UiT
THE ARCTIC
UNIVERSITY
OF NORWAY
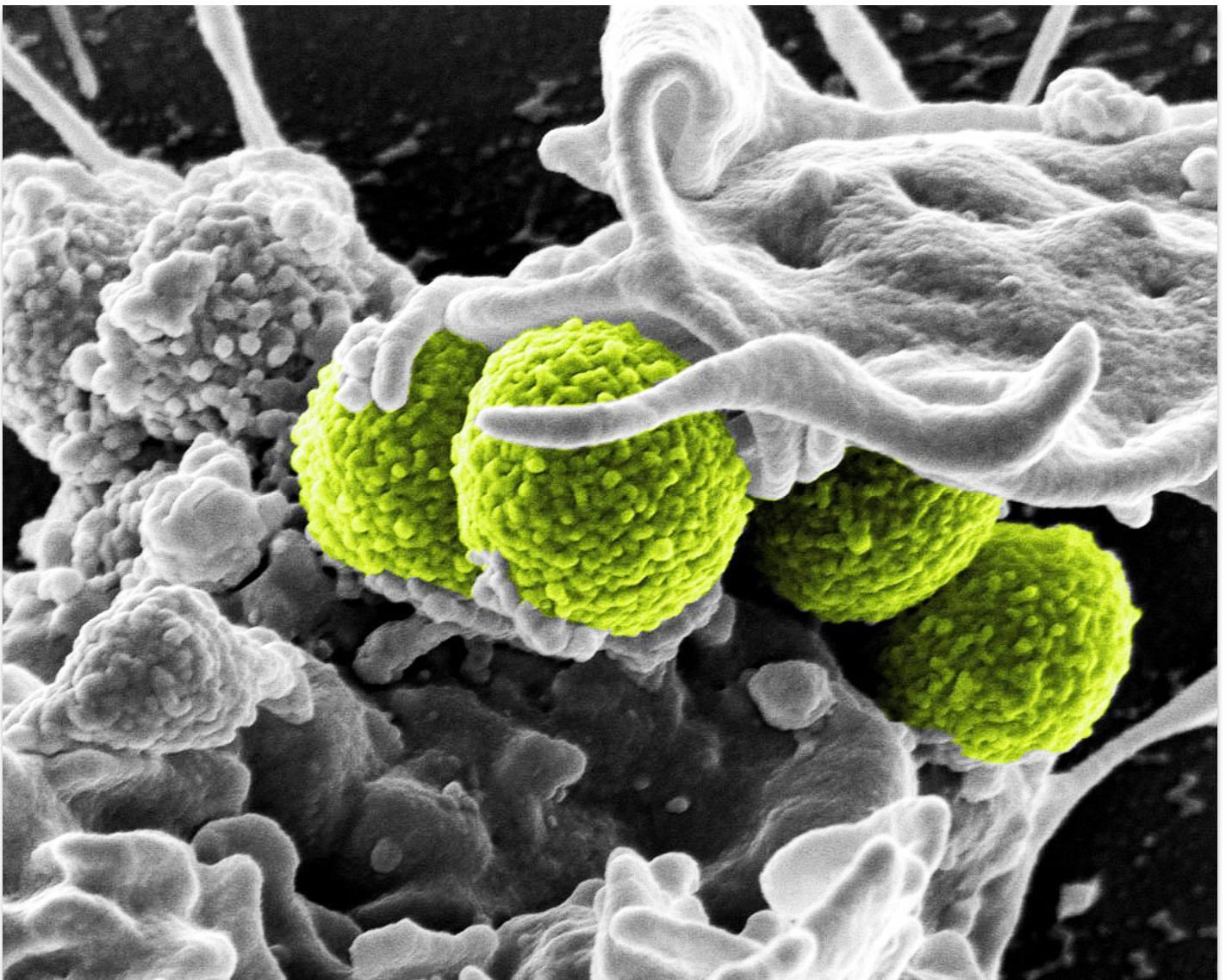
Faculty of Health Sciences

Harnessing Innate Immunity
Complement and TLR Inhibition in Experimental Models of Gram Positive and Polymicrobial Bacteremia and Sepsis
—
Espen Waage Skjeflo
A dissertation for the degree of Philosophiae Doctor – June 2017

UNIVERSITY OF TROMSØ

"La science, mon garçon, est faite des eurreurs, mais d'erreurs qu'il est bon de commetre, car elles mènent peu à peu à la verité"

"Science, my friend, is made up of mistakes, but they are useful mistakes, because little by little, they lead to the truth

- Jules Verne, Voyage au Centre de la Terre (1864)

Front: Scanning electron microscopy image showing macrophage phagocytosis of *Staphylococcus aureus*.

From Wikimedia Commons, the free media repository

Table of Contents

Acknowledgements

This thesis would have been impossible without the help and support of several great colleagues, friends and family. First and foremost, I would like to thank my supervisor, professor Tom Eirik Mollnes, for his unwavering support, optimism and belief in me and this project – ever since I joined his team in 2010. Tom Eirik has a nearly uncanny way of seeing hope and reason in results that are seemingly useless. His knowledge and overview of innate immunity, as well as his attention to detail in manuscript writing and graphical presentation is truly admirable. I remember Tom Eirik thanking me for joining his group, but I am ever grateful for the possibility to be a part of his productive and inspiring group!

Secondly, I'd like to thank my co-supervisor, professor Terje Espevik, for his invaluable expertise in planning the studies, deciphering and presenting the data afterwards. In spite of a tremendous workload, Terje still managed to give excellent feedback in important phases of this work. I would also like to thank him for the warm welcome in Trondheim when we first met.

My father has always been a great inspiration to me, so I am very glad we got the chance to collaborate on a large portion of this thesis together. It was particularly rewarding to set up the animal research lab with Knut. Our discussions of everything from academia to running mean a great deal to me.

I would also like to thank everyone in and associated with the Norwegian Complement Research Group for the excellent teamwork and cooperation these last seven years. I cherish the expertise and guidance of the chief laboratory technicians here in Bodø, Dorte, Hilde, Judith and Grethe, and I thank you for your patience and humor. Likewise, I would like to thank Anne, Corinna, Ole-Lars, Trent and Jørgen for the cooperation in our *in vitro* work. Equally, I would like to thank Caroline, Knut, Sturla, Bent, Sven Haakon, Miles, Markus and Andreas for the work in the animal research lab.

Petter has lead my department during my PhD and I could not wish for a better leader and now friend. You have a keen interest in improving the possibilities for research at Nordlandssykehuset and have always had time and shown interest in my work and progress. This has been a great source of inspiration to me.

Last, but not least, I would like to thank my family for their love and support. Most importantly, I would like to thank my wife and best friend, Martine, for her patience. Family life puts things in perspective and reminds me how lucky I truly am!

Espen W. Skjeflo

Bodø, June 19th, 2017

Abbreviations

AP = Alternative pathway

C = Complement

CP = Classical pathway

DAMP = Damage-associated molecular pattern

DIC = Disseminated intravascular coagulation

E. coli = *Escherichia coli*

EDTA = Ethylenediaminetetraacetic acid

ELISA = Enzyme-linked immunosorbent assay

Ig = Immunoglobulin

IL = Interleukin

LP = Lectin pathway

LPS = Lipopolysaccharide

LTA = Lipoteichoic acid

MAC = Membrane attack complex

MASP = MBL-associated serine protease

MBL = Mannose-binding lectin

NET = Neutrophil extracellular trap

PAMP = Pathogen-associated molecular pattern

PRR = Pattern recognition receptor/molecule

ROS = Reactive oxygen species

RT-qPCR = Quantitative reverse transcription polymerase chain reaction

S. aureus = *Staphylococcus aureus*

SIRS = Systemic inflammatory response syndrome

SOFA = Sequential (Sepsis-related) Organ Failure Assessment Score

TCC = Terminal complement complex

TF = Tissue factor

TNF = Tumor necrosis factor

Publications included

Paper 1: **Combined Inhibition of Complement and CD14 Efficiently Attenuated the Inflammatory Response Induced by *Staphylococcus aureus* in a Human Whole Blood Model**. Skjeflo EW, Christiansen D, Espevik T, Nielsen EW, Mollnes TE. *J Immunol*. 2014;192(6):2857–64.

Paper 2: ***Staphylococcus aureus*-induced Complement Activation Promotes Tissue Factor-mediated Coagulation.** Skjeflo EW, Christiansen D, Fure H, Ludvigsen JK, Woodruff TM, Espevik T, Nielsen EW, Brekke OL, Mollnes TE. *Submitted*

Paper 3: **C5aR1 and CD14 are Crucial for Phagocytosis of Live and Dead *Escherichia coli* and *Staphylococcus aureus* in Human Whole Blood.** Skjeflo EW, Christiansen D, Landsem A, Steinvik J, Woodruff TM, Espevik T, Nielsen EW, Mollnes TE. *Submitted*

Paper 4: **Combined Inhibition of Complement and CD14 Improved Outcome in Porcine Polymicrobial Sepsis**. Skjeflo EW, Sagatun C, Dybwik K, Aam S, Urving SH, Nunn MA, Fure H, Lau C, Brekke OL, Huber-Lang M, Espevik T, Barrat-Due A, Nielsen EW, Mollnes TE. *Critical Care*. 2015 Nov 27;19(1):415.

Summary

- Sepsis is a syndrome of life-threatening organ malfunction caused by a dysregulated host response to infection

- The complement, kallikrein/kinin, coagulation and fibrinolysis plasma cascade systems as well as the TLR system recognize and initiate the immediate responses to infection and thus play an important role in the development of sepsis

- The ubiquitous TLR co-receptor, CD14, as well as C5 have been identified as upstream, bottle-neck targets to attenuate the response to infection

- *S. aureus* is the most common cause of Gram-positive sepsis whereas *E. coli* is the most common cause of Gram-negative sepsis

- Both bacteria activate complement and coagulation, and are recognized by TLRs in human whole blood, triggering the release of inflammatory cytokines, markers of coagulation activation and markers of leukocyte activation

- In turn, combined inhibition of complement and CD14 efficiently reduced these responses to *S. aureus* and *E. coli*

- In a randomized, controlled trial of combined CD14 and C5 inhibition (treatment) versus saline (control) in porcine polymicrobial sepsis, treatment significantly improved survival

- Preemptive, combined inhibition of CD14 and C5 thus emerges as a potential anti-inflammatory regimen in both *S. aureus* and *E. coli* bacteremia

Background

Introduction

Sepsis is a fascinating and incompletely understood syndrome defined as life-threatening organ malfunction caused by a dysregulated host response to infection (Singer M et al., 2016).

Clinically, sepsis can be diagnosed if there is a change of two or more points in the sequential (sepsis-related) organ failure assessment (SOFA) score, encompassing the respiratory, hepatobiliary, cardiovascular, renal, central nervous and coagulation systems (table 1). Severe cases result in septic shock - a state of organ malfunction, refractory hypotension and hyperlactatemia demanding aggressive supportive therapy.

Table 1. Sequential [Sepsis-Related] Organ Failure Assessment Score[a]

System	Score				
	0	1	2	3	4
Respiration					
PaO_2/FiO_2, mm Hg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation					
Platelets, ×10^3/µL	≥150	<150	<100	<50	<20
Liver					
Bilirubin, mg/dL (µmol/L)	<1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular	MAP ≥70 mm Hg	MAP <70 mm Hg	Dopamine <5 or dobutamine (any dose)[b]	Dopamine 5.1-15 or epinephrine ≤0.1 or norepinephrine ≤0.1[b]	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1[b]
Central nervous system					
Glasgow Coma Scale score[c]	15	13-14	10-12	6-9	<6
Renal					
Creatinine, mg/dL (µmol/L)	<1.2 (110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440)	>5.0 (440)
Urine output, mL/d				<500	<200

From Singer M et al., 2016

Epidemiological data vary, but incidence rates of 100-1000 cases per 100 000 population per year are not uncommon (Alberti et al., 2002; Czupryna et al., 2013; Flaatten, 2004; Martin et al., 2003; Mellhammar et al., 2016). Overall mortality is close to 20%. Septic shock has recorded mortality rates of more than 40%, increasing with patient age and number of comorbidities (Flaatten, 2004; Gaieski et al., 2013; Kaukonen et al., 2014). Despite a lot of research in the field, no specific therapy exists.

Central to our current understanding of sepsis lies the notion that disease is not caused by the infectious agent, but by the body's dysregulated response to it. The infective agent merely topples the first domino in "[a] cascade that is initiated by a focus of infection or injury and ends with severe endothelial damage, profound hemodynamic derangements and, often, death" (Bone, 1991). In fact, several hallmarks of sepsis are also present in non-microbial conditions such as trauma and burns (Chen and Nuñez, 2010), and more than 80% of the sepsis transcriptome response is independent of the source and/or the causative pathogen of the underlying infection (Burnham et al., 2016; Vught et al., 2016). As the immune system is the first to act in response to infection, initial experiments (of modern time) have aimed to reduce an overzealous activation, often by pin-pointing individual pro-inflammatory cytokines and vascular mediators. In the recent years, however, the idea of a singularly exaggerated immune response has been questioned. Partly, because the promising results of immunomodulatory therapy in animal models

have failed to apply in the human setting (Deitch, 1998; Rittirsch et al., 2007), and partly because the pathogenesis of sepsis and septic shock is both pro- and anti-inflammatory (Antonelli, 1999; Frencken et al., 2017; Vught et al., 2016). Potential therapies are therefore still needed and the search continues with clinical trials ongoing (Schmidt and Clardy, 2016).

