

Upstream versus downstream inhibition of Gram-negative induced inflammation

PhD Thesis

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LIST OF PAPERS

Study I

Barratt-Due A., E.B. Thorgersen, J.K. Lindstad, A. Pharo, O.L. Brekke, D. Christiansen, J.D. Lambris, T.E. Mollnes. 2010. Selective inhibition of TNF- α or IL-1 β does not affect *E. coli*-induced inflammation in human whole blood. *Mol.Immunol.*47:1774-82

Study II

Barratt-Due A., H.T. Johansen, A. Sokolov, E.B. Thorgersen, B.C. Hellerud, J.L. Reubsact, K. F. Seip, T.I. Tønnessen, J.K. Lindstad, A. Pharo, A. Castellheim, T.E. Mollnes, E.W. Nielsen. 2011. The role of bradykinin and the effect of the bradykinin receptor antagonist icatibant in porcine sepsis. *Shock* 36:517-23

Study III

Barratt-Due A., E.B. Thorgersen, J.K. Lindstad, A. Pharo, O. Lissina, J.D. Lambris, M.A. Nunn, T.E. Mollnes. 2011. *Ornithodoros moubata* complement inhibitor (OmCI) is an equally effective C5 inhibitor in pig and human. *J.Immunol.*187:4913-19

Study IV

Barratt-Due A., K. Egge, E.B. Thorgersen, A. Sokolov, B.C. Hellerud, S. Pischke, J.K. Lindstad, A. Pharo, M.A. Nunn, H. Scott, T.E. Mollnes. Combined inhibition of Complement (C5) and CD14 attenuates inflammation, trombogenicity and hemodynamic changes in porcine sepsis. *Submitted*

SELECTED ABBREVIATIONS

Ab	Antibody
AP	Alternative Pathway
BK	Bradykinin
CLP	Cecal Ligation and Puncture
CP	Classical Pathway
CR1	Complement Receptor 1
CR3	Complement Receptor 3
C3aR	C3a Receptor
C5aR	C5a Receptor
DIC	Disseminated Intravascular Coagulation
<i>E. coli</i>	<i>Escherichia coli</i>
EIA	Enzyme Immunoassay
ICU	Intensive Care Unit
Ig	Immunoglobulin
IL	Interleukin
LP	Lectin Pathway
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MAP	Mean Arterial Pressure
MBL	Mannose Binding Lectin
MFI	Median Fluorescence Intensity
MOF	Multiple Organ Failure
mg	Milligram
mL	Milliliter
mM	Millimolar
mmol	Millimol
MPAP	Mean Pulmonary Arterial Pressure
MyD88	Myeloid Differentiation Primary Response Gene 88
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
OmCI	<i>Ornithodoros moubata</i> Complement Inhibitor
PAI-1	Plasminogen Activator Inhibitor
PBS	Phosphate Buffer Saline

RA	Ringer Acetate
SIRS	Systemic Inflammatory Response Syndrom
SVRI	Systemic Vascular Resistance Index
TAT	Thrombin-Antithrombin Complex
TCC	Terminal Complement Complex
TF	Tissue Factor
TNF- α	Tumor Necrosis Factor α
TLR	Toll-Like Receptor

INTRODUCTION

Throughout history, infectious disease has been a leading cause of death in humans. Descriptions of sepsis date back to antiquity. Hippocrates viewed it as evil and as a dangerous biological decay (1). The word “sepsis” derives from Greek, and refers to decomposition of animal or organic matter in the presence of bacteria (2). Our perspectives and, not least, knowledge have thereafter changed, but despite the entry of antibiotics, sepsis represents danger and is still a major challenge and a significant cause of morbidity and mortality. During the last part of the twentieth century, an improved understanding of sepsis pathophysiology and molecular mechanisms, including the host immune response, has emerged. One prevailing theory has been that sepsis represents an uncontrolled host-inflammatory response, as clearly formulated by L. Thomas in 1972:

The micro-organisms that seem to have it in for us [...] turn out on close examination to be rather more like bystanders [...] it is our response to their presence that makes the disease. Our arsenals for fighting off bacteria are so powerful [...] that we are in more danger from them than the invaders (3).

Hence, one of the main tasks in sepsis-related research has been to intervene and inhibit the detrimental effects of the excessive host-inflammatory response. Our growing understanding of innate immunity and its capacity to respond effectively on exogenous as well as endogenous danger has made it particularly interesting as a target for intervention. In this Thesis I will present the work done by colleagues and myself, on different target strategies in Gram-negative induced inflammation and sepsis.

Inflammation

Inflammation is primarily a protective immune response trying to restore homeostasis. The inflammatory pathways can be divided into inducers, sensors, mediators and effectors (4), which is helpful when trying to dissect and distinguish between complex networks of mediators and cascade systems that participate in these reactions. Different inducers initiate the inflammatory response by activating specialized sensors leading to the release of different mediators such as cytokines and subsequent effects. Bacteria and viruses are classical exogenous inducers, but endogenous inducers derived from tissue injury, tissue hypoxemia or other kinds of tissue stress or malfunction might well trigger the host response. Regardless of

the cause, these processes are followed by vascular dilatation, capillary leakage and recruitment of mediators and different cells. The short-term process of acute inflammation was described by Celsus (ca. 30 BC – 38 AD), who divided the process into five cardinal signs; dolor (pain), calor (heat), rubor (redness), tumor (swelling) and functio laesa (loss of function), all reflecting the pathophysiology of inflammation (Figure 1). The increased blood flow causes the redness and increased temperature, the accumulation of fluids and mediators causes the swelling and pain, whereas the loss of function is a consequence of it all. Inflammation can be classified either as acute or as chronic, with different and specific characteristics, but both are to a large extent, local and often limited processes. Uncontrolled inflammatory processes may develop systemically which represent a far more dramatic picture, as seen in severe sepsis.

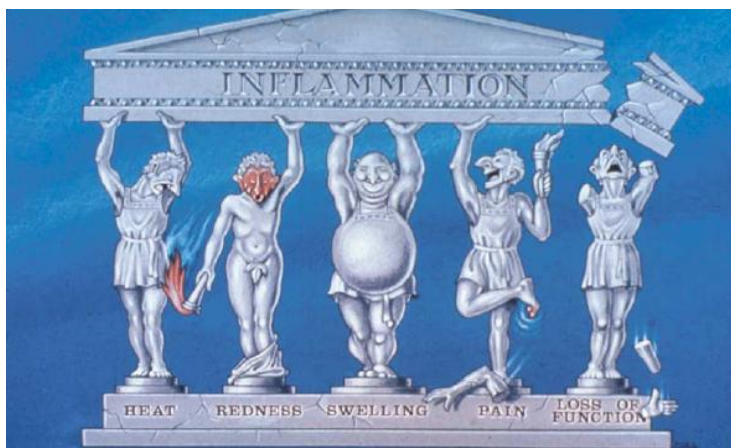


Figure 1: The five cardinal signs of inflammation: Calor, Rubor, Tumor, Dolor and Functio laesa

Innate Immunity

The principal difference between the innate and the adaptive immune system concerns the receptors used for the immune recognition (5). The adaptive immune system is organized around specialized T- and B-cells and their respective receptors, which are generated somatically through an ingenious process of clonal selection and constitute an enormous repertoire of specificities ($10^{14} - 10^{18}$ different receptors) (5). The clonally expressed receptors form the basis of adaptive immunity and are essential in triggering immunological memory. The clonal diversity and large population of lymphocytes are essential for an

efficient immune response, but the adaptive immune system is not called into play until after several days, leaving the host defenseless (6).

The receptors of the innate immune system are germ-line encoded, implying that the specificity of each of the receptors is genetically determined (7). These receptors have evolved through evolution, thus reflecting an older defense strategy. Instead of recognizing detailed specific features of a pathogen, the system relies on recognizing conserved molecular patterns of exogenous as well as endogenous origin (7,8). The recognition proteins of innate immunity are accordingly called pattern-recognition receptors (PRRs). The inflammatory inducers of non-self-derived patterns of microbes are commonly referred to as pathogen associated molecular patterns (PAMPs) (7,9). The counterpart, patterns of self, refers originally to damage -associated molecular patterns (DAMPs) or “alarmins” (10-12). In recent times, DAMPs has been given a wider meaning by also referring to danger associated molecular patterns. The terminology at this point is somewhat confusing, mixing danger and damage and needs to be elucidated shortly. According to the “danger model” introduced by Matzinger (10,13) immunity is not designed to discriminate between self and non-self, but to recognize danger-associated molecular patterns irrespective of their nature. In other words, immunity is primarily engaged in protecting the host from danger rather than in recognizing foreignness. As a consequence, a microbial derived pattern or tissue trauma may trigger similar innate immunity pathways. In the present Thesis the term DAMPs refers to danger of exogenous as well as endogenous origin.

PRRs are expressed on many effector cells, such as macrophages, dendritic cells and neutrophils but are also present as secreted proteins that act as soluble pattern recognition molecules (7). The Toll-like receptors belong to the most important class of PRRs and will be discussed later, as will the effects of the complement system, which can be regarded as a humoral “Master Alarm System” of innate immunity (14). Examples of important intracellular PRRs are the NOD-like (Nucleotide Oligomerization Domian) receptors, which respond on both microbial and endogenous danger signals (15), and the RIG-like (Retinoic acid Inducible Gene) receptors, which recognize non-self RNA species from viral infections (16). None of these effector pathways will be discussed further in this Thesis, nor will the functions of the Natural killer cells, which belong to a subpopulation of cytotoxic lymphocytes, and which constitute an important component of innate immunity (17).

