Meningococcal sepsis: Beneficial and harmful inflammatory mechanisms

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HELSE SØR-ØST

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List of papers

Paper I

Hellerud B.C., J. Stenvik, T. Espevik, J.D. Lambris, T.E. Mollnes, and P. Brandtzæg. 2008. Stages of meningococcal sepsis simulated in vitro, with emphasis on complement and Toll-like receptor activation. *Infect Immun*.76: 4183-9.

Paper II

Hellerud B.C.,A. Aase, T.K. Herstad, L.M. Næss, L.H. Kristiansen, A.M. Trøseid, M. Harboe, K.T. Lappegård, P. Brandtzæg, E.A. Høiby, and T.E. Mollnes. 2010. Critical roles of complement and antibodies in host defense mechanisms against *Neisseria menigitidis* as revealed by human complement genetic deficiencies. *Infect Immun*.78: 802-9.

Paper III

Brekke O.L.,B.C Hellerud, D. Christiansen, H. Fure, A. Castellheim, E.W. Nielsen, A. Pharo, J.K. Lindstad, G. Bergseth, G. Leslie, J.D. Lambris, P. Brandtzæg, and T.E. Mollnes. Erythrocyte complement receptor 1 binds Gram-negative bacteria and protects against phagocytosis and oxidative burst in human whole blood. *Submitted.*

Paper IV

Nielsen E.W., B.C. Hellerud, E. B. Thorgersen, A. Castellheim, A. Pharo, J. Lindstad, T.I. Tønnessen, P. Brandtzæg, and T.E. Mollnes. 2009. A new dynamic porcine model of meningococcal shock. *Shock*.32: 302-9.

Paper V

Hellerud B.C., E.W. Nielsen, E.B Thorgersen, J.K. Lindstad, A. Pharo, T.I. Tønnessen, A. Castellheim, T.E. Mollnes, and P. Brandtzæg. 2010. Dissecting the effects of lipopolysaccharides from nonlipopolysaccharide molecules in experimental porcine meningococcal sepsis. *Crit Care Med*. 38: 1467-74.

Abbreviations

ATCC	American Type Culture Collection
C	Complement factor
CD	Cluster of Differentiation
CpG	"—C—phosphate—G—", i.e. Cytosine and Guanine separated by a phosphate
CR	Complement Receptor
CVP	Central Venous Pressure
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EU	Endotoxin Unit
FITC	Fluorescein Isothiocyanate
HBSS	Hank's Balanced Salt Solution
HEK	Human Embryonic Kidney
ICAM	Inter-cellular Adhesion Molecule
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus Kinase
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MAP	Mean Arterial Pressure
MBL	Mannose Binding Lectin
MD-2	Myeloid Derived Protein-2
MFI	Median Fluorescence Intensity
mg	Milligram
mL	Milliliter

MLST	Multilocus Sequence Typing
mm	Millimeter
mM	Millimolar
mmol	Millimol
MPAP	Mean Pulmonary Arterial Pressure
MyD88	Myeloid Differentiation Primary Response Gene (88)
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NmLPS+	Wild-type *Neisseria meningitidis*
NmLPS-	LPS deficient mutant *Neisseria meningitidis*
NOD2	Nucleotide-binding Oligomerization Domain containing 2
OPA	Opsonophagocytic Activity
PaOP	Pulmonary artery Occlusion Pressure
PAF	Platelet Activating Factor
PAI-1	Plasminogen Activator Inhibitor 1
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PVRI	Pulmonary Vascular Resistance Index
RA	Ringer Acetate
SBA	Serum Bactericidal Activity
SIRS	Systemic Inflammatory Response Syndrome
SVRI	Systemic Vascular Resistance Index
STAT	Signal Transducer and Activator of Transcription
TAT	Thrombin-antithrombin complex
TNF-α	Tumor Necrosis Factor α
TLR	Toll-Like Receptor
TRIF	TIR-domain-containing Adapter-inducing Interferon-β
VCAM	Vascular Cell Adhesion Molecule

VEGF   Vascular Endothelial Growth Factor

1   Introduction

1.1   Meningococcal disease

Neisseria meningitidis, as we know the bacterium today, is thought to be a fairly new microbial pathogen which developed only a couple of hundred years ago (1). The first known descriptions of meningococcal disease are from outbreaks in Switzerland in 1805 and Massachusetts in 1806 (2-4). The first description from Africa, where the largest epidemics of this disease still appear regularly, is from Nigeria in 1905 (5). Given the characteristic appearance of meningococcal disease with hemorrhagic rash, meningitis and rapid progression to death, sometimes in epidemic outbreaks, it is suggested that the lack of description in previous medical literature, being extensive for its period, actually means that the disease did not occur (1). It is likely that harmless Neisserial commensals, adapted to inhabit the nasopharynx in humans, became transformed into virulent bacteria due to uptake of DNA from other surrounding bacteria coding for virulence factors such as the ability to make a capsule (6). Unlike other Gram-negative bacteria, *N. meningitidis* is naturally competent for genetic transformation throughout its entire life cycle (7).

N. meningitidis was first observed in the cerebrospinal fluid during an epidemic of cerebrospinal fever in Italy by Marchiafava and Celli, published in 1884 (8). They described the visual resemblance to *N. gonorrhoae* in some of the patients due to massive intracellular accumulation of bacteria. *N. meningitidis* was first isolated from the cerebrospinal fluid by Anton Weichselbaum, a pathologist in Vienna, in March 1885, published in 1887. Weichselbaum named it *Diplococcus intracellularis meningitidis* (9).

The asymptomatic carrier state of *N. meningitidis* in the throat of humans was first described in 1896 (10). Meningococci are transmitted through close contact by droplets and colonizes the nasopharynx in about 10% of the overall healthy population with peak carriage rate reaching about 25% among late adolescents estimated by throat swabs (11,12), which in fact may underestimate the carriage rates (13). In closed communities, including military barracks, the carriage rate may reach 90-100% (1,12). Carriage is an immunizing event leading to protective immunity against the microbe (14).

Only occasionally does acquisition of *N. meningitidis* lead to meningococcal disease, usually within a few days after transmission (15). A number of host factors influence the risk of

acquiring invasive disease, of which lack of protective antibodies is the most important (1). Other factors include complement deficiencies, polymorphisms of Fcγ-receptor II (Fcγ-RIIa, CD32) and Fcγ-receptor III (Fcγ-RIIIb, CD16), other genetic polymorphisms affecting the immune response, exposure to cigarette smoke, preceding airway infections or climatic changes, i.e. dust and sand that damage the nasopharynx epithelium during the dry season in sub-Saharan Africa, influence the risk of acquiring invasive disease (1,15,16). In addition, the virulence of the carried strain is of great importance. Most of the meningococci carried in the nasopharynx are devoid of a capsule and have limited pathogenic potential (1). Meningococcal virulence is related to both capsule expression, expression of other surface structures like pili and the major outer membrane protein porin PorA, secretion of IgA proteases, sialylation of LPS and the underlying genotype (14-17).

There are so far 13 serogroups of *N. meningitidis* defined based on different capsular polysaccharide structures, but only six serogroups (A, B, C, W-135, X, and Y) cause most life-threatening disease (12,14,18). Strains of serogroups B and C cause the majority of infections in industrialized countries where pathogenic strains tend to cause single cases or small clusters of disease. Strains of serogroup A and, to a lesser extent, serogroup C dominate in third-world countries where they cause large outbreaks, particularly in the meningitis belt of sub-Saharan Africa (15,18,19). However, during the last 10-15 years approximately 1/3 of all cases in USA were caused by strains expressing serogroup Y which also has occurred with increased frequency in Sweden, Israel and South Africa (16,20,21). The last decade serogroup W-135 has caused large outbreaks in Saudi Arabia and Africa, and smaller outbreaks of serogroup X has also been seen in Africa (18).

Although *N. meningitidis* may cause localized infections like pneumonia (particularly serogroup Y) or sinusitis by spreading from the nasopharynx to adjacent epithelial structures, meningococcal disease is normally initiated by meningococci breaching the nasopharyngeal mucosa in susceptible people with subsequent development of bacteremia (14). The growth velocity in the circulation is the major determinant of the clinical presentation and outcome (15). The most common clinical presentation of meningococcal disease is distinct meningitis with headache, stiff neck, fever and sometimes petecchiae. Such patients have low numbers of bacteria ($<10^3$ meningococci/mL) and low amounts of LPS (<0.5 EU/mL) in their circulation, but high bacterial numbers and high amounts of LPS in the cerebrospinal fluid (22-25). They are normally admitted to hospital within 1-2 days after onset of the first symptoms and have low mortality if given antibiotics within due time (14). A minority

rapidly develops fulminant septic shock without signs of meningitis. These patients have extremely rapid proliferation of meningococci in their blood, typically reaching $10^5 - 10^8$ bacteria/mL and LPS concentrations in plasma >10 EU/mL within 12 hours after the first onset of symptoms. The mortality is high, varying from 20 to 80% in different studies, despite rapid admittance to hospital within few hours after onset of the disease and adequate treatment (19,22-25). No other invasive bacterial disease in humans progress more frequent to fulminant septic shock with multiorgan failure. Occasionally, patients develop distinct meningitis and persistent shock simultaneously (19). Up to 30% of patients admitted to hospital neither have distinct meningitis or fulminant septicemia, but normally present with fever and a rash. They have low bacterial load and level of LPS in the circulation and the subarachnoid space, but they may develop meningitis or shock if untreated (14). Transient meningococcemia also occurs, presenting with fever and an uncharacteristic "viral" rash. Meningococci are usually detected as an unexpected finding in blood culture, and the disease may be self limiting. Chronic meningococcemia appears as a rare condition that can last from week to months with intermittent fever, arthralgia and a non-specific maculopapular rash (14). Rarely, meningococcal infections can also result in pericardial infection, purulent or immune complex arthritis, cutaneous vasculitis (particularly in sub-Saharan Africa), conjunctivitis or panophtalmitis, and infections of the urogenital tract (14,15).

1.2 Meningococcal sepsis: A brief overview of inflammatory and pathophysiological mechanisms

The circulatory collapse associated with the fulminant course of meningococcal sepsis occurs due to capillary leakage, inappropriate vascular tone, intravascular microthrombi, and myocardial dysfunction (14,19). These changes are caused by an overwhelming inflammatory response in the host.

High cytokine concentrations correlate with the severity of shock. The first cytokine to be described as an important factor in the development of sepsis was TNF-α, described by Beutler et al. in 1985 to be a key factor in the development of septic shock in a murine model of endotoxemia (26). In 1987 Waage et al. reported for the first time that TNF-α concentrations in serum correlated with mortality in meningococcal disease (27). Similar patterns were subsequently reported for other pro-inflammatory cytokines, of which IL-1β

and IL-6 are regarded as the most important, and also for chemokines like IL-8, as well as for anti-inflammatory cytokines, of which IL-10 is regarded as the most important (19,28-31).

A key function of the pro-inflammatory cytokines is to enhance leukocyte migration out of blood vessels and promote diffusion of plasma proteins like antibodies and complement to a potential site of infection (32,33). These effects are mediated by activation of endothelial cells and circulating leukocytes with up-regulation of adhesion molecules like E-selectin, P-selectin, ICAM-1 and VCAM-1, increasing the endothelial permeability, and slowing the blood flow by vasodilatation which allows tethering of neutrophils to the vessel wall (32,33). Neutrophils being adherent to endothelial cells further increase the permeability by secreting a number of inflammatory molecules including a variety of proteases acting locally (33,34). Moreover, local ischemia occurs due to small vessel obstruction caused by an increased pro-coagulant state and further increases the capillary leakage (32,35). Increased systemic procoagulant activity results primarily from up-regulation of tissue factor on monocytes and monocyte derived microparticles, activating coagulation by the extrinsic, i.e. factor VIIa dependent pathway (36-38). The adhesion of activated platelets expressing P-selectin to activated endothelial cells also contributes to the forming of microthrombi in the tissues (38) . Concurrently, reduced antithrombotic capacity appears due to reduced levels and function of antithrombin and particularly protein C as well as down-regulation of the fibrinolytic system, caused by high levels of functionally active PAI-1 released into the circulation (14,38,39). Disseminated intravascular coagulation (DIC) ultimately develops. Thrombosis occurs particularly in the vessels of the skin, adrenals, kidneys, muscles, choroid plexus, peripheral extremities, and to some extent in the lungs (6).

The inflammatory plasma contact systems involving the complement system and the kallikrein-kinin system forming bradykinin are also activated and such activation may have additional inflammatory effects including contributing to decreased vascular tone and increased permeability (14,32,40-42). Activation of complement releases potent pro-inflammatory molecules like C5a which has been suggested to have disadvantageous effects in sepsis, given the massive release, by acting through its receptor C5aR being localized on myeloid and non-myeloid cells (10,43-45). Systemic complement activation in patients with meningococcal sepsis is associated with a poor outcome (40,43). Complement also constitutes an essential part of the defense system against meningococci (46). The two main anti-bacterial activities by complement are opsonization for phagocytosis by deposition of

C3b and C4b fragments on the membrane and lytic insertion of membrane attack complex (MAC, C5b-9). Of these, insertion of C5b-9 is recognized as the most important mechanism, whereby the role of opsonization has been less clear (47-49).

In general, the various inflammatory responses described in sepsis are beneficial to fight a localized infection. For example the migration of leukocytes and the accumulation of plasma defense proteins like antibodies and complement to a site of localized infection is obviously essential to clear the pathogens. Likewise, the formation of localized blood clots can be beneficial in preventing the pathogens to spread. However, when these responses progress to affect the whole organism, inducing the systemic inflammatory response syndrome (SIRS), they easily come out of control with devastating effects.

It is becoming increasingly evident that SIRS is a result of multiple inflammatory factors acting in concert. Despite promising results from animal models of sepsis (50,51), so far no convincing therapeutic effect has been obtained by blocking individual cytokines, although there are indications that some effect may be obtained by treating selected subgroups of patients with inhibition of TNF-α (52-55). Also, clinical trials blocking other mediators in sepsis including PAF, bradykinin, prostaglandins and antithrombotic therapy by using antithrombin or a tissue factor pathway inhibitor has shown no benefit (56-61). However, in 2001 antithrombotic therapy by the use of activated protein C was reported to be beneficial in a clinical trial (62). Controversy still exists with respect to the validity of the results, but the agent has been accepted for treatment of adults with severe sepsis. Thus, with few exceptions the results of targeting specific mediators in sepsis are disappointing. Consequently, focusing on a broad range of inflammatory responses may be a more tempting approach, and a multimodal therapy targeting different pathophysiologic pathways with possible additive or synergistic effects may be more helpful than one "magic bullet" (52,63,64).

1.3 The role of LPS and other components of *N. meningitidis* in septic inflammation

LPS is a major constituent of the outer membrane of *N. meningitidis* (Figure 1). It has long been regarded to be the principal molecule inducing inflammation by Gram-negative bacteria (65). Richard Pfeiffer, a German bacteriologist, was in 1892 the first to describe the inflammatory effect of endotoxin (46). He detected that *Vibrio cholerae* contained two different toxic principles; one that caused diarrhea and could be inactivated by heating, i.e. the cholera exotoxin. The second principle was resistant to heating and caused a toxic-shock reaction in guinea pigs. It was thought to reside inside the bacterium and thus named endotoxin. Netter and Debré stated in 1911 that endotoxin represented the main toxic principle of meningococci (66).
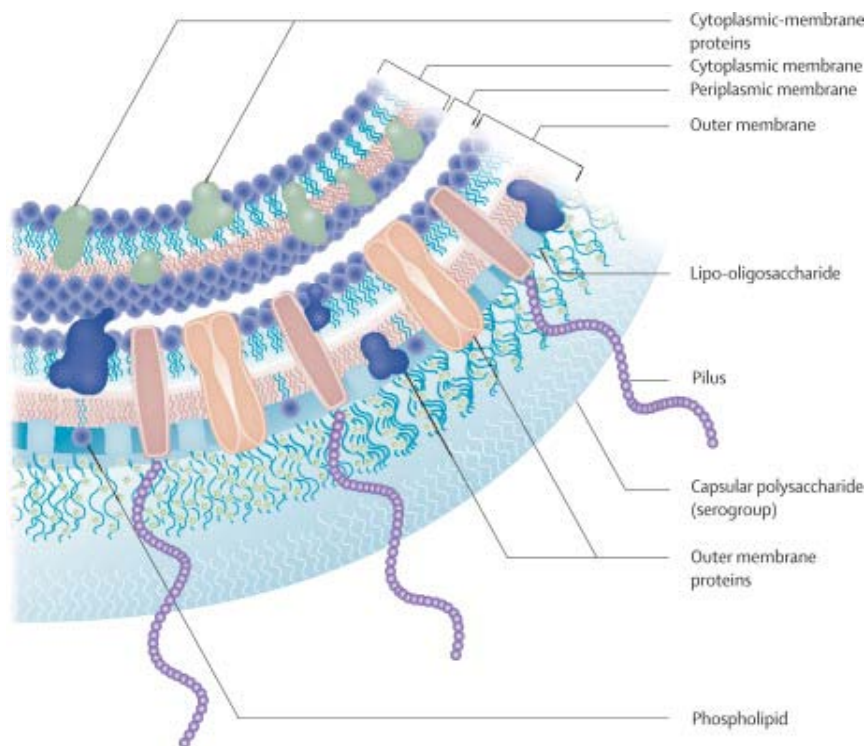
Fig. 1: **The membrane structures of *N. meningitidis*** (Stephens D.S., B. Greenwood, and P. Brandtzæg. 2007. *Lancet*. 369: 2196-2210, with permission from Elsevier)

Purification and characterization of endotoxin from lysates of Gram-negative bacteria required substantial efforts from several researchers and the term LPS was applied by Otto Lüderitz and Otto Westphal due to the presence of polysaccharide and lipid components (67). *N. meningitidis* LPS consists of lipid A, a core structure containing two 2-keto-3-deoxy-octulosonic acid (KDO) and two heptoses (L-glycero-D-manno-heptopyranoside) substituted with variable short polysaccharide α and β side chains (68,69) (Figure 2). The complete biosynthesis pathway of *N. meningitidis* LPS has been described (69). While the biological activity of enteric LPS resides in the lipid A structure alone, maximum biological activity of *N. meningitidis* LPS requires the two KDO components linked to lipid A (70). The numbers of acyl chains attached to lipid A and their pattern of attachment are important determinants of the biological activity of LPS (69,71).The predominant and biologically most active form of *N. meningitidis* LPS is symmetrically hexa-acylated (71,72). However, invasive strains of *N. meningitidis* being penta-acetylated due to mutations of lpxL1 or lpxL2 genes required for addition of secondary acyl chains have also been reported to occur being associated with less systemic inflammation and reduced activation of the coagulant system (73).
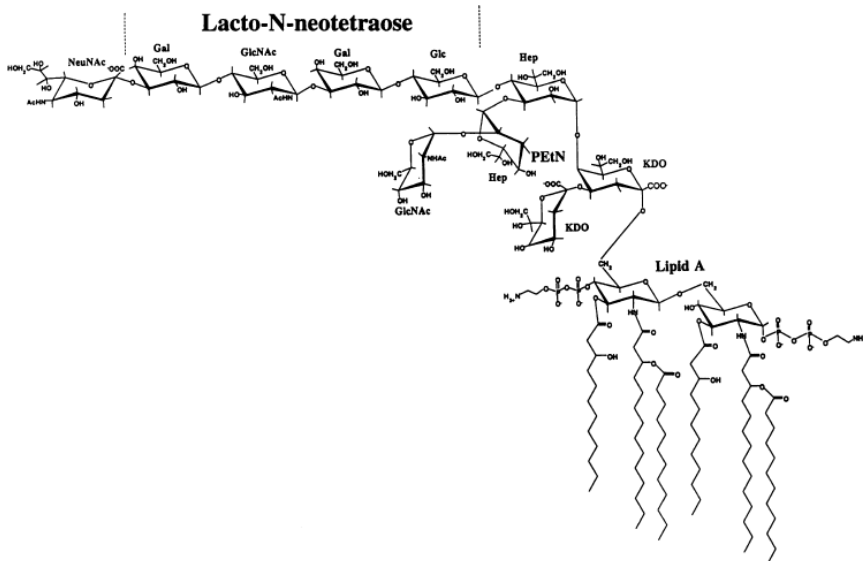
Fig. 2: **The structure of *N. meningitidis* lipopolysaccharide (LPS)** (Diaz Romero J., and I.M. Outschoorn. 1994. *Clin. Microbiol. Rev.* 7: 559-575, with permission from the American Society of Microbiology)

The lipid A structure of *E. coli* is, in contrast, asymmetrically hexa-acylated with different length of the acyl groups (72). Also in contrast to enteric LPS, *N. meningitidis* LPS has short polysaccharide chains and lack repeating O antigen units and, thus, is sometimes referred to as lipo-oligosaccharide (LOS) (68). The LOS structure is common among other mucosal pathogens, including *Bordetella pertussis, Campylobacter jejuni* and *Haemophilus* species (70). Differences in the oligosaccharide structure of LPS forms the basis of immunotyping *N. meningitidis* (L1-L12) (17). Most strains express more than one immunotype-specific epitope on their LPS (17).

The levels of LPS in blood and cerebrospinal fluid of patients infected with *N. meningitidis* are closely associated with the clinical presentation and outcome (6,68,74,75). So far, this is the only Gram-negative infection where a dose response relationship has been thoroughly documented by measurement of LPS in different human body fluids (6,68). *In vitro* examination of meningococcal shock plasma in a monocyte target assay support the role of LPS as a major activator of monocytes (75), and studies using cell lines and whole blood models challenged with wild type meningococci and an LPS-deficient mutant of *N. meningitidis* document the potency of LPS as an activator of the innate immune responses (76-78).

