Erika van der Maten

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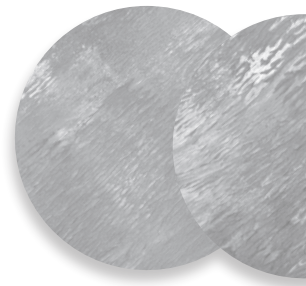
Chapter 1

General Introduction



Pneumococcal disease

Streptococcus pneumoniae (pneumococcus) is an important contributor to morbidity and mortality worldwide [1,2]. This encapsulated Gram-positive bacterium with a diplococcus shape causes diseases ranging from mild respiratory tract infections to severe diseases, such as pneumonia, sepsis and meningitis, also referred to as invasive pneumococcal disease (IPD). *S. pneumoniae* colonizes the human nasopharynx with carriage rates up to 60% in infants, mostly without causing any harm [3,4]. *S. pneumoniae* is spread from person to person via droplets or aerosols. Colonization and mucosal infections, like acute otitis media, can occasionally result in the development of severe invasive infections [5]. The significance of *S. pneumoniae* as a human pathogen is highlighted by the estimate that globally pneumococcal disease causes 0.7-1.0 million deaths annually among children aged <5 years, most of which live in developing countries [2,6]. The burden of disease of elderly in developing countries is not yet well defined [2]. In the Western world, children <2 years and elderly people are more susceptible to develop pneumococcal disease [2]. Another important group with an increased risk to develop pneumococcal disease are immunocompromised individuals, for instance those with HIV infection or immunodeficiencies [7-9].

An important virulence factor of *S. pneumoniae* is its polysaccharide capsule. There are over 90 different serotypes identified according to the chemical structures of their capsular polysaccharide [10]. The development of protein conjugate vaccines, which contains selected capsular serotypes, has significantly reduced the burden of invasive pneumococcal disease [6,11,12]. However, the plasticity of the pneumococcal genome results in adaptation to the selective pressure of vaccines. This causes serotype replacement by which pneumococci may circumvent the vaccine. The prevalence of non-vaccine types following introduction of the vaccine has substantially increased [13]. This phenomenon is of concern, since it may dampen the effectiveness of the current vaccines. In addition, widespread increase of pneumococcal resistance to antibiotics has been detected [14]. Altogether, this indicates that *S. pneumonia*e still remains an important human pathogen.

For effective prevention and treatment of pneumococcal disease it is of importance to understand the molecular mechanisms by which *S. pneumoniae* and the host interact. Recent findings in host-pathogen interactions in other infectious diseases suggest that complement factor H, a key negative regulator of the complement system, affects the susceptibility or outcome of many infectious diseases [15-17]. It is already known that *S. pneumoniae* has the ability to bind human factor H as a possible immune evasion strategy. The studies described in this thesis were designed to clarify the role of complement factor H in pneumococcal-host interactions.

The complement system as part of human host defence

The immune system

Defence against invading pathogens is mediated by intact epithelial barriers and by components of the immune system specialized in host defence and inflammatory responses. The human immune system can be divided into innate and adaptive immunity. Innate immunity uses genetic germ-line encoded receptors and secreted proteins to recognize common features of pathogens. The non-specific early defences of the innate immune system are essential to prevent or to slow infections to allow time for the adaptive immunity to develop and respond. Adaptive immunity uses a process of somatic cell gene rearrangement to recognize pathogen's unique antigens to generate an antigen-specific response to eliminate the pathogen.

S. pneumoniae colonizes the nasopharynx asymptomatically, but may become invasive and penetrate the mucosal barrier and enter the blood stream. This immediately triggers host innate immune responses on the mucosa and systemically. Mucosal immunity at mucosa-associated tissues (MALT) has an important barrier function. Soluble mediators present in the mucus, such as collectin, lactoferrin, lysozyme and defensins prevent that microorganisms pass the mucus. Although differences exist between mucosal and systemic immunity, many of the immune responses described below occur at both sites.

An important innate immune response upon *S. pneumoniae* infection is activation of the complement system. The complement system consists of over 30 plasma and membrane proteins present on the mucosa and systemically. Upon activation, these proteins contribute to clearance of the pathogen. The importance of the complement system is apparent in individuals with genetic complement deficiencies who are more susceptible for invasive infections [18]. In the next paragraph we describe the role of complement activation in inflammation and phagocytosis.

Role of complement in inflammation

Most invading pathogens induce inflammatory responses upon recognition by the host immune system. The induction of cytokines and chemokines conveys important signals to other immune cells needed for activation and coordination of host immune responses. Innate immune receptors play an important role in the first recognition of an invading pathogen. An important group of pattern recognition receptors (PRRs) are Toll-like receptors (TLRs), which detect micro-organisms through the recognition of conserved molecular motifs. Among the various TLR receptors, TLR2 recognizes lipoteichoic acid that is present in bacterial membranes, including pneumococci [19]. Furthermore, TLR9 detects pneumococcal DNA containing unmethylated CpG motifs within endosomes and TLR4 has been found to be activated by the pneumocooccal pneumolysin [19]. Another group of pattern recognition receptors (PRRs) are cytosolic NOD-like receptors and DNA sensors, which also contribute to the recognition of *S. pneumoniae* infection [19]. These PRRs regulate the production of proinflammatory mediators, including TNF-α, IL-1β, IL-6 (IL-12). These secreted cytokines induce the acute-phase response resulting in elevation of the body temperature, vasodilatation and increased vascular permeability. In addition, acute-phase proteins, such as C-reactive protein (CRP) and mannose binding lectins, are released. Furthermore, the inflammatory response stimulates the recruitment of immune cells such as neutrophils and macrophages, and shapes the adaptive immune response to control the infection. Another immune activation mechanism is recognition of the Fc part of antibody bound to their target by Fc-receptors on surfaces of immune cells such as monocytes, macrophages and neutrophils [20]. Fc-receptor binding of these immune complexes stimulates cytokine production, release of inflammatory mediators and phagocytosis [20] (**Figure 1**).

In addition to the activation of multiple innate immune receptors, pathogens also activate the complement system. Crosstalk between the complement system, Toll-like receptors (TLRs) and Fcγ receptors modulates the extent of the proinflammatory cytokine responses [21-26] (**Figure 1**). In the cascade of complement activation, activation products C3a and C5a, also called anaphylatoxins, are released. Various studies report that C5a binding to its receptor (C5aR) (also called CD88) modulates the inflammatory response induced by different bacterial pathogens, including *Escherichia coli*, *Staphylococcus aureus* and *Neisseria meningitidis* [27-29]. In addition, C3a and the more potent C5a, function as chemoattractant for neutrophils to migrate towards the site of infection [21].

However, the inflammatory response can be excessive and contribute to local tissue damage in focal infections or can be detrimental in patients with sepsis. High inflammatory responses by massive secretion of cytokines and excessive complement activation may result in fever, vascular permeability, tissue damage and organ failure. It is therefore of importance to study how the complement system is regulated and how its activation affects the host inflammatory response. In addition, therapies targeting the complement system may reduce the extremely high inflammatory response associated with the severe outcome of sepsis.

Role of complement in phagocytosis

Phagocytic white blood cells, such as macrophages and neutrophils, are of major importance for killing Gram-positive pathogens, such as *S. pneumoniae*, since these innate immune cells are able to engulf and kill invading bacteria. Mature monocytes leave the circulation to migrate into tissues where they further differentiate into macrophages. Neutrophils are abundant in blood, but can also migrate into tissues upon infection. Importantly, these phagocytes need to recognize the bacterium. This process is mediated by opsonins such as antibodies, complement factors and CRP (**Figure 1**). Several studies demonstrate that opsonisation by antibodies and by complement components is required for protective immunity against *S. pneumoniae* [30,31]. However, it has also been shown that neutrophils can kill pneumococci in the absence of opsonins. This killing is mediated by phagocyte receptors that recognize certain proteins on the pathogen surface [32,33].

The Fc-region of antibodies bound to the bacteria is recognized by Fc receptors on phagocytes which facilitates killing [34,35]. Five different antibody isotypes exist namely IgM, IgG, IgA, IgE and IgD. Antibodies of isotype IgG are most abundant in blood, followed by IgA and IgM. Each class plays a different role in the immune defence. IgA is the predominant class in extravascular secretions, whereas IgG can cross the placenta and provide protection to the fetus. IgM is a pentamer or hexamer that is released by plasma cells in the early immune response. Antibodies that are produced without prior immune activation are commonly referred to as natural antibodies. These types of antibodies may be present without antigenic exposure. In contrast to this, immune antibodies arise after specific immune exposure or vaccination [36].

Another important opsonin is the deposition of complement C3 on the bacterial surface [37]. Complement activation coats a pathogen surface with complement fragments, such as C3b, and its inactive derivate iC3b (**Figure 1**). These opsonins are recognized by complement receptors (CRs) on phagocytes and thereby

promote uptake and removal (**Figure 1**). Various complement receptors, including CR1 and CR3, are known to be present on macrophages, monocytes and neutrophils [38]. In addition, complement C5a binding to the C5aR on macrophages/monocytes and neutrophils activates the cell for phagocytosis of opsonised pathogens (**Figure 1**) [39]. Activation of the complement system by one of the three complement activation routes is mediated in various ways. The binding of antibodies to the bacterial surface plays an important role in initiation of the classical pathway. C1q recognizing the Fc region of bound IgG and IgM antibodies results in classical pathway activation and leads to C3b deposition on the bacterial surface. Due to its polymeric structure, IgM is particularly effective in classical complement pathway activation [40].

It is important to realize that the complement system is not functioning alone, as various immune responses interact to induce and regulate inflammation and phagocytosis. This is illustrated in **Figure 1**, which shows how complement activation contributes to phagocytosis and to the induction of inflammatory responses.

Complement system and its role in diseases

Evolution and discovery of complement

The complement system is an evolutionary ancient immune response. A primitive version composed of the central protein C3 and two activation proteases is proposed to be established in the common ancestor of Cnidaria (e.g. sea anemones) more than 1 billion years ago [41]. It is suggested that a version more similar to the human complement system has developed by the time of the teleost/mammalian divergence around 500 million years ago [41]. The human complement system involves many plasma and membrane bound proteins. Part of its function was first discovered in the late 19th century. Jules Bordet was awarded the 1919 Nobel Prize in Physiology or Medicine, for his discovery that bacterial killing of Gram-negative *Vibrio cholera* required heat labile components of the serum in addition to the heat stable antibodies. The heat stable antibodies against the bacteria developed following previous exposure to the pathogen, which was named "immunization" [42,43]. In contrast, heat labile components were already present before immunization and named "complement". Subsequently, many complement proteins were detected and named in the order in which they were discovered. The antibody dependent classical pathway was identified first followed by the alternative pathway [44]. The lectin pathway was discovered more recently, approximately two decades ago [45,46]. Nowadays, the critical role of the complement system is increasingly acknowledged not only in infections but also in many other inflammatory diseases and clearance of the body's cellular debris.

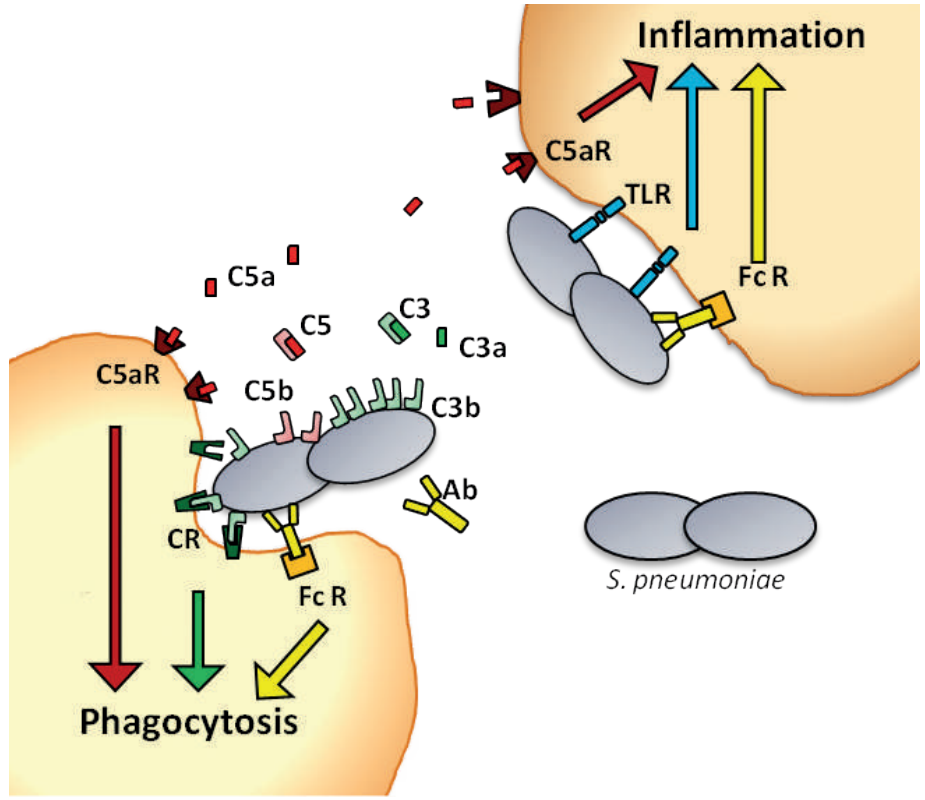


Figure 1. This figure schematically illustrates the function of complement in phagocytosis and inflammation. Antibodies (Ab) that recognize antigens on the bacteria bind to the bacterial surface, which contributes to complement activation resulting in cleavage of C3 into the opsonin C3b. In addition, complement activation products are released, C3a and C5a, of which C5a binding to C5aR is especially potent in stimulating phagocytosis and inflammation. Fc-receptors (Fc R) on phagocytes, such as monocytes, macrophages and neutrophils recognize antibodies bound to the bacteria. In addition, complement receptors (CR) recognize C3b deposited on the bacterial surface. Binding of these receptors stimulate engulfment and clearance of the pathogen by phagocytosis. In addition, complement activation modulates the inflammatory response. Crosstalk between complement C5a binding to the C5aR, Toll-like receptor (TLR) binding and Fc receptors recognizing antibodies bound to the pathogen, modulate the host inflammatory cytokine response.

Complement activation upon pneumococcal infection

The three main pathways for complement activation are the classical, lectin and alternative pathway. IgG and IgM binding to the bacterium enable C1q binding to the Fc-region of the antibodies. This results in a cascade of reactions classified as the classical pathway. Another important classical pathway activator is the acute

phase protein CRP, which binds phosphocholine residues on the pneumococcal surface [47]. Other danger signals such as bacterial DNA, RNA, lipopolysaccharides, and certain small polysaccharides are also able to induce classical pathway activation [48]. The initiation of the classical pathway by binding of C1q generates an enzyme capable of cleaving C4 and C2 which leads to the formation of a C3 convertase (C4bC2a) (**Figure 2**). The lectin pathway is activated by mannose-binding lectin (MBL) binding to mannose-containing surface proteins on the pathogen. In addition, binding of ficolins can initiate the lectin pathway [49]. Similar to the classical pathway, lectin pathway activation results in cleavage of C2 and C4 which results in the formation of a C3 convertase (C4bC2a) (**Figure 2**).

C3 convertase cleaves the central complement protein C3 into C3a and C3b (**Figure 2**). C3a is an anaphylatoxin and diffuses, whereas C3b binds to the bacterial surface aiding phagocytosis [50-52]. High concentrations of locally deposited C3b lead to the formation of C5-convertases, which cleaves C5 into C5a and C5b. The highly proinflammatory peptide C5a diffuses whereas C5b reacts with C6-C9 resulting in formation of the membrane attack complex (MAC) by the incorporation of C5b-9 in the membrane [53]. The MAC is functionally involved in killing of Gram-negative bacteria by lysis, whereas in Gram-positive bacteria, such as *S. pneumoniae*, the MAC can be detected on the bacterial surface but seems not to affect the viability [54]. This distinction between Gram-negative and Gram-positive is due to difference in cell surface composition. Gram-negative bacteria consist of an inner and an outer membrane separated by a periplasmic space and a thin peptidoglycan layer, whereas the cell wall of Gram-positive bacteria only has one membrane surrounded by a thick peptidoglycan layer [55]. Thus, the membrane attack complex required for bacterial cell lysis cannot reach the cell membrane of Gram-positive bacteria, making these microbes resistant to lysis.

The alternative pathway and its amplification loop

In contrast to the classical and lectin pathway, the alternative pathway is continuously activated at low levels. Spontaneous hydrolysis of the internal thioester bond of C3 generates a C3b-like molecule, C3(H_2O). The alternative pathway can also amplify complement activation initiated by the classical, lectin and alternative pathway (**Figure 3**). Subsequent binding of factor B to C3(H_2O) and cleavage by factor D makes a short lived soluble C3 convertase (C3(H_2O) Bb complex) that can cleave C3 to C3b. Moreover, surface bound or fluid phase C3b interacts with factor B in a Mg^{2+}-dependent manner and cleavage by factor D results in an alternative pathway C3 convertase (C3bBb) that can cleave more C3. This alternative pathway amplification loop multiplies the initial C3b deposition by all three pathways by

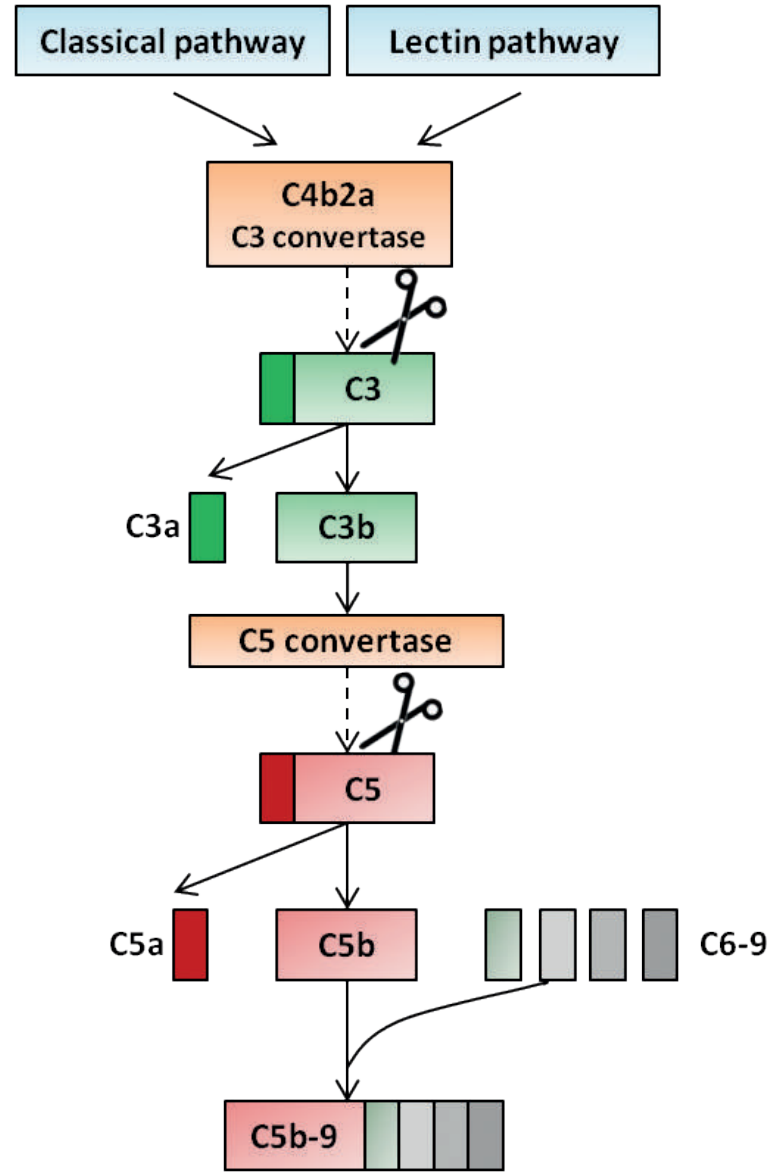


Figure 2. Simplified scheme of complement activation by the classical and lectin pathway. Activation leads to a cascade of reactions leading to the formation of C4b2a, which is a C3 convertase. Cleavage of C3 by the convertase results in the release of C3a and the C3b, which serves as an opsonin when bound to a nearby surface. Cell surface bound C3b can be formed into a C5 convertase, which cleaves C5 into C5a and C5b. C5a together with C3a are anaphylatoxins, whereas C5b reacts with C6-9 to form the membrane attack complex.

generating alternative pathway C3 convertase from any C3b generated (**Figure 3**). The alternative pathway convertase is very labile and spontaneously dissociates with a half life of about 90 s at 37 °C. Binding of the positive alternative pathway regulator properdin stabilises the enzyme and extends its half-life more than 10-fold [17,44]. Complement factor H is a key negative regulator of alternative pathway activation, both in the plasma as well as on the cell surfaces. Whereas, properdin stabilises the alternative pathway C3 convertase, factor H accelerates its decay. In addition, factor H acts as a co-factor for factor I mediated inactivation of C3b in to iC3b [56-59]. iC3b cannot form an active convertase and thereby inhibits the alternative pathway amplification loop. Factor H is an abundant plasma protein containing binding sites for glycosaminoglycans (GAGs) to protect self tissues.

The importance of the alternative pathway has long been underestimated. However, this pathway is now acknowledged as a critical pathway since the amplification loop may contribute up to 80% of the total complement activation [60-62]. Others demonstrated the importance of both the classical and lectin pathway in *S. pneumoniae* infections using animal models [63,64]. It appears that both the classical pathway and the lectin pathway are important in initiating complement activation, whereas the alternative pathway determines the final amount of C3 deposition on the bacterial surface [63,65].

Alternative pathway activity and disease

Complement regulators on cells and in plasma control activation of the alternative pathway. However, an imbalance of alternative pathway activation has been associated with diseases. Combinations of common polymorphisms in genes encoding alternative pathway proteins are known to affect the alternative pathway activity [17,66]. These genetic polymorphisms, also referred to as 'complotypes', are found in components of the alternative pathway amplification loop, C3, factor B and factor D, or in the control proteins such as factor H and factor I [17]. It has been proposed that alternative pathway enhancing polymorphisms lead to a predisposition in chronic inflammatory diseases, such as haemolytic uremic syndrome, age-related macular degeneration, and dense deposit disease [17]. In addition, reduced alternative pathway activity may increase an individual's susceptibility for infections [17].

Variation in complement factor H seems to play an important role in an individual's alternative pathway activity. The crucial function of factor H to regulate the alternative pathway activity is apparent in rare cases of complete factor H deficiency in humans in which uncontrolled alternative pathway activation

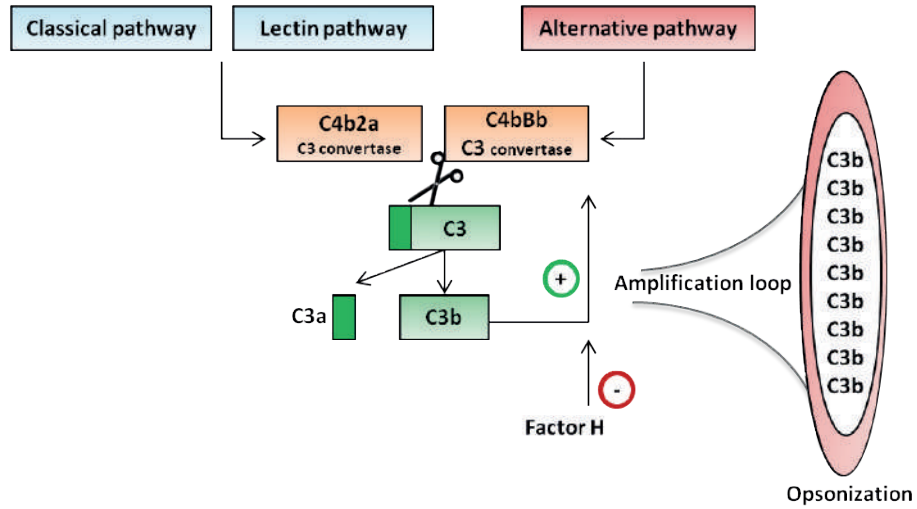


Figure 3. Simplified scheme of the alternative pathway amplification loop. In contrast to the classical and lectin pathway, the alternative pathway is continuously activated at low levels. The alternative pathway can amplify complement activation initiated by the classical, lectin and alternative pathway, known as the alternative pathway amplification loop. Factor H is a key inhibitor of the alternative pathway and the alternative pathway amplification loop.

results in a secondary C3 deficiency [67]. These individuals are susceptible to meningococcal infections, C3 glomerulopathy and haemolytic uraemic syndrome (HUS) [67]. Additionally, genetic variation in factor H has been associated with diseases such as age-related macular degeneration, atypical haemolytic uremic syndrome (aHUS), C3 glomerulopathy, including dense deposit disease and C3 glomerulonephritis [66,68-70]. Polymorphisms may affect factor H binding to host cells, regulation of alternative pathway activity, or factor H expression levels [17,71,72].

Interestingly, a genome wide associations study (GWAS) for meningococcal disease identified risk polymorphisms in the complement factor H gene (*CFH*) and complement factor H related protein 3 (*CFHR3*) [15]. Others demonstrated that a single-nucleotide polymorphism in the promoter region of *CFH* increases serum factor H levels, resulting in reduced bactericidal activity against *N. meningitidis* [16]. This suggests that polymorphisms in *CFH* may affect factor H expression and an individual's susceptibility for disease. Within the human population, factor H plasma levels show a large variation (range, 63.5-847.6 µg/mL) [16,73-76]. This variation is due to both environmental factors (e.g. smoking) and genetic factors [74].

Pneumococcal complement evasion

Evolutionary models propose that *S. pneumoniae* evolved from a pneumococcus-like bacteria presumably pathogenic to the common immediate ancestor of hominoids [77,78]. This suggests a long history of pneumococcal adaptation to the human host. *S. pneumoniae* is mainly isolated from humans and not from other mammals. However, *S. pneumoniae* infection has been diagnosed in wild chimpanzees in Taï National Park, Côte d'Ivoire [79]. The ability to regulate natural competence for genetic transformation and uptake of DNA from other pneumococci or other pathogens provides *S. pneumoniae* into a successful human pathogen with mechanisms to evade human immune defences including the complement system.

The pneumococcal capsular serotype

The polysaccharide capsule is a major pneumococcal virulence factor and is an important antiphagocytic component. The more than 90 capsular serotypes all contain structurally unique polysaccharide capsules with varying degrees of resistance to phagocytosis [33,80,81]. Studies comparing isolates from patients with invasive disease and carriage isolates from the same region and time period suggest that a strain's invasive disease potential largely depends on the capsular serotype [82-84]. Serotypes, such as 1, 4 and 7F, are more often found in invasive disease whereas other serotypes are more frequently found in carriage [84]. Infections by serotypes with a low invasive disease potential were often found in patients with underlying diseases and are associated with high mortality rates [8,84]. These serotypes are referred to as more opportunistic, whereas serotypes found in previously healthy individuals are indicated as primary pathogens [8]. Importantly, both pneumococcal virulence and the host immune response determine the risk and outcome of infection.

The pneumococcal capsule affects resistance to phagocytosis by various mechanisms [85]. Absence of a capsule increases surface C3 deposition compared to its encapsulated wild-type strain [85]. In addition, increased binding of classical pathway mediators such as IgG and C-reactive protein (CRP) to non-capsulated strains has been observed [85]. The capsule masks subcapsular antigens and thus reduces antibody binding and CRP binding. In accordance, non-capsulated mutants show reduced virulence and enhanced clearance from the circulation in animal models [86]. Moreover, gene expression for capsular synthesis is a dynamic and complex process [87]. Within a serotype, spontaneous opaque/transparent phase variation affects polysaccharide capsule thickness and pneumococcal resistance to complement [88,89]. Opaque phase pneumococci with increased amounts of capsular polysaccharide show enhanced complement resistance, which has been associated with better survival in blood [90-92]. Whereas transparent phase pneumococci are more often carriage isolates [89]. Similar to non-capsulated strains, transparent phase pneumococci show increased antibody binding to subcapsular antigens and increased binding of CRP [88]. Even in the absence of opsonins, the capsular serotype affects the interaction with neutrophils [33].

Pneumococcal proteins involved in complement evasion

In addition to the important role of the pneumococcal capsule, pneumococcal genotype affects complement resistance and its invasive disease potential [84,93]. Significant differences in complement C3 deposition between isolates within the same serotype have been observed, indicating that the strain genetic background affects complement resistance [94]. It is therefore of interest to gain more insight into how genetic variation affects complement resistance and aids pneumococcal virulence.

Pneumococcal surface proteins interact with the complement system to reduce complement deposition. Various pneumococcal proteins play a role in complement evasion, including pneumolysin, pneumococcal histidine triad proteins (Pht), pneumococcal surface protein A (PspA) and pneumococcal surface protein C (PspC) [95-98]. Pneumolysin is a pneumococcal virulence protein released during infection. It has been suggested that pneumolysin activates the immune system by its interaction with C1q, which keeps the complement deposition away from the pneumococcal surface [98]. Pneumococcal surface protein A (PspA) contributes to pneumococcal virulence in mice and has been suggested to play a role in complement resistance, as PspA has been shown to reduce C3 deposition on the bacterial surface [96,97]. PspA may compete with the binding of C-reactive protein to cell-surface phosphocholine and thereby affect complement activation [97]. Pht proteins PhtA, PhtB, PhtD, and PhtE are a family of surface proteins that may play a role in complement evasion. Pht deletion resulted in increased C3 deposition on a serotype 4 strain but not on serotype 2, 3, and 19F strains [99]. The mechanism by which Pht proteins mediate complement resistance remains unclear.

Sequestering of host inhibitory complement regulators is an evasion mechanism often seen in invasive pathogens. *S. pneumoniae* binds complement alternative pathway inhibitor factor H through pneumococcal surface protein C (PspC, also referred to by CbpA, SpsA, PbcA and Hic) [100-104]. This is considered as a mechanism to evade host alternative pathway activation because factor H increases C3 cleavage into iC3b, circumventing the formation of a C3 convertase and thereby reducing

C3b deposition on the bacterial surface (**Figure 4**). However, the function of PspC binding factor H varies between different serotypes. In some serotypes, loss of PspC results in decreased complement deposition, whereas in other serotypes loss of PspC increases complement deposition or has no effect [105,106] [80]. These differences in function of PspC in complement evasion may be the result of capsular serotype differences or other genetic differences, for example antigenic variation in PspC [105]. Importantly, factor H binding to PspC is species specific as mice or rat factor H do not bind pneumococcal PspC [107].

Factor H binding by *S. pneumoniae* strains is not only dependent on the presence of PspC, but also influenced by capsular serotype. Capsule switch experiments, where different capsules are expressed by the same strain, demonstrated the ability to bind factor H and to evade opsonisation is largely capsule dependent [80]. Nevertheless, within one serotype large variation in factor H binding was observed between different clinical isolates suggesting that other factors besides capsule serotype affect factor H binding [80]. Interestingly, PspC itself is heterogeneous and has been classified into different PspC types based on sequence comparisons. However, it is not known whether variation in PspC type, independent of capsule differences, affects pneumococcal factor H binding and its ability to evade complement deposition.

