Targeting innate immune pathways in acute infectious and sterile inflammation

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The role of complement

PhD Thesis

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Table of contents

Acknowledgements....5

List of papers....7

Abbreviations....8

1 Background....9

1.1 Introduction....9

1.2 Innate immunity....9

1.3 The complement system....10

1.3.1. Initiating pathways....12

1.3.2 Central and terminal pathways....13

1.3.3 Complement regulators....14

1.3.4 Complement receptors....15

1.4 Toll-like receptors and CD14....16

1.4.1 Toll-like receptors....16

1.4.2 CD14....17

1.5 Crosstalk and redundancy of complement and TLRs....17

1.6 Complement in health and disease....18

1.6.1 Complement in bacterial infections and sepsis....20

1.6.2 Complement in ischemia-reperfusion injury....21

1.6.3 Complement in antiphospholipid syndrome....22

2 Aim....24

3 Materials and methods....26

3.1 LPS and bacteria....26

3.2 Inhibitors....26

3.3 Analysis....27

3.3.1 ELISA....27

3.3.2 Multiplex technology....28

3.3.3 Flow cytometry....28

3.4 *In vitro* experiments......29

3.4.1 Whole blood model......29

3.4.2 Whole blood HUVEC model......29

3.5 *In vivo* experiments......30

3.5.1 Porcine myocardial ischemia-reperfusion model......30

3.6 Clinical case report......32

3.7 Statistical considerations......32

4 Summary of the main results......34

5 Discussion......37

5.1 Exogenous and endogenous activation......37

5.2 Models and human disease......38

5.2.1 *In vitro* whole blood model and bacteria-induced inflammation...38

5.2.2 Porcine *in vivo* model and ischemia-reperfusion injury......42

5.3 Complement in antiphospholipid syndrome......44

5.4 Modulation of innate immune pathways – future perspectives......45

5.4.1 Treatment strategy......45

5.4.2 Targets and bottlenecks......46

5.4.3 Timing the intervention......48

5.4.4 Inhibitor design......48

5.4.4 Monitoring the intervention......50

6 Conclusion......52

7 References......53

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List of papers

Paper 1: 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. Gustavsen A, Nymo S, Landsem A, Christiansen D, Ryan L, Husebye H, Lau C, Pischke SE, Lambris JD, Espevik T, Mollnes TE. *J Infect Dis.* 2016; 214 (1): 140-150.

Paper 2: Human endothelial cell activation by *Escherichia coli* and *Staphylococcus aureus* is mediated by TNF and IL-1β secondarily to activation of C5 and CD14 in whole blood. Nymo S, Gustavsen A, Nilsson PH, Lau C, Espevik T, Mollnes TE. *J Immunol.* 2016; 196(5): 2293-2299.

Paper 3: Complement factor 5 blockade reduces porcine myocardial infarction size and improves immediate cardiac function. Pischke SE, Gustavsen A, Orrem HL, Egge KH, Courivaud F, Fontenelle H, Despont, A, Bongoni AK, Rieben R, Tønnessen TI, Nunn MA, Scott H, Skulstad H, Barratt-Due A, Mollnes TE.

Paper 4: Eculizumab before caesarean section in severe antiphospholipid syndrome - effects on mother and infant. Gustavsen A, Skattum L, Bergset G, Lorentzen B, Floisand Y, Bosnes V, Mollnes TE, Barratt-Due A.

Abbreviations

aHUS: atypical Haemolytic Uremic Syndrome

APS: Antiphospholipid Syndrome

β2-GP1: β2-Glycoprotein 1

CAPS: Catastrophic Antiphospholipid Syndrome

CCP: Complement Control Protein domain

CR: Complement Receptor

DAMP: Damage Associated Molecular Pattern

E. coli: Escherichia coli

GPI: Glycosylphosphatidylinositol

HUVEC: Human Umbilical Vein Endothelial Cell

ICAM-1: Intercellular Adhesion Molecule-1

Ig: Immunoglobulin

IL: Interleukin

LPS: Lipopolysaccharide

MAMP: Microbial Associated Molecular Pattern

MASP: MBL Associated Serine Protease

MBL: Mannose Binding Lectin

MFI: Median Fluorescence Intensity

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

S. aureus: Staphylococcus aureus

PAMP: Pathogen Associated Molecular Pattern

PBS: Phosphate Buffered Saline

PCI: Percutaneous Coronary Intervention

PNH: Paroxysmal Nocturnal Haemoglobinuria

SLE: Systemic Lupus Erythematosus

TLR: Toll-Like Receptor

TNF: Tumor Necrosis Factor

TTC: Triphenyl Tetrazolium Chloride

1 Background

1.1 Introduction

An effective defence system is a key requisite for survival and is present in all creatures, from the basic unicellular organism to the complex multicellular human organism. Traditionally, the human immune system is divided into two separate branches, the ancestral and “basic” innate immune system and the “more sophisticated” adaptive immune system. However, these systems have not developed independent of each other (1). Increasing knowledge of the intricate, multifaceted and highly redundant inflammatory response shows that there is a marked crosstalk and mutual dependence between the two systems. Thus, there is no clear cut division between the innate and the adaptive immunity. Even the memory aspect which previously was a key feature of the adaptive immune system, is now shown to apply also for the innate immune system (2). The essential role of the immune system is to protect against situations that threaten the host. A microbial attack is an obvious threat, however tissue damage of sterile origin, dysfunctional cells and debris must also be cleared and resolved. The ultimate purpose of the immune system is to resolve pending or ongoing threats, independent of their nature, and to re-establish homeostasis.

As we understand more of how the immune system functions in health, and just as important what goes wrong when the inflammatory response results in tissue damage and disease, the potential to treat and cure inflammatory diseases increases. Research on the immune system has resulted in the development of drugs targeting different mediators and immune pathways, which already alleviate symptoms in a broad range of inflammatory diseases. Recently, also the complement system was included as a drug target when the complement inhibitor eculizumab reached the clinic.

1.2 Innate immunity

The traditional view of the innate immune system was an always present, germline encoded sensing and acting first line protection against microbial infection. In 1994, Matzinger proposed the “danger theory”, stating that pending danger to the host is met

with a response that does not differentiate between exogenous and endogenous origin (3). Indeed, the innate immune system reacts with a germline encoded set of pattern recognition molecules, also termed pattern recognition receptors, that recognize patterns of pending danger (4), and it is evident that damaged self can activate innate immune pathways similar to that of pathogens (5). These danger patterns are evolutionary highly conserved molecules which in the case of bacteria, viruses and other infectious, exogenous intruders are termed pathogen associated molecular patterns (PAMPs) while endogenous molecules originated from damaged self are termed damage associated molecular patterns (DAMPs).

The innate immune system is built up of soluble and membrane bound receptors, intercellular signalling molecules, intracellular signalling pathways, and immune cells ready to respond to an activating stimulus. The pattern recognition molecules include a wide variety of molecules, including complement system proteins, toll-like receptors (TLR) and NOD-like receptors. The sensing and binding of PAMPs or DAMPs can be regarded as the upstream initiation of inflammation which induces activation of extracellular cascade systems and intracellular signalling pathways that lead to downstream effects including cytokine production, chemotactic attraction and activation of immune cells and phagocytic cells for clearance and ultimately restoration of homeostasis (6). Importantly, the variety of these effector systems of the innate immune system work together in concert to resolve the triggering event.

1.3 The complement system

The complement system consists of a still growing list of more than 60 components, including complement recognition molecules, proteases, enzyme complexes and biologically active split products as well as soluble and membrane bound receptors and regulators. The major source of complement proteins found in plasma is the liver, with the exception of C1q, properdin and C7 which are predominantly synthetized in bone marrow derived cells and factor D synthetized in adipocytes (7-10). However, extrahepatic complement protein synthesis is evident in several cell types and organs including immune cells, endothelial cells, epithelial cells, neurons and fibroblasts (11). This enables the system to act locally, and even intracellularly, in addition to the main

systemic blood born localisation (12). Although several components are soluble and appear in fluid phase, the activation of complement takes place on surfaces. These surfaces include bacteria, crystal structures, micro particles, foreign surfaces, medical catheters or implanted devices and unprotected host cells.

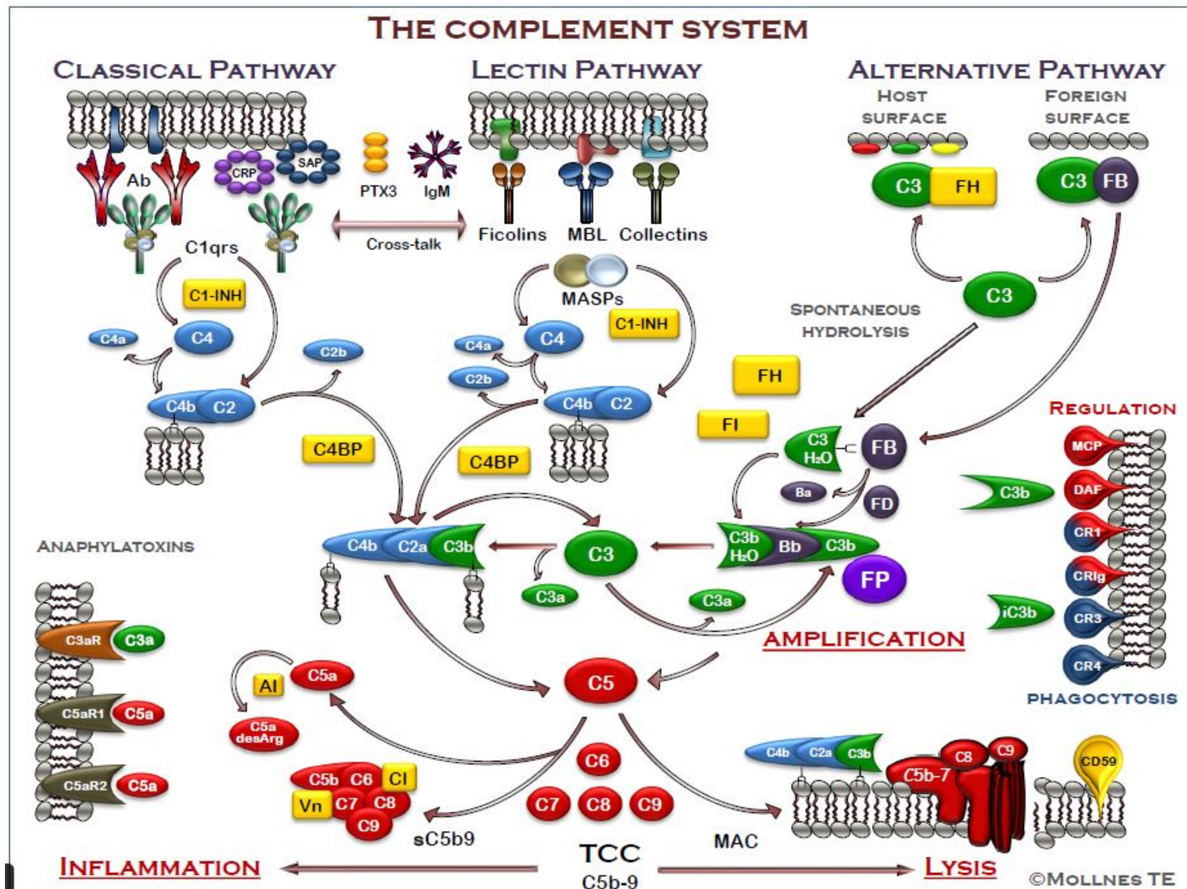

Figure 1: The complement system

The complement system functions as a cascade system activated by recognition of pathogens or damage motifs by the pattern recognition molecules of the three initiating pathways: the classical, lectin and alternative pathway. Initial activation leads to enzymatic cleavage of complement factors and amplification of central and terminal components, though tightly regulated by the regulators of complement activation. Important effector functions of the complement system are opsonisation of pathogens, dead cells and debris for phagocytosis, recruitment and activation of immune cells and even direct lysis of certain pathogens, however several other effector functions are now evident which I will elaborate in the following sections.

1.3.1 Initiating pathways

The pattern recognition molecule of the classical pathway is C1q. When IgM and IgG bind to antigens, conformational changes to the antibodies are induced, enabling C1q-binding (13). C1q binds efficiently to single IgM molecules, but requires hexamers of IgG molecules for similar efficient binding (13). Although the classical pathway was first discovered as an antibody-dependent pathway, further insight has established antibody-independent activation via bacterial products, pentraxins (including CRP) and apoptotic and necrotic cell components like annexins, DNA and histones (14). Subsequent conformational changes of C1q facilitates association of the serine proteases C1r and C1s in a calcium-dependent manner and enables this C1qrs molecular complex to cleave C4 into C4a and C4b, and further association and cleavage of C2, leading to assembly of the C3-convertase C4b2a.

The lectin pathway is based on pattern recognition molecules of the lectin type. Mannose binding lectin (MBL) was first discovered, and later several other PRMs including collectin 11 and 12 and ficolin 1-, 2- and 3 (15). These molecules recognize carbohydrate structures, typically found on bacteria, virus and fungus. However, they can also bind self-structures including mitochondria and DNA and participate in clearance of apoptotic cells (16-18). Following pattern recognition, the molecules associate with the MBL-associated serine proteases (MASPs) -1 and -2 in a calcium-dependent manner which cleave C4 and C2, again resulting in the assembly of the C3-convertase C4b2a.

The alternative pathway initiation is different from the classical and lectin pathway. Spontaneous hydrolysis (tick-over) of C3 produces C3(H_2O), a C3b-like molecule, with hydrolysis of the internal thioester of C3. Factor B is recruited to bind in a magnesium-dependent manner and subsequently cleaved by factor D to Bb and Ba, resulting in the formation of the alternative pathway C3-convertase C3(H_2O)Bb. The tick-over ensures that the complement system is always "on" with C3(H_2O) ready to react (19). Alternatively, this pathway uses C3b produced by classical or lectin pathway C3 convertase, again binding factor B to form the C3 convertase C3bBb. Subsequent binding of properdin stabilizes the convertase. Controversy exists regarding the role of properdin as a recognition molecule, and new data reveals the lack of properdin

binding when C3 is blocked, suggesting that properdin does not bind without preceding C3b-binding (20). The alternative pathway is reckoned as the amplification loop as a positive feedback of C3bBbP results in further cleavage C3 and production of additional C3bBb. Consequently, this pathway is responsible for the vast majority of C3b and C5b-9 formation independent of the initiating pathway (21).

1.3.2 Central and terminal pathways

Irrespective of which initial pathway was activated, C3 is cleaved to generate C3b and C3a. C3b, the major opsonin, binds covalently to unprotected surfaces via a thioester domain exposed after cleavage (22). C3b and its subsequent cleavage products (iC3b, C3c, C3dg) are further recognized by complement receptors with a range of effector functions described later in this section. C3b assembles with the C4b2a and C3bBb convertases to generate the C5 convertases C4b2a3b and C3bBbC3b. The addition of C3b shifts the preferential binding of the convertases from C3 to the C5 molecule which is subsequently cleaved into C5b and C5a (23).

The anaphylatoxins C3a and C5a are small peptides with important signalling properties. They bind to their respective receptors, C3aR, C5aR1 and C5aR2 on immune cells, however also found in several other cell types (24). The two former are classical G protein-coupled receptors, while the latter is uncoupled from intracellular G-proteins (25). The following effector function depends on the cell type expressing the receptor. C3a, formerly thought of as a strictly pro-inflammatory molecule, was recently shown to also exert anti-inflammatory properties especially in neutrophils, while still showing pro-inflammatory effects on monocytes, eosinophils and mast cells (26, 27). C5a is a highly potent, but short-lived peptide, regarded as the dominant pro-inflammatory effector signalling molecule of the complement system. It acts as a chemotactic agent on neutrophils and monocytes, induces oxidative burst, release of enzyme-containing granules and cytokines and induces upregulation of adhesion molecules (28). However, C5a has also been shown to exert anti-inflammatory and immune regulatory effects, mainly through the recently discovered C5aR2 which may counteract the pro-inflammatory effects driven by binding to C5aR1 (25). Still, the role of C5aR2 is controversial and definite function of this receptor is not clearly elucidated

yet. The anaphylatoxins are degraded by carboxypeptidases into C3a-desArg and C5a-desArg with lower affinity for anaphylatoxin receptors (29).

Finally, the terminal C5b-9 complex (TCC) is assembled: C5b subsequently associates with C6, C7, C8 and multiple C9 molecules. When C7 is bound, the complex associates tightly to surfaces and the addition of C9 forms a pore, which can lyse cell and bacterial membranes (30). This complex also occurs in a soluble form, sC5b-9. Some bacterial strains like Neisseria are susceptible to C5b-9 lysis although the majority of bacteria are killed by complement via C3-opsonization and subsequent phagocytosis. However, lysis is not the only effector function of C5b-9. Sub lytic C5b-9 can induce pro-inflammatory responses including NLRP3-inflammasome activation and degranulation, most likely by calcium-influx or association with other membrane-bound receptors (31, 32).

1.3.3 Complement regulation

Complement system activation cannot be described without addressing complement regulation. These two properties of the system are closely linked and act simultaneously, enabling an optimal response at the right site and time. Several regulators counteract the activation of the system by cleaving or co-factoring the decay of convertases and split products. Healthy host cells present complement regulators on their surface to prevent complement attack.

C1-INH is a serine protease inhibitor which inactivates C1r, C1s and the MASPs in classical and lectin pathway. However, C1-INH also inactivates serine proteases of other cascades as the contact system, coagulation system and fibrinolytic system, and is therefore not a strict complement inhibitor (33). C1-INH-deficiency results in hereditary angioedema, where the angioedema attacks are mediated by bradykinin (34).

Factor I is a fluid phase serine protease which cleaves and inactivates C4b and C3b in a cofactor-dependant manner (35). The inactivated products (C4c, C4dg, iC3b, C3c and C3dg) can no longer assemble to be part of the convertases to further drive the cascade, however do bind to several complement receptors or regulators. C4b binding protein

(C4BP) regulates C4 in fluid phase by binding and exerting cofactor activity for factor I-mediated cleavage of C4b and also fluid phase C3b and furthermore accelerates the decay of the classical and lectin pathway C3 convertase (36). Membrane cofactor protein MCP (CD46) also acts as a cofactor for factor I-mediated cleavage of C4b and C3b, however this protein is as its name states a membrane bound regulator, expressed on all cell types except erythrocytes (37).

Factor H is an important regulator of the alternative pathway, both of the spontaneous activation and the amplification properties in fluid phase and on host surfaces. It is a fluid phase protein built up of 20 complement control protein (CCP) domains. The N-terminal four CCPs (CCP 1-4) constitutes the complement regulatory part while the C-terminal end (CCP19-20) binds to C3b and host surfaces via glycosaminoglycan or sialic acid (38, 39). Factor H binds and protects self surfaces from complement deposition by mediating cofactor activity for factor I-mediated cleavage of C3b in addition to binding and preventing C3b to associate with factor B and accelerating the decay of the alternative pathway convertase (40).

Decay accelerating factor (DAF, CD55), a membrane bound regulator, binds to and dissociates the alternative pathway convertase. Finally, CD59 prevents final assembly and subsequent lysis effect of C5b-9 on cell membranes (37).

1.3.4 Complement receptors

Five distinct complement receptors (CR) are identified. CR 1 (CD35) and 2 (CR2, CD21) share the CCP domains typical of several of the complement regulators, CR3 (CD11b-CD18) and 4 (CD11c-CD18) are members of the integrin family while complement receptor immunoglobulin-like (CRIg) is a member of the Ig superfamily (41). These bind C3b or its degradation products iC3b, C3c and C3dg. CR1, CR3, CR4 and CRIg participate to different degrees in the binding of opsonised particles to immune cells and subsequent phagocytosis (42). CR1 is found abundantly on erythrocytes which shuttle immune complexes and opsonised particles via this receptor to the liver and spleen for clearance (43). Further, CR1 serves as a cofactor for factor I-mediated cleavage of C3b (44). CR3 is an important granulocyte phagocytosis receptor for iC3b-

opsonised particles, and in addition plays a role in leukocyte-endothelial cell interactions as it binds to the endothelial adhesion molecule ICAM-1 (45). CR2, in contrary to the other receptors, is a B-cell co-receptor important for antigen presentation and B-cell responses (46).

1.4 Toll-like receptors and CD14

1.4.1 Toll-like receptors

Toll-like receptors (TLR) are a class of membrane bound pattern recognition receptors which, upon ligand binding, activate intracellular pathways that ultimately result in release of cytokines, chemokines and other inflammatory molecules (47). Ten different human TLRs have been identified which localise on the plasma membrane or membrane of intracellular vesicular compartments as heterodimers (TLR2/1, TLR2/6) or homodimers (TLR3, 4, 5, 7, 8, 9 and 10).

TLR4 is one of the most studied TLRs. In the late 1990-ies, Poltorak *et al* and Qureshi *et al* discovered the LPS-sensing role of TLR4 (48, 49). However, multiple other molecules have been demonstrated to activate TLR4, in CD14 dependent and independent manners. Although LPS contamination is important to rule out when studying other stimulating molecules, several exogenous, endogenous (heat shock proteins, high mobility group box-1 protein), and synthetic peptides are added to the list of TLR4 activators (50, 51). Activation of TLR4 is dependent on association with another membrane bound protein, MD2, forming the TLR4-MD2-complex. Ligand binding to this complex results in recruitment of intracellular signalling domains (TIR-domains) and engagement of adaptor proteins (MyD88 and TRAM/TRIF), resulting in activation of intracellular signalling pathways that activate key transcription factors for cytokine production, including NF-κB and interferon regulatory factor 3 (IRF3) (47). The net effect of these pathways is production and release of pro-inflammatory cytokines.

1.4.2 CD14

CD14 is a pattern recognition receptor found abundantly on myeloid cells, but increasingly evident also on other cell types including endothelial cells (52, 53). The receptor occurs in two forms; a glycosylphosphatidylinositol- (GPI) anchored membrane protein and a circulating soluble molecule, sCD14 (54). It serves as a co-receptor for TLR4 where it presents the LPS molecule to the LPS binding site on the TLR4-MD2 complex (55). CD14 has also been shown to interact with human TLR2, and TLR3, TLR7 and TLR9 at least in mice (56-58). However, TLR-independent signalling by CD14 leading to calcium-influx has recently been shown in dendritic cells (59). The molecular structure of CD14 enables it to bind to a wide range of ligands, thus explaining the promiscuity of CD14 (60).

1.5 Cross talk and redundancy of complement, TLRs and CD14

As already mentioned, both complement and TLRs recognise a variety of PAMPs and DAMP. Some evidently activate both systems and there is increasing evidence of substantial cross-talk and synergy between these pathways. LPS, found in the outer membrane of Gram-negative bacteria, activates TLR4 in a CD14-dependent manner, although CD14-independent activation of TLR4 is reported for high concentrations of LPS (61). LPS is a poor activator of complement in fluid phase, but in high concentrations can induce assembly of sC5b-9 in plasma (62). The fungal cell wall protein zymosan is a potent complement activator, but is also a ligand for TLR2, again exemplifying the redundancy of the innate immune response. Raby *et al* showed that signalling via TLRs could increase C5aR-mediated responsiveness to C5a in PBMCs via downregulation of C5aR2 expression (63). Further, Zhang *et al* showed how signalling via C3aR and C5aR enhances TLR-mediated cytokine production (64). Experiments in whole blood have demonstrated how some inflammatory readouts induced by Gram-negative bacteria are predominantly dependent on complement, including phagocytosis and oxidative burst, others are increasingly dependent on CD14, including selected cytokines, while several acute inflammatory and coagulation markers are dependent on both (65).

The cross talk and redundancy of these pathways should be considered when targeting innate immunity to alleviate the inflammatory process as merely targeting one pathway may not be a sufficient approach.

1.6 The complement system in health and disease

As already mentioned, the complement system is an important defence strategy for surveillance and protection of the host against pathogens, but is also important for clearance of dead cells, debris and immune complexes. In addition, complement emerges with an important role in adaptive immunity in interplay with B- and T-cells (66-68). C3d-opsonised antigens bind to CR2 on B-cells and reduces the threshold for B-cell receptor signalling and antibody production (46). Further, CR2 is implicated in maintenance of memory B-cells (67). Studies in mice show that T-helper 1 cells require signalling via CD46, C3aR and C5aR1 for induction of normal INF-γ production (12). However, a recent study in C3-deficient patients showed that these individuals mount IFN-γ-responses, but showed abnormal IgG-subclass patterns after vaccination, most strinkingly a lack of IgG4 (69). Collectively, this shows that the complement system also participates in adaptive immune responses and long term immunologic memory.

Recently, a novel function of the complement system was established, namely intracellular complement activation. Liszewski *et al* published data showing an intracellular C3 activation system with cleavage of intracellular C3 (70). The resulting C3a was shown to engage with intracellular C3aR leading to effector functions important for T-cell homeostasis. Initially discovered in T-cells, further analysis in several other cell types including monocytes, neutrophils, endothelial cells and epithelial cells revealed similar intracellular stores of C3 and generation of C3a. This new role of complement may have broader implications in cellular homeostasis and shows that many aspects of the complement system are still not yet fully understood (71). Other research groups have linked complement to tasks related to embryonal development (72) and neuronal pruning (73). These latter insights show how the complement system also plays an important role in other settings than pure innate defence.

Given the important roles of complement regarding defence, clearance, homeostasis and immunologic memory, there is no surprise that complement also is involved in a multitude of diseases. Individuals with deficiencies of specific complement components or regulators have increased our knowledge of these roles of complement (74). The complement system balances between activation and regulation for fine-tuned and appropriate responses to triggers. This balance seems to be of crucial importance as an imbalance in this finely regulated response can lead to inappropriate activation and disease processes. Genetic polymorphisms affect an individual's balance and have been addressed as the complotype (75). Reduced potential for activation or too strict regulation increases the risk of infections while escalated activation or reduced regulation will predispose for inflammation, tissue damage and autoimmune diseases. In the following paragraphs, I will present some examples.

Complement defects resulting in insufficient activation, typically are associated with an increased risk of infection, mainly by bacteria (76). Deficiency of the central C3 molecule leads to early onset of serious infections by encapsulated bacteria, while deficiency of terminal complement components almost exclusively increases the risk of *Neisseria* infection. MBL-deficiency is associated with increased risk of infection in young children when the protection by maternally-derived antibodies fade until an efficient antibody repertoire is established (77). Likewise, in other immunodeficiency states including immune suppressive treatment, MBL-deficiency may contribute to infection frequency and severity (78-81).

The role of complement for clearance of immune complexes and cellular debris is highlighted by the fact that defects in early components of the classical pathway are associated with systemic lupus erythematosus (SLE) (82). Up to 90 % of patients with C1q-deficiency are reported to develop SLE or SLE-like disease, followed by 80 % of patients with C4 deficiency (76). Interestingly, C3 deficiency is mainly not associated with SLE or other autoimmune diseases, which can be explained by the important role of C3 for the processing of antigens in T cells, thus C3 deficiency may protect against the development of autoreactive T cells (68).

Defects in complement regulators or increased activity of the C3 convertase are found in atypical haemolytic-uremic syndrome (aHUS) and several complement-related

kidney diseases (83). In aHUS, the most common defect is mutation in factor H or autoantibodies to factor H, decreasing the complement regulating function of this molecule (84). A triggering event leading to complement activation induces a sustained and uncontrolled activation resulting in thrombotic microangiopathy most prominent in the kidneys and subsequent renal failure in these patients (85).

In paroxysmal nocturnal haemoglobinuria (PNH), the glycosylphosphatidylinositol (GPI) anchor is missing on a clone of haematopoietic stem cells due to a somatic mutation in a the *PIG A-gene* (86). Consequently, affected erythrocytes lack complement regulators CD55 and CD59 and are prone to complement-mediated lysis, leading to anaemia. In addition, these patients also have an increased risk of thrombosis.

1.6.1 Complement in bacterial infections and sepsis

The most studied effector function of complement is protection against pathogens, most importantly bacteria. The immune response in bacterial infection aims to induce an appropriate inflammatory response to confine, resolve and clear the infection and subsequently repair the tissue damage caused by the infection.