The first protein specifically binding LPS was described as LPS-binding protein (LBP) in 1986 (79). Concomitantly, in 1990 CD14 was also shown to be essential for LPS sensing (80). The discovery of the transmembrane signaling pattern recognition receptors TLRs in the late 1990's, as proposed by Charles Janeway Jr in 1989 (81,82), implied a new era in our understanding of the sensing of microbial structures like LPS by the innate immunity. The first TLR described to be activated by microbial structures was TLR2, in 1998 reported to be the receptor of LPS (83,84). A couple of years later it was shown that these results were due to contamination of the LPS preparations with lipoproteins (85). At the same time mutations in the TLR4 gene of mice were reported to be responsible for defective LPS signaling (86,87). Short time later MD-2 being in complex with TLR4 was shown to be a prerequisite for LPS-mediated signaling (88). Activation of TLR4 induces the MyD88 dependent intracellular signaling pathway being common for all TLRs except TLR3, ultimately activating the transcriptional factor NF-κB (early phase) leading to increased transcription of a range of inflammatory molecules (89). Additionally, TLR4 also activates the TRIF intracellular pathway which is used only by TLR3 and TLR4, leading to production of

β-interferon in addition to activation of late phase NF- κB (89). LPS from different Gram-negative bacteria activate the MyD88 dependent and the TRIF intracellular pathways to various extents. *N. meningitidis* LPS is a potent inducer of both pathways in monocytes (90,91). In contrast, *Salmonella* LPS predominantly activates the TRIF intracellular pathway while *E. coli* (55:B5) and *V. cholerae* LPS predominantly activate the MyD88 dependent pathway (90). Stimulation of the TRIF intracellular pathway with synthesis of β-interferon and subsequently activation of the JAK/STAT signaling pathway seems to be responsible for activation of a large group consisting of more than 2000 genes induced by *N. meningitidis* in monocytes being particular LPS-sensitive according to micro array studies (91)*.*

Although LPS has been shown to be the most potent inflammatory molecule of *N. meningitidis*, non-LPS bacterial compounds (Figure 1) have also been shown to have the capacity to induce pro-inflammatory cytokines, and complement activation by meningococci occurs independently of LPS (76-78,92,93). Also the cell adhesion molecule E-selectin has been shown to be up-regulated independently of LPS at high bacterial concentrations, while the adhesion molecules ICAM and VCAM were shown to be up-regulated strictly dependent on LPS (94). TLR2, primarily associated with ligands from Gram-positive bacteria (95,96), was in 2000 the first receptor described to mediate inflammatory signaling also by meningococcal structures other than LPS (78). Of these, the interaction between outer membrane protein porin B and TLR2 has been described in most detail (97-99). Also peptidoglycan, constituting a thin layer between the outer membrane and the cytosolic membrane of Gram-negative bacteria and shed during bacterial growth, has been demonstrated to be a ligand of TLR2, as well as intracellular NOD2 receptors (84,95,100,101). The effects of such activation seem to be additive to TLR4 mediated activation (97). Unmetylated CpG sequences in DNA molecules, being more abundant in bacteria than in vertebrates, activate innate immunity by being a TLR9 ligand (102,103). Also, native IgA1 protease, a putative virulence factor of *N. meningitidis,* has been shown to be a potent stimulus for the secretion of pro-inflammatory cytokines by monocytes, although the mechanism has not been fully established (104). However, in general it appears from experiments in various *in vitro* systems that the potency of non-LPS structures of meningococci to induce inflammation is much weaker than LPS but until now this has been scarcely tested *in vivo* (76,78).

1.4   The viable *N. meningitidis* devoid of LPS as a valuable research tool

An LPS-free mutant of *N. meningitidis*, described in 1998, was constructed by the research group of Peter van der Ley by insertional inactivation of the enzyme LpxA which is responsible for the first committed step in the lipid A biosynthesis (105). In fact, the wild-type parent reference strain 44/76 which was transformed to the LPS deficient mutant 44/76*lpxA-* was originally isolated from a woman admitted to Ullevål Hospital (presently Oslo University Hospital) in the late 1970ies (106). *N. meningitidis* is the only known Gram-negative bacterium being viable without LPS in the membrane. This mutant bacterium has paved the way for more detailed studies of the specific role of LPS versus other bacterial compounds in the inflammatory response, and has been essential for several of the studies referred to above concerning LPS-specificity of the inflammatory response elicited by *N. meningitidis*.

1.5   Experimental meningococcal sepsis: Animal models

Humans are the only natural hosts for *N. meningitidis*. This is partly due to the specificity of surface proteins of meningococci in their interaction with surface proteins of human cells, including the interaction of pili with human CD46 and Opa and Opc with human CEACAM1 (CD66) (107). Such interactions are crucial for the colonization of nasopharyngeal mucosa and invasion of the host. Moreover, neisserial iron uptake systems bind only human iron transport proteins, such as transferrin and lactoferrin (108). Thus, meningococci can only proliferate in human blood. Still, animal models for meningococcal disease have a long history. Early attempts used monkeys, rabbits and guinea pigs but the first useful model was developed in the 1930s where mice were infected by the intraperitoneal route. Coadministration of mucin, which has later been shown to serve as an exogenous source of iron available for the meningococci, was necessary for progression to meningococcal disease (109). Variants of the mouse model have in the passing years found some use in meningococcal research. More recently a transgenic mouse has been developed expressing human CD46. These mice can develop meningococcal sepsis and meningitis by nasal administration of meningococci (109). In 1999 Hazelzet et al. published a live porcine model

and documented the potent biological effects of LPS-containing outer membrane vesicles when infused as a bolus dose (110).

2 Aims of the present studies

I. To examine the involvement of TLRs and complement in inflammation induced by escalating doses of *N. meningitidis* stimulating whole blood and transfected cell lines

II. To examine the effects of congenital deficiencies of complement factors C2 and C5 on phagocytosis and killing of *N. meningitidis* in whole blood and serum models

III. To examine the effect of opsonization by complement on the binding of Gram-negative bacteria to erythrocytes by complement receptor CR1, and the effect of such binding and the formation of C5a on phagocytosis in whole blood

IV. To develop a new large animal model challenged with exponentially increasing doses of heat-killed *N. meningitidis* simulating fulminant meningococcal septicemia in man

V. To study the effects of LPS in heat-killed wild type *N. meningitidis* (44/76) versus other inflammation inducing molecules of meningococci using the isogenic knock out mutant 44/76lpxA- completely lacking LPS in the new porcine shock model

The general aim of this thesis was to investigate key elements in the interaction between the meningococcus and the immune system. Meningococcal sepsis represents a prototypical example of how the immune response elicited by a pathogen is crucial to protect against disease on the one hand, but can be detrimental for the host on the other hand (111). A profound understanding of either of these dual aspects is of fundamental importance to

understand the nature of meningococcal disease. Also, although meningococcal sepsis distinguish itself from most other septic conditions by the exceptionally rapid growth of bacteria, this disease can be used as a model to gain novel insight into common principles of how pathogens trigger inflammation, and the impact of the inflammatory responses on the host as well as the pathogen. The studies in this thesis were performed *in vitro* to investigate the inflammatory response and defense mechanisms against *N. meningitidis* at a molecular level, while the *in vivo* studies were performed to investigate pathophysiological mechanisms and the inflammatory response of the whole organism in meningococcal disease.

Ad study I

In this paper we first focused on the inflammatory response to *N. meningitidis* as regards activation of the innate immune system. TLR-mediated cell activation and activation of complement represent two main branches of innate immunity (111). Inflammation induced by activation of these two systems after exposure to meningococci has only been investigated separately in previous studies. However, there is substantial cross-talk between the two branches (111). Thus, we were interested in the relative role they play and how they interact. We aimed to simulate the different stages of meningococcal sepsis *in vitro*, with the numbers of meningococci present ranging from the low numbers typically found in low-grade meningococcemia with subsequent development of meningitis to the high numbers typically found in fulminant sepsis (23-25). The studies were therefore performed with different numbers of meningococci added, paralleling the numbers found in patients with various clinical manifestations of meningococcal disease. We also intended to compare the relative role of the two branches in the presence and absence of LPS to gain more information about how this important inflammatory molecule specifically influences the innate immune responses.

Ad study II

We then focused on the role of complement in the defense against systemic meningococcal disease taking advantage of whole blood and serum from human beings genetically completely deficient of C2 and C5, respectively. The C5 deficient donor and its control individual were also deficient of MBL. The donors differed in their respective titer of anti-meningococcal antibodies, and thus we also focused on the specific role of antibodies. Deficiencies of the complement system in the alternative pathway, C3 and the terminal

pathway are predominantly associated with increased susceptibility to meningococcal disease (112,113). Acquisition of serum bactericidal antibodies correlates with protection (114,115). Opsonophagocytosis of meningococci opsonized with C3 and C4 split products may also be important in the defense against meningococcal disease, but the relative role of these two complement mediated defense mechanisms in the presence of various amounts of anti-meningococcal antibodies is not fully clarified (47-49). First, we studied to what extent meningococci can survive and proliferate in whole blood from the respective complement deficient donors and controls. Subsequently, we studied how serum bactericidal activity and opsonophagocytosis each was influenced by the respective defects of complement in the presence of high and low titers of anti-meningococcal antibodies.

Ad study III

The immune adherence phenomenon was originally described in 1953 by Nelson as binding of Gram-positive bacteria opsonized with C3 and C4 split products to CR1 on erythrocytes (116). Other particles like complement opsonized virus and immune complexes can also adhere to erythrocytes by binding to CR1 (117), and such binding can probably be an important mechanism for clearing of hazardous agents from the circulation by shuttling them to macrophages in the liver and spleen for safe deposition (116-118). Binding of Gram-negative bacteria to erythrocyte CR1 has not been demonstrated before. Also, the influence of such binding on phagocytosis by circulating leukocytes has not been investigated previously. Thus, we performed experiments to demonstrate binding of *Eschericia coli* and *N. meningitidis* to human erythrocyte CR1 and how binding to CR1 affects the phagocytosis of these bacteria by granulocytes and monocytes.

Ad study IV

We established a large animal model of meningococcal sepsis to elucidate inflammatory responses to *N. meningitidis* and how these responses result in the clinical state of meningococcal sepsis with development of circulatory failure and subsequently organ dysfunction. Such a model could also be useful for further more detailed studies of specific inflammatory and pathophysiologic aspects of meningococcal sepsis, as well as to investigate new potential therapeutic principles. For this purpose a porcine model simulating the fast growth of bacteria in the circulation was established.

<u>Ad study V</u>

We investigated the specific role of LPS and non-LPS structures of *N. meningitidis* in triggering inflammation and associated pathophysiological changes *in vivo*. The effect of administrating wild-type and the LPS-deficient mutant *N. meningitidis* in the previously established porcine model of meningococcal sepsis was compared. A previous study of mortality in mice demonstrated that high numbers of the LPS-deficient mutant meningococci, i.e. about 2 $\log_{10}$ higher numbers than the wild-type strain, were able to kill mice (76). Apart from this study, not other more detailed studies have previously been performed with the LPS-deficient mutant *N. meningitidis in vivo*.

3 Materials and Methods

3.1 Bacteria and LPS

In all papers in this thesis the international reference strain *N. meningitidis* 44/76 (also denoted H44/76 and herein called NmLPS+) isolated from a patient with meningococcal sepsis was used (106). The bacterium is phenotypically characterized as B:15:P1.7,16:L3,7,9. This characterization is based on differences in the polysaccharide capsule (serogroup B), the outer membrane protein por B (serosubtype 15), the outer membrane protein por A (serosubtype P1.7,16) and the oligosaccharide structure of LPS (immunotype L3,7,9). The strain belongs to the MLST 32/ET-5 clone, based on genotypical classification.

In paper I *N. meningitidis* 151/85 (strain collection, National Institute of Public Health, Oslo, Norway, called NmC in this thesis) was also used. This bacterium is characterized as C:2a:P1.2:L3,9. It is a representative strain of the MLST 11/ET-37 clone and was isolated from a six month old boy who died from fulminant meningococcal septicaemia in 1985. Pathogenic meningococci belonging to the MLST 32/ET-5 and MLST 11/ET-37 clones have been isolated all over the world.

The mutant *N. meningitidis* 44/76*lpxA-* (also denoted H44/76*lpxA-* and herein called NmLPS-) was used in paper I, III and V (105). The expression level of the integral outer membrane proteins by the LPS-deficient mutant is slightly higher than that of the wild-type strain. The outer membrane phospholipid composition is altered, with a switch to mostly short-chain, saturated fatty acids (119).

The meningococci were grown by the National Institute of Public Health (Oslo, Norway) on Colombia-agar and resuspended in sterile PBS after overnight growth. The bacteria used in paper I, III, IV and V were heat inactivated at 56°C for 30 minutes and then frozen at -70°C until used. The membrane structures of heat killed *N. meningitidis* are as biological active as those of viable meningococci of the same strain (102,120). However, such treatment of the bacteria reduces TLR9 signaling by CpG DNA (102). In paper II viable meningococci were used.

In paper III experiments were also performed with *E. coli* strain LE392 from ATCC (Manassas, VA; ATCC Number 33572). *E. coli* were grown by the research laboratory at Nordlandssykehuset, Bodø. The bacteria were first grown overnight on a Lactose dish, and then transferred to LB-medium (Becton Dickinson, Franklin Lakes, NJ) and grown overnight. They were then resuspended and washed once with PBS, heat inactivated for 1 hour at 60°C and stored at -80°C. After thawing the bacteria were washed six times with PBS to remove extracellular LPS. Bacteria intended for Alexa-staining were removed and the rest were washed additional three times. Bacteria were counted in Truecount tubes (Becton-Dickinson) using flow cytometry after staining with SytoBC (Invitrogen Molecular Probes Carlsbad, CA).

In the phagocytosis assay in paper III, Alexa 488 labeled *E. coli* and *N. meningitidis* were used. NaHCO$_3$ and heat inactivated bacteria were added together with Alexa FLUOR® 488 carboxylic acid and succinimidyl ester (10 mg/mL) in DMSO (Invitrogen). The tube was packed in tinfoil and rotated for one hour before the bacteria were washed three times, resuspended in PBS and counted as described above.

The purified LPS used in paper I was extracted from 44/76 by the phenol extraction method (121).

3.2 Analyses

3.2.1 Quantification of bacterial concentrations

DNA was isolated with robotized equipment (BioRobot 48; Qiagen Inc, Valencia, CA) based on absorbance to magnetic silicia particles (MagAttract DNA Blood M96 kit 951436; Qiagen). Genome quantification of *N. meningitidis* was performed by quantitative real-time PCR (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany) based on detection of the meningococcal capsular transfer gene (*ctrA*) (23,122). Lower detection limit of the assay was 1×10^3/mL. In paper II the numbers of live *N. meningitidis* in whole blood were also determined by plating ten-fold dilutions of whole blood, and counting plates with 25-250 colony forming units (CFUs) after 24 hours growth.

3.2.2 Quantification of LPS

LPS concentrations were determined by the Pyrochrome® *Limulus* Amoebocyte Lysate (LAL) assay (Associates of Cape Cod, East Falmouth, MA), an endpoint chromogenic method using a diazo-coupling assay kit. The samples were diluted in depyrogenated Pyrotube-D tubes with LAL Reagent water. The diluted samples were heat-treated at 75°C for 10 minutes, mixed with Pyrochrome dissolved in a Glucashield β-glucan inhibiting buffer, and incubated in a 96-well Pyroplate on a dry block incubator. After incubation, the procedure was followed according to the instructions from the manufacturer. Lower detection limit of the assay was 3.13 EU/mL.

3.2.3 Complement activation

TCC was measured in EDTA plasma by ELISA (123). The assay is based on a monoclonal antibody (aE11) recognizing a neoepitope exposed in C9 after it has been inserted in the TCC. Values are given in arbitrary units (AU) defined by a serum standard activated with zymosan and defined to contain 1000 AU/mL.

3.2.4   Quantification of inflammatory mediators

In paper I TNF-α, IL-1β, IL-6 and IL-8 were measured in EDTA plasma on a Bio-Plex Array Reader (LUMINEX 100, Bio-Rad Laboratories, Hercules, CA) using a Bio-Plex Human Cytokine panel.

In paper IV and V TNF-α, IL-1β, IL-6, IL-8 and IL-12 were analyzed in EDTA plasma with porcine ELISA kits (R&D Systems, Minneapolis, MN). IL-10 was analyzed in EDTA plasma with BioSource Swine Immunoassay Kit (Invitrogen, Carlsbad, CA). VEGF was analyzed in EDTA plasma by a R&D Quantikine Human Immunoassay kit (R&D Systems, Minneapolis, MN), known to cross-react with porcine VEGF (124).

3.2.5 Transient transfection and luciferase assay

In paper I Human embryonic kidney (HEK) 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (Euroclone, Milano, Italy), L-glutamine and 10 μg/mL ciprofloxacin (Cellgro, Manassas, VA) at 37°C and 8% CO_2. Transient transfection was done using GeneJuice^{TM} transfection reagent (Novagen, Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. In short, cells were plated at a cell density of $1x10^4$ cells/well in 96-well dishes and grown to 50% confluency. Plasmids used were the NF-κB dependent luciferase reporter plasmid pELAM-luc (125), human CD14 in pcDNA3 kindly provided by Dr. D. Golenbock (University of Massachusetts Medical School), human MD-2 in pEFBOS kindly provided by Dr. K. Miyake (University of Tokyo), human TLR4 in pcDNA3 kindly provided by Drs. R. Medzhitov and C. Janeway (Yale University, New Haven, CT), and human TLR2 in pRK7 kindly provided by Dr. C. Kirschning (Technical University of Munich). Each plasmid was transfected at a dosage of 25 ng/well and pcDNA3 (Invitrogen) was used to adjust the total amount of plasmid to 100 ng/well. All plasmids were isolated using the EndoFree plasmid kit (Qiagen Inc., Valencia, CA). The cells were incubated for 24 hours before the experiments were performed as described later. HEK293 cells stably transfected with TLR9 were provided by Eisai Pharmaceuticals (Andover, MA). The positive controls were stimulated with 10μM phospothioate CpG DNA 2006 (Tib Molbiol, Berlin, Germany). Cytoplasmic extracts were prepared and luciferase activity was measured using the Luciferase Assay System kit according to the manufacturer's recommendations (Promega, Madison, WI) and Victor^{3TM} 1420 multilabel counter (PerkinElmer, Waltham, MA). Results from triplicate wells (duplicate for the TLR9 transfected cells) are given as fold induction relative to PBS treated negative control.

3.2.6 Opsonophagocytic activity (OPA) and oxidative burst

In paper II OPA was measured as oxidative burst in granulocytes using live meningococci (126). The bacteria were grown on Colombia horse blood agar for about 18 hours at 37°C in 5% CO_2 before suspension in HBSS (pH 7.2) with 0.1% bovine serum albumin. Two-fold dilution series of the various serum samples were mixed with meningococci and incubated for 30 minutes at 37°C under agitation. In some of the experiments the bacteria were first incubated with heat inactivated serum, followed by 10 minutes incubation with a separate

complement source. Granulocytes from heparinized whole blood where the red cells were removed by ammonium chloride lysis were primed with dihydrorhodamine 123 (Invitrogen) and finally added before incubation continued for another 10 minutes. The results were measured by a CyFlow® ML flow cytometer (Partec GmbH, Münster, Germany), looking for fluorescence within the granulocyte population. The percentage oxidative burst positive granulocytes were recorded and OPA titers were expressed as the reciprocal of the final serum dilution giving oxidative burst in ≥50% of the granulocytes.

In paper III phagocytosis by granulocytes and monocytes was measured as internalization of the bacteria using the Phago test kit (Orpegen Pharma, Heidelberg, Germany). Alexa-stained bacteria were incubated in lepirudin anticoagulated whole blood for 10 minutes and the blood was then processed according to kit instructions. Quenching solution was used to avoid interference of surface-bound bacteria (127). In these experiments phagocytosis was expressed as median fluorescence intensity (MFI) of the whole granulocyte or monocyte population. Samples were analyzed on a LSRII flowcytometer (Becton Dickinson). Oxidative burst was measured separately using the Burst test kit (Orpegen Pharma). Whole blood samples were incubated with bacteria for 10 minutes before dihydrorhodamine 123 was added and incubation continued for another 10 minutes. The samples were then lysed and washed according to kit instructions and oxidative burst in granulocytes and monocytes was measured by flow cytometry. Results were expressed as MFI of the whole gated cell population.

3.2.7 CD11b expression

In paper II the expression of CD11b on granulocytes was measured by the CyFlow® ML flow cytometer (Partec GmbH) by using a mouse anti-human CD11b antibody (Serotec, Dusseldorf, Germany).

3.2.8 Serum bactericidal activity (SBA)

SBA was measured in paper II using meningococci grown overnight at 37°C in 5% CO_2. The overnight growth was plated onto Colombia horse blood agar plates and incubated for four hours to reach log phase before suspension in HBSS (pH 7.2) with 0.1% bovine serum

albumin (128). Then, 25% human serum was added as an exogenous source of human complement. Heat-inactivated sera known to contain specific antibodies against the target strain were diluted two-fold in microtiter plates (starting at a serum dilution of 1:2) and incubated for 30, 60, and 90 min at 37°C in air with bacteria and complement. After plating onto agar plates and incubation overnight at 37°C, CFU were counted with Sorcerer colony counter (Perceptive Instruments, Suffolk, United Kingdom), and SBA antibody titers were expressed as the reciprocal of the final serum dilution giving ≥50% killing of inoculum compared to controls.

