Differential influences of complement on neutrophil responses to *Neisseria meningitidis* infection

Unterschiedliche Einflüsse von Komplement auf Reaktionen neutrophiler Granulozyten auf die Infektion mit *Neisseria meningitidis*

Doctoral thesis for a medical doctoral degree

at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg,

Section Infection and Immunity

submitted by

Sören Krüger

from

Fürth

Würzburg 2021

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Table of contents

1 Introduction

1.1 Neisseria meningitidis

Neisseria meningitidis (*Nme*) is a capsulated, gram-negative diplococcic bacteria. Historically, *Nme* are grouped into serogroups according to differences between polysaccharide capsule antigens. 13 different serogroups have been described (A, B, C, D, H, I, K, L, W135, X, Y, Z and 29E) based on serologic differences in agglutination tests using specific antisera. (1) More recently, polymerase chain reactions (PCR) for specific capsule gene sequences in the region A of the *Nme* genome appeared as an alternative diagnostic tool and multi-locus sequence typing (MLST) is currently applied for more differentiated molecular typing of *Nme* into clonal complexes. (2, 3)

Unencapsulated, nonserogroupable *Nme* strains exist, too, but they are non-pathogenic in most cases and are often found as commensal colonizers in the human nasopharynx. Lack of capsule expression can be due to gene inactivation by mobile genetic elements, phase variation in capsule expression or loss of genes required for capsule synthesis (capsule null locus). (4-6)

Nme is a human-specific bacterium and is transmitted by respiratory aerosol droplets. (7) The typical site of asymptomatic colonization is the upper nasopharynx and approximately 10 % of the population are transiently colonized. (8) Certain population groups living in areas of high population density and with more frequent physical contact such as adolescents, university students and military recruits have higher colonization rates of up to 35 %. (9-12)

The polysaccharide capsule is the most important virulence factor of pathogenic *Nme* inhibiting both serum bactericidal activity and phagocytes. Moreover, *Nme* possess a variety of other virulence factors for iron acquisition, adhesion, invasion of host cells, immune evasion through complement regulation, variable gene expression through phase variation and *Neisseriae's* natural competence to acquire new DNA. For these purposes, iron binding proteins (lactoferrin binding protein, transferrin binding protein), outer membrane proteins (Opa, Opc, PorA,

PorB, Pili), lipopolysaccharide and complement binding proteins (factor H binding protein, porA, Msf, NspA) are most important. (7, 13-15)

1.2 Invasive meningococcal disease

Under some circumstances *Nme* can disseminate from the nasopharyngeal mucosa into the bloodstream and cause invasive meningococcal disease (IMD) presenting as meningococcal meningitis, meningococcal sepsis or a combination of both, affecting mostly children under the age of five. (16) In general, meningococcal disease is a rare disease in most parts of the world with incidence rates of 0.4 cases per 100000 population per year in Germany. Among infants and toddlers the incidence rate is higher with up to 6.1 cases per 100000 population. (17) Other developed countries have similarly low incidence rates, e.g. 0.28 and 1.2 cases per 100000 population in the US and Australia, respectively. However, meningococcal disease is endemic in parts of Africa and the Middle East also referred to as the “African meningitis belt” and occasional outbreaks with incidence rates of 1000 cases per 100000 population in the affected region have occurred in the past. (18, 19) The burden of disease in these countries remains very high with several hundred thousand cases and up to 100,000 deaths attributed to meningococcal disease per year. (20)

Of the 13 capsule serogroups only six have been reported to cause human disease (A, B, C, W135, X, Y). (1) In Germany and worldwide, serogroup B is the predominant causative serogroup for meningococcal disease, followed by serogroups C and Y. (17, 18) However, serogroup A is predominant during outbreaks of disease in the African meningitis belt. (21) Additionally, IMD cases caused by unencapsulated have been reported although they remain very rare and are often associated with host immunodeficiencies. (22-24) General risk factors for meningococcal disease include smoking, hereditary or acquired complement deficiencies, asplenia, young age, viral upper respiratory infections and crowded living conditions. (16, 25, 26) Moreover, the dry and dusty weather conditions found in the African meningitis belt have been shown to facilitate disease transmission. (27)

IMD is a severe illness that rapidly progresses and has the potential to cause death within hours of the onset of symptoms. Meningococcal meningitis typically presents with a sudden onset of fever, headache, neck stiffness and neurological complications such as seizures and loss of consciousness due to brain oedema. Infants and toddlers may present with less typical symptoms. Meningococcal sepsis usually begins with a rash that can progress to petechiae and purpura due to dysfunctional microcirculation. (16) Capillary leakage and disseminated intravascular coagulation can cause multiorgan dysfunction and failure. The fulminant meningococcal sepsis also known as Waterhouse-Friderichsen syndrome is characterized by hypotonic septic shock with multi-organ failure and bilateral adrenal haemorrhage. (28, 29)

In the pre-antibiotic era, the case fatality rate (CFR) of IMD reached 80 %. (30) With current treatment regimens including intravenous antibiotics, aggressive fluid management and vasoactive amines the overall CFR can be reduced to below 10 %. Patients with meningococcal meningitis have a better prognosis than patients with meningococcal sepsis with mortality rates of 1-5 % and up to 40 %, respectively.(16, 28) However, up to a quarter of IMD survivors experience serious sequelae including hearing loss, cognitive impairment, amputations, skin scarring and chronic pain. (31, 32) In the long-term IMD survivors often have lower education and socioeconomic status than healthy controls. (33)

Current treatment guidelines recommend the intravenous administration of a third-generation cephalosporin for antibiotic therapy. Aggressive fluid management and vasoactive amines are necessary in meningococcal sepsis to counter hypotonia and capillary leakage. Supportive treatment with corticosteroids is applied in any suspected case of bacterial meningitis to reduce hyperinflammation and tissue damage, although steroid treatment does not lead to a significant reduction of CFR and disability in meningococcal disease. However, CFR and disability can be significantly improved with steroids in patient with bacterial meningitis caused by other pathogens. (32, 34)

To limit the spread of disease, case management includes chemoprophylaxis for close contacts of the patient including family, partners, friends and hospital staff

involved in high-risk procedures. Oral rifampicin or ciprofloxacin or intramuscular ceftriaxone are used most frequently for chemoprophylaxis. (16, 35)

Vaccination against IMD is currently available for serogroups A, B, C, W and Y and have reduced the burden of disease in many parts of the world. Vaccines against A, C, W and Y exist as polysaccharide vaccines or polysaccharide and protein conjugate vaccines whereas the vaccine against serogroup B is protein-based since the polysaccharides in the serogroup B capsule are poorly immunogenic. The introduction of routine vaccinations against serogroup C in the UK and Germany has led to a decrease of IMD caused by serogroup C. (36, 37) Preliminary data from the UK and Canada on the recently introduced vaccine against serogroup B also showed a reduction of serogroup B IMD and carriage, especially in young children. (38, 39) Moreover, vaccination campaigns such as MenAfriVac directed against serogroup A in the African meningitis belt have decreased both meningitis cases and carriage rates successfully. (40, 41)

1.3 The complement system

The complement system is an important part of human humoral innate immunity that consists of about 30 soluble and membrane-bound proteins. (42) The most prominent function of the complement system is to recognize stereotypical surface structures of pathogens and opsonize them, thus marking the pathogens for phagocytes, and lysing them by forming a pore called the membrane attack complex (MAC). Moreover, the complement system is involved in many other physiological processes such as adaptive immunity, apoptosis, angiogenesis, elimination of neoplastic cells and coagulation. (43)

There are multiple pathways that lead to the activation of the complement system and eventually to the opsonization and lysis of cells. The three known pathways that distinctly activate the complement system are the classical pathway, the alternative pathway and the lectin pathway which all converge into the formation of a C3 convertase. (see Figure 1)

The classical pathway depends on IgG or IgM antibody binding to a pathogen's surface. The amount of antibody bound to the target surface has to reach a certain density to allow the C1 complex to bind to two adjacent Fc-regions.

Therefore, on a molecular level one pentameric IgM antibody is sufficient to activate complement, but several monomeric IgG antibodies are necessary. Differences in the IgG heavy chains of different subclasses cause variable potency to activate the complement system. (IgG3 > IgG1 > IgG2; IgG4 = no activation). (44) After C1 complex binding to the Fc-region, C1q is activated through a conformational change, then activating C1r that cleaves and activates C1s. C1s then cleaves soluble C4 and C2 into C4a and C4b and C2a and C2b, respectively. C4a and C2b go into solution whereas C4b forms a covalent bond with the pathogens surface and C2a binds to surface-bound C4b to form the C3 convertase of the classical pathway C4bC2a. (45)

The lectin pathway can be initiated by several proteins belonging to the family of ficollins and collectins. [Ficollin-1, 2, 3, mannose binding lectin (MBL) and collectin 11]. These proteins bind to a variety of carbohydrates often found as terminal monosaccharides of glycosylated structures on surfaces of microbes including mannose, fucose, N-acetyl-D-glucosamine, N-acetyl-mannosamine, N-acetyl-D-galactosamine and other molecules such as lipoteichoic acid, DNA and β-1’3-D-glucan. (42) These pathogen-associated molecular patterns (PAMP) allow for rapid complement activation in the absence of specific immunity. Ficollins and collectins can associate with MBL-associated serine proteases (MASP) after binding to a target surface. MASP1 activates MASP2 which subsequently cleaves C4 and C2 to generate the same C3 convertase as in the classical pathway C4bC2a. MASP2 can also directly cleave C3 into C3a and C3b. (46)

The alternative pathway does not require specific activation but is autoactivated through “tickover” of C3. (47) Conformationally altered C3 can then induce a conformational change in soluble factor B rendering it susceptible to cleavage by factor D into Ba and Bb. While Ba goes into solution, Bb remains associated with C3 and cleaves it into C3a and C3b. The C3b generated in this process has a very short half-life to form a covalent bond with a cell surface before inactivation through water. Surface-bound C3b can then bind factor B that is subsequently cleaved by factor D and form the C3 convertase of the alternative pathway C3bBb. This C3 convertase is more unstable than the other C3 convertases and

can be stabilized by properdin binding to it. (48) To prevent autoactivation of complement through the alternative pathway on host cells and subsequent tissue damage, host cells bind complement regulatory proteins to their surface such as factor H, other proteins of the factor H family and membrane cofactor protein CD46 that act as cofactors for factor I-mediated cleavage of C3b into inactive products. Genetic defects reducing the proper function of factor H lead to atypical haemolytic uremic syndrome which is characterized by intravascular lysis of erythrocytes and thrombosis of small blood vessels due to uncontrolled intravascular complement activation. (49)

Upon assembly of the different C3 convertases, C3 is cleaved into C3a and C3b and C3b. The alternative pathway serves as an amplification loop for C3b deposition, since C3b generated by the classical and lectin pathways can initiate the formation of the alternative pathway C3 convertase which is a self-propagating process. Eventually, this amplification loop provides most of cleaved C3. (50) Moreover, an additional C3b molecule can associate with the C3 convertase forming the C5 convertases C4bC2aC3b and C3bBbC3b, thus initiating the terminal complement complex (TCC). The C5 convertases cleave C5 into C5a and C5b. C5a is a potent, soluble anaphylatoxin with pleomorphic effects on immune cells, vasculature and other cells. (51) C5b associates with soluble C6 and C7 which binds covalently to the target surface and this complex allows C8 and C9 to associate with it. During the association of the membrane attack complex (C5b-C9), several C9 molecules form a pore that is inserted into the cell membrane of the pathogen. Subsequently, influx of water lyses the cell. (52)

Besides pathogen elimination through lysis, complement activation aids in opsonization of pathogens by generation of C3b and its fragments iC3b and C3d which leads to phagocytosis through interaction with the complement receptors 1-4 (CR1-4) on immune cells and the soluble molecules C3a and C5a are important chemokines attracting immune cells and modulating inflammatory responses. (51, 53, 54)

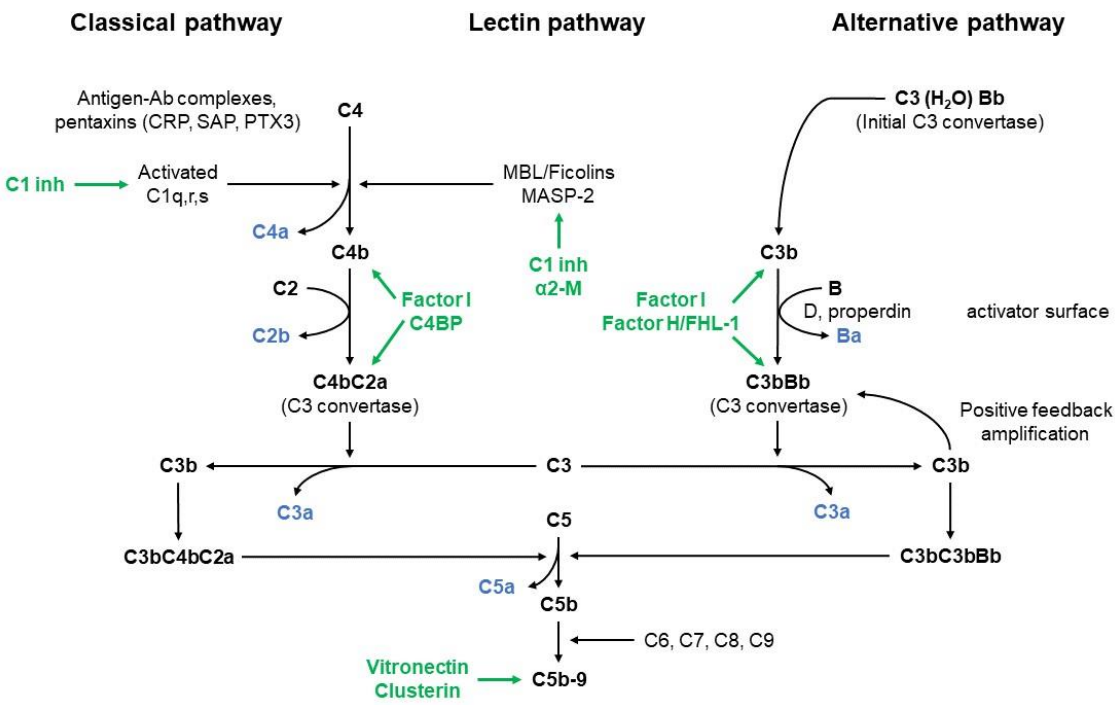

Figure 1: The complement cascade modified after Lewis and Ram (42)

1.4 Neisseria meningitidis and the complement system

Beyond its role in infectious diseases in general, the complement system is particularly important for protection against IMD. The protective properties of complement in healthy individuals become evident in the absence of a functional complement system, since people with hereditary and acquired complement deficiencies have a considerably increased risk to acquire IMD. Compared to healthy individuals the risk for IMD can increase as much as 10000-fold and multiple episodes of IMD can occur, which is very unusual in complement-sufficient individuals. Consequently, meningococcal infection is the single most frequent infection in complement-deficient individuals. (55) Depending on the deficient complement component the risk of disease can be as high as 80 %. (56) Complement deficiencies also predispose to infections with *N. gonorrhoeae* and other encapsulated bacteria such as *S. pneumoniae* and *H. influenzae* but to a lesser extent than IMD. (57, 58)

Defects of complement components in the alternative pathway, which generates most of complement deposition as described above, predispose to IMD more

frequently than defects in the classical pathway which are more commonly associated with autoimmune diseases. (42, 59) Antibody-dependent complement activation is initiated mainly through the classical pathway, but antibodies also enhance the alternative pathway which provides enough redundancy for adaptive humoral immunity to protect from IMD in classical pathway deficiencies.. (60)

Defects of C3 as the central component of the complement cascade result in a lack of opsonophagocytosis, chemotaxis and bacteriolysis. Most individuals suffer from recurrent episodes of infections with encapsulated bacteria, mostly *Nme*. Despite, the higher susceptibility the severity of these infections is comparable to infected complement-sufficient individuals. (56, 58)

Defects in the TCC (C5-C9) are almost exclusively associated with infections with pathogenic *Neisseria meningitidis* and *gonorrhoeae*. (55, 56, 61) Individuals with these complement defects have impaired serum bactericidal activity but opsonophagocytic killing is not impaired. This appears to protect sufficiently from infections with encapsulated bacteria *S. pneumoniae* and *H. influenzae*, which are not associated, but not from pathogenic *Neisseriae*. Despite the unimpaired generation of specific antibodies and retained opsonophagocytic killing the risk for IMD is up to 10000 times higher than for healthy individuals and half of affected individuals experience more than one episode of IMD.(56, 62-64) Interestingly, the severity of IMD seems to be attenuated and mortality is lower in individuals with TCC defects compared to healthy individuals and individuals with defects in earlier steps of the complement cascade. (42, 58)

Besides hereditary defects of the complement system there are several acquired conditions that can lead to insufficient complement activation. Autoimmune diseases that cause complement consumption, loss of complement in nephrotic syndrome and treatment with C5-blocking antibody eculizumab. (56, 58) Eculizumab is administered to patients with autoimmune diseases and paroxysmal nocturnal hemoglobinuria a disease characterized by spontaneous complement activation on leukocyte surfaces and intravascular coagulation due to a lack of surface-bound complement inhibitors. Treatment with eculizumab has

been linked to meningococcal disease, disseminated gonococcal disease and impaired vaccination efficacy. (65-68)

With regards to *Nme*, many virulence factors of pathogenic strains feature complement evasion as the main immune evasion strategy. Foremost, the polysaccharide capsule prevents complement activation, but other virulence factors related to complement are found in pathogenic strains as well. Sialylation and antigen modification of LPS increases human factor H binding and reduces C3 binding sites to prevent alternative pathway activation. (69-71) Some outer membrane proteins such as fHbp, porin B and neisserial surface protein A (NspA) inhibit complement in a fH-dependent manner as well and increase serum resistance. Additionally, some strains have Porin A variants that bind C4 binding protein (C4BP) which inhibits the classical and lectin pathways through prevention of C4b deposition and decay acceleration. (72-74) Furthermore, some *Nme* strains inhibit the TCC and MAC by capturing vitronectin with opacity protein Opc or meningococcal surface fibril (Msf). Vitronectin inhibits proper MAC insertion and clustering into bacterial membranes thus enhancing serum resistance. (75)

These multiple, redundant strategies of pathogenic *Nme* highlight the importance of complement in protection against IMD and illustrate that resistance to complement is a key step in the transition from colonization to invasive disease.

1.5 Neutrophil granulocytes

1.5.1 Role of neutrophil granulocytes in infectious disease

Neutrophil granulocytes are polymorphonuclear cells (PMNs) forming the predominant leukocyte population in human blood and bone marrow. PMNs have a short lifetime of hours to several days during which they reside in blood and marginalized pools in several organs such as the bone marrow and the lung. Upon organ infection changes in chemokine release and expression of adhesion molecules of the endothelium and the organ tissue lead to the recruitment of PMNs as first responders to the site of infection. Besides their role in infection

control, PMNs' involvement in other physiological processes such as sterile inflammation, cancer and tissue regeneration has been demonstrated. (76)

In infection, PMNs follow a gradient of chemokines to the site of infection, where they recognize PAMPs such as LPS with pattern recognition receptors (PRR) and deploy a vast arsenal of antimicrobial strategies against pathogens. PMNs release reactive oxygen and nitrogen species and antimicrobial proteins, phagocytise pathogens and trap them in neutrophil extracellular traps. (77, 78) Furthermore, PMNs release cytokines such as IL-8 and TNF-α to recruit more neutrophils and macrophages to the site of infection and signal to cells of the adaptive immunity. (79-81)

The importance of neutrophils as the first line of the immune system becomes evident in patients with hereditary defects of PMN functions or acquired neutropenia due to chemotherapy, bone marrow failure or immunosuppressive therapy. Patients with neutropenia or defective neutrophil effector functions have increased susceptibility for life-threatening bacteraemia with both gram-positive and gram-negative unencapsulated bacteria such as *S. aureus, S. epidermidis and P. aeruginosa*. (82, 83) Moreover, fungal infections with *Aspergillus spp.* and *Candida spp.* are common. (84-86) Increased susceptibility to encapsulated bacteria and viruses are unusual. (57)

Despite normal susceptibility towards encapsulated bacteria in neutropenic individuals, neutrophils contribute to a proper immune response towards these pathogens in the event of infection. They are important in the immune defence against the encapsulated gram-positive bacteria *S. pneumoniae* that causes a similar spectrum of disease manifestations as *Nme* including meningitis, sepsis and otitis media. Clearance of *S. pneumoniae* by phagocytes in the spleen is particularly important in this context. (87) Reduced amounts of neutrophils in the respiratory tract increase the risk of acquiring pneumococcal pneumonia and sepsis. Increased susceptibility to pneumococcal infection in connection with cigarette smoke exposure is caused by reduced recruitment of neutrophils to respiratory tissues. Once infection is established, mortality and bacterial burden are higher in neutrophil-deficient individuals. (25, 88-91) In pneumococcal

meningitis neutrophils demonstrate protective functions as well. Migration of neutrophils into the cerebrospinal fluid (CSF) reduces mortality in acute infection and long-term sequelae. (92-94) However, some aspects of neutrophil activation such as the formation of NETs and excessive neutrophil-derived cytokine production have been linked to brain tissue damage. (95-97)

Despite their essential beneficial role in immune defence, the inflammatory response of PMNs has been demonstrated to contribute to excessive host tissue damage and disease pathology in obesity, atherosclerosis and sepsis. (98-100)

1.5.2 Neutrophil granulocytes and the complement system

Neutrophil granulocytes and the complement system represent a cellular and a humoral first line of the immune system, respectively. There is extensive interaction between these two parts of the immune system. PMNs express several receptors for different complement factors. PMN CR1 and CR3 bind C3b, C4b, iC3b, C3c and C3d enabling efficient phagocytosis of particles and pathogens opsonized with these complement factors. (53, 101) Furthermore, PMNs express C3a receptor (C3aR), C5a receptor (C5aR) and C5a receptor-like 2 (C5L2) for the two most important anaphylatoxins of the complement system. C3a and C5a have been shown to trigger the oxidative burst and increase phagocytosis in neutrophils via their respective receptors and C5a induces upregulation of neutrophil complement receptor CR3. Additionally, C5a is a powerful chemokine influencing neutrophil migration. (51, 66, 102)

On the other hand, PMNs secret complement factors from their granules under inflammatory condition. The granules contain C3, Properdin and Factor B increasing alternative pathway activation and opsonization, which in return facilitates phagocytosis. Moreover, proteases from neutrophil granules have been reported to activate the alternative complement pathway directly. (103, 104)

1.5.3 Neutrophil granulocytes and meningococcal disease

In meningococcal disease, recruitment of neutrophil granulocytes can be observed at early stages of disease. Large numbers of PMNs especially in the

cerebrospinal fluid but also in peripheral blood are typical and inform diagnosis. (105, 106) However, PMNs' role in clearance of the infection remains unclear.