Bacteria can be recognised by all three initiating pathways leading to C3b-opsonisation (87). Production of anaphylatoxins and cytokines recruit and activate professional phagocytes, typically granulocytes and monocytes, which express receptors including CR3 that bind to C3b-opsonised bacteria and initiate phagocytosis. Erythrocytes also play an important role with CR1-mediated binding and subsequently transport of opsonised bacteria to resident macrophages in the liver and spleen (43). Deposition of C5b-9 can lyse certain Gram-negative bacterial strains, however most importantly *Neisseria* species. This is evident from nature's own experiments as patients deficient of C5, C6, C7, C8 or C9 are almost exclusively prone to *Neisserial* infections, included patients treated with the C5-inhibitor eculizumab (76). The important role of complement to protect against bacteria is also evident by evasion strategies developed by several bacteria to evade complement mediated attack, as reviewed by Laarman *et al* for *Staphylococcus aureus* (*S. aureus*) and group A *Streptococcus* which express and

secrete several proteins that inhibit complement recognition, activation and amplification (88).

When the inflammatory response to bacteria is kept locally and controlled, the infection can be cleared and tissue homeostasis resolved. In contrast, a systemic inflammatory activation may lead to a detrimental and host-threatening situation. Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (89). The true incidence worldwide is unknown, however estimates point out that sepsis is a leading cause of mortality and critical illness (90). Despite extensive treatment with antibiotics and supportive therapy in intensive care units, morbidity and mortality remains high, reflecting the lack of effective treatment strategies once the syndrome is acknowledged. Complement activation is implicated as part of the pathophysiology, evident from a long list of *in vivo* animal studies and demonstrated by profound complement activation in sepsis patients (91-93). However, other innate immune pathways and adaptive immunity are evidently involved in the pro- and anti-inflammatory responses characterizing sepsis, in addition to coagulation, neurohormonal and metabolic pathways, underscoring that the multifaceted and complex pathophysiology is not yet fully understood (89, 94, 95).

1.6.2 Complement and ischemia-reperfusion injury

The tissue damage caused by abrogation followed by restoration of blood supply to tissues or organs is termed ischemia-reperfusion injury. The abrogation of blood flow to part of or a whole organ halts oxygen delivery and results in ischemia. Depending on the length of ischemia, this oxygen deprivation will lead to cellular damage and subsequent cell death. Therefore, rapid restoration of blood flow is a crucial method for salvaging still vital cells. However, the ischemia and subsequent reperfusion leads to tissue damage and release of DAMPs that activate innate immune pathways. Although important for the healing process, this sterile inflammatory process causes detrimental effects and increases the final tissue or organ injury in a wide range of conditions, including myocardial infarction and solid organ transplantation (96, 97). Complement activation was demonstrated in infarcted myocardium of rats in 1971 by Hill and Ward (98), and later research has revealed the contribution of complement

activation to the ischemia-reperfusion injury in various organs (99). All three initiating pathways have been linked to the activation of complement in ischemia-reperfusion injury, although the lectin pathway recognition molecules and natural occurring IgM antibodies binding to neo-epitopes or extracellular matrix proteins exposed during ischemia-reperfusion have gained most attention (100, 101). The following complement activation and C5a production recruits neutrophils which generate reactive oxygen species that contribute to cellular injury and cell death (102). Subsequent recruitment of other immune cells including monocytes, dendritic cells and T-cells, contribute to inflammation and remodelling with both detrimental and beneficial effects (103). Finding strategies to target and reduce the detrimental effects while keeping the beneficial healing and remodelling steps has proven difficult, however, complement is still regarded as an attractive target (104).

1.6.3 Complement and antiphospholipid syndrome

Antiphospholipid syndrome is characterized by arterial, venous or small-vessel thrombosis or pregnancy morbidity (recurrent abortions, fetal losses, pre-eclampsia, and placental insufficiency) in the presence of persistent antiphospholipid antibodies (anti-cardiolipin antibodies, anti-β2 glycoprotein 1 (β2-GP1) antibodies and lupus anticoagulant) (105). The disease occurs either as a primary disorder, or secondary in other autoimmune diseases, typically SLE. Thrombosis is linked to inflammation in these patients, although the exact mechanism by which the antiphospholipid antibodies induce thrombosis or pregnancy morbidiy is not clearly elucidated. However, research points to β2-GP1 as the most pathogenic target (106). The complement system has been implicated in the pathogenesis of the disease based on in vivo studies in mice where complement inhibition was shown to reduce obstetric complications and thrombosis (107, 108). The binding of antiphospholipid antibodies is proposed to activate the classical complement pathway, linking complement and coagulation in this disease, underscored by the discovery of a non-complement-fixing anti-β2GP1-antibody which reduces thrombus formation in rats and pregnancy loss in mice (109). C5-inhibition and C6-deficieny also reduced the two outcomes, suggesting a role of C5b-9 in the pathogenesis. Intriguingly, a study by Oku *et al* showed signs of

complement activation and consumption of C3 in patients with primary antiphospholipid syndrome (110).

Although rare, some patients develop the devastating and life-threatening catastrophic antiphospholipid syndrome (CAPS), featured by multi-organ thrombosis and up to 50 % mortality rate (111). CAPS manifests as a systemic inflammatory response syndrome with clinical and histopathologic evidence of thrombotic microangiopathy (112). Typical triggering factors are infection, surgery, withdrawal of anticoagulant therapy and pregnancy/puerperium (113). The contribution of complement in the pathogenesis of CAPS was elucidated in a recent case report from our group (114). A patient developed CAPS with rapid clinical deterioration and cardiac arrest. High levels of sC5b-9 detected in plasma samples resolved after eculizumab administration, simultaneously with clinical improvement in the patient. Withdrawal of eculizumab induced a disease flare which was efficiently abolished when eculizumab was reinstituted. This case elucidates how systemic activation of complement might be a driving event in CAPS, at least in this patient, even though the mechanism of how complement activation and thrombosis are related is not yet clear.

2 Aim

The main aim of this thesis was to study the role of complement in models of acute inflammatory diseases.

Complement, as part of the rapid and immediate innate immune response to pending danger, contributes to the pathophysiology of many human diseases. However, the individual contribution of complement activation is not always clearly elucidated. This knowledge has the potential to result in new therapeutic approaches, as the first complement inhibitor has reached the clinic and several others are on the verge (115).

Specific aims:

1. **To study the cross talk and redundancy of complement and the TLR/CD14-system in acute whole bacteria-induced inflammation**.

   As the TLR4-MD2 antagonist eritoran failed in a phase three sepsis trial, we used an established *in vitro* whole blood model to investigated the inhibitory effect of this drug compared to inhibition of CD14, and the combination of each of these TLR-system inhibitors with a complement inhibitor on the inflammatory response induced by Gram-negative and Gram-positive bacteria.

2. **To study the role of endothelial cells in acute whole bacteria-induced inflammation.**

   We developed the whole blood model further to include the interaction between whole blood and endothelial cells. This model was used to study the contribution of complement and the TLR/CD14-system in endothelial cell activation by whole Gram-negative and Gram-positive bacteria, aiming to elucidate the mechanisms of interaction between endothelial cells and whole blood in acute bacteria-induced inflammation.

3. **To study the role of complement activation in a model of acute sterile inflammation.**

   Complement inhibition failed to reduce mortality in a phase three trial of percutaneous coronary intervention treated myocardial infarction (APEX-AMI), however concerns were raised regarding the inhibition as sC5b-9 increased similarly in the treatment and placebo groups. We used a porcine model of

myocardial ischemia-reperfusion injury to investigate to what extent complement activation contributes to the final myocardial infarction size.

4. **To study the effects of complement inhibition in an autoimmune disease were complement is implied as part of the pathophysiology.**

   Complement inhibition emerges in the clinic. In the case of a pregnant woman with antiphospholipid syndrome and in high risk of developing a catastrophic variant of the disease, we followed the effect of complement inhibition in the mother and child to study the efficacy and safety of this off-label prophylactic treatment approach.

3 Materials and methods

This chapter contains an overview and some considerations regarding the materials and methods used in the papers of this thesis. Closer details are found in the methods sections of the included papers.

3.1 LPS and bacteria

LPS: LPS varies among different strains of Gram-negative bacteria. In paper 1, we used ultrapure LPS from *E. coli* strain 0111:B4, a smooth type LPS.

Bacteria: In paper 1 and 2, we used *Escherichia coli* (*E. coli*) strain LE392 with smooth LPS (ATCC 33572) and *S. aureus* strain Cowan 1 (ATCC 12598). The bacteria were inactivated by heat and counted by flow cytometry to achieve the correct concentration.

3.2 Inhibitors

Paper 1: We chose to target complement at the C3 level to inhibit C3a, C5a and C5b-9-mediated responses and used the small peptide C3-inhibitor compstatin analogue Cp40. TLR4-MD2 was inhibited by the blocking lipid A analogue eritoran (E5564), used in human sepsis trials and therefore relevant for bacteria-induced inflammation. CD14 was inhibited by the blocking anti-human CD14 antibody clone r18D11, the original clone developed by Terje Espevik in Trondheim and the recombinant version developed and tested in our laboratory. This antibody is endowed with a human IgG2/4 hybrid heavy chain, and thus, inert with respect to major IgG effector functions; Fc-mediated complement activation and Fcγ-receptor binding (116). An IgG2/4 isotype antibody and a control peptide were added as controls.

Paper 2: As increasing evidence emerge regarding pro-inflammatory properties of C3a, we chose to target complement at the C5-level, blocking C5a and C5b-9-responses, but leaving the C3-molecule active. We therefore used the C5-blocking IgG2/4 antibody eculizumab. In addition, we added a specific C5aR1-receptor agonist to differentiate between C5aR1 and C5b-9 mediated responses. CD14 was inhibited by the anti-human

CD14 IgG2/4 antibody (r18D11) and TLR4-MD2 inhibited by eritoran. Cytokine inhibition was achieved by the TNF-blocking antibody infliximab and the IL-1β–blocking antibody canakinumab, respectively. The CD20-blocking antibody rituximab was used as a control antibody.

The final concentrations of the inhibitors were based on previous dose-response experiments in whole blood to ensure complete inhibition. Controls were used in equimolar final concentrations.

Paper 3: In this porcine model, we used the recombinant bacterial Ornithodoros moubata Complement Inhibitor (OmCI), a 16.8 kDa protein also known as coversin, which cross-reacts with porcine C5. The inhibitor was diluted in saline and saline only was used as placebo. The concentrations of the bolus and continuous infusion were based on previous experiments ensuring complete C5-blockage throughout the experimental time course. One animal receiving coversin did not show complete C5-inhibition for unknown reasons, and was therefore excluded from further analysis as this obviously would preclude the interpretation of the data.

Paper 4: The C5-blocking Ab eculizumab was administered by 30 min infusion twice, 7 days apart, in a dose of 600 mg. The chosen dose was based on efficient C5 inhibition in two pregnant women diagnosed with PNH of approximately the same weight.

3.3 Analysis

3.3.1 ELISA

Enzyme-linked immunosorbent assays use antibodies specific for the biomarker of interest, and a subsequent step in which the detection antibody (primary) or a new antibody detecting the detection antibody (secondary) is conjugated with an enzyme. This enzyme cleaves an added substrate to produce a colour and the colour intensity reflects the amount of biomarker in the sample. By using a standard curve of known concentrations, the colour intensity translates to a concentration value.

Fluid phase C5b-9 was measured in EDTA-plasma by an in house sandwich-ELISA using a C9-neoepitope specific monoclonal antibody (clone aE11) as a capture antibody and a biotinylated C6-specific monoclonal antibody (clone 9C4) as a detection antibody. Zymosan-activated normal human serum was used as a standard. This assay cross-reacts with porcine C5b-9 and was used in paper 3. In paper 4, complexes of C5 and eculizumab in serum were measured by an in-house sandwich-ELISA using a monoclonal anti-C5 antibody as a capture antibody and an IgG4 antibody as a detection antibody. Eculizumab added to human serum *in vitro* in a known concentration was used as a standard. For additional biomarkers detected by ELISA, commercial kits were used in accordance with manufacturer's instructions. This includes leukotriene B4 (LTB4) and heart fatty acid binding protein (H FABP) in paper 3. In paper 3 and 4, complement function of the classical, lectin and alternative pathways were assessed by a commercial available ELISA (Complement System Screen Wieslab). The analysis is semi-quantitative as the pathway activity is defined as percent of a positive control and complete C5-inhibition abrogates assembly of the detected C5b-9 of all pathways.

3.3.2 Multiplex technology

Multiplex technology allows the quantification of several biomarkers simultaneously by using uniquely fluorescence coloured beads with specific detection antibodies attached. Biotinylated secondary antibodies are added, which subsequently bind to a fluorescent reporter molecule. A flow cytometer excites the beads and the reporter molecules, respectively, leading to separation of the beads in a plot, and measurements of the fluorophore intensity reflecting the amount of the biomarker. An added standard enables quantification of the signal. TNF, IL-1β, IL-6 and IL-8 were chosen as cytokine readouts as these are proven robust from previous publications of our group and clinically relevant pro-inflammatory cytokines.

3.3.3 Flow cytometry

In paper 1, the leukocyte activation markers CD11b (subunit of CR3) and CD35 (CR1) were measured by flow cytometry after lysis of erythrocytes and staining with

fluorophore-marked antibodies against CD11b, CD35 and CD14. Monocytes and granulocytes were gated by side scatter (SSC) and high or low CD14-staining, respectively. In paper 2, the endothelial cell activation markers ICAM-1 and E-selectin were analysed by flow cytometry after briefly fixed (PFA 0,5 % for 2.5 min at 4°C), stained with fluorophore-marked antibodies against ICAM-1, E-selectin and CD146 and gently detached by trypsin-EDTA. Endothelial cells were gated by high CD146 staining and SSC.

3.4 *In vitro* experiments

3.4.1 Whole blood model

The whole blood model is an *in vitro* system designed to study acute inflammatory processes and cross talk between the entangled systems of whole blood, with all the components of whole blood in place (117). However, adding an anticoagulant is a prerequisite as the coagulation process inevitable initiates when whole blood is drawn. By using lepirudin, a thrombin inhibitor, the coagulation cascade is kept open down to the final step of thrombin activation, thus most of the coagulation factors are preserved as active contributors in the inflammatory process. In brief, whole blood was drawn into tubes containing lepirudin at a final concentration of 50 µg/ml. Aliquots were preincubated with PBS or selected inhibitors and subsequently added a stimulating agent or PBS as a control and left at 37°C for incubation for the designated time depending on the readout while gently rotated to prevent sedimentation. Six different healthy blood donors were included for each readout if not otherwise stated.

3.4.2 Whole blood HUVEC model

The whole blood human umbilical vein endothelial cell (HUVEC) model was designed to study the acute inflammatory processes of whole blood keeping in contact with the endothelial cells that line the blood vessels. Endothelial cells are not a passive barrier, but rather an active contributor and player in the inflammatory process (118). The whole blood HUVEC model enables us to study the interactions between whole blood

and endothelial cells, thus taking the whole blood model a step closer to the *in vivo* situation. In brief, human umbilical cord vein endothelial cells of early passages (0-5) were grown in monolayers to confluence in cell culture well plates. Endothelial cell medium was removed and the monolayer gently washed with sterile PBS at 37°C. Fresh whole blood anticoagulated with lepirudin was added in addition to PBS or selected inhibitors. Subsequently, a stimulating agent was added and plates left in a CO_2 incubator chamber, 37°C, for four hours with gentle shaking to prevent sedimentation. An incubation time of four hours was titrated to allow upregulation and detection of both the endothelial cell activation markers E-selectin and ICAM-1. Again, six different blood donors were included for each readout unless otherwise stated.

3.5 *In vivo* experiments

3.5.1 Porcine model of myocardial ischemia-reperfusion

This model enabled us to study the contribution of complement in an *in vivo* model of ischemia-reperfusion injury. An ischemia time of 40 min was chosen as this is comparable to four hours of ischemia in man (119). A reperfusion time of 240 min enabled us to use triphenyl tetrazolium chloride (TTC) staining for infarction assessment. The relative short observation time however limited us from drawing conclusions regarding long-term effects of complement inhibition, including the effect on cardiac remodelling. Sixteen pigs of 20 kg were included and block randomized (four animals per block) to either coversin bolus + infusion or placebo bolus + infusion, eight in each group and blinded to the investigators. In addition, three animals underwent a sham procedure and served as negative controls. After initial anaesthesia, surgery and instrumentation with monitoring equipment, the heart was exposed. Microdialysis catheters were placed in the LAD-perfused area and a control area of the heart as a method to assess local inflammation. A silicon thread was placed around the left anterior descending (LAD) coronary artery distal to the second diagonal branch allowing complete and reversible occlusion of coronary blood. Coversin or placebo was given intravenously 20 min after occlusion and throughout reperfusion. Arterial blood samples were obtained at specific time points for blood gas analysis, serum, and EDTA-plasma preparation, and microdialysis samples were obtained simultaneously.

Microdialysis is a technique where dialysate fluid circulates past a semipermeable membrane in a small probe inserted in a tissue or organ and is collected in vials. Small molecules pass through the membrane and can be detected in the collected fluid. Although the technique classically is used to detect glucose, lactate and other metabolites related to local ischemia, also immunologic biomarkers are detectable given appropriate molecular weight and charge. This enables us to detect local inflammatory biomarkers, as opposed to the plasma levels of circulating biomarkers which not necessary reflect the local processes. We measured TNF, IL-1β, IL-6, IL-8 and IL-10 in microdialysis fluid sampled at designated time points from the area at risk and a control region. A limitation of the method is the unpredictable probe life resulting in cessation of dialysate fluid circulation. The method failed in three animals before 120 min of reperfusion and these were excluded from further analysis.

Cardiac function was evaluated by echocardiography performed before induction of ischemia and prior to euthanization, based on measurements of systolic velocity and displacement, analysed by an experienced cardiologist.

Infarct size was evaluated by histological staining and MRI. Evans blue, which stains circulated tissue, was infused after reocclusion of LAD and immediately prior to euthanization and delineated the area at risk as this area remained unstained. The excised heart underwent MRI scanning for infarction size determination as compared to the total left ventricular volume. The left ventricle was isolated and the area at risk excised and divided in horizontal slices which were stained with TTC and subsequently fixed with formaldehyde before digital scanning. TTC stains dehydrogenases in viable tissue red, while infarcted tissue remains white due to the washout and no new production of dehydrogenases. Infarct size was determined as a percentage white tissue of the area at risk using pixel counts in Photoshop CS5.

Tissue samples from the area at risk, the border zone and a control area were obtained for subsequent immunofluorescence staining and tissue homogenate analysis. E-selectin and FGL-2, markers of endothelial cell activation, C5b-9 deposition and myocardial LTB4 were analysed revealing local effects of coversin treatment.

3.6 Clinical case report

Case reports have important shortcomings as they lack controls, are not generalizable and cannot establish certain causality. However, for rare diseases where randomized clinical trials are difficult to conduct, singles cases can certainly generate hypotheses about disease pathophysiology and treatment strategies, in addition to their educational value. In this case, in addition to close clinical monitoring, blood samples were obtained from the patient before and during eculizumab treatment to monitor treatment effects. In addition, blood samples were obtained from the umbilical artery and vein immediately after cord clamping and from the infant two hours after birth, to evaluate the effect of eculizumab on the new-born.

3.7 Statistical considerations

Statistical significance is a theoretical concept, initially used to consider whether an experiment, given the results, is worthy of repetition. A p-value of 0.05 connotes that there is a 5 % chance of getting the present or a more extreme result given that the null hypothesis is true. Statistical significance is not equal to biologic relevance, and we are generally interested in large differences in order to appreciate biological relevance of our findings. In the *in vitro* and *in vivo* experiments of this thesis, we operate with a small number of observations. Hence, differences must be consistent and relative large to be calculated as statistical significant. When comparing three or more groups or time points, a post-hoc analysis is required to decrease the risk of making type 1 errors (rejecting the null hypothesis when it is true). These are conservative, and thus increase the risk of making type 2 errors (failure to reject the null-hypothesis when it is false), especially when the means are positively correlated.

In paper 1, parametric statistics were used, and in the bacteria-induced readouts only the inhibitors of interest were compared to decrease the risk of type-2 errors. In paper 2, non-parametric statistics were used as the results in particular of the endothelial cell activation markers did not show a normal distribution and were better presented with the median than the mean. In paper 3, both parametric and non-parametric statistics were included. Parametric analyses are used for normally distributed data and compare the group means, however with small sample sizes the distribution pattern

can be difficult to evaluate. Non-parametric analyses compare the group medians and typically have less power than parametric statistics, but are recommended for small sample sizes. There might not always be a clear answer to which method to choose. The sample size in the *in vitro* and *in vivo* experiments results from limited amounts of unique reagents and from the animal ethical perspective stating that efforts should focus on reducing the number of animals down to a minimum, however still including enough to be able to draw conclusions from the study. A power analysis was conducted before the *in vivo* experiments in paper 3 to calculate the minimum sample size required to likely detect a possible effect of the intervention. This power analysis concluded with a sample size of six animals in each group, however, we increased the group size to eight to include a margin.

4 Summary of the main results

Paper 1: 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

The aim of this study was to investigate the effect of single TLR4-MD2 inhibition by using eritoran, compared with the effect of CD14 inhibition alone and combined with the C3 complement inhibitor compstatin (Cp40), on Gram-negative and Gram-positive bacteria-induced inflammatory response in human whole blood.

We found that ultrapure LPS-induced cytokines (TNF, IL-1β, IL-6 and IL-8) and monocyte activation markers (CD11b and CD35) were efficiently abolished by both anti-CD14 and eritoran. Further, when including whole bacteria, we found that although anti-CD14 and eritoran were equally effective in attenuating *E. coli*-induced cytokine response, anti-CD14 was more effective than eritoran in attenuating *E. coli*-induced upregulation of the monocyte activation markers and neither showed effect in attenuating *S. aureus*-induced cytokine response or leukocyte activation markers. The addition of C3 inhibition was required to attenuate *S. aureus*-induced inflammation and combining C3 inhibition with anti-CD14 was significantly more effective than combining C3 inhibition with eritoran in reducing monocyte activation markers induced by both *E. coli* and *S. aureus*.

In conclusion, this study showed that whole Gram-negative and Gram-positive bacteria-induced inflammation was inhibited more efficiently by anti-CD14 than by eritoran, particularly when combined with complement inhibition.

Paper 2: Human endothelial cell activation by *Escherichia coli* and *Staphylococcus aureus* is mediated by TNF and IL-1β secondarily to activation of C5 and CD14 in whole blood

The aim of this study was to examine the relative importance of upstream complement and TLR/CD14-systems, and downstream mediators in Gram-negative and Gram-

positive bacteria-induced endothelial cell activation in a human model, using endothelial cells incubated with whole blood.

The endothelial cells were minimally activated, assessed by the activation markers E-selectin and ICAM-1, when incubated with bacteria in serum, whereas a substantial activation was seen when incubated with bacteria in whole blood. *E. coli*-induced activation was largely CD14-dependent, whereas *S. aureus* mainly caused a C5aR1-mediated response. Combined CD14 and C5 inhibition reduced E-selectin and ICAM-1 expression efficiently for both *E. coli* and *S. aureus*. Finally, endothelial cell activation by both bacteria was completely abolished by combined inhibition of TNF and IL-1β.

In conclusion, *E. coli* and *S. aureus* activated endothelial cells in a CD14- and C5-dependent manner, respectively, with subsequent leukocyte secretion of TNF and IL-1β mediating the effect. A combined inhibitory approach upstream (complement C5 and CD14 inhibition) or downstream (TNF and IL-1β inhibition) could efficiently attenuate endothelial cell activation for both bacteria.

Paper 3: Complement factor 5 blockade reduces porcine myocardial infarction size and improves immediate cardiac function

The aim of this study was to examine the effect of inhibiting complement activation before reperfusion in a porcine model of myocardial ischemia-reperfusion injury when assessing the final myocardial infarction size and myocardial function. We used the C5 inhibitor coversin which cross reacts with porcine C5, and in addition binds leukotriene B4.

In 20 kg pigs, the left anterior descending coronary artery was occluded for 40 min and subsequently reperfused for 240 min. Coversin or placebo was infused 20 min after occlusion and throughout reperfusion in 16 blindly randomized pigs. Coversin ablated plasma C5 activation throughout the reperfusion period and abolished myocardial C5b-9 deposition, while neither plasma nor myocardial LTB4 were significantly reduced.

We found that coversin reduced myocardial infarction in the area at risk assessed by TTC staining and by using magnetic resonance imaging. Tissue Doppler echocardiography showed increased systolic displacement and increased systolic velocity in coversin treated pigs, implying improved cardiac function. Myocardial IL-1β and E-selectin expression were reduced in the non-infarcted area at risk by coversin treatment.

In conclusion, coversin reduced the size of infarction, improved ventricular function, and attenuated local inflammatory markers in this porcine myocardial ischemia-reperfusion model.

Paper 4: Eculizumab before caesarean section in severe antiphospholipid syndrome - effects on mother and infant

The aim of this study was to assess the effect of complement inhibition in a pregnant patient with severe antiphospholipid syndrome prophylactically treated with eculizumab, and further assess the effects on the premature new-born infant.

We found that complement activity recovered considerably faster after eculizumab administration in the patient than anticipated. Furthermore, we carefully investigated the preterm new-born by blood sampling from the umbilical vein and artery, and from the new-born venous blood after delivery. Complement activity measured by a commercial available ELISA was completely abrogated in the infant samples, however several complement components were low in this infant born in gestational week 33. Eculizumab-C5 complexes in the infant showed 0.3% of the concentration detected in the mother, consistent with negligible placental passage of eculizumab.

In conclusion, merely trace amounts of eculizumab passed the placental barrier in gestational week 33 and eculizumab might be a safe candidate treatment option for antiphospholipid syndrome during pregnancy and delivery. We underscore the importance of close monitoring of complement inhibition in patients receiving eculizumab, and individualizing dosage regimens. Further, we documented that traditional functional complement activity tests cannot assess the effect of eculizumab in premature infants due to the very low levels of complement factors.

5 Discussion

In this thesis, we have studied how important pathways of innate immunity contribute to the acute inflammatory processes in models of infectious and sterile inflammation. Based on the increasing evidence of substantial cross talk and redundancy of the innate immune response, we have hypothesised that merely inhibiting one single downstream mediator or pathway cannot substantially attenuate the massive response in an acute, systemic activation state like sepsis (120). In contrast, blocking upstream bottleneck targets of the recognition phase, might have the propensity to more efficiently attenuate the innate immune response in settings where this response is detrimental to the host (120). Given the danger theory stating that the innate immune response does not differentiate between exogenous and endogenous origin, this approach might also be fruitful in acute, sterile inflammation.

5.1 Exogenous versus endogenous activation

Indeed, the response to exogenous and endogenous stimuli shows clear similarities, as demonstrated in the genomic expression patterns of leukocytes from human patients suffering from trauma or burn injury or subjected to low-dose bacterial endotoxin infusion (121). The authors show how these three different inducers of inflammation resulted in an early "genomic storm" affecting >80 % of leukocyte functions and pathways, demonstrating a common response pattern between the three insults which reflects the overlap of upstream receptors and signalling pathways activated. In other words, a systemic inflammatory response may follow the same patterns of activation, irrespective of an exogenous or endogenous trigger. Exogenous molecules are detected directly by the various pattern recognition molecules, however there is evidently cross-talk between the host microbiota and innate immunity enabling us to live in symbiosis with non-pathogenic bacteria colonising a large part of our body (122). Endogenous triggers of inflammation, however, must undergo alterations (altered self) to serve as DAMPs, be exposed or be released from an intracellular location to be "visible" to the pattern recognition molecules. As already mentioned, complement and TLRs recognise both PAMPs and DAMPs which activate the same pathways leading to

downstream inflammation. The results from paper 1, 2 and 3 show how complement activation is implicated in both bacteria-induced activation of whole blood, and in sterile inflammation in a myocardial ischemia-reperfusion model, and importantly, the potential of reducing this inflammatory response by adding inhibitors of complement activation. Paper 1 and 2 additionally show how combining complement inhibition with inhibitors of the TLR-CD14-system may increase the inhibitory effect.