As the immediate pathogen recognition and activation of innate immunity remains the proposed initiating event of sepsis, modulating this upstream point of origin might be an approach to alter the otherwise detrimental response – it is therefore the focus of this thesis.

Inflammation and the Pathogenesis of Sepsis

The regulated inflammation of infection is primarily a physiological and protective process meant to restore homeostasis (Medzhitov, 2008). It basically revolves around the three R's - *r*ecognition, *r*esponse and *r*esolution: The immune system recognizes the infective agent. It then initiates a proportional, well-orchestrated response aimed at destroying the agent with minimal collateral tissue damage. Upon clearance of both the infective agent and the activated immune components, homeostasis is restored. This is also the case in localized infection. In sepsis, however, the response is disproportional and without resolution.

Early on, bacterial endotoxin or lipopolysaccharide (LPS) of the Gram-negative cell wall was – and for many still is – the suspected main cause of sepsis. LPS was therefore used in several animal models. Circulating and membrane-bound receptors on the cells of the immune system, notably polymorphonuclear neutrophils (PMNs), recognize LPS and transcript and release cytokines, the "hormones" of inflammation (Cohen, 2002; Poltorak et al., 1998). The use of LPS alone, however, proved too simplistic. Bacteria stripped of LPS can initiate similar effects, albeit at higher concentrations (Hellerud et al., 2010), but more importantly, at least half of all cases of sepsis are caused by Gram-positive bacteria (Vincent et al., 2009) lacking LPS altogether. Yet, the immune system does initiate a harmful response to infection in sepsis. It starts with innate immune recognition of the infective agent's pathogen associated molecular patterns (PAMPs) – conserved and vital components of the bacterial cell wall and cytosolic components including LPS. Equally, damage-associated molecular patterns (DAMPs) exposed by the infective agent's virulence factors, e.g. exposed intracellular proteins in response to pore-forming toxins or ischemia – can trigger and aggravate the initial response (Denk et al., 2012). Today, sepsis is generally acknowledged to follow a biphasic course of initial hyper-inflammation with the release of both pro- and anti-inflammatory mediators which is then followed by a more prolonged phase of immunosuppression in both innate and adaptive immune systems with immune cell apoptosis and anergy (Hotchkiss et al., 2013). However, two recent studies revealed that only 10.9% of patients die from

secondary infections and that the balance between pro-inflammatory IL-6 and anti-inflammatory IL-10 were stable over time in patients with severe sepsis and septic shock, thus questioning the secondary phase of unique immunosuppression (Frencken et al., 2017; Vught et al., 2016). This thesis, however, is all about the initial phase.

The innate immune system - as any biological system maintaining homeostasis - consists of afferent or sensing components that register disturbances (here pathogens). The effectors or efferent components mount a response aimed at resolving these disturbances (Beutler, 2004). Together with leukocyte activation, the early activation of the innate immune system includes activation of the plasma proteolytic cascade systems: The complement system, the coagulation system, the fibrinolytic system and the kallikrein-kinin or contact system (Frick et al., 2007; Levi and Ten Cate, 1999; Markiewski et al., 2008; Vervloet et al., 1998; Ward, 2004). Indeed, all systems are so closely linked that their division seems somewhat arbitrary and their collective activation is often referred to as thromboinflammation (Ekdahl et al., 2016). The complement system and coagulation system will be reviewed both separately and together in the following sections before the discussion of the Toll-like receptors (which also cross-talk extensively with the other systems).

Role of the Complement System in Health and Disease

The complement system is a group of nearly 50 different circulating and membrane-bound proteins rapidly activated in the vicinity of PAMPs and DAMPs (Walport, 2001a, 2001b). Early complement research designated complement as "just an elegant model system" (Hobart, 1984). As research has progressed it is now regarded an important system in immune surveillance and homeostasis. It has several other functions beside that of antimicrobial defense including synapse maturation, angiogenesis, mobilization of hematopoietic stem-progenitor cells, tissue regeneration and lipid metabolism as reviewed by Ricklin (Ricklin et al., 2010). There are nearly as many regulators in this system as there are effectors, emphasizing that the system is highly potent and kept on a tight leash (Zipfel and Skerka, 2009).

The complement system has three different enzyme-driven pathways of activation upon an exogenous, foreign or endogenous, damaged structure - the classical pathway (CP), lectin pathway (LP) and alternative pathway (AP). They all merge at the formation of a C3 convertase which divides C3 to C3a and C3b (Figure 1). The classical pathway, also known as the antibody-dependent pathway, is activated by clustered immunoglobulins (IgM or IgG) - hence the name, but also by other pattern recognition molecules such as the pentraxins (including the clinically useful C-reactive protein, CRP). After C1q of the C1 complex binds, the two serine proteases C1r activate the two C1s proteases and the five-unit C1 complex splits. C1s further cleaves C4 into C4a

and C4b, the latter opsonizes the cell or bacterium in question. C1s also cleaves C4-bound C2 into C2a and C2b in formation of the classical and lectin pathway C3 convertase, C4b2b.
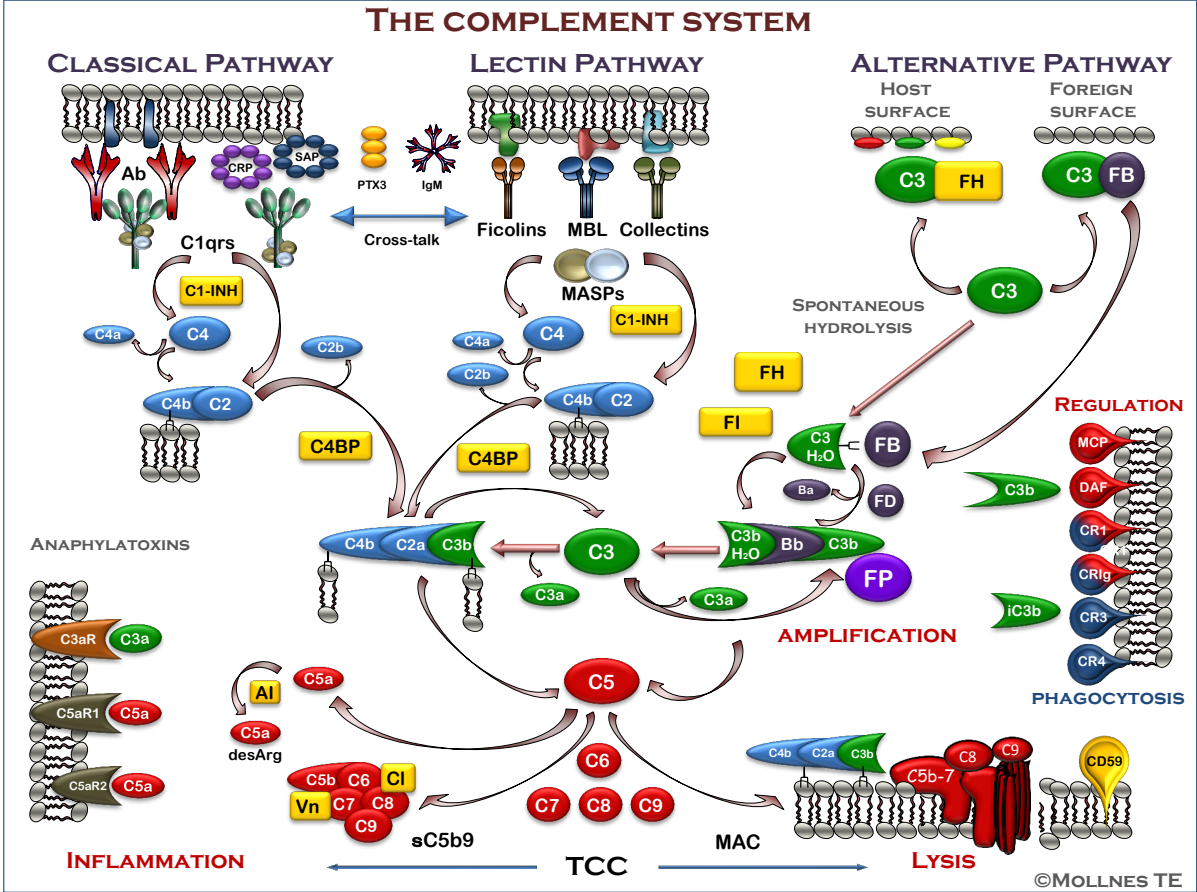

Figure 1: Overview of the complement system

The LP is similar to the CP ending in formation of the C4b2b. However, the initiating danger signal and recognition molecules differ as the lectins are carbohydrate-recognizing molecules (Fujita, 2002). Mannose-binding lectin (MBL) and ficolins – resembling C1q in structure - recognize mannose sugars on bacteria and IgA. Upon this activation, MBL and ficolins associate with the MBL-associated serine proteases (MASPs), notably MASP-2 which then cleaves C4 and C2 (Fujita, 2002).

The alternative pathway represents up to 80-90% of total complement activation (Harboe et al., 2004). The pathway is immediately activated as C3b is deposited on bacteria, foreign or apoptotic cells owing to the constant tick-over of C3; A small fraction of C3 is hydrolyzed to $C3_{H2O}$ which then binds factor B (FB). FB is subsequently cleaved by factor D (FD), forming the alternative pathway C3 convertase, $C3_{H2O}Bb$ in plasma. This generates C3b with a thioester moiety that binds amines and carbohydrates on foreign surfaces (Ricklin et al., 2010). An initially modest tagging is then greatly amplified on foreign cells and inhibited on cells of self by factor H (FH), as well as by factor I (FI) in the fluid phase. Membrane-bound C3b associates with FB which is then cleaved by FD and the convertase is further stabilized by properdin (FP), generating the AP C3 convertase, C3bBbP which then activates more C3 for greater opsonization and downstream complement activation.

As soon as AP amplification generates sufficient C3b the C3 convertases also incorporate C3 (C4b2b3b or C3bBb3b) and shift their selectivity towards C5. C5 is cleaved to C5a and b. C5b can then bind C6 through C8 and several molecules of C9. This forms C5b-9 also known as the membrane attack complex (MAC), which is able to punch holes in lipid membranes and lyse microbes, but also has effector functions at sublytic levels (Kilgore et al.,

18

1997). Soluble C5b-9 (sC5b-9) is also formed, and the two forms of C5b-9 are collectively termed the terminal complement complex (TCC).

Complement in Sepsis

The smaller fragments of complement activation, particularly C3a and C5a, have important functions in infection and inflammation (Haas and van Strijp, 2007). They are known as the anaphylatoxins because of their ability to induce smooth muscle contraction and capillary leakage. C5a is the more potent of the two. Through its two known receptors, C5aR1 and C5aR2, it functions as a powerful chemoattractant, activates phagocytic cells, and induces the release of histamine, granule-based enzymes and oxidants. C5a also activates the coagulation system and impedes vasomotor control (Guo and Ward, 2005). Recently, C5aR2 stimulation was proposed as an inhibiting step of C5aR1 activation both *in vitro* and *in vivo* (Bamberg et al., 2010; Wang et al., 2016).

The complement system is dysregulated in sepsis and detectable anaphylatoxins and TCC are proposed markers of complement hyperactivation (Markiewski et al., 2008). In this regard, C5a is suggested the primary cause of the complement-mediated effects: On the one hand, high levels of C5a shut down many essential neutrophil functions such as chemotaxis and oxidative burst. On the other hand, C5a hyper-activates macrophages leading to increased cytokine release (Ward, 2010, 2004) and was recently shown to modulate neutrophil pH directly (Denk et al., 2017). C5a also increases cytokine release from endothelial cells, increases tissue factor (TF)

expression, and compromises cardiomyocyte function (Kalbitz et al., 2016). Lastly, high levels of C5a induce thymocyte apoptosis and may contribute to the proposed immunosuppression in late-stage sepsis (Riedemann et al., 2002).

Complement activation is therefore often regarded as a double-edged sword, both in sepsis and the several immune mediated diseases it is now documented to participate in (McGeer et al., 2016; Tom E. Mollnes et al., 2002).

Role of the Coagulation and Fibrinolytic Systems in Health and Disease

In reality though, complement is not a distinct entity of plasma as it is so closely linked to the other cascade systems of coagulation and fibrinolysis. The process of hemostasis is clearly involved in the thromboinflammatory response to danger. Indeed, as with complement activation, the coagulation system balances between hypo-activation and hyper-activation, thereby preventing hemorrhage or thrombosis, respectively. However, thrombosis or intravascular clot formation has also recently been proposed involved in physiological inflammation (Engelmann and Massberg, 2013), and coagulation has been proposed to trap invading pathogens as even primitive organisms without an organized circulatory system have a rudimentary coagulation

system (Funk et al., 2009). In this regard, physiological immunothrombosis also fits the hypothesis of a regulated, localized response to infection.

The process of coagulation or blood clotting is closely related to the formation of a platelet plug in primary hemostasis. Coagulation may also occur regardless of platelet plug formation (Walter and Boulpaep, 2012). Briefly, the formation of a fibrinogen-covered platelet plug is considered the third step in hemostasis following initial vasoconstriction and increased tissue pressure whereas blood coagulation or clot formation, occurring through strictly controlled proteolysis of plasma coagulation factors, is the fourth step.