PMNs have been reported repeatedly to phagocytise non-opsonized and opsonized meningococci and effectively neutralize them and depletion of neutrophils in a mouse model of meningococcal sepsis increased mortality. (107-109) In humans, low counts of peripheral PMNs at the time of diagnosis have been associated with a higher mortality. (110) Furthermore, neutrophils appear to be particularly important in controlling meningococcal colonization of the nasopharynx, since depletion of neutrophils leads to a higher burden of colonization in a CEACAM1-humanized transgenic mouse model. (111)

Contrary to these findings, opsonophagocytic killing of *Nme* by PMNs appears to be insufficient to protect from IMD as people with terminal complement deficiencies have a thousand-fold higher risk for IMD despite unimpaired opsonization with C3. (55, 58) On the other hand, preserved opsonization and opsonophagocytosis is sufficient regarding infection with other encapsulated bacteria such as *S. pneumoniae* and *H. influenzae,* since terminal complement deficiencies do not increase susceptibility to these bacteria but exclusively to pathogenic *Neisseriae*. (58, 112, 113) Furthermore, *Nme* have resistance mechanisms against some neutrophil antimicrobial strategies such as neutrophil extracellular traps (NET) that are observed in bacterial meningitis and sepsis. (95, 114, 115) Additionally, NETs have been shown to be detrimental and impair microcirculation in a mouse model of sepsis. (98) Negative effects of excessive inflammation in both sepsis and meningitis have led to clinical trials using corticosteroids as supportive treatment to control inflammation. However, corticosteroid treatment neither reduced mortality nor frequency of sequelae in meningococcal meningitis despite beneficial results in meningitis caused by *S. pneumoniae* and *H. influenzae*. (34) Nevertheless, benefits of corticosteroid treatment in sepsis with hyperinvasive meningococcal strains have been reported. (116)

These multifaceted findings highlight that the neutrophil role in meningococcal disease is not well understood and further research is needed to understand possible beneficial and detrimental effects of PMN activation.

1.6 Ceramide in N. meningitidis infection

Ceramide is an integral component of sphingolipids in cellular membranes and an important messenger molecule in many physiological and pathological signal transmission pathways including infectious diseases. Ceramide can be cleaved from membrane sphingolipids upon certain signals by sphingomyelinases. Subsequently, ceramide accumulates in cholesterol-rich “lipid rafts”, where certain proteins cluster thus forming a platform for signal transmission. (117) Multiple pathogens have been shown to induce ceramide release via translocation of the acid sphingomyelinase (ASM) from the cytosol to the cell membrane. In infection with *P. aeruginosa*, *E. coli, Salmonella enterica* and *S. aureus* ceramide release has been demonstrated to mediate multiple effects on epithelial cells including increased internalization of bacteria into host cells, induction of apoptosis, decay of tight junctions and inflammasome activation. (118, 119) ASM-mediated ceramide release in phagocytes is important in fusion of phagosomes with lysosomes thus promoting pathogen elimination but has also been linked to increased reactive oxygen species production and tissue damage. (120-123)

Regarding pathogenic *Neisseria*, ceramide release is involved in invasion of non-phagocytic cells. Opa proteins of *Neisseria gonorrhoeae* induce ASM activation and ceramide release in epithelial cells through interaction with the CEACAM1 receptor, which subsequently leads to invasion into the cell and dissemination into tissues of the urogenital tract. (124, 125) Since neutrophils also express the CEACAM1 receptor, ceramide release is suspected to be involved in phagocytosis as well. However, neutrophils also express the CEACAM3 receptor which is restricted to neutrophils and serves as a decoy receptor to catch Opa-expressing bacteria. CEACAM3 acts via a different intracellular signalling cascades and promotes phagocytosis, oxidative burst, degranulation and a vigorous inflammatory response leading to effective bacterial killing. The overall

neutrophil response to *Neisseria gonorrhoeae* is more defined by this CEACAM3 pathway than by CEACAM1. (126-128) *Neisseria meningitidis* expresses different Opa-proteins as well and Opa interaction with epithelial CEACAM1 promotes invasion and systemic dissemination. CEACAM1 expression is upregulated by pro-inflammatory cytokines during mucosal inflammation facilitating invasion of *Nme*. This offers an explanation why epidemiologic observations show an association of IMD with preceding viral infections of the upper respiratory tract. (111, 128-130) Some meningococcal strains express Opc that binds to human cell surface heparan sulfate proteoglycans (HSPGs). Opc interaction with HSPGs triggers ASM activation and ceramide release leading to the engulfment of the bacteria by endothelial cells and transmigration through the blood-brain barrier. (131) Two clinical studies have reported hyperinvasive clusters (ST-11/ST-8) of non-Opc-expressing strains that frequently cause sepsis but rarely meningitis indicating a possible influence of Opc on the course of disease. (132-134)

Regarding neutrophils, there is insufficient evidence of the role of ceramide release in phagocytosis and other neutrophil effector functions in meningococcal disease. Since meningococcal Opa proteins do not bind to CEACAM3 but mostly to CEACAM1 and occasionally to CEACAM5 and CEACAM6, different cellular responses can be expected. (135) Since most pathogenic *Nme* are encapsulated as opposed to the unencapsulated *Ngo*, interaction between Opa proteins and CEACAM receptors could be reduced due to the capsule although CEACAM binding of encapsulated *Nme* has been described. (136, 137)

In general, Opa-CEACAM binding and ceramide release in PMN interaction with *Nme* is not well understood and requires further research.

1.7 Research hypothesis

The complement system has been established as the most important determinant for protection against IMD. Especially the bacteriolytic properties of the membrane attack complex are essential to prevent meningococcal infection since patients with terminal complement deficiencies have a high risk of acquiring IMD. However, the cellular immune response in IMD carried out by neutrophils is less

well understood. There is evidence for both beneficial and detrimental effects of PMN activation in IMD which requires further research to characterize neutrophil responses regarding phagocytosis, oxidative burst, degranulation and cytokine release in response to *Nme*. Because IMD is closely connected to the complement system and the complement system can influence PMNs through opsonization of bacteria and released anaphylatoxins, the influences of complement on these responses merit a particular focus in this context.

Therefore, the hypothesis of this project is:

"Neutrophil granulocytes react with a variety of cellular responses to Neisseria meningitidis including phagocytosis, oxidative burst, degranulation and cytokine release. Complement activation significantly influences these responses and demonstrates cooperation between humoral and cellular immune responses in pathogen clearance."

First, a whole blood model and cell culture of PMNs is to be established to analyse cellular interactions (adhesion/internalization), oxidative burst response, degranulation and cytokine release of infected PMNs. A selection of *Nme* strains will be used that represent encapsulated and unencapsulated strains and both isolates from IMD patients and from carriers.

Second, the influence of complement and specific antibodies on the aforementioned PMN cellular responses will be assessed by adding different sources of complement and antibodies to isolated PMNs. In the whole blood model, the effect of complement and antibodies will be investigated by blockade of different steps of the complement cascade and antibody-mediated phagocytosis.

Last, ceramide production in PMNs will be qualitatively assessed by immunofluorescence staining of PMNs infected with fluorescent bacteria. The interaction between bacteria and PMNs will be measured and analysed in the context of observed ceramide production.

2 Material and methods

2.1 Laboratory devices

Biosafety cabinets	LaminAir® HB 2472 (Heraeus, Hanau, Germany) MSC AdvantageTM 1.5 (Thermo Electron LED, Langenselboid, Germany)
Calculator	LCD 3112 (Olympia, Hattingen, Germany)
Centrifuges	Biofuge fresco (Heraeus, Hanau, Germany) Rotanta 460 R (Hettich, Tuttlingen, Germany)
Counter	Handstückzähler economy (unknown manufacturer, received from A. Hartenstein, Würzburg, Germany)
ELISA reader	Multiskan EX (Thermo Labsystems, Waltham, USA)
ELISA washer	NuncTM immuno Washer 12 (Nunc A/S, Roskilde, Denmark)
Flow cytometer	FACSCalibur (Becton Dickinson, Franklin Lakes, USA)
Freezers	GGU 1500 (Liebherr, Bulle, Switzerland); (-22 °C) ThermoForma -86C ULT Freezer (Thermo Scientific, (Waltham, USA); (-81 °C)
Fridges	FKS 1800 (Liebherr, Bulle, Switzerland) KS 3160 (Liebherr, Bulle, Switzerland)
Heating block	Thermochem (Liebisch, Bielefeld, Germany)
Ice machine	AF 156 (Scotsman Ice Systems, Vernon Hills, USA)
Incubators (37 °C, 5 % CO2, 90 % humidity)	B 6200 (Heraeus, Hanau, Germany) INC 246 (Memmert, Schwabach, Germany)
Magnetic stirrer	MR 3001 (Heidolph, Schwabach, Germany) RCT (IKAMAG®, Staufen, Germany)
Microscopes	Type 020-507.010 (Leica, Wetzlar, Germany) BZ-9000 Biorevo (Keyence, Osaka, Japan)
Microwave	MWS 1820 Duo (Bauknecht, Stuttgart, Germany)

Multi-channel pipette	VWR SignatureTM Ergonomic High-Performance 20-200 µl (VWR International, Darmstadt, Germany)
Neubauer counting chamber	Zählkammer Neubauer improved (unknown manufacturer, received from A. Hartenstein, Würzburg, Germany)
Photometer	CO8000 (WPA, Cambridge, UK)
Pipettes	Research® plus 0.5-10 µl / 10-100 µl / 100-1000 µl (Eppendorf, Hamburg, Germany) Reference® 0.5-10 µl / 10-100 µl / 100-1000 µl (Eppendorf, Hamburg, Germany) PIPETMAN P20 / P200 / P1000 (Gilson, Middleton, USA) Multipette® M4 (Eppendorf, Hamburg, Germany)
Pipette controller	accu-jet® pro (BRAND, Wertheim, Germany)
Scales	ABT 120-5DM (Kern, Balingen-Frommern, Germany) PFB 6000-1 (Kern, Balingen-Frommern, Germany) P1200 (Mettler, Greifensee, Switzerland)
Shaker	KL-2 (Edmund Bühler, Tübingen, Germany)
Timer	Timer SKT 338 N (Oregon Scientific, Tualatin, USA)
Vortexer	REAX 2000 (Heidolph, Schwabach, Germany)

Table 1: Laboratory devices

2.2 Consumable supplies

Blood agar plates (Columbia Agar + 5 % sheep blood)	COS 90 mm (bioMerieux, Marcy l´Etoile, France)
Cannula	Einmalkanüle 20G 1 ½ ((unknown manufacturer, received from A. Hartenstein, Würzburg, Germany)
Centrifuge tubes	15 ml / 50 ml (Greiner Bio-One, Fricken-hausen, Germany)
Closure film	Parafilm M, 4IN. X 125 Ft. (Bemis, Neenah, USA)
Cotton swab	Cotton swab 15 cm (Heinz Herenz, Hamburg, Germany)
Coverslip	Coverslips, round 12 mm/15 mm (unknown manufacturer, received from A. Hartenstein, Würzburg, Germany)
Cuvettes	Half-Micro-Cuvette (Sarstedt, Nümbrecht, Germany)
Delimiting pen	Dako-Pen (Agilent Technologies, Glostrup, Denmark)
Disinfectant	terralin® liquid (Schülke, Norderstedt, Germany)
Dialysis membrane	Dialysis tube VISKING® Type 20/32 (Carl Roth GmbH, Karlsruhe, Germany)
ELISA closure foil	Self-adhesive polyester closure foil for PCR plates (unknown manufacturer, received from A. Hartenstein, Würzburg, Germany)
FACS tubes	5 ml Polystyrene round bottom Tube (FACS) (Becton Dickinson, Franklin Lakes, USA)
Gloves	Peha-soft® nitril fino (Hartmann, Heidenheim, Germany)
Inoculation loop	Impfschlinge 10 µl (Sarstedt, Nümbrecht, Germany)
Microscope slides (glas)	(unknown manufacturer, received from A. Hartenstein, Würzburg, Germany)
Monovettes	S-Monovette® 9 ml K3E (Sarstedt, Nümbrecht, Germany) S-Monovette® 2.7 ml Hirudin (Sarstedt, Nümbrecht, Germany)
Mouthpiece	Foliodress® mask Loop (Hartmann, Heidenheim, Germany)
Pipette tips	10 µl / 200 µl / 1000 µl (Sarstedt, Nümbrecht, Germany)

Reagent reservoir	Reagent Reservoirs 50 ml (VWR International, Radnor, USA)
Reaction tubes	Eppendorf Tubes® 0.5 ml / 1.5 ml / 2.0 ml / 5.0 ml (Eppendorf, Hamburg, Germany)
Sterile filter	Filtropur S 0.2 (Sarstedt, Nümbrecht, Germany)
Syringes	1 ml TBC (Dispomed, Gelnhausen, Germany) 3 ml SOFT- JECT® / 5 ml SOFT-JECT® / 10 ml SOFT-JECT® (Henke Sass Wolf, Tuttlingen, Germany) 50 ml INFUJECT® (Dispomed, Gelnhausen, Germany)
24-well plates	24-well plate (Sarstedt, Nümbrecht, Germany)
96-well plates	Microtest plate 96 wells, R (Sarstedt, Nümbrecht, Germany)
96-well plates ELISA	Microplate, 96 well, PS, U-bottom, MICROLON® 600, high binding, clear (Greiner Bio-One, Frickenhausen)

Table 2: Consumable supplies

2.3 Reagents

2.3.1 Chemicals

Avidin peroxidase	Avidin-Peroxidase 1 mg/ml (A3151-1MG) (Sigma-Aldrich, St. Louis, USA)
Distilled water	Aqua B. Braun Ecotainer® (B. Braun, Melsungen, Germany)
Bovine serum albumin (BSA)	A1391,0100 Albumin Fraction V (pH 7.0) (PanReac AppliChem ITW Reagents, Darmstadt, Germany)
C5a	Recombinant Human Complement Component C5a (R&D Systems, Minneapolis, USA)
Compstatin	Compstatin (Tocris, Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany)
Compstatin-Cp20 analogue	Provided by John D. Lambris, University of Pennsylvania, USA
4',6-diamidino-2-phenylindole (DAPI)	DAPI for nucleic acid staining (Sigma-Aldrich, St. Louis, USA)
Dihydrorhodamine 123 (DHR123)	Dihydrorhodamine 123 (Sigma-Aldrich, St. Louis, USA)
Ethanol	Ethanol Rotipuran® ≥ 99.8 % p.a. (Carl Roth GmbH, Karlsruhe, Germany)
Fetal calf serum (FCS)	Fetal bovine serum (gibco, ThermoFisher Scientific, Waltham, USA)
Gentamicin sulfate	Gentamicinsulfat (Carl Roth GmbH, Karlsruhe, Germany)
Histology mounting medium	Fluoroshield™ (Sigma-Aldrich, St. Louis, USA)
Hydrogen peroxide	Wasserstoffperoxid 30 % (Merck, Darmstadt, Germany)
Hypotonic buffer	168 mM NH_4Cl–10 mM, $KHCO_3$–0.125 mM EDTA (prepared by Lea Strobel)
KBR buffer	10 mM HEPES, 125 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.3 (Virion-Serion GmbH, Würzburg, Germany)
Paraformaldehyde (PFA)	Paraformaldehyd reinst, DAC (Carl Roth GmbH, Karlsruhe, Germany)

Phosphate-buffered-saline (PBS)	gibco® DPBS powder without calcium chloride and magnesium chloride (life technologies Corporation, Grand Island, USA)
Phorbol 12-myristate 13-acetate (PMA)	Phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, USA)
PMX53	PMX53 (Tocris, Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany)
Polymorphprep	Polymorphprep (Progen Biotechnik GmbH, Heidelberg, Germany)
RPMI	RPMI 1640 Media (ThermoFisher Scientific, Waltham, USA)
Saponin	Saponin (Carl Roth, Karlsruhe, Germany)
Sulfuric acid	Schwefelsäure 96 % (Carl Roth, Karlsruhe, Germany)
Tween 20	Tween 20 (Merck-Schuchardt, Hohenbrunn, Germany)
Zymosan A	Zymosan A from Saccharomyces cerevisiae (Sigma-Aldrich, St. Louis, USA)

Table 3: Chemicals

2.3.2 Reaction kits

IL-8 ELISA kit	Human CXCL8/IL-8 DuoSet ELISA (R&D Systems, Minneapolis, USA)
TMB Substrate kit	TMB Substrate Kit (Thermo Scientific, Rockford, USA)

Table 4: Reaction kits

2.3.3 Antibodies

CD16 antibody	Purified anti-human CD16, Mouse IgG1, kappa (Clone 3G8) (Biozol, Eching, Germany)
CD32 antibody	CD32 monoclonal antibody, Mouse IgG1 (Clone 6C4) Functional Grade, eBioscience™ (ThermoFisher, Waltham, USA)
CD64 antibody	Purified anti-human CD64, Mouse IgG1, kappa (Clone 10.1) (Biozol, Eching, Germany
CD11b antibody (Flow cytometry)	APC anti-mouse/human CD11b, Rat IgG2b (Clone M1/70) (Biozol, Eching, Germany)
CD11b antibody isotype control (flow cytometry)	APC Rat IgG2a, kappa isotype control (Clone RTK2758) (Biozol, Eching, Germany)
Complement C3 primary antibody (Flow cytometry staining	Goat Polyclonal to Human Complement C3 (Biozol, Eching, Germany)
Complement C3 secondary antibody (Flow cytometry staining)	Donkey anti-goat IgG-alexa 488 conjugate (Jackson ImmunoResearch, West Grove, USA)
Ceramide primary antibody (Immunofluorescence staining)	mouse mAb anti-ceramide IgM (clone MID 15B4) (Enzo Life Sciences Gmbh, formerly Alexis Biochemicals, Lörrach, Germany)
Ceramide secondary antibody (Immunofluorescence staining)	Cy3-conjugated goat anti-mouse IgM (Dianova, Hamburg, Germany)
Opa antibody	Monoclonal anti-Opa protein antibody clone 4B12/C11 (138)

Table 5: Antibodies

2.3.4 Serums

Rabbit anti-*Nme* serum	produced by Kay Johswich in Toronto; polyclonal antibody, not purified; stored at -80 °C
Rabbit serum (naive)	Baby Rabbit Complement, Lyophilized (Pooled Normal Rabbit Serum from Young Rabbits) (Biozol, Eching, Germany)
Normal, non-immune human serum	Freshly obtained from healthy volunteers by venepuncture (no history of meningococcal disease or vaccination)
Human immune serum	Freshly obtained from a donor vaccinated against *Nme* serogroups A, B, C, W135, Y
Goat serum	Normal Goat Serum (Sigma-Aldrich, St. Louis, USA)

Table 6: Serums

2.4 Whole blood

Whole blood was obtained from healthy volunteers by venipuncture. This procedure was authorized by the University of Würzburg Ethics Committee on August 29, 2016 (AZ-181/16-ge) and no ethical or legal reservations were ascertained. Exclusion criteria for blood donors were acute or chronic disease, inflammatory conditions, antibiotics or corticosteroids within 14 days before venepuncture. None of the donors had a previous history of meningococcal disease or immunization. The volunteers were properly informed about this study using an information sheet and oral explanation and gave their written consent before venipuncture.

Hirudin was chosen for anticoagulation based on its property not to interfere with the complement system. The blood was obtained freshly before the experiments and used immediately.

2.5 PMN cells

2.5.1 Isolation process

Human neutrophils were isolated from whole blood through venipuncture of healthy volunteers (see chapter 2.4). Anticoagulation was performed with EDTA and 7 ml of blood was added pipetted on top of 7 ml of Polymorphprep in a 15 ml falcon using the pipette controller. The addition was carried out carefully to avoid mixture of the two liquids. The falcon was centrifugated at 700 g for 35 min at 20°C without brake. Afterwards, the PMN band was removed using a syringe and a canula and carefully resuspended in 40 ml PBS in a 50 ml falcon. The falcon was centrifugated at 500 g for 5 min at 20°C with brake. Afterwards, the supernatant was removed with the pipette controller and the leukocyte pellet was resuspended in 5 ml hypotonic buffer to lyse remaining erythrocytes. Lysis was stopped after 5 min using PBS and the cells were centrifugated at 1000 g for 5 min at 20°C. The supernatant was discarded, and the cells were suspended in 3 ml RPMI medium. 20 µl of the cell suspension were diluted 1:4, 1:16 and 1:64 in trypan blue and counted under the microscope using a Neubauer counting

chamber. Subsequently, the cell concentration was adjusted by addition of RPMI medium to the necessary concentration, mostly $5x10^5$ PMNs/ml or 10^6 PMNs/ml.

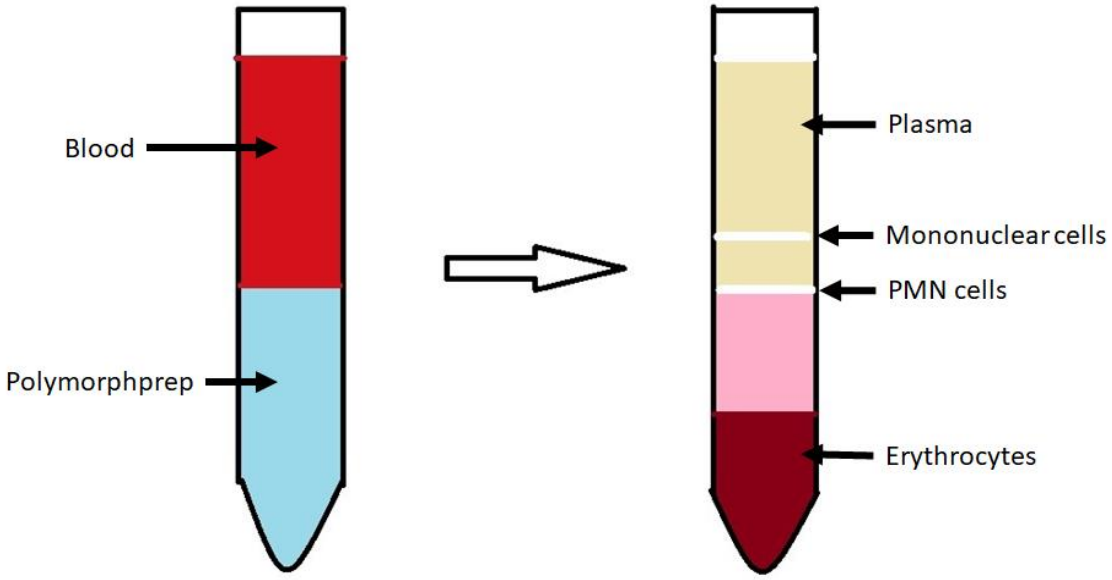

Figure 2: Polymorphprep before and after centrifugation

Left: EDTA blood was carefully pipetted on top of Polymorphprep and centrifugated for 35 minutes. **Right:** Corpuscular components of blood separated into bands and interspaced by liquid as indicated following centrifugation.