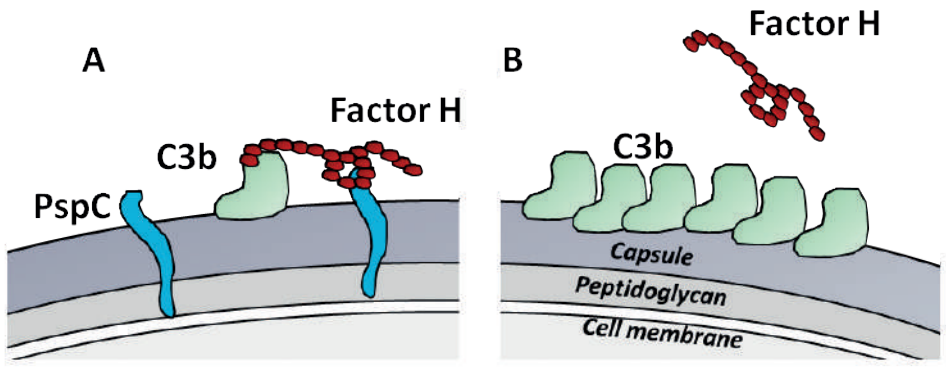


Figure 4. *S. pneumoniae* surface consisting of a cell membrane, peptidoglycan layer and a polysaccharide capsule. (**A**) PspC on the bacterial surface binds human factor H which inhibits alternative pathway mediated C3b deposition on the bacterial surface. This enhances pneumococcal complement resistance. (**B**) Whereas in the absence of PspC there is no binding of factor H which results in enhanced C3b deposition on the bacterial surface. Making the bacteria more sensitive for complement mediated phagocytosis.

Aim and outline

The overall aim of this thesis is to contribute to a better understanding of the role of complement factor H in *S. pneumoniae* infection.

More specifically, the aims are:

(i) To study the role of complement alternative pathway activity regulated by factor H on pneumococcal induced inflammation

(ii) To study whether serum factor H levels affect pneumococcal clearance from blood

(iii) To study whether differences in PspC types affect pneumococcal factor H binding and resistance to complement.

Invasive pneumococcal infections are often associated with high inflammatory responses, which contribute to the disease pathology. In **Chapter 2** we studied the role of the complement alternative pathway in pneumococcal induced proinflammatory cytokine responses by human PBMCs. Complement factor H is an important inhibitor of the alternative pathway activity. In **chapter 3** we examined whether exogenous factor H can attenuate inflammation and vascular leakage in experimental pneumococcal sepsis in mice.

Within the human population, a large variation in plasma factor H levels is observed. In **chapter 4** we show by using an experimental mouse model and a human whole blood killing assay the effects of serum complement factor H levels on resistance to pneumococcal invasive disease. In **chapter 5**, we describe the use of a whole blood killing assay that makes it possible to study bacterial clearance in human blood with an intact complement system.

In **chapter 6**, we focus on the ability of pneumococci to bind human factor H. *S. pneumoniae* binds human factor H by PspC, which is considered as a possible mechanism to evade complement deposition. PspC is a variable protein and has been classified into different PspC types based on sequence comparisons. We studied the effect of PspC type specific differences on pneumococcal factor H binding and complement evasion.

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Chapter 2

Alternative pathway regulation by factor H modulates *Streptococcus pneumoniae* induced proinflammatory cytokine responses by decreasing C5a receptor crosstalk

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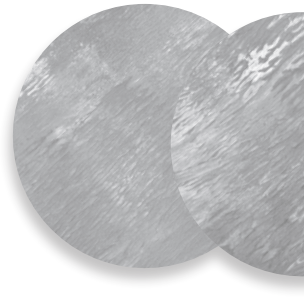

Abstract

Bacterial pathogens not only stimulate innate immune receptors, but also activate the complement system. Crosstalk between complement C5a receptor (C5aR) and other innate immune receptors is known to enhance the proinflammatory cytokine response. An important determinant of the magnitude of complement activation is the activity of the alternative pathway, which serves as an amplification mechanism for complement activation. Both alternative pathway activity as well as plasma levels of factor H, a key inhibitor of the alternative pathway, show large variation within the human population. Here, we studied the effect of factor H-mediated regulation of the alternative pathway on bacterial-induced proinflammatory cytokine responses. We used the human pathogen *Streptococcus pneumoniae* as a model stimulus to induce proinflammatory cytokine responses in human peripheral blood mononuclear cells. Serum containing active complement enhanced pneumococcal induced proinflammatory cytokine production through C5a release and C5aR crosstalk. We found that inhibition of the alternative pathway by factor H, with a concentration equivalent to a high physiological level, strongly reduced C5a levels and decreased proinflammatory cytokine production in human peripheral blood mononuclear cells. This suggests that variation in alternative pathway activity due to variation in factor H plasma levels affects individual cytokine responses during infection.

Introduction

The host innate immune system is activated immediately upon infection. Induction of inflammatory responses are essential for recruitment of immune cells and the control of adaptive immune responses. Pathogens, containing complex macromolecular surfaces, present multiple antigens to the host immune system that not only stimulate specific pattern-recognition receptors (PRRs), but also activate the complement system. Several studies indicate that there is crosstalk between the complement system, Toll-like receptors (TLRs) and Fcγ receptors, which modulates the proinflammatory cytokine responses [1-6]. It is known that complement activation product C5a is a potent inflammatory protein [7]. Addition of recombinant C5a to human peripheral blood mononuclear cells (PBMCs) stimulated with TLR ligands enhances cytokine production, whereas C5a alone has no effect [4]. In addition, it has been demonstrated that C5a binding to its receptor (C5aR) (also called CD88) modulates the inflammatory response induced by many bacterial pathogens, including *Escherichia coli*, *Staphylococcus aureus* and *Neisseria meningitides* [8-10]. Therefore it is important to study the mechanism by which complement activation contributes to the inflammatory response upon infection.

The complement cascade can be activated by three distinct pathways; (i) the classical pathway activated by C1q binding to antibody-antigen complexes; (ii) the lectin pathway activated by recognition of polysaccharide structures on pathogens; and (iii) the alternative pathway activated continuously at low levels by spontaneous hydrolysis of C3. In addition, the alternative pathway amplification loop plays a crucial role in the amplification of the initial activation of the classical and lectin pathway [11]. The alternative pathway may account for up to 80% of total complement activation, even if initially triggered by the classical pathway [12].

A key negative regulator of the alternative pathway is complement factor H, which is essential for inhibiting alternative pathway activation in the fluid phase and on cellular surfaces [13]. Factor H acts as a co-factor for factor I mediated inactivation of C3b and also accelerates the decay of the alternative pathway C3 convertase [14]. Polymorphisms in the gene encoding complement factor H (*CFH*) have been associated with human diseases such as hemolytic uremic syndrome, age related macular degeneration and dense deposit disease [13,15]. Polymorphisms may affect factor H binding to host cells, regulation of alternative pathway activity, or factor H expression levels [14-16]. Factor H plasma concentrations vary widely between individuals [17-21]. In the MRC Fenland population study of 1514 individuals, factor H serum levels ranged from 63.5 to 847.6 µg/mL (median 226.6 µg/mL) [20]. The

observed variation may be due to environmental factors (e.g. smoking) and genetic factors [13,19]. In addition, factor H serum levels may vary depending on an individual's disease state. Reduced factor H levels were observed during acute meningococcal disease compared to patients at convalescence. As noted above, the serum factor H concentration in the healthy control group in this study varied widely, ranging from 31 to 953 µg/mL (median 395 µg/mL) [17]. We have also demonstrated the importance of factor H levels and variation in alternative pathway activity on the host defense against *Streptococcus pneumoniae* [22].

In the current study, we aim to assess the role of factor H levels and alternative pathway activity on bacterial-induced proinflammatory cytokine production. We used the human pathogen *S. pneumoniae* as a model stimulus to induce proinflammatory cytokine responses by PBMCs. Our results clearly show that complement activation enhances the inflammatory response through C5a release and C5aR-mediated crosstalk. Moreover, alternative pathway inhibition by exogenous soluble phase factor H strongly reduces C5aR crosstalk and pathogen induced proinflammatory cytokine responses. Thus variation in alternative pathway activity due to variation in factor H plasma levels may affect an individual's cytokine responses during infection.

Material and Methods

Bacterial strains and growth conditions

Wild-type *S. pneumoniae* strain TIGR4 was used in all PBMC stimulation experiments [23]. In factor H binding assays only, a TIGR4 Δ*pspC* deletion mutant was used as *S. pneumoniae* has been described to bind human factor H by expressing pneumococcal surface protein C (PspC) [24]. The TIGR4 Δ*pspC* deletion mutant was constructed by allelic replacement of the target gene with an antibiotic resistance marker as described previously [25]. Briefly, overlap extension PCR was used to insert the spectinomycin resistance cassette of the pR412 plasmid between the two 500-bp flanking sequences adjacent of the target gene. The resulting PCR products were introduced by competence-stimulating peptide (CSP-2) induced transformation into TIGR4. Directed mutants were obtained by selective plating and were checked for correct integration of the antibiotic resistance cassette into the target gene by PCR using control primers located inside the gene. Subsequently, the TIGR4 wild-type strain was transformed with chromosomal DNA isolated from the mutants, to prevent the accumulation of inadvertent mutations elsewhere on the chromosome. The primer sequences are presented in table 1 of the supplementary data.

Bacteria were grown on Columbia blood agar plates (Becton Dickinson) and in Todd-Hewitt broth supplemented with 5 g/L yeast extract at 37°C and 5% CO_2 until an OD_{620} of 0.3 was reached. The number of colony forming units per milliliter was determined by plating serial 10-fold dilutions on blood agar plates. Subsequently, bacteria were heat killed at 65°C for 30 min and stored at -80°C. Heat killed pneumococci were used in order to avoid variation in bacterial numbers due to growth which could affect the host inflammatory response. Previous studies demonstrate that most TLR ligands remain functional after heat killing, although it has been shown that this can lower TLR9 dependent signaling [26].

Isolation of PBMCs and stimulation assays

After informed consent was obtained, a venous blood specimen was collected from the median cubital vein of healthy volunteers (age, 20-40 years; both males and females) into 10-mL EDTA tubes (BD). To isolate the PBMC fraction, blood was diluted in an equal volume of phosphate buffered saline (PBS), added onto 15 ml Lymphoprep (Axis Shield) and centrifuged at 800 × *g* for 20 min at room temperature. The PBMCs were harvested, washed three times in cold PBS and resuspended in culture medium (RPMI 1640 GlutaMAX-I medium, Invitrogen). Five hundred thousand cells in 100 µl were added to a round-bottom 96-well plate (Nunc) and incubated with 50 µl of stimuli and 50 µl of diluted serum resulting in a total volume of 200 µL/well. The stimuli were 10^5 heat-killed TIGR4 bacteria, or the TLR2 agonist Pam3Cys (Invivogen) (final concentration 1 µg/mL) or RPMI (negative control). The serum was diluted in RPMI to obtain a final concentration of 10% serum/well. Pooled normal human serum (NHS) (Sigma-Aldrich or GTI Diagnostics) or heat-inactivated serum (HI-NHS; 30 min at 56°C) or RPMI (negative control) was used. Specific PBMC stimulations using 10% NHS were supplemented with 0.1 or 1 µM C5a receptor antagonist, PMX53 (R&D Systems) or with 5, 25 or 50 µg/mL purified human factor H (Comp. Tech). The factor H concentration in the pooled NHS was 460 µg/mL, which in diluted serum gave a final factor H concentration of 46 µg/mL. Therefore adding 50 µg/mL of exogenous factor H doubled the amount of factor H already present in the serum. Each stimulation was prepared in duplicate. After 24 hours at 37°C and 5% CO2, the cells were pelleted by centrifugation at 650 × g at room temperature, after which the supernatants were pooled and stored at -20°C for further analysis.

Inflammatory response analysis

The concentrations of human interleukin-(IL)6, IL-1β, tumor necrosis factor (TNF-α) and IL-8 produced by the PBMCs were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Pelikine Compact, Sanquin) according to

manufacturers' instructions. Levels of complement activation product C5a were measured using a commercial human C5a ELISA kit (HK349, Hycult).

Factor H binding assay

TIGR4 heat killed or alive bacteria ($1x10^7$) were pelleted in a 96-well plate and resuspended in 10% (vol/vol) pooled normal human serum (Sigma-Aldrich) in Hanks Buffered Salt Solution (HBSS) to a total volume of 100 µL. The bacterial suspension was incubated for 30 min at 37°C in 5% CO_2. After incubation the bacteria were washed and labeled with polyclonal sheep anti-human factor H (Abcam). After a further 30 min incubation and washing, the bacteria were labeled with FITC-donkey anti-sheep IgG antibody (Jackson immunoresearch) followed by fixation in 2% paraformaldehyde. Factor H binding was measured using a FACScan flow cytometer (BD Biosciences). Data were analysed using FlowJo v10.1.

Statistics

Statistically significant differences were determined by the Wilcoxon or the Friedman test (nonparametric one-way ANOVA) followed by the Dunn's test to calculate multiplicity-adjusted P values. The data shown represents the mean ± standard error of the mean (SEM) of two or three independent experiments using PBMCs isolated from 5 or 6 different donors.

Results

Complement activation enhances pneumococcal induced PBMC cytokine production

PBMCs were stimulated for 24 hours with heat-killed *S. pneumoniae* or the TLR2 ligand Pam3Cys in the presence of normal human serum (NHS) or heat inactivated NHS (HI-NHS). HI-NHS is devoid of active complement since complement is a heat-labile component of human serum [27]. In the absence of active complement, using HI-NHS, PBMCs stimulated with *S. pneumoniae* produced proinflammatory cytokines IL-6, IL-1β, TNF-α and IL-8 (**Figure 1**). The presence of active complement in NHS significantly increased the release of proinflammatory cytokines upon stimulation with *S. pneumoniae* thus indicating that complement activation augments cytokine release by PBMCs (**Figure 1**). PBMCs stimulated with Pam3Cys induced IL-6, IL-1β, and IL-8 which was not affected by the absence or presence of active complement indicating that pneumococcal-induced complement activation was necessary for augmented cytokine release.

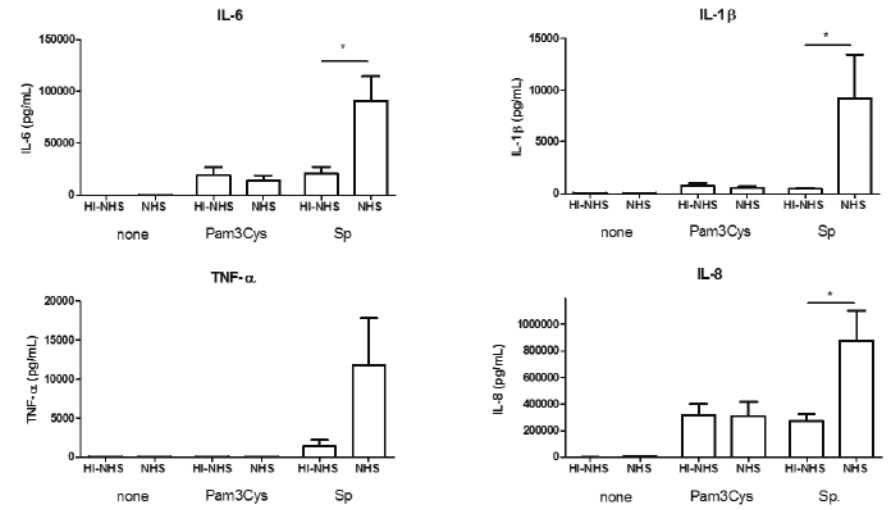

Figure 1. Complement enhances pneumococcal induced cytokine responses. Human PBMCs were stimulated with heat-killed *S. pneumoniae* TIGR4 (Sp), Pam3cys or culture media alone, in the presence of 10% pooled normal human serum (NHS) or heat-inactivated pooled normal human serum (HI-NHS) for 24 hours. Supernatants were collected and IL-6, IL-1β, TNF-α and IL-8 measured by ELISA. The data shown represent the mean and SEM of three independent experiments using PBMCs isolated from 5 donors. Statistically significant differences were determined by the paired Wilcoxon test. *$P < 0.05$

Pneumococcal induced PBMC cytokine production is enhanced through C5aR crosstalk

In order to study whether complement activation acts via the C5a-C5aR signaling pathway to enhance inflammatory cytokine release, PBMCs were stimulated with heat-killed *S. pneumoniae* in the presence of C5aR antagonist PMX53. Inhibition of C5aR in the presence of active complement led to decreased cytokine release in a dose-dependent manner to levels observed for HI-NHS. This demonstrates that the enhanced inflammatory response in the presence of active complement was largely C5aR mediated (**Figure 2**).

Alternative pathway inhibition by exogenous factor H strongly reduces C5aR crosstalk

In order to study the role of alternative pathway activation on the pneumococcal induced inflammatory response, we used the natural alternative pathway inhibitor factor H. PBMCs were stimulated with heat-killed *S. pneumoniae* in the presence of NHS with increasing concentrations of purified human factor H. As observed

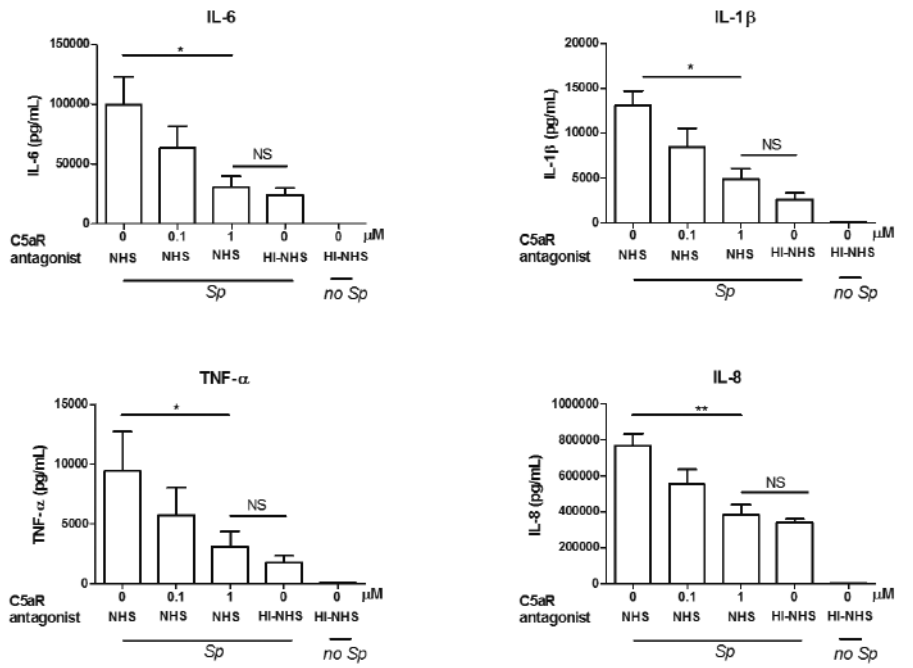

Figure 2. Complement mediated enhancement of cytokine induction is mediated by C5aR crosstalk. Human PBMCs were stimulated with heat-killed *S. pneumoniae* TIGR4 (Sp) or culture media alone, in the presence of 10% pooled normal human serum (NHS) or heat-inactivated pooled normal human serum (HI-NHS) for 24 hours. 0.1 or 1 μM of C5aR inhibitor (PMX53) was added to stimulations with 10% NHS. Supernatants were collected and IL-6, IL-1β, TNF-α and IL-8 measured by ELISA. The data shown represent the mean and SEM of three independent experiments using PBMCs isolated from 5 different donors. Statistically significant differences were determined with the Friedman test (non-parametric one-way ANOVA) followed by Dunn's test to calculate multiplicity-adjusted P values. **P* < 0.05, ***P* < 0.01. NS= not significant

previously, the presence of active complement in NHS significantly increased cytokine release by PBMCs compared to HI-NHS (**Figure 3**). Adding exogenous factor H decreased cytokine release in a dose-dependent manner, where addition of 50 μg/mL factor H, which doubles the absolute factor H serum concentration, reduced cytokine release to levels found with HI-NHS (**Figure 3**). In order to demonstrate the effect of factor H on complement activation, we measured C5a levels. Addition of exogenous factor H inhibited complement activity in a dose-dependent manner, as demonstrated by decreased C5a levels (**Figure 4**). Heat-killed *S. pneumoniae* TIGR4 used for the stimulations did not bind human factor H when incubated in serum, as opposed to live *S. pneumoniae* which bound factor H

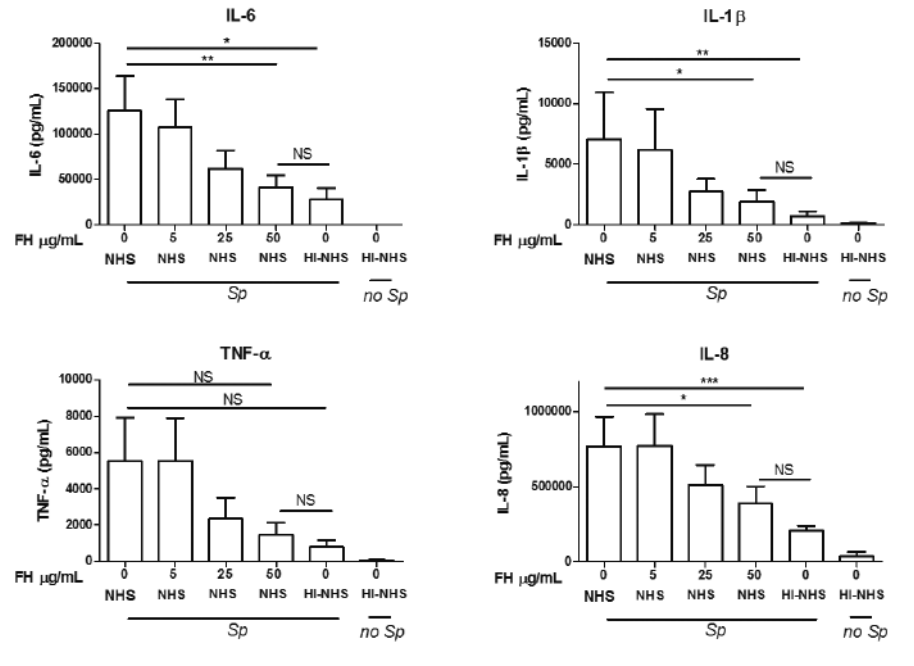

Figure 3. Alternative pathway inhibition by exogenous factor H strongly reduces pneumococcal cytokine responses. Human PBMCs were stimulated with heat-killed *S. pneumoniae* TIGR4 (Sp) or culture media alone, in the presence of 10% pooled normal human serum (NHS) or heat-inactivated pooled normal human serum (HI-NHS) for 24 hours. Increasing concentrations of purified human factor H (FH) were added to the PBMC stimulation in 10% NHS. Supernatants were collected and TNF-α, IL-1β, IL-6 and IL-8 measured by ELISA. The data shown represent the mean and SEM of three independent experiments using PBMCs isolated from 6 different donors. Statistically significant differences were determined with the Friedman test (non-parametric one-way ANOVA) followed by Dunn's test to calculate multiplicity-adjusted P values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, NS= not significant

via the pneumococcal surface protein C (PspC) (supplementary data). Use of heat-killed *S. pneumoniae* allowed us to demonstrate that only soluble phase factor H alternative pathway inhibition was sufficient to down-regulate pathogen induced proinflammatory cytokine release.

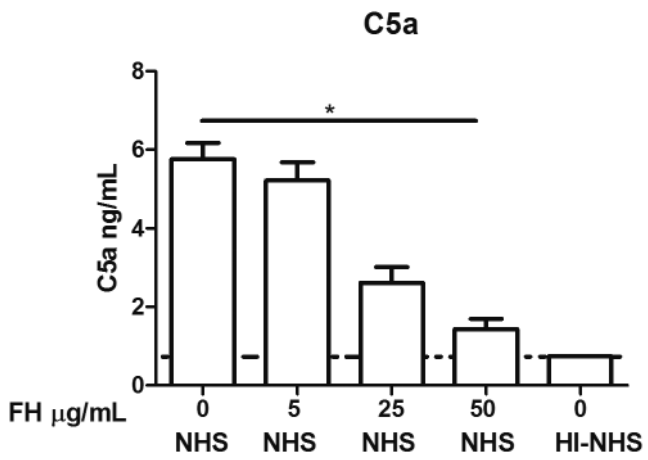

Figure 4. Exogenous factor H reduces C5a generation. Human PBMCs were stimulated with heat-killed *S. pneumoniae* TIGR4, in the presence of 10% pooled normal human serum (NHS) or heat-inactivated pooled normal human serum (HI-NHS) for 24 hours. Increasing concentration of purified human factor H (FH) were added to the PBMC stimulation in 10% NHS. Supernatants were collected and C5a measured by ELISA. The data shown represent the mean and SEM of three independent experiments using PBMCs isolated from 6 different donors. Dash line indicates lower limit of detection. Statistically significant differences were determined with the Friedman test (non-parametric one-way ANOVA) followed by Dunn's test to calculate multiplicity-adjusted P values. *$P < 0.05$

Discussion

This study demonstrates a critical role of fluid phase factor H on pneumococcal induced proinflammatory cytokine responses. Induction of proinflammatory cytokines IL-6, IL-1β, TNF-α and IL-8 were measured upon stimulation of human PBMCs with *S. pneumoniae* in the absence of active complement. This pathogen contains multiple antigens that are recognized by multiple innate immune receptors such as TLR2, TLR4, TLR9, NOD-like receptors (NLRs) and DNA sensors [26,28]. Several reports demonstrate that signaling by these innate immune receptors induces cytokine secretion [26,28,29]. We showed in our study that PBMC stimulation with the TLR2 ligand Pam3Cys induced IL-6, IL-1 β and IL-8 release to the same extent in the presence or absence of complement active serum.

Interaction of *S. pneumoniae* with human serum leads to complement activation by the classical, lectin and alternative pathway resulting in a cascade of reactions and the release of the complement activation product C3a and C5a [28,30]. Our results show the importance of complement activation and C5aR crosstalk on *S. pneumoniae* induced inflammatory cytokine responses in PBMCs. These results are in line with previous studies that demonstrated the effect of recombinant C5a on TLR ligand induced cytokine responses [4,5]. Other studies, where the inflammatory response was induced by *Neisseria meningitidis, Candida albicans* or *Staphylococcus aureus*, also demonstrate an important role for C5aR crosstalk [4,9,10].

We are the first to show that the presence of relative high factor H levels strongly reduce whole pathogen induced inflammatory responses from human PBMCs, as measured by proinflammatory cytokine production. As the heat-killed *S. pneumoniae* strain TIGR4 failed to bind human factor H to its surface (supplemental Figure), our assessment focused solely on soluble phase factor H alternative pathway regulation. Previous studies demonstrated that exogenous purified factor H acted on fluid phase complement activity as determined by haemolytic activity of serum [31].

In line with our findings, other studies have demonstrated that alternative pathway inhibition by anti-factor D reduced the oxidative burst of monocytes and granulocytes in a human whole blood model of meningococcal sepsis [10]. Furthermore, inhibition of the alternative pathway by anti-factor D in PBMCs stimulated with *C. albicans* reduced proinflammatory cytokine production [4]. Our study shows that factor H, in a dose-dependent manner, reduced C5a release and subsequent proinflammatory cytokine production, which indicated that alternative pathway-mediated C5aR crosstalk is essential for the release of cytokines by PBMCs.

Our results may help to understand inter-individual differences in inflammatory responses. Human factor H concentrations vary greatly between individuals (range, 63.5-847.6 μg/mL) and may affect an individual's alternative pathway activity [17-21]. Furthermore, combinations of polymorphisms in alternative pathway proteins have been described to influence alternative pathway activity [15]. Particular combinations of polymorphisms may result in a hyper-inflammatory state and predispose for chronic inflammatory diseases, such as haemolytic uremic syndrome, age-related macular degeneration and dense deposit disease [15]. In contrast, other combinations may result in a hypo-inflammatory state and may predispose to infections by ineffective immune activation [15]. An association

between factor H plasma levels, plasma C5a levels and inflammatory activity has been observed for clinical diseases such as anti-neutrophil cytoplasmic antibody associated vasculitis and age-related macular degeneration [16,32,33].

In conclusion, we have found that factor H, the alternative pathway inhibitor, modulates pneumococcal induced proinflammatory cytokine responses by inhibiting C5aR crosstalk. Variation in factor H levels within the physiological range of serum affected C5aR crosstalk in PBMCs stimulated with *S. pneumoniae*. This may explain the mechanism by which an individual's alternative pathway activity not only affects the susceptibility to chronic inflammatory diseases, but also the extent of the inflammatory response during infectious diseases.

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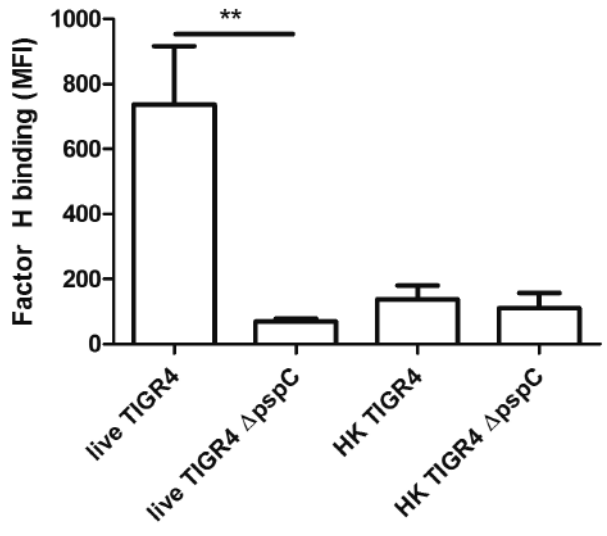
Supplementary data

Table 1. Primers used to construct the TIGR4 Δ*pspC* deletion mutant.