As with complement activation, blood clotting can be considered a "branching tree" where different pathways of activation converge on a final common pathway. Although this convention is highly simplified by omitting all the cross-talk (which will be discussed later), it does offer some perspective.

The so-called intrinsic or contact-dependent pathway is initiated by collagen, a negatively charged surface (such as that of an activated platelet or circulating microparticle) or platelet-bound high-molecular-weight kinogen (HMWK) that mediates the formation of activated factor XII (Hageman factor), i.e. FXIIa. FXIIa then converts prekallikrein to kallikrein, which accelerates the conversion of FXII to FXIIa in a positive feedback fashion, and also activates factor FXI to FXIa. Of note, kallikrein also cleaves HMWK to release bradykinin, a potent

vasodilator and pro-inflammatory mediator (Frick et al., 2007). FXIa - as FVa and FIIa - cleaves FVII to FVIIa. VIIa forms a quaternary complex with FXIa, calcium and negatively charged phospholipids on the platelet surface known as the (intrinsic) tenase. The tenase cleaves factor X to FXa.

Similarly, the extrinsic pathway is initiated through tissue factor, TF (prothrombinase or factor III) exposed to the circulation. TF is constitutively expressed in non-vascular cells and is especially inducible in circulating monocytes, neutrophils and microparticles (Mackman, 2009). It is thus exposed after endothelial damage and inflammation. TF mediates FVIIa generation and TF, FVIIa and calcium form a tertiary (extrinsic) tenase also generating FXa.

The final common pathway is initiated by FXa, which mediates FVa formation. FXa, FVa and calcium form a prothrombinase complex. As the name implies, prothrombinase generates thrombin from prothrombin. Thrombin, therefore, is at the heart of coagulation. It has three main purposes. First, it converts fibrinogen to fibrin monomers, which spontaneously and immediately polymerize, and then stabilize by FXIIIa, also generated by thrombin. Second, thrombin accelerates FVIIa and FVa generation through positive feedback and third, activates endothelium and platelets (Walter and Boulpaep, 2012).

Because of their relevance in clinical biochemistry, the two pathways of coagulation activation are often illustrated as of equal biological importance, but the physiological role of the contact or intrinsic pathway is much less than that of the tissue factor pathway. For one thing, individuals deficient in factor XII rarely suffer from bleeding disorders and inhibiting fXII does not interfere with hemostasis, but is rather proposed to protect from pathological thrombosis (Kleinschnitz et al., 2006). Recently contact activation was also proposed to participate in "capping" the otherwise oozing blood of skin wound (Cooley, 2016), but the tissue factor pathway of coagulation is still the main focus of coagulation activation in this thesis.

Fibrinolysis and Anticoagulation

The coagulation system is counterbalanced by several paracrine and anticoagulant factors, mainly originating from the endothelium. These include the tissue-factor pathway inhibitor (TFPI), which targets the prothrombinase, and antithrombin (AT), which targets thrombin and FXa. Thrombomodulin secures thrombin to the endothelial surface where it activates protein C. Activated protein C, by the aid of co-factor protein S, then inactivates factors VIIa and Va. Furthermore, as a final event of coagulation, the fibrin clots are degraded through fibrinolysis by plasmin, which itself is formed from plasminogen by either the endothelial tissue plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). Naturally, these can also be counter-balanced by two other serine protease inhibitors (serpins), namely the

plasminogen activator inhibitors 1 and 2 (PAI-I, -II) where PAI-I is the most relevant. To further complicate matters, it appears that hemostasis and anticoagulation can be highly specific to particular vascular beds in the body. So the coagulation and fibrinolysis systems exhibit considerable variability, not only between individuals, but also within one individual (Rosenberg and Aird, 1999).

Just as with complement, the clotting cascades and consequent fibrinolysis are potent biological systems that must be controlled. During inflammation and sepsis, this control is lost leading to simultaneous thrombosis and hemorrhage (Figure 2).

Figure 2: A child suffering from sepsis with DIC. (The picture is released on consent from the child's parents and the treating paediatrician, Petter Brandtzaeg)

In severe sepsis, disseminated intravascular coagulation (DIC) is characterized by microthrombi and diffuse fibrin deposition that occlude capillaries and cause multiple-organ failure, but also profuse bleeding in other areas such as the skin (Levi and Ten Cate, 1999). Thus, DIC is strongly associated with higher mortality in sepsis, and its interrelation with inflammation is complex and potentially devastating

as the two processes activate and perpetuate each other (Levi and van der Poll, 2010). However, as mentioned, the two may not be different entities. A recent review identifies the intriguing process of immunothrombosis as a novel component of the innate immune system (Engelmann and Massberg, 2013). Here, the authors propose that immunothrombosis has several different physiological functions: Pathogens are trapped and unable to disseminate as microvessels are sealed with microthrombi. In doing so, immunothrombosis generates a closed compartment ideal for pathogen recognition and killing. Furthermore, fibrinogen and/or fibrin enhance the immune response through immune cell recruitment whilst NETosis [1] is highly dependent on its procoagulant properties for optimal effect. However, the authors identify DIC as a form of aberrant immunothrombosis. Whereas microthrombi and fibrin deposition allow sufficient organ perfusion and tissue oxygenation under physiological conditions, they may strangulate these same organs in sepsis and DIC, causing multiple organ failure. What favors immunothrombosis to DIC, is still unknown.

The Toll-like Receptor System

Toll-like receptors are the prototype immune receptors and the best-studied pattern recognition receptors or molecules (PRRs) of the innate immune

---

[1]Neutrophils have recently been shown to degrade their DNA and incorporate it with histones, proteins (lactoferrin and cathepsin to name a few) and enzymes (such as myeloperoxidase (MPO) and elastase) for release to the extracellular milieu as neutrophil extracellular traps (NETs) (Brinkmann et al., 2004)

system (Kawai and Akira, 2010; Takeda et al., 2003). PRRs recognize a multitude of structures considered dangerous or foreign to the host. These structures include the essential, conserved microbial structures (PAMPs), such as the lipid A-portion of LPS in Gram-negative bacteria and peptidoglycan of Gram-positive bacteria (Medzhitov, 2001).

To date, 10 different human TLRs are known (Figure 3). These are transmembrane proteins of either the cell walls or intracellular compartments, characterized by their extracellular leucine-rich repeat (LRR) domains and intracellular Toll/IL-1 receptor (TIR) domains. TLR4 was the first receptor described and recognizes LPS whereas TLR2 is found to recognize a broad range of PAMPs through its heterodimerization with either TLR1 or TLR6 (O'Neill et al., 2013). Notably, several accessory molecules to the TLRs are described, such as CD14, essential to not only proper LPS recognition (Jiang et al., 2005; Zanoni et al., 2011) but cofactor to several other TLRs as well, such as those recognizing peptidoglycan and bacterial DNA (Lee et al., 2012).
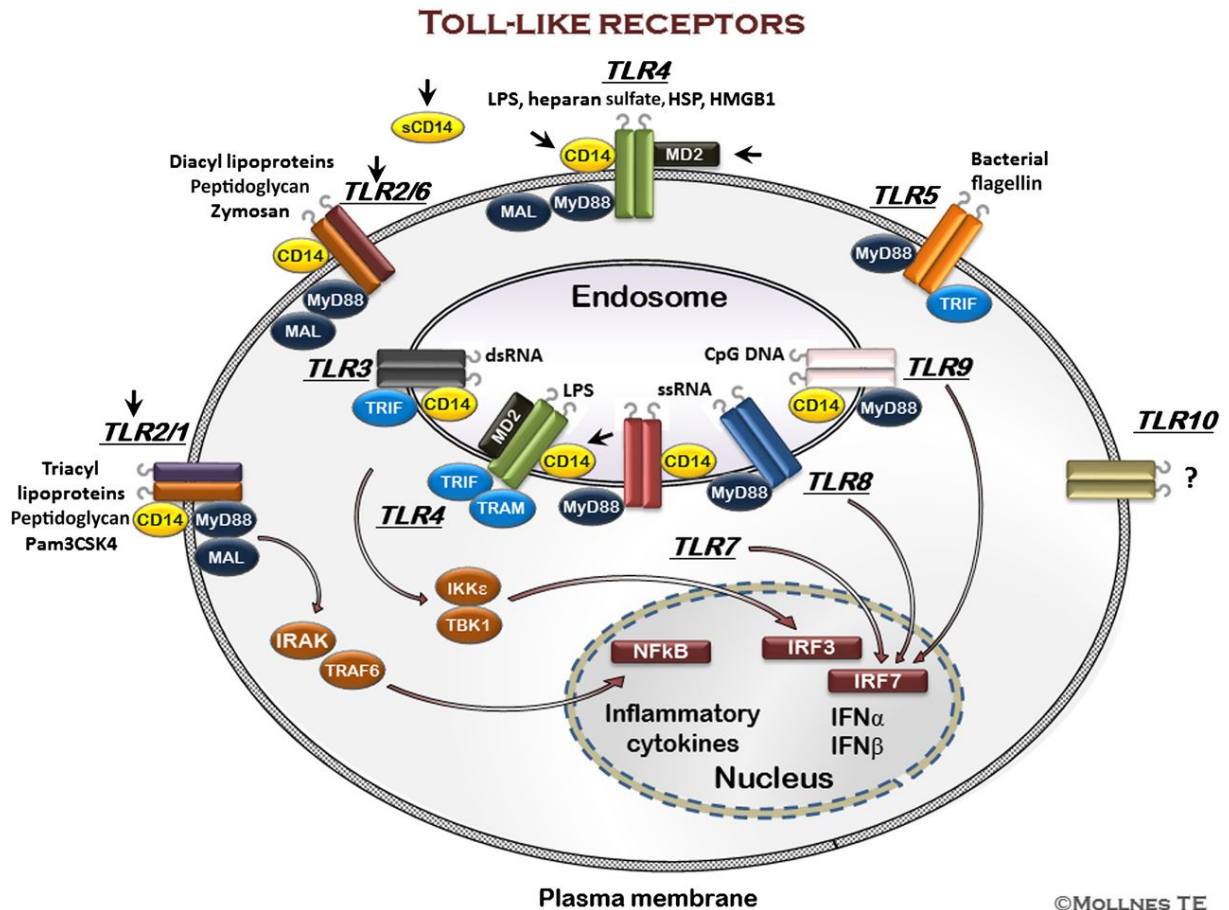
Figure 3: Overview of the Toll-like receptor system

TLR activation results in downstream signaling events culminating in increased expression of cytokines, chemokines, major histocompatibility complexes (MHCs) and co-stimulatory molecules, as well as cell-specific activation such as increased reactive oxygen species (ROS) production and phagocytic activity in neutrophils. The MyD88-dependent pathway resulting in phosphorylation and subsequent activation of nuclear factor kappa B (NF-kappa-B) is generally the most known, and presumably the most important, in the cellular innate immune response (Akira and Takeda, 2004).

The important cellular effectors of innate immunity; monocytes, macrophages and neutrophils, all express TLRs. The stimulation of these triggers several

potent mechanisms to fight and kill pathogens, both directly from intracellular signaling pathways and through the release of cytokines acting on nearby cells and promoting inflammation (Kaisho and Akira, 2006; Kolaczkowska and Kubes, 2013; Serbina et al., 2008). Opsonization, as described earlier, enables phagocytosis of cells and organisms with PAMPs or DAMPs through the activation of complement- or immunoglobulin-receptors (Dale et al., 2008). The incorporated phagosome is then brutally flooded with either reactive ROS or antibacterial proteins such as cathepsins, defensins, lactoferrin and lysozyme, as the granules containing these effectors fuse with the phagosome (Häger et al., 2010). The granules containing antibacterial proteins can also be released from the cells to attack extracellular pathogens. Additionally, on releasing their NETs, neutrophils engulf and stop pathogen spread, facilitate phagocytosis, and possibly kill the pathogen directly with the associated antimicrobial histones and proteases. Importantly, both activated neutrophils and monocytes have the ability to recruit and activate more neutrophils in a positive-feedback fashion during infection and inflammation (Sadik et al., 2011; Shi and Pamer, 2011).

In sepsis, neutrophil and monocyte function and recruitment is also disrupted (Cavaillon and Adib-Conquy, 2005; Phillipson and Kubes, 2011). Higher levels of chemokines in plasma, compared to a single foci of infection, initially increase neutrophil counts, but also downregulate chemokine receptors and therefore cell numbers in the long run (Brown et al., 2006). High levels of

cytokines from various immune cells in the early stages of sepsis, contribute to ambiguous messaging within the immune system and altered TLR signaling, which is an important cause of the altered leukocyte functions seen in sepsis (Salomão et al., 2008). Monocytes and neutrophils are shown to respond differently in sepsis, both as different cell types and according to the disease severity (Brown et al., 2006; Santos et al., 2016). Cells isolated in the early phases of disease are generally hyper-responsive to PAMPs and thus highly pro-inflammatory. Cells isolated in the later stages of disease (severe sepsis and septic shock) are hypo-responsive and more anti-inflammatory in nature according to (Salomão et al., 2009). Furthermore, the expression of different TLR receptors are modulated throughout the course of the disease (Brunialti et al., 2006). Particularly, increased expression of TLRs 2 and 4 correlated with mortality in a mouse model of polymicrobial sepsis where immunomodulation with glucan phosphate reversed the expression of the same receptors (Williams et al., 2003). However, several monocyte and neutrophil effector functions are also dynamically modulated whilst TLR2 and TLR4 expression remains the same, implying intracellular alterations of TLR signaling, and underscoring the complexity of the downstream events in sepsis. The extensive crosstalk between the different components of innate immunity, which is covered in the next section, also complicates matters further.

