having distinguishing characteristics such as cylindrical shape of body, longitudinal musculature and a thick acellular cuticle

(Hickman et al., 2001). The adult worms live in human subcutaneous tissues, reproduce and release microfilariae in peripheral blood or spinal fluid, urine, saliva, or into the alveolar space. The adult females are 40-70 mm long while the males are 30-34 mm. The produced microfilariae are 250-300 µm long and they possess a loosely bound sheath like a bag around each microfilarium. In addition to its size, it can be distinguished from other blood dwelling filarial worms by the staining characteristics, the appearance of the densely packed nuclei that extend to the tip of the rouded shape tail, and the presence of the sheath (Cook and Zumla, 2004). When isolated from human blood and examined under microscope they make whip-like movements and the viability is maintained up to two weeks when stored on ice (unpublished observation).

1.3.1.1. *Loa loa* life cycle

The blood dwelling microfilariae are ingested during a blood meal by a dipteran female vector (*Chrysops* spp.) (Figure 9). The main vectors that transmit human loiasis are Tabanidae flies belonging to species *Chrysops silacea* and *Chrysops dimidiate* (deer flies, mango flies, mangrove flies) (Gouteux et al., 1989). After entering the midgut of the vector the microfilariae lose their sheaths (Padgett and Jacobsen, 2008). Thereafter they penetrate the gut wall and move to the so called fat body in the abdomen of the arthropod vector for maturation. Here they undergo two molts into stage 1 and 2 larvae, differentiate further and increase in length from approximately 300 µm up to 700 µm. The third stage infective larvae (L3) are liberated and start to migrate from the abdomen to the head of the insect. Here the L3 infective larvae can be found from the proboscis (mouth parts) of the vector having a size of approximately 2 mm. The larvae are transmitted into the human body when the vector feeds again. The development in the vector takes approximately 8-10 days (Orihel and Lowrie, 1975) while in human subcutaneous tissues the larvae develop into adults within approximately 1 year and can live in humans up to 15 years (Gems, 2000).

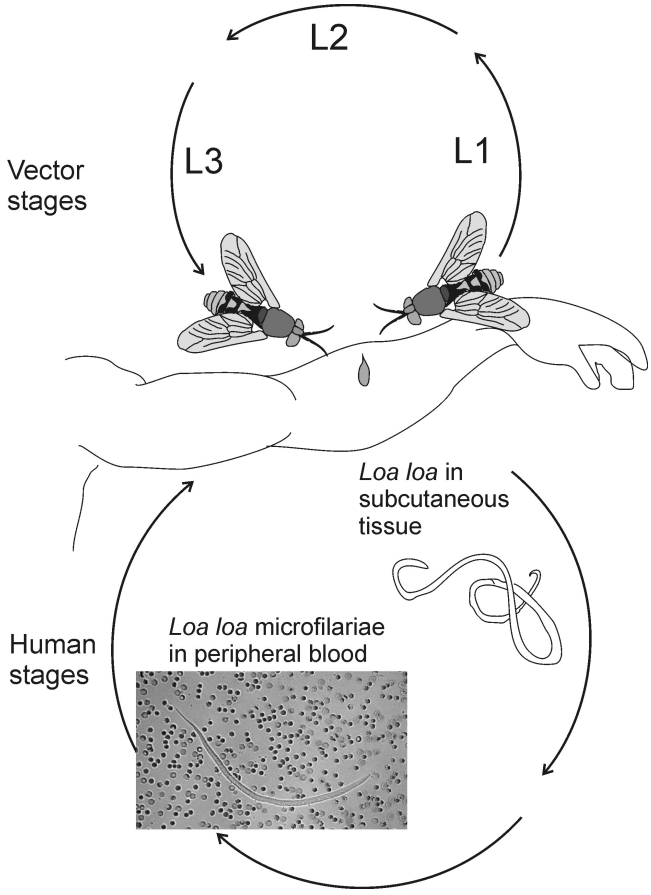

Figure 9. *Loa loa* life cycle. *Loa loa* L3 stage larvae enter human body through the bite wound when the vector *Chrysops* spp. takes a blood meal. *Loa loa* develop in subcutaneous tissues and produce sheathed microfilariae that can be found in the peripheral blood. Microfilariae enter the vector when it feeds and thereafter the larvae lose their sheaths (L1 larvae). There they develop into L2 and L3 larvae. The L3 larvae can be found from the mouth parts of the vector.

1.3.1.2 Distribution of Loiasis

Within its current endemic regions in Western and Central Africa the prevalence of loiasis can be very high being over 40% in certain areas (Boussinesq and Gardon, 1997; Zouré et al., 2011) (Figure 10). During human loiasis microfilariae live in blood (microfilaremia) and the number of microfilariae in peripheral blood has diurnal periodicity providing an explanation why they are effectively transmitted via the day biting vector only. In addition to parasite adaptation to the feeding

habits of the vector, the effective transmission is facilitated by the high density of *Chrysops* spp. in endemic areas. The vector mainly attacks moving objects and the bite is painful. Therefore the feeding is frequently interrupted which in turn increases the frequency of attacks and thereby the frequency of exposure to the parasite. Although few *Chrysops* flies carry *Loa loa* infective larvae (*e.g.* 0.7 % of *Chrysops silacea* in South-West Cameroon) the parasite load in the infected vector is high and the transmission becomes therefore effective (Nanduri and Kazura, 1989; Wanji et al., 2002). There are no effective methods to control transmission of loiasis. However, drainage of marshy areas where *Chrysops* flies live can decrease the number of the vector. Personal protection such as wearing light-colured clothing and usage of insect repellent has been shown to reduce bites by the flies.

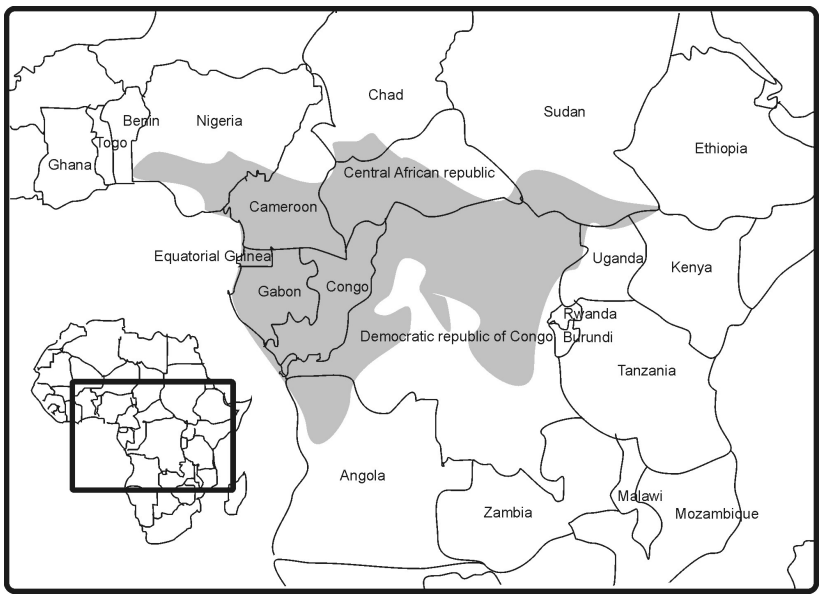

Figure 10. Distribution of loiasis. Loiasis is endemic in rain forest areas of Western and Central Africa (marked with grey). High risk level of loiasis is found at least in 10 countries. Figure modified from (Zouré et al., 2011).

1.3.1.3 Loiasis

In addition to being frequently asymptomatic loiasis can cause symptoms such as episodes of Calabar swellings (subcutaneous, non-tender oedemas that are often itching), subcutaneous and subconjunctival migration of adult worms (history of eyeworm), pruritus, and secondary dermal lesions (Noireau et al., 1990). Serious

sequelae such as renal complications have also been reported (Pakasa et al., 1997). Hypersensitivity reactions are most common within visitors to endemic areas whereas hypersensitivity within residents of endemic areas is not as frequent and less severe (Nutman et al., 1986). Loiasis is a disease of public health importance mainly because of the negative effects during ivermectin treatment that is used to control onchocerciasis (river blindness) in central Africa. Especially patients with high blood concentrations of *Loa loa* microfilariae (approximately 15,000 microfilariae/ml or more) have been reported to develop severe and sometimes fatal encephalopathic reactions after treatment with ivermectin (Chippaux et al., 1996; Gardon et al., 1997). It has been estimated that some 14.4 million people live in high risk areas where the prevalence of loiasis varies between 20% and 40%. Most of this area also overlaps with the distribution of onchcerciasis and therefore predispose these people to severe adverse reactions when treated with ivermectin (Zouré et al., 2011).

1.3.1.4 Host immunity and immune evasion by *Loa loa*

It is known that some of the *Loa loa* infected individuals carry adult worms but are amicrofilaremic while some individuals have considerable microfilaremia. In endemic areas where prevalence of loiasis is high nearly all adults have antifilarial antibodies. The antibody positivity is gained during early childhood since the percentage of children that are over five years old and positive for antifilarial IgG, IgM and IgE antibodies is similar to the corresponding percentage of adults (Goussard et al., 1984). In these areas amicrofilaremic persons may have high IgG class antibody titers indicating that antibody-mediated mechanisms could be important in controlling microfilaraemia in loiasis (Pinder et al., 1988). Moreover, a subsequent study has suggested that these antimicrofilaria IgG antibodies are effective in mediating adherence of host neutrophils on microfilariae and that both CP and AP have important roles in it (Pinder et al., 1992).

In endemic areas loiasis patients are often chronically infected. Recent studies has shown that within these patients *Loa loa* causes parasite-specific downregulation of T-cell responses more often than within *Loa loa* infected travelers that are more likely to have hypersensitivity reactions such as Calabar swellings (Steel et al., 2012). The high prevalence of chronic infection could partially explain the high transmission rate of loiasis in endemic areas. It is evident that the sheath and/or the underlying thick cuticle surrounding *Loa loa* microfilariae are most probably protective against direct complement lysis but it is still unclear how the parasites avoid host innate immune defenses until they are transmitted to the vector.

1.3.2 *Streptococcus pyogenes* (Group A streptococcus)

1.3.2.1 Infections

S. pyogenes is the causative agent of variety of infectious diseases. The most common disease caused by *S. pyogenes* is tonsillitis characterized by sore throat, fever, and white patches on inflamed tonsils. Also streptococci belonging to groups C and G can cause tonsillitis. Some *S. pyogenes* strains (certain M protein serotypes, typing being described in chapter 1.3.2.2) cause pharyngitis while some others cause mainly skin infections. Another *S. pyogenes* disease, scarlet fever, is caused by strains that produce pyrogenic exotoxins responsible for the systemic symptoms recognized by rash and strawberry tongue seen during the disease (Cunningham, 2000).

S. pyogenes skin infections impetigo and erysipelas affect the superficial layers of the skin while cellulitis affects subcutaneous tissues (Bisno and Stevens, 1996). In addition to *S. pyogenes,* streptococci belonging to groups B, C, and G can also cause erysipelas and cellulitis (Binnick et al., 1980; Eriksson et al., 1996).

Streptococcal toxic shock- or toxic shock-like syndrome (TSS, STSS or TSLS) was first described in 1987 and was shown to be similar to *S. aureus* toxic shock syndrome (Cone et al., 1987). Streptococcal pyrogenic exotoxin, SpeA, expressed by certain *S. pyogenes* strains is associated with this complication characterized by hypotension and multi organ failure (Hauser et al., 1991).

Streptococcal infection may also lead to an even more severe complication called necrotizing fasciitis where *S. pyogenes* is more commonly known by the name “flesh eating bacterium” (Kaul et al., 1997). Localized *S. pyogenes* infections may also cause complications such as septicemia, pneumonia, or meningitis.

Sometimes *S. pyogenes* infections may lead to post-infectious sequelae such as acute glomerulonephritis or rheumatic fever. Microbial factors such as type of M protein (Lindberg and Vosti, 1969), production of SpeB or streptokinase, or induction of the C3-nephritic factor (C3Nef, autoantibody against C3) by the so-called nephritogenic strains have been suggested to play a role in the pathogenesis of the post-streptococcal glomerulonephritis (PSGN) (Villarreal et al., 1979; Johnston and Zabriskie, 1986; Poon-King et al., 1993). C3Nef generated occasionally during streptococcal infections can lead to acute glomerulonephritis by causing stabilization of C3 convertases on host cells thus decreasing the pace of their decay leading to excess complement activation (Fremeaux-Bacchi et al., 1994). Rheumatic fever is an serious post-infectious condition with symptoms including fever, arthritis, and inflammation in cardiac valves leading to tissue

damage and often valve leakage (Stollerman, 1975). Streptococcal infections have also been associated in some studies with other immunologically mediated diseases such as Tourette´s syndrome, tics (Cunningham, 2000), psoriasis (Telfer et al., 1992), and narcolepsy (Aran et al., 2009). The associations, however, are controversial.

1.3.2.2 Structure and classification of *S. pyogenes*

As a gram-positive bacterium, *S. pyogenes* has a cytoplasmic membrane covered with a thick peptidoglycan layer with a number of different surface proteins (Figure 11). Within the genus *Streptococcus* the round shaped cells are typically found in chains or pairs - hence the name. The net-like structure of peptidoglycan is based on polymerization of the repeating disaccharide, N-acetylmuramic acid-(β1-4)-N-acetylglucosamine and cross linking of the polymers is mediated by dialanine bridges (Munoz et al., 1967). The peptidoglycan layer is surrounded by a capsule composed of hyaluronic acid (N-acetylglucosamine and glucuronic acid) which is poorly immunogenic in humans (Kendall et al., 1937). When observed by electron microscopy, the capsule is penetrated by filamentous structures: small fibrils, thicker and longer fimbriae or pili (Fischetti, 1989; Mora et al., 2005).