Primers for mutant generation:		
Spn2190_L1	Spn -2190 pspC	5′-TTG AGG CAA TGG TGC ACA AG-3′
EMspn2190_L2	Spn -2190 pspC	5′-CCACTAGTTCTAGAGCGGCTACACTAGCTACTCCAACAC-3′
EMspn2190_R1	Spn -2190 pspC	5′-CAAGATGAAGATCGCCTACG-3′
EMspn2190_R2a	Spn -2190 pspC	5′-GCGTCAATTCGAGGGGTATCGCTATGGAGTCAATGCCAAT-3′
EMspn2190_C	Spn -2190 pspC	5′-TCG TTC TCT GTC GCA TGA AC-3′
PBMrTn9	Spec^{res} cassette	5′-CAATGGTTCAGATACGACGAC-3′
PBpR412_L	Spec^{res} cassette	5′-GCCGCTCTAGAACTAGTGG-3′
PBpR412_R	Spec^{res} cassette	5′-GATACCCCTCGAATTGACGC-3′



Supplementary figure 1. Pneumococcal heat-killing abolishes the ability to bind factor H in human serum. A total of 1 × 10^8 colony-forming units/mL *S. pneumoniae* TIGR4 wild-type or Δ *pspC* alive or heat killed (HK) were incubated in 10% pooled human serum. After 30 minutes of incubation at 37°C, pneumococcal factor H was measured by flow cytometry. Intensity factor H binding on bacteria are indicated by mean fluorescence intensity (MFI). Each bar represents the mean ± standard error of the mean for results obtained from 3 separate experiments in duplicate. Comparisons between groups were performed using a one-way ANOVA followed by Dunn's test to calculate multiplicity-adjusted P values.**$P < .01$.

Chapter 3

Alternative Pathway Inhibition by Exogenous Factor H Fails to Attenuate Inflammation and Vascular Leakage in Experimental Pneumococcal Sepsis in Mice

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Abstract

Streptococcus pneumoniae is a common cause of sepsis. Effective complement activation is an important component of host defence against invading pathogens, whilst excessive complement activation has been associated with endothelial dysfunction and organ damage. The alternative pathway amplification loop is important for the enhancement of complement activation. Factor H is a key negative regulator of the alternative pathway amplification loop and contributes to tight control of complement activation. We assessed the effect of inhibition of the alternative pathway on sepsis associated inflammation and disease severity using human factor H treatment in a clinically relevant mice model of pneumococcal sepsis. Mice were infected intravenously with live *Streptococcus pneumoniae*. At the first clinical signs of infection, 17 hours post-infection, mice were treated with ceftriaxone antibiotic. At the same time purified human factor H or in controls PBS was administered. Treatment with human factor H did not attenuate disease scores, serum proinflammatory cytokines, or vascular permeability and did not significantly affect C3 and C3a production at 26 h post-infection. Therefore, we conclude that inhibition of the alternative complement pathway by exogenous human factor H fails to attenuate inflammation and vascular leakage at a clinically relevant intervention time point in pneumococcal sepsis in mice.

Introduction

The Gram-positive pathogen *Streptococcus pneumoniae* is a major human pathogen and one of the most common causes of pneumonia, meningitis and sepsis. Young children, the elderly and immune-compromised individuals are especially at risk to develop invasive pneumococcal disease [1,2]. Despite the availability of effective antibiotic agents, case fatality rates of pneumococcal sepsis are still high [3]. A major problem in sepsis is the ongoing inflammation and organ dysfunction following the antibiotic treatment [4].

The complement system is a major human defence and clearance system and is highly activated during sepsis [5]. Complement mediated C3 opsonisation and phagocytosis play a vital role in clearance of encapsulated Gram-positive pathogens, such as *S. pneumoniae*. Individuals with a deficiency in complement C3 activation or regulation thereof are more susceptible to invasive pneumococcal diseases [6]. Importantly, in the cascade of complement reactions, complement activation products also referred to as anaphylatoxins are released. These anaphylatoxins, C3a and in particular C5a, both promote inflammation via cross talk with Toll-like receptors [7]. Increased generation of complement activation products C3a and C5a has been associated with severity of sepsis [8-10]. Furthermore, increased complement activation has been found to enhance cytokine production, endothelial permeability and cardiac dysfunction [11-14].

The classical pathway of complement activation has long been recognized as the dominant complement activation route in host defence to *S. pneumoniae* [15]. The classical pathway is activated by C1q binding to antibody-antigen complexes on the bacterial surface. More recent the significance of the lectin pathway has been recognized [16]. This pathway is activated by binding of ficolin A or collectin 11 to the pneumococcal surface [16]. Importantly, the alternative pathway amplification loop plays a crucial role in the amplification of the initial activation of the classical and lectin pathway [17]. In the alternative pathway, spontaneous low-level hydrolysis of plasma C3 leads to deposition of C3b on the activating surface. Consequently, C3b formed by one of the three pathways can be amplified by the alternative pathway amplification loop, initiating a positive feedback loop resulting in enhanced complement activation. The quantitative contribution of alternative pathway amplification to classical pathway-induced C5a generation can be up to 80% [18]. This indicates a major contribution of the alternative pathway on the release of complement activation products [5].

The alternative pathway is closely regulated. Complement factor H is a key negative regulator of alternative pathway activation both in the plasma as well as on the cell surfaces. Factor H recognizes host cell surfaces and inhibits activity of the C3 convertase to avert injury of host tissues [19]. Factor H binds to C3b, accelerates the decay of the alternative pathway C3 convertase and acts as a co-factor for factor I mediated inactivation of C3b [19-22].

We hypothesize that after the onset of sepsis and initiation of antibiotic therapy, inhibition of alternative pathway activation is desirable to ameliorate sepsis associated inflammation and vascular leakage. Moreover, several other animal studies demonstrated the efficacy of adjuvant therapy inhibiting complement activation in animal models of sepsis [23-27]. The aim of our study was to investigate whether inhibition of the alternative pathway amplification loop by administering exogenous human factor H at a clinically relevant time point, at the first onset of clinical symptoms, combined with antibiotic treatment could attenuate inflammation and vascular leakage in a mouse model of pneumococcal sepsis.

Methods

Ethics Statement

This study was carried out in accordance with the recommendations of 'OECD Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation' (OECD Guidance Document 19, 2000). The protocol was approved by the Animal Ethics Committee of Radboud University, Nijmegen, The Netherlands (Permit Number: 2012-274).

Animals

Eight weeks old female C57BL/6 (wild-type) mice (n=36) obtained from Charles River Laboratory were used for the experiment. Mice were maintained in individually ventilated cages under a 12h light/12h dark cycle with controlled temperature (22 ± 2°C) and relative humidity (55 ± 5%). The mice had an average weight of 20.0 gram (± 1.0) before the start of the experiment.

Bacterial strains and growth conditions

A mouse-passaged *S. pneumoniae* strain TIGR4 (serotype 4) was used for infection [28]. The mouse-passaged TIGR4 strain was grown in Todd-Hewitt broth supplemented with 5 g/L yeast extract (THY) or on Columbia blood agar plates (Becton Dickinson) at 37°C and 5% CO_2 to an optical density at 620 nm of 0.2 and stored in aliquots

at -80°C in 15% glycerol. The number of colony forming units (CFU) per milliliter (CFU/mL) was determined by plating serial 10-fold dilutions on blood agar plates.

Experimental procedure

Mice were randomly divided into 4 groups; infected mice injected intraperitoneally (i.p.) with human factor H (n=10), or with i.p. phosphate buffered saline (PBS) as control treatment (n=10), uninfected mice were injected i.p. with human factor H (n=6) or i.p. PBS (n=10) respectively. The study was divided in four experiments to allow sufficient time to perform all the measurements at the end of the experiment. In each experiment 2 or 3 mice from every group were used.

Mice were infected intravenously in the tail vein with $1x10^7$ CFU in 100 µl PBS (control mice received PBS alone). Previous work showed that human factor H was able to restore complement C3 levels in homozygous factor H deficient mice demonstrating that human factor H is functional in mice [29]. Furthermore, it was demonstrated that human factor H inhibited cleavage of mouse C3 and mouse factor B in plasma [30]. Expression of human factor H completely protected homozygous factor H deficient mice from developing kidney abnormalities associated with the loss of factor H [30]. We choose to administer 600 µg purified human factor H intraperitoneally (i.p) (Complement technologies) (600 µl of the 1 µg/µL purified human factor H diluted in PBS) as i.p injection with a volume up to 600 µl was well tolerated. Earlier work documents, that a dose of 500 µg human factor H injected i.p. is sufficient to restore complement C3 levels in completely factor H deficient mice, demonstrating this dose is effective under extreme conditions [29]. Analogous to previous studies human factor H was injected i.p [29]. At the first onset of clinical signs (t=17 h) mice were injected i.p. with 600 µg human factor H in 600 µl PBS (control mice received 600 µl PBS alone). At the same time mice were injected with ceftriaxone 25 mg/kg intramuscular (Fresenius Kabi Nederland B.V.). Antibiotic treatment allowed to examine the effect of human factor H modulation of complement activity on the inflammatory response while avoiding differences in bacterial CFU counts due to potential inhibition of bacterial clearance by human factor H. At 26 h after infection the experiment was ended and CFU, vascular leakage in the liver, cytokine and complement protein levels were measured as described below. Pilot experiments demonstrated that mice in general did not reach the humane endpoint at this time point.

Measurement of disease score

During the experiment, mice were monitored for clinical signs and disease severity each hour and weighted at t=0, t=17, t=21 and t=26 hours. Mice were scored

according to their condition. The following score was used: *Ruffled coat:* showing signs of a ruffled coat (1); Dull ruffled coat, observed mildly around neck and back (2); Ruffled coat (3); *Hunched back:* Mildly hunched back (2); Hunched back (3); *Reduced mobility:* Less mobile but still being active and reacts to any handling (3); Aberrant and slower movement with back legs (4); Hardly walking, needs to be pushed to get going (5); *Weight loss:* >5 % body weight loss from t=0 (2); >10 % body weight loss from t=0 (3); >15 % body weight loss from t=0 (5); >20 % body weight loss from t=0 (15); *Skin temperature:* <35.5 °C (15); *Overall state:* no signs of disease (0); Moribund state *(15)*. Based on the disease score humane endpoints were defined. An overall disease score ≥ 15 was used as a surrogate marker of mortality and animals with a disease score ≥ 15 were killed to minimize animal pain, distress and discomfort.

Colony forming units count

At t=26 h, mice were anesthetized with 2.5% (vol/vol) isoflurane over oxygen and blood was collected by sub-mandibular bleeding. Blood was collected in Eppendorf safe lock tubes. Bacteria were recovered from the blood by plating serial dilutions on blood agar plates. Following overnight incubation of the plates at 37°C, CFU were counted.

Measurement of cytokines and complement proteins in mice

Blood samples were kept on ice and were centrifuged at 4°C 10.000 rpm after coagulation of the blood. Serum was collected, aliquoted and stored at -80°C for further analysis. To investigate the immune response in mice infected with *S. pneumoniae* TIGR4, the general inflammatory markers interleukin 6 (IL-6) and macrophage inflammatory protein 2 (MIP-2) were measured in post-infectious serum samples using ELISA assays (Mouse IL-6 ELISA, Ebioscience, 88-7064; Mouse CXCL2/MIP-2 Quantikine ELISA Kit, R&D Systems, MM200). In addition, the following complement proteins were measured with ELISA assays; mouse C3a (mC3a) (USCN life science, E90387Mu), mouse C3 (mC3) (Mybiosource, MBS564065) and human factor H (Hycult, HK342).

Measuring vascular permeability

Vascular leakage in the liver was measured by the method described by Von Drygalski *et al*., 2012 [31]. Infrared fluorescence measurements (IRF) were performed to determine Evans blue albumin extravasation to quantify vascular permeability. At t=26 h, mice were treated under anesthesia with 100 µL of 1% Evans blue dye (Sigma Aldrich) in PBS via retro orbital injection and mice remained under anesthesia. Prior to injection, the Evans blue solution was filter-sterilized (Millex, 0.22 µm; Millipore). At 15 min after the Evans blue injection the abdominal cavity and chest were opened by blunt dissection. The vena cava inferior was visualized and cut through. The liver was flushed via the portal vein with a total volume of 40 mL PBS containing heparin at 120mm Hg, thereby removing the Evans blue dye from the vasculature in the liver, followed by harvesting the liver. Mice remained under anesthesia during the whole procedure until the mice died by the perfusion and cervical dislocation. The liver was weighted and placed in a 6 wells tissue culture plate (Co-star). The liver was scanned using the Odyssey infrared imager (LI-COR, Lincoln, NE, USA) with Application software version 2.1.15, 700 channel, focal plane set at 3 mm and laser intensities set at L1.5. Area intensities from the bottom of each well were recorded as integrated fluorescence intensities (I.I) per well area. After scanning the organ was encircled and raw fluorescence intensities (RFI) were recorded and multiplied by the wet organ weight to estimate the concentration of Evans blue in the organ [31].

Statistical analysis

Data of the mice experiments are expressed as median and interquartile range. Difference between mice groups were analyzed using the Mann-Whitney test with a Bonferroni correction in case of multiple comparisons. Differences were considered statistically significant when $p < 0.05$.

Results

Exogenous human factor H fails to attenuate disease scores, inflammatory cytokine production, and vascular leakage in the liver.

At 17 hours post-infection, before the treatment with antibiotics, the pneumococcal load in the control group and the human factor H treatment group were similar (median and interquartile range) control group $5.7x10^5$ ($1.6 x10^5$ - $1.8x10^6$) CFU/mL vs. treatment group $7.1x10^5$ ($2.7x10^5$ - $4.5x10^6$) CFU/mL, *p*=0.6, Mann-Whitney test. In both groups no viable bacteria were detectable 9 h after antibiotic treatment at termination of the experiment (t=26 h). The first onset of clinical symptoms of disease occurred at around 17 h post inoculation. The clinical disease score continued to increase rapidly within the first four hours after initiation of antibiotic treatment (t=21 h) and slowly continued to increase between four and nine hours after initiation of antibiotic treatment (t=26 h) (**Figure 1A**). One infected mice of the PBS treated group reached the humane endpoint at t=22 h. At 26 h after infection clinical disease scores, serum cytokines (IL-6 and MIP-2), and vascular permeability in the liver were all significantly elevated in infected untreated animals compared

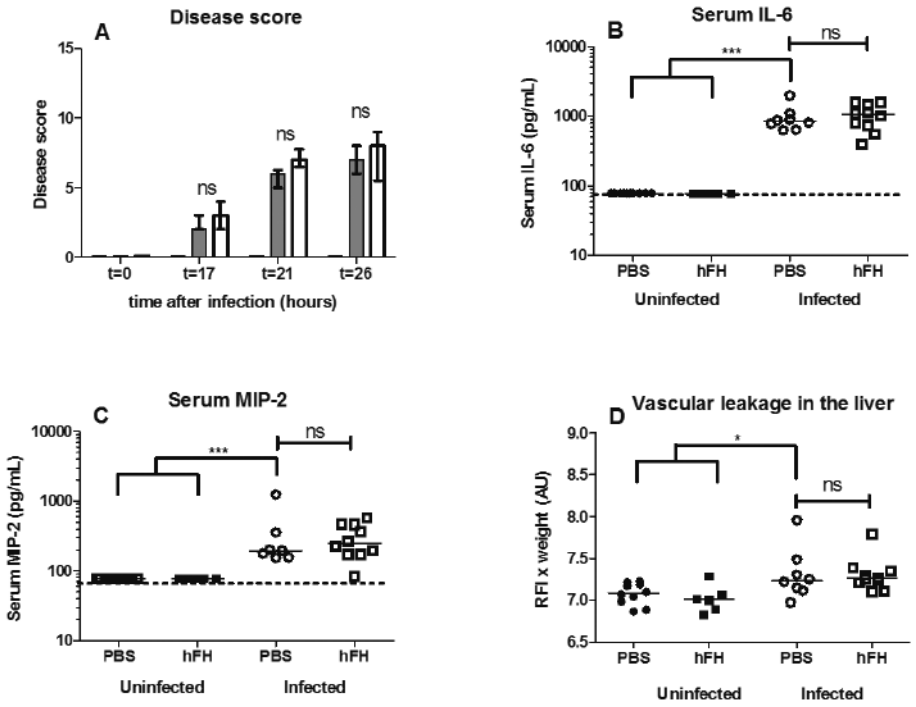

Figure 1. Exogenous human factor H fails to attenuate disease scores, inflammatory cytokine production, and vascular leakage in the liver. Mice infected with 1x10^7 CFU of *S. pneumoniae* (TIGR4) and sham infected control mice were all treated with antibiotics at t=17 h and indicated groups received an injection with human factor H (hFH) or PBS as control (n=10). The disease score was monitored at t=17, t=21 and t=26 hours after inoculation (**A**). The black bar represents uninfected mice, gray bar infected control mice and white bar human factor H treated mice. Data points represent the median value with interquartile range. At t=26 h, serum proinflammatory cytokines IL-6 and MIP-2 were measured by ELISA (**B, C**). Liver vascular leakage was measured by Evans Blue-albumin extravasation to quantify vascular permeability (**D**). Raw fluorescence intensities (RFI) were recorded and multiplied by the wet organ weight to estimate the concentration of Evans Blue in the organ. Each point depicted in graphs B,C and D indicates one mouse. One infected mice of the PBS treated group reached the humane endpoint at t=22 h and was excluded from the graphs. Furthermore one (IL-6 Figure 1B) respectively two data points (MIP-2 Figure 1C) are missing, as insufficient serum was available. In addition, one data point is missing in the vascular leakage graph, because of a technical failure during injection of Evans Blue in one mouse. Cytokine values were analyzed after logarithmic transformation; the horizontal line represents the median. Dash line indicates lower limit of detection. Comparison between groups were performed by using the non-parametric Mann-Whitney test with Bonferroni correction * $p < 0.05$ was considered significant. ** $p< 0.01$, *** $p<0.001$, ns = not significant.

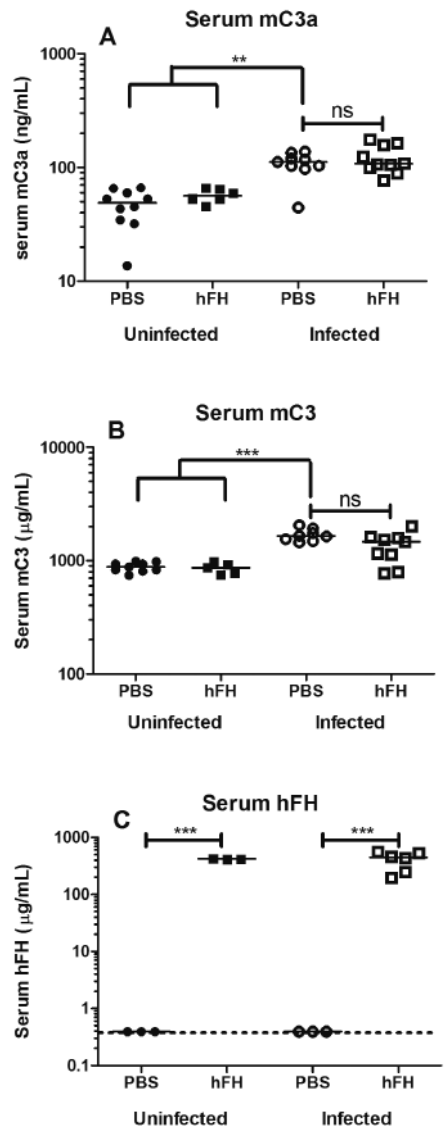

Figure 2. Exogenous human factor H administered at onset of clinical symptoms has no effect on complement activation protein levels. Mice infected with 1x10^7 CFU of *S. pneumoniae* (TIGR4) and sham infected control mice, were all treated with antibiotics at t=17 h and indicated groups received an injection with human factor H or PBS as control (n=10). At t=26 h serum mC3a, mC3 and human factor H levels were measured by ELISA. Each point depicted indicates one mouse. One infected mice of the PBS treated group reached the humane endpoint at t=22 h and was excluded from the graphs. Two data points of mC3 are missing, as insufficient serum was available to do all the measurements in all mice. Data are individual complement factor values and were analyzed after logarithmic transformation; the horizontal line represents the median. Dash line indicates lower limit of detection. Comparison between groups were performed by using the non-parametric Mann-Whitney test with Bonferroni correction * $p < 0.05$ was considered significant. ** $p< 0.01$, *** $p<0.001$, ns = not significant

with uninfected mice (**Figure 1B-D**). Treatment with human factor H did not attenuate clinical disease scores, serum proinflammatory cytokines IL-6 and MIP-2, or vascular permeability in the liver in infected mice (**Figure 1B-D**). Exogenous human factor H in uninfected control mice had no effect on disease score, serum proinflammatory cytokines IL-6 and MIP-2, or vascular permeability in the liver (**Figure 1B-D**).

Exogenous human factor H administered at onset of clinical symptoms has no effect on complement activation protein levels

In order to study the effect of exogenous human factor H on complement activation, levels of complement proteins were measured. Mouse serum C3a and C3 levels were significantly elevated in the infected mice (**Figure 2 A,B**). Exogenous human factor H had no effect on mouse C3a and C3 levels. The estimated concentration of human factor H present in mice directly after injection is about 400 µg/mL, since a dose of 600 µg intraperitoneal was given to mice with an estimated blood volume of 1,5 mL. The presence of target concentration human factor H in treated mice at the end of the experiment was confirmed by ELISA showing high human factor H levels (median and interquartile range) 445 (235-534) µg/mL serum at 9 hours after injection (**Figure 2C**).

Discussion

It is well established that excessive complement activation contributes to an enhanced inflammatory response and tissue damage in sepsis [12-14,32]. The amplification mechanism of the alternative pathway is believed to play a major role in the release of complement activation products contributing to the overwhelming inflammatory response in sepsis [18]. The main finding in this study is that inhibition of the alternative complement pathway by exogenous human factor H at a clinical relevant time point at the first onset of symptoms failed to attenuate inflammation and vascular leakage in a pneumococcal sepsis model in mice.

Complement activation is important in the clearance of endogenous and pathogen derived debris and toxins. Effective clearance of these substances may prevent ongoing induction of inflammation by this debris. In our experimental setting we did not observe increased proinflammatory activity resulting from less effective clearance of pathogen debris in mice in which alternative pathway activity was inhibited with factor H, however the duration of observation may have been too short to completely exclude this.

In previous studies, it was shown that human factor H regulates mouse alternative pathway activity [29,30]. Potentially, the effect of human factor H treatment in our study was limited by the timing of administration. Human factor H was administered at time of clinical symptoms, 17 h after infection, when physiological dysregulation already had occurred. Administration of human factor H might have greater potential when given earlier during the course of disease. Attenuation of inflammation and vascular leakage targeting alternative pathway activation by exogenous human factor H treatment may be more successful in situations where treatment can be administered prophylactic as in cardiopulmonary bypass surgery [33]. However, early human factor H treatment at time of infection is clinically not relevant for invasive pneumococcal infection because patients with sepsis will only present with apparent clinical signs and symptoms. Furthermore, others described previously that an abrogated alternative pathway activity in mice genetically lacking alternative pathway activity enhanced pneumococcal outgrowth and the severity of disease [15,34]. These studies demonstrate the importance of alternative pathway activation in the host defence against pneumococcal invasive infection and early human factor H administration may enhance pneumococcal outgrowth. Enhancement of pneumococcal outgrowth by human factor H is especially relevant as the pneumococcal virulence factor pneumococcal surface protein C (pspC) binds human factor H, as an immune evasion strategy [35]. This pneumococcal binding capacity is species specific and unique for human factor H, and pneumococci do not bind mouse factor H [36].

Other studies targeting complement activation by inhibition early in the complement cascade with C1 esterase inhibitor (C1INH) or C3 convertase inhibitor were successful in contrast to our study [25,26]. C1INH treatment was beneficial on outcome of Gram-negative bacterial sepsis and endotoxin shock in several animal studies [26,37]. Interestingly, a recent small open label clinical trial described increased survival rates in patients with surgical sepsis treated with C1INH, indicating that intervention might be beneficial in a clinical setting [38]. C1INH has many different anti-inflammatory functions, including non-complement related functions [39]. The beneficial effect of C1INH is therefore not exclusively due to complement inhibition [26].

In addition, early complement inhibition by using compstatin, a C3 convertase inhibitor, was shown protective during *E. coli* sepsis in baboons [25]. Interestingly, it was found that complement inhibition was still effective when administered

during the second stage of progressive organ failure [25]. Since compstatin blocks the C3 convertase it inhibits all three complement activation pathways and may therefore be more effective in comparison with alternative pathway inhibition alone as in the current study.

Our study targeted complement activation, aiming to decrease the activation of complement effector molecules. Potentially it may be more effective to directly block the complement effector molecules such as C3a or C5a. Recently, the potential efficacy of blocking C5a or its receptors in improving outcome in experimental sepsis models was demonstrated. Blocking C5a or its receptors preserved neutrophil function resulting in lower bacterial loads and less severe disease [23,24,40]. In cecal ligation and puncture-induced sepsis in rats, it was shown that blocking C5a was even beneficial after the onset of symptoms of sepsis [27].

Complement activity has not only detrimental effects, but is also essential for an effective host defence against invading pathogens such as *S. pneumoniae* [14,41]. Interestingly several novel experimental therapies for sepsis entail stimulating complement activation. Administration of recombinant properdin, a positive regulator of alternative pathway activation, resulted in enhanced protection against *S. pneumoniae* infection [42]. The use of properdin may be important in the context of antimicrobial resistance in sepsis, however, thus far it has not been assessed whether properdin treatment has an additive effect in combination with antibiotic treatment in sepsis. Additionally, the recombinant properdin used in that study contained a histidine tag [42]. Recently several studies raised concerns about the use of proteins containing a histidine tag, since this may convey antimicrobial activity [43,44].

To our knowledge this is the first study to assess human factor H treatment as adjuvant therapy in sepsis. Our results show that inhibition of the alternative complement pathway by exogenous human factor H at a clinical relevant time point at the first onset of symptoms failed to attenuate inflammation and vascular leakage in a pneumococcal sepsis model in mice.

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Chapter 4

Complement factor H serum levels determine resistance to pneumococcal invasive disease

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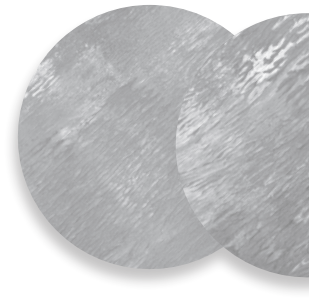

Abstract

Streptococcus pneumoniae is a major cause of life-threatening infections. Complement activation plays a vital role in opsonophagocytic killing of pneumococci in blood. Initial complement activation via the classical and lectin pathways is amplified through the alternative pathway amplification loop. Alternative pathway activity is inhibited by complement factor H. Our study demonstrates the functional consequences of the variability in human serum factor H levels on host defense. Using an *in vivo* mouse model combined with human *in vitro* assays, we show that the level of serum factor H correlates with the efficacy of opsonophagocytic killing of pneumococci. In summary, we found that factor H levels determine a delicate balance of alternative pathway activity, thus affecting the resistance to invasive pneumococcal disease. Our results suggest that variation in factor H expression levels, naturally occurring in the human population, plays a thus far unrecognized role in the resistance to invasive pneumococcal disease.

Introduction

The Gram-positive pathogen *Streptococcus pneumoniae* is one of the major human bacterial pathogens. It normally colonizes the nasopharynx, but can cause life-threatening infections such as pneumonia, meningitis and sepsis [1]. Within the population there is a large variation in the susceptibility for invasive pneumococcal disease indicating that the host immune defence against invading pathogens differs between individuals [2]. The complement system, part of the innate immunity, is of major importance in host defence against *S. pneumoniae*. Complement opsonization plays a vital role in opsonophagocytic killing of gram-positive bacteria such as *S. pneumoniae*.

The production of the major opsonin, C3b, can occur through the classical, lectin and alternative pathway. Surface C3b can be rapidly amplified through the alternative amplification loop irrespective of the pathway through which it was generated [3]. The quantitative contribution of the alternative pathway amplification loop to classical pathway-induced complement activation can be up to 80% [4].

Because alternative pathway activity has major implications for complement activation, it is tightly regulated. Complement factor H is an abundant plasma protein essential for inhibition of alternative pathway activation in the fluid phase and on cellular surfaces [5]. Factor H binds to C3b, accelerates the degradation of alternative pathway C3 convertase and factor H acts as a cofactor for complement factor I mediated proteolysis of C3b to form iC3b. The formation of iC3b stops the alternative pathway amplification loop as iC3b cannot be formed into the alternative pathway C3 convertase. Both C3b and iC3b act as opsonins for phagocytosis [6,7]. Factor H recognizes host cell surface patterns and protects against complement activation. In the fluid phase, factor H is important for regulation of spontaneous alternative pathway activation. This is apparent in individuals with complete factor H deficiency; uncontrolled alternative pathway activation results in marked secondary C3 deficiency [8]. These individuals are susceptible to meningococcal infections, C3 glomerulopathy and haemolytic uraemic syndrome (HUS) [8].

Various polymorphisms in the gene encoding complement factor H (*CFH*) have been identified to be associated with human diseases [5]. Polymorphisms may affect factor H binding to host cells, regulation of alternative pathway activity, or factor H expression levels [9-11]. These influence the susceptibility to diseases such as HUS [12,13], age-related macular degeneration, and dense deposit disease [14]. Factor H plasma

levels show a large variation (range, 63.5-847.6 µg/mL) [15-19]. This variation is due to both environmental factors (e.g. smoking) and genetic factors [17].

We hypothesized that the inter-individual variation in plasma complement factor H levels affects resistance to invasive pneumococcal disease. In the human population it is difficult to study the sole effect of factor H levels on susceptibility for infectious diseases, owing to variation in many other factors affecting complement activity, such as concentrations of opsonizing antibodies and polymorphisms in other complement components. Therefore, we compared the resistance of genetically modified mice with different factor H expression levels to invasive pneumococcal disease *in vivo*. In addition, under controlled conditions we studied the effects of variation in the factor H level in human serum on C3b opsonization and opsonophagocytic killing *in vitro*, using human factor H-depleted serum reconstituted with factor H various amounts of purified human factor H. Our data demonstrate a critical role for serum factor H levels in the host response to invasive pneumococcal infections.

Material and Methods

Ethics Statement

This study was carried out in accordance with the recommendations of 'OECD Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation' (OECD Guidance Document 19, 2000). The protocols were approved by the Animal Ethics Committee of Radboud University, Nijmegen, The Netherlands (Permit Numbers: RU-DEC 2011-050, 2012-274, 2014-156).

Animals

The development of the factor H knockout mice (*Cfh* $^{-/-}$) has been described previously [20]. C57BL/6 mice were obtained from Charles River Laboratories. During the experiment, mice were monitored and scored for clinical signs and humane endpoints were predefined. A >20% decrease in body weight from baseline, a moribund state, a skin temperature of <35°C, and a substantial reduction in motility, and animals with any of these characteristics were sacrificed to minimize animal pain, distress, and discomfort.

Pneumococcal Strains and Growth Conditions

The *S. pneumoniae* strain TIGR4 was used in all experiments [21]. The bacteria were first passaged in mice to maintain virulence, as described elsewhere [22]. The pneumococcal strain was grown in Todd-Hewitt broth supplemented with 5 g/L yeast extract or on Columbia blood agar plates (Becton Dickinson) at 37°C and 5% CO_2. TIGR4 was grown to an OD_{620} of 0.2 and stored in aliquots at -80°C in 15% glycerol. The number of colony-forming units (CFU) per milliliter was determined by plating serial 10-fold dilutions of test aliquots on blood agar plates.