Crosstalk

Activation of complement and TLRs with resultant inflammation, also activates the coagulation and fibrinolysis systems. Collectively, these processes are now often referred to as immunothrombosis (Engelmann and Massberg, 2013) or thromboinflammation (Ekdahl et al., 2016). All plasma cascade systems follow a similar pattern of recognition, activation and concurrent amplification in several pathological conditions (Foley and Conway, 2016). This is particularly evident in cases of severe meningococcal sepsis where patients present with purpura fulminans – large cutaneous bleeds as a result of DIC. Tissue factor is the central component linking inflammation to coagulation as it is readily recruited during infection and inflammation (Mackman, 2009), but Ekdahl and colleagues argue that the lectin pathway of complement and activated platelets are equally important links in this regard (Ekdahl et al., 2016). Pro-inflammatory cytokines, DAMPs, PAMPs and ROS all induce TF expression. The activated thrombin then engages the protease activated receptors (PARs) on both endothelial cells and platelets, resulting in more cytokines and more TF. Equally, inflammation triggers the release of TF-expressing microparticles from leukocytes and platelets, and NETosis provides a scaffold for coagulation with its many procoagulant components, such as histones that activate TLRs 2 and 4 (Yang et al., 2016). C3a and C5a both induce TF (Barratt-Due et al., 2013; Landsem et al., 2015; Ritis et al., 2006), as well as activates platelets and endothelial cells to procoagulant states (Foley, 2016). Furthermore,

proteins of both cascades readily co-localize on the surface of platelets, neutrophils and the endothelium resulting in increased activation (Wiegner et al., 2016). For instance, the gC1q receptor is a promiscuous receptor for both the complement system and the contact activation system, proposed to perpetuate their simultaneous activation (Ghebrehiwet et al., 2016).

Conversely, the cascades also share regulatory proteins, such as the serine protease inhibitor, serpin, C1 inhibitor (C1INH), which inhibits both the CP and LP of complement as well as the contact and clotting systems. Similarly, anti-thrombin (AT) readily inhibits serine proteases of both the lectin pathway of complement and the coagulation system, yielding detectable MASP/serpin complexes in pathological conditions (Ekdahl et al., 2016). However, plasmin, which can be generated in response to contact system activation in order to counter-balance coagulation is also proposed to activate complement which in turn activates coagulation. Such opposing pathways of activation thus result in contradictory cytokine responses, bleeding and thrombosis.

Crosstalk also occurs on the intracellular level (Hajishengallis and Lambris, 2016). Several PAMPs activate both complement and are recognized by the TLRs. Stimulation of TLR4 but also TLR2 and TLR9 in conjunction with the anaphylatoxin receptors (C5aR1, C5aR2 and to a lesser extent C3aR), modulate and often amplify the mitogen activated protein kinases (MAPK) pathways resulting in increased levels of pro-inflammatory cytokines

(Haggadone et al., 2016; Zhang et al., 2007). The role of the second C5a receptor is in this regard still unclear, but it is proposed to modulate the C5aR1 responses (Holst et al., 2012; Song, 2012; Wang et al., 2016, p. 2). Reversely, TLR stimulation is also shown to increase the transcription of complement component factor B (Zou et al., 2013). On the other hand, C4b binding protein was also shown to negatively regulate TLR1/2 responses (Morita et al., 2016). Similarly, C5a was shown to synergize with LPS and induce the anti-inflammatory programmed death-ligand 1 (PD-L1) in human whole blood, showing that bacteremia can simultaneously elicit both pro- and anti-inflammatory responses (An et al., 2016). Needless to say, the list of crisscrossing pathways in innate immunity is ever-growing.

Combined Inhibition to Attenuate the Inflammatory Response

In light of this complicated immune response to danger, the leaders of our research group have hypothesized that early, upstream intervention can attenuate the otherwise often detrimental immune response (Mollnes et al., 2008). Upstream refers to inhibition at the level of danger recognition rather than at the level of the response. Single mediator-therapy has, perhaps not surprisingly given all the crosstalk, failed in so many cases of inflammatory disease, and sepsis in particular (Barratt-Due et al., 2010; Schmidt and Clardy, 2016). So far, single inhibition and most notably combined inhibition of the key

components of complement; either C3 or C5, and the promiscuous TLR co-receptor, CD14, has efficiently reduced the levels of several inflammatory markers. We have tested the inhibitory regimen in experimental models of gram negative human whole blood bacteremia (Brekke et al., 2008), gram negative porcine whole blood (Thorgersen et al., 2009), polymicrobial murine sepsis (Huber-Lang et al., 2014) and gram negative porcine sepsis (Barratt-Due et al., 2013; Thorgersen et al., 2010). Equally, Lau has examined the transcriptome in response to gram negative human whole blood bacteremia (Lau et al., 2015). Thus, C3/C5 and CD14 emerge as suitable targets for therapeutic intervention (Barratt-Due et al., 2016) and our recent trials of this hypothesis is disclosed in the following sections (Figure 4).
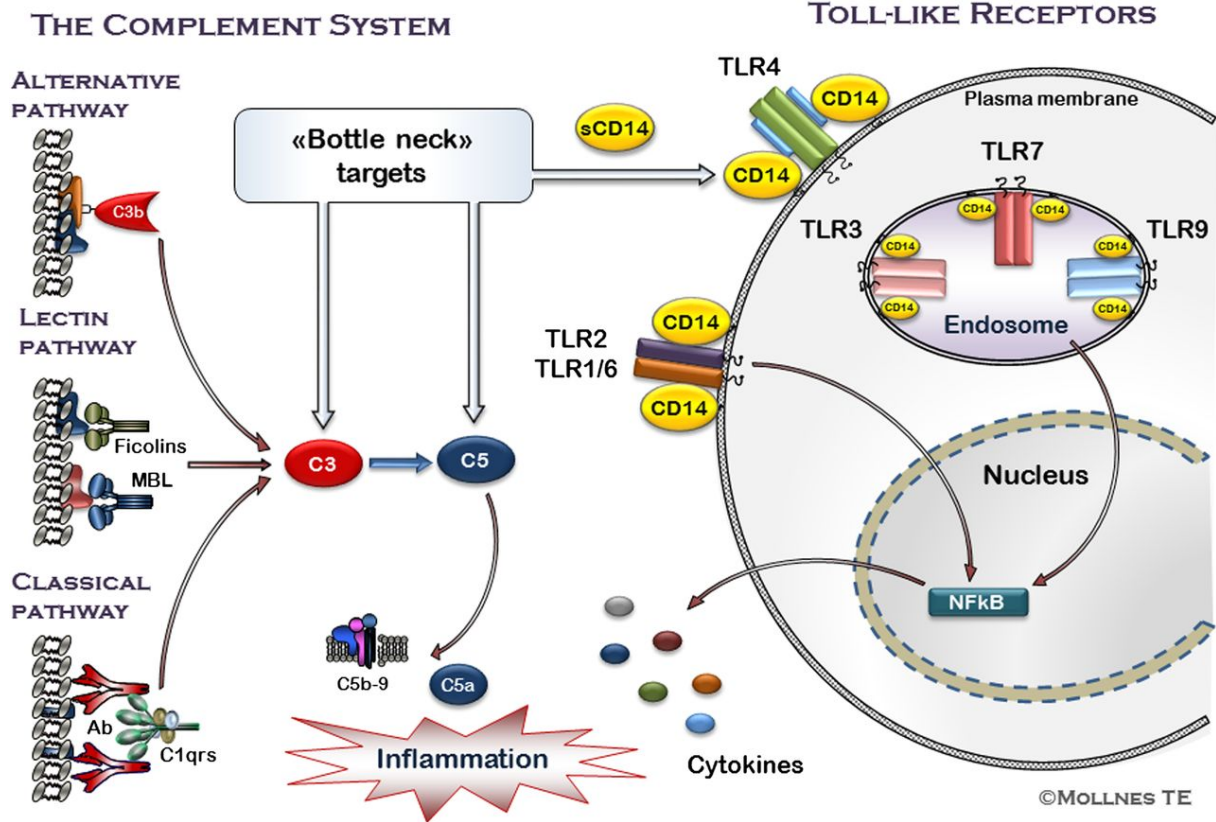
Figure 4: Combined inhibition of complement and CD14 to attenuate the inflammatory response

Aims of The Study

This thesis thus aims to measure how *Staphylococcus aureus* in particular and bacteria in general, induce inflammation through activation of the innate immune system on the level of the complement and the TLR systems at the very first contact with human whole blood *in vitro*, and during the earliest stages of porcine sepsis *in vivo*. Furthermore, it aims to show whether experimental complement and TLR inhibition at these crucial time points attenuates and balances the otherwise dysregulated inflammatory reaction, with possible benefits for the host.

Summary of Results

Paper 1: In the first paper, we first quantified the inflammatory responses, namely the levels of 27 different cytokines, the CD11b expression and oxidative burst in both neutrophils and monocytes induced by two different strains of *S. aureus* when incubated in human whole blood. Additionally, we studied and corroborated findings ruling out the staphylococcal cell wall component lipoteichoic acid (LTA) as an equivalent to Gram-negative endotoxin. Although LTA did exert pro-inflammatory effects on its own, these were insignificant when compared to the responses induced by the whole bacteria. In a second step, we documented strong inhibitory effects when pre-incubating the whole blood with inhibitors acting on both the level of C3 and C5 as well as on the level of CD14 and TLR2 on the abovementioned responses, as well as on the immediate (15 min) phagocytosis of *S. aureus* in granulocytes and monocytes. C5 inhibition was equally effective to C3 inhibition and CD14 inhibition equally effective to TLR2 inhibition in this regard. We therefore concluded that combined inhibition of C5 and CD14 efficiently attenuated the inflammatory response induced by *S. aureus* in human whole blood.

Paper 2: In the second paper, we extended the scope of our whole blood experiments to include measurements on the activation of coagulation induced by three different strains of *S. aureus* (we also included the Newman strain). As for the cytokines and leukocyte activation markers in paper 1, key markers

of coagulation activation, namely the tissue factor expression on granulocytes and monocytes, total TF mRNA levels and the levels of the prothrombin fragment 1+2 (PTF1+2), were raised in response to the bacteria. Additionally, high levels of C5a and TCC levels were quantified to relate complement activation to the activation of coagulation, indirectly indicating the level of cross-talk between the two plasma cascade systems. When inhibiting complement on the level of C5 receptor 1, we discovered that activation of this receptor was the key step for downstream tissue factor expression and subsequent activation of coagulation, as quantified by the levels of PTF1+2. This effect was potentiated by blocking either CD14 or TLR2, and not TLR4. Furthermore, by blocking tissue factor with a specific antibody, we identified tissue factor as the main inducer of PTF1+2. We thus concluded that the *S. aureus*-induced coagulation in human whole blood was mainly due to C5a-induced mRNA upregulation, monocyte expression and plasma TF activity, with subsequent downstream coagulation activation, underscoring complement as a key player in *S. aureus*-induced coagulation.

Paper 3: In the third paper, we explored the effects of complement and CD14 inhibition on the phagocytosis in granulocytes and monocytes, as well as four key cytokines (IL-1β, IL-6, IL-8, TNF, and IFN-β) in response to both live and dead *S. aureus* and *E. coli*. This was both to compare the two Gram types, as well as to address the methodological question of using live bacteria compared to the more commonly used dead bacteria. We discovered that the

phagocytosis was mainly dependent on C5a-C5aR1 interaction, but with an additional dependence on CD14, where the combination of inhibiting the two, significantly reduced the total phagocytosis and cytokine responses for all but four (24/28) of the conditions measured. The response to live bacteria was harder to reduce over time compared to heat-inactivated bacteria but remained significantly reduced for 11 out of 14 conditions measured. The response to *S. aureus* was reduced to a greater extent than the response to *E. coli*, although the responses to both were reduced. We therefore concluded that the combinatory regimen was effective towards both Gram types and life modalities of bacteria.

Paper 4: In the last paper, we therefore made the transition to a more complex biological system. We conducted a blinded, randomized trial in piglets allocated in a match-paired design, comparing the effect of the C5 binding protein, coversin, in combination with a recombinant antibody targeting porcine CD14 to the effects of saline alone in an experimental model of fecal peritonitis and sepsis. Upon unblinding, the treated group had significantly improved survival. Nine piglets survived in the treatment group and four in the control group. The treatment group also had significantly lower pulmonary artery pressure and ratio of pulmonary artery pressure to systemic artery pressure. Plasma sC5b-9 levels were significantly lower in the treatment group and correlated with mortality. Likewise, the levels of IL-8 and IL-10 were also reduced in the treatment group. We therefore concluded that combined

inhibition of C5 and CD14 significantly improved survival, hemodynamic parameters and inflammation in a blinded, randomized trial of porcine polymicrobial sepsis.