2.5.2 PMN purity

PMN purity was assessed by cytospin preparation and Diff-Quick staining. PMN purity by the isolation process above was > 90 %.(139)

2.6 Bacteria

2.6.1 Bacterial strains and handling

In this research projects several meningococcal strains were used including the strains 8013, α4, MC58, α522 and α14. Additionally, two mutants of MC58 were used, the capsule knockout MC58Δcsb and GFP-expressing MC58. Furthermore, the *N. lactamica* strain 020-06 was used in the experiments. All strains were stored at -80°C in 30 % glycerine in BHI. When working with the strains live

bacteria were only handled in a biosafety cabinet certified for the work with biosafety level 2 organisms.

Strains	Species	Serogroup (capsule)	Sequence type (clonal complex)	Invasive/carrier
8013	*N. meningitidis*	C	ST-177 (cc18)	Invasive
α4	*N. meningitidis*	B	ST-19 (cc18)	Carrier
MC58	*N. meningitidis*	B	ST-74 (cc32)	Invasive
α522	*N. meningitidis*	B	ST-35 (cc35)	Carrier
α14	*N. meningitidis*	-	ST-53 (cc53)	Carrier
020-06	*N. lactamica*	-	ST-640 (cc640)	Carrier
MC58Δcsb	*N. meningitidis*	-	ST-74 (cc32)	(mutant)
MC58-GFP	*N. meningitidis*	B	ST-74 (cc32)	(mutant)

Table 7: Bacterial strains

2.6.1.1 Meningococcal strains

The strains used in the experiments seek to represent different properties of circulating strains and their influence on pathogenicity. Both capsulated (8013, α4, MC58 and α522) and unencapsulated strains (MC58∆csb and α14) were employed in this project. Furthermore, there are two pairs of genetically related strains where one strain was isolated from an individual suffering from invasive meningococcal disease (IMD) and the other from an asymptomatic carrier. 8013 (invasive) pairs with α4 (carrier) and MC58 (invasive) pairs with α522 (carrier).

MC58-GFP is a GFP-expressing mutant of MC58 and was transformed with a plasmid carrying the GFP gene under the control of the *porA* promotor. (140)

MC58∆csb is a capsule deficient mutant of MC58 and was transformed with a plasmid containing a chloramphenicol resistance cassette in the open reading frame of the *csb* gene. The premature stop codon renders the encoded alpha-2,8-polysialic-acid transferase useless and thus no capsule can be produced. (141)

2.6.1.2 Neisseria lactamica 020-06

N. lactamica 020-06 is non-pathogenic bacteria closely related to *Nme* and *N.gonorrhoea*. *N. lactamica* species frequently colonizes the nasopharynx of children. *N. lactamica 020-06* has no polysaccharide capsule. (142)

2.6.1.3 Heat inactivation

If required, heat-inactivated bacteria were used for the experiments. For heat inactivation the required inocula were placed on a heating block for 30 min at 70 °C. After heat-inactivation serial dilutions of *Nme* were plated on blood agar plates and grown over night to exclude viability and confirm inactivation.

2.7 Time frame

All time points measured in this project were counted from the time of infection representing the time point 0h.

2.8 Infection assays

2.8.1 Inoculum

In order to obtain an inoculum of log phase bacteria *Nme* strains were plated on blood agar plates, incubated over night at 37 °C, 5 % CO_2 and transferred to a new plate in the morning and grown for 4 hours. Bacteria were suspended in RPMI using a cotton swab and the optical density of a 1:10 dilution of this stock was measured photometrically. Subsequently, the optical density was adjusted by dilution of the stock to obtain a concentration of 10^9 CFU/ml. The optical densities for each strain equalling this concentration can be found in Table 8. For the infection assays to measure adhesion, internalization and intracellular survival the strains *Nme* 8013, α4, MC58, α522, α14 and *Nlac 020-06* were used.

Strain	**Optical density equalling 10^9 CFU/ml**
8013	1.0
α4	1.0
MC58	1.0
α522	0.8
α14	1.2
N. lactamica 020-06	2.0
MC58∆csb	1.0

Table 8: Optical density

2.8.2 Infection

For infection assays $5x10^5$ PMN cells in 1 ml RPMI medium were seeded in 24-well plates. After one hour of incubation at 37 °C, 5 % CO_2 to allow attachment to the wells, the supernatant was removed using a vacuum system and replaced with 1 ml RPMI + 5 % FCS. Then the wells were infected with 50 µl of an inoculum with 10^9 CFU/ml equalling an infection dosis of $5x10^7$ CFU/ml. Well plates were placed in an incubator at 37 °C, 5 % CO_2. PMN viability was repeatedly controlled under the microscope during the incubation period.

2.8.3 Assay to measure adhesion to PMN cells

After an incubation period of 1 or 4 hours the supernatant was removed, and the wells were washed 3 times with 1 ml pre-warmed PBS to remove non-attached bacteria using the vacuum system and the ELISA washer. The washing steps were carried out very carefully to avoid detachment and loss of PMN cells. Each washing step included a 1 min waiting period after adding PBS. After the last washing step 1 ml 1 % saponin was pipetted into the wells to lyse PMN cells and the wells were incubated for 30 min. Afterwards, the content of the wells was thoroughly resuspended with a pipette and 200 µl were serially diluted 6 times and plated on blood agar plates to enumerate viable CFU counts.

2.8.4 Assay to measure internalization into PMN cells

To measure internalization an additional step was performed before the washing steps compared to the protocol for measuring adhesion above. After the incubation period the supernatant was removed with the vacuum system and 1 ml of gentamicin in RPMI + 5 % FCS (200 µg/ml) was added to the wells to eliminate extracellular bacteria. After incubation for 30 min cells were washed, lysed and plated as described before to assess the number of CFU of bacteria adherent to PMNs.

2.8.5 Gentamicin Protection Assay

To assess the viability of internalized bacteria PMN cells were infected with *Nme* and *Nlac* for 4 hours as described above. Subsequently, the medium was removed and 1 ml gentamicin in RPMI + 5 % FCS (200 µg/ml) was added to

eliminate extracellular bacteria and assess the number of intracellular bacteria over time. Cells from each donor were incubated for the time points 30 min, 1 hour, 2 hours and 4 hours during the same experiment. After the respective incubation period cells were washed with PBS, lysed with 1 % saponin and serial dilutions were plated on blood agar plates as described above.

2.8.6 Killing assay

To measure killing of bacteria by PMNs 10^6 PMNs in 100 µl RPMI + 10 % FCS were infected with an inoculum of $5x10^3$ CFU/ml and incubated for 30 min at 37 °C, 5 % CO_2. Then 20 µl of the samples were plated on blood agar plates to enumerate viable CFU counts. The normal growth control consisted of $5x10^3$ CFU/ml *Nme* growing in 100 µl RPMI + 10 % FCS without PMN cells. To investigate the effect of plasma components on PMN killing of *Nme* the experiments were also carried out in the presence of 10 % human plasma or heat-inactivated plasma.

2.9 Sources of complement and antibodies

2.9.1 Baby rabbit complement

Baby rabbit complement (BRC) was used as a source of complement since BRC is naïve serum without specific antibodies to *Nme*. BRC was used at a concentration of 10 %.

2.9.2 Rabbit polyclonal anti-*Nme* antibodies

A polyclonal antibody that was raised in rabbits against *Nme MC58* (kindly provided by Prof. Scott Gray-Owen, University of Toronto) and purified by protein G affinity chromatography was used in some experiments of this study. (109) When indicated it was used at a concentration of 1 µg/ml. Figure 3 shows the high affinity of the antibody measured by ELISA to all strains used in this study compared to naïve rabbit IgG.

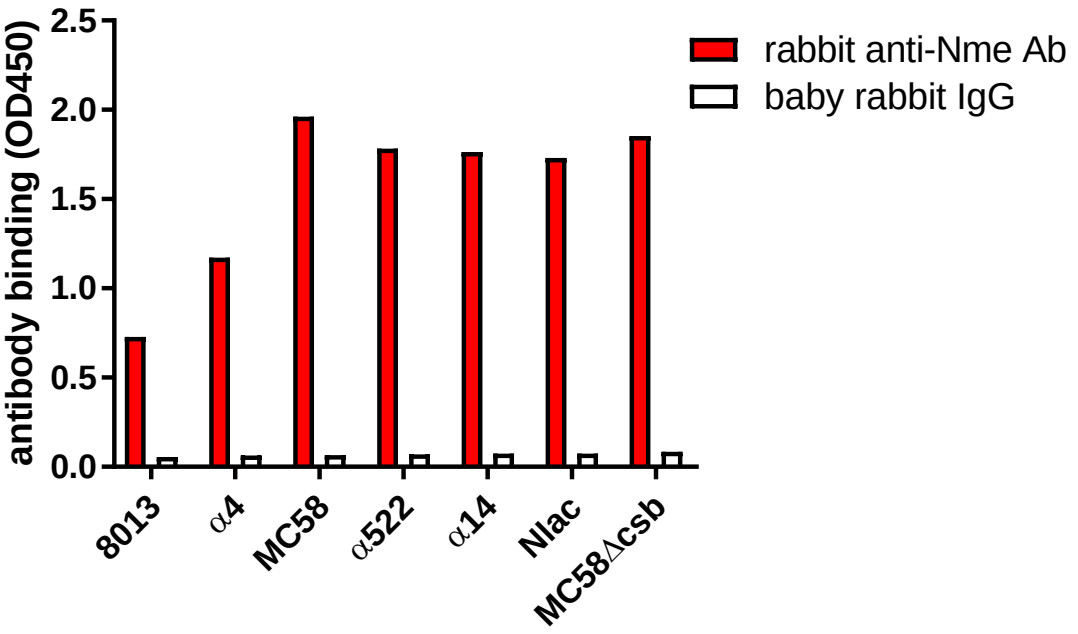

Figure 3: Affinity of rabbit polyclonal anti-Nme antibody to indicated *Nme* and *Nlac* strains compared to baby rabbit IgG

1 µg/ml of either anti-Nme ab or baby rabbit IgG were incubated on an ELISA plate coated with the indicated strains. HRP-labeled goat anti-rabbit IgG was used as a secondary antibody and colorimetric reaction was measured using TMB substrate.

2.9.3 Normal human serum

Normal, non-immune human serum (NHS) was obtained from unvaccinated donors without any history of meningococcal infection. It contains all complement factors but no specific antibodies to *Nme*. Lack of killing was verified by incubating *Neisseria* strains with serum and enumerating viable CFU counts. NHS was used at a concentration of 5 % or 10 %.

2.9.4 Human immune serum

Human immune serum (IMS) was obtained from an immunized individual with known high levels of anti-meningococcal antibodies against all serogroups used in these experiments. Furthermore, all complement factors are sufficiently available in IMS. IMS was used at a concentration of 5 %.

2.9.5 Recombinant human C5a

Recombinant human C5a was obtained from R&D Systems and used at a final concentration of 1000 nM.

2.10 Complement inhibitors

2.10.1 Compstatin and Compstatin-Cp20 analogue

Compstatin and compstatin-Cp20 analog are peptides that both inhibit C3 cleavage into C3a and C3b by binding selectively to the C3. Both peptides have 13 residues and are cyclized through a disulfide bridge. Compstatin-Cp20 analog (Ac-Ile-[Cys-Val-Trp(1-Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mIle-NH_2) has an 871-fold increased affinity compared to the original compstatin (Ile-[Cys-Val-Val-Gln-Asp-Trp-Gly-His-His-Arg-Cys]-Thr-NH_2).(143, 144) (see Figure 4)

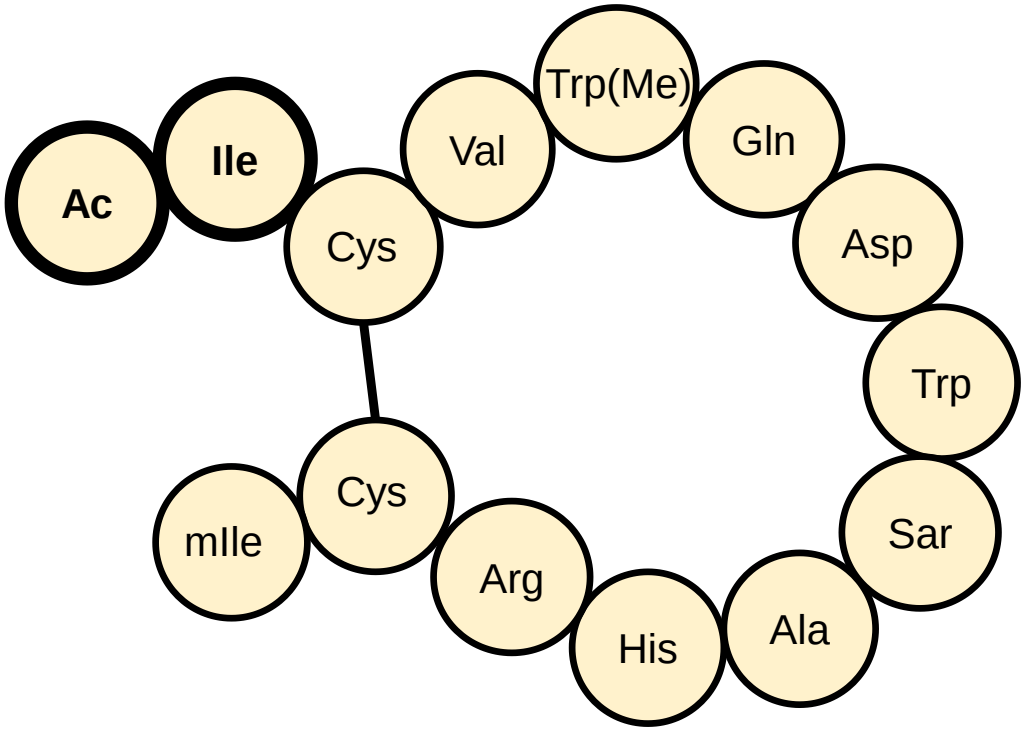

Figure 4: Structure of Compstatin Cp20

Amino acid sequence of the C3 inhibitor Compstatin Cp20 modified after Qu et al. (145)

2.10.2 C5a receptor blockade

PMX53 is a human-specific peptide inhibitor of of C5a receptor 1 (C5aR1) that antagonizes C5aR1 activation through orthosteric binding. PMX53 was used in some experiments to block the chemotactic and proinflammatory effects of the C5a/C5aR1 axis on neutrophils. (51, 146)

2.11 Flow cytometry assays

2.11.1 Measurement of *Nme* association to PMN cells

$2x10^5$ PMNs per sample were isolated as described above and suspended in 200 µl RPMI + 5 % FCS. Samples were infected with 20 µl of an inoculum with *Nme MC58-GFP* of 10^8 CFU/ml. *Nme MC58-GFP* is a mutant of strain MC58 expressing green fluorescent protein. A sample with PMNs cells but without *Nme* was taken as the negative control. After incubation for one hour at 37 °C, 5 % CO_2 the assay was stopped by adding 200µl 4 % paraformaldehyde (PFA)

to the samples. After fixation the samples were analysed by flow cytometry by gating PMN cells for their characteristic FSC/SSC pattern. GFP fluorescence was recorded in channel FL1-H.

Were indicated, 5 % NHS, heat-inactivated NHS, IMS or heat-inactivated IMS were added to RPMI instead of 5 % FCS prior to infection.

Samples were investigated seperately under the following experimental conditions: To block complement activation before C3 cleavage samples were pre-incubated 10 minutes before infection using compstatin Cp20-analog at a working concentration of 30 µg/ml. To block activation of the C5a/C5aR1 axis samples were preincubated with PMX53 at a concentration of 10 µM. To block Fcγ receptors on PMN cells antibodies against CD16 (clone 3G8; mouse IgG1; Biolegend), CD32 (clone 10.1; mouse IgG1; Biolegend) and CD64 (clone 6C4; mouse IgG1; eBiosciences) were added to the samples at 1 µg/ml each 10 minutes prior to infection. Any sodium azide in the antibodies was removed prior to the experiments by overnight dialysis.

2.11.2 Measuring the oxidative burst

2.11.2.1 PMNs

To measure the oxidative burst 10^5 PMNs were isolated as described above and suspended in 100 µl RPMI + 5 % FCS. Dihydrorhodamine 123 (DHR123) was added at a concentration of 0.2 µg/ml to the samples. DHR123 is a reactive oxygen species indicator that can pass membranes. Upon oxidation to cationic rhodamine it exhibits green fluorescence. (147) After incubation with DHR123 for 10 min cells were infected with *Nme* at 10^8 CFU/ml equalling a multiplicity of infection (MOI) of 100. Phorbol myristate acetate (PMA) was used as a positive control at a concentration of 1 µM which induces the oxidative burst independently from membrane receptors by activating protein kinase C thus demonstrating neutrophil overall responsiveness in this model. (148) After 10, 30 or 60 min the reaction was stopped by adding 300 µl 4 % PFA. After fixation PMNs were gated by their characteristic FSC/SSC pattern and DHR123 fluorescence was recorded in channel FL1-H.

When indicated 10 % BRC or autologous plasma were added prior to infection instead of 5 % FCS to analyse the effect of complement on the oxidative burst. For some experiments, a polyclonal anti-meningococcal antibody was used at 1 µg/ml and C5a was used at 1000 nM.

2.11.2.2 Whole blood

Measurements of the oxidative burst in whole blood were performed with 100 µl hirudin blood. Hirudin is an anticoagulant derived from the medicinal leech *Hirudo medicinalis* that directly inhibits thrombin and does not interfere with the complement system. After preincubation with DHR123 at 20 µg/ml for 10 minutes the blood was infected with *Nme* at 10^7 CFU/ml. PMA was used as a positive control at 1 µM. Samples were constantly rotated during incubation at 37 °C, 5 % CO_2 for one hour. After the incubation period erythrocytes were lysed by the addition of 1.4 ml cold hypotonic buffer for about 5 min until the samples appeared transparent. After centrifugation for 5 min at 4 °C, 2375 g the supernatant was removed, and the pellet was washed with 1 ml cold PBS. Then, after centrifugation for 5 min at 4 °C, 2375 g the supernatant was removed, and the pellet was resuspended and fixed with 300 µl 4 % PFA. After fixation PMNs were gated by their characteristic FSC/SSC pattern and DHR123 fluorescence was recorded in channel FL1-H.

When indicated, compstatin Cp20-analog was used at a concentration of 30 µg/ml to inhibit C3 cleavage.

2.11.3 Measuring PMN degranulation

Samples with PMN cells and with hirudin blood were prepared as outlined above in the experiments for the oxidative burst. PMA was used as a positive control and PMNs were infected with 10^8 CFU/ml and whole blood with 10^7 CFU/ml. After the incubation period of 30 min (PMN cells) or 60 min (whole blood) the samples were put on ice for further processing and 2 µg/ml APC-labeled anti-CD11b antibody clone M1/70 (BioLegend) was added. APC-labeled IgG2a antibody clone RTK2758 was used as an isotype control. Samples were incubated with the antibody on ice for 30 min. Afterwards PMN cells were centrifugated with 2375

g for 5 min at 4 °C, washed with 1 ml PBS, centrifugated again and fixed wth 300 µl 4 % PFA. Whole blood samples underwent erythrocyte lysis as described above and were fixed with 300 µl 4 % PFA. Since the erythrocyte lysis protocol already included washing steps no additional washing was performed. The cells were analysed by flow cytometry and the data was analysed with FlowJo v10 software (FlowJo, LLC). PMN cells were gated by their characteristic FSC/SSC pattern. Degranulation was measured as increase of the fluorescence intensity of APC-CD11b in channel FL1-H since CD11b is part of neutrophil granule membrane and cell surface CD11b augments upon degranulation.

2.11.4 Measuring C3-fragment deposition on *Nme* surface

Nme strains were grown overnight on blood agar plates and resuspended in KBR buffer (10 mM HEPES, 125 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.3; Virion\Serion) and adjusted to OD 0.1 (10^8 CFU/ml). 90 µl bacterial suspension were incubated with either 10 µl BRC or 10 µl heat-inactivated BRC for 1 h at 37°C on 96-well plates. After incubation the samples were put on ice for further processing and complement activity was stopped by adding 150 µl cold PBS/1 % BSA/10 mM EGTA/10 mM $MgCl_2$. Bacteria were centrifugated for 5 min at 4 °C, 4000 g and the supernatant was removed. The pellet was washed with 180 µl cold PBS/0.1 % BSA, pelleted again and resuspended in 50 µl PBS/0.1 % BSA containing polyclonal goat anti-C3 antibody at 1:1000 dilution. Goat serum was used as an isotype control.

After incubation for 45 min 130 µl PBS/0.1 % BSA was added and the samples were pelleted, washed with 180 µl PBS/0.1 % BSA and pelleted again. The pellet was resuspended in 50 µl PBS/0.1 % BSA containing Alexa 488-conjugated donkey anti-goat IgG antibody at 1:200 dilution. After 45 min incubation 130 µl PBS/0.1 % BSA was added and the samples were pelleted, washed with 180 µl PBS/0.1 % BSA and pelleted again. The pellets were fixed with 300 µl 4 % PFA. Samples were analysed by flow cytometry. C3 deposition was measured as fluorescence in FL1-H (Alexa 488) and data was analysed using FlowJo v10 software (FlowJo, LLC).

2.12 ELISAs

2.12.1 IL-8 ELISA

To measure the IL-8 release from PMNs the supernatant of infected cells was obtained after either 90 min or 4 h infection. 10^5 PMNs in 100 µl RPMI + 5 % FCS were infected with 10^8 CFU/ml. 1 µM PMA was used as a positive control. Samples were incubated at 37 °C, 5 % CO_2 and rotated overhead. After 90 min or 4 h the samples were centrifugated at 2375 g for 5 min and frozen immediately at -80 °C. Later, the samples were analysed using a commercially available DuoSet ELISA kit (R&D Systems).