Pneumococcal Invasive Disease

S. pneumoniae TIGR4 stocks were thawed, spun down by centrifugation, and resuspended in sterile phosphate-buffered saline (PBS) to the required dilution for infection (infectious dose, 1x10^7 CFU). In experiment 1, 10 *Cfh*$^{+/+}$, 10 *Cfh* $^{+/-}$ and 10 *Cfh* $^{-/-}$ C57BL/6 male mice aged 6-8 weeks were infected intravenously in the tail vein with 10^7 CFU TIGR4 in 100 µL of PBS. At 19 hour after infection, *Cfh* $^{+/+}$, *Cfh* $^{+/-}$ and *Cfh* $^{-/-}$ mice were anesthetized with 2.5% (vol/vol) isoflurane over oxygen, and blood specimens were collected by orbital puncture following cervical dislocation. The number of CFU per milliliter of blood was determined by plating serial 10-fold dilutions on blood agar plates. Residual blood was allowed to clot on ice, followed by serum separation (10 min 3,000 x *g* at 4°C), and aliquots were stored at -80°C. Levels of Mouse interleukin-6 (IL-6; eBioscience) and mouse macrophage inflammatory protein 2 (CXCL2/MIP-2; R&D Systems) in serum was measured by enzyme-linked immunosorbent assays.

In experiment 2, 20 wild-type 8-weeks-old female C57BL/6 mice were infected intravenously in the tail vein with 10^7 CFU TIGR4 in 100 µL of PBS as in experiment 1. Of these mice, 10 mice were injected intraperitoneally with 600 µg of purified human factor H (CompTech) diluted in 600 µL of PBS immediately after infection, while the other mice received PBS alone as control. Previous studies demonstrated the ability of human factor H to control mouse C3 activation [23,24]. In one of these studies, complement C3 levels in *Cfh* $^{-/-}$ mice were restored by 500 µg of exogenous human factor H [23]. In the present study, a slightly higher dose of 600 µg was chosen to inhibit C3 activation during infection. At 21 hour after infection, the number of bacterial CFU per milliliter of blood was measured, and a serum specimen was collected to determine cytokine levels as described under experiment 1.

C3 Binding Assay

Pneumococcal surface C3 opsonization was performed by incubating bacteria in murine or human serum. To assay C3 deposition on the surface of *S. pneumoniae*, TIGR4 stocks were thawed and washed in PBS. Bacteria (5x10^6) were pelleted in a 96-well plate and resuspended in 20% (vol/vol) mouse serum or 10% (vol/vol) human factor H-depleted serum in veronal buffer (Lonza) containing 5 mM

ethylene glycerol tetraacetic acid and 5 mM $MgCl_2$ to a total volume of 100 µL. The bacterial suspension was incubated for 90 min in mouse serum or for 30 min in human serum at 37°C in 5% CO_2. After C3 opsonization, complement activation was blocked by incubation in 10 mM ethylenediaminetetraacetic acid for 10 min on ice, followed by centrifugation at 3,000 x *g* at 4°C for 10 min. Bacteria were labeled with diluted fluorescein-conjugated goat IgG to mouse (1:200) or human (1:800) complement C3 (MPbio, Cappel) in PBS/2%BSA followed by fixation in 2% paraformaldehyde. C3 opsonization was measured using a FACScan flow cytometer (BD Biosciences). Data were analysed using FlowJo version X.

Pneumococcal C3b opsonization was measured in pooled serum specimens from at least 9 male C57BL/6 *Cfh* $^{+/+}$, *Cfh* $^{+/-}$, and *Cfh* $^{-/-}$ mice and in pooled *Cfh* $^{+/+}$ C57BL/6 mouse serum (Innovative Research), with or without exogenous human factor H (CompTech), with a final concentration of 25 µg/mL. In addition, pneumococcal C3b opsonization in human factor H-depleted serum (CompTech) was measured. The human factor H-depleted serum was substituted with purified human factor H, full reconstitution required 500 µg/mL human factor H. To study the effect of higher or lower factor H concentrations on pneumococcal C3 opsonization, the serum was reconstituted with 100, 200, 300, 400, 500, 600 or 1000 µg/mL human factor H (CompTech).

Pneumococcal Killing Assay

A pneumococcal killing assay was performed by incubating bacteria in human blood, in which the plasma was replaced by factor H-depleted serum reconstituted with various amounts of human factor H. After informed consent was obtained, a venous blood specimen was collected from the median cubital vein of healthy volunteers (age, 20-40 years; both males and females) into a 4-mL tube containing 50 µg/mL lepirudin (Refludan; Pharmion). A total of 100 µL of blood was added per well in a 96-well plate, followed by centrifugation at 10 min 3,000 x *g* at room temperature. Plasma was removed, 150 µl of PBS was added, and centrifugation was performed to wash the blood cells and remove residual factor H-containing fluid. The blood cells were resuspended in human factor H-depleted serum reconstituted with 0, 300, 500 or 1000 µg/mL human factor H, after which *S. pneumoniae* TIGR4 (10^6 CFU/mL) and 1 mM of $MgCl_2$ were added. The 96-well plate was incubated at 37°C for 4 hours under continuous shaking. The number of bacterial CFU was determined before incubation and 4 hours after incubation by plating serial 10-fold dilutions on blood agar plates. The percentage of bacteria that survived was calculated.

Statistical Analysis

Differences between groups of mice were analyzed using the nonparametric Mann-Whitney test a Bonferroni correction in case of multiple comparisons. Results of *In vitro* experiments performed with mouse or human serum or blood specimens were analyzed using the Student *t* test, for comparison of 2 groups, or analysis of variance with the Bonferroni correction, for analysis of multiple groups. Differences were considered statistically significant when $P < 0.05$.

Results

Clearance of *S. pneumoniae* From Plasma Is Enhanced in Heterozygous Factor H-Deficient Mice

Mice with absent (*Cfh*$^{-/-}$), reduced (*Cfh* $^{+/-}$), or normal (*Cfh* $^{+/+}$) plasma factor H levels were infected intravenously with *S. pneumoniae*. The *Cfh* $^{+/-}$ mice showed significantly reduced numbers of CFU compared to *Cfh* $^{+/+}$ mice, 19 hour after inoculation (**Figure 1A**). In contrast, *Cfh* $^{-/-}$ mice had higher numbers of CFU than *Cfh* $^{+/+}$ mice. Furthermore, 4 out of 10 *Cfh*$^{-/-}$ mice were sacrificed prior to the 19-hour time point because they had reached the predefined humane end point. Levels of the proinflammatory cytokines IL-6 and MIP-2 were significantly lower in the *Cfh* $^{+/-}$ mice (**Figure 1B and 1C**) but higher in the *Cfh* $^{-/-}$ animals.

Administration of Human Factor H to Wild-Type Mice Impaired Plasma Clearance of *S. pneumoniae*

The effect of increased factor H levels in *Cfh* $^{+/+}$ mice was studied by injection of human factor H at the time of intravenous injection of *S. pneumoniae*. *Cfh* $^{+/+}$ mice injected with human factor H showed significantly higher blood bacterial numbers of CFU than control *Cfh* $^{+/+}$ mice 21 hour after infection. In addition, these mice had significantly higher cytokine IL-6 and MIP-2 serum levels (**Figure 2 A-C**).

Pneumococcal C3 Opsonization is Enhanced in Heterozygous Factor H-Deficient Mice

We next examined whether factor H levels influenced pneumococcal C3 opsonization. We measured *S. pneumoniae* C3 opsonization by mouse sera from *Cfh* $^{+/+}$, *Cfh* $^{+/-}$ and *Cfh* $^{-/-}$ strains. C3 opsonization by *Cfh* $^{+/-}$ sera was significantly greater than that by *Cfh* $^{+/+}$ sera (**Figure 3 A-C**). No C3 deposition was observed with pneumococci incubated in *Cfh* $^{-/-}$ serum, which reflects the complete consumption of C3 in *Cfh* $^{-/-}$ sera [20]. Exogenous human factor H in *Cfh* $^{+/+}$ mouse serum significantly reduced the pneumococcal C3 deposition (**Figure 4 A-C**).

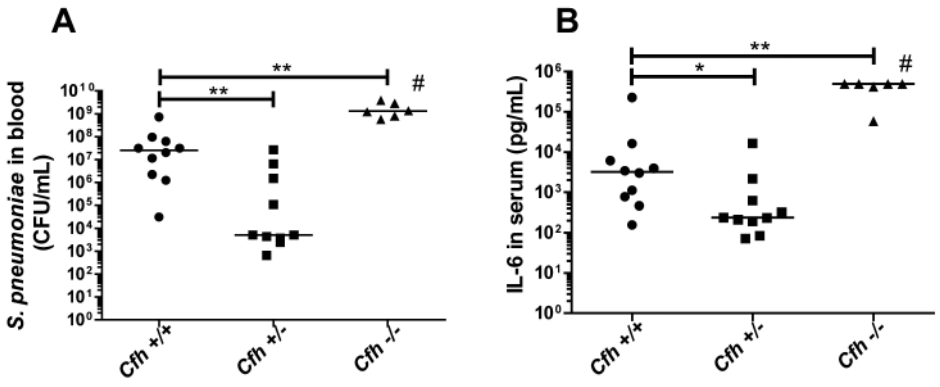

Figure 1. Clearance *of Streptococcus pneumoniae* from plasma is enhanced in mice heterozygous for the gene encoding factor H (*Cfh*). **A–C**, Wild-type (*Cfh* $^{+/+}$; n = 10), heterozygous (*Cfh* $^{+/-}$; n = 10), and homozygous (*Cfh* $^{-/-}$ n = 10) FH-deficient C57BL/6 mice were infected with 1×10^7 colony-forming units (CFU) of *S. pneumoniae* (TIGR4) and sacrificed 19 hours later. #Four of the 10 *Cfh* $^{-/-}$ animals were sacrificed before this because the predefined humane end point was reached. Nineteen hours after infection, the number of CFU per milliliter was determined in blood, and levels of proinflammatory cytokines interleukin 6 (IL-6) and macrophage inflammatory protein 2 (MIP-2) were measured in serum. Each point represents 1 mouse. Cytokine values were analyzed after logarithmic transformation; horizontal line represents the median. Comparisons between groups were performed using the nonparametric Mann–Whitney test with the Bonferroni correction. **P* < .05, ***P* < .01, and ****P* < .001.

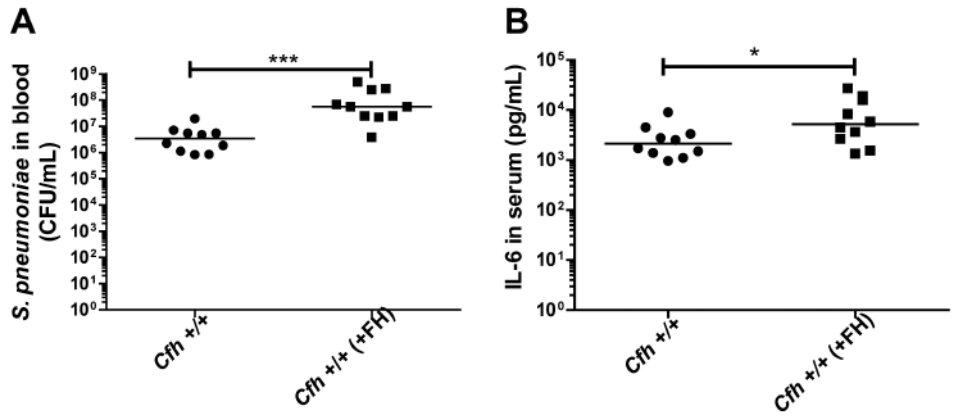

Figure 2. Administration of human factor H (FH) to wild-type mice impaired plasma clearance of *Streptococcus pneumoniae*. **A–C**, Wild-type C57BL/6 mice were intravenously infected with 1×10^7 colony-forming units of *S. pneumoniae* (TIGR4) and injected with purified human factor H (n = 10) or phosphate-buffered saline (n = 10) and sacrificed 21 hour later. Twenty-one hours after infection, the number of colony-forming units (CFU) per milliliter was determined in blood, and levels of the proinflammatory cytokines IL-6 and MIP-2 were measured in serum. Each point represents one mouse; cytokine values were analyzed after logarithmic transformation; horizontal lines represents the median. Comparisons between groups were performed using the nonparametric Mann-Whitney test with the Bonferroni correction. **P* < .05, ***P* < .01, and ****P* < .001.

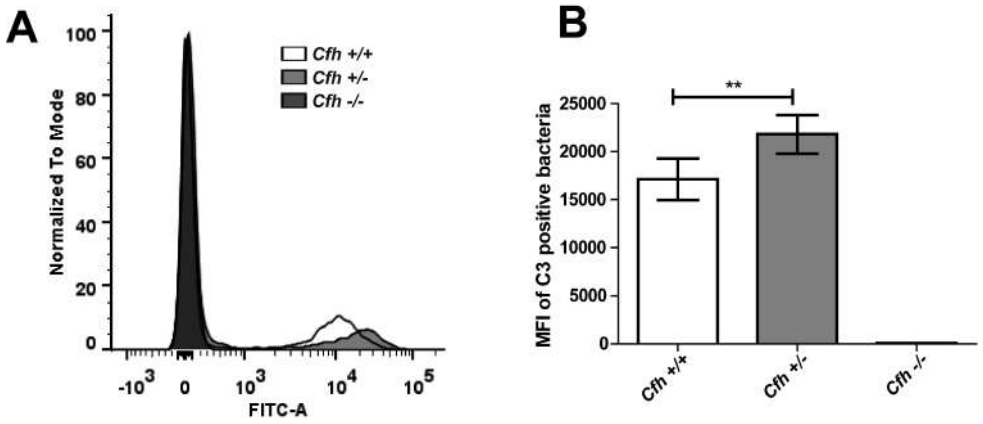

Figure 3. Pneumococcal C3 opsonization is enhanced in mice heterozygous for the gene encoding factor H (*Cfh*). C3 deposition on the surface of *Streptococcus pneumoniae* strain TIGR4 in pooled mouse serum derived from *Cfh* $^{+/+}$, *Cfh* $^{+/-}$, or *Cfh* $^{-/-}$ mice. A total of 5×10^7 colony-forming units/mL *S. pneumoniae* TIGR4 were incubated in 20% mice serum in veronal buffer containing Mg^{2+} and ethylene glycol tetraacetic acid. After 90 minutes of incubation at 37°C, pneumococcal C3 deposition was measured by flow cytometry. **A**, A representative example of a flow cytometry histogram for bacteria incubated in wild-type (*Cfh* $^{+/+}$; white peak), heterozygous (*Cfh* $^{+/-}$; gray peak), and homozygous (*Cfh* $^{-/-}$; black peak) factor H-deficient pooled mouse serum. **B**, Intensity of C3 deposition on bacteria. **C**, Proportion of C3-positive bacteria. For panels B and C, each bar represents the mean ± standard error of the mean for results obtained from 5 separate experiments. Comparisons between groups were performed using a paired Student *t* test.*$P < .05$, **$P < .01$, ***$P < .001$. Abbreviation: NS, not significant.

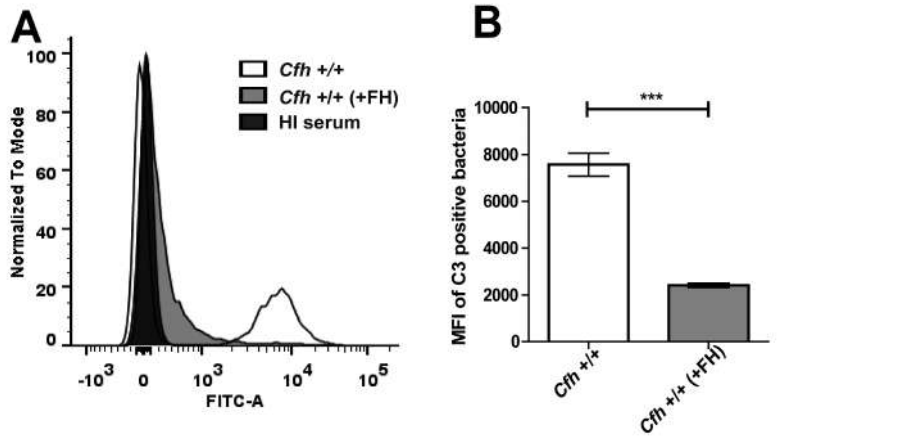

Figure 4. Pneumococcal C3 opsonization in wild-type mice serum is impaired by excess human factor H (FH). C3 deposition on the surface of *S. pneumoniae* strain TIGR4 in pooled wild-type mouse serum with additional human factor H. *S. pneumoniae* TIGR4, 5×10^7 colony-forming units/mL, were incubated in 20% mice serum in Veronal buffer containing Mg^{2+} and ethylene glycol tetraacetic acid. After 90 minutes of incubation at 37°C, pneumococcal C3 deposition was measured by flow cytometry. **A**, A representative example of a flow cytometry histogram for bacteria incubated in wild-type (*Cfh*$^{+/+}$; white peak), wild-type with exogenous human factor H (*Cfh* $^{+/+}$ [+FH]; gray peak), and heat-inactivated mouse serum (HI) as a negative control (black peak). **B**, Intensity of C3 deposition on bacteria. **C**, Proportion of C3-positive bacteria. The bars in panels B and C represent the mean ± standard error of the mean of 2 separate experiments in duplicate. Comparisons between groups were performed using a paired Student t test. *$P < .05$,**$P < .01$, ***$P < .001$. Abbreviation: NS, not significant.

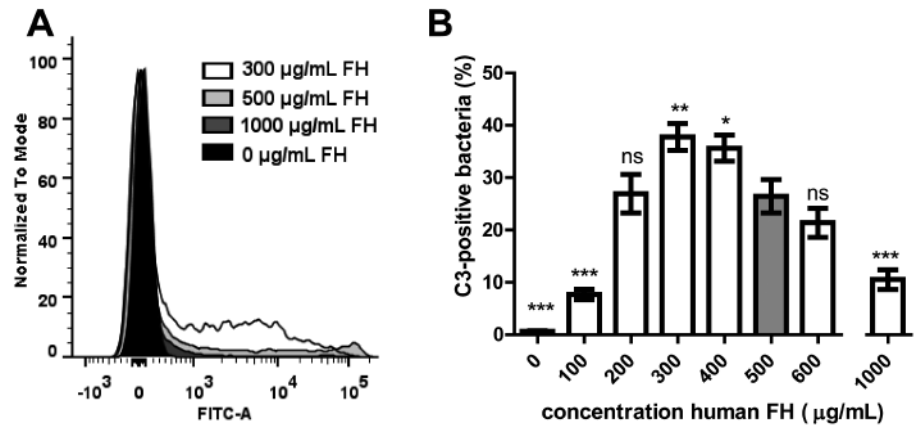

Figure 5. Alternative pathway modulation by human factor H (FH) levels determines pneumococcal C3 opsonization. C3 deposition on the surface of *Streptococcus pneumoniae* strain TIGR4 in human factor H–depleted serum reconstituted with various factor H concentrations. *S. pneumoniae* TIGR4, 5×10^7 colony-forming units/mL, were incubated in 10% human serum in Veronal buffer containing Mg^{2+} and ethylene glycol tetraacetic acid. After 30 minutes of incubation at 37°C, pneumococcal C3 deposition was measured by flow cytometry. **A**, A representative example of a flow cytometry histogram for bacteria incubated in factor H-depleted serum reconstituted with the physiological factor H concentration of 500 µg/mL factor H (light gray), or 0 µg/mL (black), 300 µg/mL (white), or 1000 µg/mL (dark gray) factor H. **B**, Proportion of C3-positive cells. Each bar represents the mean ± standard error of the mean of results obtained from 4 separate experiments. Comparison of various factor H concentrations to the physiological factor H concentration of 500 µg/mL (gray bar) were performed by using a repeated measures analysis of variance with the Bonferroni correction. **P* < .05, ***P* < .01, and ****P* < .001. Abbreviation: NS, not significant

Human Factor H Levels Influence the Degree of Pneumococcal C3 Opsonization and Killing in Human Blood

S. pneumoniae sequesters human factor H, while mouse factor H does not bind to the pneumococcal surface [25]. We performed opsonization experiments by using factor H-depleted human serum reconstituted with different concentrations of human factor H. Human serum depleted for factor H showed no C3 opsonization, owing to rapid C3 activation that occurs upon reconstitution of cations *in vitro* (**Figure 5**). Reconstitution of factor H-depleted human serum to physiological levels (500 µg/mL) resulted in opsonization of *S. pneumoniae* (mean 26.5%, ±SEM 3.2%, **Figure 5**). Interestingly, reconstitution to a lower factor H level (300 µg/mL) significantly elevated *S. pneumoniae* opsonization (mean 37.8%, ±SEM 2.6%). In contrast, reconstitution to a higher level (1000 µg/mL) significantly reduced C3 opsonization. We next examined bactericidal activity *in vitro*, using a pneumococcal killing assay, in whole blood with factor H-depleted serum reconstituted with 300,

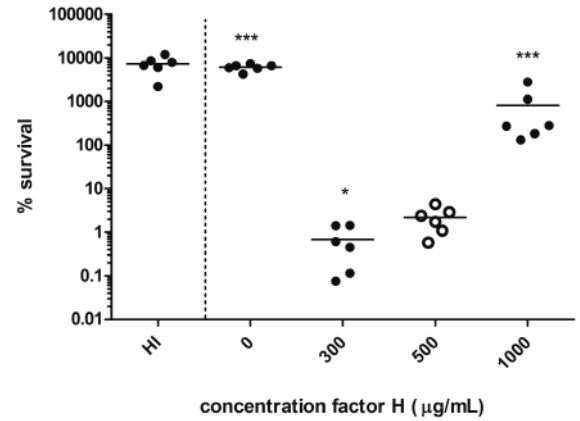

Figure 6. Alternative pathway modulation by human factor H (FH) determines pneumococcal killing in blood. Pneumococcal killing in human blood containing factor H-depleted serum reconstituted with various factor H concentrations. *Streptococcus pneumoniae* TIGR4, 1×10^6 colony-forming units/mL, were incubated in healthy donor blood in which plasma was replaced by human factor H–depleted serum reconstituted with various factor H concentrations or with heat-inactivated (HI) serum as a control. The number of colony-forming units were determined by plating serial dilutions before incubation and 240 minutes after incubation at 37°C, and the percentage of surviving bacteria was calculated. Each dot represents 1 blood donor. Comparisons of various factor H concentrations to the physiological factor H concentration of 500 µg/mL (white dots) were performed using a repeated measures analysis of variance with the Bonferroni correction. **P* < .05 was considered significant. ***P* < .01 and ****P* < .001.

500, or 1000 µg/mL factor H. The absence of factor H impaired pneumococcal clearance and resulted in pneumococcal growth (**Figure 6**). In accordance with our C3 opsonization data, 300 µg/mL factor H resulted in significantly increased bacterial clearance, compared to 500 µg/mL factor H, whereas killing was significantly impaired with 1000 µg/ml factor H.

Discussion

The aim of this study was to investigate whether variation in factor H levels influences resistance to *S. pneumoniae* infection. We demonstrated that factor H levels play a critical role in the control of alternative pathway activity and the host defense in pneumococcal invasive disease.

It has previously been shown that increased alternative pathway activity enhances pneumococcal killing. Mice injected with recombinant properdin, a positive

regulator of the alternative pathway, resulted in increased alternative pathway-mediated pneumococcal C3 opsonization and enhanced pneumococcal killing [26]. We are the first to demonstrate that decreased factor H expression in *Cfh* $^{+/-}$ mice enhances pneumococcal C3 opsonization, resulting in improved pneumococcal clearance from the blood. The observed enhanced complement activation in *Cfh* $^{+/-}$ mice is in line with recently reported excess complement activation in *Cfh* $^{+/-}$ mice resulting in age-related macular degeneration-like pathology [27]. Our study confirms that *Cfh* $^{-/-}$ mice, which have a secondary C3 deficiency, have severely impaired clearance of *S. pneumoniae* from the blood. We further show that *Cfh* $^{+/+}$ mice that received exogenous human factor H showed impaired pneumococcal clearance from blood as a consequence of reduced pneumococcal C3 opsonization. This is in line with previous findings demonstrating that factor B-deficient mice with an abrogated alternative pathway activity had increased susceptibility to *S. pneumoniae* infection after intranasal and intraperitoneal inoculation, compared to wild-type mice [28]. This is also in line with recent studies in transgenic mice expressing human factor H, which showed increased bacterial loads and disease severity in *Streptococcus pyogenes* infection [29]. In addition, we found that increased serum factor H levels in mice are associated not only with a greater pathogen burden, but also with greater elevations in levels of the proinflammatory cytokines IL-6 and MIP-2. This is consistent with findings by others [29]. We also showed that decreased expression of factor H and the resultant lower pathogen burden are associated with lower proinflammatory cytokines.

Pathogens such as *S. pneumoniae* have evolved mechanisms to evade complement-mediated killing by binding of host complement regulators, including factor H, factor H-like protein 1, C4-binding protein and plasminogen [30]. Various Gram-negative pathogens, such as *Neisseria meningitidis* and *Haemophilus influenzae*, and gram-positive pathogens, including *S. pneumoniae, Staphylococcus aureus,* and *S. pyogenes,* bind human factor H to evade complement-mediated killing [31-35]. *S. pneumoniae* binds human factor H by means of pneumococcal surface protein C (PspC) [36]. Like other pathogens, *S. pneumoniae* displays species specificity in the binding of factor H [25]. Pneumococci bind human factor H but do not bind mouse factor H [25,37,38]. Therefore, a characteristic of the mouse model is that factor H binding to the bacterial surface is limited because *S. pneumoniae* cannot sequester mouse factor H. The mice model allowed us to assess the influence of mouse factor H expression levels on the fluid-phase control of alternative pathway activity on bacterial C3 opsonization and clearance from the blood by comparing *Cfh* $^{+/-}$ and *Cfh* $^{+/+}$ mice.

Pneumococcal C3 deposition was measured using a polyclonal antibody against human or mouse C3, as described by others [39,40]. This antibody binds C3b and iC3b deposited on the bacterial surface, both of which act as opsonins for phagocytosis [6,7]. Independent of factor H, iC3b can be further degraded into C3c and C3dg and then to C3d [7]. We chose to measure C3b and iC3b deposition since our aim was to measure pneumococcal C3 opsonisation resulting in killing by phagocytosis. Importantly, we observed that pneumococcal C3 opsonization was associated with pneumococcal killing in human blood and mice.

Among humans, there is a large variation in factor H levels in blood, ranging from 63.5 to 847.6 µg/mL [15-19]. We found a delicate balance in which human factor H levels of 300 µg/mL in serum resulted in optimal pneumococcal C3 opsonization and clearance, whereas 100 µg/mL or 500 µg/mL resulted in significantly lower opsonization. A strength of our study is that we varied the human factor H concentration while keeping all other serum components, such as levels of opsonizing antibodies and other complement components, identical. Experiments were performed using the pneumococcal TIGR4 strain, since this strain is virulent in mice. Previous studies demonstrated variation in C3 opsonization and factor H binding between pneumococcal strains, owing to variation in capsular serotype, capsular expression, PspC variant, and *pspC* expression [39-43]. This may contribute to differences in virulence between strains.

Our results demonstrate that, within the normal range of human factor H levels, there is a significant difference in the ability to C3 opsonize and to kill *S. pneumoniae* TIGR4. This suggests that when pneumococci enter the bloodstream, human factor H levels are of major importance for optimal pneumococcal clearance and thus affect an individual's susceptibility to invasive pneumococcal disease and severity of infection.

Common polymorphisms in *CFH* have been described and were associated with susceptibility to various diseases. Based on the different functional effects of different types of factor H deficiencies resulting from mutations or polymorphisms known for human factor H, we propose to classify them into 3 groups: 1) polymorphisms affecting cell surface binding, either host or bacterial; 2) polymorphisms affecting the ability of factor H to regulate alternative pahtway activation; and 3) polymorphisms affecting factor H expression levels. A similar classification is already being used for properdin deficiencies [44]. The current study demonstrates the critical role of factor H serum levels in pneumococcal invasive disease. These findings are supported by previous work that demonstrated that a

single-nucleotide polymorphism in the promoter region of *CFH* increases serum levels of human factor H, resulting in reduced bactericidal activity against *N. meningitidis* [15] However, binding affinity of human factor H to the bacterial surface may also play an important role in the outcome of infectious diseases. An example of this phenomenon is a common variant of human factor H Y402H described to be associated with increased group A streptococcal killing in blood due to reduced binding of the human factor H variant to the bacterial surface [32,45]. As this human factor H variant also shows decreased binding to host cells and thus less protection of cells from complement activation, the Y402H polymorphism increases the risk of developing age-related macular degeneration presumably caused by elevated ocular complement activation [46]. Individuals carrying the Y402H variant also have lower serum levels of factor H [11]. Further research is needed to determine whether common or rare variants in *CFH* that affect the functionality or expression levels of factor H are associated with the susceptibility to infectious diseases, such as pneumococcal invasive disease.

Many genetic factors contribute to an individual's complement activity, also referred to as complotype [10]. Combinations of polymorphisms in alternative pathway proteins have been described to enhance alternative pathway activity and predispose for chronic inflammatory diseases, such as haemolytic uremic syndrome, age-related macular degeneration, and dense deposit disease. Our findings support the hypothesis that an individual's alternative pathway activity may affect not only the susceptibility to chronic inflammatory diseases, but also the susceptibility to infectious diseases [10]. Genome-wide association studies are of importance to increase the understanding of how an individual's complement activity affects the susceptibility to infectious diseases. Furthermore, this study, together with previous studies, demonstrates the importance of alternative pathway activation in the defence against invading pathogens [26]. Therefore, modulation of alternative pathway activity may be an effective therapy to enhance clearance of invasive infections. Enhanced alternative pathway activity by exogenous properdin has been demonstrated to increase pneumococcal and meningococcal clearance in mice [26]. However, therapies involving factor H may be more challenging to develop, since we demonstrate a delicate balance in which higher or lower factor H levels may impair pneumococcal clearance. Overall, a better understanding of host factors that influence the susceptibility to infection would support prediction of disease and could also contribute to the development of therapies to reduce the susceptibility to disease.

We found that human and mice factor H levels determine a delicate balance of alternative pathway activity affecting the resistance to invasive pneumococcal disease. Our results suggest that polymorphisms in *CFH* affecting factor H expression levels may play a thus far unrecognized role in the resistance to invasive pneumococcal disease.