In contrast to the adaptive immune system, innate immune pathways are fast-acting. Once activated, the effector cells or activated components will immediately perform their effector functions, such as producing and secreting signal molecules. Cytokines constitute an important class of inflammatory mediators with numerous and multifaceted activities. They activate resident cells such as fibroblasts, endothelial cells and tissue macrophages and are strongly involved in leukocyte recruitment through increased expression of cell surface molecules and chemoattraction (18). TNF- α , IL-1 β and IL-6 are regarded as primary cytokines. These play key roles in acute inflammations and sepsis, and are known to induce fever- and acute phase responses among a large variety of other effects (19). Arachidonic acid metabolites such as prostaglandins, prostacyclins, thromboxanes and leukotrienes constitute another important class of inflammatory mediators that either enhance the effects of other mediators or directly induce diverse inflammatory effects (20). Leukotriene B4 (LTB₄), which will be discussed later in this Thesis, is a potent chemotactic agent and activator of white cells, in particular neutrophils. The hemostatic system is also interconnected with innate immune responses, is activated concurrently, and is intimately connected through mutual interactions, in particular to the complement system (21,22).

The complement system

The complement system (Figure 2) is an upstream arm of innate immunity and consists of more than 30 different plasma and cell-bound proteins (23). Several of these proteins are proteases and are capable of being activated by proteolytic enzymes. The precursors, so-called zymogens, are widely distributed in body tissue and plasma, and when activated they trigger an enzyme cascade reaction. The physiological effects are diverse, including recognition and elimination of microorganisms, disposal of waste by clearance of immune complexes and apoptotic cells, and working as a bridge between innate and adaptive immunity (23). Additionally, complement also functions as an important humoral system that senses danger of exogenous as well as endogenous origin (24).

Activation is known to occur via three routes; the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP) (25), which converge and lead to the cleavage of the central complement factor C3. CP activation is initiated by the binding of C1q to immune complexes but also when binding to other molecules such as C-reactive protein (CRP) and phosphatidylserine (26,27). The LP is triggered by the binding of polysaccharide structures on

microorganisms as well as on apoptotic cells by mannose binding lectin (MBL) or ficolins (28,29). Further activation of the CP and LP is closely similar, leading to the cleavage of C4 and C2 to C4b and C2b, which together form the classical/lectin C3 convertase, C4b2b. In contrast to the CP and LP, the AP is not dependent on binding to a specific structure; instead, spontaneous hydrolysis (tickover) of C3 generates small traces of C3b, which may attach to nearby target surfaces. Target-bound C3b binds factor B, which in turn is cleaved by factor D to Bb, which remains bound to C3b forming the AP C3 convertase C3bBb. Properdin is an important stabilizer of the AP C3 convertase, but evidence suggests that it may work as PRR for de novo activation of the AP (30). The C3 convertases cleave C3 at a single site, generating C3b and C3a. C3a is an anaphylatoxin that stimulates inflammatory processes, whereas C3b covalently binds to bacterial walls and acts as opsonins, thereby facilitating phagocytosis, or binds to C3 convertase to form C5 convertases. The terminal complement pathway starts with the cleavage of C5, by the classical/lectin (C4bC3bC2b) or the alternative (C3bBb3b) C5 convertases, releasing C5a and C5b. C5b induces the assembly of the terminal C5b-9 complement complex (TCC), which can lyse certain pathogens and cells when incorporated into their lipid membranes (31). Like C3a, C5a is a potent anaphylatoxin and will be discussed later. The role of complement as a critical alarm system relies on the sensors of the distinct complement pathways, which act as soluble PRRs.

Complement activation is subjected to tight regulation by soluble and membrane-bound inhibitors limiting deposition of complement fragments on normal cells. Several inhibitors act on complement convertases through promoting degradation of activated complement fragments or by accelerating decay of the convertases (25). Pathogens are prone to opsonization due to the lack of these inhibitors, though many bacteria have evolved evasion strategies, thereby subverting complement attack (32). Excessive or inappropriate activation of complement may lead to tissue damage, and dysregulation of complement is associated with many diseases (33).

Several complement receptors are expressed on different cells, interacting with the release of complement fragments that make the immune system able to adapt into physiological changes (24). Complement receptor 1 (CR1/CD35) expressed on neutrophils and monocytes, mediates phagocytosis, but in the circulation this receptor is predominately expressed on red cells (24). Here, CR1 serves as a receptor for C3b-tagged immune complexes which are transferred to the liver and processed by macrophages. The leukocyte expressed complement receptor 3,

(CR3)(CD11b/CD18), is an important recognition receptor of innate immunity (34). It binds the inactive derivate of C3b, iC3b, which on its own acts as an opsonin. In addition, CR3 promotes leukocyte adhesion, migration and recognition of a broad range of different microbial molecules (34).

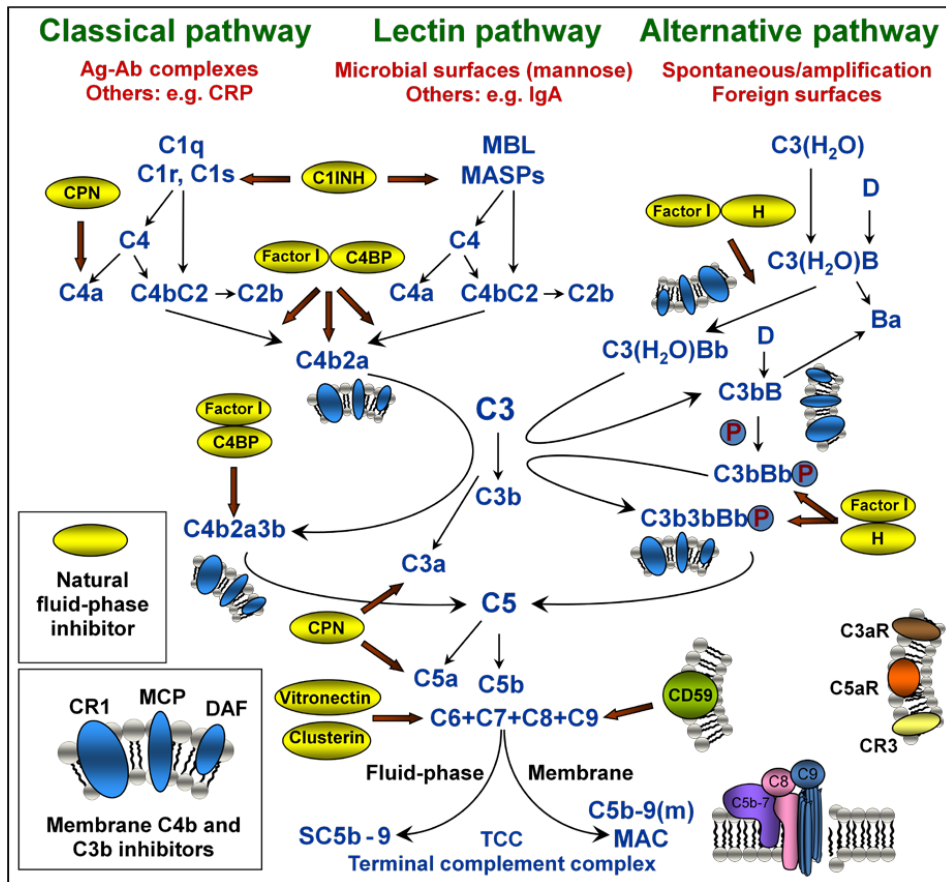


Figure 2. The complement System (Mollnes TE, Song WC, Lambris JD 2002. *Trends Immunol* 23:61-64, with permission from Elsevier)

The anaphylatoxins of complement

C3a and C5a are small split fragments of complement activation known as anaphylatoxins. They both promote inflammation, in particular C5a, which is an extremely potent inflammatory mediator that under certain clinical conditions may cause more damage than help (35). A well of C5a-effects have been described: C5a promotes phagocytosis and

oxidative burst in neutrophils and monocytes (36), upregulates adhesion molecules and the cell-surface molecule CD11b (CR3) in neutrophils (37), induces the release of granular enzymes, and is an important chemoattractant for neutrophils (35,38). C5a increases thrombogenicity by upregulation of tissue factor (TF) on endothelial and neutrophil cells (39,40). C3a causes smooth-muscle contraction (38), and both anaphylatoxins promote vasodilation and increase capillary leakage (35). Furthermore, C3a and C5a are implicated in the pathogenesis of asthma; C3a by inducing mast cell degranulation and transcription factor activating, leading to increased inflammation (41), whereas C5a seems to play a dual role by limiting allergen sensitization on the one hand, and increasing inflammation on the other (42). Interestingly, evidence suggests that the anaphylatoxins are required for the survival of liver cells during regeneration (43). The latter fact shows that C3a and C5a have pro-survival properties, and do not only take part in a tissue-injury inflammatory process. The effects of the anaphylatoxins are mediated through the C3a receptor (C3aR) and the C5a receptors (C5aR and C5L2), which are distributed on a variety of cells, such as myeloid-, endothelial-, epithelial, and smooth muscle cells, as well as on different parenchyma cells (24). The significance and biologic role of the C5L2 receptor are debated and not fully understood, though observations indicate that C5L2 may work as a “scavenger” receptor, thereby opposing the proinflammatory effects transmitted via C5aR (44,45). In contrast to the C5aR, the majority of the receptor is located intracellularly, and upon cell activation it is translocated to the cell surface (45). Interestingly, C5L2 seems to be an important ligand for the expression of the harmful sepsis mediator HMGB1 (46).

Toll-like receptors

Toll-like receptors (TLRs) are proteins that play key roles as PRRs. Most classes of TLRs are found on innate immune cells such as neutrophils, monocytes/macrophages, dendritic cells and mast cells, but they are also widely expressed on T- and B-cells as well as on a variety of other cells, including endothelial cells (47). Ten different mammalian TLRs have been identified (48). All are classified as type 1 transmembrane proteins with an extracellular leucine-rich repeat domain, a signal transmembrane α -helix portion, and a conserved intracellular TIR (Toll/IL-1R/Resistance) domain (49). Upon activation, all TLRs except for TLR3 recruit the adaptor molecule MyD88 for intracellular signal transduction, leading to the activation of the transcriptional nuclear factor- κ B (NF- κ B), or of other transcription factors such as IRF3, IRF5 and AP-1, required for transcription of a wide range of inflammatory and

immune response genes. A MyD88 independent pathway mediates the activation of interferon-regulatory factors and subsequent induction of interferon- γ (47-49).