96-well ELISA plates were coated overnight with 4 µg/ml in 50 µl PBS. Wells were washed three times with PBS + 0.05 % Tween-20 using an ELISA washer. Afterwards wells were blocked with 200 µl PBS/5 % BSA for 1 h at room temperature. The blocking buffer was discarded without washing and 50 µl of sample (pre-diluted 1:10 in PBS/0.1 % BSA) were added into each well. After 90 min incubation at room temperature the wells were washed three times with PBS + 0.05 % Tween-20 and 20 ng/ml secondary antibody in 50 µl PBS/0.1 % BSA added. After incubation for 1 h wells were washed three times with PBS + 0.05 % Tween-20 and 50 µl of avidin peroxidase (pre-diluted 1:40 from stock) in PBS/0.1 % BSA was added. After incubation for 1 h wells were washed three times with PBS + 0.05 % Tween-20 and 100 µl TMB substrate was added per well and the reaction was stopped with 100 µl 1 M H_2SO_4 once a clear colorimetric reaction was visible. For a standard curve, dilutions of human IL-8 ranging from 15 to 2000 pg/ml were used. To measure the colorimetric reaction, OD_{450} was read with an ELISA reader.

2.12.2 Anti-meningococcal antibody ELISA

To measure anti-meningococcal antibody binding in sera of unvaccinated donors ELISA plates were coated with 50 µl of heat-inactivated *Nme* at OD_{600} 0.1 overnight. Wells were washed three times with 200 µl PBS + 0.05 % Tween-20 and then blocked with 50 µl 5 % BSA in PBS per well for 1 h at room temperature. Then, the blocking buffer was discarded and 50 µl of dilutions of human serum in

1 % BSA or blank (1 % BSA) were added to each well. (see Table 9 for plate layout) Plates were incubated for 1 h at room temperature. Then, plates were washed three times as described above and 50 µl of HRP-labeled rabbit anti-human IgG antibody was added to the wells at a concentration of 1 µg/ml. Plates were incubated for 1 h and washed three times as described above. Afterwards, 100 µl of freshly mixed TMB substrate equilibrated to room temperature was added to each well. The plates were developed at room temperature until a clear colorimetric was observed in the top samples and stopped by addition of 100 µl 1 M H_2SO_4. To measure the colorimetric reaction, OD_{450} was read with an ELISA reader.

To measure antibody binding of rabbit polyclonal anti-*Nme* antibody, this assay was modified using an HRP-labeled goat anti-human IgG antibody instead of the secondary antibody mentioned above. Besides this modification, the protocols are identical.

Strains	Wells A1-H10	Wells A11-H12
Nme α4	plasma dilutions ranging from 1:31.6 to $1:10^6$	blank
Nme α14	plasma dilutions ranging from 1:31.6 to $1:10^6$	blank
Nme MC58	plasma dilutions ranging from 1:31.6 to $1:10^6$	blank
Nme 8013	plasma dilutions ranging from 1:31.6 to $1:10^6$	blank
Nme α522	plasma dilutions ranging from 1:31.6 to $1:10^6$	blank
Nlac 020-06	plasma dilutions ranging from 1:31.6 to $1:10^6$	blank
MC58∆csb	plasma dilutions ranging from 1:31.6 to $1:10^6$	blank
Uncoated wells	plasma dilutions ranging from 1:31.6 to $1:10^6$	blank

Table 9: ELISA plate layout for the anti-meningococcal antibody ELISA

Each column was incubated with a specific dilution of human serum or blank (1 % BSA). Each row was coated with a different *Nme* strain or left uncoated.

2.12.3 C3 deposition ELISA

To measure C3 deposition by ELISA, the 96-well plates coated with seven *Nme* and *Nlac* strains that were used for the previous ELISA were used here as well. (see chapter 2.12.2) Wells were washed three times with 200 µl PBS + 0.05 % Tween-20 and then blocked with 50 µl 5 % BSA in PBS per well for 1 h at room temperature. Then, the blocking buffer was discarded, and four different

conditions were tested for each strain by adding the following to the wells (see Table 10 for plate layout):

1. 50 µl 1:100 dilution of human plasma in KBR buffer (10 Mm HEPES/125 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$/pH 7.3)
2. 50 µl of blank (KBR buffer)
3. 50 µl of 1:100 dilution of human plasma in KBR buffer +20 mM EDTA
4. 50 µl of 1:100 dilution of human plasma in KBR buffer +1 µg/ml rabbit anti-Nme antibody

Next, plates were sealed and incubated for 30 min at 37 °C. Then, plates were washed three times as described above and complement activity was stopped by adding 200 µl 4 % PFA to each well for 10 minutes. Next, plates were washed three times as described above and 50 µl of 1:1000 dilution of goat anti-human C3 antibody in 0.1 % BSA/PBS was added to each well and incubated for 1 h at room temperature. Next, wells were washed three times and 50 µl of 1:10000 dilution of HRP-labeled donkey anti-goat antibody in 1 % BSA/PBS was added for 1 h at room temperature. Then wells were washed again three times and 100 µl of TMB substrate was added to each well, developed for some minutes until a clear colorimetric reaction was visible in the top samples. The reaction was stopped with 100 µl H_2SO_4 and OD_{450} was measured with an ELISA reader.

Strains	Wells A1-H3	Wells A4-H6	Wells A7-H9	Wells A10-H12
Nme α4	blank	plasma	plasma+EDTA	plasma + Abs
Nme α14	blank	plasma	plasma+EDTA	plasma + Abs
Nme MC58	blank	plasma	plasma+EDTA	plasma + Abs
Nme 8013	blank	plasma	plasma+EDTA	plasma + Abs
Nme α522	blank	plasma	plasma+EDTA	plasma + Abs
Nlac 020-06	blank	plasma	plasma+EDTA	plasma + Abs
MC58∆csb	blank	plasma	plasma+EDTA	plasma + Abs
Uncoated wells	blank	plasma	plasma+EDTA	plasma + Abs

Table 10: ELISA plate layout for C3 deposition ELISA

Three columns were incubated with either blank, plasma dilutions, plasma + EDTA or plasma + anti-Nme antibodies. Each row was coated with a different strain.

2.13 Western blot

Western blot was used to demonstrate Opa expression on *Nme* strains. To prepare the samples *Nme* strains were grown overnight, resuspended in PBS and the bacterial concentration was adjusted to OD_{600} equalling 6. Subsequently, two volumes of Laemmli sample buffer (4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromphenol blue, 0.125 M Tris HCl, pH 6.8) were added to one volume of sample and samples were heated at 100 °C for 10 minutes. After loading the samples on a 12 % SDS-PAGE gel, samples were run at 100 V for 1 h and blotted on a nitrocellulose membrane. Then, the membrane was blocked for 1 h at room temperature with 20 ml TBS/T + 5 % skim milk powder.

Antibody clone 4B12C11 was used for probing of Opa proteins in a 1:10000 dilution in TBS/T + 5 % skim milk powder. After a 1 h incubation period the membrane was washed three times (5 minutes each) with TBS/T. Afterwards, HRP-labeled goat anti-mouse antibody was added in a 1:10000 dilution and incubated for 1 h on the membrane. Then, three washing steps as described above followed and Western blot ECL detection reagent was used to visualize the staining.

2.14 Immunofluorescence staining of ceramide

To visualize the presence of ceramide in neutrophil cell membranes upon *Nme* infection, immunofluorescence was used to stain PMNs, *Nme* and ceramide. PMNs nuclei were stained using DAPI and ceramide was stained with the red fluorophore Cy3. To label *Nme* with the green fluorophore FITC, bacterial cultures were resuspended in PBS, centrifugated and pelleted for 1 min at 13000 rpm and then resuspended and incubated in 0.2 mg/ml FITC in PBS for one hour at 37 °C. Next, the suspensions were centrifugated three times for 1 min at 13000 rpm and washed with RPMI + 5 % FCS. Finally, bacteria were resuspended in RPMI + 5 % FCS and OD was adjusted to 1.0.

Meanwhile, 5 x 10^5 PMNs in 1 ml RPMI were seeded on round cover slips that were placed in 24-well plates to obtain a confluent cell layer. After one hour of incubation at 37 °C, 5 % CO_2 to allow attachment to the cover slips, the

supernatant was removed using a vacuum system and replaced with 1 ml RPMI + 5 % FCS. Then, PMNs were infected with *Nme MC58* at a final concentration of 5 x 10^7 CFU/ml and incubated for 1 h at 37 °C, 5 % CO_2. For some assays PMNs were incubated with acid sphingomyelinase (ASM) instead of infection with *Nme* to have a positive control for ceramide release. Next, the media was carefully aspirated, and the wells were washed with 1 ml PBS. Subsequently, the cells were fixed with 4 % PFA for 15 minutes and washed again with 1 ml PBS as described above.

Thereafter, the cover slips were taken carefully out of the wells and 50 µl of blocking buffer (PBS/1 % FCS/2 % BSA) were placed on a sheet of parafilm and the cover slip was placed upside down on top of it. Then, the cover slip was incubated in the dark for 30 minutes at room temperature and washed two times with PBS. To wash the coverslips 50 µl of PBS was pipetted on parafilm and the coverslips was placed on it upside down for a minute to allow washing through diffusion of the previous reagents into PBS. Afterwards, 50 µl of mouse anti-ceramide IgM antibody clone 15B4 at a 1:50 dilution in blocking buffer was applied to the cover slips as described above and incubated for 45 minutes at room temperature. Then, the cover slips were washed three times with PBS as described above and 50 µl of Cy3-labeled goat anti-mouse IgM at a 1:200 dilution in blocking buffer was applied. After incubating for 45 minutes, cover slips were washed three times with PBS as described above and 10 µl of DAPI was applied and incubated for 10 minutes at room temperature.

Ultimately, the cover slips were taken out, dried at room temperature in the dark and embedded in Fluoroshield™ on glass slides. The slides were dried and stored at 4 °C in the dark before observation under a fluorescence microscope with appropriate filter sets.

2.15 Statistics

Graphs and tables in this work were created using GraphPad Prism (Version 7.04) and Microsoft Excel. To compare two groups with normally distributed parametric data the unpaired, two-sided Student’s t test was applied. For parametrical, normally distributed data with more than two groups a one-way

ANOVA with either Bonferroni's post hoc test or Dunnett's post hoc test. In the latter case the other groups were compared with the negative control. Data sets with more than two groups and more than two testing conditions were analysed with a two-way ANOVA applying Turkey's post hoc test. Differences between groups were rated as statistically significant at a p value below 0.05.

3 Results

3.1 Establishment of purified neutrophils and whole blood models for *Nme* infection

In general, the role of complement in IMD has been thoroughly investigated and its protective properties are highlighted by the high risk of complement-deficient individuals to acquire *Nme* infection. (56) However, the role of neutrophils in IMD and their contribution to the host immune response remains elusive.

Therefore, this project aims to analyse the specific cellular responses of isolated neutrophils to *Nme* infection and the modulation of these responses through the complement system and antibodies considering the interaction of these factors in *in vivo* infection.

Thus, neutrophils were isolated using Polymorphprep-based purificiation of EDTA blood. (139) PMN purity was assessed as above 90 % by cytospin and Diff-Quick staining. Whole blood was anticoagulated with hirudin which was most suitable for the assays, because it does not interfere with the complement system. (149)

To reflect the multitude of existing meningococcal strains a variety of strains was used in the assays to detect possible differences in the cellular response to different strains. Two pairs of closely related capsulated strains isolated either from carriage or from disease were selected. The clonal complex (cc) ST-18 was represented by *Nme α4* (carriage) and *Nme 8013* (disease) and *Nme α522* (carriage) and *Nme MC58* (disease) were from cc35 and cc32 respectively. Additionally, the unencapsulated carriage strain *Nme α14* and the non-pathogenic *Nme* relative *N. lactamica 020-06 (Nlac)* were included in this study. (150) To assess the effect of capsule expression within a single strain on PMN responses the phase variation clone with deactivated capsule expression *Nme MC58∆csb* was included.

3.2 Meningococcal interaction with isolated PMNs

3.2.1 *Nme* association with and internalization into PMNs

The first experiments focused on the interaction between isolated PMNs and *Nme* to assess the functionality of the cell culture model following isolation of PMNs from whole blood (see chapter 2.5.1) and observe PMN association with and phagocytosis of *Nme* in the absence of any other blood components. Therefore, 5 x 10^5 isolated PMNs were seeded and adhered in 24-well plates and subsequently infected with 5 x 10^7 CFU/ml *Nme* for either 1 or 4 hours. (see chapter 2.8) During the incubation period cell vitality was controlled microscopically and by trypan blue staining. Despite a noticeable number of non-viable cells after seeding, the vital cell population remained stable throughout the experiment.

Association of *Nme* and *Nlac* to PMNs could be observed for all strains used in the experiments. To account for unspecific binding of bacteria to the wells CFU counts were compared to wells without PMNs. CFU counts were 4 to 25 times higher in the PMN containing wells compared to wells exclusively incubated with bacteria. These findings indicate that recovered bacteria were mainly associated with the PMNs and not with the well surface. (Figure 5)

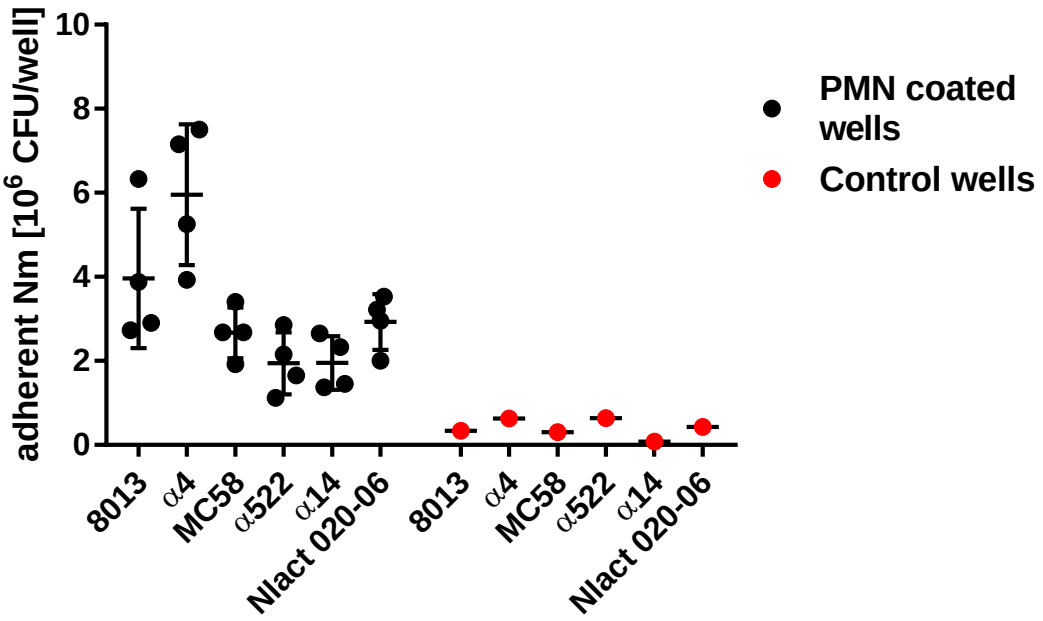

Figure 5: Association of five *Nme* and one *Nlac* strains with PMNs adhered in 24-well plates compared to unspecific binding to 24-well plates without cells

$5*10^7$ CFU/ml of *Nme* and *Nlac* added to 24-well plates with either with or without 5×10^5 adherent PMNs and incubated for 4 hours. Symbols show mean CFU recovered from individual donors. Lines show mean ± SEM of one to four different donors.

All strains including *Nlac* associated with PMNs to a similar extent after 1 hour. After 4 hours binding was several folds higher for all strains than at the previous time point and there were significant differences between the *Nme* strains. Recovered CFU of *Nme α4* were significantly higher than most other strains after 4 hours. *Nme 8013* also showed higher association but this difference was not significant. (Figure 6)

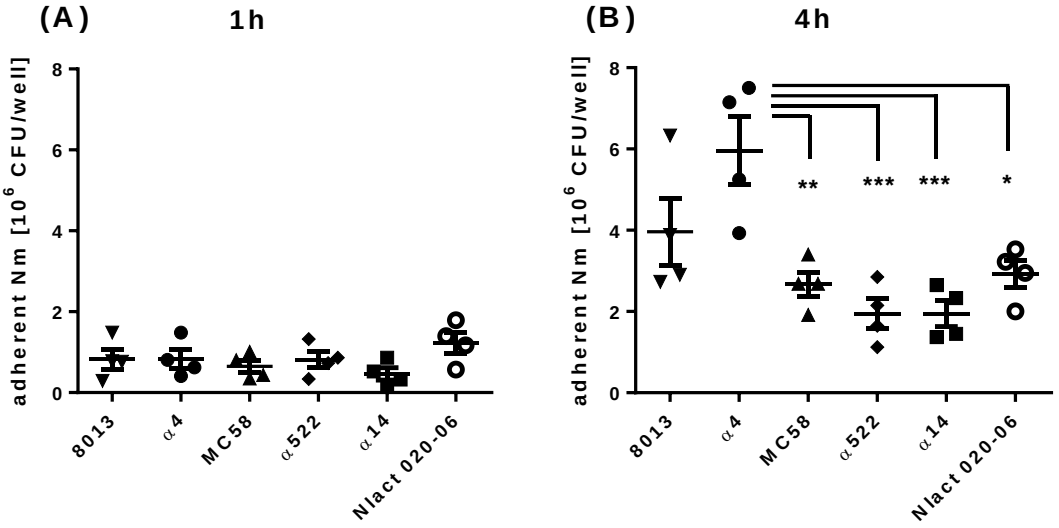

Figure 6: Association (Adhesion) of *Nme* with isolated PMNs

5 x 10^5 PMNs were adhered in 24-well plates and infected with 5 x 10^7 CFU/ml of 6 different *Neisseria* strains. **A** 1 hour time point. **B** 4 hour time point. Symbols on graph show mean CFU recovered from individual donors. Lines show mean ± SEM of four different donors. *, **, *** indicate $p<0.05$, $p<0.01$, p 0.005 respectively, in one-way ANOVA with Turkey's post hoc test.

To assess internalization (phagocytosis) of *Nme* into PMNs, cells were additionally treated with extracellular gentamicin to eliminate extracellular bacteria before lysis with saponin. After 1 hour *Nme α522* was internalized significantly more than the other capsulated strains *Nme 8013, α4, MC58*. Internalization of the unencapsulated strains *Nme α14* and *Nlac* appeared to be higher as well, but these differences were not significant due to scattering of the data.

At the 4-hour time point internalization of the unencapsulated strain *Nme α14* was significantly higher compared to most capsulated strains. A trend towards higher internalized CFU counts was observed for the unencapsulated *Nlac* strain as well, but this was not significant due to scattering of data. Differences between capsulated strains seen at the 1-hour time point were no longer significant. (see Figure 7)

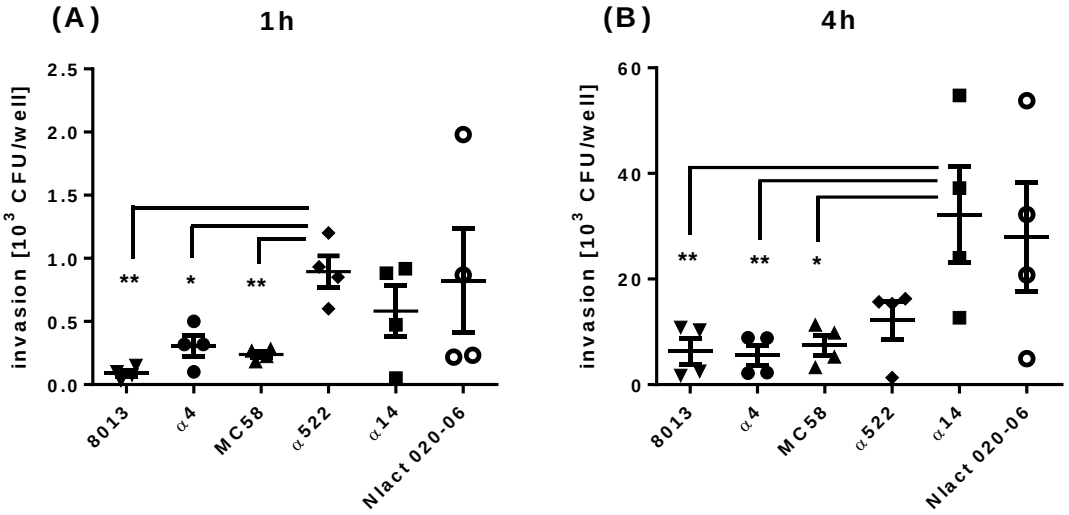

Figure 7: Internalization of *Nme* into isolated PMNs

5 x 10^5 PMNs were adhered in 24-well plates and infected with 5 x 10^7 CFU/ml of 6 different *Neisseria* strains. **A** 1 hour time point. **B** 4 hour time point. Symbols on graph show mean CFU recovered from individual donors. Lines show mean ± SEM of four different donors. *, ** indicate $p<0.05$, $p<0.01$ respectively, in one-way ANOVA with Turkey's post hoc test.

This data suggests that unencapsulated strains are taken up more easily by PMNs which was expected since the capsule is a protective bacterial factor against phagocytosis. (137, 151) Moreover, internalization appeared to be inefficient in general since the majority of *Nme* associated with PMNs remained extracellular after 4 hours. This is evident since internalized CFU counts were at least 100-fold lower than associated CFU counts. Since association and internalization were highest in different strains, these phenotypes appear to be driven by different bacterial factors and do not necessarily coincide in the same strain.