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Chapter 5

A versatile assay to determine bacterial and host factors contributing to opsonophagocytotic killing in hirudin-anticoagulated whole blood

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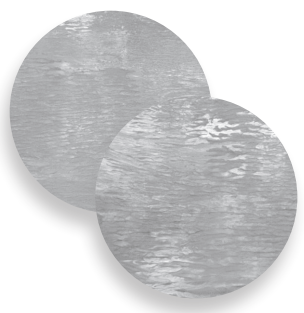

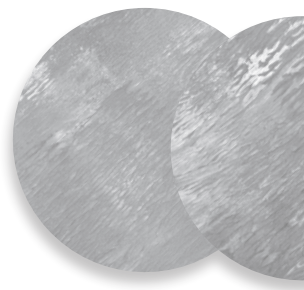

Abstract

Most bacteria entering the bloodstream will be eliminated through complement activation on the bacterial surface and opsonophagocytosis. However, when these protective innate immune systems do not work optimally, or when bacteria are equipped with immune evasion mechanisms that prevent killing, this can lead to serious infections such as bacteremia and meningitis, which is associated with high morbidity and mortality. In order to study the complement evasion mechanisms of bacteria and the capacity of human blood to opsonize and kill bacteria, we developed a versatile whole blood killing assay wherein both phagocyte function and complement activity can easily be monitored and modulated. In this assay we use a selective thrombin inhibitor hirudin to fully preserve complement activity of whole blood. This assay allows controlled analysis of the requirements for active complement by replacing or heat-inactivating plasma, phagocyte function and bacterial immune evasion mechanisms that contribute to survival in human blood.

Introduction

Blood is normally sterile, but in cases when epithelial barriers are compromised and the immune system is not optimally equipped to fight pathogens, bacteria can be present in the blood, which is called bacteremia. Bacteria have evolved various mechanisms that prevent opsonophagocytosis, contributing to their ability to colonize their host, but also occasionally resulting in severe infections. Overall, Gram-positive bacteria are protected from complement-mediated lysis by the presence of a thick outer cell wall consisting of peptidoglycan, which prevents the bacterial membrane from lysis by the pore-forming membrane attack complex [1]. Conversely, Gram-negative bacteria, which are characterized by an outer membrane surrounding the bacterial cell wall, are vulnerable to complement-mediated killing due to assembly and insertion of the membrane attack complex on the bacterial surface [2]. Several bacterial species express a polysaccharide capsule, that protects them from recognition by opsonizing antibodies and in Gram-negative bacteria such as *Haemophilus influenzae* from insertion of the membrane attack complex [3].

Besides a protective capsule, which can be found on both Gram-positive and Gram-negative bacteria, many invasive bacteria are able to hijack human complement regulatory proteins, thereby decreasing complement activation on their bacterial surface. For instance, *Streptococcus pneumoniae*, *H. influenzae*, *Escherichia coli* and *Neisseria meningitidis* are able to bind human factor H [4-7], which decreases alternative complement activation and thereby reduces C3 opsonization.

In order to study the complement evasion mechanisms of bacteria, or the capacity of complement to opsonize and kill bacteria, most *in vitro* studies performed to date are using serum, plasma or baby rabbit complement containing active complement for complement opsonization. For opsonophagocytosis, isolated phagocytes or phagocyte-like cell lines such as HL-60 are used [8-11]. However, this is by no means representative to the real live situation in whole blood. For instance, the isolation of neutrophils leads to priming, which affects the ability of the neutrophils to form reactive oxygen species and changes their responses to cytokines [12]. In addition, serum has altered levels of coagulation proteins compared to plasma in whole blood. An example is plasminogen [13], which can bind to the bacterial surface of *S. pneumoniae* and is involved in bacterial virulence [14,15]. Another example is fibrinogen, shown to bind to *Streptococcus pyogenes* M protein, which decreases C3b deposition and opsonophagocytosis [16,17].

To circumvent these limitations in order to study complement-mediated opsonophagocytosis of bacteria, we explored the possibility to use whole blood directly after venous puncture for use in opsonophagocytosis assays. Here, we describe a versatile and easy to perform whole blood killing assay in which both phagocyte function and complement activity can be monitored and modulated. We used a selective thrombin inhibitor hirudin, which preserved complement activity of whole blood, in contrast to lithium heparin, sodium heparin, EDTA or sodium citrate.

Material and methods

Ethics statement

After informed consent, a venous blood specimen was collected from the median cubital vein of healthy volunteers (age, 20–40 years; both males and females). Collection of blood was approved by the Ethics Committee of the Radboud University, Nijmegen, the Netherlands and experiments were carried out in accordance with local guidelines and regulations and complies with the Declaration of Helsinki and the Good Clinical Practice guidelines.

Bacterial growth conditions

Streptococcus pneumoniae strain TIGR4 [18], *Streptococcus pneumoniae* strain TIGR4Δ*pspC* [19], *Klebsiella pneumoniae* RUMC-KP01 (Clinical isolate Medical Microbiology, Radboud UMC Nijmegen, the Netherlands), *Staphylococcus aureus* strain NCTC 8178 (National Collection of Type Cultures), *Escherichia coli* BL21 DE3 (Agilent), *Neisseria meningitidis* serogroup B strain H44/67 [20], Pseudomonas aeruginosa ATCC15692 (American Type Culture Collection), *H. influenzae* type A strain ATCC 9006 (American Type Culture Collection), *H. influenzae* type B strain ATCC 10211 (American Type Culture Collection), non-typeable *H. influenzae* (NTHi) strain R2866 [3], NTHi strain 3655 [21] and NTHi strain 11P6H [22] were used in this study. *H. influenzae* was grown under shaking conditions at 37°C in brain heart infusion (BHI) broth (Becton Dickinson) supplemented with 10 µg/mL haemin (Sigma-Aldrich) µg/mL β-nicotinamide adenine dinucleotide (Merck) (sBHI). *S. pneumoniae* was grown under static conditions at 37°C with 5% CO_2 in Todd-Hewitt broth supplemented with 5 g/L yeast extract. *N. meningitidis* was grown on blood agar plates and collected directly from overnight plates. *K. pneumoniae*, *S. aureus*, *E. coli* and *P. aeruginosa* were grown under shaking conditions at 37°C in Luria-Bertani (LB) broth.

IgG, IgM and C3 opsonization assays

Blood for serum collection was collected in SST II *Advance* tubes (BD, Ref 367953). Tubes were inverted after blood was drawn, incubated for 15 minutes at room temperature to clot, centrifuged with 3000g for 15 min at room temperature and serum was stored in small aliquots at -80°C. Blood for plasma preparation was collected in K2E (EDTA) tubes (BD Ref 367864), Trisodium citrate tubes (BD Ref 363047), Sodium heparin tubes (BD Ref 367869), Lithium heparin tubes (BD Ref 368496) or S-Monovette r-Hirudin tubes (Sarstedt, Ref 04.1944.001). Tubes were inverted after blood was drawn, centrifuged with 3000 x *g* for 15 min at 4°C and plasma was stored in small aliquots at -80°C.

For human IgG, human IgM and human C3 binding, bacteria (1.10E7 in 100 µL) were incubated with 10% plasma or serum in Hank's Balanced Salt Solution (HBSS) without phenol red containing Ca^{2+}/Mg^{2+} + 0.1% gelatin (HBSS3+) for 30 min at 37°C. Bacteria were washed and incubated with 1:500 diluted FITC-labelled polyclonal goat anti-human C3 (MP biomedicals), 1:100 diluted FITC-labelled Fc-specific goat anti-human IgG (Sigma-Aldrich) or 1:100 diluted FITC-labelled µ-chain-specific goat anti-human IgM (Sigma-Aldrich) in PBS with 2% BSA for 30 min at 4°C. Bacteria were washed and fixed for 20 min with 2% paraformaldehyde. Bacteria were taken up in PBS for flow cytometry.

Whole blood killing assay

After informed consent was obtained, a venous blood specimen was collected from the median cubital vein of healthy volunteers (age, 20–40 years; both males and females) into S-Monovette r-Hirudin tubes (Sarstedt). Blood was kept at room temperature on a roller bench until used. For the whole blood killing assay, 100 µL of hirudin-anticoagulated blood was added per well in a 96-well plate. Bacterial suspensions in PBS, containing 1.10E5 colony forming units (CFU), were added in a maximum volume of 5 µL and immediately mixed with the blood. The 96-well plate was incubated for the indicated time at 37°C under continuous shaking. The number of bacterial CFU was determined at start and after incubation by plating serial 10-fold dilutions. The percentage of bacteria that survived was calculated.

For plasma inactivation, 100 µL of hirudin-anticoagulated blood was added per well in a 96-well plate and centrifuged at 1000 x *g* for 5 min. Plasma was removed and heat-inactivated for 20 min at 56°C. Blood cells were washed by adding 100 µL PBS and centrifuged with 1000 g for 5 min. PBS was removed and heat-inactivated plasma was mixed with the pelleted cells and used for the killing assay. To examine the effect of plasma alone on bacterial clearance, 200 µL hirudin-anticoagulated

blood was centrifuged 1 min at 16.000 x g and 100 µL plasma was used for the killing assay in the absence of blood cells. For 50%, 25% and 10% active plasma, 50 µL, 25 µL and 10 µL active plasma was mixed with 50 µL, 75 µL and 90 µL heat-inactivated plasma, respectively, and was mixed with the pelleted blood cells and used for the killing assay. For plasma replacement, 100 µL of hirudin-anticoagulated blood was added per well in a 96-well plate and centrifuged at 1000 x g for 5 min. Plasma was removed and cells were washed by adding 100 µL PBS and centrifuged at 1000 x g for 5 min. PBS was removed and pooled hirudin-anticoagulated plasma was mixed with the pelleted cells and used for the killing assay. For C6-depleted serum (CompTech) and C6-deficient patient serum [23], serum was diluted in PBS to 10%. Reconstitution of C6 was performed by supplementing 6.4 µg/mL purified C6 (CompTech) in 10% serum because manufacturer's product description states full reconstitution of serum was achieved with 64 µg/mL.

Inhibitor cytochalysin D (cyto D) (Sigma-Aldrich), anti-complement receptor 3 (CR3) subunit CD11b antibody clone 44a (α-CD11b) (Gift from Prof. Leo Koenderman), 4-hydroxytamoxifen (4-OHT) (Sigma-Aldrich), factor H (FH) (CompTech) or an equal volume of PBS were added to the hirudin-anticoagulated blood before adding the bacteria.

Phagocytosis of CFSE-loaded *S. pneumoniae*

S. pneumoniae was grown in Todd-Hewitt broth supplemented with 5 g/L yeast extract to OD_{620} = 0.2, washed with PBS and labelled with carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich) as previously described [24]. Five microliter (~1.10^6 CFU) CFSE-labelled bacteria were added to 100 uL hirudin-anticoagulated whole blood and incubated for 30 min. Red blood cells were lysed in ice-cold NH_4Cl solution (8.3 g/L NH4Cl, 1 g/L KHCO3 and 37 mg/L EDTA) and washed once with ice-cold NH_4Cl solution followed by a wash with PBS. Cells were stained with 1:200 diluted Alexa647-labelled α-CD16 (BD biosciences), 1:50 diluted V500-labelled α-CD3 (BD biosciences), 1:50 diluted PE-Cy7-labelled α-CD14 (Biolegend), 1:100 diluted BV421-labelled α-CD66b (BD biosciences) for 15 min. at room temperature. Cells were washed with PBS and analyzed by flow cytometry using a FACS LSR II (BD Biosciences). Data were analyzed using FlowJo v10.1.

Results and discussion

Hirudin-anticoagulated blood is optimal for complement preservation

We used *Streptococcus pneumoniae* as model organism to set-up a whole blood killing assay because this bacterium is causing bacteremia in immune competent individuals [25,26]. In order to survive in blood, this bacterium has developed various mechanisms that inhibit recognition by the immune system [27]. For efficient opsonophagocytic killing, C3b opsonization of the bacterial surface of *S. pneumoniae* is required [28]. To determine which anticoagulants preserved complement C3b opsonization capacity, we determined IgG, IgM and C3 binding to the bacterial surface of *S. pneumoniae* after 30 minutes with 10% human serum or 10% human plasma anticoagulated with hirudin, lithium heparin, sodium heparin, EDTA or sodium citrate (**Figure 1 A-C**).

Binding of IgG to the bacterial surface of *S. pneumoniae* incubated with 10% hirudin or EDTA anticoagulated human plasma was slightly increased compared to 10% human serum, whereas no significant differences for IgM were observed. More striking were the differences in C3 opsonization. Here, hirudin anticoagulated plasma showed the highest C3 opsonization of *S. pneumoniae*, whereas all other anticoagulants showed a significant decrease in C3 opsonization. Complement activity was preserved for at least 2 hours when blood was kept at room temperature (**Figure 1D**). From these data, we conclude that hirudin anticoagulated plasma is superior in preserving complement activity.

Previously, Ison *et al* determined killing of *Neisseria meningitidis* in citrate and heparin-anticoagulated whole blood [29]. In this study, heparin-anticoagulated whole blood was superior in killing *N. meningitidis* serogroup A compared to citrate-anticoagulated whole blood. In subsequent experiments, the same group compared this whole blood killing assay to serum bactericidal assay with blood from vaccinated children and consistently showed increased sensitivity for the whole blood killing assay [30,31]. Also, they showed a reduction of survival of *N. meningitidis* in the whole blood killing assay with increasing age of patients [32]. Whole blood killing of *N. meningitidis* has also been performed with hirudin-anticoagulated whole blood. Welsch *et al* showed efficient killing of *N. meningitidis* serogroup B with whole blood from adults [33]. A slightly modified whole blood killing assay, with 25% heat-inactivated serum, showed increased killing with post-immunization serum compared to pre-immunization serum [34]. Comparisons in whole blood killing between huridin and other anticoagulants have not been studied previously.

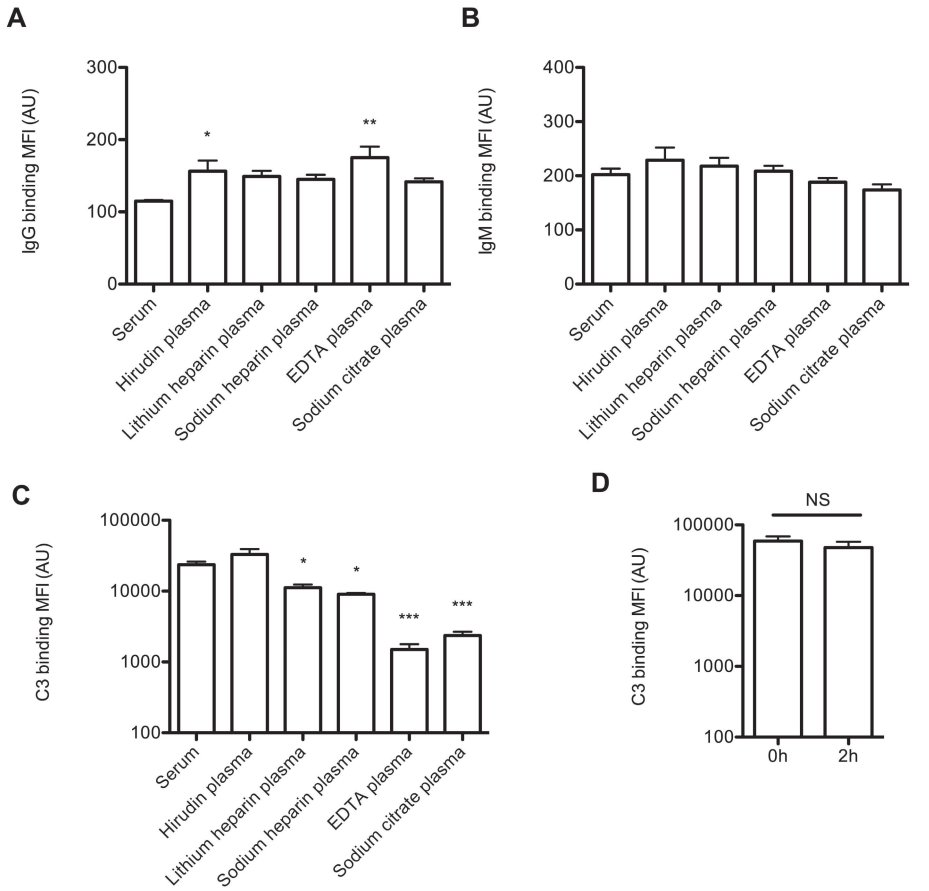

Figure 1. Plasma and serum IgG, IgM, C3 opsonization of *S. pneumoniae*. Bacteria (1.10E7) were incubated for 30 minutes in HBSS3+ containing 10% plasma anticoagulated with hirudin, lithium heparin, sodium heparin, EDTA or sodium citrate or serum from the same donor and binding of (**A**) IgG, (**B**) IgM, and (**C**) C3 was determined by flow cytometry (n=3). One-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison Test was used for statistical analysis. * = $p<0.05$, ** = $p<0.01$. (**D**) Hirudin anticoagulated blood was immediately (0h) or after 2 hours rolling on a roller mixer (2h) centrifuged and plasma was stored. Bacteria (1.10E7) were incubated for 30 minutes in HBSS3+ containing 10% of the plasma that was immediately or after 2 hours stored , and binding C3 was determined by flow cytometry (n=3). A one-tailed student t-test was used for statistical analysis. NS = not significant.

The differences in complement activity preservation can largely be explained by the function of the different anticoagulants. Lithium heparin and sodium heparin induce a conformational change of antithrombin III to accelerate the inhibition of thrombin and factor Xa, thus preventing thrombin activation and the generation of fibrin. However, heparin is known to bind different proteins in the complement cascade [35], as well as calcium and magnesium ions [36], thereby affecting complement activity. Sodium citrate prevents blood from clotting through chelation of calcium ions by forming calcium citrate and EDTA scavenges bi-valent cations, such as calcium and magnesium, both are also required for complement activation. In contrast, hirudin (also known as lepirudin) is a highly specific thrombin inhibitor that does not interfere with complement activation [37]. Hirudin has previously also been used in whole blood stimulation assays [33,38-40]. This enables to determine the contribution of cross-talk between complement and other factors such as cytokine release [38], oxidative burst [40] and phagocytosis [39].

Even though thrombin is not directly involved in complement activation, there are some reports where it has shown to modulate complement activity. For instance, in C3-/- mice, thrombin was overexpressed and showed to cleave C5 into C5a and C5b [41]. In these studies, hirudin reduced acute lung inflammatory injury in C3-/- mice, but had no effect in C3+/+, indicating that thrombin-mediated cleavage of C5 only contributed to acute lung inflammatory injury when C3 is absent.

Whole blood killing assay

Many bacterial pathogens such as *S. pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae N. meningitidis* and *H. influenzae* frequently cause invasive disease, including sepsis [42-44]. When present in the blood, bacteria need to withstand the bactericidal activity of the complement system, and phagocytosis by peripheral blood neutrophils. We used hirudin anticoagulated blood to determine the survival of invasive bacterial pathogens in blood. For these experiments, we used *S. pneumoniae* strain TIGR4, originally isolated from the blood of a 30-year-old male [18,45].

The whole blood killing assay is an easy-to-use opsonophagocytic assay to determine survival of bacterial pathogens in blood. Bacteria are added to 100 µL hirudin anticoagulated blood in a 96-wells round bottom plate and incubated at 37°C while shaking to prevent sedimentation. Different inoculums (10^3-10^5 CFU / 100 µL blood) were tested and all showed a decrease in CFU counts over time (data not shown). For subsequent experiments, 10^5 CFU / 100 µL blood were used.

We determined killing of *S. pneumoniae* in whole blood and observed significant killing already after 1 hour, which increased further in time (**Figure 2A**). In order to determine the role of complement activity and phagocyte function, we performed the whole blood killing assay with either heat-inactivated plasma (see Material and Methods section for procedure) or with only plasma containing active complement. Whereas *S. pneumoniae* was killed in blood, no killing was observed with plasma only, indicating that phagocytes are required for efficient killing (**Figure 2B**). When heat-inactivated plasma was mixed with blood cells, S. *pneumoniae* was able to grow very rapidly. This indicates that active complement is required for effective opsonophagocytosis as well, but also shows that whole blood contains sufficient nutrients for fastidious growth. The contribution of active complement in *S. pneumoniae* opsonophagocytosis is known for a long time [46-48], and our results are consistent with these studies.

We determined whole blood killing after 1 hour for different pathogens that cause bacteremia [42-44]. Survival of *S. pneumoniae* strain TIGR4 was 20%. Similar survival was found for *K. pneumoniae* (16%) and *P. aeruginosa* (7%), whereas survival for *S. aureus* (85%) or *H. influenzae* serotype B (41%) were higher. Survival of *H. influenzae* serotype A (2%), *E. coli* (0.03%) and *N. meningitidis* (0.1%) was much lower. Survival of NTHi was strain dependent, 9% for R2886, but only 0.2% and 0.3% for strains 3655 and 11P6H, respectively. These strain-dependent differences in survival are probably due to variance in complement resistance since we have previously shown that survival in pooled human serum was much lower for NTHi strains 3655 and 11P6H as compared to strain R2866 [4].

In order to compare complement-mediated killing and opsonophagocytic-dependent killing for Gram negative and Gram positive bacteria, we determined survival of *S. pneumoniae* and NTHi strain 3655 with heat-inactivated plasma, plasma and whole blood. Both plasma and whole blood showed significant killing of NTHi strain 3655, whereas this was only the case with whole blood for *S. pneumoniae* (**Figure 2D**). These data clearly indicate that killing of Gram negative, unencapsulated, NTHi strain 3655 was largely dependent on complement-mediated killing, whereas killing of *S. pneumoniae* was dependent on complement activation and opsonophagocytosis.

Modulation of bacterial, cellular and humoral factors contributing to whole blood killing.

With this whole blood killing assay, bacterial factors as well as host cellular and humoral factors can be modulated to determine their contribution to

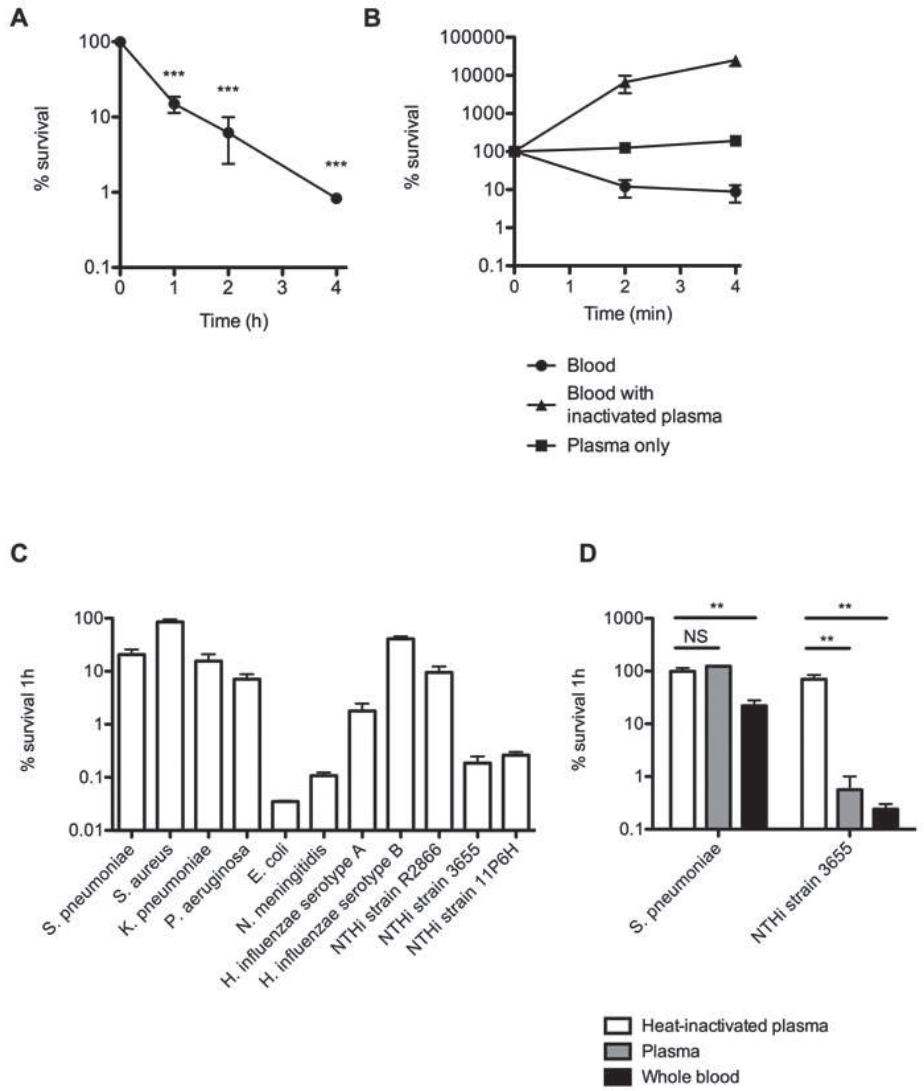

Figure 2. Phagocytes and active complement are required for efficient opsonophagocytic killing of *S. pneumoniae* in whole blood. (**A**) Bacterial survival in hirudin anticoagulated whole blood was determined after 1, 2 and 4 hours incubation (n=3). One-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison Test was used for statistical analysis. *** = $p<0.001$. (**B**) Bacterial survival in whole blood, blood with heat-inactivated plasma and plasma only was determined after 1, 2 and 4 hours incubation (n=7). (**C**) Killing in hirudin anticoagulated whole blood of *S. pneumoniae*, *S. aureus, K. pneumoniae*, *P. aeruginosa*, *E. coli*, *N meningitidis* and *H. influenzae* were determined after 1 hour incubation (n=3). (**D**) Killing of *S. pneumoniae* and non-typeable *H. influenzae influenzae* (NTHi) strain 3655 was determined with heat-inactivated (HI) hirudin plasma, hirudin plasma and hirudin anticoagulated whole blood after 1 hour incubation (n=2).

opsonophagocytic killing. For instance, blocking complement receptor 3 (CR3) with α-CD11b antibody 44a decreased killing of *S. pneumoniae* (**Figure 3A**), indicating that recognition of C3b on the bacterial surface by phagocytes contributes to killing. The contribution of the CR3 in opsonophagocytosis is *S. pneumoniae* by neutrophils and macrophages is widely investigating [49-51], and our results are consistent with these studies.

In addition, treatment of blood with cytochalysin D, an inhibitor for actin polymerization, also decreased killing of *S. pneumoniae* (**Figure 3A**), indicating that killing was dependent on phagocytosis.

Recently, Corriden *et al* showed that tamoxifen augmented neutrophil-mediated killing of *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* through enhancing several pro-inflammatory pathways in human neutrophils, including chemotaxis, phagocytosis and neutrophil extracellular trap (NET) formation [52]. Here, we show that adding 10 μM 4-hydroxytamoxifen significantly augmented killing of *S. pneumoniae* in whole blood (**Figure 3B**).

Killing of *S. pneumoniae*, but also other pathogens, is affected by the presence of opsonizing antibodies and the overall complement activity. To determine the role of complement activity, we used whole blood of which plasma was removed by centrifugation and replaced with 50%, 25% or 10% plasma containing active complement (see Material and Methods section for procedure). Replacement of the total amount of active plasma with 50% active plasma clearly decreased the killing capacity, which was even more apparent for 25% and 10% active plasma (**Figure 3C**), indicating that decreasing the level of active complement reduces the capacity to clear *S. pneumoniae* from blood in a dose dependent manner.

Previously, we have used the whole blood killing assay to assess the contribution of human factor H in controlling complement activity and killing of *S. pneumoniae* by replacing plasma with factor H-depleted serum and supplementation with different concentrations of purified human factor H. In this assay, we showed that increasing human factor H to blood increased survival of *S. pneumoniae*, whereas decreasing factor H levels increased killing [53]. Adding 100 μg/mL factor H to whole blood decreased killing of *S. pneumoniae* significantly (**Figure 3D**), which is in accordance with the findings that higher factor H levels decreased bacterial killing [53]. Binding of factor H to the bacterial surface was shown to protect many bacteria from complement-mediated opsonization [4,5,7,54-58]. Pneumococcal surface protein C (PspC) of *S. pneumoniae* is known to bind human factor H. In order to determine

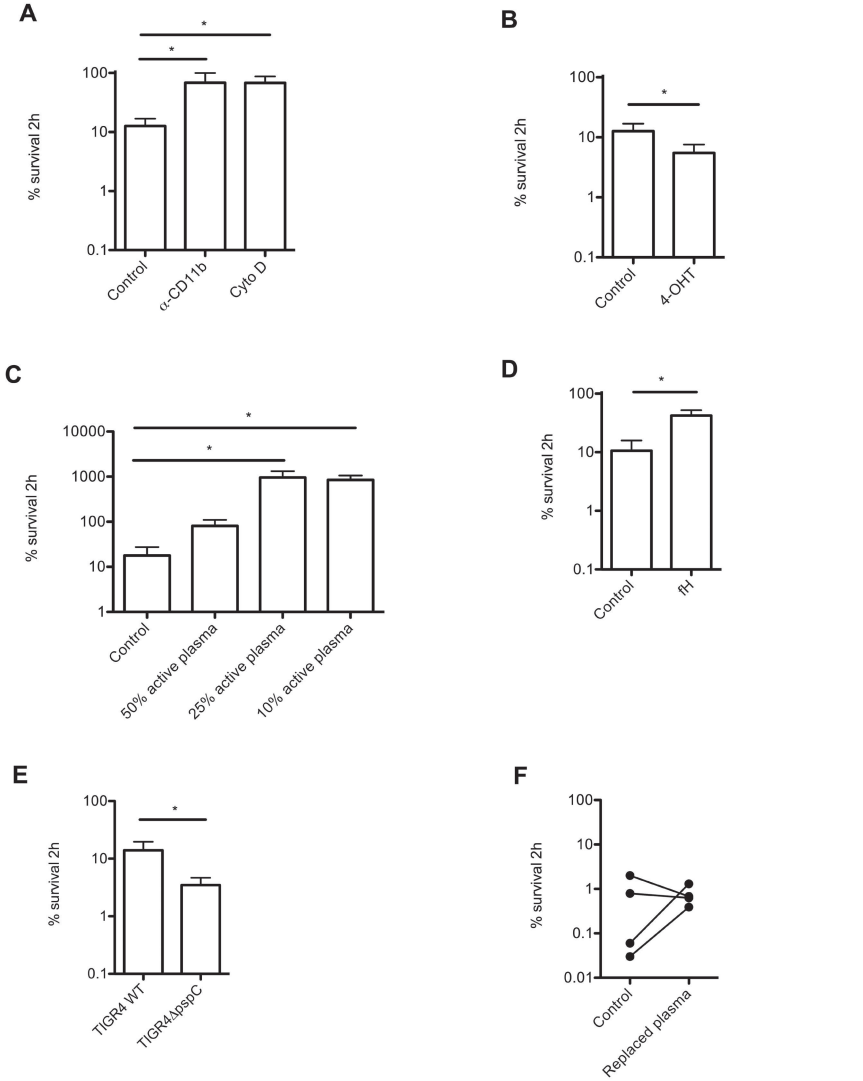

Figure 3. Modulation of *S. pneumoniae* killing by modulating phagocytosis or complement activity. Bacterial survival in hirudin anticoagulated whole blood was determined after 2 hours incubation in the presence of (**A**) 10 mg/mL CD11b blocking antibody (α-CD11b), 10 mM actin polymerization inhibitor cytochalysin D (CytoD), (**B**) 10 mM 4-hydroxitamoxifen (4-OHT) or (**D**) 100 mg/mL human factor H (fH). (**C**) Bacterial survival in hirudin anticoagulated whole blood and blood with 50%, 25% and 10% active plasma was determined after 2 hours incubation (n=4). (**E**) Bacterial survival of TIGR4 wild-type (WT) and TIGR4Δ*pspC* were determined after 2h in hirudin anticoagulated whole blood. (**F**) Bacterial survival of TIGR4 was determined after 2h in hirudin anticoagulated whole blood with or without plasma replacement. One-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison Test was used for statistical analysis (**A and C**). A one-tailed student t-test was used for statistical analysis (**B, D, E and F**). * = $p<0.05$.

the role for factor H binding in whole blood killing we determined survival of a Δ*pspC* mutant and found that this mutant, as expected, had a decreased survival in whole blood (**Figure 3E**). Overall, this demonstrates several possibilities in studying functions of complement in bacterial clearance using the whole blood killing assay.