The ability of certain streptococci to cause hemolysis of red blood cells was first noted by Alexander Mamorek in 1895 as reviewed here (Cinader and Pillemer, 1950). In a throat swab sample cultured on blood agar plates *S. pyogenes* appears as β-hemolytic colonies unlike the normal oral flora. Differentiation of *S. pyogenes* from other β-hemolytic streptococci is based either on its sensitivity to bacitracin or on agglutination by antiserum against the group A specific carbohydrate (Lancefield, 1933) composed of N-acetylglucosamine and rhamnose (Krause and McCarty, 1961). Within the *S. pyogenes* species the bacteria can be classified according to the antigenic differences in the main surface protein, M protein (Lancefield, 1928). Currently, instead of M-typing based on antisera, also M protein gene *emm* typing is widely used. This is based on sequencing of the 5´ end of the *emm* gene that encodes the hypervariable (HVR) region of M protein responsible for the M type specificity (Beall et al., 1996; Facklam et al., 1999). Other classifications such as typing according to the T antigen or the substance increasing serum opacity (opacity factor, OF) are also used in research and epidemiology as reviewed here (Cunningham, 2000). M protein is one of the most diverse surface proteins of *S. pyogenes* and it has been clearly shown that antibodies against a certain M protein are protective against infections by the *S. pyogenes* strains carrying that M type (Lancefield and Todd, 1928).

M protein is a 50-60 nm long fibrillar membrane anchored molecule having a dimeric α-helical coiled coil structure (Figure 11) (Phillips et al., 1981; McNamara et al., 2008; Macheboeuf et al., 2011). The carboxyl-terminal end that anchors the molecule onto the cell membrane consists of a hydrophobic domain and a conserved LPXTGX motif common for most gram-positive surface proteins (Fischetti et al., 1990). The extracellular region close to the membrane is called a proline- and glycine-rich region (Pro/Gly). Further out from the membrane are first D-repeats followed by C-repeats that all contain conserved amino acid sequence repeats common in all *S. pyogenes* serotypes and typical for coiled coil structures. The middle B-repeat region varies between different *S. pyogenes* serotypes while the region furthest from the membrane is the amino-terminal A-repeat region containing the serotype specific HVR region. This is the target for the protective antibody production against *S. pyogenes* infections (Jones et al., 1985; Hollingshead et al., 1986; Fischetti et al., 1988; Mouw et al., 1988; Fischetti, 1989). The number of repeating units in the A and B regions are mainly responsible for the size variation among different M proteins (Hollingshead et al., 1987). Division of M proteins into class I and class II molecules is based on the recognition of specific antibodies against the C-repeat region. The serotypes expressing the class I M protein are OF negative while the class II M protein serotypes are OF positive (Bessen et al., 1989; Bessen and Fischetti, 1990). Although overall organization of M protein is generally conserved, major differences exist such as the size and number of A and B repeats. Therefore M proteins can be divided in patterns A-E according to these differences as reviewed here (Smeesters et al., 2010).

The *emm* gene coding the M protein is positively regulated by the *mga*-regulon (multiple gene regulator in group A streptococcus)(Caparon and Scott, 1987) that also regulates transcription of *scp*A gene of C5a peptidase (or ScpA) (Simpson et al., 1990). Furthermore, the locus regulates transcription of genes encoding *S. pyogenes* M-like proteins (mrp, fcr and enn) (Podbielski et al., 1995), OF (McLandsborough and Cleary, 1995) and SIC (Kihlberg et al., 1995). Expression of M protein depends on the growth phase of the bacteria. For example the *emm*6 gene is most actively expressed in late log phase or after 8 h growth and is increased by elevated CO_2 levels (Caparon et al., 1992), $NaCl_2$ and temperature, while it is decreased during vigorous gas exchange and iron limitation (McIver et al., 1995). Although the *emm* gene encoding the M protein (type I or II) is present in the chromosome of all known *S. pyogenes* strains the presence of the genes encoding the other factors that are in the control of the regulon varies between the strains (Bessen and Hollingshead, 1994; Hollingshead et al., 1994). M protein is an important virulence factor of *S. pyogenes* as it is needed for bacterial adhesion and invasion and is required for resistance to complement attack and phagocytosis (Scott et al., 1986; Perez-Casal et al., 1992).

1.3.2.3 Bacterial colonization

Several *S. pyogenes* surface proteins mediate adhesion to host cell surfaces while some of these interact with host plasma proteins. Moreover, certain secreted enzymes and toxins are involved in bacterial invasion and spread between host tissues. Several virulence factors are not constitutively expressed but are induced by environmental changes such as changes in oxygen levels and pH. When present, these factors significantly assist the bacterial colonization and thereby influence the pathogenicity of the microbe as reviewed here (Nobbs et al., 2009).

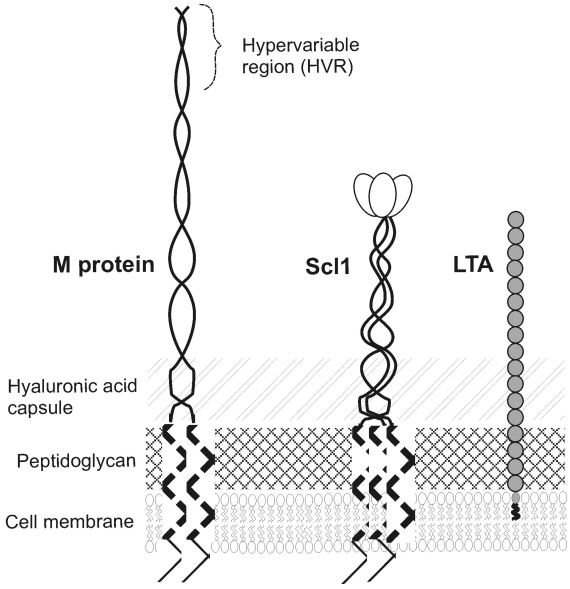

Figure 11. Virulence factors on *S. pyogenes* surface. M protein and streptococcal collagen-like (Scl) protein are surface structures penetrating through the thick peptidoglycan layer and the cell membrane having a cytoplasmic tail while lipoteichoic acids (LTA) are attached to the outer leaflet of the membrane. M protein has mostly fibrillar coiled-coil structure and its most amino-terminal region is the hypervariable region (HVR) having considerable sequence dissimilarity between the *S. pyogenes emm* types. Picture modified from (Fischetti, 1989; Xu et al., 2002; Weidenmaier and Peschel, 2008).

Adhesion is the first step essential for *S. pyogenes* colonization. Bacterial colonization is a dynamic process where the microbe has to face challenges such as nutritional and other environmental requirements including pH, oxygen level, and the attack of the host immune system. If the growth conditions are unfavorable

the bacterium may detach from the surface at this point and retry colonization elsewhere as reviewed here (Nobbs et al., 2009). The surface of the bacterium is coated with fibrils such as M proteins and lipoteichoic acids (LTA) (figure 11) that mediate adhesion onto the epithelial cell surfaces (Beachey and Ofek, 1976; Okada et al., 1994).

Some *S. pyogenes* strains have been shown to interact with extracellular matrix (ECM) components such as fibronectin or fibrinogen thus promoting bacterial colonization. These protein adhesins include for example FBP54 (Courtney et al., 1994), Protein F/SfbI (Hanski and Caparon, 1992; Hanski et al., 1992; Talay et al., 1994), PFBP (Rocha and Fischetti, 1999), OF/SfbII, (Kreikemeyer et al., 1995), streptococcal surface GAPDH (SDH or Plr binds also plasminogen, lysozyme, myosin) (Pancholi and Fischetti, 1992), M protein (Carlsson et al., 2005; Macheboeuf et al., 2011) and streptococcal collagen-like protein 1, Scl1 (Figure 11) (Caswell et al., 2010). Including the proteins mentioned above, over 35 surface associated *S. pyogenes* adhesins have been described to interact with fibronectin or fibrinogen as well as with several other host molecules such as collagen, plasminogen, plasmin, laminin, immunoglobulins, and complement proteins as reviewed here (Nobbs et al., 2009).

1.3.2.4 Invasion

In order to invade into deeper tissues, *S. pyogenes* expresses virulence factors that break host barriers themselves or activate host enzymes. *S. pyogenes* streptokinase, plasminogen-binding group A streptococcal M-like protein (PAM), and streptococcal surface enolase (SEN, Eno) have been shown to interact with plasmin/plasminogen. It has been suggested that PAM is responsible for binding of plasminogen on *S. pyogenes* surface causing activation of plasminogen by streptokinase leading to breakage of physical host barriers and migration of the bacteria into the tissues (Reddy and Markus, 1972; Berge and Sjöbring, 1993; Pancholi and Fischetti, 1998). A secreted cysteine protease SpeB expressed by various *S. pyogenes* strains cleaves human fibronectin and degrades S-protein (vitronectin) that can facilitate microbial invasion and spread between tissues (Kapur et al., 1993). Invasion into cultured human cells by certain *S. pyogenes* strains has been described to be mediated by M protein (Fluckiger and Fischetti, 1997) and Scl1 (Caswell et al., 2010). Whether this occurs *in vivo* is still unclear. However, the bacteria surviving inside host cells have been hypothesized to be the reservoir of *S. pyogenes* in recurrent infections (Österlund et al., 1997).

1.3.2.5 Toxins

Several *S. pyogenes* strains secrete toxins that are involved in systemic toxicity. Some of these are produced by most of the strains (*e.g.* streptolysin O, SLO) and some by strains associated with certain streptococcal infections or symptoms in streptococcal syndromes. These include superantigens that over stimulate T-cells by binding nonspecifically to major histocompatibility complex class II (MHCII) molecules outside the peptide-binding site and simultaneously to the Vβ chain of the T-cell receptor (TCR). The overstimulation liberates large amounts of inflammatory cytokines leading to a systemic inflammatory reaction with multiple symptoms. These kinds of superantigens include, for example, streptococcal pyrogenic exotoxins (*e.g.* SpeA, SpeB, SpeF, SpeJ, SpeG, SpeH), streptococcal mitogenic exotoxins Z (SMEZ, SMEZ-2, SMEZ-3) (Fast et al., 1989; Norrby-Teglund et al., 1994a; Norrby-Teglund et al., 1994b; Kamezawa et al., 1997; Proft et al., 1999; Gerlach et al., 2000), and streptococcal superantigen (SSA) (Mollick et al., 1993). The first streptococcal pyrogenic exotoxin was isolated from blood and urine of a patient with scarlet fever and the toxin was therefore called scarlet fever toxin (Trask and Blake, 1924) while the streptococcal superantigen has also been described from *S. pyogenes* strains causing streptococcal toxic shock-like syndrome (Mollick et al., 1993). SLO is a hemolysin that causes damage to the target cell by forming large transmembrane pores analogous to complement C5b-9 (Bhakdi et al., 1985) and permits another toxin, *S. pyogenes* NAD glycohydrolase (NADase, SPN), to enter the host cell (Madden et al., 2001). This in turn induces apoptosis of the target cell (Bricker et al., 2002).

1.3.2.6 Immune escape

Facing the immune system is clearly the biggest challenge for microbial survival in the human body. The innate immunity threatening bacteria consists mainly of the complement system and phagocytic cells. These players form the first line defense against the invading streptococci. In order to survive and multiply a bacterium has to produce virulence factors evading these systems (Figure 12). Immune escape by *S. pyogenes* involves several virulence factors. For example, interference with opsonophagocytosis is mediated via secreted cysteine proteases Mac-1 (also known as IdeS or MspA) (von Pawel-Rammingen et al., 2002), Mac-2 (Söderberg et al., 2008), SpeB, and endoglycosidase EndoS (Collin and Olsen, 2001), that all cleave IgG bound to the bacterial surface. Also, by expressing a surface bound C5a cleaving enzyme, C5a peptidase, the bacterium can inactivate complement-mediated chemotactic activity and subsequently inhibit opsonophagocytosis and thereby lower number of phagocytes at the site of invasion (Wexler et al., 1985). Furhermore, several *S. pyogenes* M proteins (Sir, Arp, Mrp) and SfbI have been

shown to bind IgG and/or IgA through the Fc part of the antibodies thereby putatively avoiding Ig-mediated opsonisation (Stenberg et al., 1992; Medina et al., 1999).

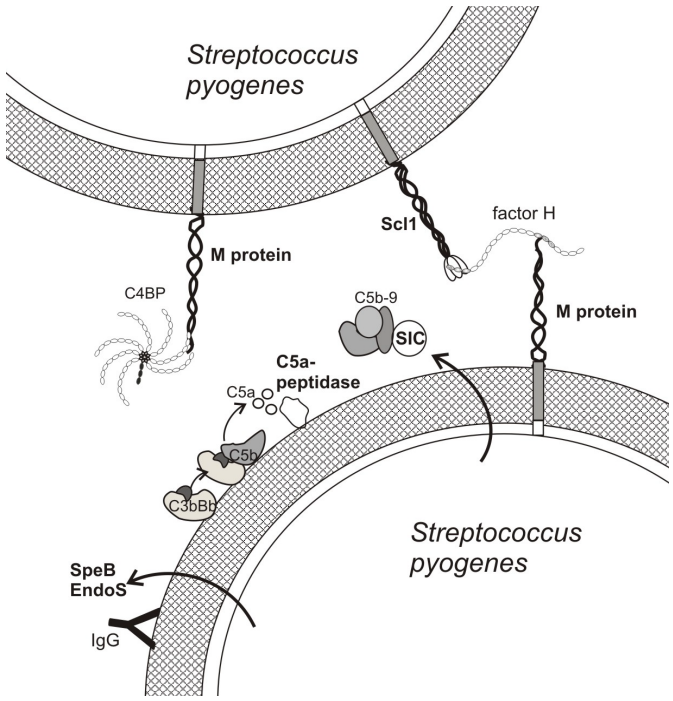

Figure 12. *Streptococcus pyogenes* immune escape. Several *S. pyogenes* factors act against the host immune system. SpeB, and EndoS cleave IgG while C5a-peptidase interferes with chemotaxis by cleaving C5a. SIC binds to C5b-7 and prevents MAC formation. M protein binds complement regulators C4BP and CFH while Scl1 binds only CFH. These interactions promote complement resistance of *S. pyogenes*. EndoS, endoglycosidase S; Scl1, streptococcal collagen-like protein 1; SIC, streptococcal inhibitor of complement; SpeB, streptococcal pyrogenic exotoxin B;

S. pyogenes adhesion on keratinocytes is mediated via the interaction between the bacterial M protein and the complement regulator MCP on the cell surface (Okada et al., 1995). The binding site for the M6 protein on MCP is located between domains 3 and 4 at a site distinct from the cofactor activity region of the molecule (Giannakis et al., 2002). In addition to bacterial adhesion, it has been suggested that the M protein interaction with MCP on human lung epithelial cells promotes intracellular entry of *S. pyogenes* (Rezcallah et al., 2005). On the other hand, on T-

cells MCP-M protein contact induces generation of regulatory T-cells leading to down regulation of T-cell responses (Price et al., 2005).