5.2 Models and human disease

There is a large leap from *in vitro* and animal *in vivo* model systems to human disease. In this thesis, I have used in vitro models with human blood, also including human endothelial cells to study bacteria-induced inflammation in paper 1 and 2. This is evidently far from the septic patient in the intensive care unit. Similarly, the porcine myocardial ischemia-reperfusion model used in paper 3 cannot elucidate the complex pathophysiology of an atherosclerotic myocardial infarction in man. An important question is thus how these models reflect the human diseases of which we intend to increase our knowledge and develop new treatment strategies for. I will argue that, given the obvious limitations discussed later, these models can reveal some important points regarding human inflammatory disease and treatment considerations. Lastly, I have included the case report of a young pregnant woman treated with eculizumab to prevent a life-threatening complement-related complication to her underlying antiphospholipid syndrome. Although no definite conclusions can be drawn regarding the treatment effect, the case reveals important considerations regarding complement inhibition in the clinic and contributes to the management approach of a rare, but devastating disease.

5.2.1 *In vitro* whole blood model and bacteria-induced inflammation

The whole blood model enables us to study the complement system and cross talk with all components of whole blood actively present, closely resembling the physiological contents. The thrombin inhibitor lepirudin does not interfere with the complement

cascade, in contrast to heparin, a commonly used anticoagulant, which can activate and inhibit complement activation, depending on the concentration (123). Spontaneous background activation increases by time in this system due to reactions with the plastic of the tubes and thus limits the observations time in the model. In addition, as a closed compartment system there is no new supply of mediators or cells to the reaction in the tube. Still, the model can give us valuable information on the acute inflammatory process in whole blood. The whole blood model allows us to investigate and compare different activating agents on the acute inflammatory response of human whole blood. Although the exact mechanism behind the activation might be difficult to reveal in detail, by adding inhibitors of innate immune pathways, we can study the effect and redundancy of these different pathways.

In sepsis, history reveals a huge challenge in translating treatment strategies showing promise in the laboratory, to effective therapy in patients (124, 125). Administration of LPS (endotoxin) to animals, and even human volunteers (126), is a hallmark of the endotoxaemia model and has been extensively used as an animal sepsis model. This approach induces fever, increased heart rate and leucocytosis, all part of the former sepsis criteria, and release of pro-inflammatory mediators including TNF, IL-1β and IL-6, however, increasingly acknowledged not to reflect the pathophysiology of human sepsis (125, 127). Whole bacteria contain a diversity of PAMPs that will bind various pattern recognition molecules of the human innate immune system, in contrast to the pure TLR4 agonist LPS. In paper 1, we used LPS as well as whole Gram-negative and Gram-positive bacteria to elucidate whether the combination of different PAMPs on whole bacteria affects the mode of activation in our system compared to LPS alone. The Gram-negative bacteria *E. coli* contains LPS in addition to other PAMPs which activate both the TLR-system and to a lesser degree complement (65). The Gram-positive bacteria *S. aureus* is a highly effective complement activator in addition to activating certain TLRs (2 and 8) (128). All three activators induced monocyte activation and a pro-inflammatory cytokine response. Whole bacteria also induced granulocyte activation, which LPS did not in this fixed concentration. Not surprising, LPS activated whole blood in a purely TLR4-MD2-manner, while whole bacteria showed a broader activation pattern including complement and CD14-dependent pathways.

In sepsis research, inhibiting single downstream molecules like TNF and IL-1β was promising based on preliminary findings, however, clinical trials could not reproduce the effect (129, 130). In line with history, also the TLR4-MD2 antagonist eritoran failed in a phase three trial of sepsis patients (131). We added eritoran to our inhibitor panel to investigate the effect of inhibiting this single TLR4-MD2 pathway compared to the effect of the combined inhibition of complement and CD14. Our results reproduce the efficacy of eritoran in abrogating LPS-induced inflammation, and even effective in many *E. coli*-induced inflammatory readouts. However, eritoran did not affect the *S. aureus*-induced inflammatory readouts. Consequently, we concluded that although eritoran is efficient in abrogating LPS-induced inflammation, the effect fades when activation is induced by whole bacteria, especially Gram-positive. A variety of microbes can trigger sepsis, and several reports describe increasing incidence of Gram-positive and polymicrobial sepsis (132). Based on the findings from paper 1, one could expect that the effect of eritoran would be more pronounced in patient with Gram-negative sepsis, while an effect in Gram-positive sepsis is less likely. Importantly, the causative agent in sepsis is usually not known at the time of initiating treatment. This underscores the importance of targeting upstream and broad-acting pattern recognition systems when trying to abrogate the overwhelming immune dysregulation that develops in these patients.

The findings from paper 1 also underscore the importance of using whole bacteria when trying to dissect the acute inflammatory responses to a bacterial infection. LPS could not reproduce all the activating properties of *E. coli,* thus not fully representative of Gram-negative inflammation. We used heat inactivated bacteria in paper 1 and 2, thereby leaving out the colonizing and replicating aspect of bacterial infection. However, in this closed system with no new supply of mediators and cells, close to all the leukocytes have up-regulated CD11b within 20 min, thus replication and further increase of the bacteria number would not have altered the main findings. In addition, whole heat inactivated bacteria are equally potent activators of innate immune pathways as live bacteria. *E. coli* and *S. aureus* are the leading Gram-negative and Gram-positive causative agents in bloodstream infections, and thus highly relevant species for research (133). We propose the whole blood model as a readily available and easy

tool for screening and evaluating the effect of innate immune modulators on different activating agents.

A limitation of the whole blood model is the absence in this model of all the other cellular systems of the organism. Endothelial cells, which line the vasculature, control the transport of blood borne leukocytes into the tissues and organs and are acknowledged to play an important role in inflammation (134). Endothelial activation and dysfunction is evident in sepsis, trauma-induced systemic inflammation and ischemia-reperfusion injury (135-137). We therefore developed the whole blood model further to also include endothelial cells. This enabled us to study the interactions between whole blood and endothelial cells as well, which can give further insight into the effects of innate immune modulation. HUVECs are primary cells acquired from fresh umbilical veins of new-borns. Although not fully representative for all the diverse functions of endothelial cells in general, they are readily available, easy to culture and have been extensively studied. Importantly, and in contrast to microvascular endothelial cells, HUVECs do not display AB0 antigens and matching blood donors are therefore not a prerequisite. In paper 2, we showed how Gram-negative and Gram-positive bacteria activate complement and TLR-pathways in different manners, however in both settings leading to the release of TNF and IL-1β from leukocytes which subsequently activate the endothelial cells. These results link the inflammatory process in whole blood to endothelial cell activation. Inhibition of the two downstream cytokines efficiently abrogated endothelial cell activation, as did upstream inhibition with anti-CD14 and eritoran for *E. coli* and C5 and C5aR1-inhibition for *S. aureus*. In contrast, complement inhibition alone did not affect *E. coli*-induced endothelial cell activation while CD14 inhibition did not affect *S. aureus*-induced activation, again underscoring the differential recognition by PRMs of different PAMPs.

To summarize the conclusions from paper 1 and 2, targeting single pathways or single mediators could attenuate some responses depending on what activating agent was used. However, the immune response showed a clear redundancy where only a combined inhibitory approach was efficient when all responses by both Gram-negative and Gram-positive activation were viewed as a whole, especially combining complement and CD14 inhibition.

5.2.2 Porcine *in vivo* model and ischemia-reperfusion injury

The step up from *in vitro* to *in vivo* models resolves several limitations of the whole blood model, now including the presence of a whole organism including the continuous production, circulation and recruitment of inflammatory cells and mediators. The pig as a model animal for human disease has several advantages compared to the more readily available and commonly used rodents. The size of the animal enables us to use medical instruments developed for human use, the anatomy and physiology resembles that of humans and importantly the innate immune system of pigs share structural and functional similarities with humans (138). This is pointed out in a study concluding that the porcine immune system more closely resembles humans for >80% of analysed parameters, whereas mice were more similar to humans in <10% (139). However, now in a non-human system, the inhibitors used must cross react with porcine complement. Coversin is an equal efficient C5 inhibitor in pigs and humans (140), in contrast to eculizumab which is highly specific to human C5 and does not cross react with C5 of other species (141).

Our group has previously shown how combined inhibition of C5 and CD14 attenuates inflammation and hemodynamic changes in a bacteria-induced sepsis model in pigs (142). Now, we wished to study the effect of complement inhibition in a model of endogenous activation of innate immunity. The myocardial ischemia-reperfusion model represents a sterile inflammatory process in a part of the myocardium which in this setting is termed the area at risk. Animals receiving coversin infusion during ischemia and before reperfusion showed smaller infarct sizes and improved ventricular function compared to their saline controls. Coversin treatment significantly reduced C5b-9-deposition, E-selectin expression and IL-1β in the area at risk, suggesting an effect on the local inflammatory process induced by reperfusion. Results from a rat model of renal ischemia-reperfusion points to C5b-9 as an important mediator of the post-ischemic injury (143). However, in ischemia-reperfusion models of other organs and species, an important role of C5a is demonstrated (144, 145). By targeting C5, both effector systems are blocked.

In addition to blocking C5, the coversin molecule contains an internal binding pocket which captures leukotriene B4 (146). Leukotrienes are important multifunctional

mediators of inflammation and promote neutrophil chemotaxis (147). Interestingly, inhibition of phospholipase A2, followed by accompanying reduction in LTB4, reduced infarction size and improved ventricular function in a preclinical model of myocardial IR injury (148). The plasma concentrations of LTB4 showed a slight reduction by coversin treatment, however, the myocardial LTB4 concentrations were not affected. Consequently, the effect of LTB4-capture on the final inflammation and infarct size in our study is uncertain.

Complement inhibition with the C5-inhibitor pexelizumab during percutaneous coronary intervention (PCI) neither reduced myocardial infarction nor decreased mortality in the clinical phase three APEX-trial (149). However, concerns regarding sufficient complement inhibition were raised retrospectively as C5b-9 values increased similarly in treatment and placebo groups (150). A recent review of animal models, preclinical and clinical trials of complement inhibition in acute myocardial infarction underscores the importance of choosing suitable models including both ischemia and reperfusion, and selecting inhibitors which secure long enough therapeutic inhibition (104). The authors conclude that the complement complement, despite disappointing clinical trials, still is a promising intervention target.

TLRs, in particular TLR2 and 4 have also been studied as contributors to ischemia-reperfusion injury (151, 152). Further, based on evidence from bacteria-induced inflammation, addition of CD14-blockage might have the propensity to enhance the effect of complement inhibition. This was not addressed in paper 3, but is clearly of interest for further research.

A limitation of this model is the short observation time. We can therefore not draw conclusions of the long-term effects of the complement inhibition, including the effect on cardiac remodelling. However, a strength of the model design is that drug delivery was timed after the ischemic event, before reperfusion, mimicking a clinical setting in which the standard treatment of ST-elevation myocardial infarctions (transmural infarctions), namely reperfusion by PCI, can be preceded by drug administration. Today, these patients are diagnosed with prehospital electrocardiography and receive platelet inhibitors during the ambulance transport. This window is open for administering additional drugs targeting inflammation before in-hospital reperfusion.

Ischemia-reperfusion injury is evident in solid organ transplantation and is shown to increase the risk of reduced organ function and rejection (153). This is again a setting where inhibition of innate pathways readily can be translated to the clinic, either by perfusing the *ex vivo* organ with inhibitors of local inflammation, or treating the recipient prior to organ implantation or both (154).

5.3 Complement in antiphospholipid syndrome

The development of new treatment approaches from the lab bench via animal models to new therapy in human disease, is a long and winding road, and many new ideas meet a definite stop before clinical use. There is no doubt that basic science is of great importance for the understanding of the immunologic physiology and the pathophysiology of immune disease. However, a different approach to find new therapeutic strategies is to investigate the effect of established immune modulators in new diseases with resembling pathophysiology. Eculizumab was established for PNH and the safety of the drug evaluated (155). In aHUS, a thrombotic microangiopathy that predominantly affects the kidneys, dysfunctional complement regulation was determined to be an important underlying prerequisite for disease development. Accordingly, eculizumab proved as an effective treatment approach which has saved lives in a disease that formerly resulted in 25 % mortality and 50 % end stage renal disease (85, 156).

Catastrophic antiphospholipid syndrome emerges as a complement related disease, with systemic inflammation and thrombotic microangiopathy as hallmarks of the disease. Although the link to complement activation has not been proven as clear as in aHUS, complement inhibition has been suggested as a treatment option based on evidence of complement system contribution from *in vitro* and *in vivo* studies, and reports of effect from single cases (113). The crosstalk between complement and coagulation has been reported in several studies (157). Both cascade systems are based on serine proteases which cleave and activate the next step of the cascade while at the same time tightly regulated. Activated products of one system can even cleave products of the other, as shown for thrombin cleavage of C5 and MASP cleavage of fibrinogen, factor XIII and thrombin (158, 159). Further, studies have shown how

tissue factor, the main activator of the extrinsic pathway of coagulation, can be upregulated on endothelial cells and leukocytes by C5a (160, 161). The anticoagulant heparin, a cornerstone in the treatment of CAPS and the only treatment which is shown to significantly improve prognosis (162), also regulates complement activation, suggesting that the effect of heparin might also by related to the complement inhibiting properties (163).

In paper 4, a young pregnant woman with severe antiphospholipid syndrome was treated with eculizumab to safely prolong pregnancy and reduce the risk of developing catastrophic antiphospholipid syndrome (CAPS) in conjunction with caesarean section and puerperium. As a single case, the effect of eculizumab in preventing the development of CAPS can clearly not be proven. The incidence of CAPS is low, even in high risk settings. However, the case reveals how complement inhibition might evolve as a treatment option in clinical settings where complement is implied in the pathophysiology, and especially administered before a known triggering event.

5.4 Modulation of innate immune pathways – future perspectives

5.4.1 Treatment strategy

The treatment strategy clearly depends on the pathophysiology of the disease. To what degree the complement system plays an important part in the pathophysiology differs from disease to disease. PNH results from the lack of complement regulators on erythrocytes, resulting in complement dependent lysis – an example of a strictly complement mediated disease. At the other end of the scale are the inflammatory responses in ischemia-reperfusion injury and sepsis with activation of a broad innate immune response. Consequently, the mode of therapy would clearly differ in these different situations. The results from paper 1 and 2 show how combining upstream inhibition of complement and CD14 is more efficient in attenuating the complex inflammatory response, compared to inhibiting single pathways. It is tempting to suggest that this broad and upstream inhibitory approach could be beneficial in clinical settings where an overwhelming or dysregulated inflammatory response contributes

significantly to the disease pathophysiology, at least if administered early in the course of disease.

5.4.2 Targets and bottlenecks

In addition to determining the broadness of the inhibitory approach, also the level or target of inhibition is of importance. The longevity of the treatment will obviously influence the inhibitory target, due to the potential side effects of blocking important pathways of innate immunity. In the complement system, C5a and C5b-9, as described, mediate important effector functions in the inflammatory process. Independent of initiating pathway, inhibition of C3 or C5, abrogates these effector functions. Consequently, although C3 and C5 can be regarded as downstream in the complement cascade, they are still upstream in the inflammatory process. Experience from eculizumab now approaching a decade since clinical approval, suggests that inhibition of C5 even long-term can be considered safe, given *Neisseria* prophylaxis by vaccination or antibiotics.

Two concerns are raised regarding C3-inhibition. Firstly, C3-opsonisation is a crucial step in microbe resistance and mounting B and T cell responses, evident by the increased risk of infection in C3-deficient patients (74). Secondly, there is evidence of C5-activation bypassing C3 with direct cleavage of C5 by thrombin, at least in purified and plasma based systems as well as in C3-knockout mice (159, 164). The former concern is of less importance when considering short term inhibition. The latter might be merely a theoretical concern as the contribution of this C5-activation mode currently is not known *in vivo* in humans (159). As the anti-inflammatory properties of C3a are increasingly acknowledged, this may be an additional advantage in keeping this molecule active.

Recently, a concern regarding C5-blockage has been expressed, namely the possibility of residual C5-activity during strong complement activation despite C5-inhibition (165). The authors propose a mechanism by which C5 bound to either eculizumab or coversin can be recruited to C3b-clusters in the presence of high densities of C3b, and

subsequently cleaved. However, in the presence of two C5-inhibitors, or a regulator of complement activation at the C3-level, this mechanism is prevented.

Currently, eculizumab is the only complement inhibitor available for clinical use. However, a wide range of complement therapeutics are currently being tested in preclinical and clinical trials, targeting proteins of the initiating pathways, convertases, C3, C5 and C5aR1 to mention some, with a range of indications (166). New inhibitors with slightly different targets will provide better opportunity to target complement inhibition to the disease-causing step of the cascade in the wide range of complement-mediated diseases. Still C3, and even more C5, emerges as promising complement bottleneck targets in acute systemic inflammation.

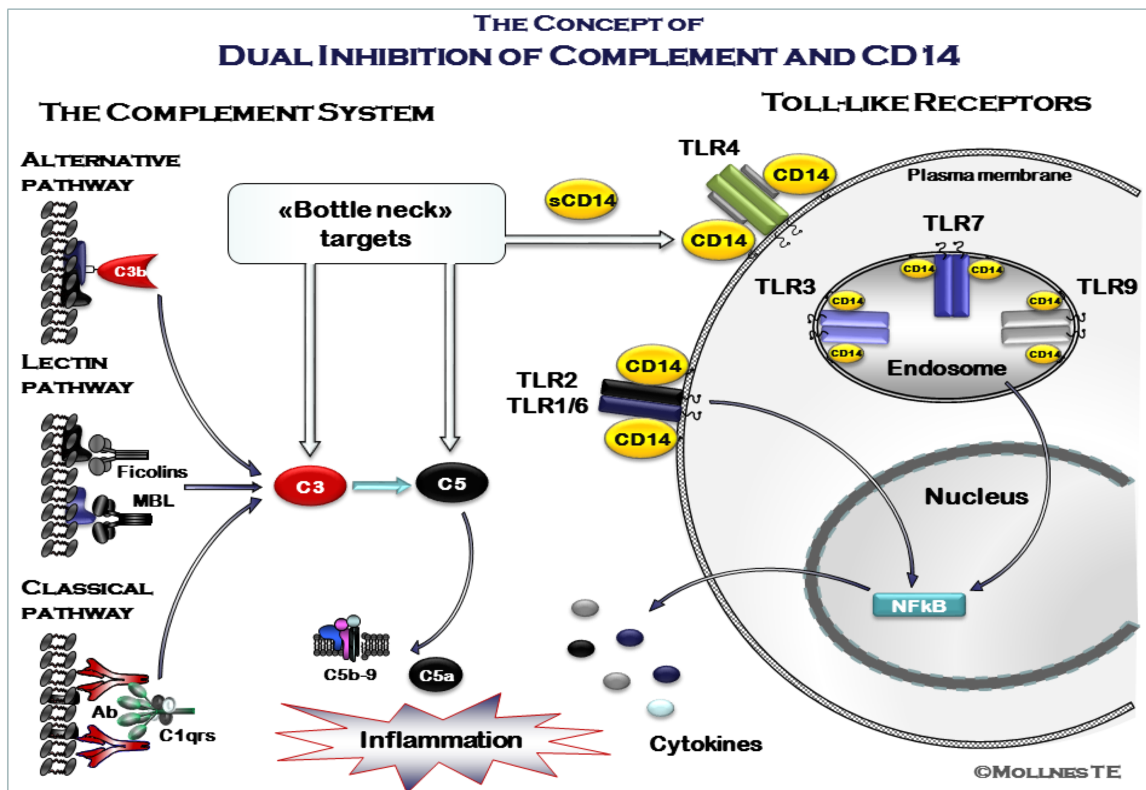

Figure 2: Dual inhibition of complement and CD14

As previously mentioned, the TLR-system is built up in a different manner than the complement system and consists of a variety of receptors which activate different intracellular signalling pathways. By blocking the common co-receptor CD14, several TLRs are blocked at the recognition phase. As evident from paper 1, this results in

broader inhibitory potential than solely inhibiting one TLR. This is important when the inflammatory process is induced by a plethora of PAMPs and DAMPs, which have the potency to activate several TLRs, as in a polymicrobial infection. As already mentioned, both TLR2 and 4 are implied in ischemia-reperfusion injury, and further research might include additional TLRs. Hence, CD14 emerges as a bottleneck target of the TLR-system.

5.4.3 Timing the intervention

One of the great challenges in developing new treatment strategies in sepsis, is providing drugs targeting the dysregulated inflammatory process at an early enough time point. This aspect is somewhat different in ischemia-reperfusion injury, where reperfusion often is a result of in-hospital treatment, leaving a window of opportunity for inflammation-targeted therapy open. As already discussed, both myocardial infarctions and solid organ transplantation are clinical settings with optimistic translational value, where inhibitors can be provided before reperfusion. In some conditions, certain events can trigger the development of a pathologic process. CAPS triggered by surgery, as was feared in the patient in paper 4, is a setting where the prophylactic use of an inhibitor is feasible. The inflammatory process can be attenuated by administrating inhibitors later in the time course of the inflammatory reaction. This was shown for the whole blood model where inhibitors of C3 and CD14 were added 5, 15 or 30 minutes after the activating agent *E. coli* (167). This post-challenge inhibition attenuated the cytokine response and C5b-9 levels. Further, the sC5b-9 levels declined and clinical condition improved in a patient with CAPS treated with eculizumab (114). However, preventing the pathologic process, when possible, is even more appealing.

5.4.4 Inhibitor design

An extra consideration was evident in paper 4, namely how the intervention would affect the baby in a pregnant patient. As previously mentioned, complement is implicated in development, and simultaneous inhibition of the unborn baby’s complement system was not desired. Some evidence already exists on the safety of

eculizumab (168), and we showed in paper 4 that only trace amounts passed the placenta in gestational week 33. This results from the IgG2/4 chimer construct of the drug, which reduces the binding capacity to Fcγ-receptors (169). Thus, the construct or design of the inhibitory agent is of importance when assessing the clinical application. The inhibiting CD14-antibody used in paper 1 and 2 is constructed in the same manner as eculizumab, with a IgG2/4 chimer construct, consequently with similar properties. In contrast, coversin as used in paper 3, is a small peptide of 16.8 kDa. This peptide is therefore cheaper to produce in large scale, but has a short plasma half-life if not bound to C5; therefore, the drug requires frequent dosing to maintain C5-inhibition (170). A modified version of coversin, PAS-coversin, however, shows increased non-C5 bound plasma half-life (165). Eculizumab and coversin bind C5 at different sites. This difference is of clinical relevance in a few patients with mutations in C5 which prevents binding of eculizumab, and consequently are resistant to this drug (171). Here, coversin or other C5 inhibitors targeting different parts of the C5 molecule would be expected to be effective.

Another factor important for the structure of the inhibitor, is whether the complement activation intended to inhibit occurs systemically or locally. In paper 3, the inflammatory process was located to the myocardium. Consequently, the complement inhibitor must penetrate the tissue to act on any locally produced complement products. Strategies of delivering complement inhibition to the site of inflammation are being tested, thereby reducing potential side effects. This includes local application of complement inhibitors as intravitreal in the retinal disease age-related macular degeneration (172), or modifying drugs like the CR2-FH hybrid TT30 or mini-factor H. TT30 is a fusion protein combining the iC3b/C3dg binding part of CR2 with the complement regulating part of FH (CCP 1-5) (173). Mini-factor H consists of CCP 1-4 and 19-20 of factor H, keeping both the complement regulating part and the C3b/iC3b/C3dg binding part (174). Both constructs enable the drug to target a site with ongoing complement activation and at the same time regulate the alternative pathway by C3 convertase decay and cofactor activity for factor I-mediated cleavage of C4b and C3b (175).

5.4.5 Monitoring the intervention

A major concern regarding the disappointing clinical trials of myocardial infarction where the C5-inhibitor pexelizumab was administered during PCI, was that sC5b-9 increased also in the intervention group. This suggests an incomplete blockage of complement activation and thus precludes the findings of the study. In PNH-patients treated with eculizumab, some patients experienced incomplete complement inhibition and breakthrough haemolysis requiring higher doses of eculizumab or shortened dosing intervals (176). In paper 4, we surprisingly found that the patient's complement activity recovered considerably faster than anticipated. These observations underscore the importance of monitoring complement activity in *in vivo* experimental studies and in clinical trials as well as in patient treatment. However, as described in two small studies of patients treated with eculizumab for complement-mediated renal diseases, eculizumab accumulation is evident for some patients (177, 178). The current guidelines on eculizumab treatment instruct the same dosing regimen for all adults ≥ 18 years of age. A registration of annual treatment costs revealed that eculizumab was the second most expensive treatment per year in Norway in 2012 (179). Consequently, in addition to risk of relapse and breakthrough symptoms with too low dosing regimens and the risk of side effects with drug over-exposure, there is also an economic incentive for individualizing dosing regimens, thereby increasing drug intervals or reducing the dose in selected patients.

Several strategies are available for monitoring eculizumab treatment, including analysing complement function by the classical haemolytic assays (CH50 and AH50) or the functional complement ELISA (Wieslab Complement system Screen COMPL 300), monitoring complement activation products , monitoring excess or through levels of eculizumab by different ELISA assays (177, 180, 181) or more disease specific assays as the proposed endothelial cell-based C5b-9 deposition assay to monitor complement activity in aHUS patients (182). Importantly, paper 4 revealed how complement assays must be carefully interpreted, in order to draw the correct conclusions. The functional complement activity ELISA demonstrated less than 1% activity in all pathways in the infant serum. However, this was not a result of C5-inhibition by eculizumab, but low levels of several complement factors in the premature infant. There is still no clear consensus on the ideal monitoring strategy for eculizumab treatment in different

diseases. However, aiming on individualized therapy taken into account the individual patient characteristics, the ongoing or threatening disease process and optimising the drug target, dosing and monitoring, complement therapy could be increasingly valuable for a larger number of patients in the future.

6 Conclusion

Complement activation obviously contributes to the inflammatory processes in infectious and sterile inflammation, which is can be detrimental when the activation is overwhelming or dysregulated. The contribution of complement varies according to the disease process and there is a substantial cross talk and redundancy in the innate immune system, especially between complement and TLRs. This implies that a combined inhibitory strategy might be superior to that of inhibiting selective pathways when a broad range of PAMPs and DAMPs are responsible for the induction of inflammation. The timing of inhibition i.e. before versus after the insult can be of importance regarding the potential effect. In some clinical settings there is a window of opportunity where inhibitors targeting innate immune pathways might be administered before the event i.e. before reperfusion in myocardial infarction and solid organ transplantations and before triggering events of CAPS in patients with antiphospholipid syndrome. Monitoring the effect of complement inhibition is crucial, to prevent residual complement activation which might hamper the treatment effect.

7 References

1. Danilova N. The evolution of immune mechanisms. Journal of experimental zoology Part B, Molecular and developmental evolution. 2006;306(6):496-520.
2. Hamon MA, Quintin J. Innate immune memory in mammals. Semin Immunol. 2016.
3. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol. 1994;12:991-1045.
4. Medzhitov R, Janeway CA, Jr. Decoding the patterns of self and nonself by the innate immune system. Science. 2002;296(5566):298-300.
5. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007;81(1):1-5.
6. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-20.
7. Morgan BP, Gasque P. Extrahepatic complement biosynthesis: where, when and why? Clin Exp Immunol. 1997;107(1):1-7.
8. Wirthmueller U, Dewald B, Thelen M, Schafer MK, Stover C, Whaley K, et al. Properdin, a positive regulator of complement activation, is released from secondary granules of stimulated peripheral blood neutrophils. J Immunol. 1997;158(9):4444-51.
9. White RT, Damm D, Hancock N, Rosen BS, Lowell BB, Usher P, et al. Human adipsin is identical to complement factor D and is expressed at high levels in adipose tissue. J Biol Chem. 1992;267(13):9210-3.
10. Wurzner R, Joysey VC, Lachmann PJ. Complement component C7. Assessment of in vivo synthesis after liver transplantation reveals that hepatocytes do not synthesize the majority of human C7. J Immunol. 1994;152(9):4624-9.
11. Laufer J, Katz Y, Passwell JH. Extrahepatic synthesis of complement proteins in inflammation. Mol Immunol. 2001;38(2–3):221-9.
12. Freeley S, Kemper C, Le Friec G. The "ins and outs" of complement-driven immune responses. Immunol Rev. 2016;274(1):16-32.
13. Diebolder CA, Beurskens FJ, de Jong RN, Koning RI, Strumane K, Lindorfer MA, et al. Complement is activated by IgG hexamers assembled at the cell surface. Science. 2014;343(6176):1260-3.