3.2.9 Flow cytometry of erythrocytes carrying bacteria and free bacteria in plasma

In paper III the proportion of bacteria being adherent to erythrocytes and free in plasma was measured in whole blood incubated with Alexa-stained *E. coli* or *N. meningitidis.* The bacteria were fixed with 0.25% (v/v) PFA for four minutes at 37°C. Samples were then diluted 1:320 with PBS in Truecount tubes to avoid coincidences with erythrocytes not carrying bacteria. Whole blood added 10 mM EDTA served as a control for coincidences. Samples were run on a FACSCalibur or LSRII flow cytometer (Becton Dickinson). Gates were set around the beads, the erythrocytes and the free bacteria and calculations were made. The erythrocyte population was verified in control experiments using anti-Glycophorin A (Dako, Glostrup, Denmark). To verify that the bacteria/erythrocyte-conjugates did not appear because of PFA we performed control experiments without PFA and similar results were obtained.

3.2.10   Flow cytometry of bacterial opsonization

In paper III C1q, C3 and C4 opsonization of *E. coli* and *N. meningitidis* was analyzed by flow cytometry using rabbit anti-human C1q, FITC-conjugated rabbit anti-human C3c (F0201) and rabbit anti-human C4c mAbs, respectively. FITC-conjugated rabbit anti-mouse Ig was used as control. All antibodies were from Dako. Results are expressed as MFI.

3.2.11 Microscopy

In paper III smears of whole blood incubated with Alexa-stained *E. coli* and *N. meningitidis* was examined using either the transmitted light observation microscopy or the reflected fluorescence procedure using appropriate filter for Alexa. Combined reflected fluorescence and transmitted light was performed according to the microscope instructions using very weak transmitted light intensity. The Olympus BX51TRF microscope (Olympus, Tokyo, Japan) was equipped with a ColorView IIIu digital camera with 5 megapixel resolution and was controlled by the CellP program (Soft imaging system, Münster, Germany).

Immunofluorescence histology was performed on cryosections obtained from the lung, liver and spleen of the pigs given Alexa-stained *E. coli*. Cryosections (5 µm thick) were cut from tissue embedded and snap-frozen in O.C.T Compound (Tissue-Tek; BDH, Lutterworth, UK). Sections were air dried and fixed for 10 minutes in ice-cold acetone. Fc receptors were blocked by incubating the sections for 30 minutes with PBS containing 5% pig serum and 5% goat serum. To identify tissue macrophages, a pretitrated anti-porcine CD45 monoclonal antibody (a kind gift from Karin Haverson, University of Bristol, Bristol, UK) was applied and incubated for two hours. Slides were washed thoroughly three times with PBS for five minutes. An isotype-specific goat anti-mouse antiserum (Southern Biotechnology, Birmingham, AL) conjugated to Texas Red was then applied and incubated for one hour. The slides were washed three more times, and the nuclear dye DAPI was applied and incubated for 10 minutes. After a final wash, the sections were mounted in Fluoromount (Vector Laboratories, Burlingame, CA) and sealed with nail varnish. Stained slides were examined using a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) fitted with a combined excitation and emission filter block specific for the applied fluorescence staining.

3.2.12 Hematological parameters

In paper IV and V hematological parameters were analyzed by the hospital's routine hematology laboratory (CELL-DYN 4000 flowcytometer, Abbot Diagnostics, IL). Neutrophil granulocytes were fully discriminated, but monocytes and lymphocytes merged as one cell population designated mononuclear leukocytes.

3.2.13   Hemostatic parameters

In paper IV and V thrombin activation was measured in citrate plasma as thrombin-antithrombin complex (TAT) by a human ELISA kit known to cross-react with porcine TAT (Dade Behring, Marburg, Germany) (129). Plasminogen activator inhibitor 1 (PAI-1) was measured by a porcine PAI-1 activity ELISA (Molecular Innovations, Novi, MI).

3.2.14   Capillary leakage

In paper IV and V the amount of protein leakage out of the intravascular compartment was estimated by calculating the relative changes of albumin content in plasma from the starting point of the experiment. Relative changes of plasma volume (Δplasma) at each time point (Tx) compared to the starting point (T0) of the experiment were accounted for by the use of hematocrit (hct): (Δplasma) = $\{(100 - hctTx) - (100 - hctT0)\}we/(100-hctT0)$ (130). Relative changes in albumin content from T0(%) = $[\{AlbuminTx + (AlbuminTx \times \Delta plasma) - AlbuminT0\}/AlbuminT0] \times 100$. The amount of fluid accumulation in the tissues was estimated by wet/dry ratio. A muscle biopsy was taken at 0 and 240 minutes, and biopsies from both lungs and the jejunum were taken at 240 minutes. All biopsies were placed in pre-weighed containers. The biopsies were then weighed and dried at 70° C until stable weight before the wet/dry ratio was calculated.

3.3   Experimental models used in the thesis

3.3.1 Complement activation in serum

In paper I complement activation in serum by NmLPS+ at increasing concentrations was examined. Based on preliminary experiments with 10-fold increasing concentrations, the concentration of NmLPS+ was increased two-fold from 1×10^6 to 5×10^8 bacteria/mL. Sera from three of the donors were activated simultaneously with NmLPS- at similar concentrations and conditions. Additional experiments were also performed with NmC at similar concentrations in sera from three of the donors. Serum was activated by the bacteria for 30 minutes at 37°C before further activation was stopped by placing the samples on ice and adding EDTA. The serum was then frozen at $- 70^{o}$C until analysis for complement activation by soluble terminal C5b-9 complement complex (TCC). TCC was chosen as

readout because it reflects overall complement activation well and we possess a well established kit for this analysis. ELISA analysis was also performed with the C3 split product C3bc in some of the samples. The results were similar to TCC but the background concentrations were higher. Thus, only TCC was used in the paper.

3.3.2 Inflammatory responses in whole blood

In paper I cytokines and complement activation were measured in lepirudin anticoagulated freshly drawn whole blood from healthy donors incubated with NmLPS+, NmLPS- and NmC at 10-fold increasing concentrations from $1x10^3$ to $1x10^8$ bacteria/mL. Lepirudin was used as anticoagulant as this substance inhibits thrombin specifically, and thus all biological systems apart from the final coagulation step are left functionally intact (127). The experiments were performed without inhibition, with a CD14 blocking antibody (ATCC Number HB-247) and an isotype mAb, and with complement inhibition by compstatin or a C5aR peptide antagonist (131). The peptide compstatin blocks complement activation by binding to C3 and thus, inhibit cleavage of this key complement factor (132). CD14 and complement was blocked separately and simultaneously. The number of donors included was eight for NmLPS+ at $10^5 – 10^7$ bacteria/mL, five for other concentrations of NmLPS+ as well as NmLPS- and three for NmC. After 2 hours incubation with bacteria at 37^oC the samples were placed on ice before EDTA was added to stop further reactions. Plasma was then collected after centrifugation and frozen at -70^oC for later analysis.

3.3.3 The role of individual TLRs and CD14 in the inflammatory response induced by *N. meningitidis*

In paper I the specific role of key receptors for stimulation of the cellular innate immune system was examined by the activity of NF-κB in HEK293 cells expressing TLR4/MD-2 with and without CD14, TLR2 with and without CD14, and TLR9. The transfected HEK293 cells were incubated with NmLPS+ or NmLPS- at 10-fold increasing concentrations from $1x10^4$ to $1x10^8$ bacteria/mL or purified LPS at 10-fold increasing concentrations from 0.1 ng/mL to 1 µg/mL (1.4 – 14000 EU/mL) for 18 hours. HEK293 cells expressing TLR9 were incubated with 10^8 bacteria/mL or 1µg LPS/mL. NF-κB activity was then measured by a luciferase assay after lysis of the cells (125).

3.3.4 Survival and proliferation of *N. meningitidis* in whole blood deficient of complement factors C2, C5 and MBL

In paper II the influence of different parts of the complement system on the survival and proliferation of meningococci in whole blood was examined by adding an inoculum of 10^5 live meningococci/mL to lepirudin anticoagulated blood freshly drawn from donors genetically deficient of C2 and C5/MBL. Parallel experiments were performed with blood reconstituted with the deficient factors and blood from two control donors, of which the control donor matching the C5/MBL deficient donor was also deficient of MBL (133). Meningococci were incubated in the blood for time intervals ranging from 10 minutes to 24 hours before samples were withdrawn for analysis of CFU and genome DNA quantification with quantitative real-time PCR. Two similar experiments were performed on two consecutive days.

3.3.5 Influence of complement and antibodies on phagocytosis

In paper II the influence of complement and antibodies on OPA was analyzed by using serum from the complement deficient donors and the matching controls. Three different protocols were examined. First, experiments were performed using two-fold titration of test sera (complement-deficient, reconstituted, and control sera) to reveal the OPA of test sera depending on its own IgG and complement content. The second protocol entailed two-fold titration of heat-inactivated test sera following the addition of 10% homologous serum passed through a protein G column to remove IgGs to provide fixed amount of complement. By using this approach, uncertainty about whether antibodies or complement was the limiting factor at higher dilutions was avoided, since the complement concentration was kept constant. The third protocol consisted of two-fold titration of heat-inactivated serum from a subject immunized with a *N. meningitidis* 44/76 outer membrane vesicle vaccine using each of the test sera preparations as the complement source (10%) after passing them through a protein G column. By this method, the antibody coating of the meningococci was standardized.

3.3.6 Influence of cell preparation on the expression of CD11b on granulocytes

In paper II heterologous granulocytes were used in the OPA experiments. A previous study showed no up-regulation of CD11b on the surface of granulocytes from the C5 deficient

donor when whole blood was incubated with bacteria, and consequently no phagocytosis (133). As phagocytosis apparently occurred in the present study also with serum from the C5 deficient donor, we examined whether handling of the heterologous granulocytes ex vivo up-regulated CD11b before they were stimulated by the bacteria. CD11b expression on granulocytes was measured in whole blood kept at 4°C and after preparation of the granulocytes, as used in the OPA assay.

3.3.7 Influence of complement and antibodies on SBA

In paper II the influence of complement and antibodies on SBA was examined by using serum from the complement deficient and the matching controls in the SBA assay. The complement deficient sera, reconstituted sera, sera from the two matching control donors or serum from an individual known to have all complement factors intact but no SBA antibodies were used as exogenous sources of complement. Heat-inactivated sera known to contain specific antibodies against the target strain 44/76 or a monoclonal antibody against the P1.16 epitope of this strain were diluted two-fold and then incubated with bacteria and complement. CFU were counted after plating onto agar plates and incubation overnight, and SBA antibody titers were expressed as the reciprocal of the final serum dilution giving ≥50% killing of inoculum compared to controls.

3.3.8   Binding of Gram-negative bacteria to erythrocytes and the influence of such binding on phagocytosis and oxidative burst

In paper III binding of Alexa-stained *E. coli* and *N. meningitidis* (NmLPS+) to erythrocytes, phagocytosis in granulocytes and monocytes and oxidative burst in granulocytes were analyzed by flow cytometry after incubation of the bacteria in lepirudin anticoagulated whole blood. First, the dose-response effect of *E. coli* binding to erythrocytes, phagocytosis and activation of complement was investigated by adding increasing doses of *E. coli* to the blood and incubate for 10 minutes. Next, the role of complement on the binding of *E. coli* and *N. meningitidis* to erythrocytes and phagocytosis was investigated by adding bacteria to the blood and incubate for 10 minutes in the presence of mAbs blocking complement factors C2 and factor D (anti-C2/D) and an isotype control mAb (134), the C5aR antagonist also used in paper I and its corresponding control peptide or EDTA. The experiments with *N. meningitidis* were also performed with compstatin as a complement inhibitor.

Subsequently time course experiments of *E. coli* binding to erythrocytes and granulocyte phagocytosis were performed with up to 120 minutes incubation of the bacteria in whole blood to investigate the dynamic interaction between binding of the bacteria to erythrocytes and phagocytosis. To further investigate the role of complement in these processes the experiments were also performed with complement inhibitors compstatin and the C5aR antagonist. The role of complement receptor CR1 in the binding of *E. coli* and *N. meningitidis* to the surface of erythrocytes were then investigated by incubating the bacteria in blood with the CR1 blocking antibody mAb 3D9 (135). Dose-response experiments were performed initially with increasing doses of the antibody present in blood incubated with a fixed concentration of *E. coli* ($7.2x10^7$ bacteria/mL). Phagocytosis and oxidative burst were analyzed in granulocytes from the same experiments to examine the effect of bacteria binding to CR1 on these mechanisms. Finally, experiments with an optimal dose of the CR1 blocking antibody present was performed with *E. coli*, NmLPS+ and NmLPS- to examine the influence of CR1 on the binding of the two Gram-negative bacteria, and the influence of LPS.

3.3.9 Complement mediated opsonization of *E. coli* and *N. meningitidis*

In paper III opsonization of *E. coli* and *N. meningitidis* with deposition of complement opsonins C3b and C4b on the surface of the bacteria was analyzed using flow cytometry after the bacteria were incubated 10 minutes in lepirudin plasma. The experiments were also performed with compstatin and the C5aR antagonist as complement inhibitors.

3.3.10   Microscopy of whole blood smears

In paper III human whole blood was supplemented with PBS containing Alexa-stained *E. coli* or *N. meningitidis* (7.2×10^7/mL final concentration) and incubated 10 min at 37^oC. Whole blood smears were then made and immediately air dried. As a control, a portion of the whole blood was diluted with PBS and examined as wet preparation by fluorescence microscopy. The smears were stored in the dark before they were examined using the transmitted light observation microscopy or the reflected fluorescence procedure alone or in combination as previously described.

3.3.11 Porcine model of meningococcal sepsis

The experiments were performed in adherence to the Norwegian laboratory animal regulations and the study protocol was approved by the University Animal Care Committee. Norwegian landrace pigs of either sex with a body weight of 30 ± 2.5 kg were investigated. The pigs were kept in the animal department with free access to water for one day ahead of the experiments. Ketamine (20 mg/kg), atropine (1 mg), and azaperone BPVet (80 mg) were given intramuscularly to induce anesthesia. Supplementary doses of pentobarbital sodium and morphine were administered intravenously through an ear vein to obtain surgical anesthesia. The pigs were tracheotomized in supine position and 1% isoflurane in air-oxygen inhalation anesthesia was administered through a Siemens Kion anesthesia apparatus. Respirator settings were adjusted to maintain a pH of 7.40 and arterial oxygen saturation above 94%. A 16F pulmonary artery catheter with heating filament and pulse oximetry for recording of continuous cardiac output, central venous oxygen saturation, central venous pressure (CVP) and pulmonary artery occlusion pressure (PaOP) (Baxter Edwards Laboratories, Irvine, CA), was inserted via the right external jugular vein and guided into a distal pulmonary artery by pressure wave-form analysis. Pressure transducers were zero referenced to mid-chest level. Three central venous lumina of the pulmonary artery catheter introducer were used for volume therapy, continuous infusion of 1 mg/kg/h morphine, and 0.5 mg/kg/h of the muscle relaxant vecuronium bromide. A Secalon® T (18G, 1.2X90 mm) was inserted in the right common carotid artery for intermittent blood sampling and continuous recording of arterial pressure. A urinary catheter for continuous urinary output measurements was inserted through an open cystostomy. A patient warmer (Warm Touch, DRE, Inc. Louisville, KY) and pre-warmed Ringer's acetate (RA) were used to maintain a core temperature of 38-39ºC. After surgery, the animals were placed in the right lateral position and allowed to stabilize. During the 60-90 minutes of surgery and stabilization, and before the start of the experiments, the animals received 30 mL/kg RA as volume therapy to compensate for a fluid deficit caused by transport and the acclimatization period.

After the stabilization period, the pigs were randomly allocated into the respective study groups. Heat inactivated meningococci were infused intravenously at exponentially increasing rate from a starting dose estimated in pilot experiments, doubling the infusion rate every 30 minutes during 4 hours. All pigs received Ringer acetate (RA) 180 mL/hour. The sepsis pigs were given RA 1000 mL/hour in addition and extra bolus infusions when needed to keep mean arterial pressure (MAP) above 65 mmHg. Norepinephrine was also

administered intravenously in cases of volume refractory hypotension. Physiological data were registered and blood samples drawn at 0, 30, 60, 120, 180 and 240 minutes. Hemodynamic data were recorded from Edwards Vigilance Monitor (Baxter Edwards Laboratories, Irvine, CA) and Drägers Infinity Delta (Dräger Medical, Lübeck, Germany). Respiratory and anesthesiological data were from Siemens KION and the Sc9000XL Monitor (Siemens, Erlangen, Germany). Extended blood gas parameters were recorded from Radiometer ABL800 Flex (Radiometer, Copenhagen, Denmark). At the end of the experiments the animals were euthanized by intravenous injection of 500-1000 mg pentobarbital, 30 mg morphine and 40 mmol potassium chloride. After the animals were dead biopsies from selected organs were taken for later analysis of wet/dry ratio, quantification of bacterial numbers by real-time PCR and LPS content by LAL-assay, immunhistology, cytokines and studies of gene transcription by micro array and real-time PCR.

In paper IV a total number of 10 pigs were included in the sepsis group. All pigs were given NmLPS+, of which five pigs received $5.7x10^{10}$ and five pigs received $1.1x10^{11}$ bacteria in total. Six pigs were included in a control group which did not receive bacteria, but an equivalent amount of 0.9% sodium chloride. Three additional pigs, not receiving bacteria, were examined to exclude a possible effect of volume therapy and norepinephrine treatment by itself. Two of the three pigs received the same volume of RA as in the sepsis group, and the third pig received escalating doses of norepinephrine over four hours reaching 0.54 µg/kg/minute for the last hour.

In paper V two groups of eight pigs each received NmLPS+ or NmLPS- intravenously. In order to examine differences dependent on bacterial load, pigs received various total numbers of meningococci and were matched into pairs where the two pigs in each pair received equal numbers of either NmLPS+ or NmLPS-. A total of 5.7×10^{10} meningococci was administrated to the pair receiving the lowest numbers of bacteria; the successive pairs received two-fold, three-fold, four-fold, five-fold, six-fold, 10-fold and 20-fold this number of meningococci. Two pigs serving as negative controls received only 0.9% soidum chloride. Six additional similar pigs from the previous study served as historical reference controls.

In paper III four pigs were also included to supplement the *in vitro* experiments. Two of the pigs received Alexa-stained *E. coli* and the remaining two pigs received Alexa-stained

N. meningitidis. A total amount of 5.7 x 10^{10} heat-inactivated bacteria were infused intravenously during the first 120 minutes. The initial bacteria dose was 4.5 x 10^{8} bacteria/hour and the infusion rate was doubled every 30 min up to 120 min. After 120 min, a bolus containing 9.2 x 10^{10} bacteria was injected during approximately one minute. Thereafter the pigs were observed for a maximum of one hour or until death. In addition to the routine analysis, blood samples were taken during the experiment for bacterial genome quantification by real-time PCR in whole blood, plasma, buffy coat and erythrocyte fractions. Also, whole blood smears were prepared for microscopy as described previously.

3.3.12 Statistical considerations

Parametric statistics was applied to compare the groups in paper I, III, IV and V as the data passed criteria for normal distribution. No statistics was applied to the data in paper II and the experiments with HEK293 cells in paper I, as the character of these experiments with a couple of parallel experiments "speaking for themselves" did not warrant statistical analysis to be performed (133).

When two groups were statistically compared the Student's *t*-test was performed, as when the effect of the two complement inhibitors compstatin and the C5aR antagonist was compared in paper I and the wet/dry ratios were compared in the porcine model studies. Paired *t*-test was used when parallel results were obtained from the same individual, while unpaired *t*-test was used when results were obtained from different individuals.

When more than two groups were statistically compared ANOVA was used. Repeated measurement ANOVA was used when the results compared was obtained from the same individual. This applied to most of the results in paper I, where also Bonferroni post-test analysis was performed. The ANOVA analysis of the inhibition studies in that paper had to be performed separately at each bacterial concentration included, while the analysis of the cytokine response after incubation with NmLPS+ and NmLPS- without inhibition was performed as an overall analysis with two-way ANOVA. In paper III repeated measurements ANOVA with Holm-Sidak post-test analysis was performed. In paper IV and V repeated measurements ANOVA was applied to include the whole time course in the same analysis. As the experimental set-up in paper V consisted of three individual groups receiving NmLPS+, NmLPS- and the control group, respectively, it would be desirable to include a

post-test analysis to separate significant effects by each of the groups by using only one test. However, we decided to use covariates in the analysis to account for different baseline values of several parameters at the beginning of the experiments influencing the later results, and also to account for different numbers of bacteria given to individual pigs. Thus, post-test analysis could not be performed. Instead, two separate tests were performed for each parameter to allow for identification of LPS-specific effects by comparing the groups given NmLPS+ and NmLPS- and to allow for identification of effects by non-LPS structures of the meningococci by comparing the group given NmLPS- and the control group. A professional statistician approved this way of applying the analyses.

In general a two-tailed p of ≤ 0.05 was considered significant.

4   Summary of the main results

4.1   Paper I

This paper demonstrates dose-dependency of complement activation and cytokine secretion with increasing numbers of *N. meningitidis*. Also, the study describes how different inflammatory mechanisms operate with different numbers of *N. meningitidis* present.

Complement activation by means of soluble TCC in serum increased to a significant threshold at 3×10^7 meningococci/mL and as the bacterial concentration was increased further TCC increased dose-dependently. All three strains investigated, i.e. NmLPS+, NmLPS- and NmC activated complement to the same extent.

Pro-inflammatory cytokine secretion in whole blood incubated with NmLPS+ and NmC started to increase at $10^3 – 10^4$ bacteria/mL. There was a dose-dependent increase in cytokines with increasing bacterial concentrations up to 10^7 bacteria/mL. The threshold for cytokine secretion in whole blood incubated with NmLPS- was 10^7 bacteria/mL and the secretion increased substantially when the concentration of NmLPS- was increased to 10^8 bacteria/mL.