While performing our whole blood killing experiments, we observed large inter-patient differences in *S. pneumoniae* survival (% survival 0.03 - 2.00) (**Figure 3F**). To determine whether these differences can mainly be attributed to differences in plasma content or phagocyte function, we used whole blood from which plasma was removed by centrifugation and replaced it with pooled plasma in which the concentrations of opsonizing antibodies and the complement activity are constant (see Material and Methods section for procedure). In this assay, killing of *S. pneumoniae* was more consistent (% survival 0.39 - 1.30), compared to survival in blood from the four single donors (% survival 0.03 - 2.00) (**Figure 3F**). These data indicate that mainly differences in plasma components (opsonizing antibodies and complement activity) between these four donors attribute to the inter-donor variation in whole blood killing capacity. This approach can also be used to determine vaccine-induced protection. Previously, Welsch *et al* showed that supplementation of whole blood with 25% heat-inactivated post-vaccination serum increased killing of *N. meningitidis* compared to pre-immunization serum [34]. This approach enables comparison of whole blood killing of pathogens with different serum samples in combination with a single fresh blood donor.

Contribution of C6 in opsonophagocytic-mediated killing of *N. meningitidis.*

Patients with deficiencies in the terminal complement components are more susceptible to invasive infections by *N. meningitidis* [59]. To mimic this in our whole blood killing assay, we replaced plasma with 10% C6-depleted serum and determined survival of *N. meningitidis* serogroup B strain H44/76 after 30 minutes. The presence of 10% heat-inactivated C6-depleted serum showed 9.1% survival (**Figure 4A**), indicating that *N. meningitidis* serogroup B strain H44/76 is killed by complement-independent mechanisms, which has been described in literature previously. For instance, *N. meningitidis* serogroup C was killed by antibody-dependent cell-mediated antibacterial activity [60] as well as opsonin-independent phagocytosis [61]. Opacity (Opa) proteins have been implicated to be important in opsonin-independent phagocytosis of *N. meningitidis* [62,63] through neutrophil surface receptors CD66 and CR3 [64,65], whereas macrophages bind unopsonized *N. meningitidis* almost exclusively via the class A macrophage scavenger receptor [66].

Although not investigated in detail, we show that *N. meningitidis* serogroup B strain H44/76 is efficiently killed through complement-independent mechanism.

To study the contribution of complement in addition to complement-independent mechanisms, we used 10% C6-depleted serum in the whole blood killing assay. When 10% C6-depleted serum was used, survival was significantly lower (4.0%) compared to heat-inactivated serum (**Figure 4A**). Since C6-depleted serum is not able to form a membrane attack complex, this increased killing is likely due to complement-dependent opsonophagocytosis. In order to restore terminal complex activity, we supplemented C6-depleted serum with C6 and observed a significant increase in killing, implicating that formation of membrane attack complex, next to opsonin-dependent and opsonin-independent killing, contributed to overall clearance of *N. meningitidis* from whole blood.

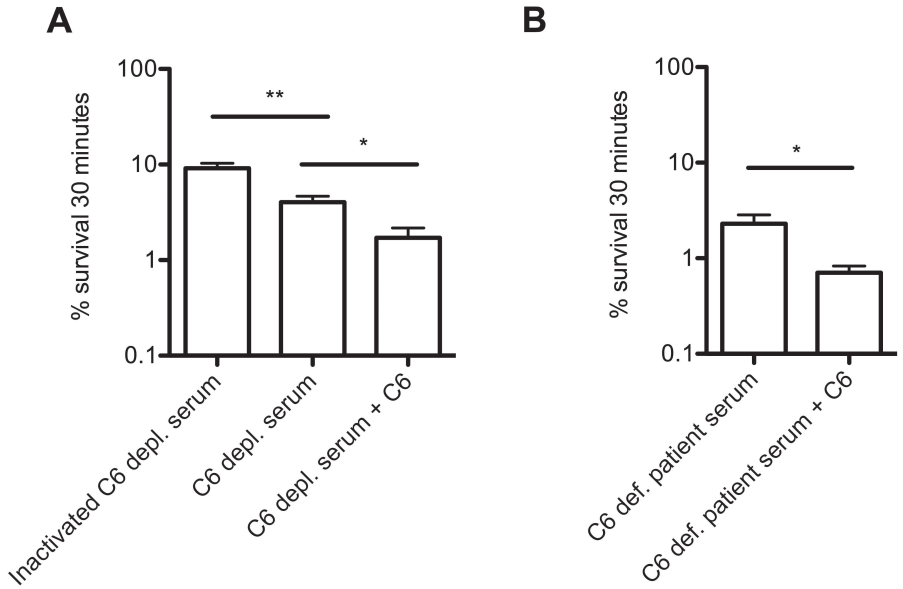

Figure 4. Contribution of C6 in opsonophagocytic-mediated killing of *N. meningitidis*. Bacterial survival of *N. meningitidis* strain H44/76 was determined after 30 minutes in hirudin anticoagulated whole blood with (**A**), 10% heat-inactivated serum, 10% C6-depleted serum, 10% C6-depleted serum supplemented with normal concentration C6 (see Material and Methods), (**B**), 10% C6-deficient patient serum, 10% C6-deficient patient serum supplemented with normal concentration C6. A one-tailed student t-test was used for statistical analysis. * = $p<0.05$, ** = $p<0.01$.

Previously, we have described a patient with a novel heterozygous missense mutation in the *C6* gene. Next to this novel heterozygous C6 mutation, a known heterozygous splice site variation was also identified, resulting in a C6 molecule that is 14% shorter due to a premature stop codon, but can still be build into the terminal complement complex, can kill bacteria, and is hemolytically active [67,68]. But, both mutations resulted in a lower (5%) C6 protein level. Normal immunoglobulin levels (IgG/IgA/IgM/IgE) and other complement factors (C3, C4) were found. When 10% C6-deficient patient serum was used, survival was low (2.3%) (**Figure 4B**), which was consistent with results obtained with C6-depleted serum. Survival was significantly lower when C6 was reconstituted (0.7%) (**Figure 4B**), indicating that C6 supplementation increased bacterial killing in whole blood.

Altogether, these results with C6-depleted serum and C6-deficient patient serum obtained similar results; decreased killing as compared to C6-reconstituted serum, which is consistent with the clinical phenotype of these patients.

Monocytes and neutrophils contribute to opsonophagocytosis of *S. pneumoniae.*

In order to address which cell type was predominantly responsible for opsonophagocytosis of *S. pneumoniae* in whole blood, we labelled *S. pneumoniae* with CFSE as previously described [24]. CFSE-labelled bacteria were added to whole blood in the absence or presence of cytochalysin D to block phagocytosis. Especially monocytes (74%) and neutrophils (72%) were found to bind and phagocytose *S. pneumoniae*, which was only 9% for lymphocytes (**Figure 5A**). Cytochalysin D decreased *S. pneumoniae* association to monocytes, neutrophils and lymphocytes to 24%, 42% and 5%, respectively, indicating that half of the cells in the control condition actually phagocytosed *S. pneumoniae*, whereas the other half of the CFSE-labelled bacteria were cell-associated. When the total percentage of *S. pneumoniae* association with cells was determined, most of them, 85%, were neutrophils, 10% monocytes and 5% lymphocytes (**Figure 5B**), indicating that neutrophils are the most important cell type for opsonophagocytosis of *S. pneumoniae* in whole blood. These results are consistent with previous literature where phagocytosis experiments showed efficient uptake of opsonized *S. pneumoniae* by both macrophages and neutrophils [69-71]. The important role for neutrophils in opsonophagocytic killing and protection against pneumococcal disease is supported by *in vivo* models wherein neutrophils were depleted [72,73].

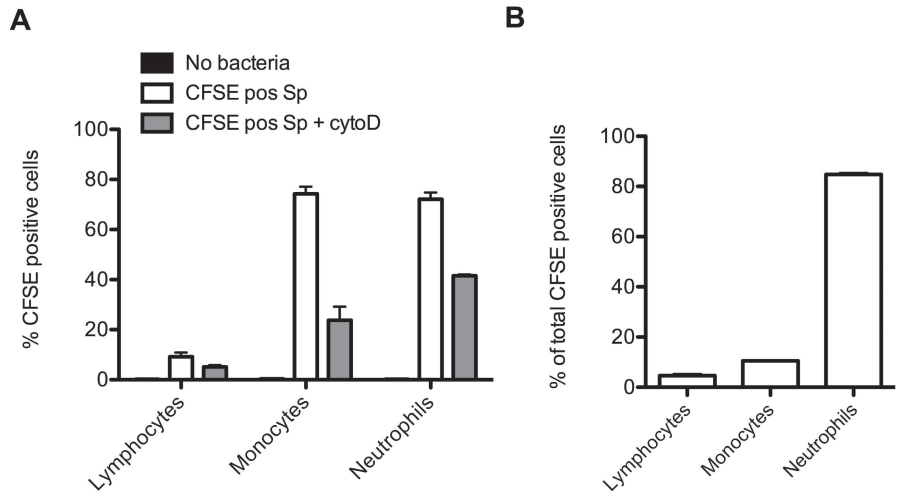

Figure 5. Whole blood killing of *S. pneumoniae* is mainly dependent on neutrophil-mediated opsonophagocytosis. (**A**) *S. pneumoniae* was loaded with 10 mM CFSE and incubated 30 min in hirudin anticoagulated whole blood. Erythrocytes were removed by hypotonic shock and the percentage CFSE positive lymphocytes (CD3 positive), monocytes (CD14 positive / CD16 negative) and neutrophils (CD16 positive / CD66b positive) were determined by flow cytometry. (**B**) Percentage of CFSE positive cells were determined.

Conclusion

The use of hirudin-anticoagulated whole blood enabled us to study the contribution of both bacterial and host factors in the killing of several pathogens, including *S. pneumoniae*, *K. pneumoniae*, *S. aureus* and *H. influenzae*. Complement activity preservation of hirudin was superior compared to lithium heparin, sodium heparin, EDTA or sodium citrate. Altogether, we describe a versatile assay to determine bacterial and host factors affecting opsonophagocytic killing of bacteria in hirudin-anticoagulated whole blood as a model for bacteremia.

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Chapter 6

Streptococcus pneumoniae PspC-subgroup prevalence in invasive disease and difference in contribution to complement evasion

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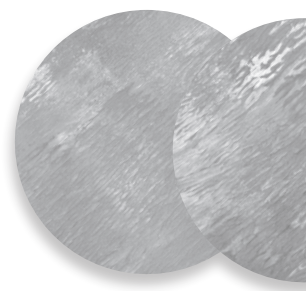

Abstract

Pneumococcal capsular serotype is an important determinant of complement resistance and invasive disease potential, but other virulence factors have also been found to contribute. Pneumococcal surface protein C (PspC), a highly variable virulence protein that binds complement factor H to evade C3 opsonization, is divided into two subgroups: choline-bound subgroup I and LPxTG-anchored subgroup II. The prevalence of different PspC subgroups in invasive pneumococcal disease (IPD) and functional differences in complement evasion are unknown. Prevalence of PspC subgroups in IPD isolates was determined in a collection of 349 sequenced strains of *S. pneumoniae* isolated from adult patients. *PspC* deletion and isogenic *pspC*-switch mutants were constructed to study differences in factor H binding and complement evasion in relation to capsule thickness. Subgroup I *pspC* was far more prevalent in IPD isolates than subgroup II *pspC*. Increased capsular thickness was associated with a greater ability of bound factor H to reduce complement opsonization. Pneumococcal subgroup I PspC bound significantly more factor H and showed more effective complement evasion compared to subgroup II PspC in isogenic encapsulated pneumococci. Variation in PspC subgroup, independent of capsule serotypes, affects pneumococcal factor H binding and its ability to evade complement deposition.

Introduction

Streptococcus pneumoniae is an important human pathogen that colonizes the upper respiratory tract. This pathogen is also an important cause of invasive diseases such as pneumonia, sepsis and meningitis. The pneumococcal polysaccharide capsule affects complement resistance and protects against phagocytic killing [1,2]. Epidemiological studies found that particular capsular serotypes are dominant in invasive disease whereas others are associated with nasopharyngeal carriage [3,4]. Besides the important role of the pneumococcal capsule, the genotype also affects complement resistance. Within the same serotype, significant differences in complement C3 deposition between isolates have been observed, indicating that the genetic background of the strain also affects complement resistance [5]. A recent study suggests that within the same serotype and clonal complex, genetic differences in virulence genes encoding pneumococcal surface protein A and C (PspA and PspC), affect the invasive disease potential [6]. It is therefore of interest to gain more insight into how genetic variation in these genes affects complement resistance and contribute to pneumococcal virulence.

The complement system is an essential component of the host defense against *S. pneumoniae* [7]. Complement activation by one of the three pathways: the classical, the lectin and the alternative pathway, leads to opsonization of the bacterial surface with C3 activation products C3b and iC3b. These opsonins mediate phagocytosis mainly through complement receptors CR1 and CR3. Importantly, the alternative pathway amplifies the initial complement activation [8]. C3b deposited on the bacterial surface is formed into an alternative pathway C3 convertase cleaving more C3, which enhances C3b opsonization [9]. The importance of the alternative pathway in complement activation is emphasized by the fact that many pathogens possess mechanisms to inhibit alternative pathway activation by binding of the host alternative pathway inhibitor factor H [10-14].

S. pneumoniae binds human factor H by PspC, also referred to as CbpA, SpsA, PbcA and Hic [15-19]. Factor H binding by PspC is a mechanism to evade complement deposition. In addition, PspC acts as an adhesion molecule by interacting with the secretory component of human IgA, the laminin receptor and epithelial/endothelial polymeric immunoglobulin receptor (pIgR), which may be an entry vehicle for PspC mediated invasion [16,17,20-25]. *In vitro* studies using human serum demonstrate that factor H binding by *S. pneumoniae* strains is dependent on the presence of PspC, but that the level of binding is influenced by the capsular serotype [1,26]. In addition, within a serotype, spontaneous opaque/transparent phase variation

occurs, which affects complement deposition [27,28]. An important characteristic of the pneumococcal phase variation is the difference in capsule thickness. Opaque phase pneumococci with increased amounts of capsular polysaccharide show enhanced complement resistance, which has been associated with better survival in blood [29-31]. Other differences between opaque and transparent phases, besides the capsule thickness, have been described. For example transparent phase pneumococci have higher levels of lipoteichoic acid with phosphorylcholine residues[27,32].

The *pspC* gene shows large allelic variation. Eleven different types of *pspC* have been identified based on clusters of sequence homology. PspC consists of a C-terminal repeat region, a proline-rich domain, and a N-terminal α-helical domain [33]. A factor H binding region of 121 amino acids has been identified at the N-terminal region (residues 38-158), containing multiple epitopes for factor H binding [21]. At the C-terminal region, a major difference in anchor sequence has been identified dividing *pspC* into two subgroups; allelic variants with a choline binding domain (classical, subgroup I) or a LPxTG anchoring domain (non-classical, subgroup II) [33].

The prevalence and distribution of the different PspC subgroups and types in invasive disease or carriage isolates has not been characterized thoroughly. Though, Iannelli *et al* demonstrated a predominance of subgroup I PspC (74%) in a collection of 43 strains containing randomly chosen clinical isolates, standard laboratory strains and American Type culture Collection strains [33]. However, it is not known whether variation in PspC type, independent of capsule differences, affects pneumococcal factor H binding and its ability to evade complement deposition. Here we describe a far greater prevalence of choline bound subgroup I PspC types as compared to LPxTG anchored subgroup II PspC types in invasive pneumococcal disease isolates. In addition, using isogenic *pspC* switch mutants, we demonstrate that subgroup I PspC is more effective in complement evasion than subgroup II PspC. These findings indicate that PspC-specific differences contribute to intra-serotype variation in complement resistance.

Material and Methods

Pneumococcal strain collection, sequence typing and PspC typing

The prevalence of various *pspC* types was studied in 349 sequenced *S. pneumonia*e isolates from invasive pneumococcal disease (IPD) patients in Nijmegen, the Netherlands, (2001 -2011; All bacteraemia isolates from patients with pneumonia (n=312), meningitis (n=30) and endocarditis (n=4)) [34,35]. The median age was 67 years (53-78 interquartile range), male/female ratio 0.86. This observational cohort study was approved by the Local Medical Ethics Committees of both participating hospitals. Genome sequences, serotypes and sequence types were obtained from Cremers *et al.,* 2015 [34]. PspC coding genes were identified from the genome sequences by aligning the 40 amino acid conserved N-terminus of PspC against all protein coding sequences of the genomes using BLASTP [36]. PspC types were determined by aligning the PspC coding proteins against the known PspC protein sequences [33] using BLASTP and the blast results were manually inspected.

Construction of *pspC* deletion and isogenic *pspC*-switch mutants

Four different *pspC* types were selected from the bacteraemia cohort and constructed into an isogenic background. The *pspC* types have been described by Ianelli *et al*., 2002 [33]. Based on this description we selected two PspC types with a choline binding anchor and two PspC types with a LPxTG binding anchor at the C-terminal domain with differences in the N-terminal factor H binding domain. To obtain these PspC types, we searched our strain collection and found isolates containing *pspC* genes belonging to the previously described PspC types. *PspC* type 2 was obtained from strain PBCN0094 (10050_2#53) and was 100% identical to the previously described *pspC* 2.2 [33], also found in TIGR4. *PspC* types 6 and 9 were both obtained from strain PBCN0133 (10050_2#83) and were cloned separately into an isogenic background. The *pspC* 9 showed 91% identity with previously described *pspC* 9.1 with all differences in the repeat region and *pspC* 6 showed 100% identity with the previously described *pspC* 6.1. The *pspC* 11, also known as hic, was found in strain PBCN0031 (10050_2#18) and showed 97% identity with previously described *pspC* 11.3. The pspC types found in these strains were cloned into an encapsulated TIGR4 strain and an unencapsulated R6 strain.

TIGR4Δ*pspC* and R6Δ*pspC* deletion mutants were constructed by allelic replacement of the target gene with an antibiotic resistance marker as described previously [37]. The various *pspC* types were cloned into the *pspC* deletion mutants to avoid recombination with the original *pspC* gene. Again, a megaprimer PCR product was constructed, but contained besides the *pspC* flanking regions and the spectinomycin cassette, the selected *pspC* type originated from the above mentioned clinical isolates. The spectinomycin cassette and the right flank were PCR amplified from the TIGR4Δ*pspC* containing the spec cassette. The megaprimer PCR product was used to transform R6Δ*pspC* (kanamycin resistant). Directed mutants were obtained by selective plating (spectinomycin) and were checked for

correct integration of the antibiotic resistance cassette and *pspC* variant gene by PCR using control primers located inside the genes. A PCR product from the left flank to the right flank containing the inserted *pspC* type and the spectinomycin cassette was used to transform TIGR4Δ*pspC* (kanamycin resistant). Mutants were obtained by selective plating and were checked for correct integration of the antibiotic resistance cassette and *pspC* variant gene by PCR using control primers located inside the genes and sequencing. The primer sequences (obtained from Biolegio, Nijmegen, The Netherlands) are indicated in supplementary table 1. Correct integration of the *pspC* gene was confirmed by sequencing.

Bacterial strains and culture conditions

Bacteria were grown on Columbia blood agar plates (Becton Dickinson) and in Todd-Hewitt broth supplemented with 5 g/L yeast extract (THY) at 37°C and 5% CO_2 to an OD620 of 0.2 was reached. The number of colony forming units per milliliter was determined by plating serial 10-fold dilutions on blood agar plates. To obtain opaque and transparent phase TIGR4 wild-type and Δ*pspC*, frozen stock cultures were plated on THY agar supplemented with 5000 U of catalase (Sigma) and incubated at 37°C and 5% CO_2. Colony morphology of variants on THY-catalase plates was determined as described previously [28]. Opaque and transparent variants were selected and subcultured two times and grown in THY broth as described above.

Factor H binding and C3 deposition assay

TIGR4 or R6 strains wild-type or mutant ($1x10^7$) were pelleted in a 96-well plate and resuspended in 10% (vol/vol) pooled normal human serum (GTI Diagnostic) in Hanks Buffered Salt Solution containing Ca^{2+} and Mg^{2+} to a total volume of 100 µL. The bacterial suspension was incubated for 30 min at 37°C in 5% CO_2. After incubation, the bacteria were washed and labelled with polyclonal sheep anti human factor H (Abcam; cat#8842) or with the FITC-conjugated goat anti-human C3 (Cappel; cat#55197) diluted in PBS + 2% BSA. After 30 min incubation, bacteria were washed and the anti-factor H antibody was labelled with FITC-donkey anti-sheep IgG antibody (Jackson immunoresearch; #713-095-003). Bacteria were fixed with 2% paraformaldehyde. Factor H binding and C3 deposition were measured using a LSR II flow cytometer (BD Biosciences). Data were analysed using FlowJo v10.1.

Real-time RT-PCR

RNA from overnight grown strains of TIGR4 and R6 expressing *pspC* type 2, 6, 9 or 11 was extracted using RNAeasy Minikit (Qiagen) and DNA was removed with Turbo-DNAse (Ambion). RNA (1µg) was reverse transcribed in cDNA using iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed using SYBR green chemistry (Bio-Rad) and PspC specific primers on a CFX-96 real-time PCR machine (Bio-Rad) (**supplementary table 1**). GyrA was used as reference gene. Quantification cycle (Cq) values and relative expression values were calculated using the CFX Manager software (Bio-Rad).

Statistics

Differences between strains were analyzed using repeated measures ANOVA with Bonferroni corrections for multiple comparisons. Differences were considered statistically significant when $P < 0.05$.

Results

PspC subgroup I is most prevalent in invasive pneumococcal disease isolates

Analysis of the 349 invasive disease strains demonstrated that PspC subgroup I was present in 298 isolates (85,4%) and in an additional 19 isolates (5,4%) that contained both subgroup I and subgroup II PspC. Only 22 of the isolates (6,3%) contained subgroup II and 10 isolates (2,9%) had no *pspC* (**Table 1**). Strains containing two *pspC* genes were mainly of serotypes 2, 6A, 6B, 19A and 19F. Strains containing subgroup II were mostly serotype 3 with sequence type (ST) 180 (18 out of 22). Within a serotype, we found large variations in PspC types, whereas some serotypes contained only a single PspC type. Most sequence types were associated with a specific PspC type, though also variation in PspC type within sequence types was observed (**supplementary Table 2**). We observed no association between PspC subgroups and disease outcome.

Table 1. PspC subgroup I is most prevalent in invasive pneumococcal disease isolates. The prevalence of various *pspC* subgroups was studied in 349 sequenced *S. pneumoniae* isolates from invasive pneumococcal disease (IPD) patients in Nijmegen, the Netherlands [34,35].

	Number of isolates (n= 349)	Percentage of total
PspC subgroup I Choline binding domain	**298**	**85.4**
PspC subgroup II LPXTG binding anchor	22	6.3
PspC Subgroup I and II	19	5.4
No PspC	10	2.9

Capsular thickness and PspC-mediated factor H binding contributes to complement evasion

Capsular polysaccharide is known to inhibit complement opsonization [29]. In addition, factor H binding by PspC is influenced by capsular serotype [1,2]. We assessed the effect of pneumococcal capsule thickness on factor H binding to PspC. We compared factor H binding to wild-type and Δ*pspC* in thick encapsulated TIGR4 (opaque phase), thin encapsulated TIGR4 (transparent phase), unencapsulated mutant TIGR4 (TIGR4 Δ*cps*) and in unencapsulated R6. In all backgrounds, deletion of *pspC* resulted in significantly reduced factor H binding measured by flow cytometry following incubation in pooled human serum (**Figure 1A,B**), demonstrating that PspC is the main factor H binding protein. Factor H binding to transparent phase, unencapsulated TIGR4 or R6 was significantly higher compared to the opaque phase TIGR4.

We assessed the effect of pneumococcal capsule thickness and *pspC* expression on complement resistance by comparing C3 opsonization of TIGR4 wild-type and *pspC* deletion mutants in the opaque and transparent phase and of unencapsulated TIGR4 and R6. Opaque TIGR4 showed significantly reduced C3 opsonization compared to the opaque TIGR4 Δ*pspC* mutant (**Figure 1C**). This demonstrates that for TIGR4 with a thick capsule, factor H binding contributes to complement evasion by reducing C3 opsonization. Transparent phase TIGR4 bacteria showed significantly enhanced C3 deposition compared to the TIGR4 opaque phase bacteria. Loss of *pspC* had no effect on C3 opsonization in TIGR4 transparent phase, as no significant difference in C3 deposition was measured between transparent TIGR4 wild-type and Δ*pspC* (**Figure 1C**). This indicated that factor H binding to PspC of TIGR4 with a thin capsule did not contribute to complement resistance. This was further confirmed with unencapsulated TIGR4 and the unencapsulated R6 strain, as loss of *pspC* did not enhance complement C3 deposition (**Figure1D**). R6Δ*pspC* even showed a slightly reduced C3 deposition compared to the wild-type R6.

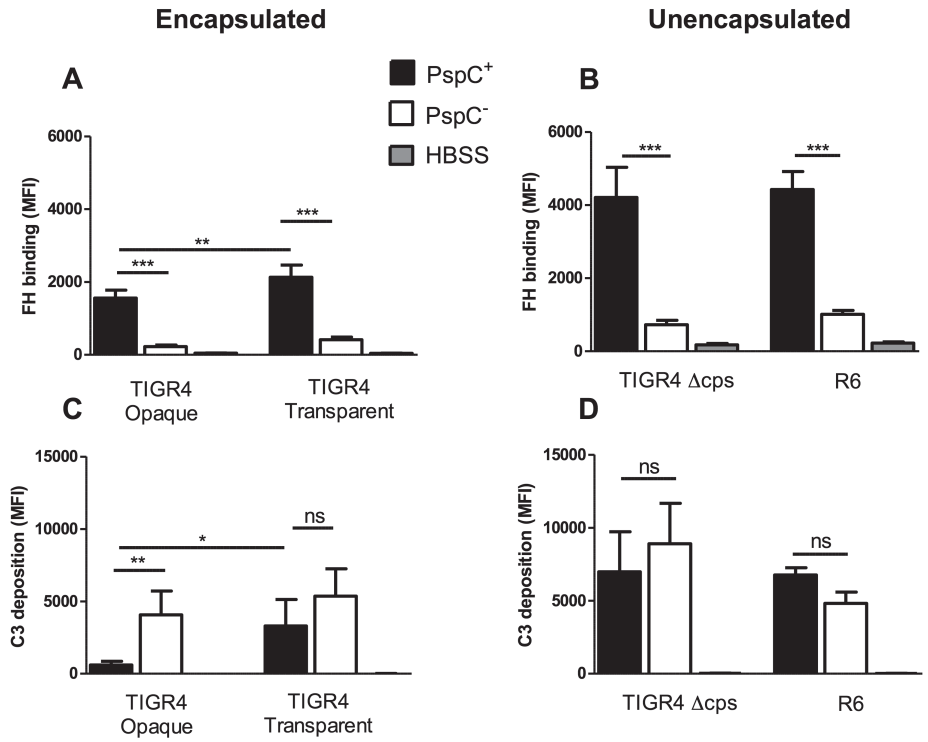

Figure 1. The effect of factor H binding on complement deposition is dependent on capsule thickness. Factor H binding and C3 deposition on the surface of *Streptococcus pneumoniae* strain TIGR4 wild-type opaque/transparent phase, unencapsulated TIGR4 (Δcps) and unencapsulated R6 and its *pspC* deletion mutants (pspC$^-$) was measured. Factor H (FH) binding and C3 deposition were measured by flow cytometry following incubation of the bacteria for 30 min at 37 °C in 10% pooled human serum diluted in HBSS. Panel **A** and **B** represent the factor H binding and panel **C** and **D** the C3 deposition both in mean fluorescence intensity (MFI). Each bar represents the mean ± standard deviation for results obtained from three or four separate experiments. Comparisons between strains was performed using an ANOVA for repeated measurements with a Bonferroni correction for multiple comparisons. *$P < .05$, **$P < .01$, ***$P < .001$. Abbreviation: ns, not significant

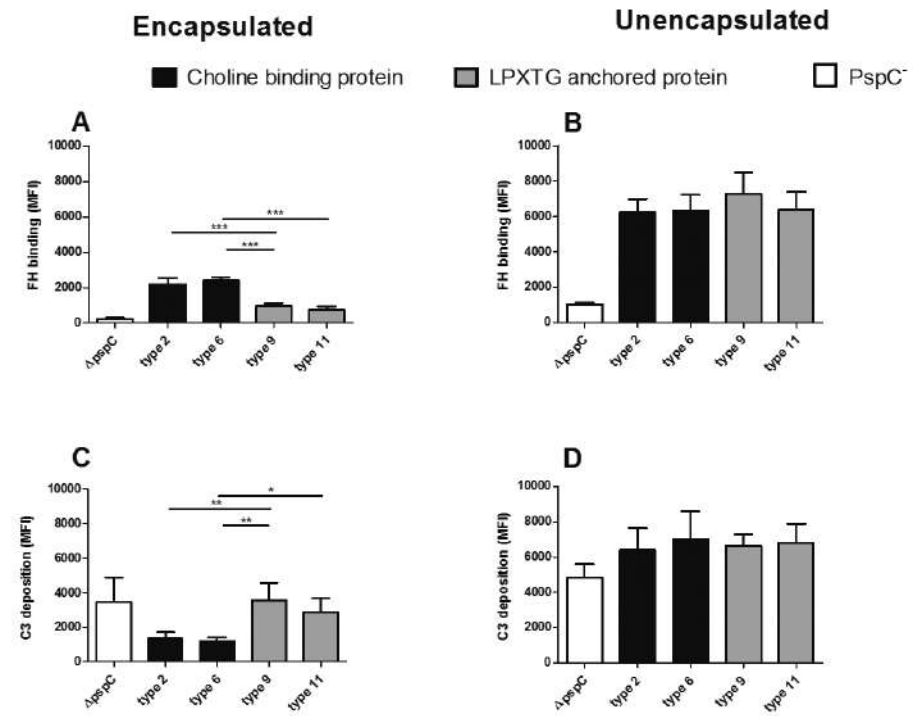

Figure 2. PspC subgroup specific differences affect factor H binding and complement resistance in encapsulated TIGR4 but not in unencapsulated R6. Factor H binding and C3 deposition on the surface of *Streptococcus pneumonia* TIGR4 Δ*pspC* (white bars) or TIGR4 containing *pspC* type 2 and 6 of subgroup I (black bars) and PspC type 9 and 11 of subgroup II (gray bars). Factor H (FH) binding and C3 deposition were measured by flow cytometry following incubation of the bacteria for 30 min at 37 °C in 10% pooled human serum diluted in HBSS. Panel **A** and **B** represent the factor H binding and panel **C** and **D** the C3 deposition both in mean fluorescence intensity (MFI). Each bar represents the mean ± standard deviation for results obtained from three separate experiments. Comparisons between *pspC* types were performed using an ANOVA for repeated measurements with a Bonferroni correction for multiple comparisons. **P* < .05, ***P* < .01, ****P* < .001. Abbreviation: ns, not significant

PspC subgroup specific differences in factor H binding and complement resistance

PspC types 2, 6, 9 and 11 were selected because PspC type 2 and 6 (subgroup I) have a choline binding domain and PspC type 9 and 11 (subgroup II) have an LPxTG binding anchor and these types are heterogeneous in their factor H binding region. The four *pspC* types were cloned into TIGR4 replacing the original *pspC* gene in order to study PspC type specific differences in complement evasion within the same genetic background. We found significantly enhanced factor H binding to pneumococci expressing subgroup I PspC types (2 and 6), compared to subgroup II PspC types (9 and 11) in the TIGR4 genetic background (**Figure 2A**). Consistently, factor H binding inversely correlated with C3 deposition on the bacterial surface, thus subgroup I PspC types 2 and 6 showed significantly reduced C3 deposition compared to subgroup II PspC types 9 and 11 in the encapsulated mutants (**Figure 2C**). This demonstrated that PspC-specific differences affected factor H binding and complement resistance in encapsulated pneumococcus. In an unencapsulated background, no difference in factor H binding between the various PspC types of subgroup I and II was found (**Figure 2B**). In addition, no difference in C3 opsonization was found between the subgroup I and subgroup II PspC types in the unencapsulated R6 strains (**Figure 2D**). qPCR confirmed that *pspC* expression levels of the 4 different PspC types within TIGR4 or R6 were equal (**Supplementary figure 1**).