Several *S. pyogenes* strains, but not all, bind host fluid phase complement regulators CFH and C4BP to restrict complement activation targeted onto the bacterial surface. C4BP binding has been demonstrated from several different *S. pyogenes* strains and the interaction is most presumably mediated via the HVR of Ig-binding M proteins (Stenberg et al., 1992; Stenberg et al., 1994; Thern et al., 1995; Morfeldt et al., 2001). The M protein-binding site on C4BP α-chain has been localized to the interface between domains one and two (Accardo et al., 1996; Villoutreix and Dahlback, 1998) and is overlapping, but not identical, with the binding site for C4b (Blom et al., 2000a). Since C4BP has a total of seven α-chains (and perhaps due to only partial overlap) this interaction is not supposed to interfere with C4BP regulatory activity. This is supported by a study showing that the interaction between C4BP and M22 protein contributes to phagocytosis resistance (Berggård et al., 2001). It has also been postulated that certain M proteins that do not bind C4BP bind fibrinogen so that M protein-bound fibrinogen could inhibit complement via the classical pathway similarly to C4BP (Carlsson et al., 2005).

Acquisition of CFH onto *S. pyogenes* is mediated at least by three different surface proteins, indicating that CFH binding is important in *S.pyogenes* immune evasion. Among these, the interaction between CFH and *S. pyogenes* M proteins has been most extensively studied. The binding site for CFH on M6 protein was first localized to the conserved C-repeat region (Fischetti et al., 1995). Later, binding studies with whole bacteria and their M protein lacking C-repeat region was used to locate the binding site outside the C-repeat, probably to the HVR (Sharma and Pangburn, 1997). Also CFHL-1 binding studies to purified M5 and chimeric M5-emm22 proteins (Johnsson et al., 1998) indicates that the HVR interacts with CFH. In any case it has been shown that CFH binding via M protein leads to AP evasion and phagocytosis resistance of *S. pyogenes* (Horstmann et al., 1988). According to current knowledge the binding site for M protein on CFH is located at the domain seven (Blackmore et al., 1998a) where is also the site for the common Y402H polymorphism (see section 1.1.3.1.).

The second *S. pyogenes* protein interacting with CFH and CFHL-1 is Fba (Pandiripally et al., 2002) that also mediates invasion of *S. pyogenes* into cultured human cells (Terao et al., 2001). Scl1 is the third *S. pyogenes* protein that has been found to interact with CFH. Binding has been shown with two M types, M6 and M55, (Caswell et al., 2008) and the binding site for Scl1 on CFH is located within domains 18-20 (Reuter et al., 2010).

In addition to acquiring host complement regulators some *S. pyogenes* strains express SIC protein that directly inhibits complement activation. This protein inhibits MAC formation on the bacterial surface (Åkesson et al., 1996) by binding to C5b-7 and C5b-8 complexes in fluid phase (and to a minor extent to C5b-9 as well) (Fernie-King et al., 2001). Moreover, the cysteine protease SpeB that cleaves IgG and fibronectin degrades also S-protein (vitronectin) (Kapur et al., 1993). However, as previously mentioned, it is unlikely that cleavage of the TP components or regulators has a significant role in complement evasion of the gram-positive bacterium.

2 AIMS OF THE STUDIES

The aims of the studies in this thesis were to analyze the following four issues:

1. Can capture of host CFH by a pathogen be observed *in vivo*?

2. Does the common amino acid substitution Y402H in CFH domain seven affect opsonization, complement evasion, and multiplication of *S. pyogenes in vitro*?

3. Could complement evasion and survival of *S. pyogenes* in human blood be inhibited using a non-functional recombinant CFH fragment that inhibits bacterial CFH binding?

4. Does the polymorphism Y402H of CFH contribute to susceptibility of human individuals to streptococcal infections?

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Proteins and antibodies

CFH and C3 proteins were isolated from EDTA plasma obtained from healthy consented laboratory workers using methods modified from Koistinen et al. 1989 (Koistinen et al., 1989) (study II). First the plasma was mixed with buffer A (Table 3), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 M benzamidine followed by precipitations with 10% and 30% polyethylene glycol 4000 (PEG) in buffer B, respectively. The second precipitate was diluted in buffer C, run through anion exchange (Q-sepharose-FF) column, and eluted with salt gradient (from 100% buffer C to 100% buffer C2). The eluted fractions containing C3 were then precipitated again with 30% PEG in buffer B and then dissolved in buffer C while eluted fractions containing CFH were pooled and stored in -70°C. After dissolving the precipitate, C3 sample was run through cation exchange (SP-sepharose-FF) column where the proteins were eluted with salt gradient (buffers D and D2).

Table 3. Special buffers used in CFH isolation (study II)

Buffer	Compounds
A	1 M KH_2PO_4; 0.2 M EDTA; 0,2 M benzamidine; pH 7.4
B	0,1 M NaH_2PO_4; 15 mM EDTA; 0.15 M NaCl; 0.01 M benzamidine; 0.02% NaN_3; pH 7.4
C	0.015 M NaH_2PO_4; 5 mM EDTA; 0.025 M NaCl; 0.02% NaN_3, pH 8.0
C2	0,015 M NaH_2PO_4; 5 mM EDTA; 0.4 M NaCl; 0.02% NaN_3, pH 7.5
D	0.015 M NaH_2PO_4; 5 mM EDTA; 0.025 M NaCl; 0.02% NaN_3, pH 6.0
D2	0.015 M NaH_2PO_4; 5 mM EDTA; 0.4 M NaCl; 0.02% NaN_3, pH 6.0
E	0.02 M NaH_2PO_4; 2 mM EDTA; 0.02% NaN_3, pH 6.0
E2	0.02 M NaH_2PO_4; 2 mM EDTA; 0.02% NaN_3; 0.35 M NaCl, pH 6.0
F	0.01 M NaH_2PO_4; 5 mM EDTA; 0.02 M NaCl, pH 7.5
F2	0.01 M NaH_2PO_4; 5 mM EDTA; 0.3 M NaCl, pH 7.5
PBS1	75 mM phosphate-buffered saline, pH 7.0
PBS2	2 M NaCl in 75 mM phosphate-buffered saline, pH 7.0

Stored CFH fractions were thawed and then precipitated again with 30% PEG and dissolved in buffer E. Some CFH was lost since all the precipitate could not be dissolved easily. The solution was centrifugated and run through a cation exchange (SP-sepharose-FF) column using salt gradient elution (buffers E and E2). Thereafter, the CFH containing fractions were mixed and run again through a smaller volume cation exchange (SP-sepharose) column and eluted with PBS containing 0.5 M NaCl. CFH was then separated from high and low molecular weight contaminants using gel filtration (Sephacryl S-300) in E buffer. Finally, the sample was run into MonoQ anion exchange column (buffers F and F2) using ÄKTA purification system and thereafter dialyzed against PBS.

Detection of which elution fractions contained CFH or C3 was done by dot-blotting using specific CFH or C3 antibodies with horseradish peroxidase (HRP) conjugated secondary antibody detection (Table 4). Before use, all the proteins were dialyzed against PBS and run by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for Western blotting and Coomassie blue or silver staining. The purities of different CFH allotypes were estimated to be similar in the stained SDS-PAGE gels. Protein concentrations were measured using BCA (bicinchoninic acid) protein assay reagent (Thermo Fischer Scientific). Isolated and ^{125}I-labeled proteins were stored at -70°C (CFH) or on ice (C3) before use.

Table 4. Purchased and/or obtained antibodies and other proteins

Protein	Supplier	Antibody	supplier	Secondary antibody*
CFH	Calbiochem	goat α-CFH	Calbiochem	donkey α-goat
factor I	Calbiochem	rabbit α-C1q	Dako	goat α-rabbit
CR1	T Cell Sciences	mouse α-MBL	Statens Serum Institute	rabbit α-mouse
C4b	Calbiochem	goat α-C4	Cappel	donkey α-goat
iC3b	Calbiochem	rabbit α-C3c	Dako	goat α-rabbit
		sheep α-C3	ICN Biochemicals	donkey α-sheep
C5	Quidel	goat α-C5	Quidel	donkey α-goat
		mouse α-C5b-9	Quidel	rabbit α-mouse
C4BP	Prof. A. Blom;	rabbit α-C4BP	A. Blom	goat α-rabbit
C4BP	Complement Technologies			
		α-C9 neoepitope	Hycult Biotech	-
		goat anti-C7	Cappel	donkey α-goat

* Supplier for the antibodies conjugated with horseradish peroxidase (HRP) was Jackson Immunoresearch; supplier for Alexa 488-conjugated antibodies was Molecular Probes

C3b was prepared from plasma-purified C3 (study I) as described earlier (Koistinen et al., 1989) by incubating C3 with trypsin (Sigma genosys) at a ratio of 100:1 (w/w). C4b was purchased from Calbiochem. To obtain iC4b (study I), C4b was inactivated by incubating 100 µg/ml of the protein with 2 µg/ml of factor I, 1 µg/ml of C4BP and 5 µg/ml of soluble CR1 (Table 4) for 2 h at +37°C. Plasma purified C4BP in complex with protein S and the rabbit α-C4BP antibody were obtained as a kind gift from Prof. Anna Blom (Department of Laboratory Medicine, Medical Protein Chemistry, Lund University, Sweden).

3.1.2 Recombinant proteins

The following CFH recombinant constructs having the domains 5-7 of full length CFH were made: CFH5-7(402Y), CFH5-7(402H) and CFH5-7(402H/420H) (studies II and III). First, a cDNA library was used as a template for PCR amplification by using specific primers (Table 5). The PCR amplicons were run on a 1% agarose gel, extracted from the gel (Qiagen), inserted and ligated into pCR2.1 vectors and further transformed into competent *E. coli* cells using a chemical transformation procedure (TOPO TA Cloning®, Invitrogen). Transformants were selected on the next day according to blue/white colony screening. Selected bacteria were grown in appropriate media, the plasmids were isolated for sequencing using a Plasmid DNA purification kit (Qiagen), after which the constructs were recloned into pPICZα vectors. Thereafter, mutagenesis to CFH5-7 DNA was performed by using a Site-directed Mutagenesis kit (Stratagene). In corrigendum of study II mutagenic primers were used to obtain CFH5-7 constructs CFH5-7(402Y) and CFH5-7(402H) both having an insertion of a the three amino acid stop-codon. In study II a single amino acid mutation in CFH5-7(402H) was done to obtain the double mutant CFH5-7(402H/420H) (Table 5). The vector was isolated from the selected colonies for sequencing and transformed into *Pichia pastoris* X-33 strains. Methanol induction was used for protein expression and subsequently the supernatants of the *P. pastoris* expression media were harvested at 96 h, filtered and run through heparin affinity chromatography on ÄKTA purification system using salt gradient elution (buffers PBS1 and PBS2) (Table 3).

Table 5. DNA and oligonucleotides used (study II*, III#)

Name	Supplier	Sequence
pPICZαC vector*	Invitrogen	¶
pPICZαA vector*,#	Invitrogen	¶
pPCR2.1 vector*,#	Invitrogen	¶
SCR5 forward primer for pPICZαC vector*	Sigma-Genosys	GAATTAGAAAAATCATGTGATAA
SCR5 forward primer for pPICZαA vector *,#	Sigma-Genosys	GAATTCGAAAAATCATGTGATAATCC
SCR7 reverse primer*,#	Sigma-Genosys	TTCTAGAGCTGTTTGACACGGATGCATC
Y420H mutagenic primer*	Sigma-Genosys	CCATCCTGG(T/C)CACGCTCTTCCAAAAGCGCAGA
Y402H mutagenic primer§	Sigma-Genosys	CCCTGTACAAACTTTCTTCCAT(A/G)ATTTTGATTATATCCATTTTC
mutagenic primer with a stop codon§	Sigma-Genosys	GATGCATCCGTGTCAAAACA(TGA)GCTCTAGAA
human liver cDNA library*,¶	Stratagene	

*used in study II, #used in study III, §corrigendum of Study II, ¶can be found in suppliers manual

3.1.3 Microbes

S. pyogenes strains (studies II and III) were kindly provided by Dr. Jaana Vuopio from National Public Health strain collection (National Institute for Health and Welfare, Helsinki, Finland) or Dr. Wezenet Tewodros (Spartan Health Science University, Spartan Medical School, St. Lucia, West Indies) or obtained from the Helsinki University Central Hospital Laboratory (HUSLAB), Helsinki, Finland. The *emm* types, *emm* subtypes (*emm* sequence variants), and sequence types (*emm* typing not yet validated by all of the streptococcus reference laboratories) had already been determined according to the Centers for Disease Control and Prevention (CDC) GAS *emm* typing protocol (http://www.cdc.gov/ncidod/biotech /strep/strepindex.htm). The strains were either reference strains or clinical isolates from throat, skin, or blood of patients (Table 6). One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) were used for cloning the CFH5-7 DNA

sequences while *P. pastoris* strain X-33 (Invitrogen) was used as the host organism for CFH5-7(402Y), CFH5-7(402H) and CFH5-7(402H/420H) protein expressions. The microbes were stored at -70˚C in milk glycerol tubes before inoculation to appropriate media for growing (Table 6).