14. Martin M, Leffler J, Blom AM. Annexin A2 and A5 serve as new ligands for C1q on apoptotic cells. J Biol Chem. 2012;287(40):33733-44.
15. Garred P, Genster N, Pilely K, Bayarri-Olmos R, Rosbjerg A, Ma YJ, et al. A journey through the lectin pathway of complement-MBL and beyond. Immunol Rev. 2016;274(1):74-97.
16. Martin M, Blom AM. Complement in removal of the dead - balancing inflammation. Immunol Rev. 2016;274(1):218-32.
17. Brinkmann CR, Jensen L, Dagnaes-Hansen F, Holm IE, Endo Y, Fujita T, et al. Mitochondria and the lectin pathway of complement. J Biol Chem. 2013;288(12):8016-27.
18. Jensen ML, Honore C, Hummelshoj T, Hansen BE, Madsen HO, Garred P. Ficolin-2 recognizes DNA and participates in the clearance of dying host cells. Mol Immunol. 2007;44(5):856-65.
19. Nilsson B, Nilsson Ekdahl K. The tick-over theory revisited: is C3 a contact-activated protein? Immunobiology. 2012;217(11):1106-10.
20. Harboe M, Johnson C, Nymo S, Ekholt K, Schjalm C, Lindstad JK, et al. Properdin binding to complement activating surfaces depends on initial C3b deposition. Proc Natl Acad Sci U S A. 2017.
21. Harboe M, Ulvund G, Vien L, Fung M, Mollnes TE. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. Clin Exp Immunol. 2004;138(3):439-46.
22. Law SK, Minich TM, Levine RP. Binding reaction between the third human complement protein and small molecules. Biochemistry. 1981;20(26):7457-63.
23. Jore MM, Johnson S, Sheppard D, Barber NM, Li YI, Nunn MA, et al. Structural basis for therapeutic inhibition of complement C5. Nat Struct Mol Biol. 2016;23(5):378-86.
24. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Kohl J. The role of the anaphylatoxins in health and disease. Mol Immunol. 2009;46(14):2753-66.
25. Li R, Coulthard LG, Wu MC, Taylor SM, Woodruff TM. C5L2: a controversial receptor of complement anaphylatoxin, C5a. FASEB J. 2013;27(3):855-64.
26. Coulthard LG, Woodruff TM. Is the Complement Activation Product C3a a Proinflammatory Molecule? Re-evaluating the Evidence and the Myth. J Immunol. 2015;194(8):3542-8.

27. Wu MC, Brennan FH, Lynch JP, Mantovani S, Phipps S, Wetsel RA, et al. The receptor for complement component C3a mediates protection from intestinal ischemia-reperfusion injuries by inhibiting neutrophil mobilization. Proc Natl Acad Sci U S A. 2013;110(23):9439-44.
28. Guo RF, Ward PA. Role of C5a in inflammatory responses. Annu Rev Immunol. 2005;23:821-52.
29. Matthews KW, Mueller-Ortiz SL, Wetsel RA. Carboxypeptidase N: a pleiotropic regulator of inflammation. Mol Immunol. 2004;40(11):785-93.
30. Morgan BP, Walters D, Serna M, Bubeck D. Terminal complexes of the complement system: new structural insights and their relevance to function. Immunol Rev. 2016;274(1):141-51.
31. Triantafilou K, Hughes TR, Triantafilou M, Morgan BP. The complement membrane attack complex triggers intracellular Ca2+ fluxes leading to NLRP3 inflammasome activation. J Cell Sci. 2013;126(Pt 13):2903-13.
32. Morgan BP. The membrane attack complex as an inflammatory trigger. Immunobiology. 2016;221(6):747-51.
33. Davis AE, 3rd, Lu F, Mejia P. C1 inhibitor, a multi-functional serine protease inhibitor. Thromb Haemost. 2010;104(5):886-93.
34. Zuraw BL, Christiansen SC. HAE Pathophysiology and Underlying Mechanisms. Clin Rev Allergy Immunol. 2016;51(2):216-29.
35. Roversi P, Johnson S, Caesar JJ, McLean F, Leath KJ, Tsiftsoglou SA, et al. Structural basis for complement factor I control and its disease-associated sequence polymorphisms. Proc Natl Acad Sci U S A. 2011;108(31):12839-44.
36. Ermert D, Blom AM. C4b-binding protein: The good, the bad and the deadly. Novel functions of an old friend. Immunol Lett. 2016;169:82-92.
37. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. Nat Rev Immunol. 2009;9(10):729-40.
38. Makou E, Herbert AP, Barlow PN. Functional anatomy of complement factor H. Biochemistry. 2013;52(23):3949-62.
39. Hyvarinen S, Meri S, Jokiranta TS. Disturbed sialic acid recognition on endothelial cells and platelets in complement attack causes atypical hemolytic uremic syndrome. Blood. 2016;127(22):2701-10.

40. Medjeral-Thomas N, Pickering MC. The complement factor H-related proteins. Immunol Rev. 2016;274(1):191-201.
41. Dustin ML. Complement Receptors in Myeloid Cell Adhesion and Phagocytosis. Microbiology spectrum. 2016;4(6).
42. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. Front Immunol. 2015;6:262.
43. de Back D, Kostova E, van Kraaij M, van den Berg T, Van Bruggen R. Of macrophages and red blood cells; a complex love story. Front Physiol. 2014;5(9).
44. Lambris JD, Lao Z, Oglesby TJ, Atkinson JP, Hack CE, Becherer JD. Dissection of CR1, factor H, membrane cofactor protein, and factor B binding and functional sites in the third complement component. J Immunol. 1996;156(12):4821-32.
45. Wiesmann C, Katschke KJ, Yin J, Helmy KY, Steffek M, Fairbrother WJ, et al. Structure of C3b in complex with CRIg gives insights into regulation of complement activation. Nature. 2006;444(7116):217-20.
46. Heesters BA, van der Poel CE, Das A, Carroll MC. Antigen Presentation to B Cells. Trends Immunol. 2016;37(12):844-54.
47. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11(5):373-84.
48. Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med. 1999;189(4):615-25.
49. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282(5396):2085-8.
50. Wang Y, Su L, Morin MD, Jones BT, Whitby LR, Surakattula MM, et al. TLR4/MD-2 activation by a synthetic agonist with no similarity to LPS. Proc Natl Acad Sci U S A. 2016;113(7):E884-93.
51. Molteni M, Gemma S, Rossetti C. The Role of Toll-Like Receptor 4 in Infectious and Noninfectious Inflammation. Mediators Inflamm. 2016;2016:6978936.
52. Jersmann HP. Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells. Immunol Cell Biol. 2005;83(5):462-7.

53. Di Gioia M, Zanoni I. Toll-like receptor co-receptors as master regulators of the immune response. Mol Immunol. 2015;63(2):143-52.
54. Lee CC, Avalos AM, Ploegh HL. Accessory molecules for Toll-like receptors and their function. Nat Rev Immunol. 2012;12(3):168-79.
55. Zanoni I, Granucci F. Role of CD14 in host protection against infections and in metabolism regulation. Frontiers in cellular and infection microbiology. 2013;3:32.
56. Lee HK, Dunzendorfer S, Soldau K, Tobias PS. Double-stranded RNA-mediated TLR3 activation is enhanced by CD14. Immunity. 2006;24(2):153-63.
57. Baumann CL, Aspalter IM, Sharif O, Pichlmair A, Bluml S, Grebien F, et al. CD14 is a coreceptor of Toll-like receptors 7 and 9. J Exp Med. 2010;207(12):2689-701.
58. Weber C, Muller C, Podszuweit A, Montino C, Vollmer J, Forsbach A. Toll-like receptor (TLR) 3 immune modulation by unformulated small interfering RNA or DNA and the role of CD14 (in TLR-mediated effects). Immunology. 2012;136(1):64-77.
59. Zanoni I, Ostuni R, Capuano G, Collini M, Caccia M, Ronchi AE, et al. CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. Nature. 2009;460(7252):264-8.
60. Kim J-I, Lee CJ, Jin MS, Lee C-H, Paik S-G, Lee H, et al. Crystal Structure of CD14 and Its Implications for Lipopolysaccharide Signaling. J Biol Chem. 2005;280(12):11347-51.
61. Lloyd-Jones KL, Kelly MM, Kubes P. Varying importance of soluble and membrane CD14 in endothelial detection of lipopolysaccharide. J Immunol. 2008;181(2):1446-53.
62. Brekke OL, Christiansen D, Fure H, Fung M, Mollnes TE. The role of complement C3 opsonization, C5a receptor, and CD14 in E. coli-induced up-regulation of granulocyte and monocyte CD11b/CD18 (CR3), phagocytosis, and oxidative burst in human whole blood. J Leukoc Biol. 2007;81(6):1404-13.
63. Raby AC, Holst B, Davies J, Colmont C, Laumonnier Y, Coles B, et al. TLR activation enhances C5a-induced pro-inflammatory responses by negatively modulating the second C5a receptor, C5L2. Eur J Immunol. 2011;41(9):2741-52.
64. Zhang X, Kimura Y, Fang C, Zhou L, Sfyroera G, Lambris JD, et al. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. Blood. 2007;110(1):228-36.

65. Lappegard KT, Christiansen D, Pharo A, Thorgersen EB, Hellerud BC, Lindstad J, et al. Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. Proc Natl Acad Sci U S A. 2009;106(37):15861-6.
66. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. Front Immunol. 2015;6:257.
67. Carroll MC, Isenman DE. Regulation of humoral immunity by complement. Immunity. 2012;37(2):199-207.
68. Scott D, Botto M. The paradoxical roles of C1q and C3 in autoimmunity. Immunobiology. 2016;221(6):719-25.
69. Pekkarinen PT, Heikkila N, Kisand K, Peterson P, Botto M, Daha MR, et al. Dysregulation of adaptive immune responses in complement C3-deficient patients. Eur J Immunol. 2015;45(3):915-21.
70. Liszewski MK, Kolev M, Le Friec G, Leung M, Bertram PG, Fara AF, et al. Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. Immunity. 2013;39(6):1143-57.
71. Arbore G, Kemper C. A novel "complement-metabolism-inflammasome axis" as a key regulator of immune cell effector function. Eur J Immunol. 2016;46(7):1563-73.
72. Hansen SW, Ohtani K, Roy N, Wakamiya N. The collectins CL-L1, CL-K1 and CL-P1, and their roles in complement and innate immunity. Immunobiology. 2016;221(10):1058-67.
73. Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, et al. The classical complement cascade mediates CNS synapse elimination. Cell. 2007;131(6):1164-78.
74. Botto M, Kirschfink M, Macor P, Pickering MC, Wurzner R, Tedesco F. Complement in human diseases: Lessons from complement deficiencies. Mol Immunol. 2009;46(14):2774-83.
75. Harris CL, Heurich M, Cordoba SRd, Morgan BP. The complotype: dictating risk for inflammation and infection. Trends Immunol. 2012;33(10):513-21.
76. Skattum L, van Deuren M, van der Poll T, Truedsson L. Complement deficiency states and associated infections. Mol Immunol. 2011;48(14):1643-55.

77. Sumiya M, Tabona P, Arai T, Summerfield JA, Super M, Levinsky RJ, et al. Molecular basis of opsonic defect in immunodeficient children. The Lancet. 1991;337(8757):1569-70.
78. Garred P, Madsen HO, Hofmann B, Svejgaard A. Increased frequency of homozygosity of abnormal mannan-binding-protein alleles in patients with suspected immunodeficiency. Lancet. 1995;346(8980):941-3.
79. Radnay ZB, Udvardy M, Papp M, Harsfalvi J, Rejto L, Pal I, et al. Evaluation of Mannose-Binding Lectin is a Useful Approach to Predict the Risk of Infectious Complications Following Autologous Hematopoietic Stem Cell Transplantation. Transplant Proc. 2016;48(10):3397-405.
80. Chalmers JD, Fleming GB, Hill AT, Kilpatrick DC. Impact of mannose-binding lectin insufficiency on the course of cystic fibrosis: A review and meta-analysis. Glycobiology. 2011;21(3):271-82.
81. Fevang B, Mollnes TE, Holm AM, Ueland T, Heggelund L, Damas JK, et al. Common variable immunodeficiency and the complement system; low mannose-binding lectin levels are associated with bronchiectasis. Clin Exp Immunol. 2005;142(3):576-84.
82. Cook HT, Botto M. Mechanisms of Disease: the complement system and the pathogenesis of systemic lupus erythematosus. Nat Clin Pract Rheumatol. 2006;2(6):330-7.
83. Ricklin D, Reis ES, Lambris JD. Complement in disease: a defence system turning offensive. Nature reviews Nephrology. 2016;12(7):383-401.
84. Nester CM, Barbour T, de Cordoba SR, Dragon-Durey MA, Fremeaux-Bacchi V, Goodship TH, et al. Atypical aHUS: State of the art. Mol Immunol. 2015;67(1):31-42.
85. Waters AM, Licht C. aHUS caused by complement dysregulation: new therapies on the horizon. Pediatr Nephrol. 2011;26(1):41-57.
86. Brodsky RA. Paroxysmal nocturnal hemoglobinuria. Blood. 2014;124(18):2804-11.
87. Daha MR. Role of complement in innate immunity and infections. Crit Rev Immunol. 2010;30(1):47-52.
88. Laarman A, Milder F, van Strijp J, Rooijakkers S. Complement inhibition by gram-positive pathogens: molecular mechanisms and therapeutic implications. J Mol Med (Berl). 2010;88(2):115-20.

89. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016;315(8):801-10.
90. Fleischmann C, Scherag A, Adhikari NK, Hartog CS, Tsaganos T, Schlattmann P, et al. Assessment of Global Incidence and Mortality of Hospital-treated Sepsis. Current Estimates and Limitations. Am J Respir Crit Care Med. 2016;193(3):259-72.
91. Angus DC, van der Poll T. Severe sepsis and septic shock. N Engl J Med. 2013;369(9):840-51.
92. Unnewehr H, Rittirsch D, Sarma JV, Zetoune F, Flierl MA, Perl M, et al. Changes and regulation of the C5a receptor on neutrophils during septic shock in humans. J Immunol. 2013;190(8):4215-25.
93. Ward PA. New approaches to the study of sepsis. EMBO Mol Med. 2012;4(12):1234-43.
94. Levi M, van der Poll T. Coagulation and sepsis. Thromb Res. 2016;149:38-44.
95. Bosmann M, Ward PA. The inflammatory response in sepsis. Trends Immunol. 2013;34(3):129-36.
96. Ibáñez B, Heusch G, Ovize M, Van de Werf F. Evolving Therapies for Myocardial Ischemia/Reperfusion Injury. J Am Coll Cardiol. 2015;65(14):1454-71.
97. Eltzschig HK, Eckle T. Ischemia and reperfusion--from mechanism to translation. Nat Med. 2011;17(11):1391-401.
98. Hill JH, Ward PA. The phlogistic role of C3 leukotactic fragments in myocardial infarcts of rats. J Exp Med. 1971;133(4):885-900.
99. Montero RM, Sacks SH, Smith RA. Complement-here, there and everywhere, but what about the transplanted organ? Semin Immunol. 2016;28(3):250-9.
100. Gorsuch WB, Chrysanthou E, Schwaeble WJ, Stahl GL. The complement system in ischemia–reperfusion injuries. Immunobiology. 2012;217(11):1026-33.
101. Zhang M, Carroll MC. Natural antibody mediated innate autoimmune response. Mol Immunol. 2007;44(1-3):103-10.
102. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc Res. 2004;61(3):481-97.
103. Liu J, Wang H, Li J. Inflammation and Inflammatory Cells in Myocardial Infarction and Reperfusion Injury: A Double-Edged Sword. Clin Med Insights Cardiol. 2016;10:79-84.

104. Emmens RW, Wouters D, Zeerleder S, van Ham SM, Niessen HW, Krijnen PA. On the value of therapeutic interventions targeting the complement system in acute myocardial infarction. Transl Res. 2016.

105. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4(2):295-306.

106. Chighizola CB, Raschi E, Borghi MO, Meroni PL. Update on the pathogenesis and treatment of the antiphospholipid syndrome. Curr Opin Rheumatol. 2015;27(5):476-82.

107. Romay-Penabad Z, Carrera Marin AL, Willis R, Weston-Davies W, Machin S, Cohen H, et al. Complement C5-inhibitor rEV576 (coversin) ameliorates in-vivo effects of antiphospholipid antibodies. Lupus. 2014;23(12):1324-6.

108. Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS, et al. Complement C3 Activation Is Required for Antiphospholipid Antibody-induced Fetal Loss. The Journal of Experimental Medicine. 2002;195(2):211-20.

109. Agostinis C, Durigutto P, Sblattero D, Borghi MO, Grossi C, Guida F, et al. A non-complement-fixing antibody to beta2 glycoprotein I as a novel therapy for antiphospholipid syndrome. Blood. 2014;123(22):3478-87.

110. Oku K, Atsumi T, Bohgaki M, Amengual O, Kataoka H, Horita T, et al. Complement activation in patients with primary antiphospholipid syndrome. Ann Rheum Dis. 2009;68(6):1030-5.

111. Cervera R, Font J, Gomez-Puerta JA, Espinosa G, Cucho M, Bucciarelli S, et al. Validation of the preliminary criteria for the classification of catastrophic antiphospholipid syndrome. Ann Rheum Dis. 2005;64(8):1205-9.

112. Rodriguez-Pinto I, Espinosa G, Cervera R. Catastrophic antiphospholipid syndrome: The current management approach. Best Pract Res Clin Rheumatol. 2016;30(2):239-49.

113. Rodriguez-Pintó I, Espinosa G, Cervera R. Catastrophic antiphospholipid syndrome: The current management approach. Best Practice & Research Clinical Rheumatology. 2016;30(2):239-49.

114. Barratt-Due A, Floisand Y, Orrem HL, Kvam AK, Holme PA, Bergseth G, et al. Complement activation is a crucial pathogenic factor in catastrophic antiphospholipid syndrome. Rheumatology (Oxford). 2016;55(7):1337-9.

115. Thurman JM, Le Quintrec M. Targeting the complement cascade: novel treatments coming down the pike. Kidney Int. 2016.
116. Lau C, Gunnarsen KS, Hoydahl LS, Andersen JT, Berntzen G, Pharo A, et al. Chimeric anti-CD14 IGG2/4 Hybrid antibodies for therapeutic intervention in pig and human models of inflammation. J Immunol. 2013;191(9):4769-77.
117. Mollnes TE, Brekke OL, Fung M, Fure H, Christiansen D, Bergseth G, et al. 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. 2002;100(5):1869-77.
118. Xiao L, Liu Y, Wang N. New paradigms in inflammatory signaling in vascular endothelial cells. Am J Physiol Heart Circ Physiol. 2014;306(3):H317-25.
119. Hedstrom E, Engblom H, Frogner F, Astrom-Olsson K, Ohlin H, Jovinge S, et al. Infarct evolution in man studied in patients with first-time coronary occlusion in comparison to different species - implications for assessment of myocardial salvage. J Cardiovasc Magn Reson. 2009;11:38.
120. Barratt-Due A, Pischke SE, Nilsson PH, Espevik T, Mollnes TE. 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. 2016.
121. Xiao W, Mindrinos MN, Seok J, Cuschieri J, Cuenca AG, Gao H, et al. A genomic storm in critically injured humans. J Exp Med. 2011;208(13):2581-90.
122. Thaiss CA, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. Nature. 2016;535(7610):65-74.
123. Logue GL. Effect of heparin on complement activation and lysis of paroxysmal nocturnal hemoglobinuria (PNH) red cells. Blood. 1977;50(2):239-47.
124. Dyson A, Singer M. Animal models of sepsis: why does preclinical efficacy fail to translate to the clinical setting? Crit Care Med. 2009;37(1 Suppl):S30-7.
125. Kingsley SM, Bhat BV. Differential Paradigms in Animal Models of Sepsis. Curr Infect Dis Rep. 2016;18(9):26.
126. Martich GD, Boujoukos AJ, Suffredini AF. Response of man to endotoxin. Immunobiology. 1993;187(3-5):403-16.
127. Anel R, Kumar A. Human endotoxemia and human sepsis: limits to the model. Crit Care. 2005;9(2):151-2.

128. Skjeflo EW, Christiansen D, Espevik T, Nielsen EW, Mollnes TE. Combined inhibition of complement and CD14 efficiently attenuated the inflammatory response induced by Staphylococcus aureus in a human whole blood model. J Immunol. 2014;192(6):2857-64.
129. Abraham E, Laterre PF, Garbino J, Pingleton S, Butler T, Dugernier T, et al. Lenercept (p55 tumor necrosis factor receptor fusion protein) in severe sepsis and early septic shock: a randomized, double-blind, placebo-controlled, multicenter phase III trial with 1,342 patients. Crit Care Med. 2001;29(3):503-10.
130. Opal SM, Fisher CJ, Jr., Dhainaut JF, Vincent JL, Brase R, Lowry SF, et al. Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group. Crit Care Med. 1997;25(7):1115-24.
131. Opal SM, Laterre PF, Francois B, LaRosa SP, Angus DC, Mira JP, et al. Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial. JAMA. 2013;309(11):1154-62.
132. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med. 2003;348(16):1546-54.
133. Luzzaro F, Ortisi G, Larosa M, Drago M, Brigante G, Gesu G. Prevalence and epidemiology of microbial pathogens causing bloodstream infections: results of the OASIS multicenter study. Diagn Microbiol Infect Dis. 2011;69(4):363-9.
134. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol. 2007;7(10):803-15.
135. Opal SM, van der Poll T. Endothelial barrier dysfunction in septic shock. J Intern Med. 2015;277(3):277-93.
136. Lord JM, Midwinter MJ, Chen YF, Belli A, Brohi K, Kovacs EJ, et al. The systemic immune response to trauma: an overview of pathophysiology and treatment. Lancet. 2014;384(9952):1455-65.
137. Singhal AK, Symons JD, Boudina S, Jaishy B, Shiu YT. Role of Endothelial Cells in Myocardial Ischemia-Reperfusion Injury. Vasc Dis Prev. 2010;7:1-14.
138. Mair KH, Sedlak C, Kaser T, Pasternak A, Levast B, Gerner W, et al. The porcine innate immune system: an update. Dev Comp Immunol. 2014;45(2):321-43.
139. Dawson HD. Comparative assessment of the pig, mouse, and human genomes: A structural and functional analysis of genes involved in immunity. In: McAnulty PA,

Dayan, A., Hastings, K.H., Ganderup, N.-C., editor. The Minipig in Biomedical Research. Boca Raton, FL: CRC Press.2011. p. 321-41.
140. Barratt-Due A, Thorgersen EB, Lindstad JK, Pharo A, Lissina O, Lambris JD, et al. Ornithodoros moubata complement inhibitor is an equally effective C5 inhibitor in pigs and humans. J Immunol. 2011;187(9):4913-9.
141. Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. Nat Biotechnol. 2007;25(11):1256-64.
142. Barratt-Due A, Thorgersen EB, Egge K, Pischke S, Sokolov A, Hellerud BC, et al. Combined inhibition of complement C5 and CD14 markedly attenuates inflammation, thrombogenicity, and hemodynamic changes in porcine sepsis. J Immunol. 2013;191(2):819-27.
143. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y, et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. The Journal of Clinical Investigation. 2000;105(10):1363-71.
144. Heller T, Hennecke M, Baumann U, Gessner JE, Meyer zu Vilsendorf A, Baensch M, et al. Selection of a C5a Receptor Antagonist from Phage Libraries Attenuating the Inflammatory Response in Immune Complex Disease and Ischemia/Reperfusion Injury. The Journal of Immunology. 1999;163(2):985-94.
145. De Hoog VC, Timmers L, Van Duijvenvoorde A, De Jager SC, Van Middelaar BJ, Smeets MB, et al. Leucocyte expression of complement C5a receptors exacerbates infarct size after myocardial reperfusion injury. Cardiovasc Res. 2014;103(4):521-9.
146. Roversi P, Ryffel B, Togbe D, Maillet I, Teixeira M, Ahmat N, et al. Bifunctional lipocalin ameliorates murine immune complex-induced acute lung injury. J Biol Chem. 2013;288(26):18789-802.
147. Le Bel M, Brunet A, Gosselin J. Leukotriene B4, an endogenous stimulator of the innate immune response against pathogens. J Innate Immun. 2014;6(2):159-68.
148. Yano T, Fujioka D, Saito Y, Kobayashi T, Nakamura T, Obata JE, et al. Group V secretory phospholipase A2 plays a pathogenic role in myocardial ischaemia-reperfusion injury. Cardiovasc Res. 2011;90(2):335-43.
149. Investigators AA, Armstrong PW, Granger CB, Adams PX, Hamm C, Holmes D, Jr., et al. Pexelizumab for acute ST-elevation myocardial infarction in patients

undergoing primary percutaneous coronary intervention: a randomized controlled trial. JAMA. 2007;297(1):43-51.

150. Martel C, Granger CB, Ghitescu M, Stebbins A, Fortier A, Armstrong PW, et al. Pexelizumab fails to inhibit assembly of the terminal complement complex in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention. Insight from a substudy of the Assessment of Pexelizumab in Acute Myocardial Infarction (APEX-AMI) trial. Am Heart J. 2012;164(1):43-51.

151. Arslan F, Houtgraaf JH, Keogh B, Kazemi K, de Jong R, McCormack WJ, et al. Treatment with OPN-305, a humanized anti-Toll-Like receptor-2 antibody, reduces myocardial ischemia/reperfusion injury in pigs. Circ Cardiovasc Interv. 2012;5(2):279-87.

152. Fang Y, Hu J. Toll-like receptor and its roles in myocardial ischemic/reperfusion injury. Med Sci Monit. 2011;17(4):Ra100-9.

153. Fremeaux-Bacchi V, Legendre CM. The emerging role of complement inhibitors in transplantation. Kidney Int. 2015;88(5):967-73.

154. Banz Y, Rieben R. Role of complement and perspectives for intervention in ischemia-reperfusion damage. Ann Med. 2012;44(3):205-17.

155. Dmytrijuk A, Robie-Suh K, Cohen MH, Rieves D, Weiss K, Pazdur R. FDA report: eculizumab (Soliris) for the treatment of patients with paroxysmal nocturnal hemoglobinuria. Oncologist. 2008;13(9):993-1000.

156. Taylor CM, Machin S, Wigmore SJ, Goodship TH. Clinical practice guidelines for the management of atypical haemolytic uraemic syndrome in the United Kingdom. Br J Haematol. 2010;148(1):37-47.

157. Wiegner R, Chakraborty S, Huber-Lang M. Complement-coagulation crosstalk on cellular and artificial surfaces. Immunobiology. 2016.

158. Gulla KC, Gupta K, Krarup A, Gal P, Schwaeble WJ, Sim RB, et al. Activation of mannan-binding lectin-associated serine proteases leads to generation of a fibrin clot. Immunology. 2010;129(4):482-95.

159. Krisinger MJ, Goebeler V, Lu Z, Meixner SC, Myles T, Pryzdial EL, et al. Thrombin generates previously unidentified C5 products that support the terminal complement activation pathway. Blood. 2012;120(8):1717-25.

160. Ikeda K, Nagasawa K, Horiuchi T, Tsuru T, Nishizaka H, Niho Y. C5a induces tissue factor activity on endothelial cells. Thromb Haemost. 1997;77(2):394-8.