Methodological Considerations

Experimental Models of Bacteremia and Sepsis

Our research group has developed a unique human whole blood model where only thrombin is incapacitated by the specific anticoagulant, lepirudin, and all other plasma components, nucleated and non-nucleated cells are left free to interact in response to a chosen challenge, in this case *S. aureus* and *E. coli* (Tom Eirik Mollnes et al., 2002). However, given the many forms of crosstalk where thrombin is involved, such as through PAR-stimulation, potentiation of coagulation, activation of fibrinolysis and possibly direct complement activation, this model is unable to fully reveal the results of bacteremia. Yet, all *ex vivo* systems face the challenge of spontaneous contact activation during sample collection and hence the need for an anticoagulant. Common anticoagulants like EDTA and citrate interfere with several of the plasma cascade systems so lepirudin, and perhaps the corn trypsin inhibitor, are the best options currently available according to Ekdah and colleagues (Ekdahl et al., 2016). Heparinization is one option to reduce contact activation and some groups use this in both tubular and slide-model systems. Specific fibrinogen inhibition is also a tempting option, but given the immunostimulatory properties of fibrin, this system would also have its shortcomings. Equally tempting is the use of endothelial cell coating, most often human umbilical vein endothelial cells (HUVECs), which not only grant some insight to the role of the endothelium, but also provide natural anticoagulation. Yet, all such model

systems still lack the circulation and natural replenishment of blood components as in real life. Therefore, the whole blood model used surely has room for improvement and its results show only parts of the entire picture. On the other hand, we have extended the experiments from the bench to the operating table in our porcine models, complementing the whole blood model nicely. Arguably, swine serve as the next-best alternative to actual human testing as swine in particular share so many physiological and anatomical features with man (Swindle and Smith, 1998). It seems that larger animal models are seldom put forward as alternatives to rodents and whole blood models. On the downside, these models demand more resources, have higher levels of biological variation and require more subjects and careful deciphering of the data. It is undoubtedly a large step to make the transition to animal studies. We therefore devoted a great deal of time and care in establishing the porcine model in the new animal laboratory we had set up in Bodø, based on the many years of experience with experiments at Rikshospitalet in Oslo (Barratt-Due et al., 2013; Hellerud et al., 2010; Nielsen et al., 2009; Thorgersen et al., 2010). Collectively, we argue that the combined approach and results of *in vitro* and *in vivo* experiments in this thesis, is one of its strongest points.

Bacteriology

Another concern in this study was transitioning from Gram-negative bacteria and LPS, particularly *E. coli*, to the Gram-positive *S. aureus* in our lab. It turns out that *S. aureus* is more fragile and rapidly disintegrates when stored compared to *E. coli*. It also clots easily *in vitro* and was therefore difficult to culture, wash and count accurately in the flow cytometer. However, in time we learned how to adequately estimate the numbers of staph and prepare them for our experiments. Equally pressing was the question of using heat-inactivated, dead bacteria. Initial trial experiments revealed little difference in the cytokine response to live versus dead *S. aureus* in human whole blood, and dead bacteria were potentially more accurately quantified. However, increased interest in vita-PAMPs and numerous virulence factors in *S. aureus* led us to retest this methodological question in paper 3.

Assays of Thromboinflammation

Thromboinflammation is the pathophysiological hallmark of sepsis, with derangements in immune and coagulation systems resulting in tissue hypoperfusion and tissue hypoxia. As mentioned, this process is initiated and mediated by a multitude of inductors, sensors, and effectors (Medzhitov, 2008), of which many can be measured in order to quantify the reaction.

In accordance with our main hypothesis, total complement activation is one such measure, and the enzyme-linked immunosorbent assay (ELISA) for the TCC developed by professor Mollnes is therefore key (Mollnes et al., 1985). Indeed, ELISA is the bread-and-butter analysis for the thromboinflammatory biomarkers measured in this thesis; cytokines, complement activation products, markers of coagulation and leukocyte activation, as well as functional assays. When quantifying the biomarkers an indirect sandwich ELISA was most often used, where wells pre-coated with antibodies for a particular antigen were covered with sample. A second epitope was then detected by a secondary antibody, which in turn was detected by a third, enzyme-conjugated antibody. The third antibody then catalyzed the formation of a fluorescent chemical from the substrate added, with the optical density corresponding to the amount of antigen in the particular sample.

More complex (and equally more expensive), were the bead-based analyses allowing the detection of not one but several different antigens. Multiplex analyses can simultaneously measure up to 500 different proteins (or genes) in a single plate well! Each bead or microsphere is distinguished from the others by internal color-coding, giving them a unique spectral address. Phycoerythrin-emitting antibodies targeting the antigen of interest, are conjugated to the bead surface and both the type of bead and the fluorescence intensity from bound antigen is determined by flow cytometric technology.

We also assayed various biomarkers of inflammation by flow cytometry in this thesis. Similar to the multiplex system, the presence of antigen was reported by fluorescence, and quantified by its intensity in the leukocytes and bacteria that we gated for. The exceptional quality of flow cytometry is the ability to individually measure the characteristics of minute particles ranging from .5 to 150 microns based on how the laser light is scattered by the cell or particle as it passes through a focus generated by the hydrodynamics of the sheath fluid. In addition to quantifying cytokines, i.e. actual protein levels, we also quantified the potential levels as messenger RNA for the different cytokines through the real-time-quantitative polymerase chain reaction (RT–qPCR). This was a three-step process, starting with the simultaneous lysis and preservation of the nucleic acids from the whole blood using the listed commercial reagents. Second, total RNA was isolated from the sample obtained in the first step, using magnetic beads. The RNA levels were then finally quantified in the actual RT-qPCR with commercially available primers for the sequences of interest. Importantly, the entire transcriptome of each whole blood experiment and porcine sample was analyzed. The principle of the RT-qPCR is also based on fluorescence intensity; a probe matching the RNA sequence of interest, is labeled with a fluorochrome in one end and a quencher in the other. The probe connects to the complementary strands in the annealing phase of the PCR, and the probes are released and distanced from their quenchers in the sequential polymerization phase thus freeing the fluorochrome, allowing the

fluorescence intensity to increase correspondingly to the amount of sequence gene amplified.

Statistics

The overarching null hypothesis of this thesis was simple enough; No differences exist between samples or subjects randomized to either combined inhibition of complement and TLR or buffer/saline. Given the simplicity of this question, and in return the great potential for incomprehensibly complex statistics, I sought to analyze my data in a straightforward and universally comprehensible way. Much thanks to a well-cited and embraced paper by Matthews and colleagues, I was able to easily summarize serial measurements made for papers 3 and 4, and perform side-by-side comparisons (analyses of variance; ANOVA, and T-tests) in GraphPad Prism - a program equally suited for the beginner statistician. (The data in papers 1 and 2 did not require any summarization.)

Importantly, we seldom compared more than 6-12 subjects (n) in these papers. This has made me question whether the assumption of normally distributed values was valid. By default, less than 20 subjects are hardly considered to be normally distributed. On the other hand, we do assume that the data are sampled from a population with normally distributed data, in theory making our data normally distributed. Equally, using tests for normality and log-transformation consistently yielded data suitable for parametric

statistical analysis. Furthermore, the less powerful nonparametric methods gave similar results for the comparisons in paper 4, where one of the referees questioned whether our data was normally distributed. However, an automated switch from parametric to non-parametric statistical methods is not straightforward (Motulsky, 2014). Indeed, when working with as little as 6 samples per group, nonparametric methods have little to no power, drastically increasing the likelihood for type II errors. Likewise, using parametric methods to test for statistically significant differences between small groups, requires these differences to be consistently large, making us confident that they actually are (Pezzullo, 2013). Actually, when considering the whole blood model system used in papers 1-3, even biostatisticians allow us to question the use of statistics altogether: "Some scientists ask fundamental questions using clean experimental systems with no biological variability and little experimental error. If this describes your work, you can heed these aphorisms:

- If you need statistics to analyze your experiment, then you've done the wrong experiment.

- If your results speak for themselves, don't interrupt!"

- Harvey Moutulsky (Motulsky, 2014)

Is the P value really just a $p$ermission to publish fundamental, biomedical research then? (Baker, 2016) Regardless, we argue that the consistent effects

of complement and CD14 inhibition described in this thesis cannot be overlooked.

The Candidate's Role

In this thesis, I handled the whole-blood experiments alone and the porcine experiments in cooperation with my co-authors. I also did a variety of ELISAs by myself, but not all. The flow cytometry was most often done with the help and expertise of DC, JKL did the multiplex analysis, and HF did the actual RT-qPCR, whereas I completed the first step (RNA stabilization) and contributed to the second step (RNA isolation) in the mRNA analysis. I did the subsequent data handling and statistical analyses and drafted all papers firsthand, whereas TEM often contributed to the final submission.

General Discussion

This thesis explores a concept of reducing the innate immune response through targeted complement and CD14 inhibition. As mentioned, this was formally hypothesized in 2008 as a general regimen to attenuate the inflammatory response (Mollnes et al., 2008). Not only to attenuate the response to infections, but to act as a general dimmer of inflammation induced by danger to the host (Matzinger, 2002). Indeed, as inflammation is practically a prerequisite for disease, there is a wide search for anti-inflammatory therapies. For instance, targeted mediator therapy, especially anti-TNF, has revolutionized the field of rheumatology and several immune-mediated diseases (Kuek et al., 2007). Concerning our combined approach, Barratt-Due recently reviewed our progress so far (Barratt-Due et al., 2016). The aim of this thesis was thus to explore the effects of the combinatory regimen on the responses induced by *S. aureus* as a Gram-positive bacterium, and then combine Gram-positive and Gram-negative species in polymicrobial sepsis, where all targeted therapy in general, and anti-inflammatory treatments in particular, have failed so far (Williams, 2012). In spite of this, we have showed data in favour of the combined approach to the pathogen-induced responses. However, the impact of our findings is limited to the model systems used, and the applicability of our results beyond that thus deserve some further discussion.

Firstly, we have tested both pathogenic and non-pathogenic strains of bacteria, with and without endotoxin, and compared these to each other, revealing rather similar patterns of activation. However, the responses to *S. aureus* was less potent than those to *E. coli,* and clearly more complement-dependent. *S. aureus* is especially good at evading innate immunity and complement in particular. It expresses numerous virulence factors that target complement pathway convertases and key proteins C3 and C5 (Amdahl et al., 2013; Jongerius et al., 2010; Laarman et al., 2011; Spaan et al., 2013). It is also able to hijack complement regulatory proteins to its surface and escape leukocyte phagocytosis (Amdahl et al., 2013; Hair et al., 2010; Ko et al., 2013; Sharp et al., 2012; Sharp and Cunnion, 2011). In this sense, too much complement activation seems unfavourable for *S. aureus*, whereas we argue that it is also unfavourable for the host. However, there is a time and place for everything, complement and innate immune evasion by staphylococci is essential in the initial phases of its infection, but upon further growth and dissemination within the host, the bacteria cannot hide and thus trigger a dysregulated response to the infection. Judging by our results there was very little effect of complement evasion proteins on the responses induced in whole blood. In fact, the responses to viable bacteria were just as potent and somewhat harder to attenuate than the response to heat-inactivated bacteria. Equally, *E. coli,* which has few ways to evade innate immunity (Billips et al., 2008), showed very similar responses in our model, indicating that the whole blood model of bacteremia triggers a universal inflammatory pathway.

Arguably, the whole blood responses documented should thus give an idea on how the general responses induced by these large classes of bacteria in whole blood are. However, there is no focus of infection in this model, and patients infected with *S. aureus* who develop bacteremia would undoubtedly present very different symptoms and time courses of infection compared to patients infected with *E. coli*. For instance, in a model of mammary endothelial cell infection, *E. coli* was shown to induce a much more acute inflammatory response than *S. aureus*, with particularly greater increases in IL-1a and TNF (Günther et al., 2011). However, Günther et al used equal amounts of bacteria whereas our data indicate that the equipotent dose of *S. aureus* is a full log greater than that of *E. coli*. Actually, when using heparinized whole blood, Beran and colleagues showed that gram-positive and gram-negative bacteria differ in their dose-dependent patterns of induction of TLR2 and TLR4, but not in cytokine expression (Beran et al., 2011). This paper also documents the discrepant potencies of Gram-negative and Gram-positive bacteria nicely.

Indeed, septic patients are an extremely heterogeneous group, and it is perhaps adequate to turn the tables and postulate that the main problem with modelling sepsis is that we are unsure what to model. Despite an effort to simplify the definition and clinical criteria of sepsis in the recent "Sepsis-3" consensus (Singer M et al., 2016), prominent researchers argue that we still don't really know what sepsis *is*, i.e. we still lack a gold standard for making

the diagnosis (Deutschman, 2016). Clinicians also argue that the transition from the "old" criteria with the systemic inflammatory response syndrome (SIRS) to that of (q)SOFA is dangerous and may cost patient lives as it focuses on patients that are obviously sick instead of those at the verge of becoming so (Simpson, 2016). A recent study by Mellhammar and colleagues shows that traditional SIRS criteria have greater sensitivity (and poorer specificity) than the new qSOFA, and that the two identify somewhat different patient cohorts without any other obvious gain made in changing the criteria (Mellhammar et al., 2016). According to Simpson, potentially septic patients have been saved by the high sensitivity of the SIRS criteria. Perhaps you really should "never change a winning team"?