Inhibition of CD14 reduced the cytokine secretion induced by NmLPS+ and NmC to baseline levels at bacterial concentrations up to 10^6/mL. At higher bacterial concentrations the cytokine secretion was still reduced substantially by inhibition of CD14. IL-6 proved to be most dependent on CD14 as the secretion was reduced by 90% at 10^6 and 70% at 10^7 bacteria/mL while IL-8 proved to be least dependent on CD14 as the secretion was reduced by 60% at 10^6 and 35% at 10^7 bacteria/mL. Inhibition of CD14 when NmLPS- was incubated in whole blood also had most effect on IL-6 being reduced by 60% at 10^7 and 10^8 bacteria/mL, and least effect on IL-8 being reduced only at 10^8 bacteria/mL (20%).

Complement inhibition in blood incubated with NmLPS+ and NmC influenced the cytokine secretion at bacterial concentrations from 10^5 to 10^7/mL. IL-1β, TNF-α and IL-8 was most dependent on complement and was reduced up to 40%, while IL-6 was not influenced significantly by complement inhibition. IL-1β, TNF-α and IL-8 were also most dependent on complement inhibition when whole blood was stimulated with NmLPS-. The effect of

complement inhibition was relatively larger with NmLPS- than with NmLPS+ and NmC. Complement inhibition at the C3 level by compstatin had equal effect as C5aR inhibition. Combined inhibition of CD14 and complement in blood incubated with NmLPS+ and NmC reduced cytokine secretion more than either of the inhibitors alone at 10^6 and 10^7 bacteria/mL. At 10^6 bacteria/mL the secretion of cytokines was almost completely abolished by the combined inhibition while at 10^7 bacteria/mL the secretion was reduced by 60 to 80%. In blood incubated with NmLPS- the combined inhibition reduced the cytokine secretion by 50 to 70% at 10^7 bacteria/mL and by 70-90% at 10^8 bacteria/mL.

Activation of transfected HEK293 cells expressing TLR4/MD2 and CD14 with NmLPS+ up-regulated NF-κB activity dose dependently from 10^4 to 10^8 bacteria/mL while incubation of cells expressing TLR4/MD2 without CD14 up-regulated NF-κB activity only at 10^7 and 10^8 bacteria/mL. At 10^8 bacteria/mL CD14 had no influence on NF-κB activation. Activation of HEK293 cells expressing TLR2 and CD14 in combination or TLR2 without CD14 up-regulated NF-κB activity equally and dose dependently from 10^7 to 10^8 bacteria/mL. Activation of cells expressing TLR9 induced only negligible up-regulation of NF-κB activity.

HEK293 cells were only activated by NmLPS- when expressing TLR2. Additional expression of CD14 did not influence the NF-κB activation by NmLPS- and no up-regulation of NF-κB activation was seen in cells with TLR4/MD2 or TLR4/MD2 and CD14.

When HEK293 cells expressing TLR4/MD2 and CD14 was activated by purified *N. meningitidis* LPS there was an almost equally high response throughout the whole range of LPS concentrations while a dose dependent response became evident when cells expressing TLR4/MD without CD14 was activated by LPS.

4.2 Paper II

This paper demonstrates critical roles of C2 and C5 in the major defense mechanisms against meningococci, i.e. phagocytosis and serum bactericidal activity. Also, the study demonstrates how the level of anti-meningococcal antibodies can influence these two defense mechanisms differently.

When meningococci were incubated in C2- and C5-deficient whole blood the number of bacteria increased throughout the incubation period with final increase in CFU of ~2 $\log_{10}$ and a final increase in the number of DNA copies of 4 to 5 $\log_{10}$. When meningococci were incubated with reconstituted C2-deficient blood and with blood from either of the two control individuals, the number of CFU initially decreased ~2 $\log_{10}$ but after two hours started to increase, ending up with similar numbers of bacteria as with the C2- and C5-deficient blood. When meningococci were incubated with reconstituted C5-deficient blood no CFU was detected after two hours and, accordingly, there was no increase in the numbers of DNA copies.

Granulocytes incubated with C2-deficient serum had no detectable OPA while C2 reconstitution restored OPA to the level of the C2 control. Granulocytes incubated with C5-deficient serum had high OPA, similar to the level seen after reconstitution with C5 and MBL and the OPA seen with the positive control (post-vaccination serum). OPA with serum from the C5 control individual, being MBL-deficient like the C5-deficient individual, was similar to the OPA seen with the reconstituted C2-deficicent serum, i.e. lower than the OPA seen with serum from the C5-deficient individual. Reconstitution with MBL had no effect on OPA.

When standardizing the amount of complement factors in all titrations by first titrating heat-inactivated sera against meningococci and then adding a fixed volume of IgG-depleted homologous serum as the complement source, similar results were obtained as in the previous experiments where native sera were titrated against meningococci, i.e. absence of OPA only with the C2-deficient serum.

When the amount of antibodies was kept equally high inn all experiments by titrating heat-inactivated postvaccination serum against meningococci before adding IgG-depleted test sera as the complement source, the OPA-titer was similarly low with the C2-deficient serum as in the negative control where complement was absent. The other test sera had increased OPA, being equally high with all different sera.

Notably, we found that CD11b was spontaneously up-regulated on the heterologous granulocytes used in this experiment when the cells were prepared including lysis of the whole blood.

SBA was not observed with any of the native test sera. However, after reconstitution of the C5-deficient serum SBA was markedly increased. MBL reconstitution had no effect on SBA.

When serum without antibodies was added to heat-inactivated test sera titrated against meningococci, SBA was also markedly increased only with the C5-deficient serum. Experiments were also performed with the C2-deficient serum in the presence of high levels of anti-meningococcal antibodies to investigate whether SBA could occur under such conditions by a C2-bypass mechanism. We found no evidence for such a mechanism to occur.

4.3 Paper III

This paper demonstrates binding of Gram-negative bacteria to erythrocyte CR1 and that such binding reduce phagocytosis and oxidative burst.

Approximately 80% of *E. coli* and *N. meningitidis* added to human whole blood were bound to erythrocytes after 10 minutes incubation. Dose-response experiments showed a linear relationship between the concentrations of *E. coli* and the number of free bacteria in plasma and bacteria bound to erythrocytes. A non-linear relationship between the pahgocytosis of *E. coli* and the added bacteria concentration was found. There was a linear relationship between the number of *E. coli* added and complement activation measured by TCC. Fluorescence microscopy of blood smears confirmed binding of *E. coli* and *N. meningitidis* to erythrocytes. When microscopy was performed on diluted whole blood in wet fluid preparations, the bacteria were mainly found on erythrocytes, moving with the bacteria bound to their surface.

Complement inhibition by anti-factor C2/D or compstatin significantly reduced the binding of bacteria to erythrocytes and simultaneously increased the number of free bacteria in plasma. Complement inhibition with the same inhibitors also completely blocked phagocytosis after 10 minutes incubation. Inhibition with the C5aR antagonist did not influence the binding of bacteria to erythrocytes but blocked phagocytosis by granulocytes completely by monocytes partially after 10 minutes incubation.

Time course studies showed that the binding of bacteria to erythrocytes was time-dependent and slowly decreased with time, although most bacteria were still bound after two hours.

Compstatin increased the release of bacteria from erythrocytes. Phagocytosis by granulocytes was initially completely inhibited by compstatin and the C5aR antagonist, but subsequently increased.

C3 opsonization of *E. coli* and *N. meningitidis* was efficiently reduced by compstatin, whereas C4 opsonization increased. LPS had no effect on the opsonization, as the wild-type *N. meningitidis* and the LPS-deficient mutant strain were equally opsonized.

The anti-CR1 blocking antibody dose-dependently decreased the binding of *E. coli* and *N. meningitidis* to erythrocytes and simultaneously increased the number of free bacteria in plasma. At the same time phagocytosis and oxidative burst by monocytes and granulocytes also increased when binding to CR1 was inhibited. LPS had no effect on the binding to CR1, as the wild-type *N. meningitidis* and the LPS-deficient mutant strain behaved equally.

In the *in vivo* experiments with the porcine sepsis model five minutes after the bolus injection of bacteria 24% of *E. coli* was located to the erythrocyte fraction while 42% was in plasma and 34% was in the buffy coat fraction. Approximately one hour later 14% was in the erythrocyte fraction while 30% was in plasma and 56% was in the buffy coat. Immunofluorescence histology of the lungs, spleen and liver from the pigs showed that the Alexa-bacteria were mainly located in the lungs. The distribution of *N. meningitidis in vivo* was equal to *E. coli.*

4.4 Paper IV

The main achievement of this paper was to establish a large animal model of meningococcal sepsis being suitable to study pathophysiological and immunological aspects of this disease experimentally *in vivo*, and which also could be used for possible experimental therapeutic interventions.

Of cardiovascular parameters MAP was kept stable during most of the observation period by intensive fluid resuscitation, but gradually declined towards the end, simultaneously with a decline in SVRI. An abrupt and prominent rise in PVRI and mean MPAP was seen

approximately 30 – 45 minutes after start of bacterial infusion. Lactate increased significantly.

The intravascular albumin content in the sepsis group decreased substantially during the course of the experiment and the final plasma albumin content was only about 50% of the initial content. The wet/dry ratio in muscle increased significantly from start to end of the experiment in the sepsis group and the wet/dry ratio in the gravity dependent right lung (all pigs were in the right decubital position) and jejunum differed significantly between the sepsis and the control group.

The number of granulocytes declined markedly in the sepsis group and platelets also declined significantly.

TAT and the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, the chemokine IL-8 and the anti-inflammatory cytokine IL-10 all increased in the sepsis group.

Soluble TCC, C-reactive protein, transforming growth factor β1, heart fatty acid binding protein and liver fatty acid binding protein were below detection limit.

4.5 Paper V

This paper documents the specific role of LPS versus other membrane components of meningococci in the evolvement of meningococcal sepsis.

Of the cardiovascular parameters SVRI decreased only in response to NmLPS+, while kept stable or even increased in response to NmLPS-. There was also a decrease in MAP towards the end in response to NmLPS+ which was not seen with NmLPS-. Lactate, indicating hypoperfusion of the tissues, increased only in response to NmLPS+. MPAP and PVRI increased in response to NmLPS+ and NmLPS-, but the increase appeared earlier in response to NmLPS+.

The fluid requirement of the pigs receiving NmLPS+ was considerably higher than the requirement of the pigs receiving NmLPS-. However, even the latter group required significantly more fluid than the control group to keep the blood pressure, and the pigs receiving the lowest numbers of NmLPS+ had comparable need for fluid as the pigs receiving the highest numbers of NmLPS-. There was a substantial decrease of intravascular albumin due to capillary leakage in both groups receiving bacteria, but the decrease appeared more immediate in the NmLPS+ group. The mean final decrease of intravascular albumin was 40% in the NmLPS+ group and 30% in the NmLPS- group. Peak inspiratory pressure, reflecting increased stiffness of the lungs due to pulmonary edema, increased substantially only in the NmLPS+ group. Wet/dry ratio in the left lung and jejunum was higher with NmLPS+ than NmLPS- while wet/dry ratio of the right lung and the muscle was only increased in the NmLPS+ group compared to the control group.

Hemoglobin increased temporarily in the NmLPS+ group, while a continuous increase towards the end was seen in the NmLPS- group. Granulocytes were virtually depleted from the circulation in the NmLPS+ group, but did not change in the NmLPS- group except for in the pig given the highest numbers of NmLPS- where a marked decrease appeared at the end of the experiment. Platelets decreased in both groups receiving bacteria.

TAT and PAI-1 increased in both groups receiving bacteria, but most in the NmLPS+ group.

All the inflammatory cytokines analyzed, i.e. TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12, increased substantially more in response to NmLPS+ than in response to NmLPS-. IL-8 was only increased in the NmLPS+ group. There was a dose-dependent increase of IL-1β, IL-6 and IL-8. The pigs receiving the lowest numbers of NmLPS+ bacteria had comparable cytokine responses to the pigs receiving the highest numbers of NmLPS- bacteria.

5   Discussion

The last decade has brought comprehensive new insight into the concept of pathogen associated molecular patterns (PAMPs), i.e. conserved microbial structures, and their interactions with the innate immune system (136). This great extension of our insight into such mechanisms was initiated by the discovery of Toll-like receptors as key players in the innate immunity and how the activation of TLRs by various PAMPs led to activation of leukocytes and other cells taking part in the inflammatory response (137). In turn, this has brought increased attention to the role of innate immunity mechanisms in inflammation in general and how the different parts of the innate immune system interact with each other as well as with the adaptive immune system (136). The failure of previous trials to show advantageous effects of blocking single inflammatory mediators in septic patients has also encouraged a broader perspective of the inflammatory response in sepsis (63,138-140). The primary achievement of this Thesis has been to gain new insight into details about the interaction between pathogen structures of meningococci and the immune system leading to inflammatory responses and development of the clinical state of sepsis on the one hand, but also being essential for the host defense against meningococcal disease on the other hand. A major focus of the studies has been how specific meningococcal pathogen structures are involved when the inflammatory networks are triggered and sepsis develops, and also the dose-dependency of such interactions. In most of the *in vitro* and *in vivo* models used in the studies we have taken advantage of previous investigations showing how the growth rate of meningococci and thus, the concentration of PAMPs being present, determines the course of meningococcal disease after the bacteria have managed to invade from the nasopharyngeal mucosa into the blood (23-25,74).

5.1   Dose dependency of inflammatory mechanisms triggered by *N. meningitidis*

The first paper, modeling different stages of meningococcal disease *in vitro* by adding increasing numbers of meningococci to serum, whole blood and transfected HEK293 cells, demonstrates how the transition from a low-grade bacteremia to fulminant sepsis is associated with a parallel transition from a non-detectable inflammatory response to a

massive inflammatory state with high levels of pro-inflammatory mediators present. The experiments demonstrate how the inflammatory responses are differentiated with respect to the different mechanisms and triggering pathogenic structures involved. CD14 dependent TLR4 mediated signaling initiated by the binding of LPS was shown to be the most sensitive mechanism in our systems, provoking inflammatory responses in the whole blood system as well as in the transfected HEK293 cells at concentrations of meningococci from $10^3 - 10^4$ bacteria/mL. This mechanism proved to be the dominant inflammatory mechanism up to 10^6 bacteria/mL. The threshold for appearance of complement dependent inflammation, mainly mediated by C5a, was $10^5 - 10^6$ bacteria/mL, the concentrations being equal to the lower range seen in patients developing fulminant sepsis (23-25). Signaling by TLR2 in the HEK293 cell system was not evident before the bacterial number reached the upper range seen in patients with fulminant sepsis, i.e. 10^7 bacteria/mL. While CD14 was a prerequisite for the activation of TLR4 at low to medium bacterial concentrations, our experiments demonstrated that with the bacterial numbers increasing up to the extremely high concentrations associated with fulminant sepsis, TLR4 can be activated independently of CD14. This indicates that the role of CD14 in meningococcal disease is primarily to enhance the ability of the immune system to respond to LPS in the initial phase of the disease, while TLR4 can readily be activated without the facilitating mechanism of CD14 when the amount of LPS becomes abundant. These findings are in line with previous findings by Sprong et.al. (141). Also, our experiments with HEK293 cells indicate that the facilitating effect of CD14 in TLR2 mediated signaling by meningococci is limited, although some effect may be present according to our whole blood experiments with NmLPS-. CD14 may be more important in TLR2 mediated signaling by other pathogens, activating TLR2 at lower concentrations than meningococci.

In the serum model in paper I increased TCC was not detected until the bacterial concentration was substantially increased to 10^7 bacteria/mL. The TCC detected in these experiments were in the soluble form in plasma. The experiments in paper 2 and other studies of SBA demonstrate clearly SBA to be apparent, i.e. efficient insertion of MAC into the bacterial membrane occurs, at lower bacterial concentrations (142). Thus, complement activation with formation of the terminal C5b-9 complex appears to be well controlled and directed to the target bacterial membrane up to very high numbers of meningococci, at the level of 10^7 bacteria/mL. Beyond this level activation of complement seems to be exaggerated with a large amount of soluble TCC being formed in plasma. Our experiments

indicate that activation of complement is primarily directed towards killing meningococci but with limited systemic inflammatory effects as long as the bacterial concentration does not exceed the level seen in low to moderate grade meningococccemia. When the bacterial numbers are increased to the high levels seen in severe meningococcal sepsis a breakdown of complement homeostasis appears, similar to the breakdown of the homeostasis of other plasma cascade systems like the coagulation system (143). Together with an exaggerated TLR mediated inflammatory activation, overriding the regular signaling mechanisms like the CD14-dependency, such activation probably leads to a "point of no return" where the inflammatory response finally results in the patient succumbing. Targeting inhibition of upstream mechanisms in this response, like the initial TLR signaling and the activation of complement, could be a future approach in the treatment of sepsis (63).

5.2   The role of C2, C5, MBL and antibodies for bacterial survival and proliferation, phagocytosis and serum bactericidal activity

Although the interaction between the innate and adaptive immune systems has become a field of recent interest, our understanding of some mechanisms of such interactions also dates long time back in the history of science. Antibody dependent activation of the classical complement pathway is an excellent example of this, being described for the first time about 100 years ago (144). Paper two describes novel insight into details of the role played by complement and antibodies in the defense against meningococci, which is of special interest since this pathogen is strikingly inclined to cause disease in individuals with certain complement deficiencies (112,145). These results were obtained by experiments using whole blood and serum from two individuals being genetically completely deficient of complement factor C2 and C5, respectively (133). The experiments demonstrated C2 as well as C5 to be critical for the killing of meningococci in whole blood, indicating that none of the defense mechanisms against meningococci in peripheral blood, phagocytosis and serum bactericidal activity could operate efficiently without these two factors. C2-dependency with no influence of MBL, as we found, implies that classical complement activation is the essential initiating step, in accordance with previously published results (146,147). However, a role of the lectin pathway by involvement of ficolins cannot be completely ruled out by these experiments since MBL-independent activation by ficolins might occur. These results do not exclude the

alternative pathway to be involved, as indicated by the increased susceptibility of individuals with deficiencies of the alternative pathway like properdin and factor D to acquire meningococcal disease and the protective role of the alternative pathway inhibitory factor H binding to meningococci (112,148,149). Involvement of the alternative pathway probably occurs mainly through amplification of the complement cascade after initial activation by the classical pathway (150). C2 bypass mechanisms, as has been proposed to occur in certain instances (151), were not observed in our experiments even when the classical pathway activation was enforced by adding high concentrations of anti-meningococcal antibodies.

C5-dependency indicates that this factor is not only needed to form the terminal lytic complex but also is a prerequisite for efficient phagocytosis of meningococci and oxidative burst to occur. This is in accordance with previous experiments where C5a was found to be critical for the up-regulation of CD11b and the formation of H_2O_2, being essential for these mechanisms (44,127,133), although an excess of C5a as can be formed in sepsis has also been shown to shut down the granulocyte function (44). However, in the present study we could not verify C5a dependency in the OPA experiments with heterologous granulocytes. This was probably due to artificially up-regulation of CD11b during their preparation ex vivo, as demonstrated in a separate experiment.

The results of this study also indicate that while a high level of antimeningococcal antibodies is necessary for serum bactericidi to occur, phagocytosis can occur even in the presence of low background antibody levels. It is reasonable to speculate that efficient serum bactericidal activity requires abundant insertion of lytic MAC complexes into the meningococcal membrane, particularly in encapsulated strains as we used in these experiments, which in turn requires high levels of specific antibodies. In contrast, even in the presence of background antibody levels sufficient classical complement activation seems to take place to give some opsonic coating of meningococci and adequate up-regulation of CD11b. These results suggest phagocytosis to be a primary defense mechanism against intruding meningococci in non-immunized individuals having low SBA, corresponding to an apparent protection against meningococcal disease in the absence of a sufficient antibody titer for SBA to occur in some individuals (49). The specificity of antimeningococcal antibodies probably also determines whether phagocytosis or bactericidal effects will be effective (47).

The results also indicate an independent role of antibodies to initiate phagocytosis without activating complement, probably by the involvement of Fc-receptors, but only when the antibodies are present in large amounts as after immunization. Our experiments do not indicate any role of MBL in the defense mechanisms against meningococci. However, we cannot exclude that involvement of MBL, as has been suggested by others (152,153), can be present under certain conditions like in the case of reduced sialylation of LPS, supposed to occur by phase switching after the meningococci breach the pharyngeal mucosa (154), since sialic acid is thought to hide MBL-binding targets on the bacterial surface (147,155,156).

5.3 The influence of erythrocyte CR1-binding of Gram-negative bacteria on phagocytosis and oxidative burst, and the handling of the bacteria *in vivo*

Phagocytosis and oxidative burst are normally studied with isolated populations of leukocytes, primarily granulocytes (157). In the third paper we utilized the whole blood model with the specific thrombin inhibitor lepirudin as anticoagulant to study the interaction of Gram-negative bacteria with erythrocytes and leukocytes. *N. meningitidis* and *E. coli* opsonized with C3 and C4 split products bound rapidly to erythrocyte CR1 receptor. By this interaction with the erythrocytes phagocytosis by granulocytes and monocytes were impeded, as demonstrated by the increased phagocytosis and oxidative burst occurring when CR1 binding of the bacteria to erythrocytes was blocked. Thus, it appears that binding of these bacteria to erythrocytes protects against phagocytosis and oxidative burst by circulating leukocytes. These findings are in line with previous studies where binding of immune complexes to erythrocyte CR1 has been found to inhibit activation of granulocytes (158,159).