S. pyogenes strains were grown at +37°C with 5% CO_2 overnight on blood-agar plates supplemented with colistin and oxolinic acid and thereafter in Todd-Hewitt broth (Table 6) until the optical density (OD_{600}) was between 0.6-0.8. This represented the late log phase growth of *S. pyogenes* in Todd-Hewitt broth as determined from 8 h growth of different *S. pyogenes* strains, where samples were taken each hour for OD_{600} measurement and CFU counting. After OD_{600}=0.8 the proportion of viable bacteria was clearly reduced. For the binding assays (study II) the bacteria were washed and diluted in PBS or veronal-buffered saline (VBS; 142 mM NaCl, 4.9 mM Na 5,5-diethylbarbiturate, pH 7.3-7.5) to reach a bacterial concentration from 3×10^7 to 1×10^{10}. For analysis of the alternative pathway activation in plasma using C3a and Bb enzyme immunoassays (EIA) and a C5b-9 enzyme-linked immunosorbent assay (ELISA) the bacteria were pelleted by centrifugation and stored in milk-glycerol solution at -70°C (study III). For the assay measuring multiplication of the bacteria in blood, the bacteria were further diluted in media and grown until OD_{600} =0.15 (studies II and III).

Zymosan A prepared from *Saccharomyces cerevisiae* (Sigma-Aldrich) was used as a positive control for surface deposition of MBL, C5, C5b-9, C3b, and iC3b molecules and as a negative control for C1q deposition in study I. For this, 1 % Zymosan A in 0.15 M NaCl was activated according to manufacturer's instructions and thereafter diluted in a concentration of 100µg/ml (0.01%). In study III 1 mg/ml Zymosan A was incubated in normal human serum (NHS) for 60 min at +37°C to obtain a serum sample where AP had been fully activated (i.e. 1000 AU/ml). A dilution series of Zymosan A-treated NHS was used to prepare the standard curve for the C5b-9 ELISA.

Table 6. Microbial strains and growth conditions

Species	*emm* type, *emm* subtype or sequence type	Study	Origin or supplier**
*S. pyogenes**	*emm*11	II,III	Rfs NCTC 100068
*S. pyogenes**	*emm*12	II,III	Rfs NCTC 100085
*S. pyogenes**	*emm*2	II,III	Rfs NCTC 100064
*S. pyogenes**	*emm*28	II,III	Rfs ATCC 12962
*S. pyogenes**	*emm*3	II,III	Rfs ATCC 12384
*S. pyogenes**	*emm*4	II,III	Rfs NCTC 8326
*S. pyogenes**	*emm*5.8193B	II,III	Rfs NCTC 8193
*S. pyogenes**	*emm*6.2 (+24nt)	II,III	Rfs ATCC 12348
*S. pyogenes**	*emm*76 (+54nt)	II,III	Rfs NCTC 12058
*S. pyogenes**	*emm*78	III	Rfs NCTC 12056
*S. pyogenes**	*emm*8	II,III	Rfs ATCC 12349
*S. pyogenes**	*emm*1	III	Spain 1995
*S. pyogenes**	*emm*22	II,III	Blood isolate
*S. pyogenes**	*emm*28	II,III	Blood isolate
*S. pyogenes**	*emm*53.1 (-39nt)	II,III	Blood isolate
*S. pyogenes**	*emm*58	II,III	Blood isolate
*S. pyogenes**	*emm68.*3	II,III	Blood isolate
*S. pyogenes**	*emm*77	II,III	Blood isolate
*S. pyogenes**	*emm*87	II,III	Blood isolate
*S. pyogenes**	*emm*89	II,III	Blood isolate
*S. pyogenes**	*st369*	II,III	Blood isolate
*S. pyogenes**	*emm*1.2b	II,III	AGN skin isolate
*S. pyogenes**	*emm*18.8	II,III	AGN skin isolate
*S. pyogenes**	*emm*55	II,III	AGN skin isolate
*S. pyogenes**	*emm*74.0	II,III	AGN skin isolate
*S. pyogenes**	*emm*89	II,III	AGN skin isolate
*S. pyogenes**	*emm*95.0	II,III	AGN skin isolate
*S. pyogenes**	*st212*	II,III	AGN skin isolate
*S. pyogenes**	*st221.0*	II,III	AGN skin isolate
*S. pyogenes**	*st212.0*	II,III	AGN skin isolate
*S. pyogenes**	*st3757.0*	II,III	AGN skin isolate
*S. pyogenes**	*st62.0*	II,III	AGN skin isolate
*S. pyogenes**	*st6735.0*	II,III	AGN skin isolate
*S. pyogenes**	*stG653*	III	AGN skin isolate
*S. pyogenes**	*stL1376*	II,III	AGN skin isolate
*S. pyogenes**	*emm*25.1	III	AGN throat isolate
*S. pyogenes**	*emm71.0*	II,III	AGN throat isolate
*S. pyogenes**	*st206.0*	II,III	AGN throat isolate
*S. pyogenes**	n.t. [¶]	II	Blood isolate
*S. pyogenes**	*emm*10/12	II	Blood isolate
Pichia pastoris[#]	*Pichia pastoris* X-33	II	Invitrogen
E. coli[§]	One Shot® TOP10	II	Invitrogen

**growth conditions: +37°C, 5% CO_2; either on blood-agar supplemented with colistin and oxolinic acid or in Todd-Hewitt broth*

[#]growth conditions: +33°C, either on Yeast Peptone Dextrose-agar or in Yeast Peptone Dextrose or in buffered minimal methanol media. All supplemented with Zeocin

[§]growth conditions: +37°C, either on Luria-Bertani-agar or in Luria-Bertani media. Both supplemented with ampicillin

[¶]n.t. seven not emm typed clinical isolates

***Abbreviations: Rfs, reference strain; AGN, acute glomerulonephritis; NCTC, National Collection of Type Cultures ; ATCC, American Type Culture Collection*

3.1.4 Biological samples

The microscopy-based laboratory diagnosis of *Loa loa* infection in one patient was done by diagnostic laboratory personnel at the Unit of Parasitology, University Central Hospital Laboratory (HUSLAB), Helsinki, Finland. After the diagnostic procedures the remaining venous EDTA (ethylene diamine tetraacetic acid) and Heparin anticoagulated blood samples, obtained from the microfilaremic patient, were kindly donated for the scientific experiments with the permission from the patient. Samples containing live microfilariae were stored on ice for direct protein-binding assays, immunofluorescence microscopy and iC3b/C3b deposition assays. For Western blotting and cofactor assays the samples were frozen and stored at -130°C (study I).

Loa loa microfilariae were isolated from venous blood samples using Percoll gradient method where the 1:2 diluted blood sample was centrifuged through a percoll-sucrose gradient (study I). Within 35 min of centrifugation (400 × g) the live microfilariae were collected from the top of the undermost percoll-sucrose layer and washed thoroughly with phosphate-buffered saline (PBS). For Western blotting assays the microfilariae were subjected to several steps: low pH, washing with PBS, elution with reduced Laemmli-buffer, and thereafter further to mechanical homogenization on ice using a homogenizer (UniForm homogenizer, Jencons). Samples were collected at each step for analysis. The concentration of a homogenate from approximately 150 microfilariae (110 µg/ml) was comparable to the concentration of Zymosan A that was used as a positive control in study I.

3.1.5 Genotype data

The complete CFH Y402H genotype data of individuals diagnosed for erysipelas, non-erysipelas controls, and individuals diagnosed for recurrent tonsillitis were kindly provided by Dr. Jaana Syrjänen (Department of Infectious Diseases, Tampere University Central Hospital, Finland) and Dr. Jari Suvilehto (Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Finland and Department of Otorhinolaryngology, Lohja Hospital, Helsinki University Hospital, Hospital District of Helsinki and Uusimaa, Finland). The studies on the erysipelas and tonsillitis patients were approved by the Ethical Review Boards (study III). Blood donor control material was obtained from previously published data (Seitsonen et al., 2006) with the approval of Prof. Irma Järvelä (Department of Medical Genetics, Haartman Institute, University of Helsinki, Finland).

3.2 Methods

3.2.1 Immunological experiments

3.2.1.1 Detection of complement proteins by Western blotting (study I)

Noncovalently bound complement components C1q, MBL and C5 were eluted from isolated *Loa loa* microfilariae and Zymosan A control particles by using low-pH buffer (0.1M glycine-HCl pH 2.3). Reduced Laemmli-buffer was used to detach the fragments of inactivated C4b bound to surface of microfilariae. To detect the membrane associated polymerized C9 complexes, C3b, C4b and fragments of inactivated C3b or C4b the microfilariae were further homogenized before subjecting samples to SDS-PAGE. Also the presence of CFH and C4BP on microfilarial surface was analyzed from the homogenate. Diluted NHS and pure complement proteins (Table 4) were used as controls for detection of the corresponding components on the microfilarial eluate or homogenate. Samples were run into 5% or 10% gels or 5-15% gradient gels, followed by transfer of proteins to nitrocellulose membranes and blocking the membranes for 1 h with 3% skimmed milk in PBS. After washing, membranes were incubated for 17 h at +4°C or for 2 h at +22°C with the primary antibodies diluted 1:2000-1:10000. After washing for three times with PBS and incubation with 1:10000 diluted HRP-conjugated secondary antibody, the proteins were detected using the enhanced chemiluminescence (ECL) method.

3.2.1.2 Immunofluorescence microscopy (study I)

Isolated microfilariae were washed with PBS prior to incubation for 1h at +37°C with primary antibodies (2 µg/ml). After washing with 0.5% BSA in PBS and incubation for 30 min at +37°C with 1:200 diluted Alexa 488-conjugated antibodies the microfilariae were further subjected to three washes. Analysis of CFH and C4BP binding on microfilariae was performed using Olympus BX50 fluorescence microscope. Samples that were incubated only with the secondary antibodies were used as background controls.

3.2.1.3 Immunofluorescence quantitation (study I)

To detect C3b and iC3b formation on microbial surfaces approximately 150 microfilariae, 100 µg/ml Zymosan A, and 0.01% red blood cells in PBS were incubated for 1 h at +37°C together with increasing amount of (0%-20%) NHS in PBS. Samples were washed twice with PBS containing 0.025% Tween 20, incubated with the primary antibody for 1 h at +22°C, washed again and thereafter incubated with 1:100 diluted Alexa 488-conjugated secondary antibody for 30 min at +22°C. After final washings the pelleted microbes were resuspended in 25% glycerol and transferred into 96 well plates. The fluorescence intensity of each sample was counted using a 1423 Victor-2 multilabel counter (Wallac, Finland).

3.2.1.4 Measuring AP activation in serum and plasma (study III)

Thawed samples of bacteria were washed three times with PBS and adjusted to OD_{600}=0.6 to obtain 2 × 10^8 bacteria/ml. The bacteria were pelleted by centrifugation and incubated in a Thermomixer (Eppendorf) with 50% NHS diluted in ice cold PBS supplemented with 10 mM $MgCl_2$ and 10 mM ethylene glycol tetraacetic acid (EGTA) for 30 min at +37°C . Bacteria that were incubated with 50% NHS in the presence of 10 mM EDTA were used as a negative control. Since EDTA prevents complement activation PBS-EDTA mixture was used to evaluate the background of AP activity. Complement activity in the samples was stopped by adding 15 µl 0.2 M EDTA. The supernatants were separated by centrifugation and stored -70°C for EIA and ELISA analysis. Commercial EIA kits (Quidel) were used to measure the formation of C3a and Bb in 1:2000 and 1:200 diluted samples, respectively. To measure C5b-9 formation 96-well plates were coated with 2 µg/ml of α-C9 neoepitope antibody (Table 4) diluted in a high pH coating buffer (0.2 M Na_2CO_3, pH 10.6) for 17 h at +4°C. Wells were blocked for 2 h at +22°C with 0.5% BSA in PBS and washed with PBS containing 0.05% Tween 20. Samples were diluted 1:400 and added into the wells. The plates were incubated for 2 h at +4°C, washed five times, and 1:1000 diluted α-C7 antibody was added followed by incubation for 1 h at +22°C. Wells were then washed and incubated with a secondary HRP-conjugated antibody for 1 h at +22°C. OPD (o-phenylenediamine) tablets (DAKO) were used as a substrate for detection of the HRP-conjugated antibodies according to manufacturer's instructions. Arbitrary units/ml (AU/ml) where calculated from a standard curve obtained by activating 1 ml NHS with 1 mg Zymosan A for 60 min at +37°C (normalized to 1,000 AU/ml). Absorbances obtained using EIA and ELISA assays were determined using Spectramax ELISA reader.

For analyzing Bb and soluble C5b-9 (SC5b-9) formation in blood, OD_{600} = 0.15 grown bacteria were diluted 1:10000 in PBS and incubated in a Thermomixer with PBS or 20 µg CFH5-7 for 15 min at +37°C. Thereafter 1.2 ml of Refludan® (Schering AG)- anticoagulated blood was added to the tubes for 1 h incubation at +37°C. Samples were taken at three time points, the reaction was stopped in the samples with 30-50 mM EDTA. Plasma samples were separated by centrifugation and stored at -70°C for Bb EIA and C5b-9 ELISA analysis.

3.2.2 Microbiological experiments

3.2.2.1 Opsonophagocytosis assays (studies II and III)

To see the effect of CFH5-7 inhibition on bacterial multiplication in study III the bacteria were grown to OD_{600}=1.5 and diluted 1:10000 followed by incubation with CFH5-7 or PBS and thereafter mixed with 1.2 ml of blood. Depending on the growth rate of the strain in bood (study II) a total of 100-700 CFU of bacteria were diluted in broth and mixed with 1.3-1.6 ml fresh anticoagulated blood obtained from donors genotyped homozygous for CFH(402Y) or CFH(402H). In another assay (study II), the bacteria were either mixed with fresh blood or blood depleted of phagocytes, complement, or both. Complement depleted blood was prepared by heat-inactivating the separated plasma for 30 min at +56°C that was then re-mixed with the blood cells. Depletion of phagocytes from blood was done by first using Percoll gradient separation of phagocytes from other blood cells according to manufacturer's instructions (Fluka Biochemica) followed by addition of the washed other cells with heat-inactivated or activated plasma. Bacteria (studies II and III) were incubated in blood in an orbital rotator for 3 or 5 h at +37°C and samples were taken at 4 or 6 time points and mixed with EDTA. The taken volume was replaced with corresponding fresh blood at each (study II) or at 60 and 90 min (study III) time points. Samples, or their dilutions, were inoculated onto agar plates, incubated for 17 h, the CFUs were counted according to the pour plate method and the multiplication factors at each time point were calculated.