TLRs are located both on the cell surfaces and intracellularly on endosomal compartments; thus, ligands of extra- and intracellular DAMPs are recognized. A broad range of different ligands are recognized by TLRs: bacterial cell wall components and DNA, viral, parasitic, and fungal products, as well as endogenously derived products such as DNA, intracellular matrix, and proteins (47). Specific accessory proteins or co-factors regulate TLRs by contributing to ligand discrimination and receptor signaling (48,50). In 1998 TLR4 was demonstrated to be the PRRs for lipopolysaccharide (LPS) of Gram-negative bacteria (51). However, TLR4 signaling is facilitated by the co-receptors, MD2 and CD14 (48,50). MD2 is a soluble protein associated with the extracellular domain of TLR4. *In vivo* experiments show that LPS responsiveness is dependent on MD2 (52), and crystal structures of TLR4-MD2 in complex with LPS have displayed how MD2 facilitates and potentiates TLR4 signaling by bridging two TLR4 molecules (53).

CD14 - the key accessory molecule

CD14 is present as a soluble protein in the blood, or as membrane bound protein to myeloid cells. This accessory molecule enhances LPS responsiveness by facilitating the transfer of LPS from LPS-binding protein to TLR4-MD2 complexes. There are two different types of LPS, rough and smooth, and the latter is the most common form expressed by Gram-negative bacteria. CD14 seems to be required for transmission of both types of LPS, whereas rough LPS imparts the ability to transfer TLR4 signaling independent of CD14 (54). Besides interacting with TLR4, CD14 works as an accessory molecule in relation to other TLRs (TLR2, TLR3, TLR5, TLR7 and TLR9) and has the ability to bind a variety of microbial products (48). Thus, CD14 is a unique upstream and promiscuous molecule, implicated in different TLRs-signaling reacting with a number of ligands with low affinity, and transferring the ligand to receptors with a higher degree of specificity and affinity.

The presence of TLRs on endothelial cells and mucosal epithelial cells in the respiratory and gastrointestinal tract is of great importance in host defense. Activation leads to upregulation of adhesion molecules, and to secretion of chemokines which are required for adequate migration of neutrophil cells.

Hemostasis and Contact activation

Hemostasis is a sensitive and tightly regulated process, involving the coagulation, the fibrinolytic system, and platelet activation, as well as endothelial cells. TF is a transmembrane glycoprotein that, *in vivo*, is regarded as the key initiator of coagulation (55). TF is expressed by subendothelial cells that are not normally exposed to circulating blood, but disruption of vascular integrity exposes TF and initiates the coagulation cascade (56). The hemostasis is intimately connected to host defense, especially to the complement system, as several interactions exist between these two cascade systems (21). As previously mentioned, the anaphylatoxin C5a upregulates TF on endothelial and white cells, by contrast, activated clotting factors such as thrombin are able to cleave C5 independent of the C5 convertases (57). The mutual interactions and almost concurrent activation of these systems force and favor host defense. For example, local formation of thrombi to or around the infected area provides a protective barrier and prevents bacterial spreading. However, uncontrolled systemic activation may induce widespread thrombi formation and accompanying microvascular dysfunction.

The contact system, also known as the intrinsic pathway of coagulation, or the kallikrein-kinin system, can be considered an integrated part of innate immunity (58). The system plays only a secondary role in hemostasis but the release of kinins has a broad specter of activities including induction of proinflammatory reactions and regulation of blood pressure (59). Activation liberates the potent proinflammatory peptide bradykinin (BK) and its metabolites desArgBK from high molecular-weight kininogen. BK participates in the cardinal feature of inflammation, producing vasodilation and increased vascular permeability, triggering the release of proinflammatory mediators such as histamine, prostaglandins, leukotrienes, cytokines, and promoting neutrophil chemotaxis (60). Kinins mediate effects through two different receptors, the B1 and B2 receptors – the former with high affinity to kinin metabolites, the latter with high affinity to native kinins such as BK (61). The B2 receptor is constitutively expressed in most tissue, particularly on endothelial cells (62), but can be upregulated upon inflammatory stimuli such as LPS (63). BK has a very short half-life of less than 30 seconds, partly due to degradation by angiotensin-converting enzyme.

Cross-talk and redundancy of host defense

The immune system is characterized by numerous different signaling pathways that constitute a robust and redundant host defense. The innate immune responses serve not only as a forceful first line defense but initiate and serve as a bridge to the adaptive immune system (9). In response to antigen presenting cells TLR signaling is important for promoting CD4+ and CD8+ T lymphocyte activation (64), and certain PRR-induced cytokines (IL-12 family) may polarize the T cell response (65). Downstream mediators of complement activation, such as iC3b and C3d, enhance B cell immunity via the complement receptor CR2, and different mechanisms for how complement regulates T cells are also described (66). It was recently it was shown that T cells deficient in C3aR and C5aR displayed a weak and limited allogeneic host response, and blockade of C5aR reduced morbidity in a relevant mice model of acute graft-versus-host disease (67).

Infections are likely to induce complement- and TLR activation at the same time, suggesting that these responses are interconnected. In fact, these pathways are closely related through an extensive cross-talk (65,68). Previously it was shown that CD14 and CR3 cross-talk and promote phagocytosis of mycobacteria (69), and furthermore that C5a and C3a enhance TLR-induced formation of proinflammatory cytokines (70). Evidence suggests a reciprocal interaction between C5a and TLRs (71), implying that compensatory mechanisms obviously play an important role in host defense.

Sepsis

Definition

In 1992, an international consensus conference provided a conceptual framework describing systemic complications induced by infections, and defined sepsis as the presence of infection and systemic inflammatory response syndrome (SIRS) (72). Two out of four criteria fulfilled the definition of SIRS: Temperature > 38 or < 36 °C, heart rate > 90 beats/min, respiratory rate > 20 breaths/min or $p\text{CO}_2 < 4.2$ kPa, WBC $> 12 \times 10^9/\text{L}$ or $< 4 \times 10^9/\text{L}$, or < 10 % immature forms. According to the severity of the disease sepsis was further categorized into severe sepsis and septic shock. Severe sepsis was defined as sepsis plus organ dysfunction, tissue hypoperfusion or hypotension, and septic shock as severe sepsis with hypotension but

refractory to fluid resuscitation. These general definitions are widely used clinically and have served as the basis for several clinical trials. They are, however, too sensitive and non-specific; thus, randomized clinical trials are colored by heterogeneous patient populations (73). Later attempts to strengthen the sepsis criteria have been made, but no evidence has been found to change the definitions apart from an extended list of signs and symptoms (74). It is utterly important to reduce patient heterogeneity in future sepsis trials. This may be achieved by a different staging-system based on predisposition, insult, response and organ dysfunction (PIRO) (75). With respect to the biomarkers, their time course may be more reliable than the absolute values (76), and we definitely need more knowledge related to this dynamic process. We still know too little about the different clinical phenotypes and associated biochemical profiles. This knowledge can only be achieved by large clinical trials (75).

Sepsis epidemiology

There is a relatively large variability associated with epidemiological data and sepsis. This is partly related to retrospective estimates and to the heterogeneous patient population. At the end of the last millennium the incidence of sepsis in the USA was estimated to 240 cases per 100.000 (77), and that of severe sepsis to 95 per 100.000 (78). In two prospective studies, from Finland and from Australia and New Zealand, the incidence of severe sepsis was calculated to 38 per 100.000 adult per year and 77 per 100.000 adult per year, respectively (79,80). The ICU mortality was found to be 15.5 % in the Finnish study, while 1-year mortality was 40.9 %. Other reports show a higher ICU-mortality, ranging from 26 % to above 50 % (80,81). The mortality reflects the severity of illness, which is clearly demonstrated in the large pan-European study, Sepsis Occurrence in Acutely Ill (SOAP), where the ICU-mortality was less than 20 % among patients with one organ failure and above 60 % among patients with more than four organ failures (81). Different reports document that the proportion of patients with severe sepsis is increasing (78,82). This represents great clinical and economical challenges.

Pulmonary infection is the primary site of infection followed by intra-abdominal infections (81,82). Isolation of Gram-negative bacteria is frequent and account for above 35 % of the isolates, and *E. coli* is one of the most frequent causative bacteria.

Innate immunity and sepsis pathophysiology

The pathophysiology of sepsis is complex. The current view suggests that sepsis is unleashed by an infection-induced systemic inflammatory response (83,84). Under normal physiological conditions innate immune pathways are intended to work locally and serve host defense. By contrast, a systemic activation of these immune defending systems may lead to a host-threatening situation that induces a counterproductive and detrimental inflammatory response. In other words, a systemic activation of the sensors of innate immunity, PPRs, implies loss of control, and a rapid and vast nonspecific response may harm the host. For instance, one of the primary roles of proinflammatory mediator release is to enhance leukocyte migration from within the blood to the site of infection. Successful activation and migration of neutrophils is dependent on a fine-tuned interplay with endothelial cells, involving upregulation of different adhesion molecules and their respective ligands (85). However, in sepsis sequestration of neutrophils also strikes organs not affected by infection due to impaired migration (86). As a consequence, neutrophil-mediated tissue injury with release of cytokines, reactive oxygen species, proteinases, and other cell-derived content also affects innocent or remote organs (87). The lungs are particularly susceptible, and impaired lung function correlates with the intensity of neutrophil infiltrates (88).

It is not possible to account for the complex network and myriads of mediators that play a part in the concert of sepsis. The picture is not uniform. In some studies the levels of proinflammatory mediators seem to correlate with the outcome of sepsis (89,90), and consumption of contact factors has been associated with a bad prognosis (91), indicating that septic patients are in a hyperinflammatory state. However, the heterogeneous pool of septic patients display different inflammatory profiles, and in some cases even a blunted response (92). This implies that some patients are in a hypo- rather than a hyperinflammatory state, reflecting the complex sepsis entity or syndrome. The release of C5a is thought to play a central role in the vast inflammatory drive, but generation of this anaphylatoxin is also associated with apoptosis of lymphocytes and other lymphoid cells (93). Although pro- and anti-inflammatory responses are activated initially, the proinflammatory responses are thought to predominate in the early phase of sepsis, whereas later phases of sepsis is featured by a predominant anti-inflammatory response (94). The immunosuppressive state of sepsis is characterized by increased susceptibility to acquiring secondary infections, as well as reactivation of otherwise harmless virus infections (94-96).