Interestingly, in the first study of the immune adherence phenomenon with bacteria by Nelson from 1953 it was shown that the phagocytosis of pneumococci by guinea pig macrophages increased in the presence of human erythrocytes (116). Subsequent studies have confirmed the particular importance of the mononuclear phagocyte system of the liver for the removal of circulating bacteria and apparently only a minor proportion of circulating bacteria are phagocytosed by circulating leukocytes (160-162). These findings are in line with a recently published study demonstrating a functional role of erythrocyte CR1 in clearance of pneumococci from the circulation by facilitating the transfer of pneumococci to

liver macrophages (118). Interestingly, the proportion of bacteria being associated with the erythrocyte fraction in the *in vivo* studies we performed using the porcine model of sepsis was substantially lower than we found in the *in vitro* experiments with human whole blood. Notably, the erythrocytes of pigs probably do not possess CR1 or similar receptors, as these are restricted to primates (163). Thus, the nature of bacterial binding to porcine erythrocytes is not clear. We speculate that the low proportion of bacteria bound to erythrocytes in the pigs could partly explain why a majority of the bacteria was removed from the circulation by the lungs and not by the liver. It appears that erythrocyte CR1 binding of complement opsonized bacteria can serve as a mechanism to protect against systemic inflammation through directing the pathogenic agents particularly to the liver where they can be more safely deposited by the mononuclear phagocyte system with less systemic inflammatory responses. This implies a supplementary role of complement to its commonly regarded primary functions of mediating phagocytosis and serum bactericidal activity and can have implications for a potential use of complement inhibition as suggested as adjuvant therapy in sepsis (44,164)

The experiments performed with different complement inhibitors supply information about how different parts of the complement system are involved in mediating phagocytosis. Interestingly, we found that when activation of C3 was inhibited, the deposition of C4b on the surface of the bacteria not being inhibited was increased. This increase was probably due to more space for C4b deposition on the complement binding membrane structures of the bacteria and such C4b deposition probably explains why some binding of bacteria to erythrocytes was seen despite complement inhibition. As expected, inhibition of the C5a receptor did not affect opsonization of the bacteria, but significantly reduced phagocytosis. This is in accordance with the results of previous studies where C5a was found to be a key factor for up-regulation of CD11b/CD18 (CR3) being essential for phagocytosis in granulocytes (127,133,134).

This study also supply additional information about expected effects of complement inhibition in sepsis, suggested as a potential new therapeutic approach to attenuate the inflammatory response in such patients (44,164). By using a complement inhibitor acting early in the complement cascade, i.e. at the C3 level or previous steps, the bacterial opsonization would be decreased, leading to reduced bacterial binding to erythrocytes and consequently increased numbers of free bacteria in plasma. Decreased opsonization and

decreased up-regulation of CR3 would also lead to reduced phagocytosis by peripheral leukocytes and probably also tissue macrophages, further increasing the number of free bacteria in plasma. Decreased phagocytosis may decrease those parts of the septic inflammatory response being related to granulocyte activation and oxidative burst, i.e. release of reactive oxygen metabolites and other products thought to be important in some of the pathophysiological changes of sepsis, particularly capillary leakage (33,159). However, increased concentrations of circulating pathogens, even if they are dead due to adequate antibiotic treatment, will probably also increase the exposure of PAMPs to the inflammatory system and consequently give disadvantageous increased inflammatory response. An alternative strategy is to inhibit complement at a later step like C5a, which implies that the bacteria will still be fully opsonizied and may bind to erythrocyte and leukocyte CR1, as well as leukocyte CR3. According to our results, although phagocytosis by granulocytes is inhibited, phagocytosis by monocytes and thus, probably also tissue macrophages, can still be relatively effective when C5a is inhibited. Thus, inhibition of C5aR may be the most suitable approach when searching for a complement inhibitor to be used in sepsis.

5.4   The new porcine model of meningococcal sepsis

An important object of this thesis has been to unite the *in vitro* and *in vivo* approach towards experimental studies of meningococcal sepsis. While *in vitro* studies are needed to investigate specific details, studies *in vivo* are necessary to prove effects on the whole organism which is of course of fundamental relevance to understand how meningococcal disease develops. A suitable *in vivo* model is also a necessity when new potential therapeutic principles are to be explored. Accordingly, we developed a new porcine model of meningococcal sepsis. Such a large animal model is superior to murine models in several aspects: Porcine and human cardiovascular physiology are relatively similar (165). The pig can be monitored with the same instruments as used for surveillance of patients and the large blood volume in pigs allows for a broad range of samples at several time points during the experiments. The fluid resuscitation used in this model was similar to that used in human septic patients. Furthermore, human and porcine LPS responsiveness are similar, in contrast to mice and rats which are relatively resistant if not presensitized to LPS (166,167). The model was designed to simulate the rapid and exponential growth of meningococci in

patients with fulminant meningococcal sepsis. No such animal model of meningococcal sepsis has been developed previously.

Although compressed with regard to time, a broad spectrum of changes including cardiovascular parameters, vascular leakage, cytokine release, and changes in hematological and coagulation parameters paralleled those observed in patients. The study gained insight into the early events of the development of meningococcal shock. Leakage of plasma out of the vascular compartment was found to appear from an early stage when the bacterial concentrations in blood still were relatively low. As the bacterial concentration increased towards $10^5 - 10^6$ bacteria/mL, which is comparable to what is found in patients with fulminant meningococcal sepsis, the capillary leakage became more profuse. However, as long as the intravascular volume deficit was sufficiently substituted by intravenously administrated fluid, hypotension was avoided. When the peripheral resistance decreased during the final hour of the experiments, large volumes of administrated fluid were not sufficient to stabilize the circulation and neither was the heart able to compensate by increasing the cardiac output. Thus, hypotension apparently evolved as a result of decreased vascular resistance in combination with a relative compromised cardiac performance.

The abrupt increase observed in pulmonary hypertension may be more pronounced than in humans with sepsis and is probably due to specific features of the porcine lung endothelial lining with particular abundance of macrophages (168,169).

Of the pro-inflammatory cytokines measured, TNF-α was the first to increase, followed by a decline during the observation. Such a pattern of TNF-α is also seen after bolus injections of LPS (166), and probably occurred due to a rapid down-regulation of this cytokine in response to the high bacterial load. Additionally, since we measured the amount of protein using ELISA assay, binding of TNF-α to soluble receptors might cover detection epitopes and thereby interfere with the assay. The absolute increase in TNF-α, being in the order of 10 ng/mL, was in fact somewhat more pronounced than observed in patients with meningococcal sepsis commonly having concentrations of TNF-α less than 1 ng/mL (29). IL-1β is particularly associated with severe meningococcal sepsis and increased in our model to about the same level as seen in patients with such disease (29,170). The concentration of IL-6 was in the same range as seen in patients with meningococal sepsis (29), and the level of IL-8 in the septic pigs was in the lower range of that seen in patients with sepsis (28). The

maximum level of the important anti-inflammatory cytokine IL-10 (171) was in the range of 0.3 ng/mL, similar to the porcine *E. coli* sepsis model also developed by our group (172). This is considerably lower than in patients with meningococcal sepsis at the time of admittance to hospital, normally having IL-10 concentrations in the range of 1-100 ng/mL (30,173). *In vitro* studies suggest that IL-10 is released with a slower kinetics than most of the pro-inflammatory cytokines reaching peak levels between 8 and 24 hours (174). Given the four hour time span of the experiments IL-10 was not up-regulated to the same extent as observed in patients with fulminant septicemia where the time from onset of the disease symptoms to hospital admission last median 12 hours and rarely <8 hours (19,30,173,175). In this respect the new model is not reflecting the powerful anti-inflammatory response observed in patients with fulminant meningococcal septicemia (30,75,171,173,175). Taken together, our results and the previous *in vitro* and clinical studies indicate that IL-10 is in fact released slower than the pro-inflammatory cytokines although fast enough to reach high peak values within the time frame of admittance to hospital.

5.5 The inflammatory role of LPS and non-LPS molecules of *N. meningitidis* examined *in vivo* in the porcine model of meningococcal sepsis

The porcine model was applied to perform experiments documenting for the first time in a large animal model the specific role of LPS, for long time assumed to be the essential PAMP of meningococci, versus other meningococcal compounds in the development of sepsis. The results confirmed that *N. meningitidis* LPS is by far the most potent group of outer membrane molecules that trigger the innate immune system and subsequent inflammatory responses. However, it demonstrated that non-LPS molecules in meningococci are capable of inducing complex pathophysiological responses leading to marked organ dysfunction.

The most apparent LPS-specific circulatory difference was the divergent changes seen in peripheral resistance. Decreased SVRI, as appeared only in the NmLPS+ group, occurred despite the administration of norepinephrine, indicating LPS-induced decreased sensitivity to catecholamines as a causative factor of vasoplegia in septic shock (176,177). In contrast, PVRI increased to the same level in both sepsis groups, although significantly earlier in the

NmLPS+ group. This indicates that non-LPS components of meningococci also are important inducers of pulmonary vasoconstrictors, like thromboxane A2 and endothelin (178)

Capillary leakage, thought to be a major pathophysiological event in meningococcal sepsis (179), was in paper IV found to be an early event leading to hypovolemia. All indicators of capillary leakage used in the present model demonstrated LPS to be the most important, although not the sole molecule responsible for this event. Even in the NmLPS- group intravascular albumin decreased and there was a substantial dose-dependent need for extra fluid replacement. Also, a continuous hemoconcentration appeared in the NmLPS- group despite the high volumes of fluid infused, strongly supporting leakage of large plasma volumes out of the circulation.

Regarding the leukopenia characteristically seen in fulminant meningococcal sepsis, caused by up-regulation of various adhesion molecules on endothelial cells and the leukocytes themselves (14), our results clearly show that LPS is the major molecule responsible for this event. However, at high concentrations other molecules of meningococci may contribute to such up-regulations, as the pig receiving the highest number of NmLPS- bacteria also had marked decrease in the number of circulating granulocytes.

DIC, being a characteristic phenomenon in severe sepsis, develops as a result of increased systemic coagulation due to up-regulation of tissue factor and decreased fibrinolytic capacity (36,37,39). By measuring TAT and PAI-1 both these aspects of changes in coagulation were studied. In addition, the consumption of platelets was also measured. The results emphasize LPS as the most important causative factor of hemostatic pathology, although other molecules may contribute to a lesser extent.

LPS proved to exert a dominant influence on all cytokines measured, i.e. TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12. However, TNF-α, IL-1β, IL-6 and IL-10 also increased in the NmLPS- group, but mostly in the pigs receiving the highest numbers of mutants. Interestingly, IL-8 was found to be the most LPS independent cytokine in our *in vitro* studies, but in the *in vivo* study IL-8 was the only cytokine being increased solely in the pigs receiving NmLPS+. This discrepancy does not relate to different species being studied, as other *in vitro* studies with porcine whole blood also have demonstrated LPS independency of

IL-8 (180). Rather, such discrepancy indicates substantial differences in the regulation of this cytokine *in vitro* and *in vivo*.

The concentrations of TNF-α, IL-1β and IL-6 were comparable in the pigs receiving the highest numbers of NmLPS- and the lowest numbers of NmLPS+, indicating that wild type meningococci stimulate the pro-inflammatory cytokine secretion with about 10 to 20 fold higher potency than the mutant strain *in vivo*. Such difference in potency between the effect of meningococci with and without LPS corresponded well with our clinical observations of the pigs. In fact, this difference is markedly less than the difference we found in cytokine secretion when whole blood was incubated with NmLPS+ and NmLPS- in paper I, and also less than the difference in *in vitro* experiments performed by others (76-78). Thus, although the difference between LPS and other meningococcal structures in their capacity to induce cytokine secretion and inflammatory changes is clearly evident even in our *in vivo* model, it appears that the difference may be less pronounced *in vivo* than in various *in vitro* models. This emphasizes the role of the various organs in the inflammatory response, a field which have been scarcely studied so far.

The effects observed with the NmLPS- strain could not be explained by a modest concentration of LPS in this preparation. The strain is completely devoid of LPS (119) and the minimal LPS activity of the bacterial suspension used for infusion (2 EU/10^{10} bacteria), regarded as contamination from the growth medium, is far below the amount needed to induce any biological response. This is also in accordance with the fact that no LPS were detected in the blood at the end of the experiments in the NmLPS- group.

6 Conclusions

6.1 Bacterial load

The complexity of the inflammatory response to *N. meningitidis* is highly dependent on the bacterial load. At low to moderate bacterial concentrations in the blood CD14 dependent signaling via TLR4 is the sole pathway being activated. At higher concentrations of meningococci other inflammatory mechanisms including complement activation and CD14 independent signaling via TLR4 and TLR2 become increasingly important.

6.2 Complement activation

Activation of complement is well balanced and directed towards killing meningococci by deposition of opsonic fragments and insertion of the C5b-9 lytic complex into the bacterial membrane as long as the concentration of meningococci does not exceed the levels seen in low to moderate grade meningococcal sepsis. When the bacterial numbers are increased to the high levels seen in severe meningococcal sepsis, the inflammatory effects of complement activation become more pronounced and ultimately a breakdown of complement homeostasis appears.

6.3 Phagocytosis and serum bactericidi

Phagocytosis and serum bactericidal activity contribute collectively in the defense against intruding meningococci. Both mechanisms are dependent on complement activation by the classical pathway and probably also activation of C5. Phagocytosis occurred in the presence of low background levels of antibodies but increased after immunization. Serum bactericidal activity was dependent on the presence of antibodies in the serum.

6.4   Erythrocyte CR1 binding

Gram-negative bacteria opsonized with C3 and C4 complement factors bind to erythrocyte CR1 and this interaction with the erythrocytes decreases phagocytosis by granulocytes and monocytes and probably influence the handling of the bacteria *in vivo*.

6.5   *In vivo* model

The porcine model we established proved to be suitable to study a broad range of pathophysiological and immunological aspects of meningococcal sepsis. Characteristic pathophysiological changes occurred in relation to the release of large amounts of inflammatory cytokines.

6.6   Pathophysiology

Capillary leakage was found to be an early event in the porcine model of meningococcal sepsis, increasing with increasing bacterial load. However, hypotension was avoided with sufficient volume replacement as long as there were no alterations in the vascular resistance. Circulatory failure with hypotension evolved as a result of decreased vascular resistance in combination with a relative compromised cardiac performance.

6.7   LPS

LPS was demonstrated to be the primary molecule of *N. meningitidis* to induce inflammation *in vitro* and *in vivo*. However, the difference between LPS and non-LPS membrane structures appeared to be more pronounced *in vitro* than *in vivo* with a difference in potency of about 3 $\log_{10}$ *in vitro* and about 1 $\log_{10}$ *in vivo*.

7 Future perspectives

Despite increasingly detailed insight into the inflammatory response to *N. meningitidis*, this has not led to advances in the treatment of meningococcal sepsis. Accordingly, more research is needed to obtain a better general understanding of the underlying inflammatory mechanisms leading to the pathophysiologic changes in this disease. In order to search for potential new therapeutic approaches, one should in our opinion look for inhibitors acting on a broad range of the inflammatory mediators being induced in sepsis, as previous studies targeting single mediators have not proven successful. This is in accordance to the present view of the septic inflammatory response involving a broad range of particularly the innate immune system being triggered by pattern recognition mechanisms.

In vitro studies are normally performed with cultured cell lines, isolated leukocytes, whole blood or serum. Further investigations on how the whole organism and individual organs contribute to the septic inflammatory state are warranted. We will perform such investigations based on material from the porcine model. In addition we intend to study post mortally the aggregation of LPS and meningococci in various organs from humans having died from meningococcal sepsis, and the corresponding organ specific inflammatory response. We have also started further investigations to clarify the interaction between complement and antibodies in phagocytosis and serum bactericidal activity, and the role of C5a in the regulation of phagocytosis and oxidative burst by use of serum and whole blood from Norwegian individuals recently detected to be C5 deficient.

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9   Papers

II

III

Erythrocyte complement receptor 1 binds Gram-negative bacteria and protects against phagocytosis and oxidative burst in human whole blood

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Running heads: Binding of Gram-negative bacteria to erythrocytes reduce phagocytosis

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Abstract

Erythrocytes play a pivotal role in transport of opsonised agents via complement receptor 1 (CR1), but how Gram-negative bacteria interact with erythrocytes, and the implications thereof, has not previously been studied. We therefore investigated the interaction of *Escherichia coli* and *Neisseria meningitidis* with erythrocytes and how this interaction affected phagocytosis and oxidative burst in human whole blood. Bacteria free in plasma, erythrocyte-bound or phagocytosed were quantified using flow cytometry. Most bacteria (80%) bound immediately to erythrocytes. The binding gradually declined with time, with a parallel increase in phagocytosis. C3 inhibition reduced erythrocyte binding and bacterial opsonization. C5a receptor blockade abolished phagocytosis, but binding of bacteria to erythrocytes was not influenced. CR1-blocking dose-dependently inhibited erythrocyte bacterial binding to nil, with subsequent increased phagocytosis and oxidative burst. Similar results were obtained with an LPS-deficient *N. meningitidis* mutant. *In vivo* porcine experiments demonstrated limited association of bacteria with erythrocytes, consistent with absence of erythrocyte CR1-like receptors in non-primates, and the bacteria were mainly deposited in the lungs. In conclusion, binding of Gram-negative bacteria to human erythrocyte CR1 decrease phagocytosis and oxidative burst by leukocytes in whole blood and may direct the bacteria to more safe deposition in the liver and spleen.

Introduction

Erythrocytes are generally viewed as simple hemoglobin carriers involved in oxygen transport in humans. However, the first observation that erythrocytes may bind microorganisms and play a role in the pathogenesis of bloodstream infections was in 1930 using serum-opsonized trypanosomes.[1] Nelson later demonstrated that opsonized particles and Gram-positive pneumococci bind to erythrocytes in a complement dependent manner.[2] Subsequently, the erythrocyte complement receptor 1 (CR1) was identified as the high-affinity binding site of C3b with lower affinity binding to iC3b, C4b,[3] C1q and mannan-binding lectin (MBL)[4] linking complement-coated bacteria to erythrocytes.[5] A number of later studies have examined the binding of immune complexes (IC) to erythrocyte CR1 and the involvement in IC clearance.[5]

CR1 is a large transmembrane glycoprotein consisting of several homologous motifs.[5] It is expressed in varying numbers, from approximately 100-1000 per human erythrocyte[5] and occurs in clusters on the erythrocyte membrane after ligation.[6,7] CR1 is also found on human monocytes, granulocytes[8] and B-lymphocytes.[5] Immune complexes opsonized with C3b and C4b bind to erythrocyte CR1 and can then be cleared from the circulation and destroyed in the liver and spleen.[9,10] Another interesting feature of CR1 is its ability to inhibit complement activation by function as a co-factor for factor I which cleaves C3b and C4b to inactive forms.[11] Through the binding of C3b and C4b, CR1 also accelerates the decay of the alternative[12] and classical pathway C3 convertases.[11] However, erythrocyte binding of Gram-negative bacteria and the implications of such binding for the fate of the bacteria in human whole blood has not, to our knowledge, been previously studied.

Escherichia coli (E. coli) and *Neisseria meningitidis* (*N. meningitidis*) are important Gram-negative pathogens causing sepsis.[13,14] The organisms activate complex inflammatory mechanisms, involving the innate as well as the adaptive immune systems.[15] Activation of complement is a key feature, being important for the defense mechanisms opsonophagocytosis[16] and serum bactericidal activity to occur. Complement activation also have potent inflammatory effects through the release of anaphylatoxins including C5a in sepsis[17] and excessive activation of complement in meningococcal disease is related to disease severity.[18]

In this study we investigated the interaction of *E. coli* and *N. meningitidis* with erythrocytes and how such interaction influence phagocytosis in a human whole blood model. The specific thrombin inhibitor lepirudin was used as anticoagulant since it does not affect complement activation in contrast to calcium-binding anticoagulants and heparin.[16] Experiments were also performed *in vivo* by intravenous administration of fluorochrome-labeled bacteria in a porcine model of sepsis, since porcine erythrocytes lack CR1. The data shed new light over the interaction of Gram-negative bacteria with the various blood cells and indicate that erythrocyte binding protects the bacteria against phagocytosis and reduces leukocyte oxidative burst in human whole blood.

Materials and methods

Reagents

All equipment including polypropylene tubes from Nalgene NUNC (Roskilde, Denmark), and tips used in the whole blood experiments was endotoxin-free. Phosphate-buffered saline (PBS) with or without Ca^{2+} and Mg^{2+} were obtained from Life Technologies (Paisley, UK). Lepirudin (Refludan®) was obtained from Hoechst (Frankfurt am Main, Germany). Protein G Spin Kit columns (0.2 mL) for antibody purification were obtained from Thermo Fisher Scientific (Pierce, Rockford, IL). The Burst test and Phago test kits were both obtained from ORPEGEN Pharma (Heidelberg, Germany). Alexa 488, the BacLight green kit for direct fluorescent staining of unlabeled bacteria and dimethylsulphoxide (DMSO) were obtained from Invitrogen Molecular Probes (Eugene, OR). Ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin were obtained from Sigma-Aldrich (St. Louis, MO).