Discussion

In this study we found that subgroup I PspC types are more prevalent in IPD isolates than subgroup II PspC types. Expression of different PspC types in isogenic encapsulated pneumococci showed that subgroup I PspC types are more effective in complement evasion compared to subgroup II PspC types. In addition, we found that capsule thickness affects PspC-mediated complement evasion. Previous studies examining the role of PspC in complement evasion have been performed in encapsulated, opaque phase, strains [1,26]. Different serotypes showed large variation in the effect on complement deposition upon loss of PspC, even though all serotypes were shown to bind factor H by PspC [26]. In line with this, we found that pneumococcal factor H binding is mainly PspC dependent, although some residual factor H binding was observed. This may be explained by factor H binding to elongation factor Tu (Tuf), which was recently found to bind human factor H [38]. In this study, we demonstrate that absence of a capsule or transparent phase capsule within the same genetic background, voids the ability of factor H binding to reduce complement deposition. These findings are in line with previous studies demonstrating that transparent phase pneumococci as well as unencapsulated strains are more sensitive for complement deposition [2,29]. This might be explained by the fact that transparent phase capsule or the absence of a capsule enhances antibody binding to subcapsular antigens and increased binding of complement mediators such as C-reactive protein, both known to activate the complement classical pathway [2,27]. Enhanced classical pathway activation may minimize

the effect of alternative pathway inhibition by factor H binding on the overall complement deposition. This may explain why factor H binding had no effect on complement C3 deposition for the unencapsulated strains [2,29].

The current study demonstrates that phase variation affects factor H binding and its role in complement evasion. Even though opaque phase TIGR4 bound less factor H as compared to the transparent phase TIGR4, the binding of factor H resulted in reduced complement deposition, suggesting that complement evasion by factor H binding is mainly of importance in the opaque phase. This is further supported by the observation that opaque phase variants are often found in invasive infections, in which pneumococcal complement evasion is vital for survival [27,29-31]. Carriage isolates, however, are mostly transparent phase bacteria [28]. We found increased factor H binding for transparent phase pneumococci, which is consistent with the observation by others that carriage isolates bind more factor H compared to systemic isolates [39]. However, our findings indicate that factor H binding to transparent phase pneumococci does not reduce C3 deposition on the bacterial surface. During nasopharyngeal carriage, other functions of PspC have been indicated by previous reports showing its importance in adherence [16,17,20-23,40]. In addition, our observation that PspC binding of factor H by unencapsulated strains had no effect on complement resistance may help to understand the loss of PspC in naturally occurring unencapsulated *S. pneumoniae* strains [41,42]. Other genes in the naturally occurring unencapsulated strains, such as the PspC-like protein PspK, were shown to play a role in colonization and adherence, but does not bind factor H [43-45].

We demonstrate that genetic differences in *pspC* affect complement resistance. Previous studies found differences in pneumococcal factor H binding between clinical isolates [1]. This could not, however, only be attributed to differences in *pspC* alone since also other factors such as the capsule type varied [1,21,26]. In the current study, four PspC types selected from a cohort with IPD isolates were cloned into an isogenic background, isolating the effect of PspC type difference on factor H binding and complement resistance. Remarkably, in the absence of capsule, we found no differences in factor H binding between strains expressing the various PspC types, even though the sequence of the previously defined 121 amino acid long factor H binding region varied extensively [21]. This indicates that all four PspC types have the same ability to bind factor H, but PspC in combination with the serotype 4 capsule resulted in differences in factor H binding. The length of the coding sequence varies between the PspC types, as *pspC* type 2 and 6 are 2082bp and 2046bp, respectively, whereas *pspC* group 9 and 11 are 1458bp and 1245bp, respectively. This differences in lengths may explain the observed differences in factor H binding in combination with capsule thickness. Some clinical isolates contain both PspC 6 and 9. Our findings demonstrate that both are able to bind factor H for unencapsulated strains, but in the presence of capsule, PspC type 6 binds more factor H and decreases C3 deposition to the bacterial surface to a larger extent compared to PspC 9.

Another important difference between the high and low factor H binding PspC types in our study is the PspC C-terminal domain, as PspC types 2 and 6 (subgroup I) have a choline binding domain and PspC types 9 and 11 (subgroup II) have a LPXTG binding anchor. Interestingly, the IPD isolates cohort described in this paper consists mainly of PspC types of subgroup I, whereas a much lower percentage of the PspC types belonging to subgroup II. Analysis of strain collections including carriage and invasive isolates from the same region and time period may help to further dissect the contribution of PspC subgroup and types to invasive disease potential. Additionally, more insight in epidemiological differences in PspC type prevalence and their contribution to invasive disease may have implications for vaccine design because PspC is an important vaccine candidate [46-48]. Our study demonstrates PspC subgroup and type distribution in invasive disease, in analogy to serotype distribution in invasive disease. No comparative data from a large set of clinical invasive disease isolates has been published to date. Though, Iannelli *et al* demonstrated a predominance of subgroup I PspC (74%) in a collection of 43 strains containing randomly chosen clinical isolates, standard laboratory strains and American Type culture Collection strains [33].

A strength of our study is that we studied various PspC types in an isogenic background. Others have demonstrated that capsule type affect factor H binding to PspC and complement evasion in capsule switch mutants [1]. Our findings are complementary to these data and demonstrate that genetic differences in *pspC,* using *pspC*-switch mutants, affects complement resistance in the presence of polysaccharide capsule. This is in line with a previous pediatric study in which genetic variation in *pspC* was suggested to explain differences in invasiveness within the same serotype and clonal complex [6].

In conclusion, we found a higher prevalence of subgroup I PspC types compared to subgroup II PspC types in IPD isolates. Expression of different PspC types in isogenic encapsulated pneumococci showed that subgroup I PspC types are more effective in complement evasion compared to subgroup II PspC types. In addition, we show that capsule thickness affects PspC-mediated complement evasion. These findings indicate that PspC type-specific differences contribute to intra-serotype variation in complement resistance.

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Supplementary table 1. Primers used to construct pneumococcal mutants.

Strain or primer	Relevant features or nucleotide sequence (5'to 3')[a]	Reference/ source or target gene[b]
PBCN0094	Serotype 4	[34]
PBCN0133	Serotype 6B	[34]
PBCN0031	Serotype 3	[34]
TIGR4	Serotype 4	[49]
R6	Unencapsulated	[50]
TIGR4Δcps	Unencapsulated TIGR4 with kanamycin	[51]
TIGR4ΔpspC (spec)	Serotype 4	This study
TIGR4ΔpspC (kan)	Serotype 4	This study
TIGR4 pspC2	Serotype 4	This study
TIGR4 pspC6	Serotype 4	This study
TIGR4 pspC9	Serotype 4	This study
TIGR4 pspC11	Serotype 4	This study
R6 pspC2	Unencapsulated	This study
R6 pspC6	Unencapsulated	This study
R6 pspC9	Unencapsulated	This study
R6 pspC11	Unencapsulated	This study
Primers mutant construction		
Spn2190_L1	TTG AGG CAA TGG TGC ACA AG	TIGR4 *pspC*; left flank
EMspn2190_L2	CCACTAGTTCTAGAGCGGCTACACTAGCTACTCCAACAC	TIGR4 *pspC*; left flank; overlap Spec^{res} cassette
EMspn2190_R1	CAAGATGAAGATCGCCTACG	TIGR4 *pspC*; right flank
EMspn2190_R2a	GCGTCAATTCGAGGGGTATCGCTATGGAGTCAATGCCAAT	TIGR4 *pspC;* right flank; overlap Spec^{res} cassette
EMspn2190_C	TCG TTC TCT GTC GCA TGA AC	TIGR4 *pspC*; control
PBMrTn9	CAATGGTTCAGATACGACGAC	Spec^{res} cassette; control
PBpR412_L	GCCGCTCTAGAACTAGTGG	Spec^{res} cassette; pR412 plasmid
PBpR412_R	GATACCCCTCGAATTGACGC	Spec^{res} cassette; pR412 plasmid
HBKanF4	GGAATTCGATATCAAGCTTA	Kan^{res} cassette; pR410 plasmid
HBKanR	AGGTACTAAAACAATTCATCC	Kan^{res} cassette; pR410 plasmid
HBKanR3	ATCCACATCGGCCAGATCGT	Kan^{res} cassette; control
EMspn2190_pspC_kan_L2	TAAGCTTGATATCGAATTCCTACACTAGCTACTCCAACAC	TIGR4 *pspC*; left flank; overlap Kan^{res} cassette
EMspn2190_pspC_kan_R2	GGATGAATTGTTTTAGTACCTGCTATGGAGTCAATGCCAAT	TIGR4 *pspC;* right flank; overlap Kan^{res} cassette
EM_pspC_2_3_L2 new	CTTTCGCTTTTTGATGCAAACATGTTTATTTCCTTCTATATTT	TIGR4; left flank; overlap *pspC* PBCN0094
EM_pspC_6_11_L2	CTTTCGTTTTTTGATGCAAACATGTTTATTTCCTTCTATATTT	TIGR4; left flank; overlap *pspC* PBCN000133 & PBCN0031
EM_pspC_9_L2	CGCTCATGATTTGATTTAAACATGTTTATTTCCTTCTATATTT	TIGR4; left flank; overlap *pspC* PBCN0133
EM_pspC_2_3_Fw	ATGTTTGCATCAAAAAGCGAAAG	PBCN0094 *pspC*; forward
EM_pspC_6_11_Fw	ATGTTTGCATCAAAAAACGAAAG	PBCN0133 & PBCN0031 *pspC*; forward
EM_pspC_9_Fw	ATGTTTAAATCAAATCATGAGCG	PBCN0133 *pspC*; forward
EM_pspC_2_3_6_R2_spec	CAATGGTGAATGGGTAAACTAAGCCGCTCTAGAACTAGTGG	TIGR4; right flank; overlap PBCN0094 & PBCN0133
EM_pspC_9_11_R2_spec	CTTGCTAAGAAAAGAATGAAATAGGCCGCTCTAGAACTAGTGG	TIGR4; right flank; overlap PBCN0133 & PBCN0031
EM_pspC_2_3_6_Rev	TTAGTTTACCCATTCACCATTG	PBCN0094 & PBCN0133 *pspC; reverse*
EM_pspC_9_11_Rev	CTATTTCATTCTTTTCTTAGCAAG	PBCN0133 & PBCN0031; pspC reverse
EM_pspC_2_Crev	GGTACTTGGGTAGCTCCCTC	*pspC2*; control
EM_pspC_3_9_Crev	CTGCTTGGGTACTTCCCTC	*pspC6*; control
EM_pspC_6_Crev	CGTGGAGTTATTCCCAATTCT	*pspC9*; control
EM_pspC_11_Crev	GCTACTTGGGTAGTTACCTC	*pspC11*; control
Mutant validation by qPCR		
Q-pspC-V2-F	CAGGCAGAACAAGGAGAACA	*pspC2*; forward
Q-pspC-V2-R	GCATAGCTCTCACCCACTATTT	*pspC2*; reverse
Q-pspC-V6-F	ACCGTAACTACCCAACCAATAC	*pspC6*; forward
Q-pspC-V6-R	CGAGATTCCTTAGCTTCCTCTTT	*pspC6*; reverse
Q-pspC-V9-F	TGGAAGTCAGGCAGAACAAC	*pspC9*; forward
Q-pspC-V9-R	GCATAGCTCTCACCCACTATTT	*pspC9*; reverse
Q-pspC-V11-F	TGGTTCATGCGACAGAGAAG	*pspC11*; forward
Q-pspC-V11-R	CATCGACTTGTTTAGCAGCTTTC	*pspC11*; reverse
Q-Sp-gyrA-F	AATGAACGGGAACCCTTGGT	*gyrA*; forward
Q-Sp-gyrA-R	CCATCCCAACCGCGATAC	*gyrA*; reverse

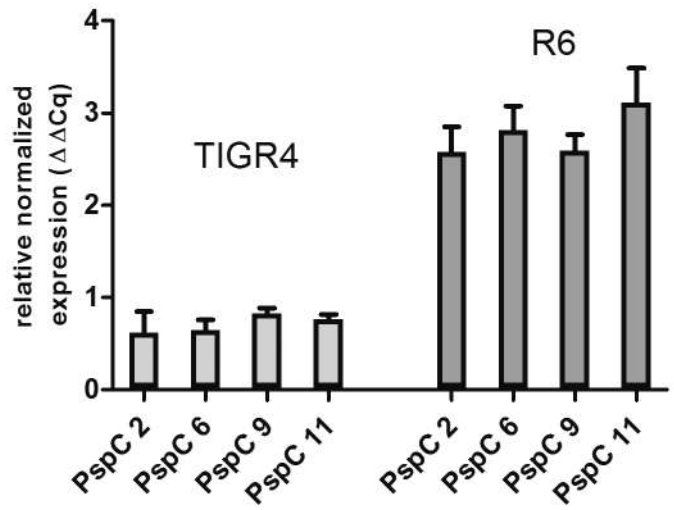
a Underlined sequences are complementary to primers used for amplification of antibiotic resistant cassette or the selected pspC variant.

b Left flank and right flank indicate positions relative to the target gene.

Supplementary Table 2.

Serotype	Sequence type	Number of isolates	pspC number in genome	best hit
1	9	1	1	Variant_4
1	304	5	1	Variant_2
1	306	27	1	Variant_2
1	350	1	1	Variant_2
3	180	4	0	
3	180	18	1	Variant_8
3	260	1	1	Variant_3
3	505	1	1	Variant_10
3	1220	3	1	Variant_3
3	6014	1	1	Variant_11
3	207	1	2	Variant_10 and variant_3
3	1377	1	2	Variant_3 and variant_9
4	162	1	1	Variant_3
4	205	12	1	Variant_2
4	206	1	1	Variant_2
4	246	3	1	Variant_2
4	247	9	1	Variant_2
5	289	2	0	
5	289	2	1	Variant_3
8	53	3	1	Variant_6
8	53	34	1	Variant_3
8	944	1	1	Variant_3
13	70	1	1	Variant_3
13	923	1	1	Variant_3
14	9	1	0	
14	124	1	0	
14	9	11	1	Variant_4
14	124	18	1	Variant_4
14	409	1	1	Variant_3
20	235	1	1	Variant_3
27	1475	1	1	Variant_3
27	4676	1	1	Variant_3
34	478	1	1	Variant_5
999	113	1	1	Variant_3
999	162	1	1	Variant_3
11A/D	62	1	1	Variant_3
12A/F	218	2	1	Variant_3
12A/F	989	1	1	Variant_3
15A	58	2	1	Variant_3
15B/C	1262	1	0	
15B/C	199	1	1	Variant_5
15B/C	3976	1	1	Variant_1
16F	3450	1	0	
18C	113	6	1	Variant_3
18C	NA	1	1	Variant_3
19A	66	1	1	Variant_3
19A	199	2	1	Variant_3
19A	230	1	1	Variant_5
19A	416	1	1	Variant_3
19A	667	1	1	Variant_3
19A	1201	1	1	Variant_3
19A	1848	1	1	Variant_4
19A	3017	3	1	Variant_3
19A	994	1	2	Variant_3 and variant_9
19A	3009	1	2	Variant_9 and variant_3
19F	51	1	1	Variant_9
19F	79	1	1	Variant_3
19F	162	1	1	Variant_3
19F	179	1	1	Variant_3
19F	230	1	1	Variant_5
19F	309	3	1	Variant_3
19F	NA	1	1	Variant_6
19F	51	1	2	Variant_9 and variant_3
19F	1045	1	2	Variant_3 and variant_9
19F	5827	1	2	Variant_10 and variant_4
22A/F	433	12	1	Variant_4
22A/F	3705	2	1	Variant_5
22A/F	4110	1	1	Variant_4
23A	42	3	1	Variant_3
23A	97	1	1	Variant_5
23B	439	1	1	Variant_5

23B	1602	1	1	Variant_3
23F	36	7	1	Variant_6
23F	37	1	1	Variant_5
23F	60	1	1	Variant_5
23F	311	3	1	Variant_5
23F	1011	2	1	Variant_3
23F	1011	1	1	Variant_6
24A/B/F	53	1	1	Variant_3
24A/B/F	230	1	1	Variant_5
33A/F	60	2	1	Variant_5
33A/F	673	2	1	Variant_3
33A/F	2705	1	1	Variant_3
35B	162	1	1	Variant_3
35B	309	1	1	Variant_3
35B	446	1	1	Variant_5
35B	1635	2	1	Variant_5
6A	53	1	1	Variant_3
6A	138	1	1	Variant_3
6A	329	1	1	Variant_1
6A	690	1	1	Variant_3
6A	NA	1	1	Variant_3
6A	207	2	2	Variant_3 and variant_10
6A	327	1	2	Variant_7 and variant_5
6A	490	1	2	Variant_3 and variant_9
6A	NA	1	2	Variant_3 and variant_5
6B	176	1	1	Variant_3
6B	176	2	1	Variant_6
6B	176	7	2	Variant_6 and variant 9
6C	1379	2	1	Variant_4
7F	191	36	1	Variant_3
7F	1589	2	1	Variant_3
7F	2331	2	1	Variant_3
7F	NA	1	1	Variant_2
9N	66	4	1	Variant_3
9V	53	1	1	Variant_3
9V	60	1	1	Variant_5
9V	162	14	1	Variant_3
9V	162	1	1	Variant_7
9V	165	1	1	Variant_3
9V	8038	1	1	Variant_3
9V	8138	1	1	Variant_3
9V	43	1	2	Variant_9 and variant_3



Supplementary figure 1; Real-time RT-PCR using pspC specific primers with a similar binding efficiency and GyrA as reference gene. Level of expression is shown as normalized expression ΔΔCq – relative quantity normalized to relative quantity of the reference gene.

Chapter 7

General discussion

The human pathogen, *Streptococcus pneumoniae* (pneumococcus), is an important contributor to morbidity and mortality worldwide [1,2]. This encapsulated Gram-positive bacterium causes diseases ranging from mild respiratory tract infections to pneumonia, sepsis and meningitis, also referred to as invasive pneumococcal disease (IPD). The complement system plays an important role in controlling infections, including those by *S. pneumoniae*. This has become evident in humans with complement deficiencies and in experiments with complement deficient animals. Interestingly, more subtle differences in complement activity by common genetic polymorphisms may affect an individual's susceptibility for infections. Complement factor H is a key regulator of the alternative pathway. In a large genome-wide association study (GWAS) polymorphisms in the complement factor H gene (*CFH*) region were identified, which were associated with susceptibility for meningococcal infection [3]. This suggests that polymorphisms in the *CFH* region affect an individual's susceptibility for infections. In addition, factor H levels, measured in blood, vary greatly within the human population [4-8]. This thesis aims to enhance our understanding of the biological role of complement factor H in pneumococcal-host interactions.

The effect of alternative pathway activity regulated by factor H on pneumococcal induced inflammatory responses

Several studies indicate that crosstalk occurs between the complement system, Toll-like receptors (TLRs) and Fcγ receptors, leading to modulation of the immune response towards a proinflammatory state [9-14]. Complement activation product C5a binding to its receptor (C5aR) (also called CD88), modulates the inflammatory response induced by various pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Neisseria meningitidis* and *Candida albicans* [12,15-17]. The alternative pathway amplification loop plays a crucial role in the amplification of the initial activation of the classical and lectin pathway. It may account for up to 80% of the total complement activation, even if initially triggered by the classical pathway [18,19].

In **chapter 2**, we demonstrate that the alternative pathway, modulated by factor H levels, plays an important role in pneumococcal induction of proinflammatory cytokine responses in human peripheral blood mononuclear cells (PBMCs). Inhibition of the alternative pathway by factor H concentrations in the high physiological range strongly reduce C5a levels and decrease proinflammatory cytokine production by human PBMCs. This suggests that inter-individual variation in alternative pathway activity due to variation in factor H plasma levels affects the level of cytokine production during infection. The alternative pathway activity is determined by a delicate balance between activation and inhibition. The extent of this balance is determined by an inherited set of common genetic variants of alternative pathway components and regulatory proteins. This can be referred to as the complotype [20,21]. The complement activity is an important determinant for the risk of inflammatory diseases, such as age-related macular degeneration (AMD), dense deposit disease (DDD) and atypical haemolytic uremic syndrome (aHus)[20]. In line with this, we demonstrated that modulation of the alternative pathway activity affects the extent of inflammatory responses induced by pneumococci. Overall this indicates the important role of the alternative pathway activity in human inflammatory responses.

In **Chapter 4** we show that higher serum factor H levels in mice are not only associated with greater pathogen burden, but also with increased levels of proinflammatory cytokines. Unexpectedly, we also found that decreased factor H levels resulted in a lower pathogen burden, which was associated with lower levels of proinflammatory cytokines. These experiments indicate that variation in factor H levels may affect the outcome of bacterial infections. The effect of the alternative pathway activity on the magnitude of the inflammatory response is hard to predict during natural infection in patients. Reduced alternative pathway activity by higher factor H levels may reduce cytokine induction, whereas it may also inhibit bacterial clearance and allow greater outgrowth of the bacteria resulting in a stronger inflammatory response. Despite the important role of complement activation to prevent disease, excessive complement activation may damage host cells and increase disease severity. Several studies suggest that complement inhibition may be beneficial, especially during bacteraemia, irrespective of the potential danger to impair the host defence response against invading pathogens [17,22-24].

In **chapter 3** we examined whether exogenous factor H, at the onset of symptoms, can attenuate inflammation and vascular leakage in experimental pneumococcal sepsis in mice. Factor H treatment was combined with administration of antibiotics to avoid differences in bacterial numbers and to mimic the clinical situation in which severely ill patients receive antibiotics immediately upon hospital admission. Our data show that inhibition of the alternative complement pathway by exogenous human factor H at a clinical relevant time point, at the first onset of symptoms, fails to attenuate inflammation and vascular leakage in a pneumococcal sepsis model in mice. Recently, the potential efficacy of blocking C5a or its receptors to improve outcome in experimental sepsis models was demonstrated. Blocking C5a or its receptors preserved neutrophil function resulting in lower bacterial loads and less severe disease [22,25,26]. In cecal ligation and puncture-induced sepsis models in rats, it was shown that blocking of C5a was even beneficial when initiated after the

onset of symptoms of sepsis [27]. Based on these results, it may be more effective to directly block the complement effector molecule C5a, instead of blocking the complete complement pathway.

Chapter 4, together with other studies, demonstrates the important role of the alternative pathway activation in pneumococcal opsonisation and clearance [28]. Stimulation of the alternative pathway activity may be an effective therapy to enhance clearance of invasive infections and thereby reduce the severity of sepsis. Mice injected with recombinant properdin, a positive regulator of the alternative pathway, show an increased alternative pathway-mediated pneumococcal C3 opsonization and enhanced pneumococcal killing [28]. Administration of properdin may enhance microbial clearance, although, it has thus far not been assessed whether properdin treatment affects the host inflammatory response or whether it has an additive effect in combination with antibiotic treatment in sepsis. The development of therapies involving factor H may be more challenging, since we demonstrate in **chapter 4** a delicate balance in which higher or lower factor H levels may both impair pneumococcal clearance.

Role of factor H levels in pneumococcal complement mediated phagocytosis

Mouse model

The role of the complement system and the different complement activation routes in infections by *S. pneumoniae* have been studied in animal models. Several studies demonstrate that deficiency or depletion of the central complement component C3 severely impairs pneumococcal clearance [29-33]. The importance of the classical as well as the lectin pathway activity has been illustrated in mouse models of pneumococcal invasive disease [29,34,35]. It appears that both pathways are important to initiate complement activation, whereas the alternative pathway determines the final amount of C3 deposition on the bacterial surface [29,36]. Serum of factor B deficient mice with an abrogated alternative pathway activity showed a lower intensity of pneumococcal C3 deposition compared to classical or lectin pathway deficient mice serum [29].

In **chapter 4,** we studied the function of the alternative pathway activity regulated by factor H on pneumococcal clearance. Absence of factor H in homozygous factor H deficient humans or mice leads to immediate uncontrolled alternative pathway activation and subsequent consumption of fluid-phase C3 [37,38]. It was previously shown that 8 month old mice with a homozygous factor H deficiency develop a kidney disease, named membranoproliferative glomerulonephritis, and renal injury [37]. We found that pneumococcal clearance in homozygous factor H deficient mice is impaired. This is in agreement with other reports studying complement deficient mice [29-33]. Inhibition of the alternative pathway by exogenous human factor H administered to wild-type mice also impaired pneumococcal clearance. This is due to a reduction in pneumococcal C3 opsonization. More surprisingly, we found that reduced factor H levels in heterozygous factor H deficient mice, enhanced pneumococcal C3 opsonization and clearance from blood in comparison to wild-type mice. *In vitro* experiments demonstrated that pneumococcal C3 opsonization was enhanced as a result of increased alternative pathway activity in serum of heterozygous factor H deficient mice. Others demonstrated that the increased alternative pathway activity in heterozygous factor H deficient mice results in age-related macular degeneration-like pathology [39]. This suggests that reduced factor H levels in mice can be beneficial during infection, but may increase the susceptibility for complement-mediated inflammatory diseases.

Similar to other pathogens, *S. pneumoniae* binds factor H as a complement evasion strategy, it specifically binds to human factor H, but not mouse factor H [40-42]. Therefore, the mouse model allowed us to assess the influence of mouse factor H expression levels on the fluid-phase control of the alternative pathway activity and pneumococcal clearance. However, the role of pneumococcal factor H binding to PspC in humans cannot be elucidated using the mice model. A possible way to circumvent this problem is to use a transgenic mice expressing human factor H. In addition, the use of human *in vitro* assays using human serum or blood may provide important insights into the role of factor H in pneumococcal host interactions.

Human in vitro model

Complement factor H levels vary markedly between humans [4-8]. Factor H levels measured in 1514 individuals varied between 63.5 µg/mL to 847.6 µg/mL (median 226.6 µg/mL) [7]. Not much is known whether this variation affects the host resistance to pneumococcal infections.

In **chapter 4,** we demonstrate that human and mice factor H levels determine a delicate balance of alternative pathway activity affecting the resistance to invasive pneumococcal disease. To gain more insight into how human factor H levels affect pneumococcal C3 opsonization and clearance we performed *in vitro* experiments using factor H depleted serum reconstituted with various factor H concentrations. We found that human factor H levels of 300 µg/mL in serum results in optimal pneumococcal C3 opsonization and clearance, whereas 100 µg/mL or 500 µg/mL resulted in significantly lower opsonization. This clearly indicates that effective

pneumococcal opsonization depends on a delicate balance of complement factors. In order to study whether factor H levels in human serum affect pneumococcal clearance, we used a pneumococcal whole blood killing assay (**chapter 5**).

In this whole blood model, we replaced plasma by factor H depleted serum reconstituted with various factor H concentrations. In accordance with the pneumococcal C3 opsonisation results, we found optimal pneumococcal clearance at a factor H concentration of 300 µg/mL, whereas lower or higher factor H levels reduced pneumococcal clearance (**chapter 4**). This result suggests that when pneumococci enter the bloodstream, human factor H levels are of major importance for optimal pneumococcal clearance and thus affect the individual's susceptibility to invasive pneumococcal disease and severity of infection. **Figure 1** shows in a simplified model how factor H levels affect resistance to pneumococcal infection.