From a clinical point of view, tissue hypoperfusion accompanied by multiple organ failure (MOF) is a key feature in severe sepsis and septic shock. Hypotension and capillary leakage with loss of plasma fluids to the interstitial space lead to intravascular volume deficit and deteriorated circulation. Additionally, increased interstitial fluid accumulation and tissue edema negatively affect nutritional transport and oxygenation.

Dysregulated coagulation is another typical feature characterized by disseminated intravascular coagulation (DIC) with consumptions of platelets and widespread formation of intravascular microthrombi (97,98). The endothelial system in particular is an important component to the microvascular dysfunction, as it enhances increased adhesion of platelets and leukocytes and promotes vasodilation by NO production (99). Evidence suggests an association between severe microvascular function and fatal outcome (100). Thus, to maintain adequate tissue oxygen delivery and to prevent MOF, resuscitation with fluids is essential in the treatment of septic patients (101), and targets for early goal-directed resuscitation are therefore specifically recommended in the international guidelines for management of severe sepsis and septic shock (102).

Although microbial investigations are commonly negative in these patients, early broad-spectrum antibiotic treatment and/or surgical control of the infected area is mandatory. Further treatment is primarily supportive, with the goal of maintaining an adequate blood pressure, oxygen content, blood glucose level, renal function, and acid-base balance in order to reach a reasonable homeostasis and sustain organ functions (102). Hydrocortisone is recommended as adjuvant when hypotension remains poorly responsive to fluid resuscitation and vasoactive medication. Drotrecogin alpha activated (recombinant human activated protein C), which prevents formation of thrombin and has profibrinolytic properties, was added to the list of adjunctive therapies, but has recently been withdrawn due to failure to demonstrate efficacy (103). Apart from significantly more advanced intensive medical services the causative treatment of sepsis has in general remained unchanged for the last decades.

AIM

The aim of this Thesis was to explore the efficacy of principally different target approaches in Gram-negative induced inflammation and sepsis. The sensors of innate immunity pathways and single mediators of inflammation were targeted goals. Preclinical models were used to study the inflammatory reaction and effects of inhibition, *ex vivo*, in human and pig whole blood, and, *in vivo*, during Gram-negative induced sepsis in pigs.

Study I. To examine the significance of the traditionally regarded early cytokines TNF- α and IL-1 β , compared to the central upstream arms of innate immunity, complement, and CD14, as mediators of and inhibitory candidates of *E. coli*-induced inflammation in humans.

Study II. To investigate the efficacy of the B2 receptor antagonist, icatibant, in a porcine model of *Neisseria meningitidis*-induced sepsis.

Study III. To investigate the efficacy of the *Ornithodoros moubata* Complement inhibitor (OmCI) in pigs and humans by using an *ex vivo* whole blood model for both species and an *in vivo* porcine model of *E. coli*-induced sepsis.

Study IV. To investigate the effect on inflammation, hemostasis and hemodynamics of OmCI alone, and OmCI combined with anti-CD14, in a porcine model of *E. coli*-induced sepsis.

MATERIALS AND METHODS

Introduction

Gram-negative induced inflammation in human or pig whole blood, and Gram-negative induced sepsis in pigs, are central and important topics for all papers discussed in this Thesis. Both these *ex vivo* and *in vivo* induced inflammatory processes imply liberation of countless of mediators and the involvement of numerous reactions. It is important to be aware of the fact that the applied methods and analyses do not elucidate this picture completely, and that some questions are left unanswered as a result of the selection of topics that were examined. On the other hand, the methods and analyses that are used are well known and regarded as established and reliable. The following section provides an overview of methods and analyses that were used, as well as some related considerations. For a more detailed description of equipment reagents and methods, see the material and methods sections in the individual papers.

Bacteria and complement activators

Ex vivo (paper I and III), heat-inactivated *E. coli*, strain LE392 (ATCC 33572) from the American Type Culture Collection (Manassas, VA), was used. Heat inactivation results in minor alterations to the bacterial membrane, leaving the biological activity preserved but preventing further dividing. These bacteria were counted by flow cytometry and stored at + 4 °C in batches of 1×10^9 *E. coli*/mL PBS. *In vivo* (paper III and IV), live *E. coli* of similar strain, LE392, was administered intravenously to pigs. They were stored at -70 °C until used, and could thereafter freely divide. The reference strain of *N. meningitidis*, 44/76, was used in paper II. This strain was originally isolated from a patient with fulminant meningococcal septicemia in 1976. The bacteria were heat inactivated and stored at -70 °C until used to induce meningococcal sepsis.

Heat-aggregated IgG (HAIGG) and zymosan, potent activators of the classical and lectin/alternative pathway, respectively, were used in study III to induce complement activation in whole blood.

Inhibitors

In all papers, different inhibitors and respective isotype-matched or other suitable controls were used. Several of these were purchased, such as TNF- α inhibitors etanercept and infliximab, which bind specifically to soluble and membrane bound TNF- α , and the IL-1 β receptor antagonist anakinra (paper I). All three cytokine inhibitors are widely used as anti-inflammatory therapeutics for different autoimmune disorders. The mouse anti-human CD14 F(ab')₂ was used in paper I and III. The mouse anti-porcine CD14 monoclonal antibody clone MIL-2 has in previous experiments been proven to bind CD14 on porcine granulocytes and efficiently attenuate *E. coli* LPS-induced inflammation, both *ex vivo* and *in vitro*. This monoclonal antibody was used in paper III and IV. The selective B2 receptor antagonist, icatibant (paper II), has recently been made commercially available (Firazyr®), and was donated from Jerinin AG. Three different complement inhibitors were applied in the papers; compstatin (paper I and III), which potently binds to and prevents cleavage of C3, eculizumab (Soliris®) (paper III), which is a humanized monoclonal IgG2/4k- antibody that binds to and prevents cleavage of the human complement factor C5, and finally the tick-derived *Ornithodoros moubata* Complement Inhibitor (OmCI) (paper III and IV), a small protein (16.8 kDa) that binds to and prevents cleavage of C5. The procurement of OmCI was particularly important, as it was demonstrated to be a very effective complement inhibitor in pigs and humans, and was importantly, delivered in large enough amounts to make it possible to carry out study IV.

Analyses

Enzyme immunoassay and multiplex technology

Human cytokines were analyzed using multiplex technology. With this immunoassay a range of different biomarkers can be measured simultaneously. The technology is based on colored beads, each with a unique cytokine detection antibody. The beads are incubated with the samples, a secondary biotin-conjugated antibody is added, and finally a reporter molecule that binds to biotin is added. The samples are run in a modified flow cytometer with two lasers, one that excites the beads, and one that excites the reporter molecule. In this way the beads are separated, and the amount of each of the cytokines can then be measured.

We did not have multiplex technology available for porcine cytokine analysis; instead these cytokines and plasminogen activator inhibitor-1 (PAI-1) were analyzed by standard individual commercial EIA kits. The availability of specific porcine kits is limited, making the repertoire of potential and interesting biomarkers restricted. However, thrombin activation in pig was measured in citrate plasma using a human thrombin-antithrombin (TAT) immunoassay kit that works for porcine plasma (104). In addition, the formation of TCC was analyzed by an enzyme immunoassay (EIA) previously developed in our laboratory (105). The test was designed for human complement activity, but was later shown to cross-react with pig (106). The wells were coated with the mAb aE11, which is specific for a C9 neoepitope in TCC. A biotinylated monoclonal anti-C6 was used as detection Ab. A commercial kit was used to test the functional activity of the CP, LP, and AP in human and pig serum. Formation of C5a in human plasma was analyzed by a commercial EIA. Despite several efforts with several different kits, we did unfortunately not manage to measure C5a in pig serum or plasma. Leukotriene B4 (LTB4) was measured in pig and human using the same kit.

Flow cytometry

In pig whole blood, granulocytes are clearly discriminated from mononuclear cells, but lymphocytes and monocytes cannot be separated by a forward/side scatter dot plot. Therefore, the expression of tissue factor (TF) (paper IV) was measured on granulocytes, which is a bit unfortunate since it would have been even more interesting to look at this expression on monocytes. The expression of wCD11R3 (the pig ortholog to human CD11b) was measured on granulocytes, whereas human expression of this cell surface molecule was measured on granulocytes and monocytes in paper I, but only on granulocytes in study III. In study I, oxidative burst was measured using a commercial kit.

When analyzing the expression of a surface marker on an activated cell it is important to take into account that the background activity can be increased. This may affect the result, especially for markers that are expressed at low values, which was the case for TF. In all analysis we used isotype controls to adjust, so that the increased expression observed is reliable.

Determination of BK1-5 level and icatibant

BK is an extremely short-lived effector molecule and is rapidly metabolized to inactive products (107). A reasonable approach for exploring its pathophysiologic role is therefore to measure one of its stable metabolites, BK1-5, comprising the first five amino acids of BK with a half-life around 90 minutes (108). Blood was drawn into a plastic syringe and immediately added to chilled ethanol to denature kallikrein and kinases. After one hour, the samples were centrifuged and the supernatant stored at -70 °C. The samples were spiked with the internal standard BK1-6 and thereafter eluted, before high-performance liquid chromatography technique was used to determine the content of BK1-5 and icatibant.

Histopathological evaluation

In study IV biopsies from lung and liver were formalin-fixed, and thereafter cut into thin sections, deparaffinized, and stained by haematoxylin/eosin (H&E) and saffron. A pathologist evaluated these sections in a blinded manner according to how it is described in paper IV.