3.2.2 Intracellular viability of *Nme* phagocytized by PMNs

To assess the viability of internalized bacteria, gentamicin protection assays were performed to monitor the CFU count of internalized bacteria over time. 5 x 10^5 adherent PMNs were infected with 5 x 10^7 CFU/ml of bacteria and incubated for four hours. Subsequently, gentamicin was added to kill extracellular bacteria and cells were incubated with gentamicin for 30 min, one hour, two hours and four hours, lysed and serial dilutions were plated to enumerate CFU counts. (see

chapter 2.8.5) The CFU counts decreased for all strains over time. After four hours of gentamicin treatment CFU counts were reduced by several orders of magnitude or did not recover any bacteria at all. Thus, this experiment suggests successful killing of phagocytized bacteria by PMNs. (see Figure 8)

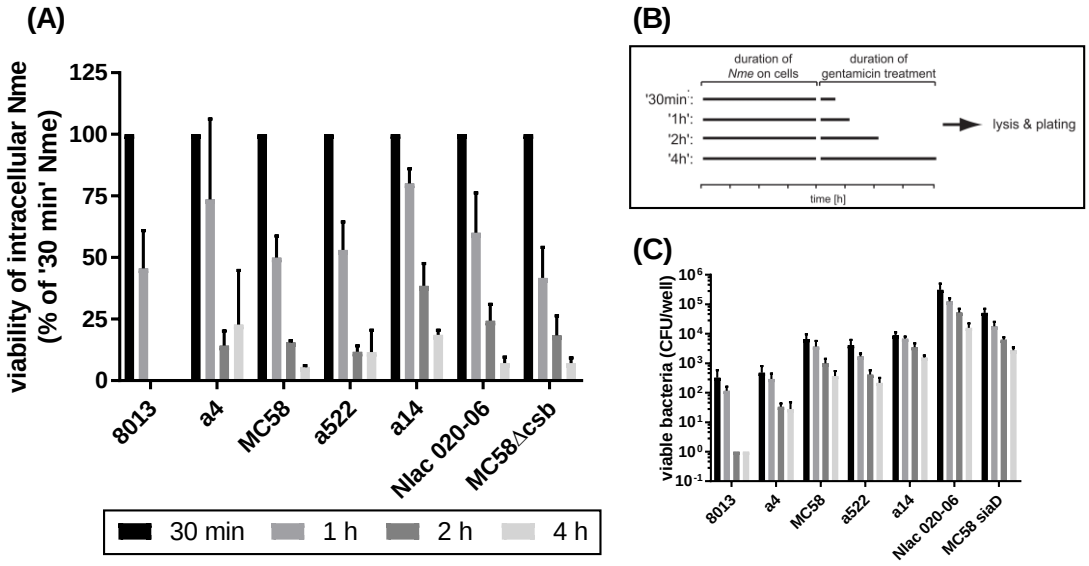

Figure 8: Intracellular survival of *Nme* after internalization by PMNs

A Viability of intracellular bacteria in PMNs. 5 x 10^5 PMNs were incubated for four hours with 5 x 10^7 CFU/ml and subsequently treated with gentamicin to kill extracellular bacteria to time points from 30 min to four hours. Data are expressed as percentage (survival) of CFU recovered for 30 min gentamicin treatment time point. Graphs show mean ± SEM of three independent experiments. **B** Graphical overview of the timely setup of the experiments for the four time points in **A** and **C** regarding the duration of the infection and of the subsequent gentamicin treatment. **C** Viability of intracellular bacteria in PMNs. Data displayed in **A** are shown in absolute numbers on a log scale. Graphs show mean ± SEM of three independent experiments.

3.2.3 Killing of *Nme* by PMNs

To assess bactericidal effects of isolated PMNs and whole blood on bacterial growth, bacteria were grown in whole blood and in the presence of PMNs supplemented with medium or serum.

10^7 CFU were incubated in 100 µl hirudin-anticoagulated whole blood for 30 minutes. The capsulated strains had a recovery rate below 50 %, but survival varied greatly from donor to donor ranging between 1 and 80 %. Less than 1 % of the inoculated unencapsulated strains strains *Nme α14* and *Nlac 020-06* survived in the presence of whole blood. (see Figure 9 **A**)

To measure the bacterial killing by PMNs, 5 x 10^3 CFU/ml of the strains *Nme α4, MC58, MC58*Δ*csb* were incubated with 10^5 PMNs for 30 minutes and CFUs were plated and counted compared to CFUs of bacteria grown in medium. To evaluate the effect of complement-mediated opsonization and lysis on killing 10 % autologous plasma was added to some samples. No significant reduction of CFUs in the presence of isolated PMNs was observed under most investigated conditions. Only PMN supplementation with 10 % autologous plasma reduced survival of the unencapsulated strain *MC58*Δ*csb* significantly. 10 % plasma did not cause significant killing of the two capsulated strains used in this assay. (see Figure 9 **B)**

This observation is most likely due to complement-mediated lysis in the absence of a capsule. The other strains presumably experience less complement deposition and killing due to capsule expression which inhibits complement activation. (69, 152) Additionally, this data shows that complement-mediated killing appears to be quantitatively more important than killing by PMNs. As previously shown only a fraction of adherent *Nme* are internalized and killed by PMNs which had a quantitatively insignificant effect on bacterial growth in this experiment. (see Figure 7, Figure 8)

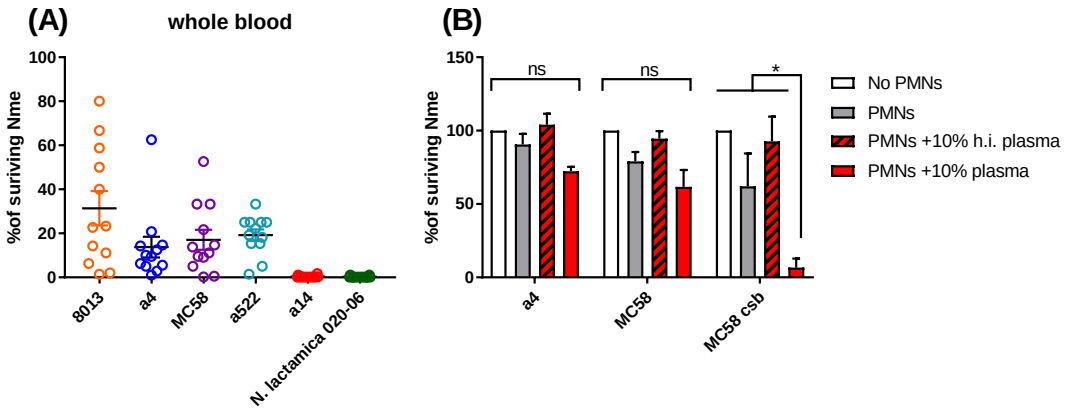

Figure 9: Killing assays with whole blood and isolated PMNs

A 100 µl hirudin-anticoagulated blood were infected with 10^7 CFU of indicated strains for 30 minutes. Serial dilutions were plated to enumerate CFUs. Data is shown as recovered percentage of inoculum. Graph shows individual data points and means ± SEM of 12 different donors. **B** 10^5 PMNs were infected with 5 x 10^3 CFUs for 30 minutes and serial dilutions were plated to enumerate CFUs. Autologous plasma and heat-inactivated (h.i.) plasma were supplemented prior to infection. Graphs show means ± SEM from three different donors. *, ns indicate $p<0.05$, not significant in two-way ANOVA applying Turkey's post hoc test.

3.2.4 The effect of Opa proteins on association and internalization

Since Opa proteins are known to play a key role in cellular interaction of pathogenic *Neisseria* with host cells, the effect of Opa protein expression on association and internalization was assessed. (136, 153) *Opa*-expressing *Nme* strain *MC58* was compared to a phase variant clone of the same strain in which *opa*-expression is shut off by phase variation (*MC58Opa*^{OFF}). 5 x 10^5 adherent PMNs were infected with 5 x 10^7 CFU/ml of either strain for one or four hours and treated as described above to measure association and internalization. (see chapter 2.8.3, 2.8.4) Regarding CFU counts of *Nme* associated with PMNs both strains did not differ neither after one nor four hours. For internalization *MC58Opa*^{OFF} showed a tendency towards lower CFU counts after one hour that was not significant but showed five times lower CFU counts after four hours which was significant. (see Figure 10)

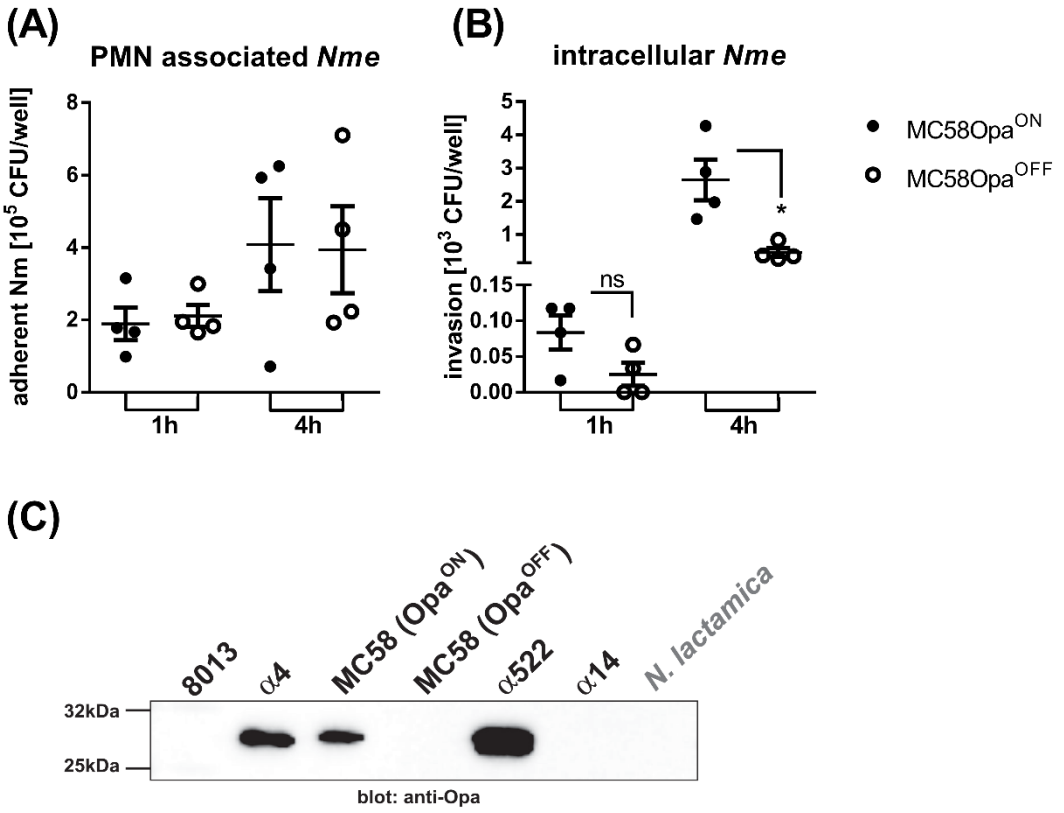

Figure 10: Effect of Opa expression on *Nme* association with and internalization into PMNs

5 x 10^5 adherent PMNs were infected with either wildtype *Nme MC58 (Opa^{ON})* or with the mutant *Nme MC58Opa^{OFF}* and incubated for one or four hours as indicated above and lysed and plated subsequently. **A** Association of *Nme* with PMNs after one and four hours. **B** Internalization of *Nme* into PMNs after one and four hours. Cells were treated with gentamicin for 30 minutes to eliminate extracellular bacteria prior to lysis. Graphs show mean ±SEM of four independent experiments. *, ns indicate $p<0.05$, not significant respectively, in Student's t-test. **C** Western blot demonstrating Opa expression of the strains used in this study. Antibody clone 4B12/C11 was used for probing.

Opa-expression was tested for all strains used in this study by Western blot using the broadly cross-reactive antibody clone 4B12/C11. Strains *α4, MC58* and *α522* were found to express Opa proteins and the other strains did not. (138) However, *opa*-expression did not correlate with high internalization which was most pronounced in *Nme α14* and *Nlac 020-06.* These strains do not have a capsule which appears to facilitate internalization. (14) Thus, these data indicate that Opa-expression may promote internalization within a strain but in comparison with other strains the contribution of factors such as capsule expression play a larger role than Opa-expression.

3.3 Effects of complement on the phagocytosis of *Nme* by PMNs

It is well established that the complement system is protective in meningococcal disease. Furthermore, it influences PMN cellular reactions through chemotactic and proinflammatory activity of anaphylatoxins and enables complement-receptor-mediated phagocytosis through opsonization of pathogens. Therefore, several experiments focused on the effect of complement on the previously described PMN responses to *Nme.*

Therefore, GFP-expressing *Nme* was used to measure *Nme* colocalization with PMNs in flow cytometry. The isolated PMNs were supplemented with media or different sources of complement and antibodies to assess their effect on phagocytosis. Since non-vaccinated individuals can acquire antibody-dependent immunity agains *Nme* through asymptomatic colonization, killing activity of serum from non-vaccinated donors was measured to identify normal non-immune serum. (154, 155) Normal non-immune human serum contains all active complement factors but no specific antibodies since the donors. Immune serum contains both complement and specific antibodies against *Nme.* Inhibitors of complement and antibody function were used as well to investigate the specific contributions of these humoral factors of immunity.

3.3.1 Complement activation increases *Nme* phagocytosis

To measure association of *Nme MC58-GFP* with PMNs, 2 x 10^5 PMNs were infected with a total of 2x 10^6 CFU *Nme* or incubated without bacteria as a control and fixed subsequently. As a source of complement 5 % of normal, non-immune human serum (NHS) and 5 % of immune serum (IMS) were used and heat-inactivated samples of these sera as controls. PMNs were gated in flow cytometry by their prominent FSC/SSC pattern and the proportion of PMNs shifting towards higher fluorescence in the GFP channel compared to control PMNs without bacteria was used to assess the extent of *Nme* association (phagocytosis). (see chapter 2.11.1)

The human sera used in these assays were obtained from healthy volunteers and freshly prepared prior to the experiment. IMS was obtained from a donor vaccinated against serogroups A, B, C, W135 and Y with known high antibody titers against the strains used in the assays. Normal, non-immune human serum was obtained from donors without any history of meningococcal disease or vaccination and without measurable serum bactericidal activity. Figure 11 **D** shows effective killing of *MC58-GFP* by 5 % concentration and no killing when NHS was used compared to CFU counts of bacteria incubated in RPMI. Some of the data in this section (and in Figure 11 **A** and **B**) are from experiments which were conducted during the laboratory rotation of Emma Eichler as part of her master's studies in Biology at the University of Würzburg.

The results from the flow cytometry assays showed that about 10 % of PMNs were GFP-positive when incubated in RPMI only. NHS did not increase phagocytosis significantly and neither did heat-inactivated NHS. Immune serum increased the number of GFP-positive PMNs up to more than 80 % and this result was highly significant ($p<0.001$). Heat-inactivation and thus complement inactivation of IMS completely abolished this effect to levels recorded for NHS and RPMI. (see Figure 11 **A**)

Considering the efficient killing of *MC58-GFP* when treated with IMS, the integrity of the GFP signal of *Nme* was verified following the treatment with different sera. For this purpose, bacteria were treated with the different sera at 5 % concentration for one hour and analysed in flow cytometry to compare GFP fluorescence intensity between the different treatments. No difference was found between these groups and GFP signal integrity could be confirmed. (see Figure 11 **E**)

Since only IMS was able to elicit a significant increase in phagocytosis of *Nme*, these data imply that both complement activity and specific antibodies are required for efficient phagocytosis by neutrophils.

3.3.2 Inhibition of complement decreases phagocytosis of *Nme*

To further assess the contribution of the complement system to efficient phagocytosis, the assay described above was performed with different inhibitors

of the complement cascade added to IMS and compared to IMS alone. Compstatin Cp20 inhibits the C3 convertase from cleaving C3 into C3a and C3b. PMX53 inhibits the C5a receptor 1 (C5aR1) which is expressed on neutrophils and binds the anaphylatoxin C5a.

In the presence of 30 µg/ml Compstatin Cp20 the proportion of GFP-positive PMNs was dramatically reduced from a mean of about 80 % to about 30 % almost to levels seen in heat-inactivated IMS.

Inhibition of the C5aR1 with 10 µM PMX53 as well lead to a reduced number of GFP-positive PMNs, which was significant but less pronounced than general inhibition of complement with Compstatin Cp20. Association of PMNs with *Nme* was still above 50 % in presence of PMX53. (see Figure 11 **B**)

These findings indicate that the early stages of complement activation are key to efficient phagocytosis through C3 cleavage and deposition on *Nme* which is needed for opsonization, efficient binding of PMNs through the neutrophil C3 receptor and enables the formation of the membrane attack complex (MAC) downstream from it. (42) The importance of these cascadic events is highlighted by the reduction of phagocytosis when inhibiting the complement cascade before C3 cleavage with Compstatin Cp20. Blockade of the C5aR1 with PMX53 does not interfere with opsonization of the bacteria and MAC formation but decreases neutrophil degranulation, oxidative burst and chemotaxis. (102) These effects of C5a seem to contribute to more efficient phagocytosis but not to the extent earlier stages of complement activation do.

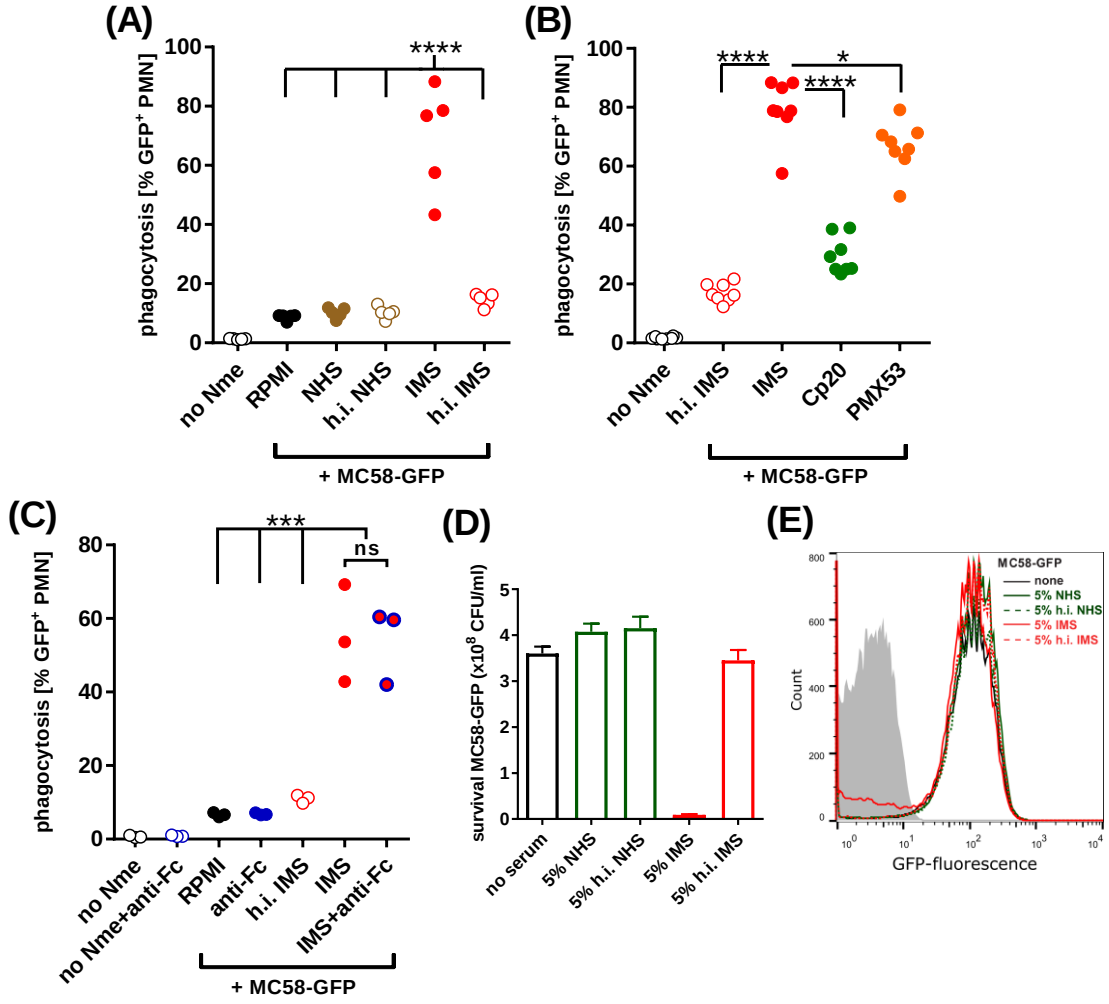

Figure 11: Phagocytosis of *Nme MC58-GFP* by PMNs under the influence of complement

A 2 x 10^5 PMNs were infected with 2 x 10^6 CFU/ml of *MC58-GFP* or incubated without bacteria ('no Nme') for one hour and subsequently fixed with 4 % PFA. PMNs were incubated in medium only ('RPMI') or 5 % normal, non-immune human serum ('NHS') or 5 % immune serum ('IMS'). Heat-inactivated ('h.i.') sera with no complement activity were used as controls. PMNs were gated in FSC/SSC with flow cytometry and *Nme* association was measured as percentage of PMNs shifting in the GFP channel relative to the control PMNs without bacteria. **B** The same assay was conducted as in panel **A** and IMS was used as a control to evaluate changes in phagocytosis in the presence of 30 µg/ml complement factor C3 inhibitor Compstatin Cp20 and 10 µM C5a receptor 1 antagonist PMX53. **C** The same assay was conducted as in panel **A** and IMS was used as a control to evaluate the effect of Fc receptor blockade with 1 µg/ml of antibodies against CD16 (clone 3G8), CD32 (clone 10.1) and CD64 (clone 6C4) (all mouse IgG1 antibodies). **D** Survival of *Nme* exposed to different sera. **E** GFP fluorescence of bacteria from panel **D** to control for the integrity of GFP expression after *Nme* exposure to serum. All graphs: ****, **, *, ns indicate p<0.001, p<0.005, p<0.05, not signifcant in one-way ANOVA with Bonferroni's post hoc test. The data in panels **A** and **B**, were generated by Emma Eichler as part of her laboratory rotation for Biology master's students.

3.3.3 Fc receptor blockade has no effect on phagocytosis

Since neutrophils can bind pathogens via multiple mechanisms including both C3b mediated binding and binding of antibody Fc regions through Fc receptors, the specific contributions of these two ways of opsonophagocytosis were evaluated. As demonstrated above, C3 deposition is very important for efficient binding of PMNs to *Nme*. (156) The contribution of Fc receptor (FcR) mediated binding to the enhanced binding of PMNs in immune serum was assessed by performing the assay described above with antibodies directed against FcγRIII (CD16), FcγRII (CD32) and FcγRI (CD64) added to IMS and compared to IMS alone.

With 1 µg/ml of each antibody added to IMS there was no significant difference in *Nme* association with PMNs compared to IMS alone and the majority of PMNs was GFP-positive in the presence of FcR blockade. Controls with PMNs, *MC58-GFP* and FcR antibodies in the absence of IMS showed no unspecific signal resulting from the blocking antibodies alone. (see Figure 11 **C**)

Thus, FcR-mediated binding seems to contribute less to uptake of *Nme* in IMS than complement deposition through the classical pathway and C3-dependent phagocytosis. Concurrently, heat-inactivated IMS neither shows a strong association of *Nme* with PMNs.

3.4 PMN oxidative burst response to *Nme*

The production and release of reactive oxygen species (ROS) is one of the most important cellular responses of PMNs to neutralize extracellular and intracellular pathogens. (78) Therefore, the oxidative burst both in isolated PMNs and in whole blood upon infection with *Nme* was assessed. The effects of supplementing complement and inhibition of complement with Compstatin Cp20 on the oxidative burst were evaluated as well.

For this purpose, PMNs were loaded with dihydrorhodamine 123 (DHR123) which passively diffuses across the plasma membranes and organelle membranes and is oxidized intracellularly by ROS to rhodamine 123 which exhibits green fluorescence. (147) This green fluorescence was used as an

indicator of the oxidative burst and measured after fixation of the samples using flow cytometry.