161. Ritis K, Doumas M, Mastellos D, Micheli A, Giaglis S, Magotti P, et al. A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. J Immunol. 2006;177(7):4794-802.
162. Kazzaz NM, McCune WJ, Knight JS. Treatment of catastrophic antiphospholipid syndrome. Curr Opin Rheumatol. 2016;28(3):218-27.
163. Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. Nat Med. 2004;10(11):1222-6.
164. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, et al. Generation of C5a in the absence of C3: a new complement activation pathway. Nat Med. 2006;12(6):682-7.
165. Harder MJ, Kuhn N, Schrezenmeier H, Hochsmann B, von Zabern I, Weinstock C, et al. Incomplete inhibition by eculizumab: mechanistic evidence for residual C5 activity during strong complement activation. Blood. 2016.
166. Ricklin D, Lambris JD. New milestones ahead in complement-targeted therapy. Semin Immunol. 2016.
167. Egge KH, Barratt-Due A, Nymo S, Lindstad JK, Pharo A, Lau C, et al. The anti-inflammatory effect of combined complement and CD14 inhibition is preserved during escalating bacterial load. Clin Exp Immunol. 2015;181(3):457-67.
168. Kelly RJ, Hochsmann B, Szer J, Kulasekararaj A, de Guibert S, Roth A, et al. Eculizumab in Pregnant Patients with Paroxysmal Nocturnal Hemoglobinuria. N Engl J Med. 2015;373(11):1032-9.
169. Palmeira P, Quinello C, Silveira-Lessa AL, Zago CA, Carneiro-Sampaio M. IgG placental transfer in healthy and pathological pregnancies. Clin Dev Immunol. 2012;2012:985646.
170. Kuhn N, Schmidt CQ, Schlapschy M, Skerra A. PASylated Coversin, a C5-Specific Complement Inhibitor with Extended Pharmacokinetics, Shows Enhanced Anti-Hemolytic Activity in Vitro. Bioconjug Chem. 2016;27(10):2359-71.
171. Nishimura J, Yamamoto M, Hayashi S, Ohyashiki K, Ando K, Brodsky AL, et al. Genetic variants in C5 and poor response to eculizumab. N Engl J Med. 2014;370(7):632-9.
172. Volz C, Pauly D. Antibody therapies and their challenges in the treatment of age-related macular degeneration. Eur J Pharm Biopharm. 2015;95(Pt B):158-72.

173. Fridkis-Hareli M, Storek M, Mazsaroff I, Risitano AM, Lundberg AS, Horvath CJ, et al. Design and development of TT30, a novel C3d-targeted C3/C5 convertase inhibitor for treatment of human complement alternative pathway-mediated diseases. Blood. 2011;118(17):4705-13.
174. Schmidt CQ, Bai H, Lin Z, Risitano AM, Barlow PN, Ricklin D, et al. Rational engineering of a minimized immune inhibitor with unique triple-targeting properties. J Immunol. 2013;190(11):5712-21.
175. Risitano AM, Notaro R, Pascariello C, Sica M, del Vecchio L, Horvath CJ, et al. The complement receptor 2/factor H fusion protein TT30 protects paroxysmal nocturnal hemoglobinuria erythrocytes from complement-mediated hemolysis and C3 fragment. Blood. 2012;119(26):6307-16.
176. De Latour RP, Fremeaux-Bacchi V, Porcher R, Xhaard A, Rosain J, Castaneda DC, et al. Assessing complement blockade in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab. Blood. 2015;125(5):775-83.
177. Wehling C, Amon O, Bommer M, Hoppe B, Kentouche K, Schalk G, et al. Monitoring of complement activation biomarkers and eculizumab in complement-mediated renal disorders. Clin Exp Immunol. 2017;187(2):304-15.
178. Volokhina EB, van de Kar NC, Bergseth G, van der Velden TJ, Westra D, Wetzels JF, et al. Sensitive, reliable and easy-performed laboratory monitoring of eculizumab therapy in atypical hemolytic uremic syndrome. Clin Immunol. 2015;160(2):237-43.
179. Målfrid B. 92 pasienter fikk medisin for 157 millioner. Dagens Medisin. 2013 2013-10-10.
180. Hallstensen RF, Bergseth G, Foss S, Jaeger S, Gedde-Dahl T, Holt J, et al. Eculizumab treatment during pregnancy does not affect the complement system activity of the newborn. Immunobiology. 2015;220(4):452-9.
181. Gatault P, Brachet G, Ternant D, Degenne D, Recipon G, Barbet C, et al. Therapeutic drug monitoring of eculizumab: Rationale for an individualized dosing schedule. mAbs. 2015;7(6):1205-11.
182. Noris M, Galbusera M, Gastoldi S, Macor P, Banterla F, Bresin E, et al. Dynamics of complement activation in aHUS and how to monitor eculizumab therapy. Blood. 2014;124(11):1715-26.

Paper I

The Journal of Infectious Diseases
MAJOR ARTICLE

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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

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Background. Single inhibition of the Toll-like receptor 4 (TLR4)–MD2 complex failed in treatment of sepsis. CD14 is a coreceptor for several TLRs, including TLR4 and TLR2. The aim of this study was to investigate the effect of single TLR4-MD2 inhibition by using eritoran, compared with the effect of CD14 inhibition alone and combined with the C3 complement inhibitor compstatin (Cp40), on the bacteria-induced inflammatory response in human whole blood.

Methods. Cytokines were measured by multiplex technology, and leukocyte activation markers CD11b and CD35 were measured by flow cytometry.

Results. Lipopolysaccharide (LPS)–induced inflammatory markers were efficiently abolished by both anti-CD14 and eritoran. Anti-CD14 was significantly more effective than eritoran in inhibiting LPS-binding to HEK-293E cells transfected with CD14 and *Escherichia coli*–induced upregulation of monocyte activation markers ($P < .01$). Combining Cp40 with anti-CD14 was significantly more effective than combining Cp40 with eritoran in reducing *E. coli*–induced interleukin 6 ($P < .05$) and monocyte activation markers induced by both *E. coli* ($P < .001$) and *Staphylococcus aureus* ($P < .01$). Combining CP40 with anti-CD14 was more efficient than eritoran alone for 18 of 20 bacteria-induced inflammatory responses (mean $P < .0001$).

Conclusions. Whole bacteria–induced inflammation was inhibited more efficiently by anti-CD14 than by eritoran, particularly when combined with complement inhibition. Combined CD14 and complement inhibition may prove a promising treatment strategy for bacterial sepsis.

Keywords. sepsis; complement; CD14; eritoran; treatment; TLR.

Sepsis is a severe and life-threatening systemic inflammatory response to an infection. Despite extensive treatment with antibiotics and supportive therapy, morbidity and mortality due to sepsis remain high. *Escherichia coli* is the leading gram-negative bacterial cause of sepsis and *Staphylococcus aureus* is the most frequently observed gram-positive bacterial cause, with the latter showing increasing incidence [1, 2]. However, the causative agent is often unknown at the time of diagnosis and initiation of treatment.

The current hypothesis is that the initial infection triggers proinflammatory and antiinflammatory responses by the immune system and, subsequently, may severely disturb this normally finely regulated system, leading to tissue damage, organ failure, and ultimately death [3]. This detrimental, uncontrolled activation might be attenuated by manipulating the inflammatory process as an adjunct to antimicrobial treatment. A tempting strategy is to manipulate the early stages of inflammation by targeting upstream recognition of pathogen-associated molecular patterns and damage-associated molecular patterns.

The complement system provides an important first-line defense for protecting the body against pathogens. Recognition of pathogens by any of the 3 initial pathways (the classical, lectin, and alternative pathways) leads to the activation of C3 and C5, generating C3 fragments for opsonization of bacteria and the anaphylatoxins C3a and C5a, with a range of effector functions. Finally, the terminal C5b-9 complex is assembled, which can lyse bacterial membranes, especially *Neisseria* species. Most bacteria are, however, killed by complement via opsonization of C3 fragments and subsequent phagocytosis.

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Toll-like receptors (TLRs) induce responses through the activation of specific intracellular signaling pathways [4]. TLR4 and TLR2 are the most studied receptors, with TLR4 being the key receptor for lipopolysaccharide (LPS) from gram-negative bacteria and TLR2 detecting lipoproteins from gram-positive bacteria. CD14 serves as a coreceptor for TLR4, where it presents the LPS molecule to the LPS-binding site on the TLR4-MD2 complex [5]. CD14 has also been shown to interact with other TLRs, including TLR2, TLR3, TLR7, and TLR9 [6–8]. Recently, we produced a recombinant version of the neutralizing monoclonal mouse anti-human CD14 antibody 18D11 [9]. This antibody is endowed with a human immunoglobulin G2/4 (IgG2/4) hybrid heavy chain and is thus inert with respect to the major IgG effector functions (ie, complement activation and Fcγ-receptor binding) and therefore does not deplete leukocytes.

Eritoran is a synthetic lipid A analogue that binds to the LPS binding site on MD2 and prevents further signaling via the TLR4-MD2 complex. Recent reports suggest that eritoran also binds directly to CD14 and might partly inhibit the LPS-CD14 interaction [10]. Although eritoran effectively inhibits LPS-induced inflammatory responses, no reduction in 28-day mortality was achieved in a phase 3 study, in which eritoran was administered as an adjunct to standard sepsis treatment [11].

Combined inhibition with a neutralizing CD14 antibody and a complement inhibitor is effective in attenuating the inflammatory process by inhibiting cytokine release and expression of activation markers on leukocytes to a greater extent than single inhibition with either agent alone [12–14]. Murine and porcine in vivo sepsis models have demonstrated this strategy to be superior to single inhibition with either of the inhibitors alone, with respect to both attenuation of the inflammatory response and increased survival [15–17].

The aim of the present study was to elucidate the differential effects of eritoran and anti-CD14, alone and in combination with a complement inhibitor, on the inflammatory process induced by LPS, gram-negative bacteria, and gram-positive bacteria.

MATERIALS AND METHODS

Equipment and Reagents

Endotoxin-free Cryo tubes were purchased from Nunc (Roskilde, Denmark). Ethylenediaminetetraacetic acid (EDTA), paraformaldehyde, and sterile phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, Missouri). Lepirudin (Refludan) was purchased from Celgene (Marburg, Germany). Dimethyl sulfoxide and Alexa Fluor 488 were obtained from Invitrogen Molecular Probes (Eugene, Oregon). Anti-CD11b-PE (clone D12), anti-CD35-FITC (clone E11), anti-CD45-PerCP (clone 2D1), anti-CD14-PerCP, and anti-CD14-PE (clone MϕP9) and the isotype controls PE-mouse IgG2a (clone X59) and FITC-mouse IgG1k (clone MOPC-21) were obtained from Becton, Dickinson, and Company (San Jose, California).

Inhibitors

The C3-inhibitor compstatin analogue Cp40 (D-Tyr-Ile-[Cys-Val-1MeTrp-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-meIle) and the control peptide (Sar-Sar-Trp[Me]-Ala-Ala-Asp-Ile-His-Val-Gln-Arg-mIle-Trp-Ala-NH_2) were produced in the lab of one of the authors (J. D. L.), as previously described [18]. Eritoran (E5564) was kindly provided by Eisai (Andover, Massachusetts). Recombinant anti-human CD14 IgG2/4 antibody (r18D11) and an IgG2/4 isotype control antibody were produced in our laboratory as previously described [9].

LPS and Bacteria

Ultrapure LPS from *E. coli* strain 0111:B4 (smooth type) was purchased from InvivoGen (San Diego, California) and Cy5-labelled for the human embryonic kidney (HEK) 293E cell–based experiments as described previously [19]. *E. coli* strain LE392 with smooth LPS (ATCC 33572) and *S. aureus* Cowan strain 1 (ATCC 12598) were obtained from American Type Culture Collection (ATCC; Manassas, Virginia). The bacteria were inactivated by heat and counted by flow cytometry as previously described [12, 14].

HEK-293E Cell–Based Experiments

HEK-293E cells do not express CD14, TLR4, or MD2 and were transfected with human CD14, using GeneJuice transfection reagent (Novagene, Darmstadt, Germany). The cells were preincubated for 5 minutes with increasing doses of anti-CD14 (0.007, 0.07, and 0.7 μM), eritoran (0.1, 1, and 10 μM), or an IgG2/4 isotype control antibody (0.007, 0.07, and 0.7 μM) before addition of Cy5-labeled LPS (500 ng/mL). Cy5-LPS binding to CD14 was measured by flow cytometry after 1 hour of incubation (37°C). The samples were washed in cold PBS twice and detached in Accutase solution (Sigma) before the addition of 2% fetal calf serum in PBS. The cell pellets were harvested by centrifugation (at 470*g* for 5 minutes) and resuspended in PBS before analysis on a BD LSR II flow cytometer. Data analysis was performed with FlowJo software (Ashland, Oregon). Results are given as mean fluorescence intensity.

Whole-Blood Model of Inflammation

The whole-blood model is an in vitro system for studying inflammation activity and has been described in detail previously [20]. Briefly, blood specimens were collected from healthy volunteers (both sexes were evaluated, with an age range of 30 to 58 years) and anticoagulated with the thrombin inhibitor lepirudin (50 μg/mL). Blood specimens were preincubated with the inhibitors compstatin Cp40 (20 μM), anti-CD14 (0.1 μM), eritoran (1 μM), control peptide (20 μM), IgG2/4 isotype control antibody (0.1 μM), or PBS in sterile polypropylene tubes for 5 minutes (37°C). The optimal inhibitor concentrations were determined in separate dose-response experiments (data not shown). The concentration of eritoran was comparable to the plasma concentration in a phase 2 clinical study [21], using a similar dose as that in the ACCESS sepsis trial [11]. Subsequently,

blood specimens were stimulated with LPS, *E. coli*, or *S. aureus* at concentrations specified below and incubated with gentle rotation (at 37°C) for the appropriate time, depending on which inflammation markers were to be studied.

Cytokine Analysis

LPS (10 ng/mL), *E. coli* (1×10^6 bacteria/mL), or *S. aureus* (1×10^7 bacteria/mL) were added, and samples were incubated for 120 minutes. After incubation, EDTA was added (10 mM), and the samples placed on ice and centrifuged (at 1800*g* for 15 minutes at 4°C); the resulting plasma was frozen (−70°C) until further analyzed. Analyses of tumor necrosis factor (TNF), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) were performed with Bio-Plex Pro technology (Bio-Rad Laboratories, Hercules, California), using standard procedures from the manufacturer.

Leukocyte Activation Markers CD11b and CD35

LPS (10 ng/mL), *E. coli* (1×10^7 bacteria/mL), or *S. aureus* (1×10^8 bacteria/mL) were added, and samples were incubated for 20 minutes. Cells were fixed with 0.5% paraformaldehyde and incubated with anti-CD11b-PE and anti-CD35-FITC or with isotype controls, in addition to anti-CD14-PerCP. Samples were lysed, centrifuged (at 300*g* for 5 minutes at 4°C), and analyzed on a FACSCalibur flow cytometer. Data analysis was performed with FlowJo software. Monocytes and granulocytes were gated by side scatter and CD14 expression. Results are given as median fluorescence intensity.

Phagocytosis

As previously described, whole-blood specimens were incubated with a final concentration of Alexa Fluor 488–stained *E. coli* (5×10^7 bacteria/mL) and *S. aureus* (1×10^8 bacteria/mL) for 15 minutes, and phagocytosis was assessed by Phagotest (Glycotope Biotechnology, Heidelberg, Germany), according to kit instructions [20]. Monocytes and granulocytes were gated by side scatter and CD14 expression. Results are given as mean fluorescence intensity.

Statistical Analysis

All statistical calculations were done in Prism 5 (GraphPad, San Diego, California). A *t* test was used for the HEK-293E cell experiments. All whole-blood experiments were repeated with 6 different donors, unless otherwise stated. The LPS data were analyzed by repeated measures 1-way analysis of variance (ANOVA) of all 3 groups (LPS positive control, anti-CD14, and eritoran), with comparison of the latter 2 groups by post hoc Bonferroni correction for selected multiple testing. The *E. coli* and *S. aureus* data were analyzed by repeated measures 1-way ANOVA of the groups of interest (anti-CD14, eritoran, anti-CD14 plus compstatin, and eritoran plus compstatin). Comparisons of adjacent inhibitors were estimated by post hoc Bonferroni correction for selected multiple testing. A P value of <.05 was considered statistically significant. Compstatin Cp40 alone was included in the test panel to show the contribution of complement inhibition, but it was not included in the statistical analysis [13].

Ethics

Informed written consent was obtained from each blood donor. The local ethical committee approved the study.

RESULTS

LPS-Induced Responses

To demonstrate the inhibitory effects of eritoran and anti-CD14 on the LPS-induced inflammatory responses, inhibition of LPS-induced cytokine release and upregulation of CD11b and CD35 on leukocytes were tested in human whole-blood specimens (Figure 1). Both eritoran and anti-CD14 efficiently and significantly inhibited LPS-induced release of TNF, IL-1β, IL-6, and IL-8 ($P < .01$–$P < .0001$; Figure 1*A*). Eritoran showed equivalent (for TNF, IL-1β, and IL-6) or significantly more-effective (for IL-8) inhibition when compared to anti-CD14 ($P < .05$). Similarly, both inhibitors significantly attenuated the LPS-induced increase in monocyte expression of CD11b (96% and 90% reduction, respectively; $P < .01$) and CD35 (95% and 94% reduction, respectively; $P < .05$; Figure 1*B*). Granulocytes were not activated under these conditions, as their LPS-responsiveness is much lower than seen for monocytes [22].

To investigate the inhibitory effects of eritoran on LPS-binding to CD14, HEK-293E cells transfected with human CD14 were used. The cells were preincubated with increasing doses of eritoran or anti-CD14 prior to LPS stimulation. Both eritoran and anti-CD14 inhibited LPS-binding to CD14 in a dose-dependent manner (Figure 2). However, anti-CD14 was significantly more effective ($P < .05$) when comparing concentrations sufficient to inhibit LPS-induced inflammatory responses in the whole-blood model (0.07 μM anti-CD14 and 1 μM eritoran).

The negative controls (IgG2/4 isotype control antibody and control peptide) did not inhibit any of the LPS readouts (data now shown).

E. coli–Induced Inflammatory Responses

To investigate the effects of eritoran alone, anti-CD14 alone, and the combination of each with the C3 inhibitor compstatin Cp40 in a more complex model of inflammation, *E. coli* was used to stimulate whole-blood specimens. Cytokine analysis showed that inhibition with anti-CD14 alone or eritoran alone markedly attenuated the release of TNF, IL-1β, IL-6, and IL-8, without significant differences between the 2 inhibitors (Figure 3). The combination of anti-CD14 and compstatin Cp40 inhibited the release of all cytokines down to background levels. For IL-6, this combination was significantly more efficient than the combination of eritoran and compstatin Cp40 ($P < .05$).

Inhibition with anti-CD14 alone reduced the *E. coli*–induced upregulation of monocyte CD11b and CD35 by 82% and 83%, respectively, and was significantly more effective than inhibition with eritoran alone (33% reduction [$P < .001$] and 25%

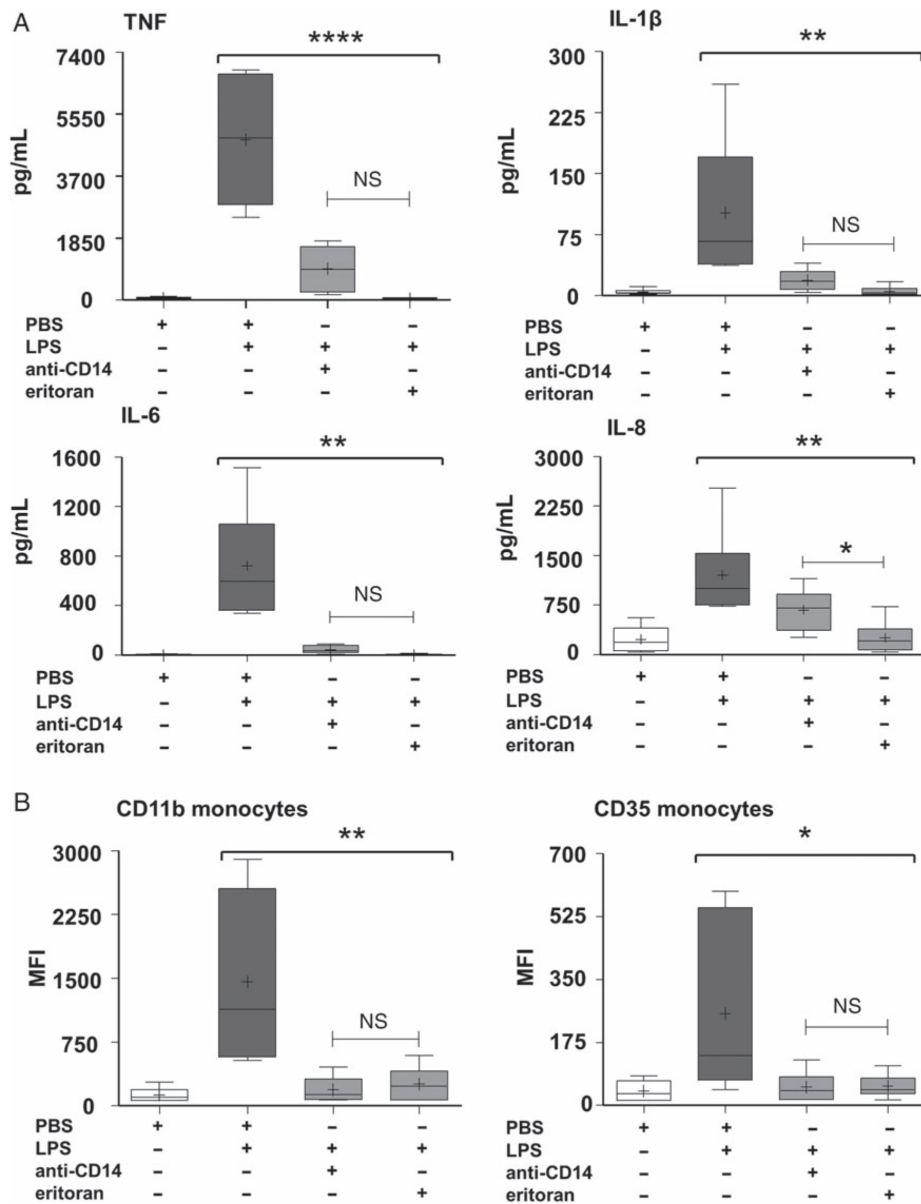

Figure 1. Effects of anti-CD14 and eritoran on lipopolysaccharide (LPS)–induced inflammation markers in human whole-blood specimens. *A*, Tumor necrosis factor (TNF), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) release in response to incubating human whole-blood specimens with ultrapure LPS (10 ng/mL) for 120 minutes after preincubation with phosphate-buffered saline (PBS), anti-CD14, or eritoran. *B*, CD11b and CD35 expression on monocytes in response to stimulation of human whole-blood specimens with ultrapure LPS for 20 minutes after preincubation with PBS, anti-CD14, or eritoran. Data are presented as box plots, with upper and lower limits of the boxes representing interquartile ranges, whiskers representing and 10th and 90th percentiles, horizontal lines within boxes representing median values, and vertical lines within boxes representing mean values; data are from 6 independent experiments, using 6 different donors. Statistical significance was estimated by using repeated measures 1-way analysis of variance (top line), and comparison between anti-CD14 and eritoran was estimated by post-hoc Bonferroni correction for selected multiple testing (closed line). $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$. Abbreviations: MFI, median fluorescence intensity; NS, nonsignificant.

reduction [$P < .01$], respectively; Figure 4). The combination of anti-CD14 and compstatin Cp40 reduced levels of both receptors to background levels, significantly more efficient than the combination of eritoran and compstatin Cp40 (45% and 34% reductions, respectively; $P < .01$). In contrast to monocytes, upregulation of granulocyte CD11b and CD35 was complement dependent (86% and 83% reductions by compstatin Cp40, respectively) and only moderately affected by inhibition with anti-CD14 alone (40% and 30% reductions, respectively) or eritoran alone (28% and 15% reductions, respectively; Figure 4). The combined treatments reduced the upregulation of both receptors down to background levels.

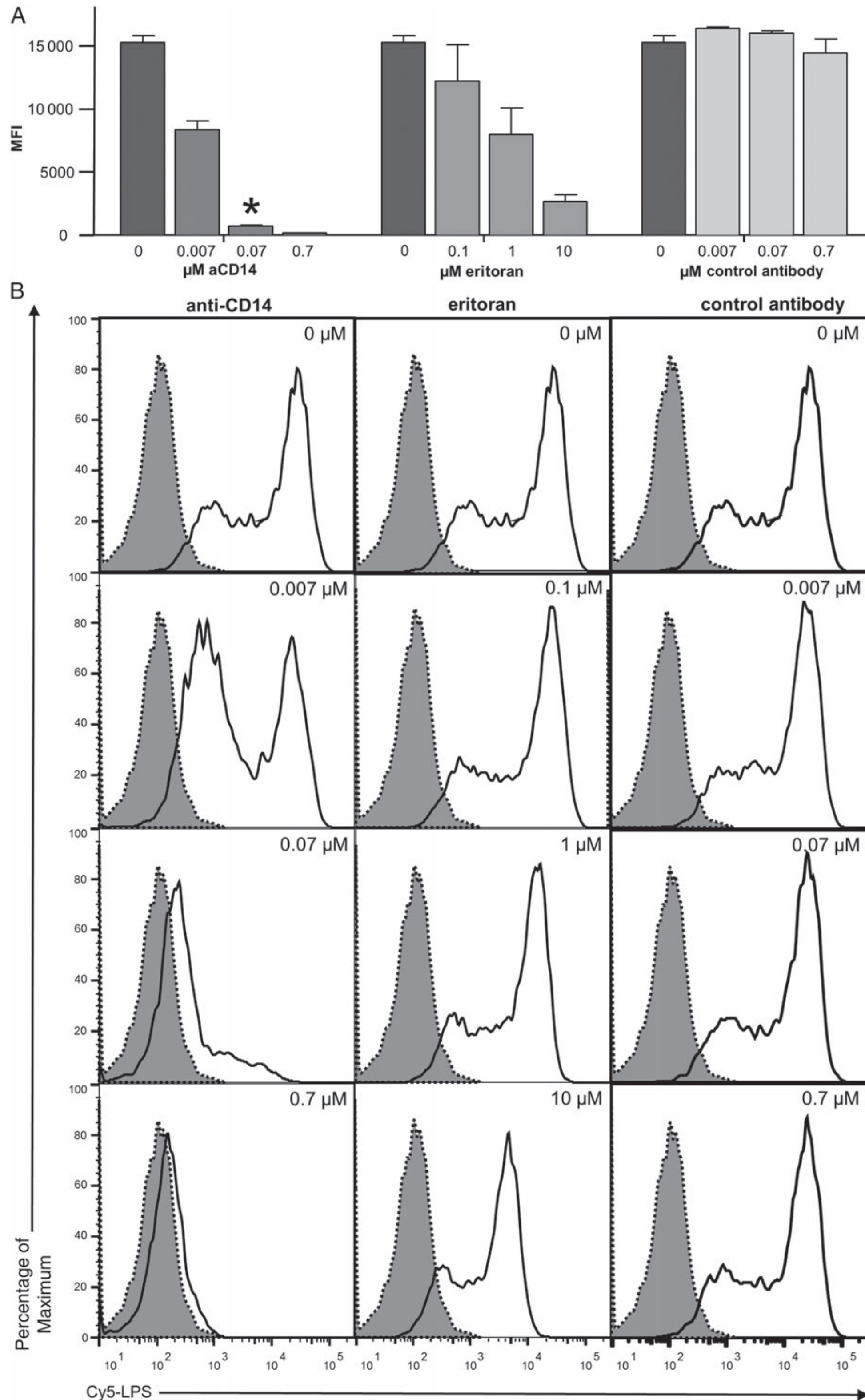

Figure 2. Effects of anti-CD14 and eritoran on lipopolysaccharide (LPS)–binding to membrane-bound CD14. *A*, Human embryonic kidney 293E cells transfected with human CD14 were incubated with Cy5-labeled LPS after preincubation with increasing doses of anti-CD14 (aCD14), eritoran, or a control antibody. Data are shown as mean fluorescence intensity (MFI) and presented as mean ± standard error of the mean of 3 experiments. Statistical significance was estimated by a parametric *t* test between anti-CD14 0.07 µM and eritoran 1 µM data. **P* < .05. *B*, Flow cytometry histograms showing data from one of the 3 virtually identical experiments presented in panel *A*.