3.2.3 Experiments with radiolabeled proteins

Purified proteins were radiolabeled with $Na^{125}I$ (Perkin Elmer) using the Iodogen method (Salacinski et al., 1981). Briefly, glass tubes were pre coated with Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenyl-glycoluril) according to manufacturer's

instructions (Thermo Scientific or Sigma). $Na^{125}I$ was then mixed in the Iodogen-tube with the purified protein in a ratio of 1 mCi/100 µg, respectively. After 10 min incubation the mixture was removed to another glass tube and thereafter the non-bound ^{125}I was separated from bound using PD-10 columns (Amersham Biosciences). The specific activities of the labeled proteins used in the assays were $0.4 \times 10^6 – 88 \times 10^6$ cpm/µg.

3.2.3.1 Direct ^{125}I-protein binding assays

For the C3b deposition assay (study II) bacteria were grown to OD_{600} = 0.6 (2×10^8 CFU/ml), washed and adjusted to a concentration of 1×10^{10} bacteria/ml in VBS containing 0.1% gelatin (GVB). 40% NHS containing 10 mM EGTA, 5 mM $MgCl_2$ (NHS-MgEGTA) and 200 000 cpm of ^{125}I-C3 was mixed with 4.5 × 10 bacteria/ml. Heat-inactivated serum instead of NHS-MgEGTA was used as negative control for C3b deposition. Zymosan A instead of bacteria was used as positive control. Samples were incubated for 2h at +37°C; reactions were stopped with 10 mM EDTA.

For direct ^{125}I-protein binding assays 3×10^7 bacteria in GVB (study II) or 40-100 microfilariae in PBS (study I) were incubated with 25 000 cpm of ^{125}I-labeled proteins for 30 min at 37°C in a Thermomixer. The bound ^{125}I-labeled proteins were separated from free label by centrifuging the samples through GVB (study II) or PBS (study I) containing 20% sucrose. Thereafter the tubes were frozen at -70°C and the tubes were cut in to two pieces and the pellets and supernatants were placed in separate counting tubes. The radioactivities in the tubes were counted using a Wallac Microbeta Trilux gamma counter (Perkin Elmer) and the ratios of pellet vs. total activities (pellet and supernatant) were calculated.

3.2.3.2 Radioligand assays

Bacteria grown to OD_{600} = 0.6 were washed and used for coating of microtiter plates with 1.2×10^8 bacteria/well. The plates were incubated at +37°C until the wells were dry. Thereafter, the wells were washed and blocked with 3% BSA diluted in PBS for 1 h at +37°C and washed again. For the dose dependent competition assay (study II), increasing concentrations (5-50 µg/ml) of unlabeled competitor proteins were added and the wells were incubated for 15 min at +37°C. Thereafter, in all assays (studies II and III) the wells were incubated with 50 000 cpm of ^{125}I-labeled proteins containing 0.1% BSA diluted in PBS and washed again. Wells were dried, separated from each other and the radioactivities were counted using a gamma counter.

3.2.3.3 Measuring CFH cofactor activity

Approximately 75 isolated microfilariae diluted in VBS were incubated with 100 000 cpm of ^{125}I-C3b and 15 µg/ml factor I for 1 h at +37°C (study I). Mixtures containing ^{125}I-C3b and factor I or ^{125}I-C3b, factor I and 10 µg/ml CFH were used as negative and positive controls, respectively. The samples were run into 10% SDS-PAGE gels, fixed with 5% acetic acid, dried using Model 583 Gel Dryer (Bio-Rad), and detected by autoradiography.

3.3 Statistical data analysis

Standard deviations for results obtained with replicate samples in two or more separate assays (studies II and III) were counted using Microsoft Excel software. In the assay where immunofluorescence based quantitation of C3b/iC3b deposition was measured (study I) the relationship between the surface deposited C3b/iC3b and NHS concentration was modeled by using linear regression analysis in Microsoft Excel software and the correlation coefficient value (r^2) was calculated.

Student's t-test in Microsoft Excel software was used to compare means between two independent samples in the direct ^{125}I-protein binding assays (studies I-III), radioligand binding assays (study II) and the assays with immunofluorescence based quantitation of C3b/iC3b deposition (study I). To compare the effect of CFH binding on C3b depositions and the role of complement and phagocytes on opsonophagocytosis between three different *S. pyogenes* strains, a multiple comparison test between pairs of means was performed (study II). This was done by using one-way analysis of variance (ANOVA) and Tukey's HSD post-test in version 15.0 of SPSS for Windows, Analytical Software (IBM®). Dependence between complement activation and binding of CFH proteins, and between SC5b-9 and Bb or C3a production (study III) was measured by using Pearson's correlation in version 15.0 of SPSS for Windows.

Allele and genotype frequencies were counted according to the Hardy-Weinberg equation (study III). Thereafter a χ^2 test was performed to compare the observed and the expected genotype frequencies within each study population and the deviations from the Hardy-Weinberg equilibrium were determined using Microsoft Excel software. Genetic associations between the patient and control groups were analyzed by Pearson's χ^2 test (study III), and the odds ratio (OR) and its 95% confidence interval (CI) of each comparison was calculated using version 15.0 of SPSS for Windows.

4 RESULTS AND CONCLUSIONS

4.1 Microbial complement evasion by binding of complement regulators *in vivo* (I)

There are several studies showing that microbial complement evasion is mediated *in vitro* via microbial binding of complement regulators CFH and C4BP. Some of these have shown that binding of the regulators promotes the bacterial survival by interfering with C3b deposition (China et al., 1993), complement mediated lysis (Ram et al., 1998b; Ram et al., 2001a), and by improving microbial phagocytosis resistance (Horstmann et al., 1988; Berggård et al., 2001). However, these studies have been done *in vitro*. Therefore, by using a blood dwelling microbe, *Loa loa*, that is extremely well adapted to the human host, we were able to study binding of complement regulators *in vivo* (study I).

Loa loa microfilariae were isolated from patient blood samples. By using Western blotting we examined the complement components that had been bound to the surface of microfilariae *in vivo* in the infected patient. The covalently bound fragments were examined from the homogenate of microfilariae. We found that complement components C1q and MBL were deposited on the surface whereas no deposited C5 fragments or MAC components were observed. Some C4 and C3 derived fragments were found on microfilariae but those fragments were mainly inactivated iC3b and iC4b (Figure 1 of study I).

In these analyses Zymosan A incubated in NHS was used as a positive control. As expected, C1q was not found on Zymosan A particles but deposition of terminal components were abundant. These findings lead to the conclusion that complement activation is initiated on the surface of the blood dwelling microfilariae *in vivo* but is limited thereafter. Next we analyzed the presence of complement regulators on the surface of microfilariae *in vivo* and clearly observed CFH and C4BP from the homogenate (Figure 3 of study I). By using immunofluorescence microscopy the binding of CFH and C4BP was localized on the sheath layer of the microfilariae (Figure 5 of study I). Incubation of the isolated microfilariae with ^{125}I-labeled proteins *in vitro* showed 8 and 18% binding of CFH and C4BP when approximately 100 microfilariae were in each sample, respectively (Figure 6 of study I).

We further examined whether the bound CFH was functionally active and whether CFH binding by microfilariae was contributing to the limited number of deposited

complement components on the microbial surface. This was done by analyzing cofactor activity of the surface bound CFH on cleavage of ^{125}I-C3b (Figure 4 of study I) and by using a quantitative method for analyzing C3b/iC3b depositions (Figure 2 of study I). In Western blotting of the cofactor assay we observed that the α´-chain of microfilariae incubated ^{125}I-C3b was cleaved by factor I indicating that the surface bound CFH was fully active. Furthermore, the deposition of C3b/iC3b on microfilariae was limited (when 7-20% NHS was used) but not totally negative when compared to red blood cells that were used as a negative control in the assay. According to study I, we were able to show that microbial acquisition of complement regulators CFH and C4BP occurs *in vivo* and can cause limited complement activation on surface of *Loa loa* microfilariae.

4.2 Effect of mutation or polymorphism in domain seven of CFH on *S. pyogenes* immune evasion (II)

The first evidence that a polymorphism in domain seven of CFH has functional consequences was found in 2005 when four separate studies showed association between CFH Y402H polymorphism and AMD (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). This was an interesting finding since the domain where the substitution is located has been shown to mediate the binding of CFH to M protein of *S. pyogenes* (Blackmore et al., 1998a; Giannakis et al., 2003). It has also been assumed that some genetic factors such as polymorphisms in TLR-4 (Liadaki et al., 2011) and Ficolin-2 (Messias-Reason et al., 2009) genes could affect the susceptibility of some individuals to *S. pyogenes* infections.

We hypothesized that if the binding between CFH and M protein is mediated via residues close to the polymorphic site in domain seven the substitution changing the charge of the site could also change the affinity of the interaction. In study I we had found that CFH binding on the microbial surface occurs *in vivo* and therefore we assumed that impaired binding of CFH on *S. pyogenes* could also have functional consequences and thereby influence infection susceptibility.

Before analyzing the impact of the CFH Y402H polymorphism on microbial evasion, we studied binding of CFH on several *S. pyogenes* strains (Figure 13). On the basis of these results, we chose three *S. pyogenes* strains with different binding affinity to ^{125}I-labeled CFH to study complement activation by the bacterium. By incubating these strains with NHS and a trace amount of ^{125}I-C3 we saw that the more the strain bound ^{125}I-CFH the less C3b opsonization was detected (Figure 1 of study II).

When the same strains were incubated in full blood the same trend in bacterial multiplication was seen. The strain that bound CFH efficiently (st369) multiplied well in the presence of complement and phagocytes while the multiplication of an intermediate CFH binding strain (*emm*5) was slower. The strain that did not bind CFH (*emm*8) did not survive in the presence of complement and phagocytes. As a control assay it was determined that this strain survived well when complement and phagocytes were absent. When only complement was absent the *emm*8 strain survived but, surprisingly, when only phagocytes were absent the bacteria failed to survive. This was interesting since the data showed not only that restriction of complement activation can lead to survival of the CFH binding bacteria but also that complement could directly limit survival of a non-CFH binding gram-positive bacterium – a property thought to be restricted to gram-negative bacteria.

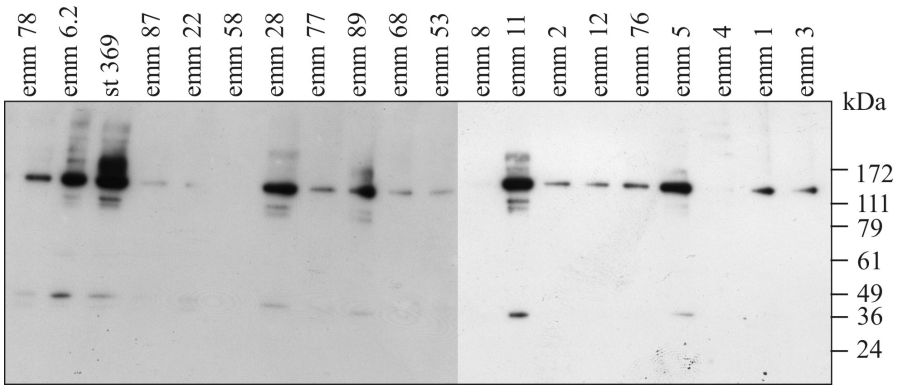

Figure 13. Binding of CFH from serum to 20 *S. pyogenes* strains. A serum bathing assay was performed to show the differential binding of CFH to several *S. pyogenes emm* types . Bacteria were incubated for 30 min in 20% normal human serum (pool from six individuals) and washed three times with PBS. The bound proteins were eluted using 0.1M glycine-HCl (pH 2.3) and detected by Western blotting as in study I where CFH was detected from the surface of *Loa loa*.

To study the effect of the Y402H polymorphism on complement evasion of *S. pyogenes* full length CFH proteins CFH(402Y) and CFH(402H) were isolated from individuals homozygous for the corresponding allele. Binding of these proteins to the same *S. pyogenes* strains was examined by direct ^{125}I-protein binding assays and radioligand assays (Figure 5 of study II). These data showed that the binding of CFH(402H) protein was clearly impaired when compared to the corresponding CFH(402Y) proteins.

The binding of CFH on *S. pyogenes* was also studied by using recombinant constructs CFH5-7(402Y), CFH5-7(402H), and CFH5-7(402H/420H). The proteins consisted of CFH domains five to seven (CFH5-7). The reason for generating three constructs was that the intended 402H mutation in the original publication (study II) was accidentally generated at position 420Y in the DNA template that already contained histidine (H) at position 402. As explained in the corrigendum to the article II, instead of using numbering from the preprotein (with 18 aminoacid signal sequence) we used numbering from the beginning of the mature protein. Later it was noticed that the specific polymorphism in the AMD association studies had been located starting from the signal sequence at position Y402H or SNP T1277C (rs1061170) that was position Y384H in the expressed protein (Clark et al., 2006). Therefore, the results in the study II have been obtained with CFH5-7 fragments CFH5-7(402H) - thought to be CFH5-7(402Y) - and CFH5-7(402H/420H) - thought to be CFH5-7(402H). In contrast, the results in the corrigendum to the article II were obtained using the fragments CFH5-7(402Y) and CFH5-7(402H) (Figure 14).