3.4.1 Oxidative burst in isolated PMNs

To assess the oxidative burst response of isolated PMNs to infection with five *Nme* and *Nlac 020-06*, 10^5 PMNs were infected with 10^7 CFU for different time points (10, 30, 60 min) in initial experiments. Phorbol-13-myristate-12-acetate (PMA) was used as a positive control, since it activates neutrophil oxidative burst and degranulation independently from membrane receptors and therefore allows to demonstrate general responsiveness of PMNs after the isolation process. (148)

Surprisingly, there were no changes in ROS production compared to the negative control even after 60 minutes of incubation despite the high infection dose. An unspecific increase in signal seen in the negative control and all the samples could be observed after 60 minutes which could be due to higher autofluorescence by cellular stress following prolonged incubation or non-specific metabolization of the fluorescent dye. In the positive control stimulated with PMA ROS production and DHR123 fluorescence was strong under all conditions at least 30 times higher than baseline. (see Figure 12)

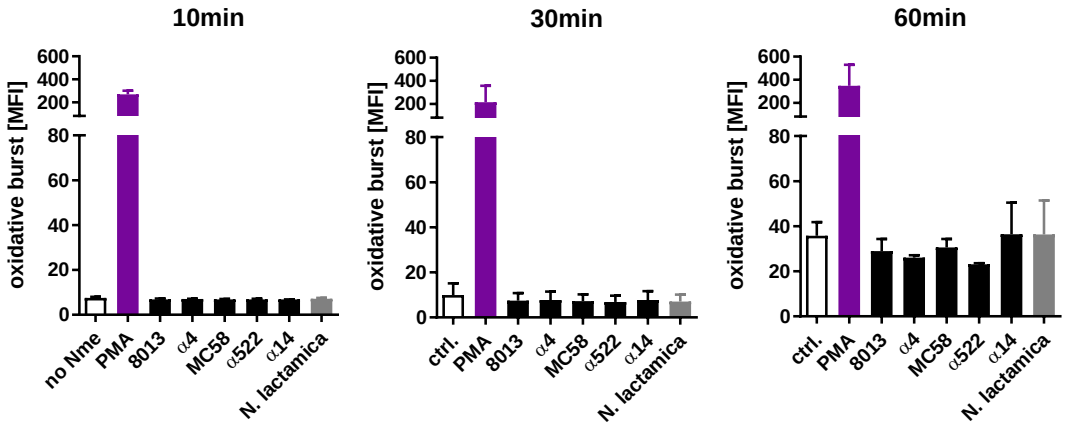

Figure 12: Oxidative burst in PMNs isolated from whole blood

10^5 PMNs were infected with 10^7 CFU *Nme* and incubated rotating overhead for 10, 30 and 60 minutes as indicated. 1 µM Phorbol-13-myristate-12-acetate (PMA) was used as a positive control demonstrate PMNs' responsiveness. Cells were fixed with 4 % PFA for 20 minutes and DHR123 fluorescence was measured with a flow cytometer in FL1-H. Graphs show mean fluorescence intensity (MFI) ± SEM from three, four and two different donors for 10, 30 and 60 min respectively.

These data demonstrate that isolated PMNs are not sufficiently activated by the mere presence of *Nme* and do not mount a substantial oxidative burst response. Since there was no significant oxidative burst after incubation for 60 minutes, 30 minutes were chosen incubation period for the following experiments.

3.4.2 PMN oxidative burst in the presence of complement

Next, the influence of complement and other serum components on the oxidative burst response was assessed, since immune serum and complement activation were previously shown to be essential for efficient phagocytosis. (see chapter 3.3) Therefore, isolated PMNs were supplemented with different serum factors including recombinant C5a, baby rabbit complement (BRC), polyclonal anti-meningococcal antibodies serum or autologous plasma and infected with *Nme* for 30 minutes. Isolated PMNs in media were used as a control and baseline for the effect of these supplements.

Since C5a can trigger the oxidative burst and enhance neutrophil chemotaxis and other responses to pathogens, it was tested as a co-stimulus that enhances neutrophil responsiveness in this assay. (102) BRC was used as a source of active complement without specific antibodies since it is obtained from

immunologically naïve animals. Conversely, purified rabbit anti-*Nme* antibodies were used to assess the effect of antibodies alone. Autologous plasma from non-immune donors however should contain active complement but no specific antibodies.

In the presence of 1000 nM C5a there was a significant increase of the oxidative burst, but this appeared to be independent from infection with *Nme* since the PMNs without bacteria also displayed oxidative burst activation upon addition of C5a. There was no C5a-dependent effect on the PMA-derived oxidative burst in the control group. (see Figure 13 **A**)

When PMNs were preincubated with rabbit anti-meningococcal antibodies, no significant increase of the oxidative burst was detected, even though antibody binding to the different strains assessed by ELISA and high affinity for all strains used in this assay was confirmed. (see Figure 3 and Figure 13 **B**)

Supplementation of 10 % baby rabbit complement triggered a significant increase of the oxidative burst response towards the unencapsulated strains *Nme α14* and *Nlac 020-06*. *MC58* capsule knockout *MC58∆csb* showed more oxidative burst as well, but this difference was not significant. (see Figure 13 **B**) A flow cytometry staining of C3 deposition after incubating the panel of strains with 10 % BRC revealed robust C3 deposition on the unencapsulated strains *Nme α14, MC58∆csb* and *Nlac* and some C3 deposition occurred on strains *Nme MC58* and *α522* as well, but was almost absent on *Nme 8013* and *α4.* (Figure 13 **C)**

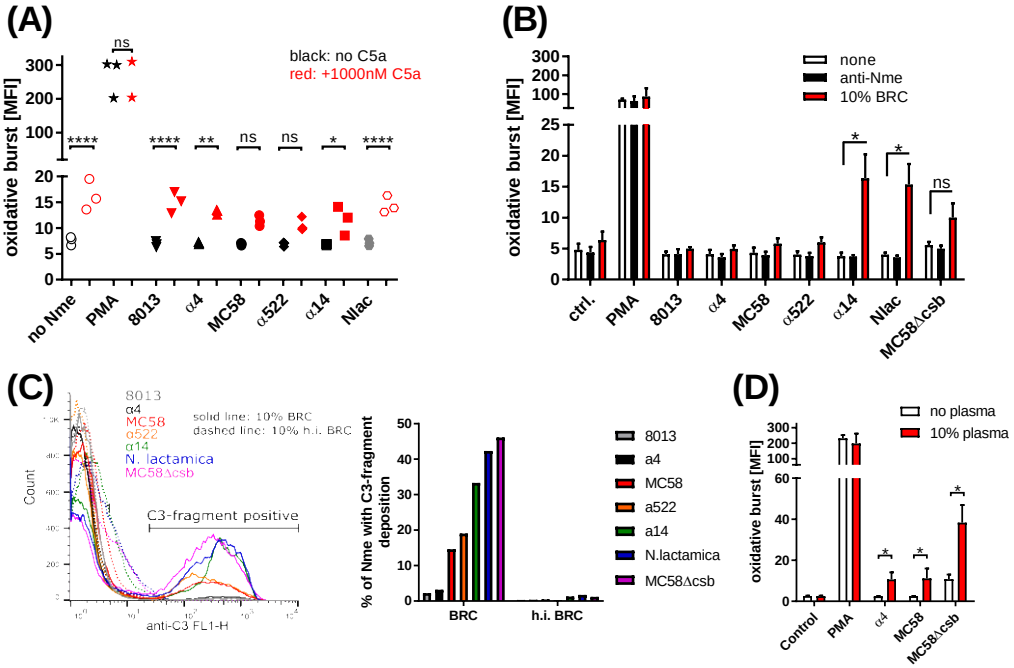

Figure 13: PMN oxidative burst under the influence of complement and antibodies

10^5 PMNs isolated from whole blood were preincubated for 10 min with either human C5a, purified polyclonal rabbit anti-meningococcal antibodies (anti-Nme), 10 % baby rabbit complement (BRC) or 10 % autologous plasma and then infected with 10^7 CFU of the indicated strains, incubated for 30 minutes and fixed with PFA. Mean fluorescence intensity of DHR123 was measured by flow cytometry. Stimulation of PMNs with PMA was used as a positive control. **A** PMN oxidative burst in the presence of 1000nM C5a. Graph shows data from samples of three different donors. ****, **, *, ns indicate p<0.001, p<0.01, p<0.05, not significant respectively in one-way ANOVA with Bonferroni's post hoc test. **B** PMN oxidative burst in the presence of either medium, 10 % BRC or 1 µg/ml of polyclonal anti-meningococcal antibodies. Graphs show mean ± SEM from samples of three different donors. *, ns indicate p<0.05, not significant, respectively, in one-way ANOVA applying Dunnett's post hoc test. **C** C3 deposition on indicated strains after one hour incubation in 10 % BRC or h.i. BRC. Left panel: Histogram showing the results for C3 staining in flow cytometry and the gate used to measure C3-positive bacteria. Right panel: Percentage of bacteria from indicated strains positive for C3 assessed by using the gate from the left panel in flow cytometry. **D** PMN oxidative burst in RPMI compared to 10 % autologous donor plasma. Graph shows mean ± SEM from samples of four different donors. * indicates p<0.05 in Student's t-test.

When 10 % autologous plasma was added to PMNs prior to infection there was a significant increase of the oxidative burst for both capsulated strains *Nme α4* and *MC58* and even more prominent for unencapsulated strain *MC58Δcsb*. (see Figure 13 **D**)

These experiments demonstrate that neutrophil oxidative burst in meningococcal infection depends on the presence of active complement. The anaphylatoxin C5a

only triggered the oxidative burst independently from infection, but BRC and autologous plasma triggered a significant increase. Since the increase of the oxidative burst in BRC correlates with the extent of C3 deposition observed for the individual strains, C3 deposition is likely to mediate the enhanced oxidative burst response. Accordingly, the unencapsulated strains in the panel displayed more C3 deposition and triggered a stronger oxidative burst than the capsulated strains, since the capsule inhibits C3 deposition in the absence of antibodies. Autologous plasma can additionally contain polyspecific antibodies even without prior immunization which could trigger the small but significant oxidative burst response towards capsulated strains. (157)

3.4.3 Oxidative burst response in whole blood

Next, experiments to measure PMN oxidative burst were conducted in whole blood to assess differences in comparison with isolated PMNs. Therefore, 100 µl of hirudin anti-coagulated blood was preincubated with DHR123 and infected with 10^7 CFU/ml of the strains for one hour. After erythrocyte lysis and washing the cells were fixed for analysis in flow cytometry.

All strains evoked a significant oxidative burst response compared to the negative control. PMA was used as a positive control. The intensity varied between the strains, but the differences were not significant.

To evaluate the effect of inhibition of the complement system, two peptides, Compstatin and Compstatin Cp20 analogue, which inhibit C3 conversion by blocking C3 binding to its convertase were used. Compstatin is the original peptide with this mechanism of action and Compstatin Cp20 analogue is a newer analogue with modified amino acid residues and a 6000-fold higher activity to C3 than the original Compstatin. (158) Both inhibitors were used at 30 µM and added to the samples 10 minutes prior to infection.

Compstatin Cp20 reduced the oxidative burst response to all strains significantly except for *Nme MC58Δcsb.* Up to 90 % of oxidative burst response could be abolished for some of the strains (*Nme 8013, α4*). The positive control and the negative control remained uninfluenced by the inhibitor as expected, since the

mechanism of action of PMA does not depend on complement. (See Figure 14 **B)**

The use of compstatin also showed a trend of reduced oxidative burst but none of the differences was significant, which reflects the lower activity of this complement inhibitor. (See Figure 14 **A)**

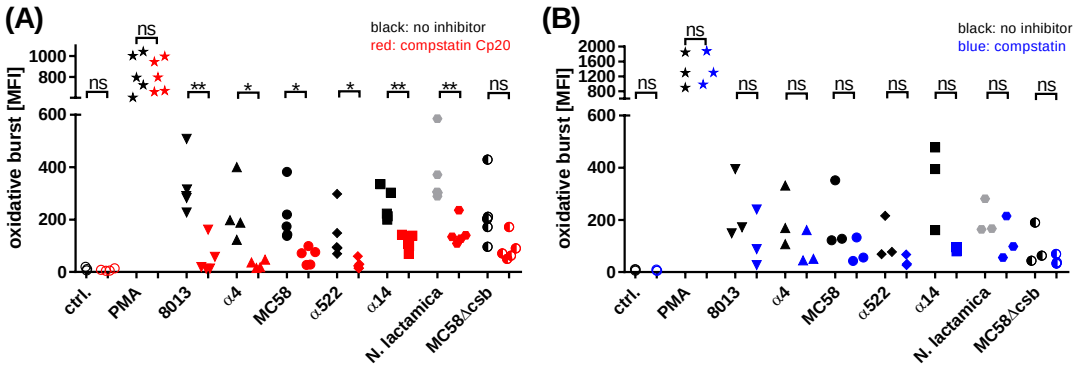

Figure 14: Oxidative burst in whole blood and the effect of complement inhibition

100 µl of hirudin anti-coagulated blood labeled with 20 µg/ml DHR123 was infected with 10^7 CFU/ml of the indicated strains or preincubated with either 30 µM Compstatin or 30 µM Compstatin Cp20 for 10 minutes and then infected. After one hour incubation erythrocytes were lysed with hypotonic buffer, cells were washed and fixed with PFA. DHR123 fluorescence intensity was assessed by flow cytometry. **A** Whole blood either with 30 µM Compstatin Cp20 analogue (●) or without (●). Graph shows data from samples of five different donors. **, *, ns indicate $p<0.01$, $p<0.05$, not significant respectively, in Student's t-test comparing the two conditions for each individual *Nme* strain. **B** Whole blood either with 30 µM Compstatin (●) or without (●). Graph shows data from samples of three different donors. ns indicates not significant in Student's t-test comparing the two conditions for each individual *Nme* strain.

This experiment shows that the robust oxidative burst response in whole blood is largely mediated by complement activation. Effective opsonization of *Nme* through C3 deposition appears to play a key role in PMNs mounting a full oxidative burst response. BRC and autologous plasma were able to induce an oxidative burst response in isolated PMNs. (see Figure 13) Vice versa, inhibition of complement in whole blood abolished the oxidative burst response to a large extent.

3.5 PMN degranulation in *Nme* infection

Previous experiments of this project demonstrated that both phagocytosis and the oxidative burst response are strongly influenced by complement activity in

Nme infection. Besides those neutrophil strategies to combat pathogens, degranulation of neutrophil granules is another important effector function which was evaluated as well. (76)

Isolated PMNs and whole blood were infected as described above in the experiments regarding the oxidative burst (see chapters 402.11.2, 2.11.3) and CD11b expression was used as a marker for degranulation. CD11b is constitutively expressed on PMN surfaces but increases in concentration upon degranulation, because it is also located in the neutrophil granule membranes which fuse with the plasma membrane during degranulation. (159)

3.5.1 Degranulation in isolated PMNs

Isolated PMNs were tested in RPMI medium and with the supplementation of 10 % autologous plasma. The cells demonstrated a significant increase in degranulation compared to the negative control when infected with strains *Nme α4, MC58,* and *MC58∆csb*. Treatment with PMA initiated a significant increase in degranulation as well. However, supplementation with 10 % autologous plasma did not cause a significant change in degranulation for any of those strains. (see Figure 15 **A**)

Thus, degranulation appeared to be triggered independently from the presence of complement or plasma in general.

3.5.2 Degranulation in whole blood

PMNs in whole blood were tested with and without 30 µM Compstatin Cp20 to evaluate the influence of complement activation on degranulation. All seven tested strains induced a significant increase in CD11b expression (degranulation) compared to the negative control. When Compstatin Cp20 was added, there was a tendency towards less degranulation, but this effect was not significant. (see Figure 15 **B**)

These results demonstrate that degranulation robustly occurs in whole blood as well but is largely independent from C3 deposition and complement activation in general.

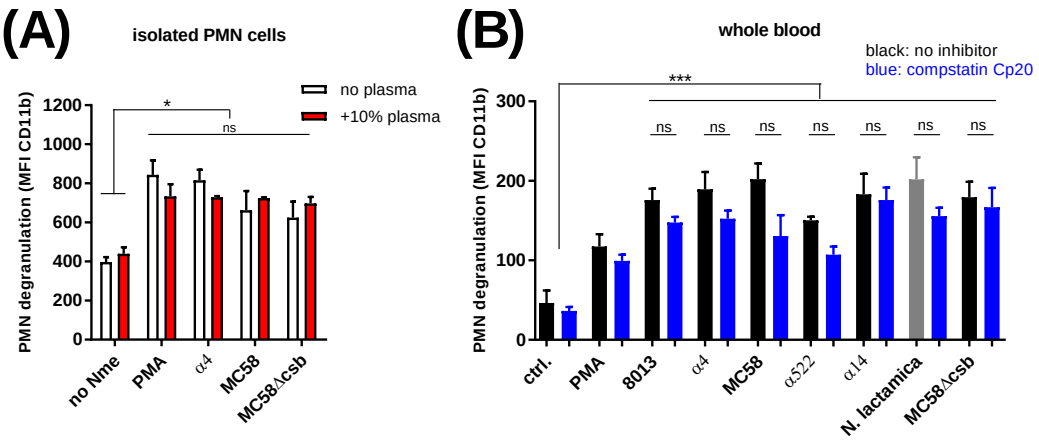

Figure 15: Degranulation in isolated PMNs and whole blood PMNs

A 10^5 PMNs in 100 µl RPMI + 5 % FCS were infected for 30 min with 10^8 CFU/ml bacteria of indicated strains. When indicated 10 % autologous plasma was added 10 min prior to infection. Subsequently, reactions were stopped by placing on ice and APC-labeled CD11b antibody was added and degranulation was measured as APC fluorescence in FL4-H in flow cytometry. Graph shows mean ± SEM from samples of three different donors. *, ns indicate $p<0.05$, not significant respectively, in one-way ANOVA applying Bonferroni's post hoc test. **B** 100 µl hirudin anti-coagulated blood was infected for 60 min with 10^7 CFU/ml bacteria of indicated strains. Where indicated, 30 µM Compstatin Cp20 was added 10 min prior to infection. After infection, reactions were stopped by placing on ice and APC-labeled CD11b antibody was added, erythrocyte lysis performed, and degranulation was measured as APC fluorescence in FL4-H in flow cytometry. Graph shows mean ± SEM from samples of four different donors. ***, ns indicate $p<0.005$, not significant respectively, in one-way ANOVA applying Bonferroni's post hoc test.

3.6 IL-8 release from PMNs in *Nme* infection

Besides phagocytosis, oxidative burst and degranulation, the release of cytokines and chemokines is an important cellular response of PMNs to pathogens. IL-8 is the chemokine that is most abundantly released by neutrophils during infections and it attracts more cells of myeloid lineage to the site of infection. (80)

To assess IL-8 release isolated PMNs were incubated with different *Nme* strains for either 90 minutes or four hours and the supernatant was obtained for IL-8 measurements by ELISA.

The influence of complement and complement inhibition on IL-8 release was assessed by adding 10 % autologous plasma in presence or absence of Compsatin Cp20 to isolated PMNs. IL-8 release was not measured in whole blood, because other cell types like monocytes produce very large amounts of chemokines which would obscure PMN-derived IL-8 release. (160)

3.6.1 IL-8 release after 90 min and 4 hours

When 10^5 PMNs in medium were infected with 10^7 CFU of different *Neisseria* strains for 90 min and 4 hours, all strains induced a significant increase in IL-8 release compared to the negative control. IL-8 concentrations in the infected samples increased tenfold between the 90 minutes and 4 hour time points. On the other hand, IL-8 concentrations remained low in the negative control over time. Differences in IL-8 release between different strains were not significant and there was a high donor-dependent variability in IL-8 release. (see Figure 16 **A-B**)

IL-8 concentrations in samples stimulated with PMA were significantly lower than in infected samples. This could be due to reduced viability of PMNs, because prolonged incubation with PMA is cytotoxic for neutrophils, thus abolishing further IL-8 release. (161)

3.6.2 IL-8 release under the influence of complement

To assess the influence of complement activation on IL-8 release PMNs where incubated with 10 % plasma and both 10 % plasma and Compstatin Cp20 for four hours. When 10 % plasma was added to isolated PMNs prior to infection there

was no significant change compared to PMNs incubated in RPMI medium. There was a trend towards lower IL-8 concentrations following inhibition of supplemented plasma with Compstatin Cp20, but this was not significant. (see Figure 15 Figure 16 **C**)

The negative control and PMA remained uninfluenced by plasma supplementation and complement inhibition.

These data demonstrate that PMNs release high concentrations of IL-8 over a long period of time in the presence of *Nme*, but IL-8 release appears to be independent from plasma components such as complement.