The Models

Ex vivo whole blood model

In study I and III a unique *ex vivo* whole blood model was used. This model is extensively studied in human whole blood (109) and has been proven to work in pig whole blood as well (110,111). When developing this model, the main goal was to keep all functional effector systems functional and able to interact, and at the same time avoid coagulation. Commonly used anticoagulants, such as EDTA and citrate, inhibit complement activation, whereas heparin enhances complement activation at low concentrations and inhibit such activation in high concentrations (112). By using the specific thrombin inhibitor lepirudin (Refludan®) no adverse effects on complement activation were seen (109). Although the experiments performed in study I and III obviously lack the complexities that arise in a living organism, they have been performed under conditions reflecting a reliable method for studies of inflammatory cross-talk, where all inflammatory mediators in whole blood, except thrombin, are able to interact mutually (109).

In vivo models

A large proportion of former sepsis research has been based on endotoxin models, reflecting the fact that LPS has been recognized as a key molecule in pathogenesis of sepsis. There are limitations to this approach. LPS is essential to virtually all Gram-negative bacteria, and is a typical archetype of conserved microbial structure inducing innate immune responses. LPS comprises three covalently linked regions; a lipid A moiety, a core oligosaccharide structure, and an O-antigen polysaccharide chain. The biosynthesis of the lipid A structure varies among different organisms and determines the biological activity and pathogenicity of LPS (113). LPS is known as the most potent molecule of *N. meningitidis*, but LPS-mutant meningococci strain has been shown to induce inflammation and increase mortality in a mouse model (114). By using the same sepsis model as used in study II, our group recently demonstrated that *N. meningitidis* completely lacking LPS, induced cardiovascular and hematological changes comparable to those induced by an intact LPS-sufficient strain, though a 10–20-fold higher dose of the LPS-deficient mutant was required (115). *Ex vivo* experiments have revealed different effects of anti-CD14 and CyP (*Cyanobacterial Product* – which derives from blue green algae and inhibits TLR4/MD2), depending on whether whole blood was activated by whole bacteria (*E. coli*) or by ultrapure LPS (110,111). The effect of both inhibitors was less pronounced by *E. coli*-induced cytokine formation. Thus, the use of whole bacteria models encompasses apparently greater biological diversity as compared to pure endotoxin models, and is more relevant in reflecting the magnitude of danger signals to which the host is exposed.

Pigs are suitable animals to use and have served as an important biomedical model for humans for decades, not least because several of the applied disease models using pigs are more relevant for the human condition. An example is the pig atherosclerosis-model, which facilitated analysis of disease progression and evaluation of different medications (116). The size of the pig allows us to use standard monitoring equipment and enables repeated blood sampling for comprehensive analysis. The size also makes it possible to take biopsies during an experiment and detailed tissue samples after euthanasia. Compared to rodents, pigs have the advantage of displaying relatively closer anatomical and physiological relations to humans, and, importantly, the pig genome has high sequence and structure homology with humans (117). As compared to mice, the immune parameters in pigs show closer resemblance to humans in more than 80 % of analyzed parameters, whereas mice show closer resemblance in less than 10 % (118).

In study II, III, and IV different models of experimental induced sepsis in pigs were used. In study II a model of porcine meningococcal sepsis was used in the main protocol (119). According to the procedure, the intravenous infusion rate of *Neisseria meningitidis* was doubled every 30 minutes to mimic the generation time of live *Neisseria* in the human disease. This forceful pathogenic model, expressed by a substantial plasma leakage and an increase in a broad specter of inflammatory parameters, parallels the changes that are observed in patients (119). Additionally, and as part of a supplementary project to this study, two pigs underwent cecal ligation and puncture (CLP)-induced sepsis, and two other pigs underwent *E. coli*-induced sepsis. The polymicrobial sepsis induced by CLP was a modification of a previously described model (120), in which autologous feces was aspirated from cecum, suspended in saline, and spread to all parts of peritoneum. The *E. coli*-induced sepsis model was also used in study III and IV. The model is well described and reflects an acute sepsis with early and rapid increase of inflammatory and hemostatic biomarkers (121). Sepsis was induced with an increasing intravenous infusion of *E. coli*, and each pig received a total of 1.075×10^8 *E. coli*/kg, corresponding to 1.1×10^6 bacteria/mL blood. The *E. coli* that was used came from the same batch and was infused exactly at the same rate as when we previously examined the effect of anti-CD14 alone (122).

The insertion of a PiCCO catheter was implemented in all models described above. This thermodilution catheter enables continuous measurements of stroke volume variation (SVV), which is a dynamic variable of fluid responsiveness and currently one of the most reliable hypovolemia predictors (123). The pigs were further extensively monitored. Blood gases were regularly taken, and respirator settings adjusted to maintain a pH of 7.40, and the pigs were monitored with ECG, artery line, central venous catheter, and pulmonary artery catheter. The latter is important since pigs have resident macrophages in the lungs, closely resembling human Kupffer cells (124), and are extremely vulnerable to pulmonary vasoconstriction (125). Increased pulmonary artery pressure (MPAP) is a typical early feature of porcine sepsis that is probably caused by local release of a broad spectrum of endogenous metabolites (125). An abrupt increase in MPAP was observed regularly, sometimes so severe that it was necessary to use norepinephrine for resuscitation. Although both the *N. meningitidis* and the *E. coli* models showed biochemical and clinical similarities, the observed increase in MPAP is a feature we do not find in patients, at least not to this extent.

Statistical considerations

In this Thesis, several experiments revealed obvious biological effects that speak for themselves. One may wonder whether statistical calculations in these cases actually provided any more information, apart from meeting the medical scientific requirements. I therefore find it wise to emphasize that a statistical test can either reject or fail to reject a null hypothesis, but never prove that it is true.

Parametric statistics were applied in the experiments performed in paper I, II, III, and IV. The results in paper I and III were statistically compared by one-way analysis of the variance between groups (ANOVA) with Bonferroni or Dunnet's post-test analysis. The post-hoc analyses are required for comparison of three or more means, but these tests are conservative, implying that the likelihood of making a type 1 error (rejecting the null hypothesis) decreases. In paper II and IV the data were examined with a repeated-measures 2-way analysis of variance (ANOVA) followed by Bonferroni's correction for multiple tests. In addition, a two-sample *t*-test for independent samples was applied in paper II, whereas the non-parametric test, Kruskal-Wallis one-way analysis of variance, was used to analyze histopathological lung changes in paper IV. Results with a *p*-value <0.05 were considered as statistically significant. The GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used for all statistical analyses.

To avoid type II error and falsely maintain the null hypothesis, performing power analyses to predict the numbers needed to be included in a study is highly recommended. For different reasons we did not do this prior to the *in vivo* studies described in paper II and IV. The numbers of animals included in the different groups were defined by several factors, including the available amount of inhibitors, costs, logistics, and animal ethical perspectives. The latter is important. The purpose of using animals is to improve our understanding of biological or medical questions related to human or animal health, but efforts should focus on keeping the use of animals down to a minimum, which we have done.

SUMMARY OF THE MAIN RESULTS

Paper I

The effect of pure rhTNF- α and rhIL-1 β as inflammatory inducers in fresh human whole blood was explored. A modest but dose dependent increase of IL-1 β , IL-6, IL-8, MIP-1 α , and IL-1ra was induced by rhTNF- α , while the effect of rhIL-1 β was limited to a modest increase of TNF- α and IL-8. The inflammatory significance of *E. coli* was substantially broader and more potent compared to pure recombinant TNF- α and IL-1 β . As potential candidates for inhibition of *E. coli*-induced inflammation, the TNF- α inhibitors, etanercept and infliximab, and the IL-1 receptor antagonist, anakinra, were explored. Etanercept and infliximab, specifically binding TNF- α , dose-dependently neutralized 10 ng/mL of rhTNF- α added to whole blood. Complete neutralization was obtained by 200 μ g/mL and 0.5 μ g/mL whole blood, respectively. Anakinra does not bind IL-1 β , and functional inhibition was measured using IL-8 as readout. The IL-8 concentration induced by 1 ng/mL of rhIL-1 β was dose-dependently decreased by anakinra and completely inhibited by 1 μ g/mL whole blood. However, inhibition of TNF- α or the receptor ligand to IL-1 β did not have any impact on oxidative burst or production and release of other cytokines, nor upregulation of the cell surface marker wCD11b. By contrast, combined inhibition of C and CD14 virtually abolished all measured inflammatory mediators induced by *E. coli*.

Paper II

The infusion of BK to anesthetized pigs caused an immediate and dose-dependent drop in MAP and SVRI. One μ g and 100 μ g of BK reduced MAP with 15 % and 48 %, respectively. CI increased, whereas HR and MPAP remained constant. All hemodynamic effects of different concentrations of BK (100, 1000 and 20 000 μ g) were completely blocked by a prior infusion of icatibant, which is a highly selective competitive B2 receptor antagonist. The effect of icatibant was then investigated in a blinded randomized controlled study model of Gram-negative induced sepsis in pigs. *N. meningitidis* was infused intravenously without any pretreatment (n = 8), or to pigs pretreated with icatibant (n = 8). Negative controls received saline only. The icatibant treated group developed the same degree of severe sepsis as did the

positive controls. Both groups had massive capillary leakage, leukopenia, and excessive cytokine release.

Measurement of BK1-5 was first conducted *in vitro*. BK1-5 levels corresponded closely to the amount of exogenous BK that was added to whole blood. *In vivo*, after giving increasing bolus doses of BK greater than 100 µg, increasing amounts of BK1-5 were detected. In the blinded randomized controlled study of *N. meningitidis*-induced sepsis, BK1-5 was not measurable in baseline samples, but increased to an average level 1.4 ± 0.8 ng/mL at 4 hours of sepsis. However, no differences in BK1-5 level were found between pigs receiving only saline, only bacteria or bacteria and icatibant. As part of a supplementary project to this study, two pigs underwent cecal ligation and puncture (CLP)-induced sepsis, and two pigs underwent *E. coli*-induced sepsis. BK1-5 was not detected in any of these pigs.