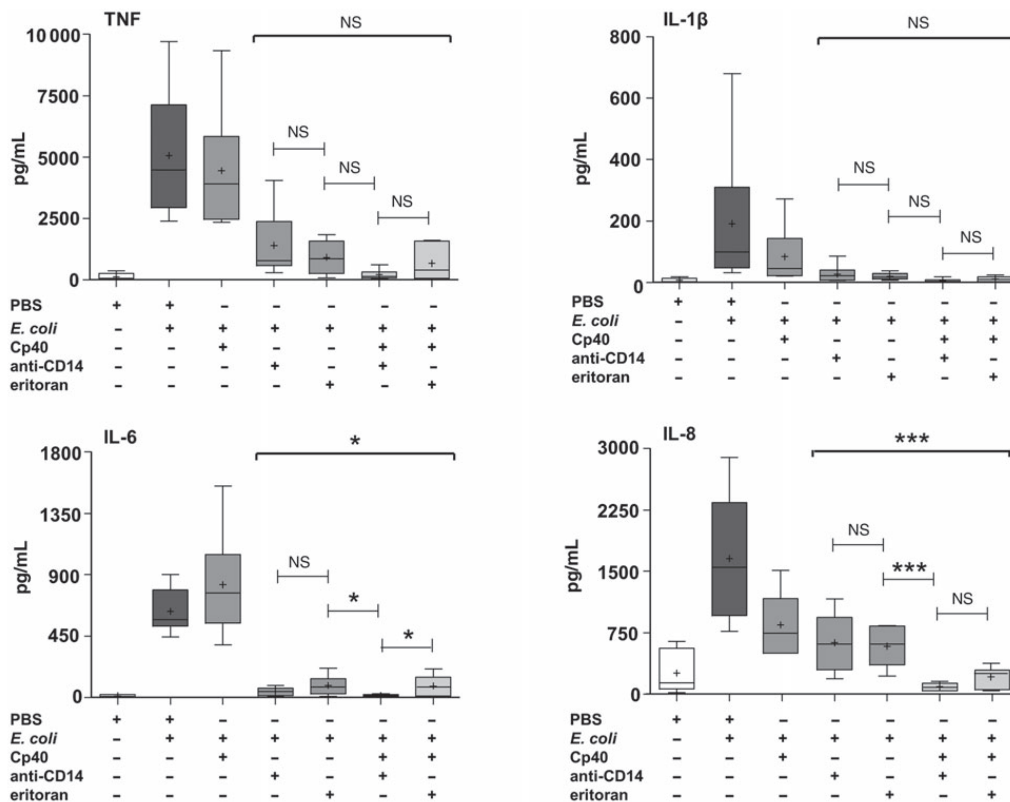

Figure 3. Inhibition of *Escherichia coli*–induced cytokine release in human whole blood. Tumor necrosis factor (TNF), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) release in response to incubating human whole-blood specimens with heat-inactivated *E. coli* (1×10^6 bacteria/mL) for 120 minutes after preincubation with phosphate-buffered saline (PBS), compstatin (Cp40), anti-CD14, eritoran, anti-CD14 plus compstatin, or eritoran plus compstatin. Data are presented as box plots, with upper and lower limits of the boxes representing interquartile ranges, whiskers representing and 10th and 90th percentiles, horizontal lines within boxes representing median values, and vertical lines within boxes representing mean values; data are from 6 independent experiments, using 6 different donors. Statistical significance between the inhibitors of interest was estimated by using repeated measures 1-way analysis of variance (top line), and comparison between adjacent inhibitors was estimated by post-hoc Bonferroni correction for selected multiple testing (closed lines). *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$. Abbreviation: NS, nonsignificant.

The negative controls did not inhibit any of the *E. coli*–induced inflammatory readouts (data not shown).

S. aureus–Induced Inflammatory Responses

We next investigated whether eritoran alone, anti-CD14 alone, and the combination of each with the complement inhibitor Cp40 would have similar effects in a model involving gram-positive induction of inflammation. *S. aureus* was used in the same experimental design as described above for *E. coli*.

Cytokine analysis showed that inhibition of TNF, IL-1β, IL-6, and IL-8 was dependent on complement to a greater extent for *S. aureus* than for *E. coli*. The attenuating effect of single inhibition with anti-CD14 or eritoran was minimal, without significant differences between the 2 inhibitors (Figure 5). The combination of anti-CD14 and compstatin Cp40 reduced the cytokine release by 60%–75%, also showing a tendency of stronger attenuation than the combination of eritoran and compstatin Cp40, although these differences did not reach statistical significance.

Upregulation of monocyte CD11b and CD35 by *S. aureus* was reduced significantly more by the combination of anti-CD14 and compstatin Cp40 (70% and 75%, respectively) than by the combination of eritoran and compstatin Cp40 (47% and 34% reduction, respectively; $P < .01$; Figure 6). Inhibition with either anti-CD14 alone or eritoran alone showed no inhibitory effect. Upregulation of granulocyte CD11b and CD35 was mainly complement dependent and reduced by 92% and 78%, respectively, by compstatin Cp40 alone (Figure 6). No further effect was seen by the combined treatments, and again, single inhibition with either anti-CD14 or eritoran showed no inhibitory effect.

The negative controls did not inhibit any of the *S. aureus*–induced inflammatory readouts (data not shown).

Bacterial Phagocytosis

E. coli

Monocyte phagocytosis of *E. coli* was reduced by 24% by inhibition with anti-CD14 alone and was significantly more

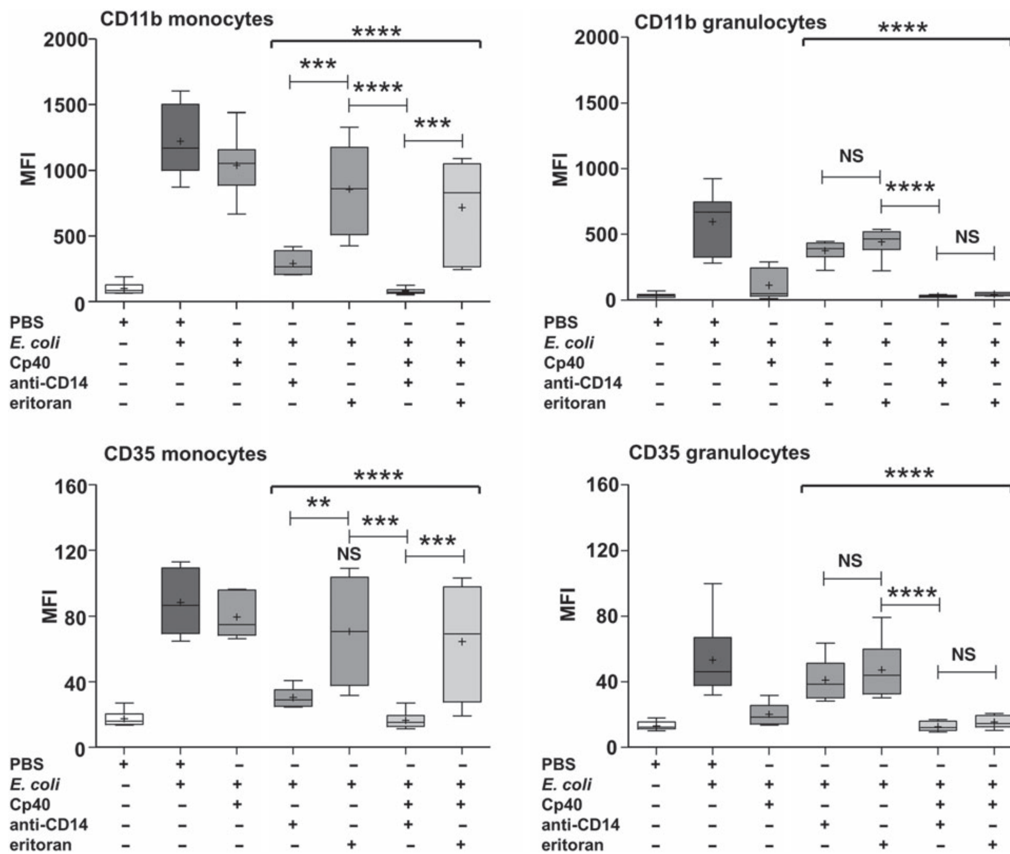

Figure 4. Inhibition of *Escherichia coli*–induced upregulation of CD11b and CD35 on monocytes and granulocytes in human whole-blood specimens. Upregulation of CD11b and CD35 on monocytes and granulocytes in response to incubating human whole-blood specimens with heat-inactivated *E. coli* (1×10^7 bacteria/mL) for 20 minutes after preincubation with phosphate-buffered saline (PBS), compstatin (Cp40), anti-CD14, eritoran, anti-CD14 plus compstatin, or eritoran plus compstatin. Data are shown as median fluorescence intensity (MFI). Data presentation and statistics are as described in the legend to Figure 3. **$P < .01$, ***$P < .001$, and ****$P < .0001$. Abbreviation: NS, nonsignificant.

effective than inhibition with eritoran alone (no reduction; $P < .01$; Figure 7*A*). The combination of anti-CD14 and compstatin Cp40 reduced the phagocytosis by 79%, which was not significantly different from the combination of eritoran and compstatin Cp40 (57% reduction). Granulocyte phagocytosis was only minimally affected by inhibition with anti-CD14 alone or eritoran alone (11% and 6% reduction, respectively; Figure 7*A*). The combination of anti-CD14 and compstatin Cp40 reduced phagocytosis by 90%, similar to the combination of eritoran and compstatin Cp40 (87% reduction).

S. aureus

Monocyte phagocytosis of *S. aureus* was complement dependent (75% reduction by compstatin Cp40; Figure 7*B*). Single inhibition with anti-CD14 showed a 20% reduction and did not significantly differ from eritoran (7% reduction). The combination of anti-CD14 and compstatin Cp40 (93% reduction) showed a minor additional inhibitory effect, although not statistical significant, compared to the combination of eritoran and compstatin Cp40 (76% reduction). Granulocyte phagocytosis was completely complement dependent, being reduced to background levels by compstatin Cp40 alone, without any effect of single inhibition of anti-CD14 or eritoran (Figure 7*B*).

Eritoran Versus the Combined Inhibition of CD14 and Complement

Since eritoran was used to treat sepsis but failed, we compared the effects of eritoran alone with that of combined anti-CD14 and complement inhibition, which we propose as an alternative treatment approach [15, 17]. Noteworthy, for 18 of 20 readouts reported for the bacteria-induced inflammatory responses (Figures 3–7), the combination of anti-CD14 and compstatin Cp40 was substantially more efficient than inhibition with eritoran alone ($P < .05$–$P < .0001$; mean $P < .0001$).

DISCUSSION

In the present study, we have demonstrated that, although eritoran was an effective inhibitor of LPS-induced inflammation, it showed no effect on *E. coli*–induced leukocyte activation markers or inflammation induced by *S. aureus*. Anti-CD14 showed a broader inhibitory effect than eritoran, as it also efficiently

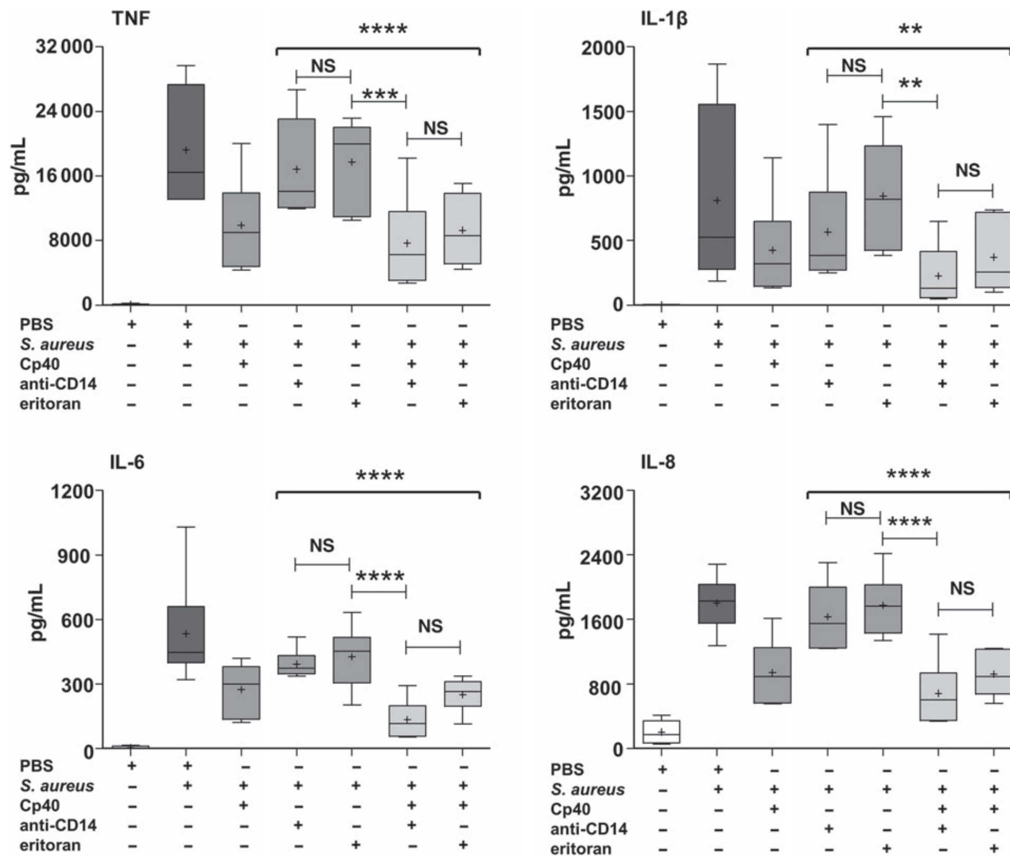

Figure 5. Inhibition of *Staphylococcus aureus*–induced cytokine release in human whole-blood specimens. Tumor necrosis factor (TNF), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) release in response to incubation of human whole-blood specimens with heat-inactivated *S. aureus* (1 10^7 bacteria/mL) for 120 minutes after preincubation with phosphate-buffered saline (PBS), compstatin (Cp40), anti-CD14, eritoran, anti-CD14 plus compstatin, or eritoran plus compstatin. Data presentation and statistics are as described in the legend to Figure 3. $**P < .01$, $***P < .001$, and $****P < .0001$. Abbreviation: NS, nonsignificant.

inhibited monocyte activation by *E. coli*. The addition of a complement inhibitor was necessary to inhibit granulocyte activation and *S. aureus* inflammatory responses. The combined inhibition of CD14 and complement was significantly more efficient than the combination of eritoran and complement inhibition, especially in terms of monocyte activation.

In addition to blocking the TLR4-MD2 complex, eritoran has also been reported to bind to soluble CD14, preventing further ligand binding to MD2 [10]. We here show that even though eritoran attenuated LPS-binding to membrane-bound CD14 dose dependently, anti-CD14 inhibited the LPS-binding much more effectively.

Both anti-CD14 and eritoran showed efficient attenuating effects on *E. coli*–induced cytokine responses, in line with the LPS-driven activation of TLR4. However, adding a complement inhibitor increased the effect. Moreover, there was a trend indicating that the combination of anti-CD14 and compstatin Cp40 was more effective than the combination of eritoran and compstatin Cp40, with the difference reaching significance for IL-6. Eritoran had minimal effect on *S. aureus*–induced cytokine responses. The different effects of eritoran on *E. coli*– and *S. aureus*–induced inflammation are not surprising, since *S. aureus* does not contain LPS and is thought to activate the inflammatory system mainly through TLRs other than TLR4, particularly TLR2 and TLR8, and the complement system [14, 23–25]. The addition of a complement inhibitor was necessary to efficiently inhibit *S. aureus*–induced cytokine release.

Leukocyte activation is a hallmark of bacterial infection. CD11b and CD35 have been shown to increase significantly on monocytes and neutrophils in patients with bacterial infections and sepsis, compared with healthy controls [26, 27]. Monocyte activation by gram-negative bacteria has previously been shown to be largely CD14 dependent [28]. This observation has been attributed to LPS-driven activation of TLR4.

In the current study, eritoran completely abolished LPS-induced expression of the 2 leukocyte activation markers CD11b and CD35 on monocytes but, surprisingly, had almost no effect on the *E. coli*–induced expression. In contrast to eritoran, inhibition with anti-CD14 alone significantly and efficiently reduced the expression of these activation markers. This suggests that

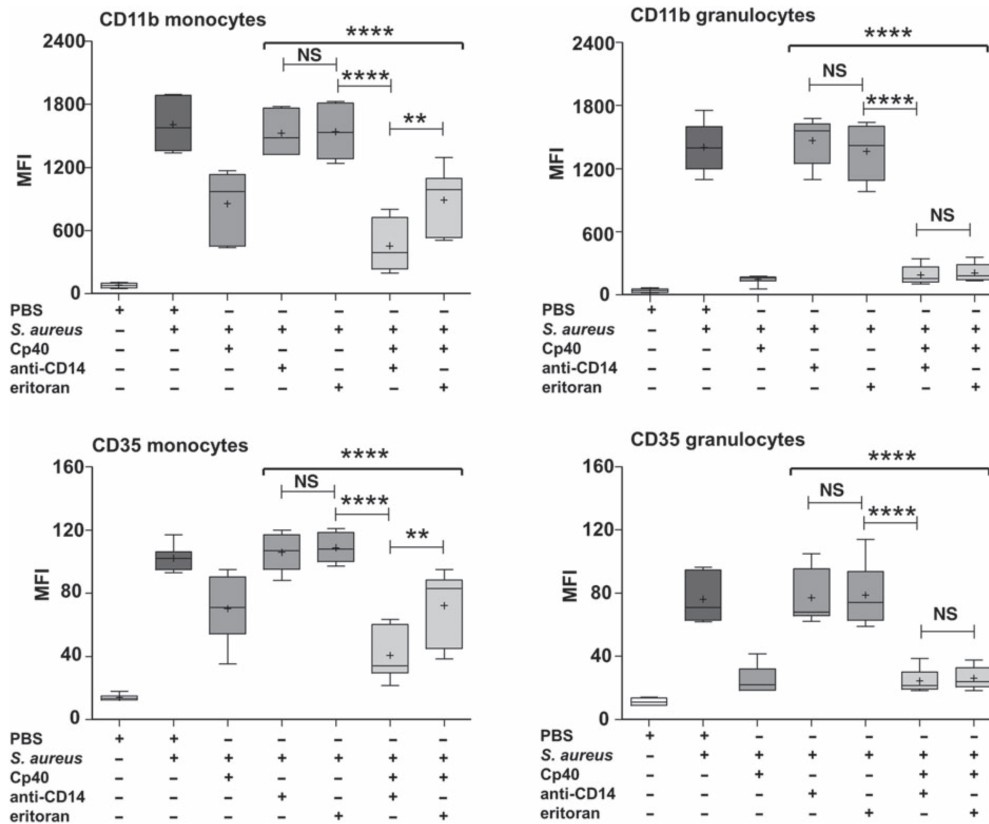

Figure 6. Inhibition of *Staphylococcus aureus*–induced upregulation of CD11b and CD35 on monocytes and granulocytes in human whole-blood specimens. Upregulation of CD11b and CD35 on monocytes and granulocytes in response to incubating human whole-blood specimens with heat-inactivated *S. aureus* (1×10^8 bacteria/mL) for 20 minutes after preincubation with phosphate-buffered saline (PBS), compstatin (Cp40), anti-CD14, eritoran, anti-CD14 plus compstatin, or eritoran plus compstatin. Data are shown as median fluorescence intensity (MFI). Data presentation and statistics are as described in the legend to Figure 3. $^{**}P < .01$ and $^{****}P < .0001$. Abbreviation: NS, nonsignificant.

redundant pathways of CD11b and CD35 upregulation that are independent of soluble LPS and TLR4-MD2 but dependent on CD14 may be activated by whole bacteria.

Similar results were obtained for *S. aureus*–induced expression of CD11b and CD35 on monocytes, where combined inhibition of CD14 and complement clearly outperformed the combination of compstatin Cp40 with eritoran, again indicating that anti-CD14 mediates effects beyond TLR4-MD2 inhibition. However, inhibition with anti-CD14 alone did not show an inhibitory effect under these conditions.

Granulocyte activation and phagocytosis of *E. coli* and *S. aureus* by monocytes and granulocytes were largely complement dependent, consistent with earlier observations [20, 28]. Interestingly, inhibition of CD14 alone was more efficient than eritoran alone in reducing monocyte phagocytosis of *E. coli*. In addition to phagocytosis, a possible effect of the various inhibitors on plasma bacterial lysis could be of importance for the effect observed on the inflammatory reaction. Working with heat-inactivated bacteria precludes use of colony-forming units as a readout. However, we have previously shown that inhibition of CD14 did not reduce the killing of live *E. coli* in porcine whole-blood specimens, whereas inhibition of C3 reduced but did not abolish bacterial killing [29]. This effect of complement inhibition might reduce the amount of plasma LPS and thus attenuate the cytokine storm.

There was much optimism expressed surrounding eritoran and its potential to reduce mortality in sepsis. Yet, again, when the phase 3 trial was concluded, no improvement was seen in 28-day mortality [11]. Savva et al pointed out that merely inhibiting a single pattern-recognition receptor could be insufficient, since other receptors might fill in for TLR4, particularly when gram-positive bacteria are involved, thus overriding the inhibitory effect [30]. We have hypothesized that a combined inhibitory approach is necessary in sepsis. Indeed, when comparing combined CD14 and complement inhibition to inhibition by eritoran alone, as tested in clinical trials, the combined approach was vastly more efficient in our model. Furthermore, our data indicate that complement and CD14 inhibition combined would be more efficient than combining a complement inhibitor with eritoran. This warrants further validation in animal studies.

Development of new treatments for sepsis has proven difficult, demonstrating the discrepancies between preclinical

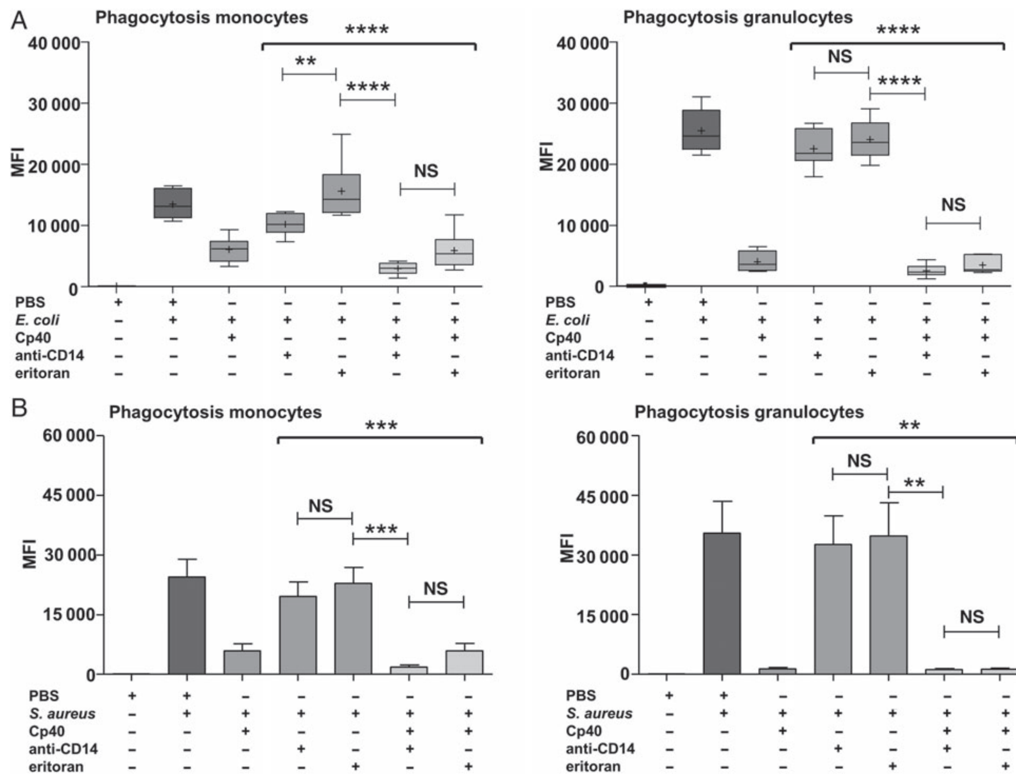

Figure 7. Inhibition of phagocytosis of *Escherichia coli (A)* or *Staphylococcus aureus (B)* by monocytes and granulocytes in human whole-blood specimens. Human whole-blood specimens were preincubated with phosphate-buffered saline (PBS), compstatin (Cp40), anti-CD14, eritoran, anti-CD14 plus compstatin, or eritoran plus compstatin and then incubated with Alexa-labeled *E. coli* (5×10^7 bacteria/mL) or *S. aureus* (1×10^8 bacteria/mL) for 15 minutes. Phagocytosis was measured by flow cytometry, and the results are indicated as mean fluorescence intensity (MFI). Data presentation and statistics are as described in the legend to Figure 3 except that these *S. aureus* experiments involved 3 different donors. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$. Abbreviation: NS, nonsignificant.

models and clinical outcomes. There are obvious shortcomings in our model, particularly the pretreatment approach, because most patients would not receive medications at such an early stage. However, finding complex human systems that can better reflect the clinical effects of treatments is important to improve the selection of therapies for clinical trials. Here we demonstrate important shortcomings of TLR4-MD2 inhibition alone, particularly in gram-positive inflammation; this conclusion corresponds to clinical findings. Thus, the whole-blood model could be a promising tool for identifying potential therapeutic approaches to complex inflammatory diseases.

Although *E. coli* and *S. aureus* are the leading causative agents in sepsis, a long list of other microbial pathogens can be found, including the setting of polymicrobial infections, underscoring the need for targeting broad-acting pattern-recognition receptors and upstream in the recognition systems. We have recently demonstrated that the importance of combined inhibition, as compared to inhibition by anti-CD14 alone, also increases with incremental doses of *E. coli* [31]. Since septic patients have an overwhelming dysregulation of the inflammatory system, one might suspect that the benefits of combined inhibition instead of single-agent inhibition would be even more pronounced in that setting. Thus, in conclusion, we have shown that a combined inhibition strategy against CD14 and complement might be a promising treatment for human sepsis.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med **2003**; 348:1546–54.
2. Luzzaro F, Ortisi G, Larosa M, Drago M, Brigante G, Gesu G. Prevalence and epidemiology of microbial pathogens causing bloodstream infections: results of the OASIS multicenter study. Diagn Microbiol Infect Dis **2011**; 69:363–9.