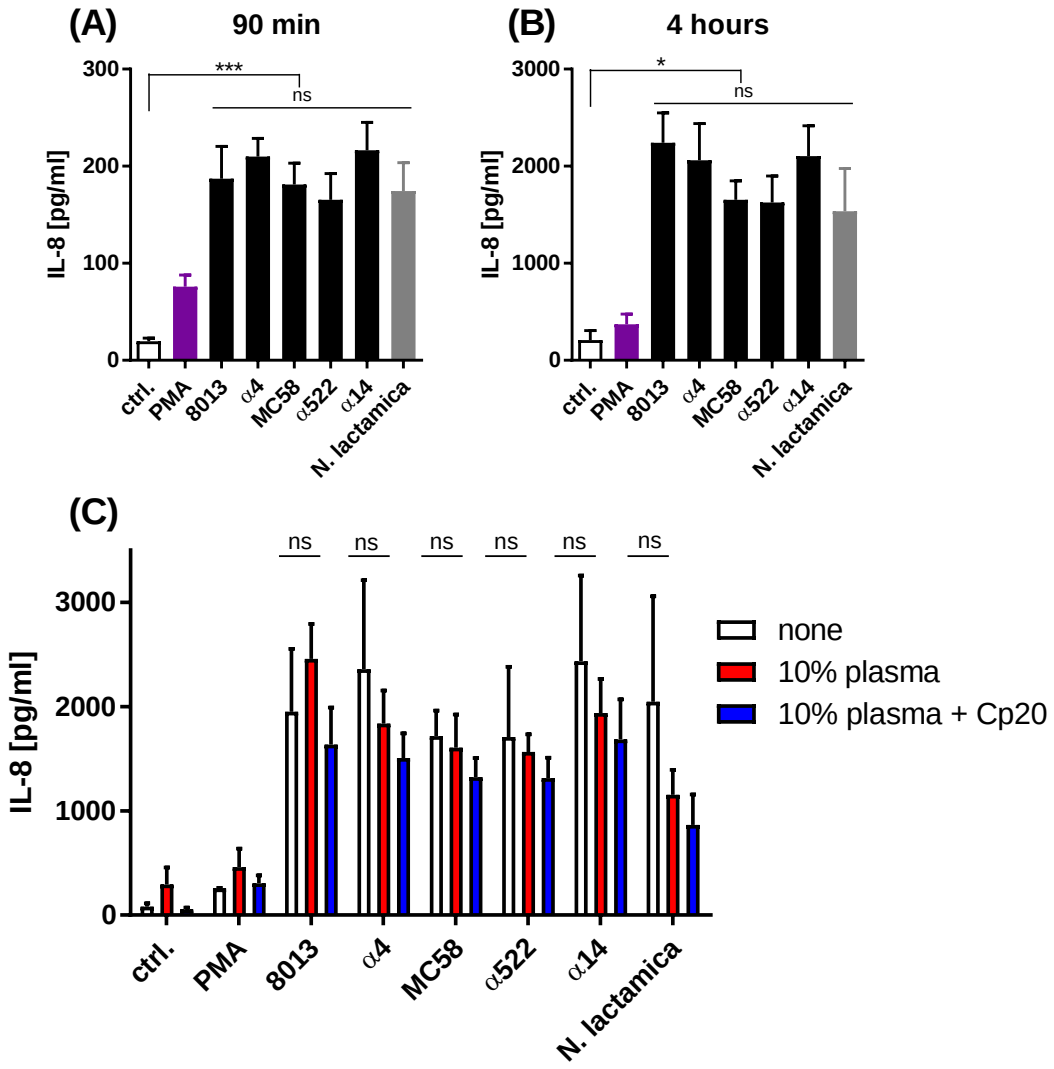

Figure 16: IL-8 release of PMNs in *Nme* infection over time and under the influence of complement

10^5 PMNs in RPMI +5 % FCS were infected with 10^7 CFU/ml of indicated strains. Supernatant was obtained for measurement of IL-8 concentrations by ELISA. **A** IL-8 release after 90 min. Graph shows means ± SEM from samples of six different donors. ***, ns indicate $p<0.005$, not significant respectively in one-way ANOVA applying Bonferroni's post hoc test. **B** IL-8 release after 4 hours. Graph shows means ± SEM from samples of seven different donors. *, ns indicate $p<0.05$, not significant in one-way ANOVA applying Bonferroni's post hoc test. **C** 10^5 PMNs were preincubated either with 10 % autologous plasma (●), with 10 % plasma and 30 µM Compstatin Cp20 (●) or without any plasma or complement inhibitor (●). Next, cells were infected and processed as in **A** & **B**. Graph shows means ± SEM from samples of three to four different donors. ns indicates not significant in one-way ANOVA applying Dunnett's post hoc test comparing the three different conditions for each *Nme* strain.

3.7 Ceramide in PMN cellular interaction with *Nme*

Neutrophil phagocytosis has previously been described to involve the activation of sphingomyelinase and generation of ceramide. (162) Moreover, some *Nme* strains induce ceramide generation in endothelial cells via the Opa protein Opc to promote internalization. (131) The expression of Opa was shown to significantly increase *Nme* internalization into PMNs in this study as well. (see Figure 10) To explore a hypothetical role of ceramide in PMN interaction with *Nme* immunofluorescence staining was used.

PMN nuclei, *Nme* and ceramide were stained with blue, green and red fluorophores, respectively, and association of these three fluorophores were counted under the microscope for several hundreds of PMNs for each strain. In this setup intracellular and extracuellular localization of bacteria colocalizing with PMNs could not be distinguished, which is why total association was measured. (see Figure 18)

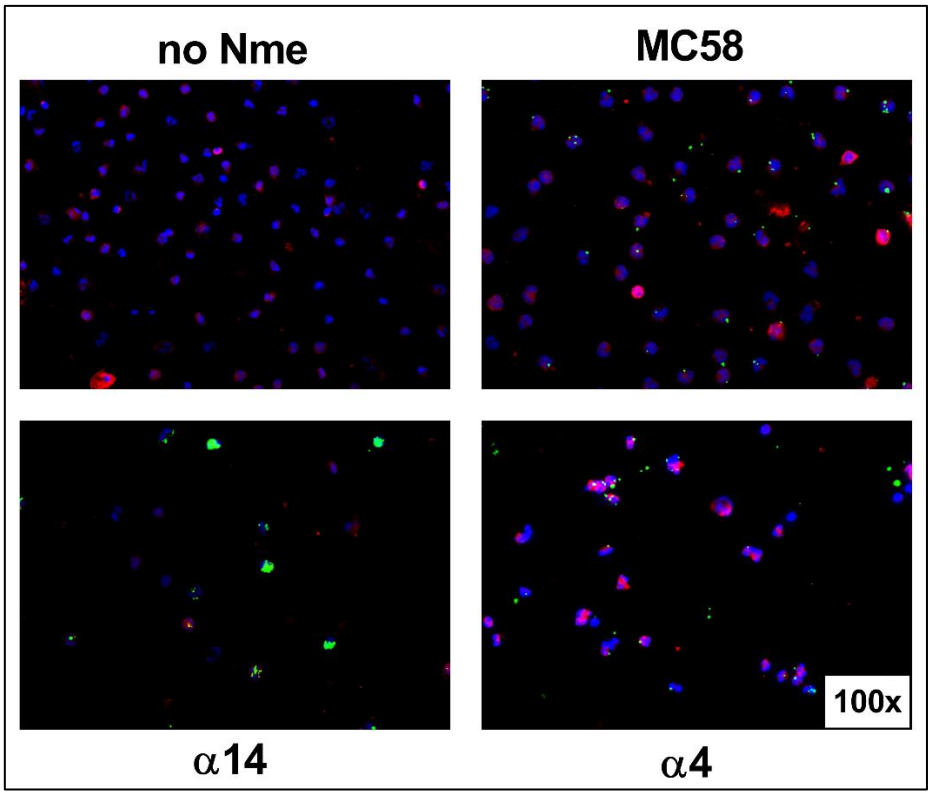

Figure 17: Immunofluorescence staining of PMNs and ceramide

Exemplary images of uninfected controls and PMNs infected with either *α4, α14* or *MC58* as indicated. Green colour (●) shows *Nme*, red colour (●) shows ceramide and blue colour (●) shows PMNs' nuclei.

Regarding the association of PMNs with different *Nme* strains, *α522* and *MC58* had the highest proportion of PMNs associated with at least one bacteria. However, the unencapsulated strains demonstrated a much higher bacterial burden per PMN despite the lower proportion of PMNs associated with bacteria. This effect was most pronounced for strain *α14* and could be due to aggregation of unencapsulated bacteria prior to phagocytosis. (see Figure 18 **A**)

Concerning the colocalization of ceramide and *Nme*, the immunofluorescence staining revelead that ceramide release in PMNs was by far not exclusive for cells in contact with bacteria. A considerable portion of PMNs was positive for ceramide without any contact to bacteria regardless of the infecting strain and even in the uninfected, negative control. A fraction of cells in contact with bacteria without any visible ceramide in their membranes was also present for all strains. However, the fraction of PMNs positive for both ceramide and bacteria appeared to be larger in samples infected with *α522* and *MC58*. (see Figure 18 Figure 18**B**)

Overall ceramide levels in the samples showed great variability and even in the uninfected, negative control more than 40 % of cells were positive for ceramide. Samples infected with bacteria or treated with acid sphingomyelinase had between 45 and 71 % of ceramide positive cells. (see Figure 18 **C**)

These data suggest that unencapsulated strains are phagocytised more abundantly since the bacterial burden of PMN cells was much higher bacterial burden for these strains. Moreover, the data suggest that some strains (e.g. *MC58* or *α522*) might trigger more ceramide release in PMN cellular membranes when interacting with them. However, the small-scale set-up of the experiment and the scattering of data allow only preliminary deductions and further investigations would be required to analyse the role of ceramide in neutrophil interaction with *Nme.*

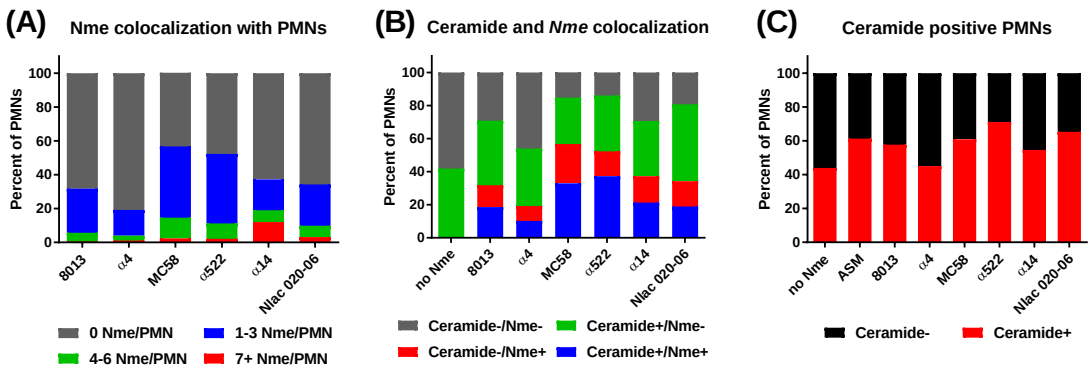

Figure 18: Ceramide release of PMNs upon *Nme* infection

5 x 10^5 PMNs were seeded on round coverslips and infected with 5x 10^7 CFU/ml of indicated FITC-labeled *Nme* strains. Cells were then fixed with PFA and ceramide was stained with Cy3 and PMNs nuclei with DAPI. **A** Association of PMNs with indicated *Nme* strains. Graph shows proportion of PMNs associated with 0, 1-3, 4-6 or 7 and more *Nme* per cell. “No *Nme*” indicates uninfected control. Each bar represents visual counting of 254 to 604 PMNs with associated *Nme*. **B** Proportion of PMNs showing signal for none of two stainings (ceramide/*Nme*), both stainings or just one of the stainings (ceramide or *Nme*). Each bar represents visual counting of 254 to 604 PMNs with associated *Nme* and ceramide signal. **C** Proportion of PMNs with a positive ceramide signal without bacteria (“no *Nme*”), infected with indicated strains or treated with acid sphingomyelinase (ASM) for one hour. Each bar represents visual counting of 254 to 805 PMNs.

4 Discussion

4.1 Neutrophil phagocytosis of *Neisseria meningitidis* and complement

The role of the complement system in meningococcal disease has been extensively researched and characterized. Its protective properties regarding IMD are evident considering the high susceptibility to IMD of complement-deficient individuals. Serum bactericidal activity conferred by the terminal complement pathway appears to be essential in prevention and clearance of pathogenic *Neisseriae* in non-immunized individuals. (56, 61) The role of neutrophils in the context of meningococcal disease is less clear and requires further investigation and characterization. Although, primary and acquired immunodeficiencies involving neutrophil cell count and neutrophil effector functions do not increase susceptibility to IMD, the disease severity and mortality are increased in case of neutropenia. However, the epidemiological association of disease severity with low blood neutrophil counts can also be explained by increased neutrophil extravasation from the peripheral circulation under highly inflammatory conditions and does not necessarily reflect a diminished neutrophil immune response. (57, 86, 163-165) A murine *Nme* sepsis model with complement-sufficient mice showed increased mortality after pharmacologically induced neutrophil depletion, which implies an overall beneficial role of neutrophils in IMD. (109) The results of this study characterize these neutrophil responses in the context of IMD and add to existing knowledge about the neutrophil contribution to infection control of IMD.

To investigate isolated PMNs several isolation procedures from whole blood have been described. Methods include spontaneous sedimentation separation, density gradient centrifugation with Ficoll, Percoll or Polymorphprep solutions, dextrane sedimentation and negative cell sorting with magnetic or fluorescent beads. Purity and viability of PMNs achieved by all these methods has been reported to be above 90 %. Isolation based on cell sorting can achieve PMN purity above 99 %, but the yield of cells is usually decreased considerably by the isolation

procedure. Alterations of PMN responsiveness and surface protein expression compared to whole blood PMNs has been reported for all isolation procedures in similar ways. Motility, responsiveness to stimuli of the oxidative burst and chemotaxis and expression of integrins can be reduced. (139, 166-170) Since the ultra-high purity achieved with cell sorting does not necessarily influence readouts of infection assays but high yields of PMNs are required to perform these assays, the density gradient centrifugation method with Polymorphprep was chosen as the isolation procedure in this project. (171)

This study demonstrated that PMN phagocytize *Nme* to a limited extent, when isolated PMNs without serum components were incubated with bacteria. To measure association and internalization of *Nme* an established infection assay was used quantifying viable CFU counts as a measure of neutrophil-meningococcal interaction. As described before, adhesion of bacteria to PMNs and non-opsonic internalization was observed. (see chapter 3.2.1, Figure 6, Figure 7) (107) However, internalization (phagocytosis) appeared to be inefficient since only a small fraction of PMN-adherent bacteria were internalized. This is in accordance with research by Read et al. that measured phagocytosis of *Nme* by isolated macrophages and observed a low ratio of internalized to adherent bacteria. (137) *Nme* internalized by PMNs in the assays appeared to be killed efficiently however, since internalized CFUs declined progressively over a period of four hours (see Figure 8). Since recovered viable CFU was used to measure internalization, phagocytosis might be underestimated, if most internalized bacteria are already unviable at the time of recovery, which was either after one or four hours. In neutrophils, onset of killing of *E. coli* has been described starting at 60 to 90 minutes after internalization. (172) In macrophages phagosome-lysosome fusion was described as early as 30 minutes after internalization with unencapsulated *Nme* being transported into phagolysosomes more efficiently and rapidly.(137) These factors could result in underestimation of internalized bacteria especially regarding the unencapsulated strains.

Concerning overall killing of bacteria by PMNs, no significant decline of CFU in the inoculum was observed after 30 minutes when *Nme* were incubated with PMNs only or in the presence of heat-inactivated serum compared to CFU counts

in RPMI medium. When 10 % autologous plasma was added to PMNs there was a 90 % reduction of CFU counts of unencapsulated bacteria. It seems likely that naivety of the donor's immune system to the encapsulated strains explains why no significant killing by plasma of these strains was observed. In hirudin anti-coagulated whole blood assays, all unencapsulated strains were eliminated completely by any donor blood sample and killing or substantial reduction of encapsulated strains was observed in most donor blood samples as well. (see Figure 9) The killing of encapsulated strains in whole blood despite the lack of killing with 10 % plasma might be due to higher concentrations of complement and polyspecific antibodies. (154) The higher bactericidal activity of whole blood and plasma compared to isolated PMNs indicates a higher efficacy of complement-mediated killing compared to the cellular responses.. This is in contrast with findings by Estabrook *et al.* who observed a 50 % reduction of inoculated *Nme* in the presence of PMNs and heat-inactivated serum and almost complete killing of opsonized bacteria with some serogroup C strains. (107) However, the strains used in this project were completely different from the ones used by Estabrook *et al*. Another publication of Estabrook et al. evaluating different strains only reports complement-dependent killing but no killing by PMNs in the presence of heat-inactivated complement. (151) Opsonization and killing by antimicrobial peptides and strain-dependent resistance to those could explain complement-independent killing in these publications and strain-dependency of this phenomenon. (173-175)

Regarding the mechanism of non-opsonic phagocytosis, Opa-CEACAM interaction was found to promote internalization of *Neisseriae* which has been demonstrated extensively for both *Nme* and *Ngo*. (111, 128, 135, 176) When comparing strain *MC58* with its Opa knockout, the expression of Opa promoted internalization significantly, but adhesion remained uninfluenced. However, no significant differences regarding internalization were observed between encapsulated strains expressing Opa and those not expressing Opa. (see Figure 7, Figure 10) Considering the abundance of Opa variants and their differential interaction with human cells, the detection of Opa expression using a panspecific Opa antibody in this study does not allow any further deductions on the effect of

specific Opa variants expressed by the individual strains on internalization. (128) The unencapsulated bacteria were internalized more extensively than other strains regardless of Opa expression which highlights that other virulence factors such as capsule and pili expression and binding through TLR-mediated pathways exert a stronger influential effect on internalization than Opa proteins. (177, 178)

In addition, this project briefly examined the involvement of the ceramide signalling pathway in neutrophil interaction with non-opsonized *Nme*, because many studies have shown that ceramide release is important in host pathogen interaction and particularly in infections with pathogenic *Neisseriae*. (119, 122, 123, 179) *Neisseria gonorrhoeae* activates the acid sphingomyelinase both in epithelial and in phagocytic cells through interaction of bacterial Opa proteins with host CEACAM receptors. While this signalling pathway promotes invasion into epithelial tissues, Opa-CEACAM interaction is also used by neutrophils to phagocytise and kill *Ngo*. (124, 125, 128) In *Nme* infection Opa-CEACAM and Opc-HSPG interactions and subsequent ceramide release are important for the invasion of epithelial cells and endothelial cells, respectively. (129, 131) Using immunofluorescence microscopy, no obvious association of neutrophil ceramide with *Nme* could be observed under non-opsonic conditions. Ceramide appeared to be equally distributed over the cell membrane and no ceramide-rich microdomains could be regularly detected next to PMN-associated bacteria. (see Figure 17) The overall proportion of PMNs with detectable ceramide on the cell surface was higher with strains *MC58* and *α522*, but this appeared to be independent from cellular contact with bacteria. On the other hand, it was demonstrated that the bacterial burden inside PMNs was higher for the unencapsulated strains including many more PMNs with multiple internalized bacteria as compared to the encapsulated strains. (see Figure 18) This is coherent with the results of the infection assays and previous studies showing more internalization and bacterial burden per PMN cell in the absence of a protective capsule. (see Figure 7) (136, 137) In neutrophil interaction with *Ngo*, ceramide release contributes to phagocytosis and a strong pro-inflammatory response promoting bacterial clearance is driven by CEACAM3 interaction with gonococcal Opa proteins. (125, 127, 180, 181) Meningococcal Opa proteins

however do not bind to CEACAM3 but mostly to CEACAM1, which could explain why no ceramide release was observed, because different CEACAM receptors can activate different intracellular signalling pathways depending on the cell type. (128, 135, 182, 183) Moreover, the expression of a capsule by some of the strains in this assay could be a steric hindrance for Opa-CEACAM interactions by covering parts of the Opa proteins resulting in less ceramide release. Opa-mediated internalization in epithelial cells occurs more efficiently with unencapsulated *Nme* and encapsulated *Nme* require higher CEACAM receptor density for internalization indicating at least some negative effect of the capsule on cellular interactions. (13, 136, 184-186) Moreover, further research is required to determine whether ceramide release is relevant beyond internalization triggered by Opa-CEACAM interaction but also in the context of opsonic phagocytosis via complement receptors and Fc receptors.

Complement was demonstrated to be essential for opsonic phagocytosis. Highly increased phagocytosis in immune serum was linked to complement activation following specific antibody binding rather than antibody binding alone, since heat-inactivation of complement abolished phagocytosis in immune serum. However, low phagocytic activity in non-immune, complement-sufficient human serum highlights that specific antibodies are still required to initiate complement activation through the classical and the alternative pathway. (60) However, serum concentrations higher than 5 % might result in increased phagocytosis in non-immune human serum as well, because alternative pathway activity increases with higher complement concentrations. The substantial reduction of phagocytosis by inhibiting opsonization with C3b through complement inhibitor Compstatin Cp20 in contrast to the inefficacy of Fc receptor blockade implicates complement receptor 3 (CR3; CD11b/CD18) on neutrophils as the main driver of phagocytosis in immune serum rather than the Fc receptors CD16, CD32 and CD64. (see Figure 11) (54, 187, 188) This explains almost complete reduction in the presence of Compstatin Cp20 which greatly reduces availability of the main CR3 ligand iC3b on *Nme*. (144, 145) The smaller reduction of phagocytosis by C5aR1 inhibition can be explained by the ability of the anaphylatoxin C5a to upregulate CR3 on neutrophils thus further promoting phagocytosis. Upregulation

of CR3 can also be triggered by other inflammatory stimuli such as TNFα and LPS. (102, 109, 189, 190) Although Fc-receptor-mediated phagocytosis occurs in other settings of infectious disease it appears to be negligible in this context. (191) Additionally, it should be considered that the internalized *Nme* are already unviable at the time of phagocytosis, since 5 % immune serum alone effectively kills the bacteria. (see Figure 11) Thus, neutrophil killing of *Nme* cannot be evaluated in the presence of immune serum but only the uptake of dead bacteria.

Furthermore, complement-mediated phagocytosis explains the better clinical outcome in humans and mouse model with normal neutrophil function and number. Even though *Nme* killing is predominantly mediated by complement bactericidal activity, the removal of *Nme* and their endotoxins from circulation could improve systemic inflammation and limit cytokine storm and organ damage. Neisserial LOS directly causes endothelial damage and increases vascular permeability which is a hallmark of sepsis leading to intravascular coagulation and reduced organ perfusion. (192-195) Moreover, LOS induces the production and release of proinflammatory cytokines such as IL-8, TNFα, MIP1α, IL-1β, IL-6 and IL-10, possibly through binding to toll-like receptor 4 (TLR4). Levels of these proinflammatory cytokines have been shown to correlate with disease severity and mortality in sepsis. (196-199) On the other hand, phagocytosis through CR3 is an immunologically more silent process and has been shown to reduce proinflammatory responses of phagocytes upon LPS stimulation and experimental inhibition of LPS signalling via TLR4 improves the clinical score of infected animals in a porcine model of meningococcal sepsis. (200-203) In the context of meningococcal meningitis, high concentrations of LOS and inflammatory cytokines in the cerebrospinal fluid have been correlated with disease severity. (195, 204) Although studies using a meningococcal meningitis model have not been conducted, studies with pneumococcal meningitis mouse models have shown that neutrophil depletion increases bacterial burden in blood and CSF leading to increased lethality and long-term sequelae from meningitis. (94, 205, 206) These findings suggest a positive role of neutrophil granulocytes in the setting of meningitis, though the results should be replicated for meningococcal meningitis. Thus, the main contribution of neutrophils to

meningococcal clearance could be the removal of complement-killed bacteria from blood and the cerebrospinal fluid and switching the immune response to an anti-inflammatory response away from the pro-inflammatory LPS/TLR4 axis that would otherwise cause excessive inflammation and organ damage.