Regardless, our group has aimed to induce a "dysregulated response to infection", but partly because of the still imprecise definitions, we cannot be sure that it is in fact sepsis. As Deutschmann adequately points out, what is a "regulated response to infection" and where and when do we transition to a dysregulated, opposing response? Bearing this in mind, we have manipulated our model systems to create an inflammatory response suitable for both the current and previous definitions, namely that of a "cytokine storm" and ample leukocyte activation in whole blood, as well as circulatory-respiratory derangements in piglets. To achieve this, we used large amounts of bacteria, and the amounts used in papers 1-3 have been questioned. On the other hand, very few papers quantifying the bacterial load in bacteremia and sepsis

have been published. Furthermore, the few that have, quantify the load in colony-forming units (CFU) (Yagupsky and Nolte, 1990). We speculate that a large proportion of bacteria in bacteremia and sepsis are dead or at least non-forming, corresponding to stationary or declining growth phases. As we see in paper 3, dead bacteria are very much capable of inducing robust inflammatory responses. This could also explain why only 30-40% of bacterial cultures are positive in septic patients, whereas 80-90% of causative agents are identified in the end (Cohen et al., 2015). Accordingly, in the randomized trial summarized in paper 4, our group was able to document a clinically relevant effect of the combined, anti-inflammatory regimen, also corroborating the similar study by Huber-Lang and colleagues performed in rodents (Huber-Lang et al., 2014). Importantly, this model had a clear focus of infection, and both Gram-negative and Gram-positive (including *S. aureus*) species grew in the blood cultures drawn.

A justified and returning objection to the clinical relevance to the early, upstream inhibition of complement and CD14, lies in its preemptiveness. Arguably, very few patients who develop sepsis have early, tell-tale signs that could make them ideal candidates for an early intervention. On the other hand, as Nymo justly points out in his thesis, the immediate, independent contact-dependent response in whole blood as seen as early CD11b upregulation on leukocytes, necessitates prophylactic intervention in (Gustavsen et al., 2016). Even so, Egge et al. identified that the inflammatory response to *E. coli* in

whole blood could still be constrained by combined complement and CD14 inhibition up to 30 minutes post challenge (Egge et al., 2014). We continued the prophylactic approach in the animal studies, but the window of opportunity is quite possibly larger in a clinical setting, as it is likely that bacteria constantly spill over to the bloodstream and engage circulating stores of the innate immune system there.

On the other hand, we are still exploring the concept of early, upstream inhibition to attenuate the inflammatory response. It is therefore reasonable to test this hypothesis in systems where we can harness the response *before* it runs wild. If we in time have sufficient and compelling data, showing that the dysregulated response to infection is indeed caused by complement over-activation and Toll-like receptor super-stimulation, a possible extension of this is that the inhibited complement and Toll-like receptor response reflects the abovementioned *regulated* response to infection. This is an intriguing thought which ultimately could allow us to identify what a regulated response *should* look like and thus what we should aim for in later stages of infection as well. Perhaps the combined regimen allows patients to have a more protracted and contained innate immune response to infection. Likewise, maybe stimulating parts of complement such as C3 deposition and MAC formation whilst blocking C5a, is a way to handle late-stage sepsis? Equally, several pathological mechanisms such as ischemia-reperfusion injuries or elective surgery can be treated beforehand, so there is a lot of ground that needs to be covered.

A last notion that deserves mention, although it lies beyond the scope of this thesis, is the growing threat of antibiotic resistance (Akova, 2016; Reardon, 2017). This is particularly evident in *S. aureus,* but is prevalent in all bacteria and thus jeopardizes immune-modulating therapies (Pantosti et al., 2007). We simply cannot reduce the (innate) immune response to infection without the security of efficacious antimicrobials. An equally important field of research is therefore to identify new and effective targeted, antimicrobial therapies. This is particularly challenging because of lukewarm interest from pharmaceutical companies, and perhaps with good reason. New antibiotics like all new medicines are extremely costly to develop, so if we were to find a new and efficient antibiotic, this would surely be saved as a backup and not generate sales for the developers. The challenge of tackling antibiotic resistance is therefore twofold, both the scientific challenge of developing selectively toxic antimicrobials with modest resistance drive, and the challenge to get sufficient funding. I hope to combine my continued research in the field of more clinically oriented complementology with that of combating antibiotic resistance. As a specific first step, it would be very interesting to develop a clinically relevant porcine model of staphylococcal infection and sepsis, test the combinatory regimen there and later explore the more long-term effects of the treatment on immune function. In this regard, new and potential candidate therapies such as targeted immune therapies merit testing in solid and clinically relevant models of staphylococcal infection. This is also well in line with the roadmap laid forth

by Cohen and colleagues of the Lancet Infectious Diseases Commission, as well as by van der Poll in a recent, solid review of the immunopathology of sepsis and potential therapeutic targets (van der Poll et al., 2017). Interestingly, the Commission both problematize the ambiguous definitions of sepsis on the one hand, and on the other, recommends more individualized approaches to cases of sepsis, as well as longer experimental models to better examine its later stages (Cohen et al., 2015).

Conclusion

In this thesis, complement activation and CD14 stimulation was identified as important components in the thromboinflammatory responses to *Staphylococcus aureus* as well as *Escherichia coli*, both corroborating and extending the scope of previous work from the Norwegian Complement Research Group. In return, this response was efficiently reduced by inhibiting said components. Furthermore, the inhibition of complement and CD14 improved survival in an experimental, yet clinically relevant model of polymicrobial sepsis. Thus, the concept of early, upstream inhibition of innate immunity remains a promising prophylactic approach to reduce inflammation and might further identify more desirable courses of innate immune responses to danger in the longer run.

References

Akira, S., Takeda, K., 2004. Toll-like receptor signalling. Nat Rev Immunol 4, 499–511. doi:10.1038/nri1391

Akova, M., 2016. Epidemiology of antimicrobial resistance in bloodstream infections. Virulence 7, 252–266. doi:10.1080/21505594.2016.1159366

Alberti, C., Brun-Buisson, C., Burchardi, H., Martin, C., Goodman, S., Artigas, A., Sicignano, A., Palazzo, M., Moreno, R., Boulmé, R., Lepage, E., Le Gall, R., 2002. Epidemiology of sepsis and infection in ICU patients from an international multicentre cohort study. Intensive Care Med 28, 108–21. doi:10.1007/s00134-001-1143-z

Amdahl, H., Jongerius, I., Meri, T., Pasanen, T., Hyvärinen, S., Haapasalo, K., van Strijp, J.A., Rooijakkers, S.H., Jokiranta, T.S., 2013. Staphylococcal Ecb protein and host complement regulator factor H enhance functions of each other in bacterial immune evasion. J Immunol 191, 1775–84. doi:10.4049/jimmunol.1300638

An, L.-L., Gorman, J.V., Stephens, G., Swerdlow, B., Warrener, P., Bonnell, J., Mustelin, T., Fung, M., Kolbeck, R., 2016. Complement C5a induces PD-L1 expression and acts in synergy with LPS through Erk1/2 and JNK signaling pathways. Sci. Rep. 6, 33346. doi:10.1038/srep33346

Antonelli, M., 1999. Sepsis and septic shock: pro-inflammatory or anti-inflammatory state? J Chemother 11, 536–40.

Baker, M., 2016. Statisticians issue warning over misuse of *P* values. Nat. News 531, 151. doi:10.1038/nature.2016.19503

Bamberg, C.E., Mackay, C.R., Lee, H., Zahra, D., Jackson, J., Lim, Y.S., Whitfeld, P.L., Craig, S., Corsini, E., Lu, B., Gerard, C., Gerard, N.P., 2010. The C5a receptor (C5aR) C5L2 is a modulator of C5aR-mediated signal transduction. J. Biol. Chem. 285, 7633–7644. doi:10.1074/jbc.M109.092106

Barratt-Due, A., Pischke, S.E., Nilsson, P.H., Espevik, T., Mollnes, T.E., 2016. Dual inhibition of complement and Toll-like receptors as a novel approach to treat inflammatory diseases-C3 or C5 emerge together with CD14 as promising targets. J. Leukoc. Biol. doi:10.1189/jlb.3VMR0316-132R

Barratt-Due, A., Thorgersen, E.B., Egge, K., Pischke, S., Sokolov, A., Hellerud, B.C., Lindstad, J.K., Pharo, A., Bongoni, A.K., Rieben, R., Nunn, M., Scott, H., Mollnes, T.E., 2013. Combined inhibition of complement C5 and CD14 markedly attenuates inflammation, thrombogenicity, and hemodynamic changes in porcine sepsis. J Immunol 191, 819–27. doi:10.4049/jimmunol.1201909

Barratt-Due, A., Thorgersen, E.B., Lindstad, J.K., Pharo, A., Brekke, O.-L., Christiansen, D., Lambris, J.D., Mollnes, T.E., 2010. Selective inhibition of TNF-alpha or IL-1 beta does not affect E. coli-induced inflammation in human whole blood. Mol. Immunol. 47, 1774–1782. doi:10.1016/j.molimm.2010.02.026

Beran, O., Potměšil, R., Holub, M., 2011. Differences in Toll-like receptor expression and cytokine production after stimulation with heat-killed gram-positive and gram-negative bacteria. Folia Microbiol 56, 138–42. doi:10.1007/s12223-011-0001-9

Beutler, B., 2004. Innate immunity: an overview. Mol Immunol 40, 845–859. doi:10.1016/j.molimm.2003.10.005

Billips, B.K., Schaeffer, A.J., Klumpp, D.J., 2008. Molecular Basis of Uropathogenic Escherichia coli Evasion of the Innate Immune Response in the Bladder. Infect. Immun. 76, 3891–3900. doi:10.1128/IAI.00069-08

Bone, R.C., 1991. The pathogenesis of sepsis. Ann Intern Med 115, 457–469.

Brekke, O.L., Christiansen, D., Fure, H., Pharo, A., Fung, M., Riesenfeld, J., Mollnes, T.E., 2008. Combined inhibition of complement and CD14 abolish E. coli-induced cytokine-, chemokine- and growth factor-synthesis in human whole blood. Mol Immunol 45, 3804–3813. doi:10.1016/j.molimm.2008.05.017

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. Science 303, 1532–5. doi:10.1126/science.1092385

Brown, K.A., Brain, S.D., Pearson, J.D., Edgeworth, J.D., Lewis, S.M., Treacher, D.F., 2006. Neutrophils in development of multiple organ failure in sepsis. Lancet 368, 157–69. doi:10.1016/S0140-6736(06)69005-3

Brunialti, M.K.C., Martins, P.S., Barbosa de Carvalho, H., Machado, F.R., Barbosa, L.M., Salomao, R., 2006. TLR2, TLR4, CD14, CD11B, and CD11C expressions on monocytes surface and cytokine production in patients with sepsis, severe sepsis, and septic shock. Shock 25, 351–357. doi:10.1097/01.shk.0000217815.57727.29

Burnham, K.L., Davenport, E.E., Radhakrishnan, J., Humburg, P., Gordon, A.C., Hutton, P., Svoren-Jabalera, E., Garrard, C., Hill, A.V., Hinds, C.J., Knight, J.C., 2016. Shared and Distinct Aspects of the Sepsis Transcriptomic Response to Fecal Peritonitis and Pneumonia. Am. J. Respir. Crit. Care Med. doi:10.1164/rccm.201608-1685OC

Cavaillon, J.-M.-. M., Adib-Conquy, M., 2005. Monocytes/macrophages and sepsis. Crit Care Med 33, S506–S509. doi:10.1097/01.CCM.0000185502.21012.37

Chen, G.Y., Nuñez, G., 2010. Sterile inflammation: sensing and reacting to damage. Nat. Rev. Immunol. 10, 826–837. doi:10.1038/nri2873

Cohen, J., 2002. The immunopathogenesis of sepsis. Nature 420, 885–91. doi:10.1038/nature01326

Cohen, J., Vincent, J.-L., Adhikari, N.K.J., Machado, F.R., Angus, D.C., Calandra, T., Jaton, K., Giulieri, S., Delaloye, J., Opal, S., Tracey, K., van der Poll, T., Pelfrene, E., 2015. Sepsis: a roadmap for future research. Lancet Infect. Dis. 15, 581–614. doi:10.1016/S1473-3099(15)70112-X

Cooley, B.C., 2016. The dirty side of the intrinsic pathway of coagulation. Thromb. Res. doi:10.1016/j.thromres.2016.06.028

Czupryna, P., Garkowski, A., Moniuszko, A., Pancewicz, S., Ciemerych, A., Zajkowska, J., 2013. Patients with sepsis in infectious diseases department in years 1997-2010 - epidemiology and clinical features. Przegl Epidemiol 67, 429–34, 535–8. doi:24340555

Dale, D.C., Boxer, L., Liles, W.C., 2008. The phagocytes: neutrophils and monocytes. Blood 112, 935–945.