3. Angus DC, van der Poll T. Severe sepsis and septic shock. N Engl J Med **2013**; 369:840–51.
4. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol **2010**; 11:373–84.
5. Zanoni I, Granucci F. Role of CD14 in host protection against infections and in metabolism regulation. Front Cell Infect Microbiol **2013**; 3:32.
6. Lee HK, Dunzendorfer S, Soldau K, Tobias PS. Double-stranded RNA-mediated TLR3 activation is enhanced by CD14. Immunity **2006**; 24:153–63.
7. Baumann CL, Aspalter IM, Sharif O, et al. CD14 is a coreceptor of Toll-like receptors 7 and 9. J Exp Med **2010**; 207:2689–701.
8. Weber C, Muller C, Podszuweit A, Montino C, Vollmer J, Forsbach A. Toll-like receptor (TLR) 3 immune modulation by unformulated small interfering RNA or DNA and the role of CD14 (in TLR-mediated effects). Immunology **2012**; 136:64–77.
9. Lau C, Gunnarsen KS, Hoydahl LS, et al. Chimeric anti-CD14 IGG2/4 Hybrid antibodies for therapeutic intervention in pig and human models of inflammation. J Immunol **2013**; 191:4769–77.
10. Shirey KA, Lai W, Scott AJ, et al. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. Nature **2013**; 497:498–502.
11. Opal SM, Laterre PF, Francois B, et al. Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial. JAMA **2013**; 309:1154–62.
12. Brekke OL, Christiansen D, Fure H, et al. Combined inhibition of complement and CD14 abolish *E.* coli-induced cytokine-, chemokine- and growth factor-synthesis in human whole blood. Mol Immunol **2008**; 45:3804–13.
13. Egge KH, Thorgersen EB, Lindstad JK, et al. Post challenge inhibition of C3 and CD14 attenuates *Escherichia coli*-induced inflammation in human whole blood. Innate Immun **2014**; 20:68–77.
14. Skjeflo EW, Christiansen D, Espevik T, Nielsen EW, Mollnes TE. Combined inhibition of complement and CD14 efficiently attenuated the inflammatory response induced by *Staphylococcus aureus* in a human whole blood model. J Immunol **2014**; 192:2857–64.
15. Barratt-Due A, Thorgersen EB, Egge K, et al. Combined inhibition of complement C5 and CD14 markedly attenuates inflammation, thrombogenicity, and hemodynamic changes in porcine sepsis. J Immunol **2013**; 191:819–27.
16. Huber-Lang M, Barratt-Due A, Pischke SE, et al. Double blockade of CD14 and complement C5 abolishes the cytokine storm and improves morbidity and survival in polymicrobial sepsis in mice. J Immunol **2014**; 192:5324–31.
17. Skjeflo EW, Sagatun C, Dybwik K, et al. Combined inhibition of complement and CD14 improved outcome in porcine polymicrobial sepsis. Crit Care **2015**; 19:415.
18. Qu H, Ricklin D, Bai H, et al. New analogs of the clinical complement inhibitor compstatin with subnanomolar affinity and enhanced pharmacokinetic properties. Immunobiology **2013**; 218:496–505.
19. Husebye H, Halaas O, Stenmark H, et al. Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity. EMBO J **2006**; 25:683–92.
20. Mollnes TE, Brekke OL, Fung M, et al. 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 **2002**; 100:1869–77.
21. Tidswell M, Tillis W, Larosa SP, et al. Phase 2 trial of eritoran tetrasodium (E5564), a toll-like receptor 4 antagonist, in patients with severe sepsis. Crit Care Med **2010**; 38:72–83.
22. Sabroe I, Jones EC, Usher LR, Whyte MK, Dower SK. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. J Immunol **2002**; 168:4701–10.
23. Muller P, Muller-Anstett M, Wagener J, et al. The *Staphylococcus aureus* lipoprotein SitC colocalizes with Toll-like receptor 2 (TLR2) in murine keratinocytes and elicits intracellular TLR2 accumulation. Infect Immun **2010**; 78:4243–50.
24. Schroder NW, Morath S, Alexander C, et al. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J Biol Chem **2003**; 278:15587–94.
25. Bergstrom B, Aune MH, Awuh JA, et al. TLR8 senses staphylococcus aureus RNA in human primary monocytes and macrophages and induces IFN-beta production via a TAK1-IKKbeta-IRF5 signaling pathway. J Immunol **2015**; 195:100–11.
26. Brunialti MK, Martins PS, Barbosa de Carvalho H, Machado FR, Barbosa LM, Salomao R. TLR2, TLR4, CD14, CD11B, and CD11C expressions on monocytes surface and cytokine production in patients with sepsis, severe sepsis, and septic shock. Shock **2006**; 25:351–7.
27. Nuutila J, Jalava-Karvinen P, Hohenthal U, et al. Comparison of degranulation of easily mobilizable intracellular granules by human phagocytes in healthy subjects and patients with infectious diseases. Hum Immunol **2009**; 70:813–9.
28. Lappegard KT, Christiansen D, Pharo A, et al. Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. Proc Natl Acad Sci USA **2009**; 106:15861–6.
29. Thorgersen EB, Pharo A, Haverson K, et al. Inhibition of complement and CD14 attenuates the *Escherichia coli*-induced inflammatory response in porcine whole blood. Infect Immun **2009**; 77:725–32.
30. Savva A, Roger T. Targeting toll-like receptors: promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. Front Immunol **2013**; 4:387.
31. Egge KH, Barratt-Due A, Nymo S, et al. The anti-inflammatory effect of combined complement and CD14 inhibition is preserved during escalating bacterial load. Clin Exp Immunol **2015**; 181:457–67.

Paper II

Human Endothelial Cell Activation by *Escherichia coli* and *Staphylococcus aureus* Is Mediated by TNF and IL-1β Secondarily to Activation of C5 and CD14 in Whole Blood

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Endothelial cells (EC) play a central role in inflammation. E-selectin and ICAM-1 expression are essential for leukocyte recruitment and are good markers of EC activation. Most studies of EC activation are done in vitro using isolated mediators. The aim of the present study was to examine the relative importance of pattern recognition systems and downstream mediators in bacteria-induced EC activation in a physiological relevant human model, using EC incubated with whole blood. HUVEC were incubated with human whole blood. *Escherichia coli*– and *Staphylococcus aureus*–induced EC activation was measured by E-selectin and ICAM-1 expression using flow cytometry. The mAb 18D11 was used to neutralize CD14, and the lipid A analog eritoran was used to block TLR4/MD2. C5 cleavage was inhibited using eculizumab, and C5aR1 was blocked by an antagonist. Infliximab and canakinumab were used to neutralize TNF and IL-1β. The EC were minimally activated when bacteria were incubated in serum, whereas a substantial EC activation was seen when the bacteria were incubated in whole blood. *E. coli*–induced activation was largely CD14-dependent, whereas *S. aureus* mainly caused a C5aR1-mediated response. Combined CD14 and C5 inhibition reduced E-selectin and ICAM-1 expression by 96 and 98% for *E. coli* and by 70 and 75% for *S. aureus*. Finally, the EC activation by both bacteria was completely abolished by combined inhibition of TNF and IL-1β. *E. coli* and *S. aureus* activated EC in a CD14- and C5-dependent manner with subsequent leukocyte secretion of TNF and IL-1β mediating the effect. ***The Journal of Immunology*, 2016, 196: 2293–2299.**

Direct bacterial tissue damage and bacteria-induced inflammation are important causes of both severe morbidity and mortality worldwide. Both Gram-negative and Gram-positive bacteria can cause severe disease. Although historically Gram-negative bacteria have been the main cause of severe infections, with *Escherichia coli* being the most common, there is an increasing prevalence of Gram-positive–induced infections, in particular with *Staphylococcus aureus* (1).

In recent years, the role of endothelial cells (EC) in inflammation has been recognized both as central and undervalued (2–4). From the basic point that EC activation is necessary for leukocyte recruitment to the recent discovery that inhibiting increased vascular permeability in sepsis seems to alleviate most of the symptoms of the condition in murine models (5–7), it has been demonstrated that a better evaluation of EC activation has a central part in the quest for understanding and modulating inflammation.

A large number of models have been used to examine EC activation, with HUVEC being a common surrogate. Using HUVEC, multiple potential activating factors have been found in in vitro studies. However, as the field of sepsis has duly demonstrated, there is a long path from simple isolated cell models to the clinic, a path riddled with wrong turns and empty promises.

To narrow this gap, we have developed a novel model to look at human EC activation in a more complex system using an already established whole blood model, that is, coincubating lepirudin anticoagulated whole blood with HUVEC monolayers (8).

Recognition of bacteria by the innate immune system is mediated by pattern recognition receptors. There are several different classes of these, where TLRs play a central role, particularly in detection of LPS from Gram-negative bacteria through TLR4, but also motifs on Gram-positive bacteria, particularly lipoproteins, recognized by TLR2 (9). CD14 is a promiscuous coreceptor to several of the TLRs, and it plays a central role in recognition both by TLR4 and TLR2 (10).

The complement system is an important recognition system comprising both fluid-phase and cell-bound components. There are three pathways of pathogen recognition (classical, lectin, and alternative pathways) that all lead to activation of the C3 convertases, which proteolyze C3 into C3a, an anaphylatoxin with a range of effector functions, and C3b, which acts as an opsonin on bacteria and other exogenous or endogenous structures. With further ac-

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Abbreviation used in this article: EC, endothelial cell.

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tivation of C3, the C5 convertase is constructed, which activates C5, leading to the release of C5a, the most potent anaphylatoxin in this system, and assembly of the terminal C5b-9 complex. This complex can either be formed in the fluid phase as sC5b-9 or assembled on a membrane as the membrane attack complex, which may lyse Gram-negative bacteria. Complement system activation is strictly regulated by endogenous inhibitors at different steps of the cascade (11–13).

We have earlier shown that combined inhibition of CD14 and one of the key complement components, at the level of either C3 or C5, can more or less completely attenuate cytokine release and leukocyte activation in whole blood after bacteria-induced activation both with Gram-negative and Gram-positive bacteria (14–16). Furthermore, in a murine sepsis model, we have recently shown that combined inhibition reduces both inflammation and mortality (17).

The aim of the present study was to evaluate the mechanisms behind bacteria-induced EC activation in our novel whole blood and HUVEC model. Using *E. coli* and *S. aureus*, we examined the effect of inhibition both at the recognition stage, inhibiting complement activation and CD14, and at the downstream mediator stage, inhibiting TNF and IL-1β.

Materials and Methods

Reagents

Sterile PBS with and without Ca^{2+} and Mg^{2+} and EDTA were purchased from Sigma-Aldrich (St. Louis, MO). CryoTubes (polypropylene) were purchased from Nunc (Roskilde, Denmark). Lepirudin (Refludan) was purchased from Pharmion (Copenhagen, Denmark) and used at a final concentration of 50 μg/ml in whole blood. A 4% stock solution made of paraformaldehyde was purchased from Sigma-Aldrich. BSA 30% was purchased from Biotest (Dreieich, Germany) and trypsin/EDTA was from Invitrogen (Carlsbad, CA). A complete EC growth medium was purchased from Cell Applications (San Diego, CA). Heat-inactivated *E. coli* strain LE392 (ATCC 33572) and *S. aureus* Cowan strain 1 (ATCC 12598) were obtained from American Type Culture Collection (Manassas, VA).

Abs used for flow cytometric detection of EC surface proteins were FITC-conjugated mouse anti-human ICAM-1 (CD54, clone BBIG-I1) and isotype control (FITC-conjugated mouse IgG1, clone 11711), PerCP-conjugated mouse anti-human MCAM (CD146, clone 128018) (from R&D Systems, Minneapolis, MN), PE-conjugated mouse anti-human E-selectin (CD62E, clone 1.2B6) and isotype control (PE-conjugated mouse IgG1, clone 15H6) (both from SouthernBiotech, Birmingham, AL).

The following inhibitors were used: the C5-blocking Ab eculizumab (Soliris; Alexion), the TNF-blocking Ab infliximab (Remicade; Janssen Biologics), the IL-1β–blocking Ab canakinumab (Ilaris; Novartis), a blocking Ab to CD14 (r18D11) that was produced and used as described previously (18), the specific C5a receptor (C5aR1) antagonist (AcF[OPd-ChaWR]), which was a gift from Prof. John D. Lambris and synthesized as previously described (19), and the TLR4/MD2-blocking lipid A analog eritoran (E5564), which was provided by Eisai (Andover, MA). The CD20-blocking Ab rituximab (Mabthera; Roche) was used as a control Ab in all experiments.

Whole blood and HUVEC

The model for whole blood and HUVEC activation was described previously (8). Briefly, a modified version of an ex vivo whole blood model (20) was used combined with HUVEC (European Collection of Authenticated Cell Cultures, Salisbury, U.K.) seeded in 48-well plates (Costar, Corning, NY). HUVEC were used in passages two through five. On the day of the experiment, fresh human whole blood was obtained from healthy donors of both genders (age of 27–83 y), who had used no medications the previous week, and was anticoagulated with the thrombin-specific inhibitor lepirudin. In experiments with human serum, three separate pooled human sera were used. Confluent HUVEC monolayers were washed twice with sterile, 37°C PBS before the addition of 100 μl whole blood, pooled normal human serum, or growth medium with 2% FBS. The inhibitors anti-CD14 (15 μg/ml), eculizumab (100 μg/ml), C5aR1 antagonist (10 μM), infliximab (100 μg/ml), canakinumab (100 μg/ml), eritoran (1μM), rituximab as a control Ab (100 μg/ml), or PBS were added in a total volume of 20 μl and incubated for 4 min at 37°C prior to addition of PBS, heat-inactivated *E.coli* (10^5 bacteria/ml unless specified otherwise) or heat-inactivated *S. aureus* (10^7 bacteria/ml, unless specified otherwise). Samples were then incubated at 37°C with 5% CO_2 for 4 h with gentle shaking. Thereafter, whole blood, serum, or medium was removed and EDTA (20 mM) was added before centrifugation for 15 min at 3000 × *g* at 4°C. Plasma or supernatant was stored at −70°C until analysis.

EC activation markers

After removal of medium, serum, or whole blood, HUVEC monolayers were gently washed twice with ice-cold PBS, fixed with 0.5% paraformaldehyde, and incubated at 4°C for 2.5 min, as described previously (21). After gentle washing with PBS, anti–ICAM-1-FITC and anti–E-selectin-PE, or their isotype controls, and anti–MCAM-PerCP were added and plates were incubated for 30 min at 4°C. Cells were washed twice with PBS, briefly trypsinated, and transferred to 5-ml polypropylene tubes (Sarstedt, Nuernbrecht, Germany), washed with PBS with 0.1% BSA, and run on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). HUVEC were gated as MCAM-positive cells. Median values were used for fluorescence intensity. Data were analyzed in FlowJo X (Tree Star, Ashland, OR).

Cytokine measurements

Analysis of plasma, serum, or medium concentrations of IL-1β, IL-6, IL-8, and TNF were done using multiplex technology. Single-plex beads were purchased from Bio-Rad Laboratories (Hercules, CA) and used according to the manufacturer's recommendations.

Statistical analysis, data presentation, and ethical approval

All data were compiled in Prism 6 (GraphPad Software, San Diego, CA). Both absolute and relative values of groups were compared using a nonparametric Friedman test with a Dunn posttest. Data were paired for each donor to eliminate donor variation, and all groups were compared with noninhibited controls unless specified otherwise. The data are presented as absolute values in the figures, but for comparison relative values have been calculated and are presented in Supplemental Figs. 2–4. Informed written consent was obtained from each donor, and the local ethics committee approved the study.

Results

Bacteria-induced EC activation

To evaluate activation of EC by Gram-negative bacteria, we incubated EC with incremental doses of *E. coli* in medium (no human serum), pooled normal human serum, or whole blood. *E. coli* in medium only could not induce EC activation. However, in the presence of human serum, 10^7 bacteria/ml caused a modest expression of E-selectin (Fig. 1A) and ICAM-1 (Fig. 1B). Notably, the sensitivity of the system was drastically increased with similar activation by 10^5 bacteria/ml in whole blood as 10^7 bacteria/ml in serum (Fig. 1A, 1B).

To evaluate activation of EC by Gram-positive bacteria, we incubated EC with incremental doses of *S. aureus* in medium (no human serum), pooled normal human serum, or whole blood. *S. aureus* did not activate EC in any bacterial concentration in medium only or in human serum. However, in the presence of whole blood, *S. aureus* caused a potent and dose-dependent upregulation of E-selectin (Fig. 1C) and ICAM-1 (Fig. 1D), although at a 100-fold increase in bacteria concentration compared with *E. coli* (Fig. 1C, 1D).

Inhibition of bacteria-induced EC activation in whole blood

We have earlier shown that the inflammatory reaction induced by both *E. coli* and *S. aureus* in human whole blood can be attenuated by combined inhibition of CD14 and one of the key complement components (14, 16). To evaluate whether bacteria-induced EC activation also would be reduced by this regimen, we incubated whole blood on EC monolayers with either *E. coli* (10^5 bacteria/ml) or *S. aureus* (10^7 bacteria/ml) in addition to blocking mAbs to CD14 and C5, alone or in combination (Fig. 2, Supplemental Fig. 2).

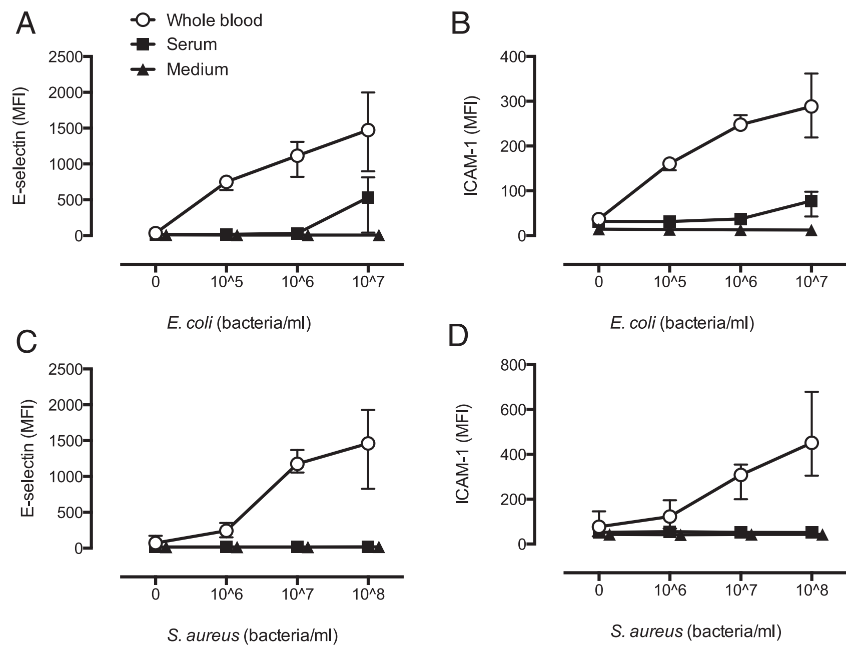

FIGURE 1. *E. coli* and *S. aureus* cause dose-dependent EC activation in whole blood. Expression of E-selectin (**A** and **C**) and ICAM-1 (**B** and **D**) on EC after incubation of *E. coli* (A and B) or *S. aureus* (C and D) in growth medium, normal human serum, or in whole blood. Data are given as mean ± range of $n = 3$ and $n = 4$ for *E. coli* and *S. aureus*, respectively.

E. coli–induced EC activation was largely attenuated by anti-CD14 alone, as evaluated by E-selectin (Fig. 2A, Supplemental Fig. 2A) and ICAM-1 (Fig. 2B, Supplemental Fig. 2B). We therefore included the TLR4/MD2 antagonist eritoran to evaluate to what extent the CD14 effect depended on TLR4 signaling. Eritoran had a comparable effect to anti-CD14 (Fig. 2A, 2B, Supplemental Fig. 2A, 2B).

S. aureus caused a time-dependent formation of C5b-9 in whole blood (Supplemental Fig. 1). Whole blood–induced EC activation was largely attenuated by complement inhibition alone, as demonstrated by the C5-blocking Ab eculizumab (Fig. 2C, 2D, Supplemental Fig. 2C, 2D). To evaluate whether this attenuating effect was due to the inhibition of C5a or C5b-9 formation, we also included a C5aR1 antagonist. The effect of the antagonist was virtually identical to that of eculizumab, indicating that the effect was mainly mediated through the C5a–C5aR1 interaction and not C5b-9 (Fig. 2C, 2D, Supplemental Fig. 2C, 2D). For both bacteria, combined inhibition of CD14 and C5 had a potent and similar degree of inhibition compared with single inhibition of either (Fig. 2, Supplemental Fig. 2).

Mediators of bacteria-induced EC activation in whole blood

As activation of EC by *E. coli* and *S. aureus* was largely dependent on whole blood, we then evaluated potential downstream, secreted mediators causing this activation. TNF and IL-1β are commonly described EC activators (22). Thus, we evaluated to what extent

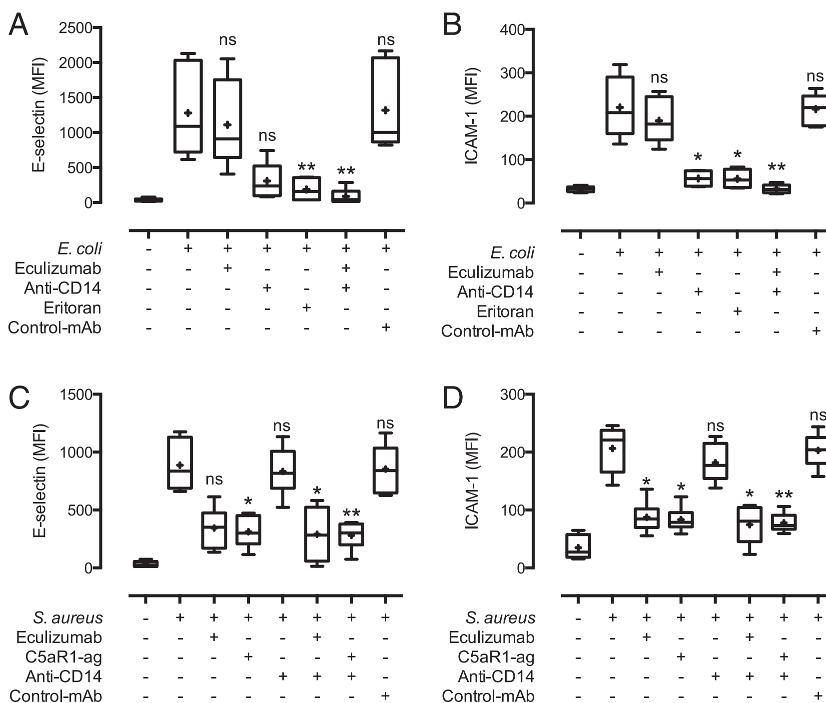

FIGURE 2. Complement (C5) and TLR (CD14 and TLR4/MD2) inhibition of whole blood attenuates EC activation by *E. coli* and *S. aureus.* Monolayers of EC were incubated with whole blood. The C5 inhibitors eculizumab or C5aR1 antagonist, anti-CD14, TLR4/MD2 antagonist eritoran, or combinations of these, as well as control Ab (rituximab) were added to the blood prior to the addition of 10^5 bacteria/ml *E. coli* (**A** and **B**) or 10^7 bacteria/ml *S. aureus* (**C** and **D**). E-selectin expression (A and C) and ICAM-1 expression (B and D) data are presented with median values (bars), mean values (+), and error bars from the 10th to 90th percentile with $n = 6$ donors. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared with bacterial incubation without inhibitors.

they could explain the whole blood bacteria-induced EC activation using Abs already in clinical use, namely infliximab (anti-TNF) and canakinumab (anti–IL-1β). TNF and IL-1β release was detectable after 2 h and increased over time after activation with both *E. coli* and *S. aureus* (Supplemental Fig. 1). Notably, although single inhibition of TNF or IL-1β only modestly reduced activation of EC, combined TNF and IL-1β inhibition completely abolished the expression of E-selectin (Fig. 3C, Supplemental Fig. 3C) and ICAM-1 (Fig. 3D, Supplemental Fig. 3D) on the EC surface to background levels. This was the case for both *E. coli*– and *S. aureus*–induced activation.

Mechanisms of complement and CD14 inhibition

To evaluate whether the effect of CD14 and C5 inhibition was due to reduced secretion of TNF and IL-1β, we analyzed cytokine release in whole blood from *E. coli* and *S. aureus* activation in the presence of HUVEC. *E. coli*–induced TNF and IL-1β secretion was reduced to background levels by combined CD14 and C5 inhibition, and it was clearly reduced by single inhibition with anti-CD14 (Fig. 4A, 4B, Supplemental Fig. 4A, 4B). We included eritoran to examine whether the CD14 effect was mainly dependent on TLR4 activation, and eritoran also reduced TNF and IL-1β secretion to background levels (Fig. 4A, 4B, Supplemental Fig. 4A, 4B), indicating that TNF and IL-1β release induced by *E. coli* was mediated through TLR4 activation. *S. aureus*–induced TNF and IL-1β secretion was reduced to background levels by combined CD14 and C5 inhibition, as well as by single inhibition of C5 (Fig. 4C, 4D, Supplemental Fig. 4C, 4D). To evaluate the mechanism behind C5-induced TNF and IL-1β release, we included a specific C5aR1 antagonist, which also reduced their release to background levels, indicating that TNF and IL-1β release induced by *S. aureus* was mainly mediated through the C5a–C5aR1 interaction and not C5b-9.

Taken together, these findings suggest that targeting key upstream recognition molecules, that is, CD14 and complement, inhibits *E. coli*– and *S. aureus*–induced EC activation by preventing blood cells from releasing the downstream inflammatory mediators TNF and IL-1β.

Discussion

In the present study, we show a mechanistic link between upstream recognition of C5 and CD14 activation and downstream TNF and IL-1β release, demonstrating that mainly C5a–C5aR1 activation combined with CD14 interaction are responsible for the TNF- and IL-1β–dependent EC activation induced by both *E. coli* and *S. aureus.* Of particular interest is that the combined inhibition of either of the two upstream molecules C5 and CD14, or the two downstream cytokines TNF and IL-1β mediators, was required to obtain a virtually complete attenuation of the EC activation.

The central role of EC in inflammatory disease is becoming increasingly apparent, and thus the importance of understanding the mechanisms behind their activation can reveal potential targets of intervention in inflammatory conditions. However, with increasing appreciation of the cross-talk between systems formerly thought of as separate, we need to develop models that at least include some of this complexity if we are to successfully translate laboratory results into clinical efficacy. To a large extent, the evaluation of EC activation has been done in vitro in isolated cell systems, where the exposure of EC to different mediators is used to evaluate the role of these mediators in EC activation. Although the list of potential activators is increasing, the relative importance of these activators in clinical inflammation remains unclear, and thus which of those provide most promise for intervention is still elusive.

We have developed a novel model of EC activation by coincubating HUVEC with whole blood. As a necessary limitation of the model, coagulation has to be prevented. However, using the thrombin-specific agent lepirudin, only the end stage of the coagulation process is inhibited, providing a more physiological environment compared with the more commonly used heparin (23). HUVEC do not express ABO Ags, and consequently ABO matching between EC and whole blood donors is not necessary (24). In this system, we can examine the effect of specific mediators on EC, but also study cell–cell interactions between EC and leukocytes and cross-talk between different blood constituents, such as platelets, the complement system, RBCs, and leukocytes, as well as evaluate interventions that might alleviate EC activation.

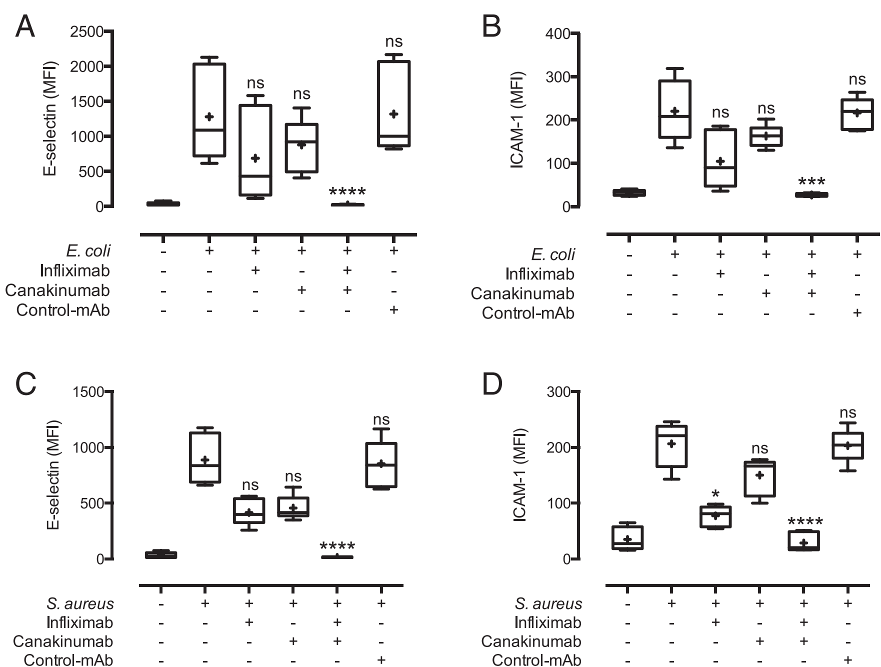

FIGURE 3. Complement (C5)- and TLR (CD14 and TLR4/MD2)-induced EC activation is mediated by TNF and IL-1β. Monolayers of EC were incubated with whole blood and the TNF-blocking Ab infliximab, the IL-1β–blocking Ab canakinumab, a combination of these, or control Ab (rituximab) prior to the addition of 10^5 bacteria/ml *E. coli* (**A** and **B**) or 10^7 bacteria/ml *S. aureus* (**C** and **D**). E-selectin expression (A and C) and ICAM-1 expression (B and D) data are presented as in Fig. 2 with $n = 6$ donors. $***p < 0.001$, $****p < 0.0001$ as compared with bacterial incubation without inhibitors.