4.2 Neutrophil oxidative burst response to *Neisseria meningitidis* and complement

The oxidative burst response of phagocytes towards *Nme* has been demonstrated in several previous studies. (190, 197) However, most studies investigating this PMN response to pathogens used whole blood or PMN samples supplemented with serum to measure the oxidative burst. In this project, experiments with isolated neutrophils in the absence of serum were included. Surprisingly, isolated neutrophils did not show any oxidative burst response to *Nme* even after prolonged incubation while whole blood assays revealed a robust oxidative burst response to all *Neisseria* strains used in the assays. The oxidative burst response could be partially restored upon addition of baby rabbit complement. (see Figure 13) This effect was only significant regarding the unencapsulated meningococcal strains. Here, complement can only be activated through the alternative pathway in the absence of specific antibodies and many studies have proven that capsular polysaccharide can decrease susceptibility to complement by limiting alternative pathway activation and complement deposition on *Nme*. (152, 207, 208) Moreover, the meningococcal capsule can redirect complement deposition to different sites on the *Nme* surface and avoid the bactericidal activity of complement despite C3b deposition. (70) In this study higher levels of C3b deposition were measured on the unencapsulated strains suggesting that the observed difference is mediated through different surface C3b levels. Upon addition of autologous plasma there was a significant oxidative burst response of PMNs to encapsulated *Nme* as well. Since the donors were not immunized against *Nme*, this effect could be mediated through the binding of poly-specific IgM or IgG in donor plasma, which can enhance complement deposition through the classical pathway. (70, 154, 209)

Considering the complement-dependent onset of the oxidative burst in isolated PMNs it is plausible that interaction of C3b with CR3 mediates the oxidative burst response and no other ligand-receptor interactions such as LOS/TLR4. LPS has been shown to cause an oxidative burst in murine neutrophils, but only when used at concentrations several log scales above concentrations observed in patients with severe sepsis and probably not present in the assays. (195, 210-212) When using LPS at lower concentrations that also occur in gram-negative infections, LPS does not induce an oxidative burst in neutrophils and monocytes, but it induces secretion of proinflammatory cytokines and upregulation of CR3, which is more pronounced in monocytes. The soluble LPS binding protein (LBP), which is present in serum, further facilitates TLR4 interaction with LPS and enhances TLR4-mediated signalling. (189, 213-215) Additionally, complement activation induces additional CR3 upregulation by C5a interaction with its receptor C5aR1 on neutrophils. As shown in this study and in previous publications, C5a does not induce an oxidative burst in *Nme* infection by itself either. (216, 217) Upregulation of CR3 has been shown to be independent from meningococcal LPS expression using LPS-knockout strains. (197) Redundant signalling to upregulate CR3 via C5aR1 and cytokine receptors in addition to TLR4 can explain this phenomenon. However, CR3 signalling has been demonstrated to induce an oxidative burst response in many studies. Inhibition of CR3 expression and signalling on neutrophil surfaces abrogates both phagocytosis and the oxidative burst. (188, 189, 218) Therefore, it seems evident that interactions between neutrophil CR3 and C3b on *Nme* surfaces promote *Nme* uptake and simultaneously stimulate the oxidative burst response. Signals via TLR4 and C5aR1 and other pro-inflammatory cytokines such as TNFα and G-CSF prime neutrophils towards binding opsonized bacteria by inducing upregulation of CR3. (213) This hypothesis is further supported by the successful inhibition of the oxidative burst by inhibiting C3 cleavage in whole blood with Compstatin Cp20 leaving all other signaling pathways intact except for complement-mediated signaling. (see Figure 14) Both CR3 engagement and C5a-mediated CR3 upregulation are inhibited in this context.

In contrast to these findings, the addition of specific antibodies alone to isolated PMNs did not elicit an oxidative burst response despite high affinity binding to all strains used in the experiments. (see Figure 13) In general, PMNs express three different Fc receptors; two low-affinity receptors, FcγRIIA (CD32) and FcγRIIIB (CD16), and one high-affinity receptor, FcγRIA (CD64). FcγRIIIB is expressed abundantly but its most important function is the endocytosis of circulating immune complexes and its downstream signaling does not induce an oxidative burst response. FcγRIIA activation can induce phagocytosis and the oxidative burst, but its binding properties to IgG are poor in resting neutrophils and neutrophils require priming e.g. by cytokines to efficiently signal via FcγRIIA. (191, 219-222) The high-affinity FcγRIA is not constitutively expressed by neutrophils but induces efficient phagocytosis and an oxidative burst response. Studies investigating the role of FcγRIA and FcγRIIA in neutrophils have used treatment with pro-inflammatory cytokines such as IFNγ to induce FcγRIA expression prior to stimulation. (223-225) Since there was no priming of neutrophils by cytokines or other serum components in the assays, the lack of oxidative burst response towards IgG-opsonized *Nme* might be due to the poor binding properties of expressed FcγRIIA and FcγRIIIB and the lack of expression of FcγRIA. Furthermore, the antibodies used to opsonize *Neisseriae* were purified rabbit antibodies. Human Fc receptors have a low affinity for most murine antibody classes which could interfere with antibody-mediated signaling. (226) The use of human anti-meningococcal antibodies would be necessary to control for this. However, it should be considered that human anti-meningococcal antibodies were investigated in the context of phagocytosis and did not show significant effects in the absence of complement. (see Figure 11) Further research with primed neutrophils and human anti-meningococcal antibodies might be worth conducting to reveal an oxidative burst response under inflammatory conditions, since those conditions might resemble conditions in meningococcal sepsis more accurately. (227)

All in all, the oxidative burst response of neutrophils appears to be a consequence of complement activation, possibly elicited by CR3-mediated phagocytosis of opsonized *Nme*. Pro-inflammatory mediators such as LPS and C5a upregulate

neutrophil capacities for efficient phagocytosis and oxidative burst to eliminate *Nme*, but do not cause the oxidative burst response and phagocytosis by themselves. Interestingly, myeloperoxidase, a key enzyme for ROS production, has been shown to limit LPS-induced toxicity and cytokine production in a mouse model. (228) It should be considered that the CR3-mediated release of ROS could have protective effects by inactivating circulating endotoxins and bacteria besides collateral tissue damage.

4.3 Neutrophil degranulation and cytokine release

Neutrophil degranulation is an important cellular response in infectious disease and especially in infection with gram-negative bacteria such as *Nme*. Degranulation is often measured by the upregulation of phagocyte receptors CR1 (CD35) and CR3 (CD11b/CD18) because they are primarily stored in the granule membranes in resting PMNs and translocated to the surface with the content of the granules upon stimulation. (159, 229) In gram-negative sepsis CD11b expression appears to be relatively higher than in gram-positive sepsis, indicating an important function in immune defence against gram-negative pathogens. (230, 231) Especially in meningococcal sepsis, early degranulation of PMNs has been described which also leads to sequestration into the peripheral circulation, because granule-derived integrins promote adhesion to activated endothelium and migration towards sites of infection. (163, 213)

In this project, neutrophil degranulation measured by CD11b surface levels was observed both in whole blood and in isolated PMNs as a robust response to all *Neisseria* strains used in the experiments. This phenomenon was independent from capsule expression and pathogenicity of the strains. Complement inhibition in whole blood or supplementation of isolated PMNs with complement did not alter the extent of degranulation significantly although significant reduction of degranulation by complement inhibition has been observed in other studies. (see Figure 15) (217) These findings can be reconciled by the fact that CD11b (CR3) upregulation as described above is not exclusively dependent on complement activation. The C5a/C5aR1 axis leads to degranulation and upregulation of CD11b but so does the LPS/TLR4 axis which is complement-independent. Thus,

these two independent signalling pathways redundantly induce degranulation in infection with gram-negative bacteria. Combined inhibition of both signalling pathways however can abrogate degranulation e.g. by blocking antibodies against C5a and CD14. (189, 215) Therefore, investigation of the effect of combined inhibition of these pathways on PMN degranulation in the presence of *Nme* is worth assessing. On the other hand, degranulation itself influences complement-mediated responses of neutrophils. Degranulation-mediated complement receptor upregulation primes and enhances neutrophil responses to complement. However, this priming is transient in the case of sepsis since overactivation of PMNs through the C5a/C5aR1 axis can lead to decreased responsiveness and systemically to a state of immune paralysis. Previous studies show that neutrophil responsiveness is dependent on dose and application time of C5a before a stimulus and decreases with higher doses and longer exposition. (102, 232) This might indicate that increased PMN responsiveness is triggered by LPS and complement activation in settings of localized infection where anaphylatoxin concentrations are lower and elevated for a shorter period of time, but in the setting of systemic infection cytokine production and complement activation become deregulated and lead to a progressive paralysis of neutrophil responses. (233, 234)

Regarding neutrophil chemokine and cytokine production this study assessed IL-8 release, since it is the most abundantly released and produced chemokine, stimulating the recruitment of further myeloid cells to sites of infection. Furthermore, neutrophils can produce a variety of other cytokines and chemokines including TNFα, IL-1β and MIP-1α. (79, 80) Both the C5a/C5aR1 axis and TLR4-mediated signalling leads to increased secretion of IL-8. (109, 235) Since monocytes produce substantially higher quantities of cytokines and chemokines per cell than neutrophils, experiments were exclusively conducted with isolated PMNs in order to avoid contamination by monocytes in whole blood and elucidate the neutrophil-specific IL-8 production. (236) PMNs infected with *Nme* displayed a vigorous and persistently increasing IL-8 release over a period of four hours. This effect was not strain-specific. Upon addition of 10 % autologous plasma, no significant differences were observed. When comparing

supplementation of 10 % plasma to plasma inhibited by Compstatin Cp20, IL-8 concentrations appeared to be reduced slightly in the setting of complement inhibition, but these differences were not significant. This could be due to a strong donor-dependent variability of IL-8 concentrations. Conducting the experiment with a larger cohort might yield enough power to show significant differences, since significant reductions of IL-8 release by complement inhibition, including the use of Compstatin Cp20, have been demonstrated in other studies. (109, 217)

Moreover, the production of IL-8 can be initiated by many inflammatory stimuli through the engagement of various pattern recognition receptors that activate NFκB. Complement and LPS are among the triggers of neutrophil IL-8 release, but they are not necessarily required to initiate this response. (194, 197, 235) Further research is required to analyse the release of other cytokines by neutrophils upon *Nme* infection in the context of complement inhibition and inhibition of additional pro-inflammatory pathways such as TLR4/CD14.

4.4 Neutrophil extracellular traps and *Neisseria meningitidis* infection

Another neutrophil strategy to neutralize invasive bacteria is the formation of neutrophil extracellular traps (NETs) which contain neutrophil DNA and antimicrobial peptides and proteins. NETs catch and inactivate pathogens and are a potent strategy in innate immune defence. (77) NET formation has been shown in meningococcal disease as well and is associated with more severe disease and disseminated intravascular coagulation. (98, 237) Moreover, *Nme* among other bacteria possess a high intrinsic resistance against NETs despite inducing NETs both *in vitro* and *in vivo*. (114, 115) In this study, NET formation was negligible in the infection assays as assessed by microscopy and flow cytometry. (see Figure 17) In flow cytometry NET formation is characterized by a shift of neutrophil FSC/SSC pattern which was not observed under the experimental conditions of this study. Considering these observations and the existing evidence for meningococcal NET resistance, NETs were not further studied in this study.

4.5 Synergies between neutrophils and complement in meningococcal infection

The findings of this study characterize effector functions of neutrophil granulocytes in *Nme* infection. *Nme* serve as a sufficient stimulus for PMNs to initiate phagocytosis, oxidative burst, degranulation and secretion of pro-inflammatory chemokines to recruit more immune cells to the site of infection. However, neutrophils depend on complement activation and opsonization of *Nme* to effectively perform phagocytosis and an oxidative burst response, because complement factors facilitate binding and induce upregulation of CR3 and other membrane receptors which are crucial to neutrophil responses. (189, 216, 217) While most of the killing of *Nme* is attributable to the bactericidal activity of complement, neutrophils also are important to reduce mortality during IMD. Depletion of neutrophils increases mortality in experimental meningococcal sepsis. (109, 228) Possibly, clearance of circulating *Nme* and their endotoxins by PMNs via CR3 aids to limit systemic inflammation and vascular and organ damage. By this, the pro-inflammatory response in sepsis might be redirected towards a more anti-inflammatory response. (200-202, 233) In individuals with terminal complement deficiencies opsonophagocytosis is the main remaining effector function of the complement system. Despite high susceptibility to IMD due to lack of bactericidal activity, vaccination of these individuals reduces the rate of infections. (62, 238, 239) In this context, neutrophils of vaccinated individuals with a TCC deficiency have been shown to effectively kill *Nme.* (240) Neutrophils are important for mucosal immunity, too, since neutrophil depletion leads to a significantly higher bacterial burden during *Nme* colonization. (111) This illustrates that potentiating opsonophagocytosis through specific antibodies can partly compensate for TCC defects which supports that phagocytes have an overall beneficial role in defence against *Nme.* Therefore, vaccination of complement-deficient individuals is of utmost importance and it is effective even if serum bactericidal activity cannot be achieved. It remains to be investigated whether vaccinations are also protective in patients with a C3 deficiency who have neither opsonophagocytic nor bactericidal activity of complement. Since C3 deficiencies are very rare there are no studies available regarding this subject.

However, there are increasing numbers of people with acquired complement deficiency due to the introduction of anti-C5 antibody eculizumab for the treatment of hematological and autoimmune diseases. Patients treated with eculizumab have both impaired bactericidal and opsonophagocytic complement activity. The impairment of opsonophagocytosis is due to the absent chemotactic activity of C5a and failure of phagocytes to upregulate CR3 effectively in the absence of C5a. (66, 218) Additionally, C5-deficiency has been reported to cause a higher susceptibility to IMD than other TCC deficiencies involving C6–C9. (241) Since primary C5 deficiency is rare and studies evaluating the effect of vaccinating TCC-deficient individuals have not included many C5-deficient individuals, it remains unclear whether C5-deficiency effectively responds to vaccination against *Nme.* (62, 242, 243) The lack of both humoral and cellular killing of *Nme* in patients treated with eculizumab leads to a high susceptibility for meningococcal disease and vaccine failure has been reported multiple times in this context. (67, 68, 244-247) Obviously, a completely functional complement system in conjunction with bactericidal antibody titers confers the best protection against IMD, but these findings illustrate that neutrophils are able to confer a basic level of protection, when parts of the complement system are still functional.

4.6 Future perspective

This project has provided insight into how neutrophils are activated by complement to effectively combat *Nme* and aid in the immune defence against meningococcal sepsis and meningitis. Severe sepsis, however, is often characterized by hyperinflammation, cytokine storm and immune paralysis. Under these conditions, PMNs responsiveness to *Nme* is reduced dramatically due to overactivation by the C5a/C5aR1 axis and other pro-inflammatory mediators such as LPS. (198, 211, 232)

Therefore, supportive therapies in addition to antibiotics that restore neutrophil functions and removal of bacteria from the circulation seem promising to improve outcome of IMD. Blockade of C5a signalling via C5aR1 and C5aR2 has demonstrated mortality reductions in animal models and merits further research to test the application in humans. (109, 217) Moreover, approaches targeting LPS

seem promising as well, because LPS also contributes to organ damage via TLR4-mediated hyperinflammation. Studies targeting LPS or LPS in combination with C5a have shown promising results in animal models and its application could be studied in clinical trials. (203, 248) One study demonstrated that inhibition of LPS/TLR4 signalling with anti-LBP antibodies combined with the application of G-CSF resulted in a substantial mortality reduction in an animal sepsis model indicating that neutrophils act beneficially, if inflammation can be limited. (249) Further studies to investigate whether functions of immune-paralyzed neutrophils can be restored in vitro by inhibition of these pro-inflammatory signals are required as an important step to clinical application.

Moreover, the role of neutrophils in the context of meningococcal meningitis should be further investigated. Neutrophil-driven inflammation of the central nervous system (CNS) has been shown to induce tissue damage and blockade of neutrophil functions has been demonstrated to improve neurological outcome both in pneumococcal and meningococcal meningitis. (96, 97, 250-252) However, complete depletion of neutrophils has been associated with increased mortality in multiple models of bacterial meningitis. (94, 109, 205) Interestingly, the blockade of some neutrophil inflammatory responses in pneumococcal meningitis has been correlated with a better neurological outcome despite an increased overall mortality which indicates a differential role of neutrophils in the cerebrospinal compartment. (206) A meningococcal meningitis model is required to evaluate the absence or presence of neutrophils in the CNS and their specific effector functions regarding the neurological outcome. Interventions focused on complement-dependent functions of neutrophils should be assessed to reduce organ damage and long-term sequelae. This approach could provide new therapeutic options besides the established combination of steroids and antibiotics in bacterial meningitis.

Additionally, treatment with eculizumab should be critically monitored considering the high risk for IMD in treated individuals. Alternative treatment options with antibodies directed against the alternative pathway or complement components C6-C9 should be considered instead of blocking C5 thus impairing both opsonophagocytic and bactericidal complement activity. (66, 245) With a

functional classical pathway and opsonization of bacteria, neutrophil activity could confer more protection in vaccinated individuals receiving complement-directed therapy.

5 Summary

The gram-negative diplococcus *Neisseria meningitidis (Nme)* is a frequent human-specific, commensal bacterium of the upper respiratory tract. Under certain conditions especially in infants, meningococci can translocate into the bloodstream and cause invasive meningococcal disease (IMD) manifesting as meningitis or sepsis or a combination of both. IMD is feared for its rapid progression and high fatality rate if it remains untreated. IMD affects up to one million people annually causing substantial morbidity and mortality worldwide. It is well-established that the complement system is an important protective factor in meningococcal disease through opsonization of bacteria with C3b and the lytic activity of the membrane attack complex although the inflammatory C5a/C5aR1 axis can aggravate IMD. The role of neutrophil granulocytes in meningococcal infection is less clear despite their abundant recruitment throughout the course of disease. This study aimed to characterize neutrophil responses to *Nme in vitro* and the influence of complement on these responses. In infection assays with whole blood and isolated PMNs, effective binding, internalization and killing of *Nme* by neutrophils was demonstrated. A significant complement-dependence of neutrophil phagocytosis and oxidative burst was observed. The opsonizing and lytic pathway of the complement cascade were found to be most relevant for these responses since blockade of C3 using inhibitor Compstatin Cp20 reduced phagocytosis and oxidative burst significantly more than the blockade of the inflammatory branch with C5aR1-antagonist PMX53. Opsonization with specific antibodies could not replicate the effect of complement activation indicating that engagement of neutrophil complement receptors, particularly complement receptor 3, is involved. Other neutrophil effector functions such as degranulation and IL-8 release were activated in a complement-independent manner implying activation by other inflammatory signals. Considering existing evidence on the overall protective effect of PMNs, further studies investigating the contribution of each neutrophil effector function to infection survival *in vivo* are required. Ideally, this should be studied in a murine meningitis or sepsis model in the context of complement activation.

Zusammenfassung

Der Gram-negative Diplokokkus *Neisseria meningitidis (Nme)* ist ein weit verbreitetes, human-spezifisches, kommensales Bakterium des oberen Respirationstraktes. Unter bestimmten Bedingungen, insbesondere bei Kleinkindern, können Meningokokken in die Blutbahn translozieren und eine invasive Meningokokkenerkrankung (IMD) auslösen, die sich als Meningitis, Sepsis oder eine Kombination beider Krankheitsbilder manifestiert. IMD werden aufgrund ihrer raschen Progression und ohne Behandlung hohen Letalität gefürchtet. IMD betreffen jährlich bis zu einer Million Menschen weltweit mit substanzieller Morbidität und Mortalität. Es ist bekannt, dass das Komplement-system bei IMD als protektiver Faktor durch die Opsonisierung von *Nme* mit C3b und Lyse mittels Membranangriffskomplex wirkt, obwohl die inflammatorische C5a/C5aR1-Achse die Erkrankung aggravieren kann. Die Rolle neutrophiler Granulozyten (PMNs) bei IMD bleibt hingegen kontrovers, obwohl eine starke Rekrutierung dieser Zellen stattfindet. Diese Studie charakterisiert die Reaktionen neutrophiler Granulozyten auf *Nme in vitro* und deren Modulation durch Komplement. In Infektionsassays mit Vollblut und isolierten PMNs konnte die Bindung, Internalisierung und Elimination von *Nme* durch PMNs demonstriert werden. Oxidativer burst und Phagozytose zeigten dabei eine ausgeprägte Abhängigkeit von Komplement. Die opsonisierende und lytische Wirkung von Komplement erwiesen sich dabei als relevant für diese Funktionen, da die Blockade von C3 durch den Inhibitor Compstatin Cp20 Phagozytose und oxidativen Burst signifikant stärker reduzierte als die Blockade der Inflammation durch C5a mittels C5aR1-Antagonist PMX53. Die Opsonisierung mit spezifischen Antikörpern konnte den Effekt der Komplementaktivierung nicht replizieren, was auf eine Signalvermittlung durch Komplementrezeptoren insbesondere CR3 hindeutet. Andere Effektorfunktionen wie Degranulation und IL-8 Freisetzung waren komplementunabhängig, was auf deren Initiierung durch andere inflammatorische Signale hindeutet. In Anbetracht bestehender Evidenz für einen insgesamt protektiven Effekt von PMN sind weitere Studien nötig, die den Effekt der einzelnen Effektorfunktionen auf das Outcome *in vivo* untersuchen. Besonders geeignet wäre dafür ein murines Sepsis oder Meningitis-Modell.

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7 Abbreviations

ASM	Acid sphingomyelinase
BRC	Baby rabbit complement
C3	Complement factor 3
C3b	Opsonizing fragment of C3
C5a	Anaphylatoxin and fragment of C5
C5aR1	C5a receptor 1
CD11b	Component of complement receptor 3 stored in neutrophil granules
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CFR	Case fatality rate
CR1-4	Complement receptors 1-4
DAPI	4’,6-diamidino-2-phenylindole (stains DNA)
DHR123	Dihydrorhodamine 123 (emits fluorence upon oxidization)
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FcγR	Antibody Fc receptor
FSC/SSC	Foreward/Sideward scatter (flow cytometry)
h.i.	Heat-inactivated
IL-8	Interleukin 8 (chemokine)
IMD	Invasive meningococcal disease
IMS	Immune serum
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAC	Membrane attack complex
NET	Neutrophil extracellular trap
NHS	Normal, non-immune human serum
Nlac	*Neisseria lactamica*
Nme	*Neisseria meningitidis*
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocyte
PMX53	Inhibitor of C5aR1
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
TCC	Terminal complement complex
TLR4	Toll-like receptor 4

8 Figures

9 Tables

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Journal of Medical Virology, January 2, 2021

Affidavit

I hereby confirm that my thesis entitled „ Differential influences of complement on neutrophil responses to *Neisseria meningitidis* infection“ is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of an other examination process neither in identical nor in similar form.

Würzburg, 28/03/21 _____

Sören Krüger

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Unterschiedliche Einflüsse von Komplement auf Reaktionen neutrophiler Granulozyten auf die Infektion mit *Neisseria meningitidis*" eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, den 28.03.2021 _____

Sören Krüger