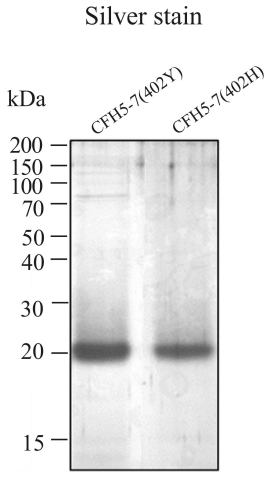

Figure 14. Silver staining of recombinant proteins CFH5-7(402Y) and CFH5-7(402H). The purified proteins were separated by SDS-PAGE and stained with Silver stain. Molecular weight standard can be seen at the left side of the figure. The CFH5-7(402Y) or CFH5-7(402H) proteins can be seen as 21 kDa bands

We examined binding of the recombinant constructs to several different *S. pyogenes* strains. In the study II the majority of the strains showed decreased binding of CFH5-7(402H/420H) compared to CFH5-7(402H) (Study II). When both the direct binding and radioligand binding assays were done with the CFH5-7(402Y) and CFH5-7(402H) fragments (Figure 15), fewer strains showed decreased binding of the CFH5-7(402H) compared to the CFH5-7(402Y) (corrigendum to study II).

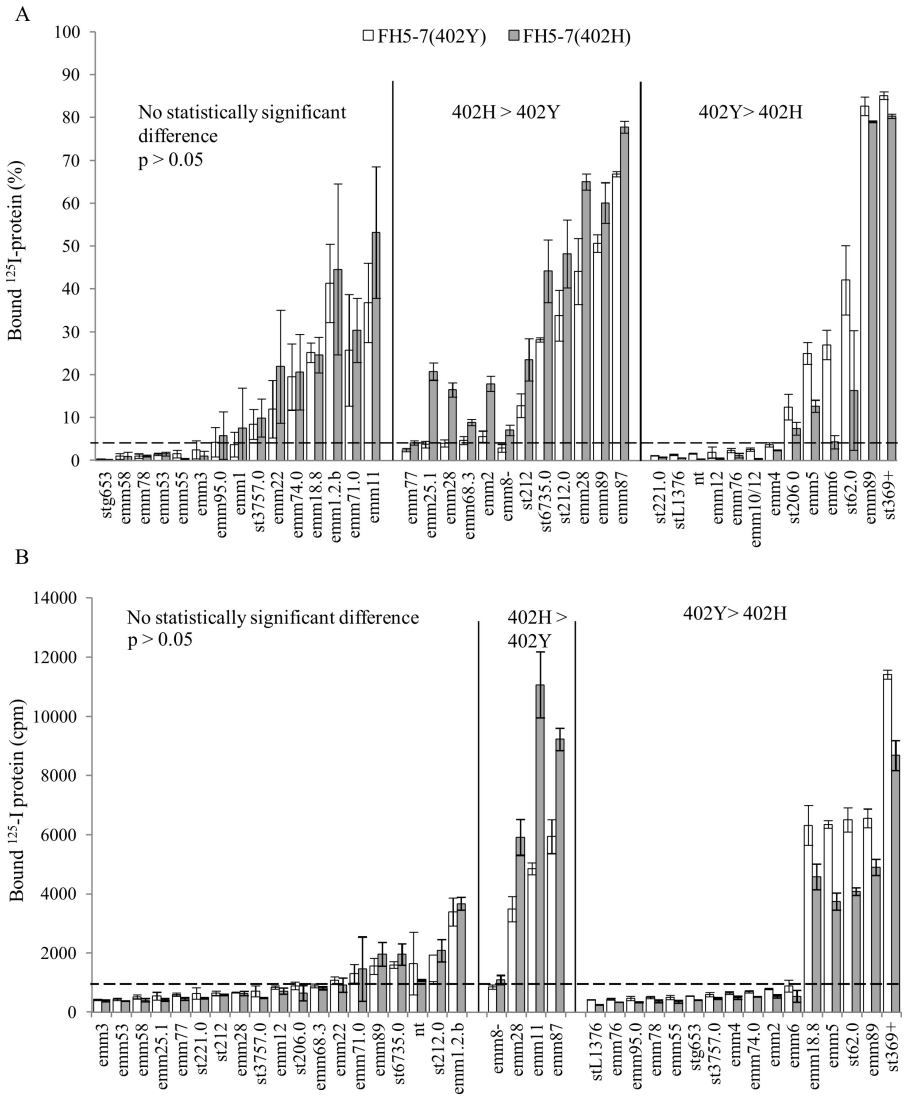

Figure 15. Binding of CFH5-7(402Y) and CFH5-7(402H) to *S. pyogenes* strains using direct binding assay (A) and radioligand assay (B). This data is the basis for the correction to fig. 6 of the article II. The strains are shown in ascending order based on the binding percentage of the CFH5-7(402Y) but have been separated into three categories by the vertical line depending on the statistically significant difference between binding of the CFH5-7(402Y) and CFH5-7(402H) recombinant fragments. The horizontal dashed line indicates the level of binding by the negative control strain *emm*8. Strain st369 is used as a positive control. Mean ±SD of triplicate samples in a representative of two individual experiments is shown. Cpm, counts per minute; nt, not typeable.

When the strains were categorized depending on the statistically significant difference between binding of the CFH5-7(402Y) and CFH5-7(402H) three groups were indentified: those strains that showed no difference, those with reduced binding to the CFH5-7(402H) and those with reduced binding of the CFH5-7(402Y). However, the results of only three strains (from which one was negative control strain *emm*8) of the latter group could be repeated in both direct binding and radioligand assays. In a competition assay, however, binding of ^{125}I-CFH5-7(402Y) on strain st369 was more decreased when incubated with CFH5-7(402Y) compared to the incubation with CFH5-7(402H) (Figure 16). According to these results, it is clear that several of the tested *S. pyogenes* strains bind CFH via domain seven in such a way that the residue where Y402H substitution is located is associated with CFH binding. Moreover, binding of CFH onto most of the strains is strongly affected by the Y420H substitution.

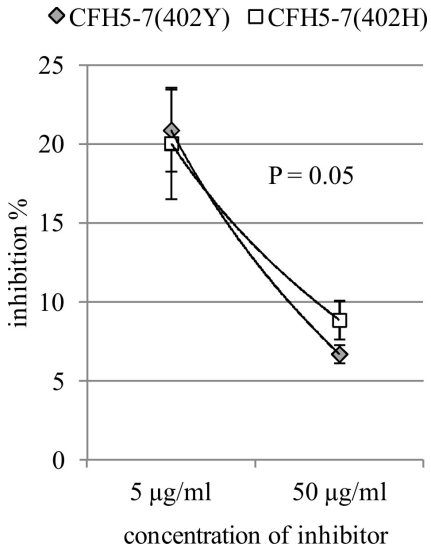

Figure 16. Dose-dependent inhibition of binding of ^{125}I-CFH5-7(402Y) to the GAS strain st369 by CFH5-7(402Y) or CFH5-7(402H). CFH5-7(402Y) or CFH5-7(402H) were added before addition of the ^{125}I-CFH5-7(402Y) protein in a radioligand assay. Percentage of bound radioactivity is shown as relative to the maximal CFH5-7(402Y) binding without the inhibitor. Mean ±SD of triplicate samples in one individual experiment is shown.

To see whether the reduced binding of CFH(402H) compared to the binding of CFH(402Y) on bacterial surface affects bacterial survival in human blood we incubated three strains (*emm*5, *emm*8 and st369) in blood obtained from individuals homozygous for either CFH(402Y) or CFH(402H). When the multiplication factors were calculated we found that within five hours of incubation the bacterial survival was significantly reduced in the blood obtained from the CFH(402H) homozygous individuals compared to the blood from CFH(402Y) individuals (Figure 7 of study II). Our main conclusion in the study II was that CFH Y402H polymorphism clearly affects binding of CFH to *S. pyogenes* surface in such a way that it has an impact in bacterial survival in blood *ex vivo*.

4.3 Multiplication of *S. pyogenes* bacteria in blood *ex vivo* can be impaired by inhibiting CFH binding with CFH5-7 (III)

In study II we had shown that CFH binding by *S. pyogenes* clearly reduces bacterial C3b opsonization and opsonophagocytosis. Since the interaction between CFH and M protein is mediated via the amino-terminal HVR of M protein (Johnsson et al., 1998), the region responsible for the *emm* type of the strain, we wanted to study this phenomenon using several strains of different *emm* types.

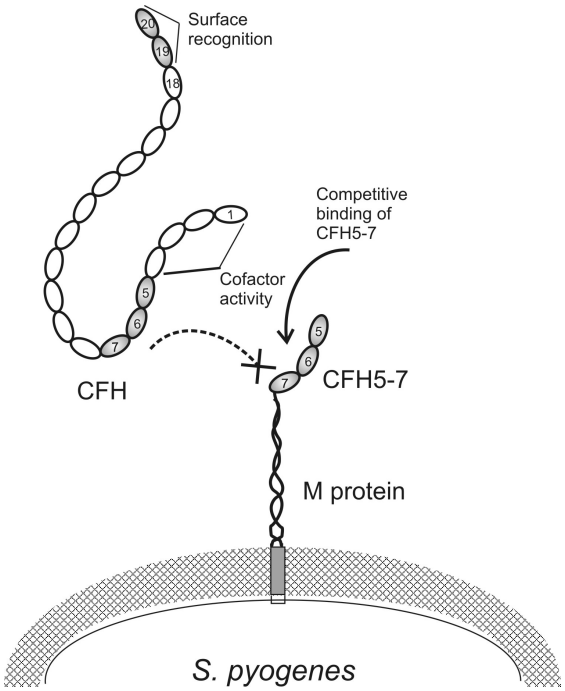

Figure 17. Inhibition of CFH binding to *S. pyogenes* M protein by CFH5-7. Recombinant CFH5-7 that does not act as a complement regulator but binds to M protein was used for competing with CFH in binding to *S. pyogenes* surface.

By incubating 38 different *S. pyogenes* strains (Study III) with NHS where only AP was active and by detecting AP activation markers from the supernatants we found that CFH binding as well as binding of CFH construct with only domains 5-7 is clearly negatively correlated with release of C3a fragment and generation and

release of SC5b-9 (Figures 1 and 2 of study III). In a whole blood incubation assay we reviewed the phenomenon in an opposite perspective. The binding of CFH was inhibited using a non-functional CFH5-7 fragment prior to inoculating the bacteria in whole blood. We hypothesized that the fragment lacking the cofactor active site 1-4 could bind competitively on the bacterial surface thus preventing bacterial immune evasion via CFH binding (Figure 17). When the activation markers Bb and SC5b-9 were analyzed from the supernatants we observed that complement activation was clearly enhanced when the bacteria were incubated in the presence vs. absence of CFH5-7 (Figure 4 of study III). CFH5-7 did not cause an increase in the level of the activation markers when the bacteria were absent, showing that the recombinant fragment did not cause excess complement activity in blood *ex vivo*. When four *S. pyogenes* strains were subjected to a multiplication assay they all multiplied well in the absence of CFH5-7 but failed to do so when CFH5-7 was present (Figure 5 of study III). All these findings were consistent with our hypothesis that binding of CFH via the domain seven is crucial for survival of *S. pyogenes* in blood.

4.4 Association of CFH Y402H polymorphism with *S. pyogenes* infections (III)

It was clear on the basis of our previous results that the CFH(402H) allotype has diminished binding to certain *S. pyogenes* strains compared to the binding of CFH(402Y). Since the decreased binding reduces bacterial survival *in vitro* we hypothesized that CFH(402H) homozygosity could be a protective phenotype against *S. pyogenes* infections. To test this we performed a genetic association study where individuals suffering from streptococcal infections were genotyped for the single nucleotide polymorphism T1277C in the CFH coding allele responsible for the CFH Y402H polymorphism. The patient data consisted of genotype data of individuals suffering from recurrent streptococcal tonsillitis (n=209) or erysipelas (n=278). Genetic data of non-erysipelas control patients (n=105) and healthy blood-donors (n=305) were used as controls.

When the allele genotype frequencies were compared between the patient and control groups we observed that the frequencies of C alleles and CC genotypes were significantly lower in the combined patient group than in the non-erysipelas or combined control group. On the basis of these results it seems that the Y402H polymorphism in CFH is associated with streptococcal tonsillitis and erysipelas, infections that both are mainly caused by *S. pyogenes*.

5 DISCUSSION

5.1 *In vivo* binding of complement regulators

Microbial CFH binding has been shown to be an important complement evasion mechanism for several pathogens *in vitro*. Before the studies presented in this thesis there was no evidence, however, of acquisition of CFH in human infection *in vivo*. It has been difficult to test this since good animal models are not available due to the species specificity of CFH binding as demonstrated for pathogens such as *S. pneumoniae* and *N. meningitidis* (Lu et al., 2008; Ngampasutadol et al., 2008). However, there is evidence that preincubation of *Streptococcus pneumoniae* with human CFH increases streptococcal survival (Quin et al., 2005) and enhances bacterial invasion *in vivo* in a mouse model (Quin et al., 2007).

It has been suggested that during loiasis high level of anti-filarial IgG has an important role in controlling the microfilaremia (Akue et al., 1997) with the help of CP of complement. Together they mediate antibody dependent neutrophil adherence to the microfilariae (Pinder et al., 1992). Since in study I we observed complement regulatory proteins C4BP and CFH on the surface of the isolated microfilariae it is likely, however, that opsonization and thereby neutrophil adherence could be restricted on the microfilariae in human blood *in vivo*.

In an earlier study where binding of CFH on *Onchocerca volvulus* microfilariae has been demonstrated (Meri et al., 2002) the main role of the surface bound complement regulators was suggested to be the prevention of the action of neutrophils, not the direct lysis of complement. The reason for this is the existence of the thick cuticle on microfilariae surface that is obviously resistant to the cytolytic effects of complement. The opsonins C3b and iC3b can further be inactivated to C3dg by CR1 in the presence of factor I providing a ligand for CR2 of B-cells thereby decreasing the threshold for B-cell activation (Morgan et al., 2005). Therefore, reduced formation of C3b and iC3b on microfilariae observed in study I could potentially also delay antifilarilal antibody production and thereby affect longevity of microfilariae in patients (Akue et al., 1997). CFH binds to the outermost sheath layer of the microfilariae (study I) but the specific CFH interacting ligand is not yet known. It also remains to be tested by using recombinant fragments of CFH which are the domains in CFH mediating the binding on *Loa loa* microfilariae.