Deitch, E.A., 1998. Animal models of sepsis and shock: a review and lessons learned. Shock 9, 1–11. doi:9466467

Denk, S., Neher, M.D., Messerer, D.A.C., Wiegner, R., Nilsson, B., Rittirsch, D., Nilsson-Ekdahl, K., Weckbach, S., Ignatius, A., Kalbitz, M., Gebhard, F., Weiss, M.E., Vogt, J., Radermacher, P., Köhl, J., Lambris, J.D., Huber-Lang, M.S., 2017. Complement C5a Functions as a Master Switch for the pH Balance in Neutrophils Exerting Fundamental Immunometabolic Effects. J. Immunol. Baltim. Md 1950. doi:10.4049/jimmunol.1700393

Denk, S., Perl, M., Huber-Lang, M., 2012. Damage- and pathogen-associated molecular patterns and alarmins: keys to sepsis?. Eur Surg Res 48, 171–9. doi:10.1159/000338194

Deutschman, C.S., 2016. Imprecise Medicine: The Limitations of Sepsis-3. Crit. Care Med. 44, 857–858.

Egge, K.H., Thorgersen, E.B., Lindstad, J.K., Pharo, A., Lambris, J.D., Barratt-Due, A., Mollnes, T.E., 2014. Post challenge inhibition of C3 and CD14 attenuates Escherichia coli-induced inflammation in human whole blood. Innate Immun. 20, 68–77. doi:10.1177/1753425913482993

Ekdahl, K.N., Teramura, Y., Hamad, O.A., Asif, S., Duehrkop, C., Fromell, K., Gustafson, E., Hong, J., Kozarcanin, H., Magnusson, P.U., Huber-Lang, M., Garred, P., Nilsson, B., 2016. Dangerous liaisons: complement, coagulation, and kallikrein/kinin cross-talk act as a linchpin in the events leading to thromboinflammation. Immunol. Rev. 274, 245–269. doi:10.1111/imr.12471

Engelmann, B., Massberg, S., 2013. Thrombosis as an intravascular effector of innate immunity. Nat Rev Immunol 13, 34–45. doi:10.1038/nri3345

Flaatten, H., 2004. Epidemiology of sepsis in Norway in 1999. Crit Care 8, R180–184. doi:10.1186/cc2867

Foley, J.H., 2016. Examining coagulation-complement crosstalk: complement activation and thrombosis. Thromb. Res. 141 Suppl 2, S50–54. doi:10.1016/S0049-3848(16)30365-6

Foley, J.H., Conway, E.M., 2016. Cross Talk Pathways Between Coagulation and Inflammation. Circ. Res. 118, 1392–1408. doi:10.1161/CIRCRESAHA.116.306853

Frencken, J.F., van Vught, L.A., Peelen, L.M., Ong, D.S.Y., Klein Klouwenberg, P.M.C., Horn, J., Bonten, M.J.M., van der Poll, T., Cremer, O.L., MARS Consortium, 2017. An Unbalanced Inflammatory Cytokine Response Is Not Associated With Mortality Following Sepsis: A Prospective Cohort Study. Crit. Care Med. 45, e493–e499. doi:10.1097/CCM.0000000000002292

Frick, I.-M.M., Björck, L., Herwald, H., 2007. The dual role of the contact system in bacterial infectious disease. Thromb Haemost 98, 497–502. doi:17849037

Fujita, T., 2002. Evolution of the lectin-complement pathway and its role in innate immunity. Nat Rev Immunol 2, 346–53. doi:10.1038/nri800

Funk, D.J., Parrillo, J.E., Kumar, A., 2009. Sepsis and septic shock: a history. Crit Care Clin 25, 83–101, viii. doi:10.1016/j.ccc.2008.12.003

Gaieski, D.F., Edwards, J.M., Kallan, M.J., Carr, B.G., 2013. Benchmarking the incidence and mortality of severe sepsis in the United States. Crit. Care Med. 41, 1167–1174. doi:10.1097/CCM.0b013e31827c09f8

Ghebrehiwet, B., Kaplan, A.P., Joseph, K., Peerschke, E.I.B., 2016. The complement and contact activation systems: partnership in pathogenesis beyond angioedema. Immunol. Rev. 274, 281–289. doi:10.1111/imr.12469

Günther, J., Esch, K., Poschadel, N., Petzl, W., Zerbe, H., Mitterhuemer, S., Blum, H., Seyfert, H.-M.M., 2011. Comparative kinetics of Escherichia coli- and Staphylococcus aureus-specific activation of key immune pathways in mammary epithelial cells demonstrates that S. aureus elicits a delayed response dominated by interleukin-6 (IL-6) but not by IL-1A or tumor necrosis factor alpha. Infect Immun 79, 695–707. doi:10.1128/IAI.01071-10

Guo, R.-F.F., Ward, P.A., 2005. Role of C5a in inflammatory responses. Annu Rev Immunol 23, 821–52. doi:10.1146/annurev.immunol.23.021704.115835

Gustavsen, A., Nymo, S., Landsem, A., Christiansen, D., Ryan, L., Husebye, H., Lau, C., Pischke, S.E., Lambris, J.D., Espevik, T., Mollnes, T.E., 2016. Combined Inhibition of Complement and CD14 Attenuates Bacteria-Induced Inflammation in Human Whole Blood More Efficiently Than Antagonizing the Toll-like Receptor 4-MD2 Complex. J. Infect. Dis. 214, 140–150. doi:10.1093/infdis/jiw100

Haas, P.-J.J., van Strijp, J., 2007. Anaphylatoxins: their role in bacterial infection and inflammation. Immunol Res 37, 161–75.

Häger, M., Cowland, J.B., Borregaard, N., 2010. Neutrophil granules in health and disease. J Intern Med 268, 25–34. doi:10.1111/j.1365-2796.2010.02237.x

Haggadone, M.D., Grailer, J.J., Fattahi, F., Zetoune, F.S., Ward, P.A., 2016. Bidirectional Crosstalk between C5a Receptors and the NLRP3 Inflammasome in Macrophages and Monocytes. Mediators Inflamm. 2016, 1340156. doi:10.1155/2016/1340156

Hair, P.S., Echague, C.G., Sholl, A.M., Watkins, J.A., Geoghegan, J.A., Foster, T.J., Cunnion, K.M., 2010. Clumping factor A interaction with complement factor I increases C3b cleavage on the bacterial surface of Staphylococcus aureus and decreases complement-mediated phagocytosis. Infect Immun 78, 1717–27. doi:10.1128/IAI.01065-09

Hajishengallis, G., Lambris, J.D., 2016. More than complementing Tolls: complement-Toll-like receptor synergy and crosstalk in innate immunity and inflammation. Immunol. Rev. 274, 233–244. doi:10.1111/imr.12467

Harboe, M., Ulvund, G., Vien, L., Fung, M., Mollnes, T.E., 2004. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. Clin Exp Immunol 138, 439–46. doi:10.1111/j.1365-2249.2004.02627.x

Hellerud, B.C., Nielsen, E.W., Thorgersen, E.B., Lindstad, J.K., Pharo, A., Tønnessen, T.I., Castellheim, A., Mollnes, T.E., Brandtzaeg, P., 2010. Dissecting the effects of lipopolysaccharides from nonlipopolysaccharide molecules in experimental porcine meningococcal sepsis. Crit Care Med 38, 1467–1474. doi:10.1097/CCM.0b013e3181de8c94

Hobart, M., 1984. Agreement now the norm. Immunol. Today 5, 121–125. doi:10.1016/0167-5699(84)90227-5

Holst, B., Raby, A.-C.C., Hall, J.E., Labéta, M.O., 2012. Complement takes its Toll: an inflammatory crosstalk between Toll-like receptors and the receptors for the complement anaphylatoxin C5a. Anaesthesia 67, 60–4. doi:10.1111/j.1365-2044.2011.07011.x

Hotchkiss, R.S., Monneret, G., Payen, D., 2013. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nat. Rev. Immunol. 13, 862–874. doi:10.1038/nri3552

Huber-Lang, M., Barratt-Due, A., Pischke, S.E., Sandanger, Ø., Nilsson, P.H., Nunn, M.A., Denk, S., Gaus, W., Espevik, T., Mollnes, T.E., 2014. Double blockade of CD14 and complement C5 abolishes the cytokine storm and improves morbidity and survival in polymicrobial sepsis in mice. J Immunol 192, 5324–31. doi:10.4049/jimmunol.1400341

Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M., Beutler, B., 2005. CD14 is required for MyD88-independent LPS signaling. Nat. Immunol. 6, 565–570. doi:10.1038/ni1207

Jongerius, I., Puister, M., Wu, J., Ruyken, M., van Strijp, J.A.G., Rooijakkers, S.H.M., 2010. Staphylococcal complement inhibitor modulates phagocyte responses by dimerization of convertases. J Immunol 184, 420–5. doi:10.4049/jimmunol.0902865

Kaisho, T., Akira, S., 2006. Toll-like receptor function and signaling. J. Allergy Clin. Immunol. 117, 979–987. doi:10.1016/j.jaci.2006.02.023

Kalbitz, M., Fattahi, F., Herron, T.J., Grailer, J.J., Jajou, L., Lu, H., Huber-Lang, M., Zetoune, F.S., Sarma, J.V., Day, S.M., Russell, M.W., Jalife, J., Ward, P.A., 2016. Complement

Destabilizes Cardiomyocyte Function In Vivo after Polymicrobial Sepsis and In Vitro. J. Immunol. 197, 2353–2361. doi:10.4049/jimmunol.1600091

Kaukonen, K.-M., Bailey, M., Suzuki, S., Pilcher, D., Bellomo, R., 2014. Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012. JAMA 311, 1308–1316. doi:10.1001/jama.2014.2637

Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11, 373–84. doi:10.1038/ni.1863

Kilgore, K.S., Schmid, E., Shanley, T.P., Flory, C.M., Maheswari, V., Tramontini, N.L., Cohen, H., Ward, P.A., Friedl, H.P., Warren, J.S., 1997. Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappa B activation. Am. J. Pathol. 150, 2019–2031.

Kleinschnitz, C., Stoll, G., Bendszus, M., Schuh, K., Pauer, H.-U., Burfeind, P., Renné, C., Gailani, D., Nieswandt, B., Renné, T., 2006. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. J. Exp. Med. 203, 513–518. doi:10.1084/jem.20052458

Kolaczkowska, E., Kubes, P., 2013. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 13, 159–75. doi:10.1038/nri3399

Ko, Y.-P.P., Kuipers, A., Freitag, C.M., Jongerius, I., Medina, E., van Rooijen, W.J., Spaan, A.N., van Kessel, K.P.M., Höök, M., Rooijakkers, S.H.M., 2013. Phagocytosis escape by a Staphylococcus aureus protein that connects complement and coagulation proteins at the bacterial surface. PLoS Pathog 9, e1003816. doi:10.1371/journal.ppat.1003816

Kuek, A., Hazleman, B.L., Östör, A.J.K., 2007. Immune-mediated inflammatory diseases (IMIDs) and biologic therapy: a medical revolution. Postgrad. Med. J. 83, 251–260. doi:10.1136/pgmj.2006.052688

Laarman, A.J., Ruyken, M., Malone, C.L., van Strijp, J.A.G., Horswill, A.R., Rooijakkers, S.H.M., 2011. Staphylococcus aureus Metalloprotease Aureolysin Cleaves Complement C3 To Mediate Immune Evasion. J Immunol 186, 6445–53. doi:10.4049/jimmunol.1002948

Landsem, A., Fure, H., Christiansen, D., Nielsen, E.W., Østerud, B., Mollnes, T.E., Brekke, O.L., 2015. The key roles of complement and tissue factor in Escherichia coli-induced coagulation in human whole blood. Clin. Exp. Immunol. 182, 81–89. doi:10.1111/cei.12663

Lau, C., Nygård, S., Fure, H., Olstad, O.K., Holden, M., Lappegård, K.T., Brekke, O.-L., Espevik, T., Hovig, E., Mollnes, T.E., 2015. CD14 and complement crosstalk and largely mediate the transcriptional response to Escherichia coli in human whole blood as revealed by DNA microarray. PloS One 10, e0117261. doi:10.1371/journal.pone.0117261

Lee, C.C., Avalos, A.M., Ploegh, H.L., 2012. Accessory molecules for Toll-like receptors and their function. Nat Rev Immunol 12, 168–79. doi:10.1038/nri3151

Levi, M., Ten Cate, H., 1999. Disseminated intravascular coagulation. N Engl J Med 341, 586–92. doi:10.1056/NEJM199908193410807

Levi, M., van der Poll, T., 2010. Inflammation and coagulation. Crit Care Med 38, S26–34. doi:10.1097/CCM.0b013e3181c98d21

Mackman, N., 2009. The many faces of tissue factor. J Thromb Haemost 7 Suppl 1, 136–9. doi:10.1111/j.1538-7836.2009.03368.x

Markiewski, M.M., DeAngelis, R.A., Lambris, J.D., 2008. Complexity of complement activation in sepsis. J Cell Mol Med 12, 2245–2254. doi:10.1111/j.1582-4934.2008.00504.x

Martin, G.S., Mannino, D.M., Eaton, S., Moss, M., 2003. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med 348, 1546–1554. doi:10.1056/NEJMoa022139

Matzinger, P., 2002. The danger model: a renewed sense of self. Science 296, 301–305. doi:10.1126/science.1071059

McGeer, P.L., Lee, M., McGeer, E.G., 2016. A review of human diseases caused or exacerbated by aberrant complement activation. Neurobiol. Aging 52, 12–22. doi:10.1016/j.neurobiolaging.2016.12.017

Medzhitov, R., 2008. Origin and physiological roles of inflammation. Nature 454, 428–35. doi:10.1038/nature07201

Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nat Rev Immunol 1, 135–45. doi:10.1038/35100529

Mellhammar, L., Wullt, S., Lindberg, Å., Lanbeck, P., Christensson, B., Linder, A., 2016. Sepsis Incidence: A Population-Based Study. Open Forum Infect. Dis. 3. doi:10.1093/ofid/ofw207

Mollnes, T.E., Brekke, O.-L.L., Fung, M., Fure, H., Christiansen, D., Bergseth, G., Videm, V., Lappegaard, K.T., Köhl, J., Lambris, J.D., 2002. Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. Blood 100, 1869–1877.