Antibodies and inhibitors

The anti-CR1 blocking mAb (clone 3D9) inhibits the binding of CR1 to C3b/C4b and has been extensively characterized previously.[19] The mAb 3D9 was purified from 50 µL sterile ascites fluid containing approximately 1 mg/mL mAb using protein G columns. The concentration of the purified 3D9 IgG1 antibody in the eluate (0.46 mg/mL) was analyzed at 280 nm using a SmartSpec™Plus Spectrophotometer from Bio-Rad (Hercules, CA). An isotype matched control IgG1 mAb (clone BH1) was purchased from Diatec (Oslo, Norway). Antibodies were tested for LPS contamination using the chromogenic LAL-assay QCL-1000 from BioWhittaker (Walkersville, MD). If necessary, LPS was removed from the mAbs using

END-X from Cape Cod (East Falmouth, MA) to obtain final LPS concentrations in the low pg/mL range. Compstatin is a 13 aminoacid cyclic peptide which binds to and inhibits cleavage of C3. We used the compstatin analogue Ac-I[CV(1MeW)QDWGAHRC]T-NH2, which is 264 times more active than the parent peptide I[CVVQDWGH HRC]T-NH2. Both compstatin and a control peptide IAVVQ DWGHHRAT-NH2 were synthesised as previously described.[20] The cyclic hexapeptide AcF[OPdChaWR], a C5a receptor antagonist (C5aRa), was synthesized as previously described.[21] The murine anti-human mAbs anti-C2 and anti-factor D including the isotype-matched control mAb G3-519 has been described in detail previously.[22,23]

Bacterial preparations

E. coli strain LE392 (ATCC 33572) was obtained from American Type Culture Collection (Manassas, VA). *E. coli* was grown overnight on a Lactose dish and 5-10 colonies were transferred to LB-medium (1% Tryptone, 0.5% (w/v) yeast extract, 1% (v/v) NaCl) from Becton Dickinson (Sparks, MD) and grown overnight. The bacteria were harvested and washed once with Dulbeccos PBS without Ca^{2+} and Mg^{2+} using centrifugation (3220g, 10 minutes, 4°C). Bacteria were aliquoted, heat inactivated for 1 hour at 60°C and stored at -80°C. A frozen ampoule was thawed at ambient temperature and washed six times with PBS (3220g, 10 minutes, 4°C) to remove extracellular LPS. Bacteria intended for Alexa-staining were removed and the rest were washed additional three times. Bacteria were stained 5 minutes with SytoBC from Invitrogen Molecular Probes. Thereafter, bacteria were counted in Truecount tubes (Becton-Dickinson) using a FACScalibur or a LSRII flowcytometer (Becton-Dickinson). The heat-inactivated *E. coli* bacteria were stored in PBS at +4°C for up to two

months. The LPS concentration in the supernatant of the bacterial preparation was unchanged during the storage period.

For use in the phagocytosis assay, heat-inactivated *E. coli* (6 x 10^{9}) or *N. meningitidis* were washed six times as described above and the supernatant discarded.[23] Thereafter, NaHCO3 (0.2 M, 600 µL, pH 8.35), sterile filtered and heat inactivated (1 hour, 60°C) was added together with 6 µL Alexa FLUOR® 488 carboxylic acid, succinimidyl ester (10 mg/mL) in DMSO. The tube was packed in tinfoil and rotated for 1 hour at ambient temperature. Bacteria were washed 3 times (8000*g*, 5 minutes), resuspended in PBS and counted as described above.

Heat-inactivated wild-type *N. meningitidis* 44/76 (also named H44/76) was obtained from the National Institute of Public Health (Oslo, Norway). This international reference strain is characterized as B:14:P1.7,16:L3,7,9 and was originally isolated from a patient with invasive meningococcal disease.[24] The *N. meningitidis* 44/76 *lpxA*-mutant strain which completely lacks LPS in the outer membrane was created by L. Steeghs and P. van der Ley, National Institute of Public Health and Environment, the Netherlands,[25] and donated to the National Institute of Public Health, Oslo, for research purposes. Both strains are encapsulated. Staining of these bacteria was performed as staining of *E. coli*.

Human whole blood sepsis model

The whole blood model has been described in detail previously.[16] Briefly, samples were drawn from healthy donors into 4.5 mL NUNC tubes containing 50 µg lepirudin/mL blood. The study was approved by the Regional ethics committee. Separate tubes with complement inhibitors or PBS controls were prepared, the blood was added immediately after sampling and

tubes were preincubated 4 minutes at 37°C. Thereafter, PBS (control) or Alexa-stained or unstained bacteria was added and samples were further incubated at 37°C. Inhibitor concentrations used: anti-C2 (71 µg/mL), anti-factor D (36 µg/mL), control mAb G3-519 (107 µg/mL), compstatin (25 µM), the control peptide and C5aRa (both 10 µg/mL), and EDTA (10 mM).

Flow cytometry of erythrocytes carrying bacteria and free bacteria in plasma

Whole blood incubated with inhibitors and Alexa-stained *E. coli* or *N. meningitidis* was fixed with 0.25% (v/v) paraformaldehyde for 4 minutes (37°C). Samples were diluted 1:320 with PBS to avoid coincidences with erythrocytes not carrying bacteria and counted using Truecount tubes. Whole blood added 10 mM EDTA served as a control for coincidences. Samples were run on a FACSCalibur or LSRII flowcytometer (Becton Dickinson) with FSC and SCC in a log mode and threshold on the green channel. Gates were set around the beads, the erythrocytes and the free bacteria and calculations were made. The erythrocyte population was verified in control experiments using anti-Glycophorin A (Dako, Glostrup, Denmark). To verify that the bacteria/erythrocyte-conjugates did not appear because of PFA we performed control experiments without PFA and similar results were obtained.

Analysis of complement activation

Complement activation was analyzed as the terminal complement complex (TCC) using ELISA as previously described.[26] Results are given as arbitrary units (AU/mL).

Flow cytometry of bacterial opsonization

Lepirudin plasma was obtained after centrifugation at $+4^{\circ}C$ (3220g, 15 minutes). Plasma was preincubated 4 minutes with PBS or the indicated inhibitors at $37^{\circ}C$ in NUNC tubes. Unopsonized *E. coli, N. meningitidis* or PBS was added and the incubation continued for 10 minutes at $37^{\circ}C$. Bacteria were washed twice (3220g, 15 minutes, $+4^{\circ}C$) and resuspended in PBS containing 0.1% (w/v) BSA. C1q, C3 and C4 opsonization was analyzed using rabbit anti-human C1q, FITC-conjugated rabbit anti-human C3c (F0201) and rabbit anti-human C4c mAbs, respectively. FITC-conjugated rabbit anti-mouse Ig was used as control. All antibodies were from Dako. Results are expressed as median fluorescence intensity (MFI).

Phagocytosis assay

Whole blood incubated with inhibitors and Alexa-stained *E. coli* or *N. meningitidis* was processed according to kit instructions and phagocytosis of bacteria was analyzed in the presence of quenching solution. The assay in the absence of quenching solution reflects both phagocytosis and extracellular binding of the stained bacteria to leukocytes. Samples were run on a LSRII flowcytometer (Becton Dickinson) with FSC and SCC in a linear mode and threshold on FSC and data expressed as MFI.

Microscopy

Human whole blood was supplemented with PBS containing Alexa-stained *E. coli* or *N. meningitidis* (72 x 10^6/mL final concentration) and incubated 10 minutes at $37^{\circ}C$. Whole blood smears were made and immediately air dried. As a control, a portion of the whole blood

was diluted with PBS and examined as wet preparation by fluorescence microscopy. The smears were stored in the dark and examined using either the transmitted light observation microscopy or the reflected fluorescence procedure using appropriate filters for FITC and Alexa.

Oxidative burst

Whole blood preincubated with inhibitors and *E. coli* was processed according to kit instructions as previously described.[16] Samples were run on a LSRII flowcytometer (Becton Dickinson) with FSC and SCC in a linear mode and threshold on FSC. Results were expressed as MFI.

In vivo model of sepsis in pigs

Pigs (Sus scrofa domesticus) were anesthesized and treated surgically as previously described.[27,28] Two pigs received heat-inactivated Alexa-labeled *E. coli* and two pigs received Alexa-labeled *N. meningitidis* intravenously. A low dose of bacteria, 5.7×10^{10} in total were infused intravenously during the first 120 minutes through a central venous catheter (CVC). The initial dose was 4.5×10^8 bacteria/hour and the infusion rate was doubled every 30 minutes up to 120 minutes. After 120 minutes, a bolus containing 9.2×10^{10} bacteria was injected during approximately 1 minute. Blood samples for cytospin preparations, qPCR of bacteria in whole blood, plasma, buffy coat and erythrocyte fractions, blood gas analysis, routine hematology and flow cytometry were collected from the pigs at the times indicated. Samples for qPCR were aliquoted and stored at -70°C. Haematological parameters including leukocyte differential count were analyzed in EDTA tubes on a CELL-DYN 4000 from Abbot

Diagnostics (Abbot Park, IL) as previously described.[27] The pigs were treated with noradrenalin, fluid and sildenafilcitrate to prevent pulmonary hypertension. The experiments were performed in adherence to the Norwegian laboratory animal regulations and the study was approved by the University Animal Care Committee.

qPCR for bacterial DNA in whole blood

Lepirudin anticoagulated whole blood was centrifuged (145g, 15 minutes at +4°C) without braking. Thereafter, the plasma, buffy coat and red cell fractions were collected and stored at -80°C. DNA from whole blood, plasma, buffy coat and red cell fractions were isolated on a MagNA Pure LC instrument (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instruction using a MagNA Pure LC DNA Isolation Kit I (Roche). Genomic DNA from *E. coli* O157, strain EDL 933 (Institute for Reference Materials and Measurements, Geel, Belgium) was diluted with water, quantified by optical density measurement and used as a standard. The standard was diluted 10-fold with whole blood anti-coagulated with lepirudin (10^8 to 10^3 *E. coli* DNA copies/mL), total DNA was isolated from 200 µL of each dilution and 5 µL DNA extract was used for qPCR. The negative control was whole blood added sterile PBS and the positive control was DNA extracted from lepirudin anticoagulated whole blood mixed with DNA from *E. coli* strain B from Sigma-Aldrich. Quantification of *E. coli* DNA was performed as previously described using qPCR on a ABI 7500 instrument (Applied Biosystems, Warrington, UK).[29] Quantification of *N. meningitidis* DNA was also performed with qPCR (LightCycler; Roche Diagnostics, Basel, Switzerland), as previously described.[30] The lower detection limit of the analysis for *E. coli* and *N. meningitidis* DNA were 1 x 10^4 and 1x10^3 DNA copies/mL, respectively.

Statistics

Results were analyzed using SigmaStat version 3.5 (SPSS Science Software Gmbh, Erkrath, Germany). Data were analyzed using one-way repeated measurements ANOVA followed by Holm-Sidak post test analysis using the bacteria plus PBS as control group. Significance was assigned where $P < 0.05^{*}$.

Results

***Initial erythrocyte binding and phagocytosis of E. coli and N. meningitidis in human whole
blood.*** The majority of the *E. coli* bacteria added to human whole blood were bound to
erythrocytes after 10 minutes incubation (Figure 1A). Dose-response experiments showed a
linear relationship between the concentrations of *E. coli* and the number of free bacteria in
plasma and bacteria bound to erythrocytes (Figure 1A). Similar results were obtained for *N.
meningitidis* (data not shown). A non-linear relationship between the phagocytosis of *E. coli*
bacteria and the added bacteria concentration was found (Figure 1B). Finally, a linear
relationship between *E. coli* concentration and complement activation in plasma, measured as
TCC, was also found (Figure 1C). Control experiments confirmed that Alexa-labeled and
unlabeled bacteria activated complement to the same extent (data not shown).

Fluorescence microscopy of smears from the blood confirmed that the *E. coli* bacteria initially
bound to erythrocytes (Figure 1D), which was also the case for *N. meningitidis* (Suppl. Figure
1A). Some phagocytosed bacteria were observed (Figure 1E) and occasionally, free bacteria
were seen in plasma (Figure 1F). Similar findings were obtained using unlabeled bacteria and
fluorescence microscopy after staining with fluorochrom-labeled anti-*N. meningitidis* or anti-
E. coli or BacLight (data not shown), indicating that Alexa-labeling of the bacteria did not
influence the binding to erythrocytes. Furthermore, when microscopy was performed on
diluted whole blood in wet fluid preparations, the bacteria were mainly found on erythrocytes,
moving with the bacteria bound to their surface. Collectively, these data show that the
majority of *E. coli* and *N. meningitidis* bacteria initially bound to erythrocytes when incubated
in human whole blood.

Effect of complement inhibitors on the binding of bacteria to erythrocytes and on phagocytosis. The effect of complement inhibitors on the binding of *E. coli* and *N. meningitidis* bacteria to erythrocytes was then examined. Approximately 80% of *E. coli* (Figure 2A,B) and at least 90% of *N. meningitidis* (suppl. Figure 2A,B) initially bound to erythrocytes. Complement inhibition by mAbs blocking factor D and C2 significantly reduced the binding of *E. coli* and *N. meningitidis* to erythrocytes and simultaneously increased the number of free bacteria in plasma (Figure 2A,B and Suppl. Figure 2A,B). Complement inhibition completely blocked phagocytosis ($p<0.05$) by granulocytes and monocytes after 10 minutes (Figure 2C,D). In comparison, an isotype control mAb had no effect. As expected, a C5aRa and a control peptide had no effect on the binding of the bacteria to erythrocytes. However, the C5aRa completely blocked phagocytosis by granulocytes and partially by monocytes after 10 minutes incubation ($p<0.05$). EDTA, blocking both complement- and cell-activation, almost completely inhibited both the binding of bacteria to erythrocytes, leaving them in plasma (Figure 2A,B and Suppl. Figure 2A,B) and efficiently blocked the phagocytosis (Figure 2C,D and Suppl. Figure 2C,D). As a control, bacteria were added in PBS buffer in the absence of blood to check the bacterial number added (Figure 2A and Suppl. Figure 2A).

Time course study on the effect of complement inhibitors on E. coli binding to erythrocytes and on granulocyte phagocytosis. The number of free *E. coli* bacteria in plasma was low at all time points, except in the presence of EDTA (Figure 3A). The binding of *E. coli* to erythrocytes was time-dependent and slowly decreased with time (Figure 3B). Complement inhibition using the C3 convertase inhibitor compstatin significantly decreased the binding to erythrocytes. The number of phagocytosed bacteria by granulocytes increased with time in the absence of inhibitor (Figure 3C). The data indicate that the complement-dependent binding of

bacteria to erythrocytes *in vitro* last several hours and the phagocytosis increases when the bacteria are released from the erythrocytes. However, neither compstatin nor EDTA completely inhibited phagocytosis after 60 and 120 minutes, indicating that the complement-independent phagocytosis of *E. coli* increased with time.

Effect of complement inhibitors on bacterial C3 and C4 opsonization. The effect of complement inhibitors compstatin and C5aRa on the C3 and C4 opsonization on *E. coli* and *N. meningitidis* bacteria were then examined in lepirudin plasma using flow cytometry (Figure 4). Compstatin efficiently reduced C3 opsonization on both *E. coli* and *N. meningitidis* (Figure 4A,C), whereas the C4 opsonization increased (Figure 4B,D). Notably, LPS had no effect on binding or inhibition of the opsonins since wild-type *N. meningitidis* and the LPS-deficient mutant behaved identical (Figure 4C,D). As expected, the C5aRa and its corresponding control peptide had no effect on bacterial opsonization. The calcium chelator EDTA efficiently blocked both C3 and C4 opsonization as expected, since it efficiently inhibits all complement activation pathways. C1q opsonization was very low and was not different from the MFI in the gamma control mAb (data not shown).

Effect of the anti-CR1 blocking mAb 3D9 on the binding of E. coli to erythrocytes*, on *phagocytosis and on oxidative burst. The anti-CR1 blocking mAb 3D9 dose-dependently increased the number of free *E. coli* in plasma (Figure 5A) and simultaneously efficiently blocked *E. coli* binding to erythrocytes (Figure 5B). Interestingly, the anti-CR1 also effectively increased the granulocyte phagocytosis of the bacteria (Figure 5C). Similar findings were obtained for monocyte phagocytosis (data not shown). This finding suggests that the binding of bacteria to erythrocytes reduce leukocyte phagocytosis by reducing the number of bacteria available to leukocyte recognition. Microscopy of whole blood smears also

indicated that bacteria alone and not bacteria bound to erythrocytes were phagocytosed by leukocytes.

The effect of anti-CR1 on *E. coli*-induced oxidative burst was then examined (Figure 5D). The anti-CR1 blocking mAb significantly increased ($p<0.05$) *E. coli*-induced oxidative burst in granulocytes. In comparison, a control mAb had no effect. The data indicate that blocking of bacterial binding to erythrocyte CR1 significantly increase phagocytosis and oxidative burst in leukocytes, implying that bacterial binding to erythrocytes protects against leukocyte oxidative burst in whole blood.

Effect of anti-CR1 mAb 3D9 on the binding to erythrocytes of N. meningitidis with and without LPS. To examine whether LPS was involved in the binding of bacteria to erythrocyte CR1, we examined the effect of the CR1 blocking mAb 3D9 on the binding of *E. coli*, wild-type 44/76 *N. meningitidis* with LPS and the LPS-deficient 44/76*lpxA*-mutant to erythrocytes. All three bacteria efficiently and similarly bound to erythrocytes after 10 minutes incubation and the anti-CR1 blocking mAb 3D9 inhibited this binding completely (Figure 6). In comparison, an isotype control mAb had no effect. The calcium chelator EDTA also completely blocked the binding of all three bacteria to erythrocytes. As a control we also added the same concentration of all three bacteria to PBS buffer only, in the absence of whole blood (right columns), and the same concentration of bacteria was found as in the whole blood samples. The binding of *N. meningitidis* 44/76*lpxA*-mutant to erythrocyte CR1 clearly shows that this binding is LPS-independent.

***In vivo* experiments with Alexa-stained *E. coli* and *N. meningitidis* 44/76 in a porcine model of sepsis.** Finally we wanted to obtain information about erythrocyte binding and the

fate of Gram-negative bacteria *in vivo* by the porcine model of Gram-negative sepsis, since pig erythrocytes lack CR1. The peak concentrations of *E. coli and N. meningitidis* were 6 x 10^7 and 2 x 10^5 bacteria/mL whole blood, respectively (Fig. 7A,B). The number of bacteria in the erythrocyte fraction, in plasma or in the buffy coat were then analyzed using qPCR of bacterial genome and the percentage in each fraction calculated (Figure 7C,D). The percentage of *E. coli* bacteria in the erythrocyte fraction was only 24% approx. five minutes after the bolus injection and decreased to 14% 55 minutes later (Figure 7C). During the same time period, the percentage of *E. coli* in plasma decreased from 42% to 30% and increased from 34% to 56% in the buffy coat fraction. In comparison, only 6% of *N. meningitidis* was in the erythrocyte fraction shortly after the bolus injection (Fig. 7D), indicating that pig erythrocytes, in contrast to humans, hardly bind Gram-negative bacteria. Notably, immunofluorescence histology of lungs, spleen and liver of the pigs after 4 hours showed that the Alexa-stained bacteria was mainly located in the lungs and not in the liver and spleen (Figure S3).

Discussion

The present study shows that erythrocytes rapidly bind the Gram-negative bacteria *E. coli* and *N. meningitidis* in human whole blood. This binding is complement-dependent, LPS-independent and occurs through CR1. Blocking CR1 led to release of bacteria to plasma with enhanced phagocytosis and subsequent oxidative burst by leukocytes. The data thus provides new insights into important mechanisms involved in the pathophysiology of Gram-negative sepsis in humans, suggesting that erythrocyte binding of the bacteria protects against intravascular phagocytosis and, thus, attenuate systemic inflammation including oxidative burst.

Since human erythrocytes express complement receptor CR1 and leukocytes express CR1 and CR3,[5] we expected that both cell types would bind complement opsonized bacteria. The finding that approximately 80-90% of the Gram-negative bacteria initially binds to erythrocytes is per se most likely due to the approx. 400-1500 fold higher concentration of erythrocytes compared to leukocytes in human whole blood. In addition, erythrocyte CR1 may bind bacteria with a higher avidity than leukocyte CR1 as shown for the binding of IC.[31] The reported ligands for human CR1 are C3b, iC3b, C4b, MBL and C1q.[5] In this study, C3 and C4, but not C1q (data not shown) was detected on the bacterial surfaces. Complement factors seem to be the most important opsonins involved in the binding of Gram-negative bacteria to erythrocytes since inhibitors of complement activation at the level of C3 significantly reduced this binding. The increased C4 opsonization found after incubation with the C3 inhibitor compstatin probably occurred due to increased space for C4 deposition on the bacterial surface when the C3 deposition was reduced. Such C4b deposition probably explains why some binding of bacteria to erythrocytes was still seen when complement was inhibited. In

comparison, EDTA abolished both C3 and C4 opsonization with almost completely absent binding of bacteria to erythrocytes as a result. The anti-CR1 mAb 3D9, which specifically inhibits the binding of C3b/C4b to CR1,[19,32] also completely blocked the binding to erythrocytes. This further indicates that C3b and C4b are the most important opsonins in the binding of bacteria to erythrocytes and that this binding occurs solely by binding to CR1. As expected, the C5aRa had no effect on the binding to erythrocytes, but significantly inhibited phagocytosis.[16] The almost linear reduction in bacteria bound to erythrocytes with time indicates that the initial rate of bacterial release from erythrocytes is relatively constant. The release process is most probably due to a time-dependent change in the number of C3b molecules, converted by factor I to the less affinity CR1 ligand iC3b, on the bacterial surface.[33,34,35] In addition, increasing phagocytosis of bacteria by leukocytes reduce the number of bacteria available for binding to erythrocytes.

The binding of bacteria to erythrocyte CR1 was LPS-independent since the *N. meningitidis* LPS deficient mutant bound as efficiently to the erythrocytes and was as efficiently released when CR1 was blocked as the wild-type strain. Both the wild-type and the LPS-deficient *N. meningitidis* mutant used in this study are encapsulated, but the opsonization probably also occurs under the capsule surface. This is in line with the observation that the opacity protein in the subcapsular outer membrane and other neisseria structures binds C3b and C4b.[36] The observation that both LPS-containing and LPS-deficient Gram-negative bacteria initially bind to erythrocyte CR1 further indicates that other bacterial structures than LPS bind C3b and C4b.