5.2 CFH binding within *S. pyogenes* strains

In this thesis and in earlier studies where CFH interaction with clinical isolates has been studied (Horstmann et al., 1988; Perez-Caballero et al., 2000; Suvilehto et al., 2008) a total of 35 *S. pyogenes* strains have been found to bind CFH (Table 7). The level of CFH binding to the bacteria, however, varies between the strains. As demonstrated in the study II it seems that CFH binding by *S. pyogenes* is mainly mediated via domains 5-7 (19 of 35 CFH binding strains). It was found that certain strains only bound CFH5-7 but not CFH. This could be explained by the folded structure of CFH (DiScipio, 1992b) where the M protein-binding site is less easily exposed when compared to the truncated molecule CFH5-7. This could also be the reason why in certain cases *S. pyogenes* binds CFHL-1 rather than CFH (Perez-Caballero et al., 2000).

As suggested by others (Perez-Caballero et al., 2000; Suvilehto et al., 2008) there is also variability in CFH binding between different isolates within the same *emm* type. In our hands the two different isolates of *emm*28 strains, that according to the sequence data share 100% similar *emm* sequences, showed completely different CFH binding: the blood isolate bound CFH clearly while the reference strain bound CFH very weakly (Table 7). It is important to note that CFH binding correlated with the complement activity caused by the bacterium indicating that CFH binding is important in complement evasion of *S. pyogenes*. Therefore, although the *emm* type can be determined at the genetic level it is still unclear whether the diminished CFH binding is due to the lack of interaction between CFH and surface bound M protein or due to the lack of M protein expression by the isolate because of, for example, phase variation. When incubated in culture media *S. pyogenes* undergoes phase variation during the stationary growth phase. Here the expression of the *emm* and *speB* genes are significantly reduced while the expression level of *slo* is unaffected changing the bacterium almost completely sensitive to phagocytosis (Leonard et al., 1998). Therefore, in this study bacteria were grown to log phase before subjecting to CFH binding assays. Since in some studies (Perez-Caballero et al., 2000; Suvilehto et al., 2008) CFH binding has been analyzed using bacteria grown for 18 h this could at least partly explain why CFH binding of different isolates of the same *emm* type differs between studies. It is also possible that CFH binding is reduced due to the action of bacterial proteases on the M protein or due to the spontaneous release of the M protein from the bacterial surface (Åkesson et al., 1994) or even due to small changes in M protein sequences that spontaneously occurs in the HVR part of the M protein.

Table 7. Formation of C3a and binding of CFH/CFH5-7 on *S. pyogenes*

	Study III	Study II and III		Previous studies
Strain	C3a	CFH	CFH5-7	CFH[#,§]
st212	-	+++	+++	
*emm*1.2b	-	+++	+++	
*st*6735.0	-	+++	+++	
*emm*71.0	-	+++	+++	
st369	+	++	+++	
st212.0	-	++	+++	
*emm*89	+	++	+++	
*emm*11*[,#]	-	+	+++	+
*emm*28*[,§]	+	+	+++	+
*emm*87	-	+	+++	
*emm*95.0	-	+++	++	
st62.0	+	+++	++	
st3757.0	++	+++	++	
*emm*18.8[#]	-	++	++	+
*emm*2	+	++	++	
st206.0	++	+++	+	
*emm*25.1	++	+++	+	
st221.0	-	+++	–	
stG653	-	+++	–	
*emm*74.0	-	++	+	
*emm*53	+	–	++	
emm22	++	–	++	
*emm*68	++	–	++	
*emm*77	+++	–	++	
*emm*55	-	++	–	
*emm*6[#,§]	+	++	–	+
*emm*5+	+++	++	–	+
*emm*3	+++	++	–	–
*emm*58	+++	+	+	
*emm*1*[#]	-	–	+	+
*emm*76	-	–	+	
*emm*28*[,§]	+++	-	+	+
*emm*12	++	+	–	–
*emm*78	+++	+	–	
stL1376	+++	+	–	
*emm*4*[#]	+++	–	–	+
*emm*8*[#]	+++	–	–	+
emm19[§]	n.a.	n.a.	n.a.	+
emm24[#,§]	n.a.	n.a.	n.a.	+
emm29[#]	n.a.	n.a.	n.a.	+
emm30[#]	n.a.	n.a.	n.a.	+
emm31[#]	n.a.	n.a.	n.a.	+

* Differences in CFH binding between isolates; na, not assayed

CFH binding shown in Reference: [#]Perez-Caballero et al., 2000, [§]Horstmann et al., 1988

5.3 CFH binding and *S. pyogenes* survival

Binding of CFH to different *S. pyogenes* strains shows a high variability – some strains bind CFH efficiently while others completely lack the ability. The amount of detected CFH binding to the different *S. pyogenes* strains shows a negative correlation with the level of complement activation induced by the bacteria. In addition, CFH binding correlates positively with bacterial survival in blood. This indicates that M protein expression level and thereby the amount of CFH bound on *S. pyogenes* surface and/or the affinity between CFH and M protein determines the efficacy of complement evasion. It seems that CFH binding may play a key role in the streptococcal survival in serum or blood at least *in vitro*. The importance of complement system in elimination of *S. pyogenes* and the function of CFH in bacterial survival can be demonstrated in an *ex vivo* survival model (study II) where depletion of complement allows growth of a non-CFH binding strain that does not survive in fresh blood. Interestingly, the presence of complement in the absence of phagocytes can be enough to decrease survival of a non-CFH binding strain indicating that complement somehow acts on this gram-positive bacterium by itself. The mechanism behind this novel observation is unknown. It remains to be studied if excessive amount of C3b deposits could interfere with, for example, bacterial metabolism or cell division.

Although the domains 5-7 are mainly mediating the binding of CFH on *S. pyogenes* surface it is evident that some strains interact with other domains in CFH using ligands other than the M protein. For example, the binding of CFH to M6 protein has been localized to CFH domain seven (Blackmore et al., 1998a) but in our hands the *emm*6.2 isolate bound CFH5-7 only weakly when compared to the negative control strain *emm*8. This isolate differed slightly from the *emm*6 CDC strain (90.4% sequence identity) in the *emm* sequence that could possibly explain the impaired binding of CFH5-7. Nevertheless, the isolate bound clearly the whole CFH protein indicating that the interaction is mediated via two ligands, probably also via Scl1, that has been shown to bind CFH via domains 18-20 (Caswell et al., 2008; Reuter et al., 2010). Because the *emm*6.2 strain showed only limited complement activation in serum when compared to the negative control strain *emm*8, the binding of CFH outside domains 5-7 could be an alternative method to evade complement attack. Binding of CFH via domain seven is, however, probably more common within *S. pyogenes* bacteria because Scl1, that has been shown to bind CFH via domains 19-20, is also expressed by strains *emm*1, *emm*28, *emm*4, *emm*77, *emm*12 and *emm*2 that do not interact with CFH (Caswell et al., 2008). It could also be that binding of CFH via domains 5-7 leads to more efficient complement evasion than binding of CFH via other domains. The results showing that the *S. pyogenes emm*6 mutant lacking M6 protein becomes sensitive to

complement and phagocytosis indicate that expression of M6 protein is important for immune evasion (Horstmann et al., 1988). In addition, introduction of the *emm*6 gene to an M protein deficient strain restores its ability to resist phagocytosis (Perez-Casal et al., 1992).

It is possible that the CFH-binding region within the different M proteins is variable since the interaction site has been localized to two distinct areas: the C-repeat region for the M6 protein (Fischetti et al., 1995; Perez-Casal et al., 1995) and HVR for M5 and probably also for M6 (Johnsson et al., 1998; Kotarsky et al., 2001). These differences are not surprising since size variation found in A and B repeat regions between different M proteins also occurs within M6 expressing laboratory isolates (Fischetti, 1989). As previously mentioned, the CFH binding does not necessarily lead to complement and phagocytosis resistance especially if the binding affinity or the amount of expressed ligand is not sufficient. For example, in study III the *emm*5 strain binds some CFH but does not diminish complement activity in human serum. Furthermore, some phagocytosis resistance by the strain can be seen when compared to a non-CFH binding strain but the multiplication in blood is fairly limited when compared to strains showing high level CFH binding. Therefore, it is likely that CFH binding does not completely explain the limited phagocytosis resistance of this particular strain. Although the CFH binding by the M5 protein has been reported to occur via HVR it looks like the B-repeat region is essential for phagocytosis resistance of an *emm*5 strain studied by others (Kotarsky et al., 2001; Sandin et al., 2006). It is, however, noteworthy that while the deletion of the B-repeat region of the M5 protein almost completely abolished bacterial phagocytosis resistance the partial deletion of the HVR clearly decreases the resistance (Sandin et al., 2006) indicating that CFH binding is partially involved in the phenomenon. The deletions done in the M protein could also partially destroy the coiled coil structure of the protein or shorten the protein to the extent that it has an influence on the studied interactions.

Certain M proteins bind CFHL-1 (Johnsson et al., 1998) and none of the assays in this thesis ruled out this possibility. On the contrary, there was one strain (*emm*87) that showed weaker binding of both ^{125}I-labeled CFH (Table 7) and CFH from serum (Figure 13) when compared to the binding of CFH5-7. CFH and CFHL-1 are identical in the structure of the first seven domains, except of four additional amino acids in the carboxyl-terminal end of CFHL-1, whose function is unknown. They are encoded by the same gene and both have cofactor activity in the four most amino-terminal domains. Because of the structural similarities it is probable that CFH and CFHL-1 share similar functions in *S. pyogenes* complement evasion. However, since the serum concentration of CFHL-1 is 10-50 times lower than the concentration of CFH it is possible that the importance of CFHL-1 binding by the bacterium is more relevant in specific locations in human body (Zipfel and Skerka, 1999).

Taking together, the high variability in CFH binding within different *S. pyogenes* strains can be explained by the binding affinity of CFH to the expressed M protein, the expression level of the M protein *e.g.* due to the limited expression in certain growth phase of *S. pyogenes,* alternative ligands for CFH binding, or inability of full CFH but not CFHL-1 or CFH5-7 to bind to M protein. Nevertheless, CFH binding by the microbe is most likely the main phenomenon that determines bacterial complement evasion and survival *in vitro* and therefore can be generalized as a common property for the studied species.

5.4 The interaction between CFH and M protein

The present knowledge on the interactions between *S. pyogenes* and CFH is mainly based on studies done using clinical *S. pyogenes* isolates or single M proteins. Therefore we cannot claim that M protein is the definitive ligand mediating CFH binding by most *S. pyogenes* strains. Since CFH5-7 mediates binding to most *S. pyogenes* strains (studies II and III) it is likely that these strains use these domains to bind CFH via M protein. In addition, the high variability in HVR of M protein between different strains gives the best explanation for the differences in CFH binding observed within these strains.

At the protein level, the main residues in CFH domain seven interacting with the M protein are at least 387R-388K, 404R-405K (Giannakis et al., 2003), and 402Y as suggested by the study II. Also, a Y to H substitution in residue 420, obtained as a result of accidental misnumbering as explained in Materials and Methods, resulted in significant decrease in CFH5-7 binding suggesting that this residue is also important for the interaction. All the experiments were done with the three CFH5-7 fragments, CFH5-7(402Y), CFH5-7(402H), and CFH5-7(402H/420H). The main message and the conclusions obtained with with CFH5-7(402Y) and CFH5-7(402H) (corrigendum to study II) were the same as those obtained with CFH5-7(402Y) and CFH(402Y/420H) and full length CFH from CFH(402Y) and CFH(402H) homozygous individuals (study II). This is logical since the residue 420 is located at the same side of the domain as the polymorphic site 402 and the residue 405 previously shown to be involved in binding to M6 protein (Figure 18) (Giannakis et al., 2003; Prosser et al., 2007). Furthermore, the mutation to the residue 420 gave additional information about the site of CFH responsible for binding to M proteins. This is important since the residue 420 seems to be the most widely used residue in binding of CFH5-7 to various M proteins and could thereby become a main target for therapeutic approach in preventing *S. pyogenes* immune evasion in future.

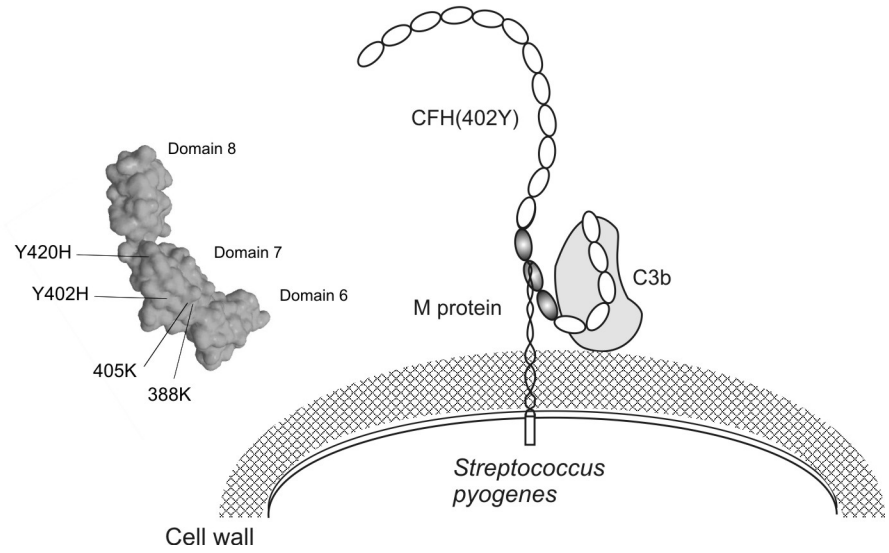

Figure 18. A schematic presentation of simultaneous binding of CFH to C3b and *S. pyogenes* M protein. According to the combined mutagenesis data including the data in study II the residues 388, 402, 405 and 420 are all involved in M protein binding. The residues are marked in the crystal structure of domains 6-8 (Prosser et al., 2007) (PDB entry 2UWN). The same domains are marked in gray in the schematic presentation of simultaneous binding of surface bound C3b, CFH, and *S. pyogenes* M protein.