Paper III

OmCI dose-dependently inhibited all three complement activation pathways similarly, and the inhibition was complete at a dose of 0.64 µM OmCI in both pig and human. The inhibitory effect of OmCI in pig and human was further demonstrated in different setups and readouts. OmCI dose-dependently inhibited *E. coli*-induced formation of TCC in whole blood. HAIGG- and zymosan-induced TCC formation in serum was completely inhibited at 0.64 µM OmCI. *E. coli*-induced upregulation of the cell surface marker CD11b and the pig ortholog wCD11R3 on granulocytes were in both species dose-dependently inhibited by OmCI. In humans, the upregulation of CD11b was reduced by > 60 %, and in pigs the wCD11R3 was completely abolished at 0.32 µM OmCI. *E. coli*-induced IL-8 formation was inhibited in pig and human whole blood by OmCI alone, whereas IL-1β was inhibited in human whole blood only. By using OmCI and different specific complement inhibitors this study demonstrates that the *E. coli*-induced LTB4 formation is complement dependent. However, it is known that OmCI captures LTB4 within an internal binding pocket, making it difficult to determine whether the reduced concentration of LTB4 is conditioned to direct binding or reduced production. Thus, increasing doses of OmCI was added to a pig and human LTB4-enriched plasma. OmCI dose-dependently decreased the signal in the assay, consistently with covering of epitopes on the leukotriene preventing it from being detected in the assay. Finally, the effect of OmCI on complement activity was evaluated in an *E. coli* sepsis model in pigs of 15 kg. All pigs received a similar continuous infusion of OmCI (0.5 mg OmCI/h), whereas

different bolus doses of OmCI were tested. A bolus dose of 0.5 mg/kg completely ablated the classical, lectin, and alternative pathways immediately after administration and remained effective throughout the experiment.

Paper IV

This study explored the effect of OmCI alone and combined with anti-CD14 on the early inflammatory, hemostatic, and hemodynamic responses in porcine *E. coli*-induced sepsis. Thirty pigs were randomly allocated to a negative control group (n=6), a positive control group (n=8), or one of two intervention groups receiving either OmCI (n=8) or OmCI and anti-CD14 (n=8). The OmCI dosing regimen completely ablated complement activation and significantly decreased the level of LTB₄ in septic pigs. Granulocyte tissue factor expression, formation of thrombin-antithrombin complexes (p<0.001), and formation of TNF- α and IL-6 (p<0.05) were efficiently inhibited by OmCI alone, and abolished or strongly attenuated by the combination of OmCI and anti-CD14 (p<0.001 for all). Additionally, the combined therapy attenuated the formation of PAI-1 (<0.05), IL-1 β , and IL-8, increased the formation of IL-10, and abolished the expression of wCD11R3 and the fall in neutrophil cell count (p<0.001 for all). Interestingly, OmCI combined with anti-CD14 delayed the increases in heart rate by 60 min (p<0.05) and mean pulmonary artery pressure by 30 min (p<0.01). Histopathology revealed a non-significant median 60 % inhibition of lung inflammatory changes in the OmCI group.

DISCUSSION

The four papers included in this Thesis explore and elucidate the significance of different target approaches in Gram-negative induced inflammation and sepsis. The *ex vivo* study presented in paper I, draws a clear line between inhibition of traditionally regarded early and central proinflammatory cytokines, versus inhibition of complement and CD14, in Gram-negative induced inflammation. Paper II is an example of single agent intervention using icatibant to inhibit the effects caused by BK, recognized as an immediate mediator of inflammation. Paper III and IV are closely interconnected and are follow-ups of paper I. The data from these preclinical *ex vivo* inflammation studies and *in vivo* sepsis studies display clear differences, and reveal a significant distinction between the efficacies of upstream versus downstream inhibition of the inflammatory response.

Upstream and downstream

Severe sepsis and septic shock are life-threatening conditions characterized by a whole-body inflammatory state and homeostatic imbalance (92). With early intervention one may reduce the companion organ failure and gain time by attenuating the detrimental inflammatory response. This has been and still is a major task in sepsis related research. From a historical perspective, much attention has been directed toward the early-appearing central proinflammatory cytokines. Due to their potential ability to cause endogenous harmful systemic effects they have been regarded as upstream mediators, but, in my opinion they belong to the downstream mediators of inflammation.

Downstream inhibition

Tumor necrosis factor-alpha (TNF- α) was regarded as the most important proinflammatory cytokine, and different observations and experimental results connected the role of TNF- α directly to the pathogenesis of sepsis. The level of TNF- α correlated with the outcome of sepsis (89,90), administration of TNF- α to animals created both cardiovascular alterations and inflammatory responses similar to what was seen in septic patients (126-128), and inhibition of TNF- α protected animals against lethal effects induced by experimental sepsis (129,130). These promising results were followed by several small and large clinical trials, but in general they failed to demonstrate the utility of anti-TNF- α therapy in septic humans (92,131,132). An

additional lack of effect was also observed when trying to inhibit the effect of IL-1 β (133,134).

Why? This can partly be explained by a mismatch between experimental data largely obtained from animal research and the human course of sepsis. In mice challenged with intravenous infusion of LPS, an increased formation of TNF- α exceeded several folds compared to what was observed in clinically more relevant mouse-CLP models (135). Experiments further showed that mice subjected to CLP-induced sepsis did not achieve improved survival by anti-TNF- α treatment (135,136). In humans, certain forms of sepsis, such as meningococcal disease, demonstrate high levels of TNF- α , whereas low levels are observed among patients with less severe forms of sepsis (137). Evidence suggests a correlation between the severity of global hypoperfusion and TNF- α level (138). This fact underscores a crucial problem related to running clinical sepsis trials characterized by a nonhomogenous patient population, and can partly explain why anti-TNF- α treatment failed to that extent. In this context it is reasonable to note that one of the largest trials, including over 2500 patients and treated with afelimobab (F(ab)₂ monoklonal anti-TNF- α fragment), significantly reduced 28-days mortality by 5.8 % among patients stratified as IL-6 positive (139).

Paper I demonstrates that TNF- α and IL-1 β are inferior inducers of inflammation compared to *E. coli*. In addition, selective inhibition of these cytokines failed to attenuate *E. coli*-induced inflammatory responses in human whole blood, whereas a profound inhibitory effect was observed by anti-CD14 and compstatin. There is therefore reason to presume that the rather disappointing effect seen by the mediator-directed therapy, i.e. proinflammatory cytokine inhibition, was due to the fact that the inflammatory network is broadly activated by upstream molecules related to recognition, limiting the effect of neutralization of single downstream mediators.

Upstream inhibition

The sensors of innate immunity, expressed as PRRs, are as far upstream in the inflammatory network as possible. As explained in the introduction they act as danger sensors, thereby initiating the first inflammatory stimuli, which subsequently lead to downstream cascade reactions. TLR-signaling is dependent on or facilitated by several accessory molecules (50). Both MD2 and CD14 are important molecules facilitating TLR4-signaling. CD14 is an

archetype of an upstream recognition molecule, and has the ability to bind different microbial products, such as LPS, peptidoglycan and DNA (48). In addition, is also implicated as an accessory molecule in relation to TLR2, TLR3, TLR5, TLR7 and TLR9 (48). Thus, the properties of CD14 as a promiscuous upstream molecule make it an ideal target for inhibition.

The recognition molecules of complement activation, C1q, MBL, and ficolins, are selectively not suitable targets for a global inhibitory effect of complement. All complement pathways converge toward the central complement factor C3, but they do not contribute equally to downstream effects. The AP amplification plays a predominant role for the effects initiated by both the CP and the LP, being responsible for more than 80 % of TCC release in the fluid phase (140,141). Inhibition of the alternative pathway by targeting factor D, thereby leaving the CP and LP functionally preserved, is an interesting therapeutic approach. A combined inhibition of factor C2 and factor D to block all three initial complement pathways, or inhibition of C3, are methods for achieving a profound and almost complete inhibition of the complement system (37,142). In addition to the key role that C3 plays in the complement cascade, it can be regarded as a recognition molecule. Through spontaneous hydrolysis or tickover of C3, traces of C3b may attach to nearby target surfaces with the consequence that subsequent amplification of C3b deposition takes place in the absence of complement regulatory proteins (143). Thus, inhibition of C3 represents an ideal upstream inhibition target.

By targeting the terminal step in the complement pathway, C5 or its receptor(s) has a vast anti-inflammatory potential due to the important biologic role of C5a, and does not affect immunoprotective and immunoregulatory functions of upstream C3 activity (144). Although C5 is regarded as downstream in the complement cascade, targeting this molecule may represent upstream inhibition. C5 is not a pattern recognition molecule but is closely connected to recognition, and a crucial molecule for downstream effects.

The individual sensor and effector pathways described above act as partly independent branches of pattern recognition. However, as mentioned in the introduction, evidence indicates a considerable cross-talk, implying that they can either compensate, synergize, or antagonize each other. Although the efficacy of selectively targeting one of these branches may induce a profound anti-inflammatory effect, real upstream inhibition is achieved by a combined inhibition due to the effect of the redundancy in host defense (68).

Single-mediator inhibition

The historical anti-TNF- α studies are already referred to as insufficient examples of single-mediator intervention. Recently, eritoran tetrasodium (anti-TLR4) and drotrecogin alfa (activated protein C) were added to the list of unsuccessful single-mediator interventions, as both treatment regimens have failed to demonstrate improvement in clinical randomized trials (103,145).

Capillary leakage and vasodilatation are cardinal features of sepsis, and lead to intravascular volume deficit, hypotension, tissue edema, and deteriorated nutritional transport and tissue oxygenation. Attenuating the capillary leakage is a tempting approach, and BK has been a relevant candidate target. First, among numerous effects BK promotes capillary leakage (60); second, evidence suggests that sepsis activates the contact system, thereby releasing excess of BK (146-148); and third, the highly selective antagonist of the B2 receptor, icatibant (Firazyr®), has been proven to effectively attenuate the capillary leakage induced by hereditary angioedema (149).