Mollnes, T.E., Christiansen, D., Brekke, O.L., Espevik, T., 2008. Hypothesis: combined inhibition of complement and CD14 as treatment regimen to attenuate the inflammatory response. Adv Exp Med Biol 632, 253–263.

Mollnes, T.E., Lea, T., Frøland, S.S., Harboe, M., 1985. Quantification of the terminal complement complex in human plasma by an enzyme-linked immunosorbent assay based on monoclonal antibodies against a neoantigen of the complex. Scand. J. Immunol. 22, 197–202.

Mollnes, T.E., Song, W.-C.-. C., Lambris, J.D., 2002. Complement in inflammatory tissue damage and disease. Trends Immunol 23, 61–64.

Morita, N., Yamai, I., Takahashi, K., Kusumoto, Y., Shibata, T., Kobayashi, T., Nonaka, M.I., Ichimonji, I., Takagi, H., Miyake, K., Takamura, S.A., 2016. C4b binding protein negatively regulates TLR1/2 response. Innate Immun. doi:10.1177/1753425916672312

Motulsky, H., 2014. When do you need statistical calculations?, in: GraphPad 6 Statistics Guide. p. 10.

Nielsen, E.W., Hellerud, B.C., Thorgersen, E.B., Castellheim, A., Pharo, A., Lindstad, J., Tønnessen, T.I., Brandtzaeg, P., Mollnes, T.E., 2009. A new dynamic porcine model of meningococcal shock. Shock 32, 302–309. doi:10.1097/SHK.0b013e31819c37be

O'Neill, L.A.J., Golenbock, D., Bowie, A.G., 2013. The history of Toll-like receptors - redefining innate immunity. Nat. Rev. Immunol. 13, 453–460. doi:10.1038/nri3446

Pantosti, A., Sanchini, A., Monaco, M., 2007. Mechanisms of antibiotic resistance in Staphylococcus aureus. Future Microbiol 2, 323–34. doi:10.2217/17460913.2.3.323

Pezzullo, J.C., 2013. Getting Statistical: A Short Review of Basic Statistics, in: Biostatistics for Dummies. John Wiley & Sons, Inc., pp. 31–51.

Phillipson, M., Kubes, P., 2011. The neutrophil in vascular inflammation. Nat Med 17, 1381–90. doi:10.1038/nm.2514

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B., 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282, 2085–8.

Reardon, S., 2017. Resistance to last-ditch antibiotic has spread farther than anticipated. Nat. News. doi:10.1038/nature.2017.22140

Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J.D., 2010. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 11, 785–97. doi:10.1038/ni.1923

Riedemann, N.C., Guo, R.-F., Laudes, I.J., Keller, K., Sarma, V.J., Padgaonkar, V., Zetoune, F.S., Ward, P.A., 2002. C5a receptor and thymocyte apoptosis in sepsis. FASEB J 16, 887–888. doi:10.1096/fj.02-0033fje

Ritis, K., Doumas, M., Mastellos, D., Micheli, A., Giaglis, S., Magotti, P., Rafail, S., Kartalis, G., Sideras, P., Lambris, J.D., 2006. A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. J. Immunol. Baltim. Md 1950 177, 4794–4802.

Rittirsch, D., Hoesel, L.M., Ward, P.A., 2007. The disconnect between animal models of sepsis and human sepsis. J Leukoc Biol 81, 137–43. doi:10.1189/jlb.0806542

Rosenberg, R.D., Aird, W.C., 1999. Vascular-Bed–Specific Hemostasis and Hypercoagulable States. N. Engl. J. Med. 340, 1555–1564. doi:10.1056/NEJM199905203402007

Sadik, C.D., Kim, N.D., Luster, A.D., 2011. Neutrophils cascading their way to inflammation. Trends Immunol 32, 452–60. doi:10.1016/j.it.2011.06.008

Salomão, R., Brunialti, M.K.C., Gomes, N.E., Mendes, M.E., Diaz, R.S., Komninakis, S., Machado, F.R., da Silva, I.D.C.G., Rigato, O., 2009. Toll-like receptor pathway signaling is differently regulated in neutrophils and peripheral mononuclear cells of patients with sepsis, severe sepsis, and septic shock. Crit Care Med 37, 132–9. doi:10.1097/CCM.0b013e318192fbaf

Salomão, R., Martins, P.S., Brunialti, M.K.C., Fernandes, M. da L., Martos, L.S.W., Mendes, M.E., Gomes, N.E., Rigato, O., 2008. TLR signaling pathway in patients with sepsis. Shock 30 Suppl 1, 73–7. doi:10.1097/SHK.0b013e318181af2a

Santos, S.S., Carmo, A.M., Brunialti, M.K.C., Machado, F.R., Azevedo, L.C., Assunção, M., Trevelin, S.C., Cunha, F.Q., Salomao, R., 2016. Modulation of monocytes in septic patients: preserved phagocytic activity, increased ROS and NO generation, and decreased production of inflammatory cytokines. Intensive Care Med. Exp. 4. doi:10.1186/s40635-016-0078-1

Schmidt, G.A., Clardy, P.F., 2016. Investigational and ineffective therapies for sepsis [WWW Document]. UpToDate.com. URL http://www.uptodate.com/contents/investigational-and-ineffective-therapies-for-sepsis (accessed 8.3.16).

Serbina, N.V., Jia, T., Hohl, T.M., Pamer, E.G., 2008. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol 26, 421–52. doi:10.1146/annurev.immunol.26.021607.090326

Sharp, J.A., Cunnion, K.M., 2011. Disruption of the alternative pathway convertase occurs at the staphylococcal surface via the acquisition of factor H by Staphylococcus aureus. Mol Immunol 48, 683–90. doi:10.1016/j.molimm.2010.11.014

Sharp, J.A., Echague, C.G., Hair, P.S., Ward, M.D., Nyalwidhe, J.O., Geoghegan, J.A., Foster, T.J., Cunnion, K.M., 2012. Staphylococcus aureus Surface Protein SdrE Binds Complement Regulator Factor H as an Immune Evasion Tactic. PLoS One 7, e38407. doi:10.1371/journal.pone.0038407

Shi, C., Pamer, E.G., 2011. Monocyte recruitment during infection and inflammation. Nat Rev Immunol 11, 762–74. doi:10.1038/nri3070

Simpson, S.Q., 2016. New Sepsis Criteria: A Change We Should Not Make. Chest. doi:10.1016/j.chest.2016.02.653

Singer M, Deutschman CS, Seymour C, et al, 2016. The third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA 315, 801–810. doi:10.1001/jama.2016.0287

Song, W.-C.C., 2012. Crosstalk between complement and toll-like receptors. Toxicol Pathol 40, 174–82. doi:10.1177/0192623311428478

Spaan, A.N., Henry, T., van Rooijen, W.J.M., Perret, M., Badiou, C., Aerts, P.C., Kemmink, J., de Haas, C.J.C., van Kessel, K.P.M., Vandenesch, F., Lina, G., van Strijp, J.A.G., 2013. The staphylococcal toxin Panton-Valentine Leukocidin targets human C5a receptors. Cell Host Microbe 13, 584–94. doi:10.1016/j.chom.2013.04.006

Swindle, M.M., Smith, A.C., 1998. Comparative anatomy and physiology of the pig. Scand J Lab Anim Sci 25, 1–10.

Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. Annu Rev Immunol 21, 335–76. doi:10.1146/annurev.immunol.21.120601.141126

Thorgersen, E.B., Hellerud, B.C., Nielsen, E.W., Barratt-Due, A., Fure, H., Lindstad, J.K., Pharo, A., Fosse, E., Tønnessen, T.I., Johansen, H.T., Castellheim, A., Mollnes, T.E., 2010. CD14 inhibition efficiently attenuates early inflammatory and hemostatic responses in Escherichia coli sepsis in pigs. FASEB J 24, 712–722. doi:10.1096/fj.09-140798

Thorgersen, E.B., Pharo, A., Haverson, K., Axelsen, A.K., Gaustad, P., Kotwal, G.J., Sfyroera, G., Mollnes, T.E., 2009. Inhibition of complement and CD14 attenuates the Escherichia coli-induced inflammatory response in porcine whole blood. Infect. Immun. 77, 725–732. doi:10.1128/IAI.01305-08

van der Poll, T., van de Veerdonk, F.L., Scicluna, B.P., Netea, M.G., 2017. The immunopathology of sepsis and potential therapeutic targets. Nat. Rev. Immunol. doi:10.1038/nri.2017.36

Vervloet, M.G., Thijs, L.G., Hack, C.E., 1998. Derangements of coagulation and fibrinolysis in critically ill patients with sepsis and septic shock. Semin Thromb Hemost 24, 33–44. doi:10.1055/s-2007-995821

Vincent, J.-L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C.D., Moreno, R., Lipman, J., Gomersall, C., Sakr, Y., Reinhart, K., EPIC II Group of Investigators, 2009. International study of the prevalence and outcomes of infection in intensive care units. JAMA 302, 2323–2329. doi:10.1001/jama.2009.1754

Vught, L.A. van, Klouwenberg, P.M.C.K., Spitoni, C., Scicluna, B.P., Wiewel, M.A., Horn, J., Schultz, M.J., Nürnberg, P., Bonten, M.J.M., Cremer, O.L., Poll, T. van der, 2016. Incidence, Risk Factors, and Attributable Mortality of Secondary Infections in the Intensive Care Unit After Admission for Sepsis. JAMA 315, 1469–1479. doi:10.1001/jama.2016.2691

Walport, M.J., 2001a. Complement. First of two parts. N Engl J Med 344, 1058–1066. doi:10.1056/NEJM200104053441406

Walport, M.J., 2001b. Complement. Second of two parts. N Engl J Med 344, 1140–1144. doi:10.1056/NEJM200104123441506

Walter, F., Boulpaep, E.L.J.B., 2012. Blood, in: Medical Physiology: A Cellular and Molecular Approach. Elsevier Saunders, pp. 458–466.

Wang, R., Lu, B., Gerard, C., Gerard, N.P., 2016. C5L2, the Second C5a Anaphylatoxin Receptor Suppresses Lipopolysaccharide-induced Acute Lung Injury. Am. J. Respir. Cell Mol. Biol. doi:10.1165/rcmb.2016-0067OC

Ward, P.A., 2010. The harmful role of c5a on innate immunity in sepsis. J. Innate Immun. 2, 439–445. doi:10.1159/000317194

Ward, P.A., 2004. The dark side of C5a in sepsis. Nat Rev Immunol 4, 133–42. doi:10.1038/nri1269

Wiegner, R., Chakraborty, S., Huber-Lang, M., 2016. Complement-coagulation crosstalk on cellular and artificial surfaces. Immunobiology 221, 1073–1079. doi:10.1016/j.imbio.2016.06.005

Williams, D.L., Ha, T., Li, C., Kalbfleisch, J.H., Schweitzer, J., Vogt, W., Browder, I.W., 2003. Modulation of tissue Toll-like receptor 2 and 4 during the early phases of polymicrobial sepsis correlates with mortality. Crit. Care Med. 31, 1808–1818. doi:10.1097/01.CCM.0000069343.27691.F3

Williams, S.C., 2012. After Xigris, researchers look to new targets to combat sepsis. Nat. Med. 18, 1001–1001. doi:10.1038/nm0712-1001

Yagupsky, P., Nolte, F.S., 1990. Quantitative aspects of septicemia. Clin. Microbiol. Rev. 3, 269–279.

Yang, X., Li, L., Liu, J., Lv, B., Chen, F., 2016. Extracellular histones induce tissue factor expression in vascular endothelial cells via TLR and activation of NF-κB and AP-1. Thromb. Res. 137, 211–218. doi:10.1016/j.thromres.2015.10.012

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., Kagan, J.C., 2011. CD14 Controls the LPS-Induced Endocytosis of Toll-like Receptor 4. Cell 147, 868–880. doi:10.1016/j.cell.2011.09.051

Zhang, X., Kimura, Y., Fang, C., Zhou, L., Sfyroera, G., Lambris, J.D., Wetsel, R.A., Miwa, T., Song, W.-C., 2007. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. Blood 110, 228–236. doi:10.1182/blood-2006-12-063636

Zipfel, P.F., Skerka, C., 2009. Complement regulators and inhibitory proteins. Nat. Rev. Immunol. 9, 729–740. doi:10.1038/nri2620

Zou, L., Feng, Y., Li, Y., Zhang, M., Chen, C., Cai, J., Gong, Y., Wang, L., Thurman, J.M., Wu, X., Atkinson, J.P., Chao, W., 2013. Complement factor B is the downstream effector of TLRs and plays an important role in a mouse model of severe sepsis. J. Immunol. Baltim. Md 1950 191, 5625–5635. doi:10.4049/jimmunol.1301903

Papers

65