Although the key results in the corrigendum are similar to those in the original published article (study II) the binding of the CFH5-7(402Y) and CFH(402H) constructs to more than 40 GAS strains showed some differences compared to the binding of CFH5-7(402H) and CFH5-7(402H/420H). Most of the strains showed impaired binding of the CFH5-7(402H) compared to the CFH5-7(402Y) recombinant fragment as expected. However, two of the strains showed the opposite results. This indicates that the residues mediating the interaction between CFH and GAS M protein are less conserved within different GAS strains than what we originally expected. However, the residue 420 could be important in several CFH-M protein interactions. The interaction between *S. pyogenes* M protein and the domain seven of CFH is mediated by multiple residues. When observing the location of the known streptococcal binding residues 402, 405 and 420 in the crystal structure of the CFH domains 6-8 (Prosser et al., 2007) it appears that the binding site follows the domain seven longitudinally. This kind of binding may allow simultaneous binding of the regulatory active domains 1-4 of CFH to C3b as represented in (Wu et al., 2009). Using the obtained information, the next aim could be to try to obtain the X-ray crystal structure of the CFH5-7:M protein complex in the future using also M proteins expressed by other *emm* types than *emm*5 and *emm*6.

5.5 CFH Y402H polymorphism and susceptibility to streptococcal infections

Certain polymorphic variants in CFH gene have been found to be associated with increased risk of developing diseases such as aHUS, MPGNII, or AMD. There is also evidence that variation in CFH gene is associated with a risk to develop invasive *N. meningitidis* infections (Davila et al., 2010). This is interesting since the complement resistance of *N. meningitidis* is mediated via binding of CFH using the surface proteins Fhbp (Madico et al., 2006) and NspA (Lewis et al., 2010). Both of these ligands bind CFH via domain seven – the same domain that mediates the interaction between CFH and *S. pyogenes* M protein. In studies where the role of CFH binding by *N. meningitidis* has been studied show that the presence of CFH *in vivo* improves the ability of *N. meningitidis* to cause bacteremia in infant rats (Vu et al., 2012) and that increased concentrations of CFH in plasma, caused by C496T homozygocity in the CFH coding gene, is associated with increased risk of developing *N. meningitidis* infections (Haralambous et al., 2006).

In this thesis we show (study II), by comparing the binding of allotypes CFH(402Y) and CFH(402H) to *S. pyogenes* strains, that the decreased binding of CFH in the presence of only the CFH(402H) allotype, also leads to functional consequences. Here, bacteria induced complement activation is enhanced and multiplication of the bacteria is decreased (Figure 19) indicating that host genetic variation in CFH affects survival of *S. pyogenes*. This is also supported by the genetic association study (study III) where Y402H polymorphism (C1277T) suggests an association with streptococcal diseases. Therefore CFH Y402H polymorphism could be important in determining the susceptibility of an individual to streptococcal diseases and could also explain why the AMD associated allele is enriched in human population despite of its harmful effect later in life.

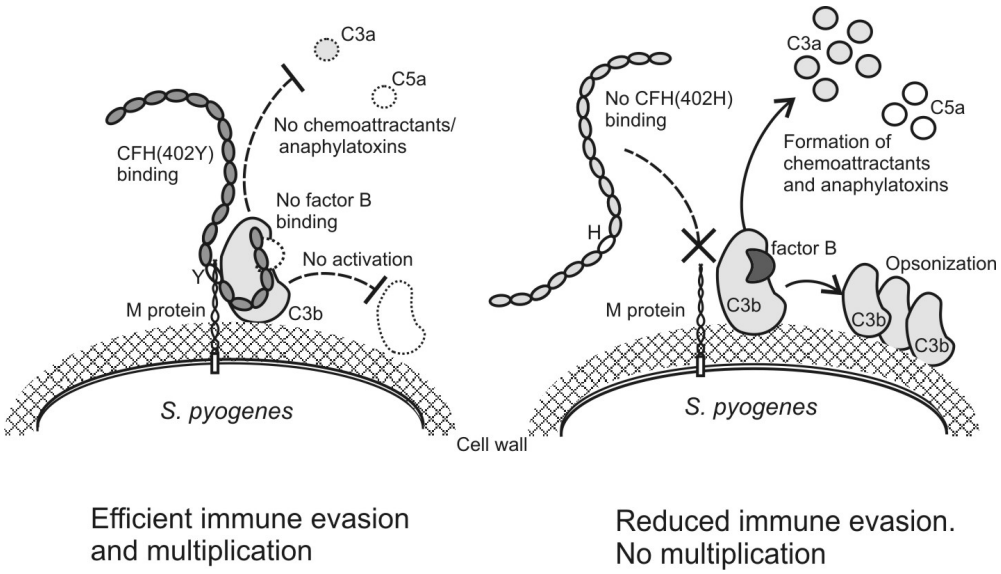

Figure 19. A hypothetical presentation of the effect of different binding properties of CFH(402Y) and CFH(402H) allotypes on survival of *S. pyogenes*. The bacterium binds allotype CFH(402Y) (dark grey) on its surface and thereby evades complement attack and survives in blood. The binding of the allotype CFH(402H) (light grey) on M protein is reduced interfering with *S. pyogenes* immune evasion and leading to decreased bacterial survival. The domain seven containing the polymorphic Y402H site is marked in white.

Whether the Y402H polymorphism could affect host susceptibility to other infections caused by a CFH domain seven binding bacterium is not yet known. A genome wide association study (GWAS) (Davila et al., 2010), however, suggests only very nominal association of the polymorphism to *N. meningitidis* infections. In this study, it is possible that the true disease variant has been lost in the statistical noise when multiple variants have been examined simultaneously. Although the study in this thesis showed only a possible association of the Y402H polymorphism in *S. pyogenes* infections the observed association was strongly supported by the functional studies where the Y402H polymorphism was shown to affect survival of *S. pyogenes* in blood.

It would be interesting to study the association of Y402H polymorphism in *S. pyogenes* diseases by using data of patients with verified *S. pyogenes* infections since erysipelas can be caused by other risk factors than the variation in CFH such as edema, broken skin and obesity (Karppelin et al., 2010). In addition, the infection can also be caused by group G streptococcus since it has often been isolated from erysipelas patients (Siljander et al., 2008). It is also possible that the

majority of the *S. pyogenes emm* types isolated from Finnish erysipelas and tonsillitis patients do not bind CFH via the domain seven. In invasive *S. pyogenes* cases the *emm* types 28, 1, 84, 75 and 89 are predominant but in the study II no CFH binding was detected to the used *emm*1 strain representing the most common *emm* type isolated in Finland in 2007 (Siljander et al., 2010). Within strains isolated from Finnish tonsillitis patients *emm*28 is probably the most common (Suvilehto et al., 2008). The results on CFH binding by two different isolates of type *emm*28 (study III) showed evidence that there are variation in CFH binding even within the same *emm* type. From the two *emm*28 strains, the reference strain did not bind CFH while the blood isolate bound CFH indicating that a spontaneous mutation in the M protein, or a decrease in M protein expression level of the reference strain had occurred. There is at least evidence that *S. pyogenes* bacteria isolated from asymptomatic patients can show limited M protein expression because of the phase variation (Leonard et al., 1998).

In future studies, it could be beneficial to collect data from patients with invasive *S. pyogenes* infections and study the association of Y402H polymorphism in *S. pyogenes* infections using additional control material where the *S. pyogenes* infection has been excluded. Furthermore, the effect of Y402H polymorphism in infections caused by CFH binding *Loa loa* microfilariae should be studied although the interacting domains in the protein are not known (study I). Previous studies have suggested that genetic factors may play a major role in susceptibility or resistance to *Loa loa* infections. These could partly explain why the prevalence of loiasis in hyperendemic regions rarely exceed 30% although these individuals are continually exposed to the parasite (Garcia et al., 1999).

5.6 CFH5-7: a drug candidate against *S. pyogenes* infections?

S. pyogenes is a common pathogen affecting individuals around the world and many of the infections may lead to harmful sequelae or complications and lethality. A lot of effort in prevention of *S. pyogenes* infections has been focused on generating M protein vaccines, which could be the most promising vaccine candidates, particularly targeted against the rheumatogenic strains. It is, however, well known that *e.g.* arthritis following *S. pyogenes* infection is based on an autoimmune reaction where M protein has been shown to induce production of antibodies against host tissues. This indicates that molecular mimicry between M protein and human proteins could play a role in the pathogenesis of this disorder (Dale and Beachey, 1985; Cunningham et al., 1997). This is further supported by

the finding that both Coxsackie B3 virus and *S. pyogenes* infections can induce autoimmunity leading to heart diseases and their proteins, VP1 and M protein, share similarities and cross reactivity with heart tissues (Cunningham et al., 1992). Although most M protein vaccine trials have shown production of protective antibodies and good tolerance among vaccinated individuals the safety of the vaccine candidate must be thoroughly analyzed (Steer et al., 2009). The possible induction of an autoimmune disorder by a vaccine is a major concern especially because of a possible long latency period. For example the interval between *S. pyogenes* infection and onset of post-streptococcal glomerulonephritis, rheumatic fever, or narcolepsy are 1-2 weeks, 3-8 weeks, and weeks to months, respectively (Aran et al., 2009).

CFH5-7 is a fragment of human CFH lacking the regulatory site but simultaneously being able to bind to several *S. pyogenes emm* types. Binding of the CFH5-7 fragment to several *S. pyogenes* strains significantly increases the complement activity against *S. pyogenes* bacteria (study III) that in turn increases the phagocytosis of the pathogen. When added in fresh human plasma the CFH5-7 fragment does not cause excess complement activation by itself. In contrast to M protein vaccine candidates, human CFH5-7 fragment is unlikely to be antigenic and therefore most probably does not induce autoimmunity. By preincubating *S. pyogenes* bacteria with CFH5-7 the binding of whole CFH is inhibited (study III). Two major findings shown in this thesis indicate that this inhibitory effect of CFH5-7 on CFH binding to *S. pyogenes* could also affect to the survival of the bacterium. First, those strains that bind CFH5-7 show increase in AP activation. Second, CFH binding by *S. pyogenes* is associated with rapid multiplication rate of the bacteria in blood while the multiplication rate decreases when the bacteria are preincubated with CFH5-7 (study III).

The CFH5-7 fragment could interfere with CFH binding not only to M protein but also to Fba on *S. pyogenes*. The reason for this assumption is that Fba binds CFHL-1 indicating that the interaction could be mediated via domains 5-7 of CFH (Pandiripally et al., 2002; Birkenfeld et al., 2011). Since at least three CFH interacting ligands have been found on *S. pyogenes* it is also probable that within some strains the efficient phagocytosis resistance requires two or more CFH ligands. In this case binding of CFH5-7 fragment to M protein or Fba could be enough to interfere with CFH binding to the extent that the bacteria become sensitive to phagocytosis.

It has been proposed that bacterial invasion of host cells could explain the recurrence of *S. pyogenes* infections (such as tonsillitis and erysipelas) as well as failures in bacterial eradication, since intracellular bacteria could avoid the effects of penicillin (Österlund et al., 1997). The recurrence of *S. pyogenes* infections has also been suggested to be caused by β-lactamase producing commensal bacteria in

oral flora (Brook, 1984). Since *S. pyogenes* Fba that also interacts with CFH has been shown to promote microbial entry into host cells (Terao et al., 2001) the CFH5-7 fragment might also interfere with bacterial invasion into epithelial cells. Therefore, if CFH5-7 was used as a drug it also might prevent relapses.

There are only two main strategies to control *Loa loa* transmission rates in endemic countries - mass treatment of endemic communities or prevention of vector bites. The risk of adverse effects in areas coendemic for loiasis and onchocerciasis creates a strong need of novel control strategies (Padgett and Jacobsen, 2008). It remains to be studied whether CFH5-7 could decrease survival of *Loa loa* microfilariae in blood and whether this knowledge could assist in development of a safer way to prevent transmission of loiasis.

The CFH5-7 fragment could also cause adverse effects because the domain seven of CFH interacts with heparin and malondialdehyde adducts (Blackmore et al., 1996; Weismann et al., 2011). Therefore, it is theoretically also possible that CFH5-7 fragment could prevent CFH interaction with host cells and thereby contribute to diseases such as AMD. The protective function of CFH5-7 against CFH binding microbes should be thoroughly investigated in further studies.

6 SUMMARY

Binding of soluble complement regulators occurs both *in vitro* and *in vivo* and is a common complement evasion strategy of several microbial pathogens. In this thesis microfilariae of *Loa loa* were shown to bind CFH *in vivo* and the binding of the regulator was localized on the surface of the parasite. A large variety of bacteria have been shown to bind CFH via domains 5-7 or 19-20. *S. pyogenes*, the common causative agent of tonsillitis and erysipelas, was shown in this thesis to interact mainly with domains 5-7 of CFH. The ability to capture host CFH varies within different *S. pyogenes emm* types as well as within different isolates and the microbial induced complement activation is most likely dependent on the amount of CFH bound by the strain.

While CFH binding by *S. pyogenes* seems to have an important role in microbial complement evasion and survival in blood, the AMD associated Y402H polymorphism could have an effect in genetic susceptibility of an individual to *S. pyogenes* infections via affecting to the binding affinity of CFH on the microbe. Since the domains 5-7 of CFH play an important role in binding of CFH by *S. pyogenes* the CFH5-7 fragment could be used in inhibiting survival of this bacterium in blood. Therefore CFH5-7 or a derivative of it that inhibits CFH binding to the bacteria might be used as a novel drug in treatment of *S. pyogenes* infections or infections caused by other CFH binding microbes.

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