The rapid binding of bacteria to erythrocytes may affect and delay the reactions of peripheral leukocytes to bacteria in the circulation, as indicated by our finding that blocking CR1

increased the *E. coli*-induced phagocytosis and oxidative burst in monocytes and neutrophils. This is in accordance with previous studies using immune complexes showing that binding of immune complexes to erythrocyte CR1 inhibited IC mediated activation of neutrophils.[37,38] Interestingly, in the first study of the immune adherence phenomenon with bacteria by Nelson from 1953 it was shown that the phagocytosis of pneumococci by guinea pig macrophages increased in the presence of human erythrocytes.[2] Subsequent studies have confirmed the particular importance of the mononuclear phagocyte system of the liver and spleen for the removal of circulating IC and bacteria and apparently only a minor proportion of bacteria in the blood are phagocytosed by circulating leukocytes.[39,40,41,] These findings are in line with a recently published study demonstrating a functional role of erythrocyte CR1 in clearance of pneumococci from the circulation by facilitating the transfer of pneumococci to liver macrophages.[42]

Interestingly, the proportion of bacteria being associated with the erythrocyte fraction in the *in vivo* studies using the porcine model of sepsis was substantially lower than we found in the *in vitro* experiments with human whole blood. Notably, the erythrocytes of pigs do not express human CR1 or similar receptors, as these are restricted to primates.[5] We speculate that the low proportion of bacteria bound to erythrocytes in the pigs, could explain why a majority of the bacteria was removed from the circulation by the lungs and not by the liver.[29] In addition, capillaries in porcine lungs are lined with macrophages probably involved in the removal of bacteria from the circulation. It appears that binding of complement opsonized bacteria to erythrocyte CR1 can serve as a mechanism in humans to protect against systemic inflammation by directing the pathogenic agents particularly to the liver and spleen where they can be more safely deposited by the mononuclear phagocyte system with less systemic inflammatory responses. This implies a supplementary role of complement to its commonly

regarded primary functions of mediating phagocytosis and serum bactericidal activity, and might have implications for a potential use of complement inhibition as suggested as adjuvant therapy in sepsis.[43]

The effects of specific complement inhibitors may have several possible implications on the bacterial binding to erythrocytes. By using a complement inhibitor acting early in the complement cascade, i.e. at the C3 level or previous steps, the bacterial opsonization would be decreased, leading to reduced bacterial binding to erythrocytes and consequently increased numbers of free bacteria in plasma as shown in this study. Decreased opsonization and decreased up-regulation of CR3[23] would also lead to reduced phagocytosis by peripheral leukocytes and probably also tissue macrophages, further increasing the number of free bacteria in plasma. Decreased phagocytosis may decrease those parts of the septic inflammatory response being related to neutrophil activation and oxidative burst, i.e. release of reactive oxygen metabolites and other products thought to be important in some of the pathophysiological changes of sepsis, particularly capillary leakage.[44] However, increased concentrations of circulating pathogens, even if they are dead due to adequate antibiotic treatment, may also increase the exposure of pathogen associated molecular patterns to their respective receptors including the Toll-like receptors and consequently increase the inflammatory response with increased secretion of pro-inflammatory cytokines.[45] Also, inflammatory cells in organs more vulnerable to inflammation than the liver and spleen, like the lungs and the kidneys may be more activated. An alternative strategy is to inhibit complement at a later step such as blocking the potent anaphylatoxin C5a by blocking C5aR, which implies that the bacteria will still be fully opsonizied and may bind to both erythrocyte and leukocyte CR1. According to the results in this and previous studies,[16] C5aR inhibition would substantially decrease granulocyte phagocytosis due to diminished up-regulation of

CR3. However, according to our results phagocytosis by monocytes and thus, possibly also tissue macrophages in the liver and spleen, would be better maintained with C5aR inhibition than with inhibition of previous steps with decreased complement opsonization. Accordingly, inhibition of C5aR may be an attractive approach when searching for a complement inhibitor to be used in sepsis since it did not affect the binding of bacteria to erythrocytes.

In conclusion, the present data provide new and important insights into the initial interaction of Gram-negative bacteria with erythrocytes and leukocytes in human whole blood and give some indications on how bacterial binding to erythrocyte CR1 influence the handling of the bacteria *in vivo*. The mechanisms described are suggested to be of clinical importance in sepsis and especially in the development of complement inhibitors as potential therapeutic agents in sepsis.

Authorship and Conflict of Interest Statements

The authors O.L.B., B.C.H., D.C., H.F., A.C., E.W.N., A.P., J.L., G.B., P.B. and T.E.M. designed research, performed research, interpreted data and participated in writing the manuscript. The authors G.L. and J.D.L. both contributed with vital reagents, planning of the experiments and writing of the manuscript. The author J.D.L. has a potential conflict of interest concerning several patent applications on complement inhibitors. None of the other authors have potential conflicts of interest.

Figure 1. Dose-response effect of *E. coli* on the early binding to erythrocytes, complement activation and phagocytosis in human whole blood. Alexa-labeled *E. coli* was added to fresh whole blood (72 x 10^6 bacteria/mL) and incubated 10 minutes at 37^oC. (A) Free bacteria in plasma (filled circles) and erythrocyte bound bacteria (open triangles) were analyzed using flow cytometry and results are given as 10^6 bacteria/mL. (B) Phagocytosis in granulocytes (open triangles) and monocytes (open circles) was analyzed using flow cytometry and given as median fluorescence intensity (MFI). (C) Complement activation was analyzed as the terminal complement complex (TCC) in plasma using ELISA and expressed as arbitrary units AU/mL (filled triangles). Data are presented as means and SD from three to six independent experiments using different blood donors each time. (D, E, F) Microscopy of Alexa-labeled *E. coli* bacteria in whole blood smears made after 10 minutes incubation (1000x magnification). Combined reflected fluorescence and transmitted light microscopy was performed according to the microscope instructions using very weak transmitted light intensity. The Olympus BX51TRF microscope was equipped with a ColorView IIIu digital camera with 5 megapixel resolution and was controlled by the CellP program (Soft imaging system, Münster, Germany). (D) *E. coli* bound to erythrocytes, (E) *E. coli* phagocytosed in a leukocyte and (F) *E. coli* free in plasma. Results from one of five representative experiments are shown.

Figure 2. Effect of complement inhibitors on the binding of *E. coli* to erythrocytes and phagocytosis in granulocytes and monocytes. Alexa-labeled *E. coli* (72 x 10^6/mL) was added to human whole blood and incubated at 37^oC for 10 minutes in the presence of mAbs blocking C2 and factor D (Anti-C2/D), an isotype control (Ctr. mAb), a C5aR antagonist (C5aRa) and its corresponding control peptide (Ctr. pep.), or EDTA. (A) *E. coli* in plasma and

(B) erythrocyte bound *E. coli* was analyzed using flow cytometry and expressed as 10^6 bacteria/mL (Bact. 10^6/mL). Bacteria added to PBS in the absence of whole blood were included as a control (hatched bar to the right). Granulocyte (C) and monocyte (D) phagocytosis of *E. coli* with (black bars) and without quenching solution (white bars) was analyzed using flow cytometry and given as median fluorescence intensity (MFI). Results are presented as means and SD of data from three to six different blood donors. *P <0.05 compared to *E. coli* alone using one-way repeated measurements ANOVA and the Holm-Sidak post test.

Figure 3. Time course of *E. coli* binding to erythrocytes, phagocytosis and effect of complement inhibitors. Alexa-labeled *E. coli* (72 x 10^6/mL) was added to human whole blood and incubated for up to 120 minutes (37^oC). (A) *E. coli* in plasma and (B) erythrocyte bound *E. coli* and (C) granulocyte phagocytosis with quenching solution were analyzed using flow cytometry. Complement inhibitors added: compstatin (black triangle), its control peptide (white triangle), C5aRa (white square) and PBS control (black circles). The calcium chelator EDTA (black diamond) was included as a control. Results are presented as means and SD (n=3) using different blood donors. *P <0.05 compared to *E. coli* plus PBS as control group analyzed after 120 minutes incubation using one-way repeated measurements ANOVA followed by the Holm-Sidak post-test.

Figure 4. Effect of complement inhibitors on *E. coli* and *N. meningitidis* C3 and C4 opsonization. The effect of complement inhibitors compstatin, its corresponding control peptide (Ctr. peptide) and a C5aRa on bacterial C3 and C4 opsonization was examined. (A,B) *E. coli* (72 x 10^6/mL), (C,D) *N. meningitidis* (72 x 10^6/mL) with LPS (black bars) or the LPS deficient 44/76*lpxA*-mutant (white bars) were incubated 10 minutes in lepirudin plasma

(+Plasma) supplemented with PBS or complement inhibitors as indicated. The calcium chelator EDTA which efficiently inhibits complement was included as control. Bacterial C3 (A,C) and C4 (B,D) opsonization was analyzed using flow cytometry and results given as median fluorescence intensity (MFI). Results are given as means and SD from separate experiments using plasma from three different healthy donors.*P <0.05 compared to bacteria in PBS alone by one-way repeated measurements ANOVA followed by the Holm-Sidak post-test.

Figure 5. Effect of the anti-CR1 blocking mAb 3D9 on free *E. coli* in plasma, *E. coli* on erythrocytes, phagocytosis and oxidative burst. The anti-CR1 blocking mAb 3D9 was added in increasing concentrations to whole blood without (open diamonds) or with 72 x 10^6 *E. coli*/mL (black circles) added. (A,B) Free *E. coli* in plasma (A) and bacteria bound to erythrocytes (B) was analyzed using flow cytometry and given as 10^6 bacteria/mL (Bact. 10^6/mL). (C) Phagocytosis with (filled symbols) and without quench (open symbols) was analyzed in granulocytes (open and filled triangles). Effect of the isotype matched control mAb G3-519 (open and filled squares) is indicated. Data from one representative of three experiments are shown. (D) Granulocyte oxidative burst in the absence of *E. coli* (open diamond) and after stimulation with *E. coli* in the presence of anti-CR1 (filled circles) or a control mAb (open squares) analyzed using flow cytometry and given as median fluorescence intensity (MFI). Data are given as means and SD (n=3). $^*P$<0.05 compared to the *E. coli* + PBS control.

Figure 6. Effect of the anti-CR1 blocking mAb 3D9 on the binding to erythrocytes of *E. coli, N. meningitidis* with LPS and *N. meningtitidis* without LPS. Whole blood was incubated with 72 x 10^6 bacteria/mL in the presence of PBS, anti-CR1 mAb 3D9 (Anti-CR1, 4

µg/mL), an isotype matched control mAb (Ctr. mAb, 4 µg/mL) or the calcium chelator EDTA. In addition, bacteria added to PBS in the absence of whole blood were included as a control (hatched bar to the right). The number of free bacteria in plasma (white bars), and bacteria bound to erythrocytes (black bars) was analyzed using flow cytometry and given as 10^6 bacteria/mL (Bact.10^6/mL). Data are given as means and SD from individual experiments with three different blood donors. [*]$P<0.05$ analyzed by one-way repeated measurements ANOVA using the Holm-Sidak post-test and multiple comparisons versus the bacteria plus PBS as control group.

Figure 7. The time course after intravenous infusion of bacteria in pigs analyzed using qPCR. (A) *E. coli* and (B) *N. meningitidis* genome were quantified in whole blood samples from two different experiments using qPCR and expressed as bacteria/mL (log scale). Results are given as means and SD. (C, D) The fraction of bacteria in erythrocyte (black bar), buffy coat (white bar) and plasma fractions (hatched bar) expressed as % of total bacteria concentration.

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Fig. 1

Fig. 2

A *E. coli* in plasma

Bact.(10⁶/mL)

B *E. coli* on erythrocytes

Bact.(10⁶/mL)