Paper II challenges the current view of bradykinin (BK) as an important mediator of edema shock and inflammation in sepsis. The stable metabolite of BK, BK1-5, did not increase in any of the pigs subjected to either meningococcal-, *E. coli*-, or CLP-sepsis. In healthy individuals the plasma concentration of BK is reported to be 2.2 fmol/mL (150) and thus very low. However, in patients suffering from the deficiency disorder of C1-inhibitor, hereditary angioedema, BK has been measured to increase 12 times the upper limit of normal level during attacks (150). Concerning sepsis, previous reports indicate excess BK release during sepsis, but these estimations are based on indirect measurements, which only state that contact activation has taken place. We do not have any exact data about the BK-level in septic conditions, but given the plethora of articles describing contact activation in sepsis we have a clear notion that sepsis induces BK release. It is therefore astounding that we did not find any increased level of BK1-5 among the septic pigs. The possibility that the measurements of BK1-5 were not reliable is present. Shortage of sample material from pigs entailed that we used human blood as surrogate matrix, which may have affected the chromatographic analysis and increased lower detection limit of BK1-5. Furthermore, a thorough stability test was not performed, which may reflect the relatively low sample yield we observed. Finally, for technical reasons several of the validation samples were frozen while waiting to be analyzed. Despite this, we still have reason to suggest that the analyses were valid. Icatibant was clearly

demonstrated in the icatibant group only. More important, all samples taken from the pig receiving exogenous BK showed BK1-5 levels as expected. Similarly, the *in vitro* experiment demonstrated that the BK1-5 level corresponded closely to the amount of exogenous BK added to whole blood. Still, this reflects an uncertainty, and it seems imperative to proceed with studies and elucidate this field more closely, and, in that connection, also include samples from septic patients.

If we assume that BK is generated during sepsis, paper II demonstrates no effect of blocking the bradykinin 2-(B2) receptor. Based on theoretical estimates, we claim that the concentration of icatibant given was high, also causing a 65 % to 85 % blockage of the B1 receptor. Did we give too much icatibant? In a previous LPS-induced sepsis study on pigs, it was found that solely inhibiting the B2 receptor increased survival ($p=0.052$), and improved all hemodynamic parameters, including a reduction of pulmonary artery pressure (151). Interestingly, inhibition of both B1 and B2 receptors led to a worse outcome compared to the inhibition of the B2 receptor only. This implies that we may have masked beneficial effects in our own study by giving too much icatibant. However, the interplay or relation between the B1 and B2 receptors is complicated and difficult to penetrate, and there are indeed conflicting results which, are partly discussed in paper II.

Notably, the significance of BK as an important mediator in capillary leakage may easily have been drowned out by the massive inflammation induced by the meningococcal shock. Although BK is an immediately generated mediator in the inflammatory process, it is only one of many. The missing effect of icatibant joins the ranks of other interventional single agent studies, which in general can refer to disappointing results.

Complement inhibition and pigs

It has been challenging to find a functional complement inhibitor that could be used large-scale in pigs. In a previous study our lab tested several candidate complement inhibitors; the synthetic serine protease inhibitor FUT-175, anti-factor B mAb, anti-factor D mAb, and the recombinant protein Vaccinia virus Complement control Protein (VCP) (152). All these candidates inhibited porcine complement activation to various degrees. For instance, factor B efficiently inhibited zymosan- and HAIGG-induced complement activation, but *E. coli*-induced complement activation was insufficiently inhibited. Factor D incompletely inhibited

all types of complement activation. VCP was the only candidate that efficiently inhibited all activators of complement, and was therefore found to be promising for *in vivo* studies. However, the absence of large-scale supplies has prevented these experiments. Our lab has also tested the C5 inhibitor, eculizumab (Soliris®), and the C3 inhibitor, compstatin, and found that none of these complement inhibitors work in pigs. The arrival of OmCI was therefore encouraging, particularly since we were able to use it on a large scale.

Paper III documents that OmCI efficiently inhibits all complement pathways in pigs and humans. As briefly discussed in paper III and IV, the effects obtained by OmCI cannot exclusively be attributed to complement-inhibitory effects of OmCI, since this molecule has bifunctional properties. Unpublished data demonstrate that the binding activities of OmCI are independent, and that the binding of C5 and LTB4 occurs on opposite faces of OmCI (Miles Nunn, personal observation). Hence, OmCI has the properties to circumvent the effects of TCC, C5a, and the effects of the LTB4. The arachidonic acid derived LTB4 is of vital importance in the early inflammatory reaction, and its action includes neutrophil chemotaxis, cytokine and chemokine production, secretions of granules, phagocytosis, and induction of oxidative burst (20,153). Evidence suggests that activities of both LTB4 and C5a are reciprocally enhanced. In a mice tumor protocol study, neutrophil recruitment by C5a required amplification via LTB4 (154), and by contrast, another mice study evaluating the effect of C5a receptor-inhibition on intestinal injury revealed reduced formation of LTB4 (155). Although it seems quite obvious that the overall inflammatory power of C5a exceeds that of LTB4, the OmCI results from both paper III and IV are influenced by an unknown LTB4-inhibitory effect. In the context of attenuating the inflammatory response, this is presumably an advantage.

Differential roles of complement and CD14

An unconditional comparison between paper I, III, and IV is difficult as the results reflect *ex vivo* and *in vivo* data, and, importantly, are based on different species, human and pig. For instance, *E. coli*-induced upregulation of wCD11R3 in pig whole blood was completely abolished by OmCI (paper III), implying that this cell surface molecule was complement-dependent. By contrast, no effect was observed with respect to OmCI when pigs were challenged with *E. coli*-induced sepsis (paper IV). Common to all three studies was the strong dependence on CD14, especially those effects related to *E. coli*-induced formation of

cytokines. *Ex vivo*, anti-CD14 alone significantly and profoundly attenuated pig and human cytokines. The efficacy of complement inhibition was less pronounced, though several of the individual cytokines were significantly reduced. Combined inhibition of complement and CD14 essentially abolished the formation of all cytokines. *In vivo* (paper IV), the anti-inflammatory effects of OmCI alone and the combined effects of OmCI and anti-CD14 mirrored to a large extent the *ex vivo* observations. An additional treatment arm, exclusively with anti-CD14, would have improved the study by revealing the impact of anti-CD14 alone. Due to a lack of access to anti-CD14 this could unfortunately not be done. However, historical data reflecting the effects of anti-CD14 alone (122) reveal effects on cytokine formation comparable to the ones observed by the combined treatment group (OmCI + anti-CD14). This gives reason to suggest that the effect of OmCI in this group was partly overwhelmed by the effect of anti-CD14. At the same time OmCI alone strongly reduced thrombogenicity and exerted pronounced anti-inflammatory effects. Despite the fact that the model was featured by a particularly strong dependence on CD14, this underscores the significance of complement and redundancy of host defense.

Different roles of complement in *E. coli*- and *N. meningitidis*-induced inflammation have been comprehensively elucidated in a human *ex vivo* whole blood study (156). This study demonstrated that several cell adhesion molecules and granulocyte enzyme release were mostly complement dependent, whereas the majority of the proinflammatory cytokines were primarily dependent on CD14. Recently, the significance of complement was also demonstrated in a model of *E. coli*-induced sepsis in baboons, as complement inhibition displayed broad anti-inflammatory effects, partially reversed microcirculatory dysfunction, and prevented systemic blood pressure falling (157).

Notably, complement may be relatively more important in other sepsis models or other types of experimentally induced inflammation. For instance, the significance of C5a has been broadly uncovered in polymicrobial sepsis in rodents, induced by CLP (cecal ligation and puncture) (93). Inhibition of C5a or one or both of its receptors are all target-strategies that have been demonstrated to reduce inflammation and increased survival among rodents subjected to this type of abdominal sepsis (158,159). Preliminary data from our own lab suggest that the efficacy of complement inhibition of Gram-positive induced inflammation is to some extent more pronounced compared to Gram-negative induced inflammation.

Tissue ischemia is a central component of several conditions such as myocardial infarction, stroke, organ transplantation, and vascular surgery. Fast restoration of adequate blood flow to the jeopardized tissue is the primary treatment goal, however, reperfusion of ischemic tissue may paradoxically exacerbate tissue injury (160). Numerous reports document that complement inhibition attenuates ischemia-reperfusion injury, indicating that complement plays a pivotal role in this tissue-pathogenesis (160-162). It may be argued that the models of Gram-negative induced inflammation and sepsis used in this Thesis are less suitable in demonstrating the significance of complement, as compared to other models. Nevertheless, paper I, III, and IV elucidate a central role of complement in the applied models that cannot be ignored.

The *in vivo* models and intervention principle

Lacking concordance between animal studies and clinical trials is regularly observed (163). Concerning preclinical sepsis models the picture is probably even worse. Due to the intrinsic complexity that arises in relation to the human syndrome of sepsis, it has been stated that “there is no single ideal model of shock or sepsis” (75). Nevertheless, among several researchers the polymicrobial model induced by CLP has been regarded as the most preferred, as it better mimics the human sepsis progression, including the hypo-inflammatory or immunosuppressive “second” state of sepsis (164). The latter is important, as modulation of the blunted immune response is an interesting target for intervention (165). However, the model of *N. meningitidis*-induced sepsis, which was used in this Thesis, mirrors to a large extent the human meningococcal disease and displays clear translational properties (119), and could not be replaced by a CLP-model. Besides, there are also several limitations with the CLP-model, not least concerning the inaccuracy with respect to controlling the magnitude of sepsis (164). In addition, the majority of these studies have been performed using rodents, which do not share the same degree of similarities with humans as pigs do (see Material and methods.)

The applied sepsis-models of either *E. coli* or *N. meningitidis* have both definite strengths. They are forceful *in vivo* models, particularly suitable for studying the initial phase of Gram-negative shock, and demonstrate an important combined investigation of inflammation, hemostasis, and physiology. Although the time frames in both models were compressed, they displayed time-dependent dynamics characteristic of shock development. Furthermore, the

models are well characterized and standardized, implying that the interpretation of the data can be integrated into a larger context. This is of significant value, and has been stated to be an explicit challenge with regard to preclinical models (166).