It has been found that factor H levels in blood are determined by both environmental and genetic factors, in which the genetic factors are most dominant [6]. In the same study, factor H levels were found to increase with age and to be lower in smokers [6]. In addition, factor H serum levels may vary depending on the disease state of an individual. Reduced factor H levels were observed during acute meningococcal disease compared to those at convalescence [4]. It has been suggested that human factor H levels play a role in the susceptibility for meningococcal disease. A functional single-nucleotide-polymophism (SNP) has been found in a presumed nuclear-factor –kB responsive element (NF-kB) in the *CFH* gene, which was associated with the susceptibility for meningococcal disease (C-496T) [4]. It was shown that individuals with the *CFH* C-496T C/C genotype are more likely to have increased serum factor H levels, which was associated with a reduced bactericidal activity against *N. meningitidis* [4]. This is in agreement with our findings in which high factor H levels reduce pneumococcal clearance **(Chapter 4)**. In addition, a large GWAS for meningococcal disease identified SNPs in *CFH* and factor H related 3 (*CFHR3*) associated with increased susceptibility for meningococcal disease [3]. Additional GWAS studies are needed to determine whether polymorphisms in the *CFH* gene are associated with other infectious diseases, such as pneumococcal invasive disease. This is one of the objectives of the European childhood life-threatening infectious diseases study (EUCLIDS), in which our group participated [43]. Several studies report that genetic variation not only in *CFH,* but also in the factor H related genes (*CFHR1-5)* are associated with disease susceptibility [44-48]. Factor H related genes 1-5 contain surface recognition sites similar to factor H, whereas none of the factor H related proteins contains the complement regulatory domains of factor H [49]. It has been suggested that the factor H related proteins modulate complement activation by competing with factor H for binding to ligands, including the bacterial surface [50]. However the concentration of factor H related proteins seems to be much lower than previously expected [51]. For instance, the molar concentration of FHR-3 is on average more than 100-fold lower compared to the factor H concentration in serum [51]. This makes it unlikely that ligand binding competition plays an important role. More research is needed to elucidate the proposed competition. In addition, we suggest to analyze how common or rare polymorphisms, may affect the expression and function of factor H and the factor H related proteins.

The strength of our study described in **chapter 4** is that we varied the human factor H concentration while keeping all other serum components, such as levels of opsonizing antibodies and other complement components, identical. This demonstrates that variation in factor H levels alone has a significant effect on pneumococcal clearance in blood. Nevertheless, it is important to answer the question whether factor H levels or the alternative pathway activity within the human population are associated with susceptibility for infections. In order to do so, factor H levels need to be determined in a large group of convalescence patients and controls. The presence of specific antibodies with high classical pathway activation may compensate for a reduction in alternative pathway activation. In addition, many genetic factors contribute to an individual's complement activity. This inherited set of polymorphisms in complement genes is named the complotype [20,21]. Certain combinations of polymorphisms in alternative pathway proteins enhance alternative pathway activity and predispose individuals for chronic inflammatory diseases, such as haemolytic uremic syndrome, age-related macular degeneration and dense deposit disease [20]. On the other hand, it has been proposed that the increased alternative pathway activity may reduce the risk for infectious diseases [20]. This hypothesis is supported by our findings, showing that increased alternative pathway activity by a reduction in factor H levels enhances pneumococcal clearance. Genetic analysis or measurement of complement levels and polymorphic variants at the protein level can provide relevant data about an individual's complotype or complement activity. Genome-wide association studies and additional *in vitro* and *in vivo* studies are of importance to increase the understanding on how an individual's complement activity affects the susceptibility to infectious diseases. Our study, together with previous studies, demonstrates the importance of alternative pathway activity in the defence against invading pathogens [28].

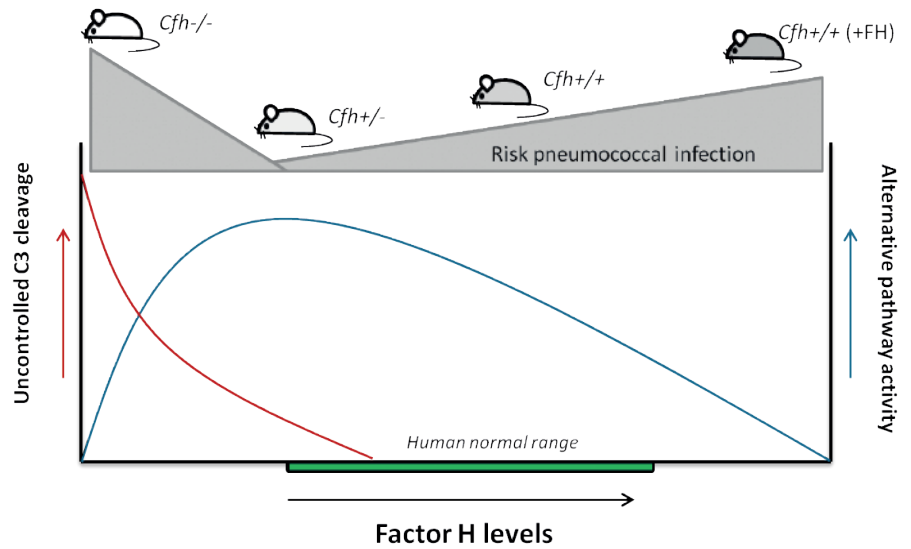


Figure 1. Schematic model summarizing how complement factor H levels affect the alternative pathway activity and the risk for pneumococcal infection**.**

Pneumococcal complement evasion by factor H binding

The pneumococcal polysaccharide capsule affects complement resistance and protects against phagocytic killing [52,53]. Epidemiological studies showed that particular capsular serotypes are dominant in invasive disease whereas others are associated with nasopharyngeal carriage [54,55]. Pneumococcal resistance to complement deposition is an important determinant of its invasive disease potential [55]. In addition to the capsule, *S. pneumoniae* has evolved other mechanisms to evade complement activation. The pneumococcal surface protein PspC binds the alternative complement pathway inhibitor factor H and thereby reduces C3 deposition. *PspC* is heterogeneous and has been classified into different PspC types based on sequence similarity. In **chapter 6** we report that variation in PspC type, independent of capsule serotypes, affects the pneumococcal ability to evade complement deposition. Others have demonstrated that the capsule type affects factor H binding to PspC and complement evasion in capsule switch mutants [52]. Our findings are complementary to these data and demonstrate that genetic differences in *pspC*, using *pspC*-switch mutants, affect complement resistance in the presence of polysaccharide capsule. We demonstrate that PspC types with a choline binding domain (subgroup I) are more effective in complement evasion than PspC types with a LPxTG binding anchor (subgroup II). These findings indicate that PspC type specific differences contribute to intra-serotype variation in complement resistance. This is in line with a previous study in which genetic variation in *pspC* was suggested to explain differences in invasiveness within the same serotype and clonal complex [56].

Pneumococcal phase variation is an important strategy to adapt to the different local environments during the course of disease pathogenesis [57,58]. Consistent with previous studies, binding of factor H to opaque phase TIGR4 resulted in reduced complement deposition [52,59]. The potential role for PspC in complement evasion in opaque phase bacteria is supported by the observation that opaque phase variants are often found in invasive infections, in which pneumococcal complement evasion is vital for survival [57,60-62]. Carriage isolates from the upper respiratory tract, where complement levels are lower than in serum, are mostly transparent phase bacteria [63-65]. Due to a thinner capsule, these bacteria may be more effective in adherence to human lung epithelial cells [58], which is an essential first step in colonization of the human host.

In **chapter 6** we demonstrate that absence of a capsule or transparent phase capsule within the same genetic background, voids the ability of factor H binding to reduce complement deposition. Other studies demonstrated that transparent phase pneumococci as well as unencapsulated strains are more sensitive for complement deposition [53,60]. A thinner capsule may unmask transmembrane protein epitopes recognized by immunoglobulins and reveal surface moieties recognized by lectin pathway factors. The enhanced classical and lectin pathway activation may minimize the effect of alternative pathway inhibition by factor H binding on the overall complement deposition [53,61]. In addition, in the absence of capsule, we found no differences in factor H binding between strains expressing the different PspC types, even though the sequence of the previously defined 121 amino acid long factor H binding region varied extensively [66]. This indicates that all four selected PspC types have factor H binding sites with similar abilities to bind factor H. Differences in factor H binding between the various PspC types was only observed in the presence of a capsule.

Interestingly, naturally occurring unencapsulated *S. pneumoniae* strains do not have PspC. Additional proteins in the naturally occurring unencapsulated strains, such as the PspC-like protein PspK, were shown to play a role in colonization and adherence, but do not bind factor H [67 68]. Our observation in **chapter 6** shows that PspC binding of factor H by unencapsulated strains has no effect on complement resistance. This may explain the loss of PspC in naturally occurring unencapsulated

S. pneumoniae strains [67,69]. In addition, it is also not known what determines the selection for certain PspC types in encapsulated strains. PspC binding of factor H contributes to complement evasion and selection may be based on optimal pneumococcal factor H binding in combination with its capsule to reduce C3 deposition. However, increased resistance to complement has been associated with a higher invasive disease potential [55]. The evolutionary advantage of a high invasive disease potential is not directly obvious for *S. pneumoniae*, since invasive disease may not promote pneumococcal transmission or colonization. It therefore makes no obvious sense to evolve PspC-mediated complement evasion to enhance invasive disease potential. It has been postulated that resistance to complement is needed for local invasion of mucosal tissues and long term colonization [64]. Local invasion may be an intermediate step between surface colonization and systemic invasion. This may explain the selection for more complement resistant strains.

Besides complement evasion PspC has other functions. For instance, PspC acts as an adhesion molecule by interacting with the secretory component of human IgA and the epithelial polymeric immunoglobulin receptor (pIgR) thus facilitating invasion of the mucosa [66,70-74]. Interaction of PspC and vitronectin plays a role in pneumococcal adherence as well as in complement evasion by inhibiting the deposition of the terminal complement complex [75]. In addition, PspC binding of factor H facilitates adherence to epithelial cells [66,76]. Markedly, increased human nasal factor H levels by presence of a virus have been associated with increased pneumococcal colonization density in the upper airway [65]. PspC binding of factor H thereby contributes to pneumococcal colonization, supporting the relevance of pneumococcal factor H binding in carriage isolates. Overall this demonstrates that PspC binding of factor H contributes to complement evasion as well as facilitates pneumococcal adherence. Not much is known about the prevalence of the different PspC types in carriage or invasive pneumococcal disease isolates.

In **chapter 6** we show a higher prevalence of PspC types with a choline binding domain (subgroup I) compared to PspC types with a LPxTG binding anchor (subgroup II) within an invasive pneumococcal disease cohort. To date, no comparative data from clinical invasive disease isolates have been published. Iannelli et al. demonstrated a predominance of subgroup I PspC (74%) in a collection of 43 strains containing randomly chosen clinical isolates, standard laboratory strains and American Type culture Collection strains [77]. It would be interesting to analyze strain collections including carriage and invasive isolates from the same region and time period to further dissect the contribution of PspC subgroup and types to the invasive disease potential. Particular PspC types may more often be found in invasive disease compared to other PspC types. More insight in epidemiological differences in PspC type prevalence and their contribution to invasive disease may have implications for vaccine design because PspC is an important vaccine candidate [78-80]. Importantly, *in vivo* PspC binding of factor H has been proposed to mask antibody binding epitopes, which could facilitate pneumococcal infection [65]. Mutations eliminating PspC binding of factor H allow antibody generation against the PspC factor H binding region which may be a strategy to enhance protective antibody responses to vaccination [65,81]. In addition, various studies examined the use of factor H fragments fused to Fc as anti-microbial immune therapy [82-85]. For this purpose, factor H fragments are needed that specifically bind bacteria with a greater avidity than the physiological factor H. This shows how knowledge on pneumococcal factor H binding can be used to develop therapies to facilitate microbial clearance. We contributed by showing PspC types specific differences in pneumococcal factor H binding and complement evasion.

Final conclusions

The complement system is an essential part of the innate immune system, with decisive roles in protection against infection and inflammatory responses. Imbalance between activation and inhibition due to excessive activation or improper regulation has pathological consequences. Complement factor H is a key regulator of the alternative pathway. The primary aim of this thesis was to clarify the role of complement factor H in pneumococcal-host interactions. Based on the studies described in this thesis, we concluded the following:

1. Inhibition of the alternative pathway by factor H strongly reduces proinflammatory cytokine production by human peripheral blood mononuclear cells. This finding suggests that inter individual variation in alternative pathway activity due to variation in factor H plasma levels affects individual cytokine responses during pneumococcal infection.
2. Inhibition of the alternative complement pathway by exogenous human factor H fails to attenuate inflammation and vascular leakage at a clinically relevant intervention time point in pneumococcal sepsis in mice.
3. Factor H levels determine the delicate balance of alternative pathway activity. The level of serum factor H affects complement deposition, and correlates with pneumococcal opsonophagocytic killing. This suggests that variation in factor H expression levels, naturally occurring in the human population, plays a thus far unrecognized role in the resistance to invasive pneumococcal disease.
4. Choline bound subgroup I PspC types showed a greater prevalence than LPxTG anchored subgroup II PspC types in invasive pneumococcal disease isolates.

Pneumococcal subgroup I PspC types bind significantly more factor H and showed more effective complement evasion compared to subgroup II PspC types in isogenic encapsulated pneumococci. This demonstrates that variation in PspC type, independent of capsule serotypes, affects pneumococcal factor H binding and its ability to evade complement deposition.

Overall, a better understanding of host factors that influence susceptibility to infection would support prediction of disease outcome and could also contribute to the development of therapies to reduce disease susceptibility. In addition, more insight in epidemiological differences in PspC type prevalence and their contribution to invasive disease may have implications for protein-based pneumococcal vaccine design.

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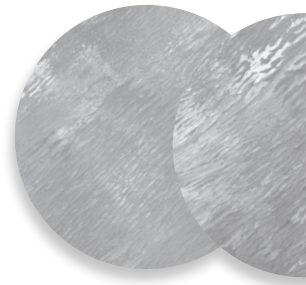
Summary in English



Complement is an essential component of the host defence against pneumococci. Complement evasion contributes to pneumococcal virulence and survival in the human host. The complement protein factor H is a key inhibitor of the alternative complement pathway. The primary aim of this thesis is to clarify the role of complement factor H in pneumococcal-host interactions.

We focused on the following questions:

- What is the role of factor H alternative pathway regulation on pneumococcal induced inflammation?
- What is the effect of natural variation in serum factor H levels on host resistance to pneumococcal infection?
- Do differences in pneumococcal PspC types affect binding of factor H and complement deposition on the pneumococcal surface?

Chapter 1 provides background knowledge of infections by *S. pneumoniae* and the host immune response. This encapsulated Gram-positive diplococcus shaped bacterium causes diseases ranging from mild respiratory tract infections to severe diseases, such as pneumonia, sepsis and meningitis, also referred to as invasive pneumococcal disease (IPD). An important innate immune response upon *S. pneumoniae* infection is activation of the complement system. The three main pathways for complement activation are the classical, lectin and alternative pathway. Complement activation contributes to pneumococcal clearance by phagocytosis and to the induction of inflammatory responses. Initial complement activation via the classical and lectin pathways is amplified through the alternative pathway amplification loop. A key negative regulator of the alternative pathway and the alternative pathway amplification loop is complement factor H. *S. pneumoniae* has the ability to bind human factor H as an immune evasion strategy. For another encapsulated bacterial pathogen, *Neisseria meningitidis*, also known as the meningococcus, a genome wide associations study (GWAS) identified risk polymorphisms in the complement factor H gene (*CFH*) and the complement factor H related protein 3 (*CFHR3*) gene associated with meningococcal disease. These GWAS results indicate that genetic variation in the *CFH* gene may affect an individual's resistance for invasive infections, including invasive pneumococcal disease. In addition, complement factor H levels vary markedly within the human population.

In **chapter 2** we demonstrate that inhibition of the alternative pathway by factor H concentrations in the high physiological range strongly reduces proinflammatory cytokine production by human peripheral blood mononuclear cells. The complement activation product C5a is known to modulate the inflammatory response by binding its receptor (C5aR) (or CD88). We show that alternative pathway inhibition by exogenous factor H strongly reduces C5aR crosstalk. Both alternative pathway activity and the plasma factor H levels show large variation between individuals. These findings suggest that inter individual variation in alternative pathway activity due to variation in factor H plasma levels may affect the individual's cytokine responses during infection.

In **chapter 3** we hypothesized that at onset of sepsis and initiation of antibiotic therapy, inhibition of alternative pathway activation is desirable to ameliorate sepsis associated inflammation and vascular leakage. The induction of cytokines and chemokines conveys important signals to other immune cells needed for activation and coordination of host immune responses. However, the inflammatory response can be excessive and can contribute to local tissue damage in focal infections or be detrimental in patients with sepsis. High inflammatory responses by massive secretion of cytokines and excessive complement activation may result in fever, vascular permeability, tissue damage and organ failure. However, mice treated with human factor H at the onset of clinical symptoms of sepsis showed no decrease in disease scores, serum proinflammatory cytokines, or vascular permeability. Factor H administration did also not significantly affect C3 and C3a production at 26 h post-infection. We therefore conclude that inhibition of the alternative complement pathway by exogenous human factor H fails to attenuate inflammation and vascular leakage at a clinically relevant intervention time point in pneumococcal sepsis in mice.

A large variation in plasma factor H levels is observed between different individuals. In **Chapter 4,** the functional consequences of the variability in human serum factor H level on host defence are demonstrated. Using an *in vivo* mouse model combined with human *in vitro* assays, we show that the level of serum factor H affects complement deposition, which correlates with pneumococcal opsonophagocytic killing. We found that plasma factor H levels determine a delicate balance of alternative pathway activity, thus affecting the resistance to invasive pneumococcal disease. Our results suggest that variation in factor H expression levels, naturally occurring in the human population, plays a thus far unrecognized role in the resistance to invasive pneumococcal disease.

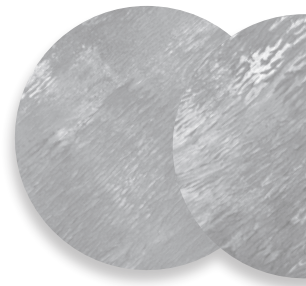
In **chapter 5**, we describe the use of a versatile whole blood killing assay which is a method that allowed us to study bacterial clearance in human blood with

an intact complement system *in vitro*. By using a selective thrombin inhibitor hirudin, complement activity of whole blood is preserved, in contrast to other anticoagulants. With this whole blood killing assay, bacterial factors as well as host cellular and humoral factors can be modulated to determine their contribution to opsonophagocytic killing. In addition, this assay allows to substitute the plasma content of one donor with serum or plasma from another source before initiating the whole blood killing assay. This enables manipulation of the concentration of different complement factors. Therefore, this assay can be used to study the requirements for active complement, phagocyte function and bacterial immune evasion mechanisms that contribute to survival in human blood.

In **chapter 6**, we focus on the ability of pneumococci to bind human factor H. *S. pneumoniae* binds human factor H mainly by PspC, which is considered a mechanism to evade complement deposition. PspC is heterogeneous and has been classified into different PspC types based on sequence similarity. We found a far greater prevalence of choline bound (subgroup I) PspC types as compared to LPxTG anchored (subgroup II) PspC types in invasive pneumococcal disease isolates. In addition, applying isogenic *pspC* switch mutants, we demonstrate that subgroup I PspC is more effective in complement evasion than subgroup II PspC. These findings indicate that PspC type specific differences contribute to intra-serotype variation in complement resistance.

In **chapter 7**, we discuss the findings of the research described in this thesis. Imbalance due to excessive activation or improper regulation of the complement system has pathological consequences. We demonstrated the importance of alternative pathway activity in the defence against invading pathogens. Increased alternative pathway activity may reduce the risk for infectious diseases, whereas it may enhance host inflammatory responses. Genetic analysis or measurement of complement levels and polymorphic variants at the protein level can provide relevant data about an individual's complotype or complement activity. Genome-wide association studies and additional *in vitro* and *in vivo* studies are of importance to increase the understanding on how an individual's complement activity affects the susceptibility to infectious diseases. A better understanding of host factors that influence susceptibility to infection would support prediction of disease outcome and could also contribute to the development of therapies to reduce disease susceptibility. In addition, more insight in epidemiological differences in PspC type prevalence and their contribution to invasive disease may have implications for protein-based pneumococcal vaccine design.

Nederlandse samenvatting



De gekapselde Gram-positieve *Streptococcus pneumoniae* bacterie (pneumokok) is een belangrijke verwekker van zowel milde luchtweginfecties als ernstige ziektes, zoals longontsteking (pneumonie), hersenvliesontsteking (meningitis) en bloedvergiftiging (sepsis). Deze ernstige ziektes worden ook wel invasieve pneumokokkeninfecties genoemd. **Hoofdstuk 1** geeft achtergrondinformatie over pneumokokkeninfectie en de afweerreacties van de mens. Een belangrijke aangeboren immuunreactie tijdens pneumokokkeninfectie is de activering van het complementsysteem. Activering van het complementsysteem resulteert in de depositie van complement eiwitten op de bacterie. Deze complementdepositie draagt bij aan het verwijderen van de bacterie door middel van fagocytose. Daarnaast draagt activatie van het complementsysteem bij aan ontstekingsreacties. De drie belangrijkste activatie routes van het complementsysteem zijn de klassieke-, de lectine- en de alternatieve route. Complementactivatie via de klassieke- en lectine route wordt versterkt door de alternatieve route. Complement factor H is een belangrijke negatieve regulator van de alternatieve route en van de amplificatie door de alternatieve route. *S. pneumoniae* kan menselijk factor H binden om immuunreacties te ontwijken. *S. pneumoniae* bindt factor H, via het bacteriële oppervlakte-eiwit PspC. Voor *Neisseria meningitidis* (meningokok), een andere gekapselde bacteriële ziekteverwekker, heeft een grote studie polymorfismen in het complement factor H gen gevonden die geassocieerd zijn met meningokokkeninfecties. Deze resultaten duiden aan dat genetische variaties in het complement factor H gen mogelijk effect hebben op de gevoeligheid voor invasieve infecties, waaronder mogelijk ook invasieve pneumokokkeninfecties. Daarnaast is er veel variatie in factor H plasma concentraties tussen personen. Het doel van dit proefschrift is om de rol van complement factor H in pneumokokkeninfecties te verduidelijken. Daarbij zijn de volgende vragen van belang:

- Wat is de rol van de alternatieve route, gereguleerd door factor H, bij ontstekingsreacties tijdens pneumokokken infectie?
- Wat is het effect van natuurlijke variatie in factor H concentraties op de afweer tegen pneumokokken infectie?
- Heeft het pneumokokken PspC type effect op de binding van factor H en de complement depositie op het oppervlak van pneumokokken?

In **hoofdstuk 2** laten we zien dat remming van de alternatieve route door toevoeging van factor H sterk de pro-inflammatoire cytokineproductie door mononucleaire cellen uit perifeer bloed (PBMCs) verlaagt. Het complementactivatie product C5a moduleert de ontstekingsreactie door middel van binding aan de C5a receptor.

Wij laten zien dat remming van de alternatieve route, door toevoeging van factor H, de ontstekingsreactie sterk vermindert via de C5a receptor. Zowel de activiteit van de alternatieve route als de factor H concentraties in plasma verschillen sterk tussen individuen. Onze resultaten suggereren dat variatie in activiteit van de alternatieve route tussen individuen door variatie in factor H concentraties mogelijk effect heeft op ontstekingsreacties tijdens infecties.

In **hoofdstuk 3** was onze hypothese dat remming van de alternatieve route activiteit, bij de eerste sepsis symptomen en antibiotica behandeling, de sterkte van ontstekingreacties en de mate van vaatlek zou verminderen. De inductie van cytokines en chemokines brengen belangrijke signalen over naar immuuncellen die nodig zijn voor de activatie en coördinatie van immuunreacties. Echter, de ontstekingsreacties kunnen overmatig zijn en daarmee weefselschade veroorzaken waardoor ze levensbedreigend kunnen zijn. Sterke ontstekingsreacties door hoge productie van cytokines en overmatige complementactivatie kunnen koorts, vaatlek, weefselschade en orgaanuitval veroorzaken. Echter, muizen behandelt met menselijk factor H, op het moment dat de eerste symptomen van sepsis zichtbaar waren, hadden geen vermindering in ziektescore, cytokines of vaatlek. Factor H toediening had 26 uur na de infectie geen significant effect op C3 en C3a productie. Daarom concluderen wij dat remming van de alternatieve route door middel van factor H toediening, op een klinisch relevant tijdstip, niet de ontstekingsreactie en vaatlek vermindert bij pneumokokkensepsis in muizen.

Er is veel variatie tussen mensen in de plasmaconcentratie van factor H. In **hoofdstuk 4** laten we de functionele consequenties van deze variatie in factor H concentratie op de afweerreacties van de gastheer zien. Door middel van *in vivo* muismodellen gecombineerd met humane *in vitro* experimenten laten we zien dat de factor H concentratie effect heeft op de complementdepositie. De mate van complementdepositie op de pneumokok komt overeen met overleving van de bacterie in bloed. We vonden dat plasma factor H concentraties belangrijk zijn in de balans van de alternatieve route activiteit en daarmee kan de factor H concentratie effect hebben op de afweer tegen invasieve pneumokokkeninfectie. Onze resultaten suggereren dat variatie in factor H expressie, die van nature voorkomt in de populatie, mogelijk een belangrijke rol speelt in de afweer tegen invasieve pneumokokken infecties.

In **hoofdstuk 5** beschrijven we het gebruik van een methode om de doding van de bacterie in bloed te bepalen, een 'killing assay'. Deze methode maakt het mogelijk om *in vitro,* in de aanwezigheid van een intact complement systeem,

de doding van bacteriën in menselijk bloed te bestuderen. Door gebruik te maken van een selectieve trombine remmer, hirudin, blijft de complement activiteit behouden, in tegenstelling tot andere antistollingsmiddelen. Met deze killing assay kunnen zowel bacteriële factoren als cellulaire of humorale gastheerfactoren gemoduleerd worden om hun rol in de doding van de bacterie door middel van fagocytose te bestuderen. Daarnaast kan in deze assay het plasma van een donor vervangen worden door het plasma van een andere donor vóór het begin van de killing assay. Dit maakt het mogelijk om de concentratie van verschillende complementfactoren te variëren. Deze test kan daarom gebruikt worden om de rol van complementactiviteit, fagocytosefunctie en bacterie immuun ontwijkingmechanismen op de overleving van de pneumokok in bloed te bestuderen.

In **hoofdstuk 6** hebben we gekeken naar het vermogen van pneumokokken om menselijk factor H te binden. *S. pneumoniae* bindt factor H met het oppervlakte-eiwit 'pneumococcal surface protein C' (PspC). Omdat factor H de alternatieve route activatie remt, wordt dit beschouwd als een mechanisme van de bacterie om complementdepositie te ontwijken. PspC is heterogeen en is gegroepeerd in verschillende PspC types gebaseerd op sequentieovereenkomsten. Wij vonden dat in een collectie van invasieve pneumokok isolaten choline bindende PspC types (subgroep I) veel vaker voorkwamen dan LPxTG geankerde PspC types (subgroep II). Daarnaast, door gebruik te maken van isogene PspC switchmutanten hebben we laten zien dat subgroep I PspC types beter complementactivatie ontwijken dan subgroep II PspC types. Deze bevindingen duiden aan dat PspC-specifieke verschillen bijdragen aan variatie in complementgevoeligheid.

In **hoofdstuk 7** bediscussiëren we de bevindingen die beschreven zijn in dit proefschrift. Wanneer het complementsysteem uit balans is, door overmatige activatie of afwijkingen in de regulatie, kan dit pathologische consequenties hebben. Wij laten het belang van de alternatieve route in de afweer tegen invasieve pneumokokkeninfecties zien. Verhoogde activiteit van de alternatieve route beschermt mogelijk beter tegen invasieve infecties. Daarentegen, een verhoogde activiteit van de alternatieve route leidt mogelijk tot sterkere ontstekingsreacties. De combinatie van genetische factoren die de complement activiteit bepalen wordt ook wel 'complotype' genoemd. Genetische analyses of het meten van complementcomponenten inclusief polymorfismen op eiwitniveau geven mogelijk relevante data over iemands complotype of complement activiteit. GWAS (genome-wide association studies) en additionele *in vitro* en *in vivo* studies zijn van belang om beter te begrijpen hoe iemands complement activiteit effect heeft op de gevoeligheid voor infectieziekten. Meer kennis over welke factoren effect hebben op iemands gevoeligheid voor infecties zouden kunnen bijdragen aan het voorspellen van het ziekteverloop en kunnen bijdragen en de ontwikkeling van therapieën. Daarnaast heeft meer inzicht in de epidemiologische verschillen in PspC types en hun rol bij invasieve ziekten mogelijke implicaties voor de ontwikkeling van pneumokokken vaccins.

List of publications

Van der Maten E, de Jonge MI, de Groot R, van der Flier M, Langereis JD. A versatile assay to determine bacterial and host factors contributing to opsonophagocytotic killing in hirudin-anticoagulated whole blood. *Sci Rep. 2017 Feb 8;7:42137. doi: 10.1038/srep42137*

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Trappetti C, **van der Maten E**, Amin Z, Potter AJ, Chen AY, van Mourik PM, Lawrence AJ, Paton AW, Paton JC. Site of isolation determines biofilm formation and virulence phenotypes of Streptococcus pneumoniae serotype 3 clinical isolates. *Infect Immun. 2013 Feb;81(2):505-13. doi: 10.1128/IAI.01033-12.*

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Curriculum Vitae

Erika van der Maten werd op 30 mei 1988 geboren in Zwolle. Ze groeide op in Heerde en behaalde haar vwo-diploma aan het Christelijke College de Noordgouw in Heerde. Van 2006 tot 2012 studeerde zij Biologie aan Wageningen Universiteit. Ze volgde tijdens haar bachelor de specialisatie dierbiologie met een minor in 'immuunreacties bij infecties'. In de daaropvolgende master fase specialiseerde ze zich in celbiologie en volgde ze vakken aan de Universiteit van Umea in Zweden tijdens een Erasmus uitwisselingsprogramma. Voor haar afstudeervak, onder begeleiding van G.P. Pijlman bij het laboratorium voor virologie in Wageningen, bestudeerde ze de interactie van het Chikungunya virus met het aangeboren immuunsysteem. Vervolgens liep ze stage bij de universiteit van Adelaide bij Prof. J.C. Paton en Dr. C. Trappetti. Tijdens deze stage bestudeerde ze de humane ziekteverwekker, *Streptococcus pneumoniae*, ofwel pneumokok. Zowel haar afstudeervak als stage hebben geleid tot publicaties. In 2012 startte ze haar promotietraject 'Role of complement factor H in pneumococcal infections' onder de begeleiding van Dr. M van der Flier, Dr. J.D. Langereis, Prof. R. de Groot en Prof. P.W.M. Hermans binnen het Laboratorium Kinderinfectieziekten van het Radboudumc. Het project is onderdeel van het EUCLIDS consortium (European childhood life-threatening infectious disease study). Dit onderzoek heeft geresulteerd in meerdere artikelen en dit proefschrift. Tijdens haar promotietraject heeft ze verschillende studenten begeleid. Erika is momenteel werkzaam als projectleider viruskweek bij MSD animal health in Boxmeer.

Dankwoord

Dan is nu het moment gekomen dat ik de laatste pagina's van dit proefschrift schrijf. Er zijn heel veel mensen die me op verschillende manieren hebben geholpen, waarvoor ik ze erg dankbaar ben.

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