C Granulocyte phagocytosis

MFI

D Monocyte phagocytosis

MFI

T10, PBS, Anti-C2/D, Ctr. mAb, C5aRa, Ctr. pep., EDTA, No blood

- *E. coli*   + *E. coli*

Fig. 3

A *E. coli* in plasma

B *E. coli* on erythrocytes

C Granulocyte phagocytosis

Fig. 4

Fig. 5

A Free *E. coli* in plasma

B *E. coli* on erythrocytes

C Granulocyte phagocytosis

D Oxidative burst
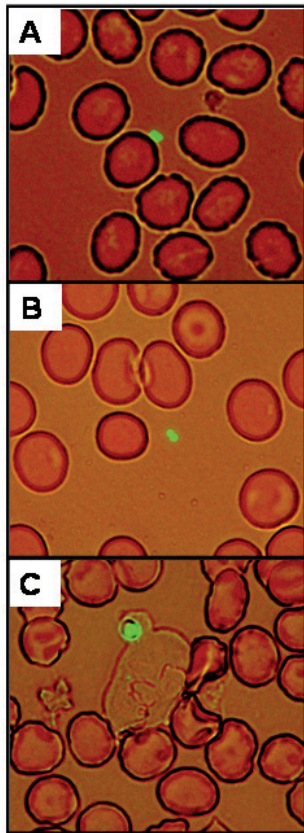
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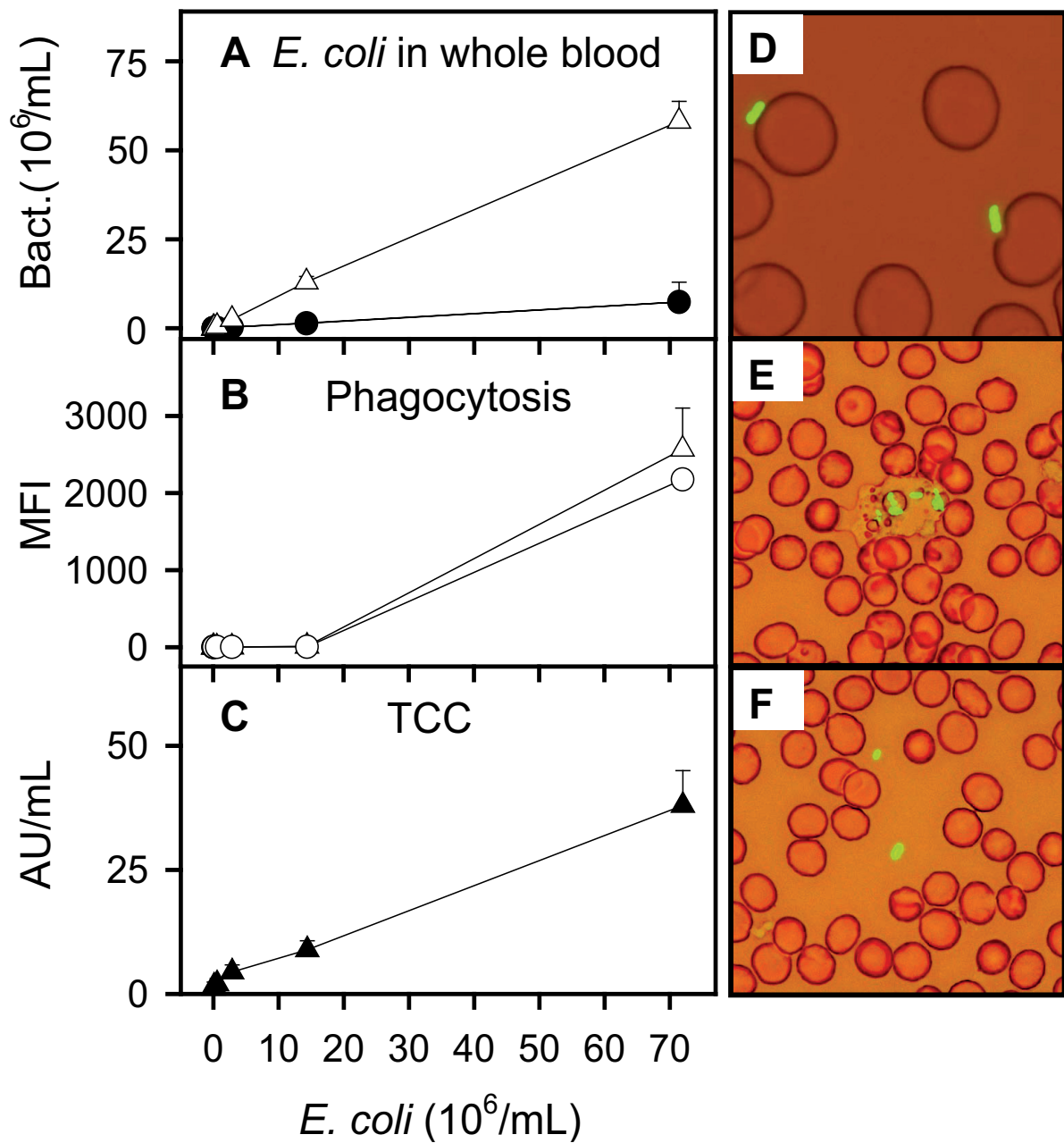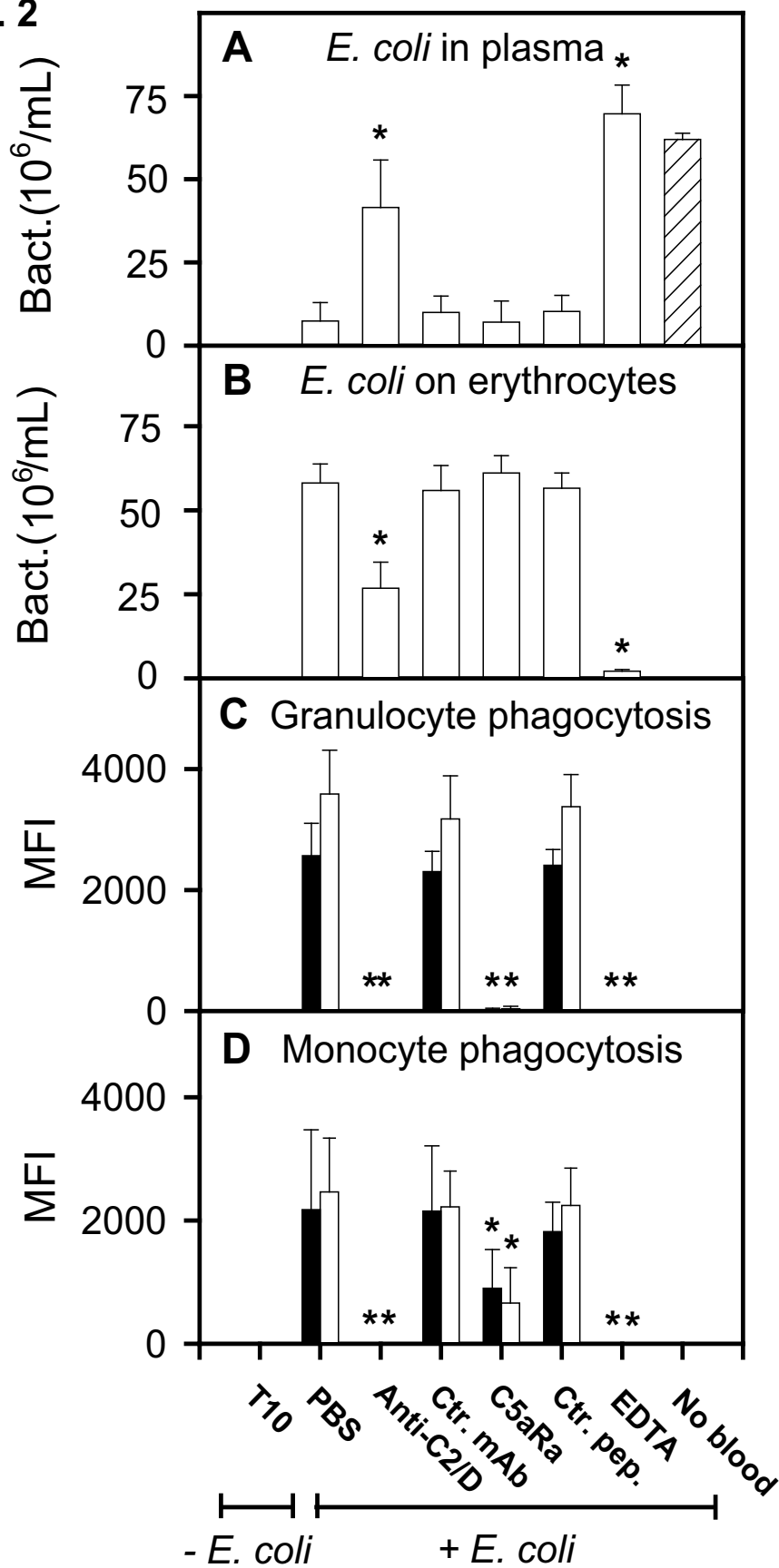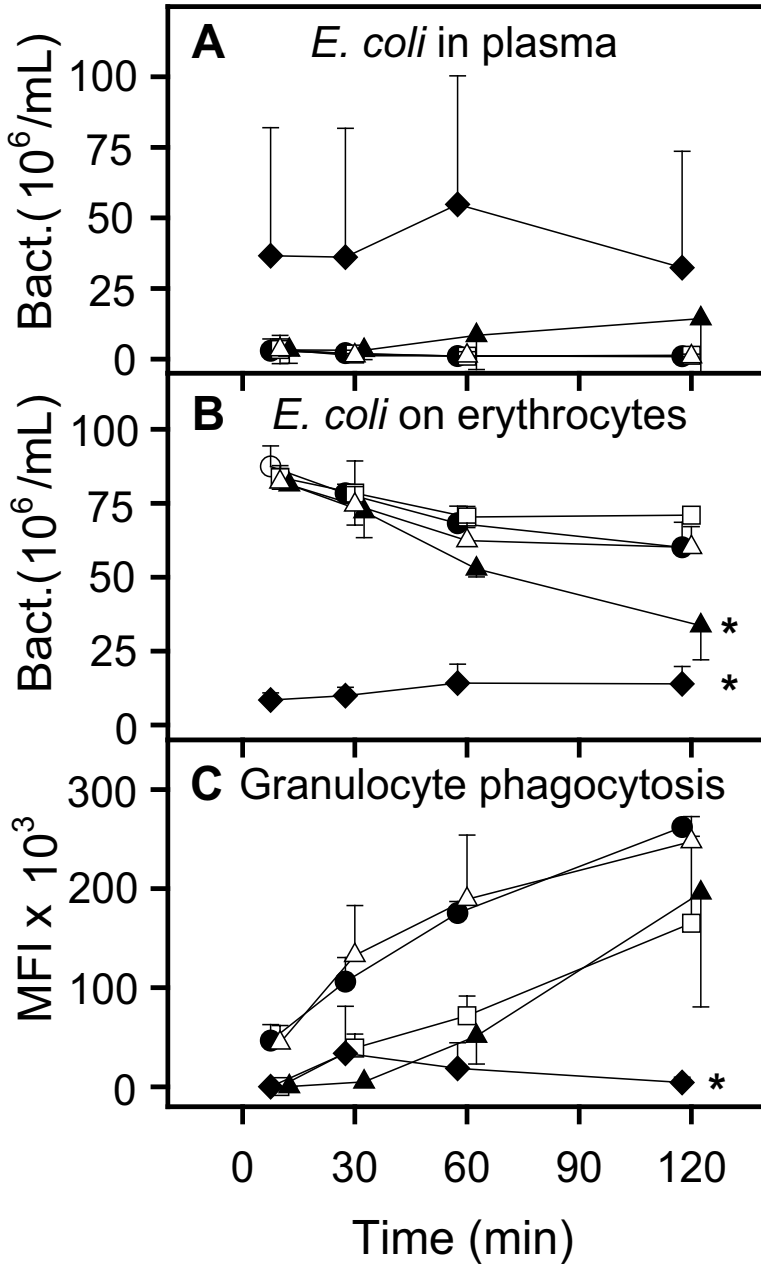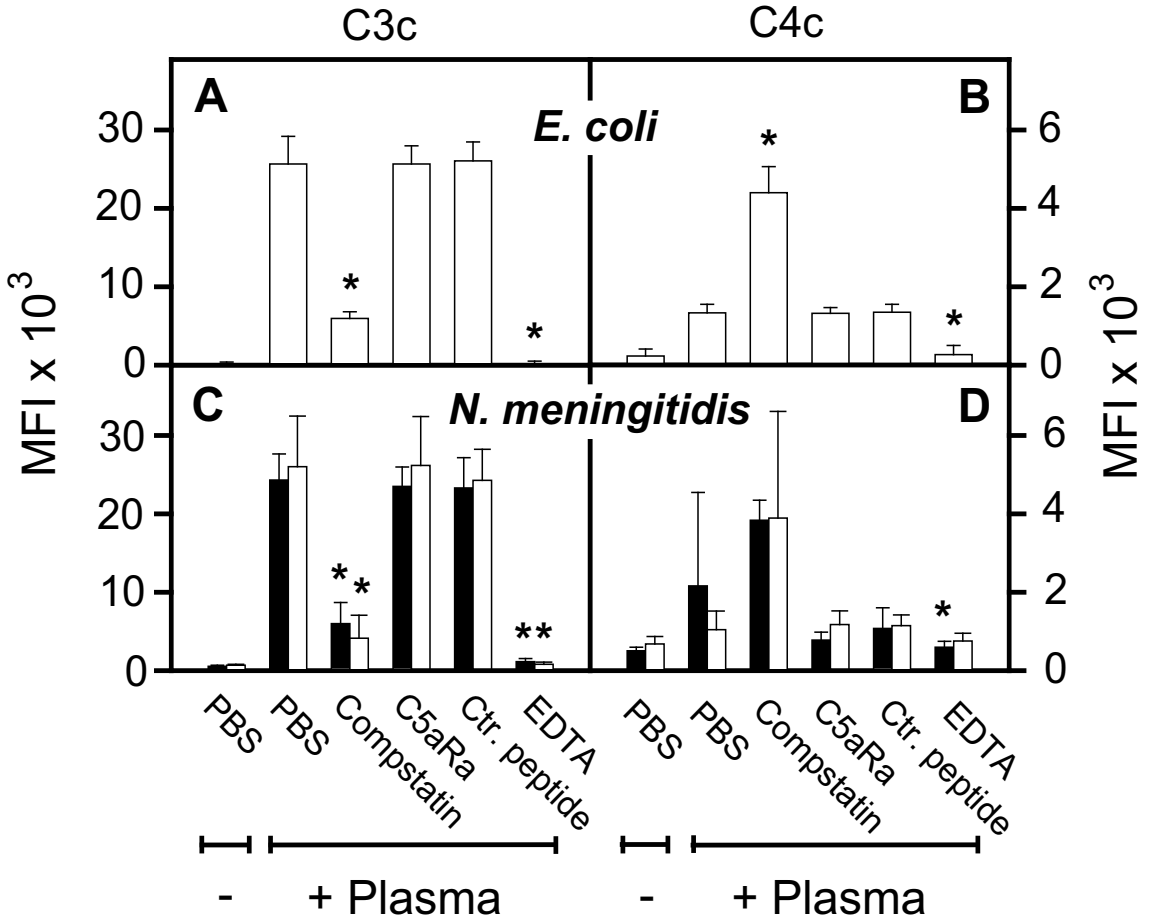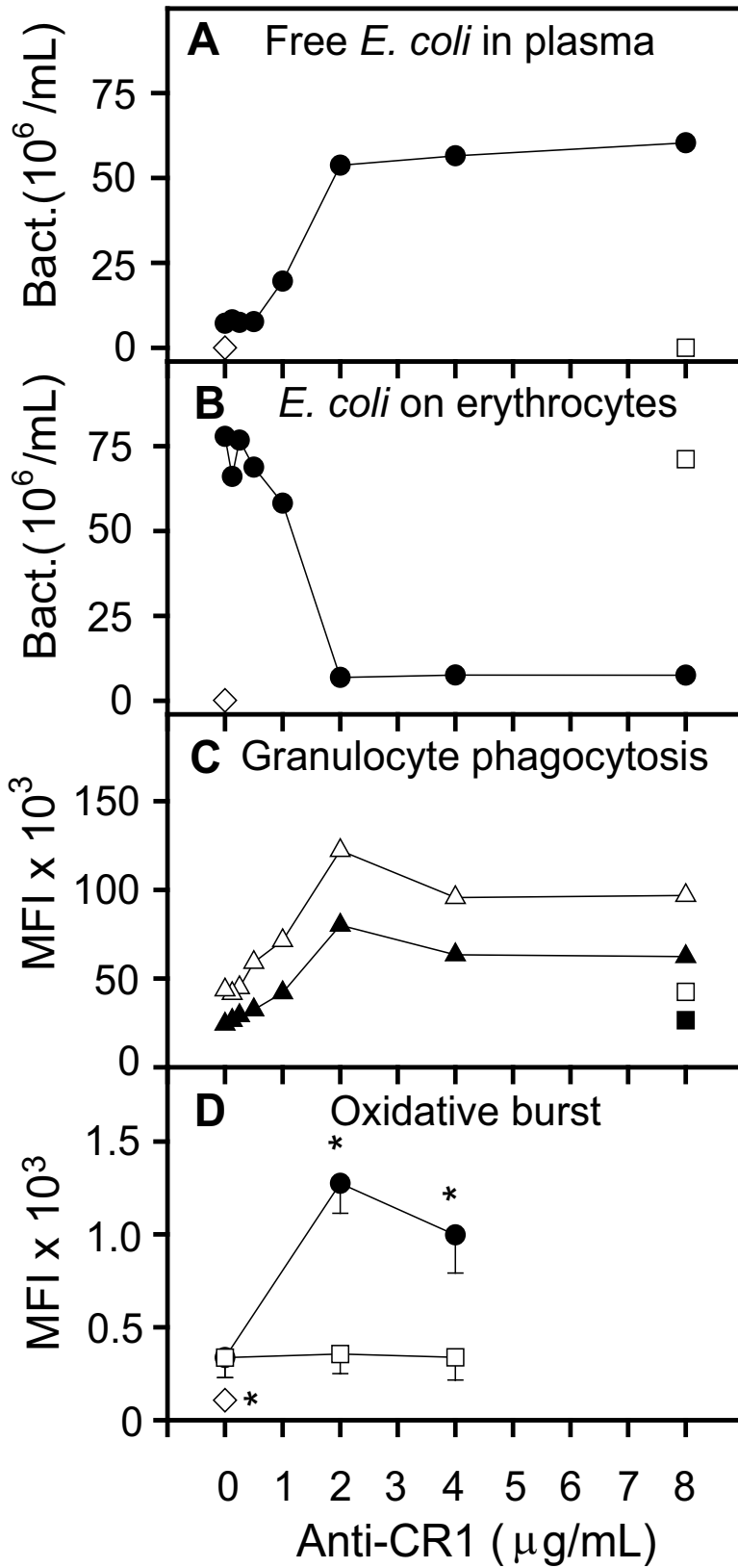
Fig. 6
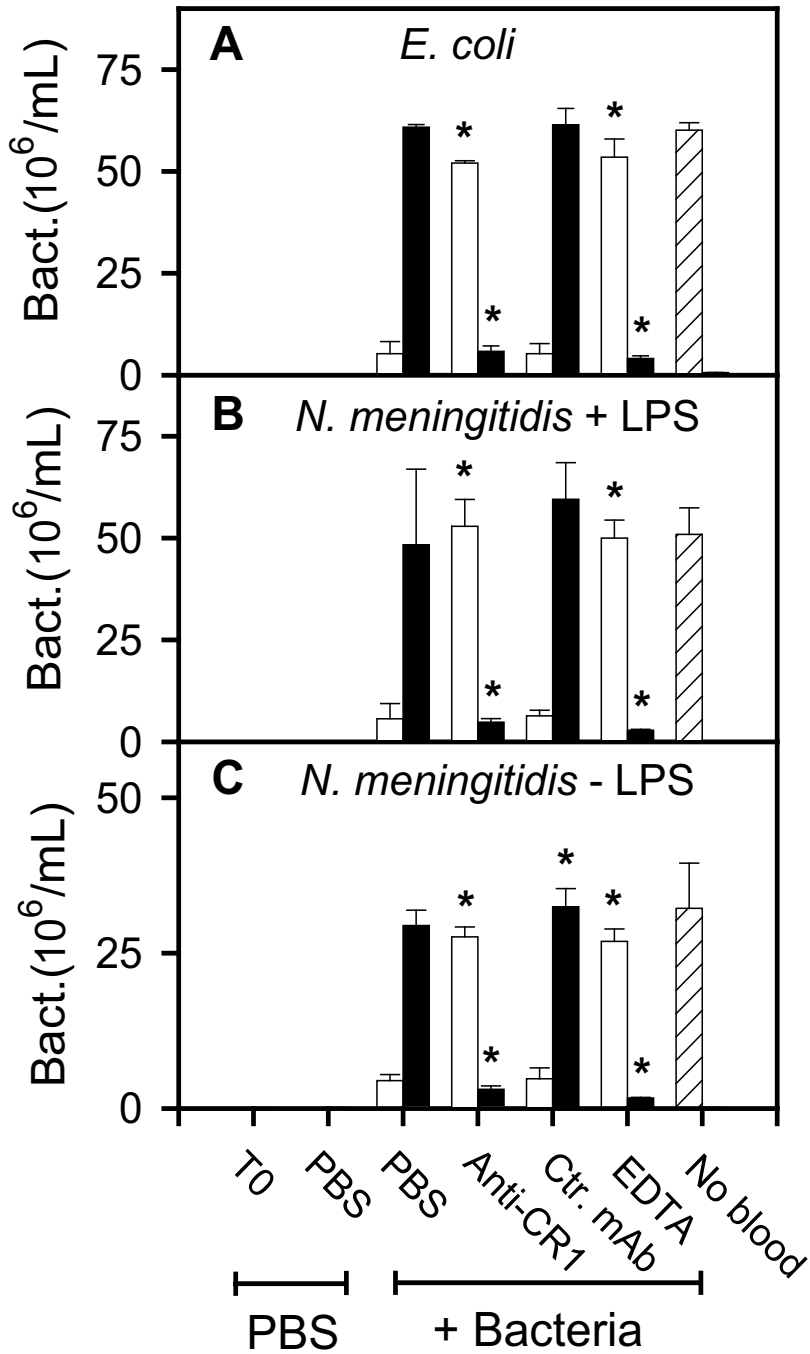
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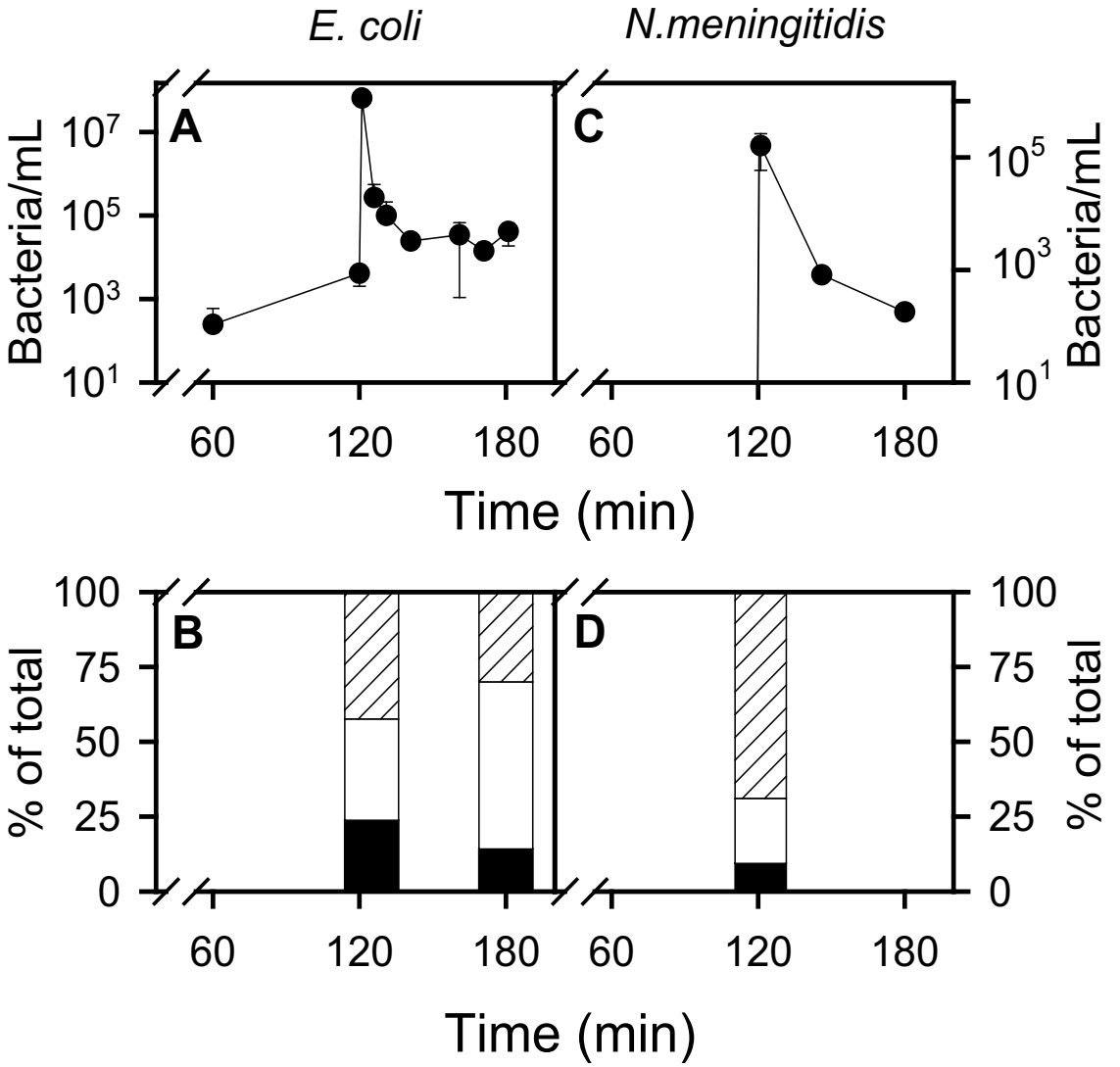
Fig. S1

Figure S1. Microscopy of *N. meningitidis* binding to erythrocytes in human whole blood smears. Alexa-labeled *N. meningitidis* (72×10^6 bacteria/mL) were added to human whole blood. After 10 minutes incubation, blood smears were made and the samples analyzed using combined fluorescence and transmitted light microscopy (1000x magnification) on a Olympus BX51TRF microscope equipped with a ColorView IIIu digital camera. (A) *N. meningitidis* bound to erythrocytes, B: *N. meningitidis* free in plasma, (C) *N. meningitidis* phagocytosed in a leukocyte. Results from one of three representative experiments are shown.
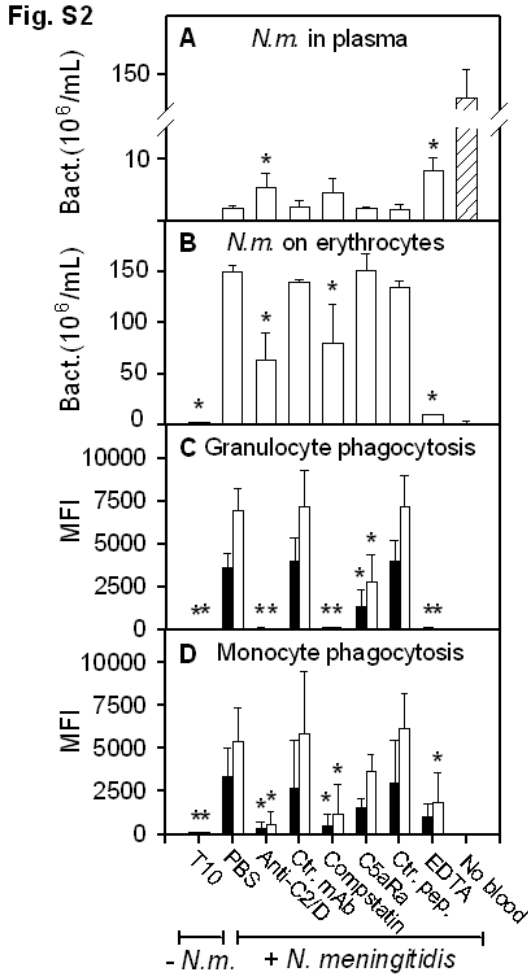
Figure S2. Effect of complement inhibitors on the binding of *N. meningitidis* **44/76 to erythrocytes.** The effect of complement inhibitors on free *N. meningitidis* (*N.m.*) in plasma, bound to erythrocytes and granulocyte and monocyte phagocytosis in human whole blood. Alexa-labeled *N. meningitidis* (72×10^6/mL) were added to human whole blood and incubated at 37°C for 10 minutes in the presence or absence of PBS, anti-C2 and anti-factor D (Anti-C2/D), its corresponding control mAb (Ctr. mAb) , compstatin, C5aRa, a control peptide (Ctr.

pep.) or EDTA. (A) *N. meningitidis* in plasma and (B) erythrocyte bound *N. meningitidis* were analyzed using flow cytometry and expressed as 10^6 bacteria/mL (Bact. 10^6/mL). Granulocyte (C) and monocyte phagocytosis (D) of bacteria was analyzed using flow cytometry and expressed as MFI. Phagocytosis was analyzed with (black bars) and without (white bars) quenching solution, reflecting phagocytosed and phagocytosed plus surface bound bacteria, respectively. Results are given as means and SD of three different blood donors. *$P<0.05$ compared to *N. meningitidis* alone using one-way repeated measurements ANOVA and the Holm-Sidak post-test.
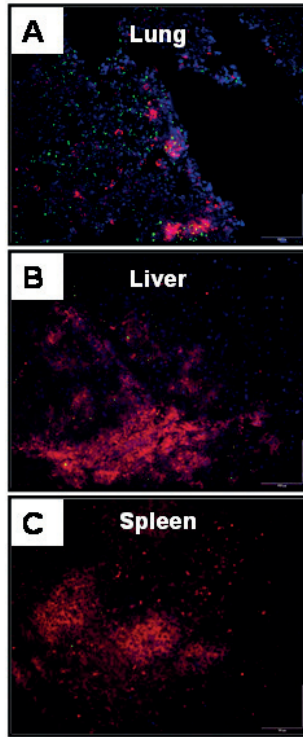
Figure S3. Microscopy of *E. coli* in porcine lung, liver and spleen in vivo. Alexa-stained *E. coli* (green color) were infused intravenously in pigs. After 4 hours, the organs were frozen and immunofluorescence histology was performed on cryosections obtained from the (A) lung, (B) liver and (C) spleen. Cryosections (5 µm thick) were cut from tissue embedded and snap-frozen in O.C.T Compound (Tissue-Tek; BDH, Lutterworth, UK). Sections were air dried and fixed for 10 minutes in ice-cold acetone. Fc receptors were blocked by incubating the sections for 30 minutes with PBS containing 5% pig serum and 5% goat serum. To identify tissue macrophages, a pretitrated anti-porcine CD45 monoclonal antibody (a kind gift

from Karin Haverson, University of Bristol, Bristol, UK) was applied and incubated for 2 hours. Slides were washed thoroughly 3 times with PBS for 5 minutes. An isotype-specific goat anti-mouse antiserum (Southern Biotechnology, Birmingham, AL) conjugated to Texas Red was then applied and incubated for 1 hour. The slides were washed three more times, and the nuclear dye DAPI was applied and incubated for 10 minutes. After a final wash, the sections were mounted in Fluoromount (Vector Laboratories, Burlingame, CA) and sealed with nail varnish. Stained slides were examined using a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) fitted with a combined excitation and emission filter block specific for the applied fluorescence staining. Samples were analyzed by fluorescence microscopy using 200x magnification. Macrophages was stained using Texas Red (red color) and cellular nuclei were stained using DAPI (blue color).

IV