There are several limitations with the *in vivo* models, which should carefully be considered. The pigs subjected to *E. coli*-induced sepsis in paper IV had an average weight of 15 kg, and are thus comparable to small children in the clinic. The data might therefore reflect pediatric sepsis more than adult sepsis. In both paper II and IV the administration of inhibitors or treatment was given prior to the challenge of sepsis and inflammation. A prophylactic treatment regimen limits the clinical utility, of course. Although prophylactic treatment as such is widely used in modern medicine, not least antibiotics in relation to surgical procedures (167,168), a prophylactic treatment for sepsis is not yet an issue. Theoretically, one can imagine the usefulness of providing a prophylactic sepsis regimen. The combined inhibition of complement and CD14/TLRs was also initially proposed as a treatment strategy to the patients at risk (68). The challenge, however, is to determine how we identify these patients. As already mentioned in the introduction, we do not have the tools to identify the patients at risk, despite the availability of a large number of biomarkers (75,169). In addition, a prophylactic treatment regimen that profoundly attenuates central arms of innate immunity must be provided with caution, and it necessitates a secure identification of the patients at risk. Thus, a prophylactic treatment regimen remains a question for the future.

When it comes to judging the usefulness of the prophylactic treatment, we must remember that the primary goals of the included studies belong to the field of science exploring the *proof of concepts*. The different studies try to answer the scientific questions regarding whether the molecules C5, CD14, TNF- α , IL-1 β , and BK are involved, and to what extent they are involved, in Gram-negative induced inflammation and/or sepsis. As part of a preclinical investigation of sepsis and the study of the underlying mechanisms, this is of great importance and a prerequisite for further investigation that may explore the effect of postinsult inhibition or treatment (166). Interestingly, delayed administration of anti-C5a antibody to mice subjected to CLP-sepsis, showing obvious clinical signs of sepsis, has shown to increase the survival rates (158). These encouraging results should be confirmed in future experiments with larger animals such as pigs, thereby providing better preclinical evidence and information about a therapeutic window.

The translational advantage of the *in vivo* models

The close relation between inflammation and coagulation (21) was clearly demonstrated in paper IV. The combined inhibition of C5 and CD14 had a striking anti-inflammatory effect, totally ablated TF expression, and prevented neutropenia. OmCI alone significantly attenuated the formation of TNF- α and IL-6, reduced the expression of TF with more than 40 %, and dampened sepsis-induced histopathological lung changes. These effects are particularly interesting and demonstrate the remarkable and potent effect of upstream inhibition of PRRs. The global effects were multifaceted and attenuated some core features of sepsis; inflammation (138), thrombogenicity (170), neutropenia, and tissue (lung) injury (171). The model's ability to show these dynamic relations is a strength and indeed of translational value.

The effects of OmCI parallel those observed in a newly published report according to which complement inhibition partially reversed sepsis-induced leucopenia, thrombocytopenia, DIC, and inflammatory response, and provided a substantial organ-protection (157). It is well established that complement promotes coagulation and thrombosis by upregulation of TF on neutrophils, monocytes and endothelial cells (21,40). It was recently shown that *E. coli*-induced TF functional activity in plasma microparticles was primarily dependent on C5aR (172). This may be of great importance, since the formation of microparticles seems to reflect the severity of DIC (97). In accordance with our observation demonstrating a total ablation of TF-expression by the combined inhibition of CD14 and complement, evidence also suggests that upregulation of TF is dependent on TLR4/MD2-signaling (173). However, other reports maintain that either CD14 or LPS is important for TF upregulation (172,174), suggesting that this must be further elucidated. I have previously commented on the anti-inflammatory effect of OmCI alone and the tremendous effect provided by combined inhibition by anti-CD14 and OmCI. The vast anticoagulant effect of the combined treatment regimen may have reinforced this anti-inflammatory effect due to reduced amount of coagulation/fibrinolysis proteases and thereby cleavage of complement factors (175).

Interpretation of the histopathological findings in paper IV appears to be somewhat difficult. OmCI alone seemed to reduce the sepsis-induced lung changes markedly, though these results were not statistically significant, whereas no similar trend was observed in the combined treatment group. Reduced thrombogenicity and inhibited LTB₄-dependent effects would in both groups partially induce the same protective effects. Attenuation of wCD11R3 by the combined treatment regimen should presumably have a tissue-protective effect, as this

integrin molecule plays a critical role in sequestering neutrophils from the circulation (85). Furthermore, the adhesions molecules VCAM and ICAM, which are important for neutrophil extravasation and inflammation, are upregulated on respiratory epithelium upon LPS-stimulation (176). The question as to why we did not see any lung-protective effect of the combined treatment regimen, remains unanswered and a subject of speculation. Neither the increased numbers of neutrophils observed in this group nor a side effect conditional on the administration of anti-CD14 can be excluded.

The abrupt increase in MPAP forced us to give norepinephrine as rescue medication, which certainly biased the hemodynamic data. It is therefore tempting to speculate that the increased HR and MPAP observed in the combined treatment group would be further delayed or only limitedly increased if we had used a less forceful model.

Thus, this *in vivo* study demonstrates that combined inhibition of key molecules belonging to the main system of recognition has the propensity to act broadly on the numerous biomarkers and reveal pronounced effects on downstream mediators far beyond what is achievable from *ex vivo* studies. With regard to sepsis, several authors have correctly claimed that single-mediator directed therapy insufficiently exerts effects, or hardly exerts any effects at all, on the complex network of mediators (177-179). This Thesis supports this notion. The *ex vivo* experiments performed in paper I confirm the downstream properties of proinflammatory cytokines, in addition, paper II questions the significance of BK in sepsis and suggests that this one is of minor importance. In contrast, upstream inhibition of central pathways of innate immunity, complement and CD14, appears to be more of a pluri-mediator directed therapy (Figure 3). Careful interpretation should always be made when evaluating preclinical and experimental data (180), however, the findings from the Thesis provide valuable insight into the inflammatory pathways, which will guide further science.

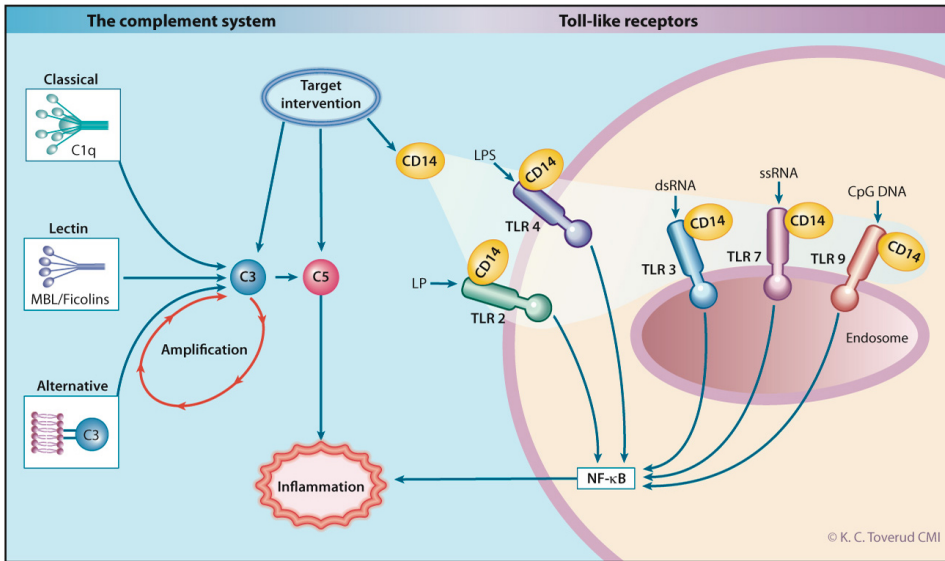


Figure 3. A proposed approach for upstream target intervention of innate immunity to attenuate inflammation.

Future perspectives

Administration of LPS to healthy humans induces differential expression of 3714 unique genes (181), reflecting the enormous complexity of the innate immune response. At the same time, the expression of innate immune responses seems to be regulated through a relatively small number of pathways, which is encouraging. In the continuing work it seems imperative to elucidate the efficacy of postinsult or rescue-inhibition of Gram-negative induced inflammation and sepsis, and explore the possibility of a potential therapeutic window. In principle this may be achieved by *ex vivo* experiments, but obviously needs further examination *in vivo*. Using a polymicrobial model induced by CLP in pigs would indeed have been tempting, but source demanding with respect to finances, time, and logistics. Still, I believe there is no way around it we are to continue down this path, and we need a more realistic or disease-like model for this purpose. In addition, it seems crucial to reveal the significance of complement and CD14 in inflammation induced by other pathogens than *E. coli*, especially Gram-positive bacteria that are the cause of a large portion of the infectious diseases. Although infections by microbes potently activate an inflammatory response, the endogenous derived inducers of inflammation may trigger a similar harmful response via the

same inflammatory pathways. As already mentioned with regard to complement, studies suggest that the TLR receptor family is implicated in myocardial ischemia-reperfusion injury, especially TLR4 and TLR2 (182,183). We have established a relevant pig model of myocardial infarction suitable for exploring the significance of the independent branches of pattern recognition, complement, and CD14. In future projects, it will therefore be interesting to explore the efficacy of the combined inhibition of complement and CD14 in this model, but also in other relevant inflammatory disease models of endogenous origin.

CONCLUSION

This Thesis clearly demonstrates a significant difference between inhibition of upstream versus downstream mediators of Gram-negative induced inflammation. Central proinflammatory cytokines such as TNF- α , IL- β , and IL-6, although early in the cytokine response, belong to the downstream actors of inflammation as compared to upstream mediators of recognition systems such as complement and CD14/TLRs. The tick-derived C5 inhibitor, OmCI, was comprehensively evaluated in pigs and humans, and the data demonstrate an effective and comparable potency of OmCI in both species. In a preclinical model of Gram-negative sepsis in pigs, pretreatment with OmCI combined with anti-CD14 displayed vast anti-inflammatory properties, reduced thrombogenicity, and delayed hemodynamic changes. Despite the model's apparent dependency on CD14, OmCI alone markedly reduced thrombogenicity and formation of central proinflammatory cytokines. Although there is still a long way to go, combined inhibition of complement and CD14 represents a potential treatment regimen in Gram-negative sepsis. Importantly, this treatment regimen may be an interesting treatment approach in other systemic inflammatory conditions, of exogenous as well as endogenous origin, as well.

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Paper I

Paper II

Paper III