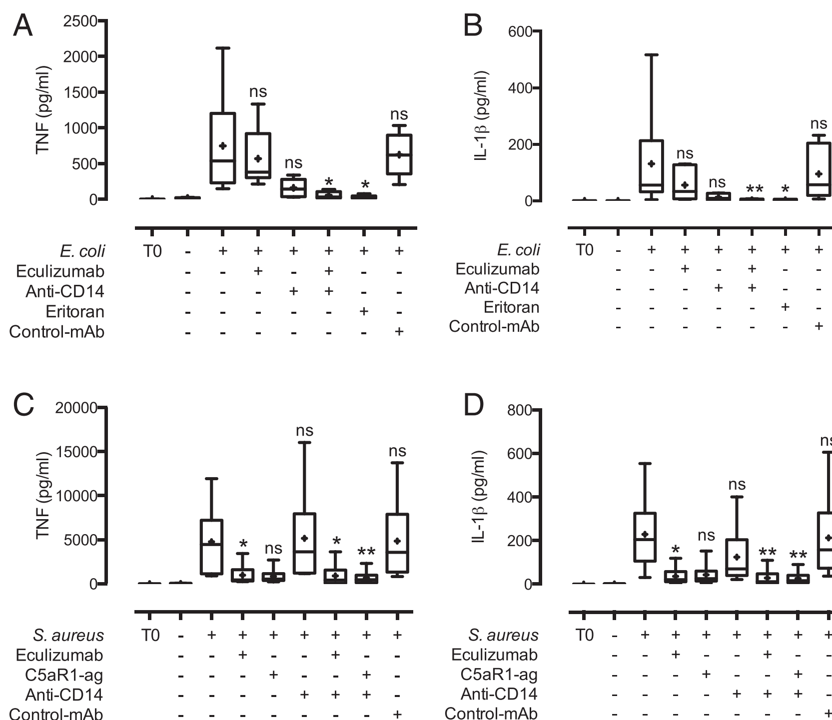

FIGURE 4. Bacteria-induced cytokine release in whole blood is attenuated by complement (C5) and TLR (CD14 and TLR4/MD2) inhibition. Plasma from whole blood incubated with monolayers of EC and 10^5 bacteria/ml *E. coli* (**A** and **B**) or 10^7 bacteria/ml *S. aureus* (**C** and **D**) and inhibitors was analyzed for TNF (A and C) and IL-1β (B and D). Data are presented as in Fig. 2 with $n = 6$ donors. $^*p < 0.05$, $^{**}p < 0.01$ as compared with bacterial activation without inhibitors.

Several studies have shown that HUVEC can react to LPS or *E. coli* directly mediated by TLR4, but the role of CD14 is less clear. Lloyd-Jones et al. (25) showed that both soluble and membrane-bound CD14 was necessary with low levels of LPS, whereas when the dose increased, the dependency of CD14 was eliminated. The potential role of TLR4 recognition by EC in vivo was further elaborated in a mouse model with TLR4 expressed exclusively in EC (26). It was found that this was sufficient to recruit leukocytes to tissue after local injection of LPS or *E. coli*, and limiting TLR4 expression to EC was beneficial, as it led to a larger number of immune cells recruited to the inflamed tissue instead of sequestered in the lungs (26). Thus, it has become clear that TLR4 on EC is capable of detecting bacterial stimuli, and this detection is sufficient to initiate a robust local inflammatory response. However, what role TLR4 activation on EC plays in inflammation in a complex, TLR4-sufficient, human system is less clear.

In our study, we found that although EC could be activated directly by *E. coli* in sufficient doses, this activation was dependent on plasma, which contains soluble CD14, and was 100-fold less potent than the response to whole blood–mediated *E. coli*–induced activation of EC. This is in line with previous studies demonstrating that plasma from *E. coli*–activated heparin anticoagulated whole blood (27) or conditioned medium with LPS-activated THP-1 cells, a monocytic cell line (28), caused ~100-fold more potent activation of EC than did activating EC directly with the same concentration of *E. coli* or LPS. Thus, it seems that although *E. coli* can directly activate EC, this does not play a significant role due to the shear scale of leukocyte-induced activation. However, as there are indications that EC-expressed TLR4 can more potently activate microvascular than macrovascular cells (29), whole blood–independent EC activation may play a larger role in the microvasculature than what we found in our model.

In a number of different models, we have previously shown that combined inhibition of CD14 and key complement components can attenuate inflammation induced by Gram-negative bacteria (15, 16, 20, 30–34), Gram-positive bacteria (14), and polymicrobial stimuli (17). We therefore intended to evaluate to what extent inhibiting whole blood inflammation would affect EC activation by using CD14- and C5-blocking Abs. We found that *E. coli*–induced whole blood–mediated activation of EC was largely CD14-dependent whereas *S. aureus*–induced activation was mediated by the complement system, mainly through C5a–C5aR1 interaction. We have earlier found that single inhibition is less efficient than combined inhibition, particularly with increased load of inflammatory stimuli (35). In the present study, we used fairly low concentrations of bacteria compared with previous studies in whole blood alone, which might explain the impressive effect of single inhibition. However, there is no doubt that a combined inhibitory approach would be superior when the causative agent is not known, as the effect of single CD14 blockage in *S. aureus*–induced inflammation or single complement inhibition in *E. coli*–induced activation is highly ineffective.

Several previous studies in less complex models have found an impressive effect of combined TNF and IL-1β inhibition in HUVEC activation. Already in 1995, Pugin et al. (36) demonstrated that combined use of a TNF-blocking Ab and an IL-1R antagonist could completely inhibit activation of HUVEC after stimulation with either plasma or highly diluted whole blood in medium incubated with the EC. Nooteboom et al. (37, 38) used media conditioned with plasma from LPS- and bacteria-activated heparin anticoagulated whole blood to look at EC expression of adhesion molecules and permeability, and Schildberger et al. (28, 39) used conditioned media from LPS-activated THP-1 cells, a monocytic cell line, to activate HUVEC. Both groups found a central role for TNF and IL-1β in EC activation.

Although these studies do take us a step closer to a clinically more relevant model of EC activation, there are some important shortcomings. Using activated plasma, mediators with short half-lives such as C5a, a central complement system anaphylatoxin postulated to activate EC (40–42), as well as cell–cell interaction between leukocytes and EC cannot be modeled. Furthermore, diluting the plasma or whole blood dramatically also entails a danger of including dilution-specific effects, as for instance the alternative pathway of the complement system is close to inactivated at plasma concentrations <6–10% (43).

We therefore evaluated to what extent TNF and IL-1β could account for the whole blood–induced activation in our model. We found a robust time-dependent release of TNF and IL-1β starting from 2 h of incubation, and that combined inhibition of TNF and IL-1β completely blocked HUVEC activation induced by bacteria-stimulated whole blood. Importantly, the central role of TNF and IL-1β seems to hold both with endogenous and exogenous stimuli, as we have previously shown in the same model that blocking TNF completely abrogated cholesterol crystal–induced activation of HUVEC (8). To examine whether the effects of C5 and CD14 blockage were mediated by their ability to eliminate the release of TNF and IL-1β, we analyzed cytokine release in the plasma from the experiments. Indeed, we found that TNF and IL-1β release from whole blood after stimulation with both Gram-negative and Gram-positive bacteria was completely eliminated by combined C5 and CD14 blockage, thus supporting the central role of these two cytokines in inducing EC activation

Thus, in our complex human model, EC are activated not directly by the bacteria, but indirectly, due to bacterial activation of leukocytes. This leads to the secretion of TNF and IL-1β, which then activate the EC. Interestingly, despite the large number of inflammatory mediators in the system, only TNF and IL-1β contributed significantly toward EC activation. One can speculate about why other inflammatory mediators did not seem to significantly contribute toward direct EC activation. EC receptors for activators such as C5a and LPS could be outcompeted by the shear number of receptors for these molecules on leukocytes. The lack of activation could also be due to a lower potency of these mediators, where the overwhelming scale of TNF- and IL-1β–induced activation drowns out their effect. No matter the mechanisms involved, our findings suggest the importance of not only describing mediators that are capable of activating the immune system, but also evaluating the relative importance of these mediators in complex systems. Although mediators are found to cause activation in in vitro single-cell systems, this might not translate into significant relevance as the complexity of the system increases.

In conclusion, this study demonstrates the ability of a novel whole blood and EC model to evaluate EC activation in a complex human ex vivo system. Doing so, we found that EC activation by both Gram-negative and Gram-positive bacteria could be eliminated in the upstream recognition phase by dual inhibition of C5 and CD14. We also found that CD14- and C5-mediated activation caused EC adhesion molecule expression due to subsequent leukocyte release of TNF and IL-1β, and blocking these mediators completely abrogated EC activation. Thus, both upstream combined targeting of C5 and CD14 and downstream combined targeting of TNF and IL-1β appear to be promising foci to modulate EC activation in inflammation.

Disclosures

The authors have no financial conflicts of interest.

References

1. Martin, G. S., D. M. Mannino, S. Eaton, and M. Moss. 2003. The epidemiology of sepsis in the United States from 1979 through 2000. *N. Engl. J. Med.* 348: 1546–1554.
2. Mai, J., A. Virtue, J. Shen, H. Wang, and X.-F. Yang. 2013. An evolving new paradigm: endothelial cells—conditional innate immune cells. *J. Hematol. Oncol.* 6: 61.
3. Harding, M., and P. Kubes. 2012. Innate immunity in the vasculature: interactions with pathogenic bacteria. *Curr. Opin. Microbiol.* 15: 85–91.
4. van Hinsbergh, V. W. M. 2012. Endothelium—role in regulation of coagulation and inflammation. *Semin. Immunopathol.* 34: 93–106.
5. Zhao, H., A. R. Anand, and R. K. Ganju. 2014. Slit2-Robo4 pathway modulates lipopolysaccharide-induced endothelial inflammation and its expression is dysregulated during endotoxemia. *J. Immunol.* 192: 385–393.
6. London, N. R., W. Zhu, F. A. Bozza, M. C. Smith, D. M. Greif, L. K. Sorensen, L. Chen, Y. Kaminoh, A. C. Chan, S. F. Passi, C. W. Day, et al. 2010. Targeting Robo4-dependent Slit signaling to survive the cytokine storm in sepsis and influenza. *Sci. Transl. Med.* 2: 23ra19.
7. Jones, C. A., N. R. London, H. Chen, K. W. Park, D. Sauvaget, R. A. Stockton, J. D. Wythe, W. Suh, F. Larrieu-Lahargue, Y.-S. Mukouyama, et al. 2008. Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. *Nat. Med.* 14: 448–453.
8. Nymo, S., N. Niyonzima, T. Espevik, and T. E. Mollnes. 2014. Cholesterol crystal-induced endothelial cell activation is complement-dependent and mediated by TNF. *Immunobiology* 219: 786–792.
9. Kumar, H., T. Kawai, and S. Akira. 2009. Toll-like receptors and innate immunity. *Biochem. Biophys. Res. Commun.* 388: 621–625.
10. Di Gioia, M., and I. Zanoni. 2015. Toll-like receptor co-receptors as master regulators of the immune response. *Mol. Immunol.* 63: 143–152.
11. Kolev, M., G. Le Friec, and C. Kemper. 2014. Complement—tapping into new sites and effector systems. *Nat. Rev. Immunol.* 14: 811–820.
12. Holers, V. M. 2014. Complement and its receptors: new insights into human disease. *Annu. Rev. Immunol.* 32: 433–459.
13. Mollnes, T. E., W.-C. Song, and J. D. Lambris. 2002. Complement in inflammatory tissue damage and disease. *Trends Immunol.* 23: 61–64.
14. Skjeflo, E. W., D. Christiansen, T. Espevik, E. W. Nielsen, and T. E. Mollnes. 2014. Combined inhibition of complement and CD14 efficiently attenuated the inflammatory response induced by *Staphylococcus aureus* in a human whole blood model. *J. Immunol.* 192: 2857–2864.
15. Lappegård, K. T., D. Christiansen, A. Pharo, E. B. Thorgersen, B. C. Hellerud, J. Lindstad, E. W. Nielsen, G. Bergseth, D. Fadnes, T. G. Abrahamsen, et al. 2009. Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. *Proc. Natl. Acad. Sci. USA* 106: 15861–15866.
16. Brekke, O.-L., D. Christiansen, H. Fure, A. Pharo, M. Fung, J. Riesenfeld, and T. E. Mollnes. 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.
17. Huber-Lang, M., A. Barratt-Due, S. E. Pischke, Ø. Sandanger, P. H. Nilsson, M. A. Nunn, S. Denk, W. Gaus, T. Espevik, and T. E. Mollnes. 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: 5324–5331.
18. Lau, C., K. S. Gunnarsen, L. S. Høydahl, J. T. Andersen, G. Berntzen, A. Pharo, J. K. Lindstad, J. K. Ludviksen, O.-L. Brekke, A. Barratt-Due, et al. 2013. Chimeric anti-CD14 IGG2/4 hybrid antibodies for therapeutic intervention in pig and human models of inflammation. *J. Immunol.* 191: 4769–4777.
19. Finch, A. M., A. K. Wong, N. J. Paczkowski, S. K. Wadi, D. J. Craik, D. P. Fairlie, and S. M. Taylor. 1999. Low-molecular-weight peptidic and cyclic antagonists of the receptor for the complement factor C5a. *J. Med. Chem.* 42: 1965–1974.
20. Mollnes, T. E., O.-L. Brekke, M. Fung, H. Fure, D. Christiansen, G. Bergseth, V. Videm, K. T. Lappegård, J. Köhl, and J. D. Lambris. 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.
21. Gräbner, R., U. Till, and R. Heller. 2000. Flow cytometric determination of E-selectin, vascular cell adhesion molecule-1, and intercellular cell adhesion molecule-1 in formaldehyde-fixed endothelial cell monolayers. *Cytometry* 40: 238–244.
22. Haskard, D. O. 2008. Endothelial Activation in Inflammation: Lessons Learned from E-Selectin. In *Vascular Complications in Human Disease: Mechanisms and Consequences.* D. Abraham, M. Dashwood, C. Handler, and G. Coghlan, eds. Springer, London, p. 77–93.
23. Bexborn, F., A. E. Engberg, K. Sandholm, T. E. Mollnes, J. Hong, and K. Nilsson Ekdahl. 2009. Hirudin versus heparin for use in whole blood in vitro biocompatibility models. *J. Biomed. Mater. Res. A* 89: 951–959.
24. O'Donnell, J., B. Mille-Baker, and M. Laffan. 2000. Human umbilical vein endothelial cells differ from other endothelial cells in failing to express ABO blood group antigens. *J. Vasc. Res.* 37: 540–547.
25. Lloyd-Jones, K. L., M. M. Kelly, and P. Kubes. 2008. Varying importance of soluble and membrane CD14 in endothelial detection of lipopolysaccharide. *J. Immunol.* 181: 1446–1453.
26. Andonegui, G., H. Zhou, D. Bullard, M. M. Kelly, S. C. Mullaly, B. McDonald, E. M. Long, S. M. Robbins, and P. Kubes. 2009. Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection. *J. Clin. Invest.* 119: 1921–1930.
27. Nooteboom, A., C. J. van der Linden, and T. Hendriks. 2004. Modulation of adhesion molecule expression on endothelial cells after induction by lipopolysaccharide-stimulated whole blood. *Scand. J. Immunol.* 59: 440–448.
28. Schildberger, A., E. Rossmanith, V. Weber, and D. Falkenhagen. 2010. Monitoring of endothelial cell activation in experimental sepsis with a two-step cell culture model. *Innate Immun.* 16: 278–287.
29. Lu, Z., Y. Li, J. Jin, X. Zhang, M. F. Lopes-Virella, and Y. Huang. 2012. Toll-like receptor 4 activation in microvascular endothelial cells triggers a robust inflammatory response and cross talk with mononuclear cells via interleukin-6. *Arterioscler. Thromb. Vasc. Biol.* 32: 1696–1706.
30. Thorgersen, E. B., B. C. Hellerud, E. W. Nielsen, A. Barratt-Due, H. Fure, J. K. Lindstad, A. Pharo, E. Fosse, T. I. Tønnessen, H. T. Johansen, et al. 2010. CD14 inhibition efficiently attenuates early inflammatory and hemostatic responses in *Escherichia coli* sepsis in pigs. *FASEB J.* 24: 712–722.

31. Hellerud, B. C., A. Aase, T. K. Herstad, L. M. Naess, L. H. Kristiansen, A.-M. S. Trøseid M. Harboe, K. T. Lappegård, P. Brandtzaeg, E. A. Høiby, and T. E. Mollnes. 2010. Critical roles of complement and antibodies in host defense mechanisms against *Neisseria meningitidis* as revealed by human complement genetic deficiencies. *Infect. Immun.* 78: 802–809.
32. Thorgersen, E. B., A. Pharo, K. Haverson, A. K. Axelsen, P. Gaustad, G. J. Kotwal, G. Sfyroera, and T. E. Mollnes. 2009. Inhibition of complement and CD14 attenuates the *Escherichia coli*-induced inflammatory response in porcine whole blood. *Infect. Immun.* 77: 725–732.
33. Brekke, O.-L., D. Christiansen, H. Fure, M. Fung, and T. E. Mollnes. 2007. The role of complement C3 opsonization, C5a receptor, and CD14 in *E. coli*-induced up-regulation of granulocyte and monocyte CD11b/CD18 (CR3), phagocytosis, and oxidative burst in human whole blood. *J. Leukoc. Biol.* 81: 1404–1413.
34. Sprong, T., A.-S. W. Møller, A. Bjerre, E. Wedege, P. Kierulf, J. W. M. van der Meer, P. Brandtzaeg, M. van Deuren, and T. E. Mollnes. 2004. Complement activation and complement-dependent inflammation by *Neisseria meningitidis* are independent of lipopolysaccharide. *Infect. Immun.* 72: 3344–3349.
35. Egge, K. H., A. Barratt-Due, S. Nymo, J. K. Lindstad, A. Pharo, C. Lau, T. Espevik, E. B. Thorgersen, and T. E. Mollnes. 2015. The anti-inflammatory effect of combined complement and CD14 inhibition is preserved during escalating bacterial load. *Clin. Exp. Immunol.* 181: 457–467.
36. Pugin, J., R. J. Ulevitch, and P. S. Tobias. 1995. Tumor necrosis factor-α and interleukin-1β mediate human endothelial cell activation in blood at low endotoxin concentrations. *J. Inflamm.* 45: 49–55.
37. Nooteboom, A., R. P. Bleichrodt, and T. Hendriks. 2006. Modulation of endothelial monolayer permeability induced by plasma obtained from lipopolysaccharide-stimulated whole blood. *Clin. Exp. Immunol.* 144: 362–369.
38. Nooteboom, A., C. J. van der Linden, and T. Hendriks. 2005. Whole blood-mediated endothelial permeability and adhesion molecule expression: a model study into the effects of bacteria and antibiotics. *J. Antimicrob. Chemother.* 55: 150–156.
39. Schildberger, A., T. Buchacher, V. Weber, and D. Falkenhagen. 2011. Adsorptive modulation of inflammatory mediators dampens endothelial cell activation. *Blood Purif.* 32: 286–295.
40. Foreman, K. E., M. M. Glovsky, R. L. Warner, S. J. Horvath, and P. A. Ward. 1996. Comparative effect of C3a and C5a on adhesion molecule expression on neutrophils and endothelial cells. *Inflammation* 20: 1–9.
41. Kilgore, K. S., J. P. Shen, B. F. Miller, P. A. Ward, and J. S. Warren. 1995. Enhancement by the complement membrane attack complex of tumor necrosis factor-α-induced endothelial cell expression of E-selectin and ICAM-1. *J. Immunol.* 155: 1434–1441.
42. Foreman, K. E., A. A. Vaporciyan, B. K. Bonish, M. L. Jones, K. J. Johnson, M. M. Glovsky, S. M. Eddy, and P. A. Ward. 1994. C5a-induced expression of P-selectin in endothelial cells. *J. Clin. Invest.* 94: 1147–1155.
43. Harboe, M., G. Ulvund, L. Vien, M. Fung, and T. E. Mollnes. 2004. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin. Exp. Immunol.* 138: 439–446.

Paper III

Complement factor 5 blockade reduces porcine myocardial infarction size and improves immediate cardiac function

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In press

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In press

Abstract

Inhibition of complement factor 5 (C5) reduced myocardial infarction in animal studies, while no benefit was found in clinical studies. Due to lack of cross-reactivity of clinically used C5 antibodies, different inhibitors were used in animal and clinical studies. Coversin (*Ornithodoros moubata* complement inhibitor, OmCI) blocks C5 cleavage and binds leukotriene B4 in humans and pigs. We hypothesized that inhibition of C5 before reperfusion will decrease infarct size and improve ventricular function in a porcine model of myocardial infarction.

In pigs (*sus scrofa*), the left anterior descending coronary artery was occluded (40 min) and reperfused (240 min). Coversin or placebo was infused 20 min after occlusion and throughout reperfusion in 16 blindly randomized pigs. Coversin significantly reduced myocardial infarction in the area at risk by 39% ($p=0.03$, triphenyl tetrazolium chloride staining) and by 19% ($p=0.02$) using magnetic resonance imaging. The methods correlated significantly ($R=0.92$, $p<0.01$). Tissue Doppler echocardiography showed increased systolic displacement (31%, $p<0.01$) and increased systolic velocity (29%, $p=0.01$) in coversin treated pigs. Interleukin-1β in myocardial microdialysis fluid was significantly reduced (31%, $p<0.05$) and tissue E-selectin expression was significantly reduced ($p=0.01$) in the non-infarcted area at risk by coversin treatment. Coversin ablated plasma C5 activation throughout the reperfusion period and decreased myocardial C5b-9 deposition, while neither plasma nor myocardial LTB4 were significantly reduced.

Coversin substantially reduced the size of infarction, improved ventricular function, and attenuated interleukin-1β and E-selectin in this porcine model by inhibiting C5. We conclude that inhibition of C5 in myocardial infarction should be reconsidered.

Keywords: ischemia/reperfusion, myocardial infarction, complement, C5, contractility, LTB4

In press

Introduction

The introduction of early reperfusion therapy of acute myocardial infarction (MI) in the clinical setting has decreased morbidity and mortality and improved post-MI cardiac function. However, a considerable part of the ischemic myocardium is still lost upon reperfusion. Ischemia and reperfusion cause liberation of damage associated molecular patterns (DAMP) from ischemic or injured cells, activating innate immune responses, a prerequisite for the healing process, currently reviewed in [19]. However, overactivation causes detrimental effects by injuring the myocardium, an effect termed ischemia/reperfusion injury (IRI) [22], leading to aggravated infarctsize and pump failure.

Complement is an upstream sensor and effector system of innate immunity, a key system for immune surveillance and homeostasis, but also implicated to play a critical role in the pathophysiology of myocardial IRI [4, 35]. Complement as a danger sensing alarm system relies on soluble pattern recognition receptors of three different activation pathways, the classical, the lectin and the alternative pathway [35]. They all converge at the central component C3, which is cleaved into C3a and C3b and subsequently leads to cleavage of C5, which generates the potent anaphylatoxin C5a and the terminal C5b-9 complement complex, both exerting proinflammatory effector functions [35].

Complement inhibition in myocardial infarction was first shown to reduce infarction size in rodents already in 1990 [47]. Experimental studies investigating complement inhibition in a clinically relevant context are rare, *i.e.* the inhibitor was given after onset of ischemia, but confirmed the protective potential of C5 inhibition [44]. Pigs are highly recognized for the translational value of results obtained [20], however C5 inhibition has not been tested as no inhibitors for pig C5 have been available. Inhibition of various other parts of the complement

In press

cascade by inhibition of complement factor 1 [21], treatment with soluble complement receptor 1 [2], protecting the endothelium with dextran sulfate [3] and tyrosine-O-sulfate [4] clearly showed the potential of complement inhibition in pigs. Clinical studies with the C5-antibody pexelizumab were therefore performed without prior preclinical testing and the results were disappointing [15, 31]. Administration of the anti-C5 antibody during percutaneous coronary intervention neither reduced myocardial infarction nor decreased mortality [23]. However, a major concern with these studies was that complement activation measured by soluble C5b-9 (sC5b-9), the final activation product that should be completely blocked by the antibody, increased similarly in the treatment and the placebo groups [31] leading to discussion whether a too low dose of the anti-C5 drug had been used.

The tick derived, specific C5 inhibitor coversin (*Ornithodoros moubata* Complement Inhibitor, OmCI), prevents equally efficiently the cleavage of C5 in humans and pigs [32] [6]. The potency of coversin in inhibiting C5 in comparison to the clinically used C5 inhibitor eculizumab, which has been derived from the same clone as its predecessor pexelizumab [43], is not known. Additionally, coversin also has an internal binding pocket for leukotriene B4 (LTB4) [39], an arachidonic acid metabolite thought to play a role in myocardial IRI [25]. However, the magnitude and effect of LTB4 binding on the physiologic effects of coversin are uncertain.

We hypothesized that the C5 inhibitor coversin could reduce infarct size and improve myocardial function in a clinically relevant porcine model of acute myocardial infarction.

In press

Material and Methods

Animal preparation

The ethics committee of the Norwegian Food Safety Authority approved this study in pigs (approval number: 68/11-3811) and all experiments were performed in concordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Housekeeping, anaesthesia, euthanasia, and recording of hemodynamic and respiratory parameters were performed in accordance to ARRIVE guidelines as shown in table (Online Resource 1) and as reported previously [5]. Briefly, anaesthesia was induced in twenty-one 20 kg pigs by intramuscular ketamine (800 mg), azaperone (80 mg), atropine (1 mg) followed by intravenous (iv) pentobarbital 1-3 mg kg^{-1} and maintained using iv morphine 1-2 mg kg^{-1} h^{-1} and isoflurane 1.0-1.5% in oxygen/air mixture. After sternotomy, a silastic occluding tape was placed around the left anterior descending (LAD) coronary artery distal to the second diagonal branch allowing reversible complete occlusion. Microdialysis catheters (CMA 71, 100 kDa cut-off, 2 cm membrane, 1 µl min^{-1} flow, M Dialysis, Solna, Sweden) were placed in the LAD dependent area and in a control region supported by the left circumflex artery (Cx).

Experimental protocol

Ischemia was induced for a total of forty minutes by LAD occlusion, except for sham animals. Twenty minutes prior to reperfusion, sixteen animals were randomized to treatment with coversin or saline (NaCl 0.9%, placebo group), n=8 in each group. Coversin (Akari Therapeutics Plc, London, UK) has a plasma half-live of about 30 h due to stable binding to C5 [18] and was diluted in saline. It was given as a 1 mg kg^{-1} bolus, and followed by a continuous infusion of 0.036 mg kg^{-1} h^{-1} [5]. The control group and the three sham animals received the same amount of saline without coversin. Fifteen minutes before euthanasia, iv magnetic

In press

resonance imaging (MRI) contrast agent gadoteric acid (0.4 mM kg^{-1}, Dotarem, Guerbet, Paris, France) was given [34]. Just before euthanasia, LAD was re-occluded and iv Evans Blue (2% in 40 ml phosphate buffered saline, Sigma Aldrich, St. Louis, MO) was given to delineate the area at risk (AAR). Euthanasia was carried out by iv injection of pentobarbital (500 mg), morphine (30 mg), and potassium chloride (50 mmol). After euthanasia, the heart was excised and rinsed in ice-cold saline.

Arterial blood samples were obtained prior to surgery, after stabilization prior to induction of ischemia, at the end of 40 min of ischemia, and every hour throughout the reperfusion period. Samples were taken for blood gas analysis, serum, and EDTA-plasma preparation and were immediately cooled and centrifuged prior to storage at -80^0C. Microdialysis samples and thermal dilution cardiac output were obtained at the same time-points. After euthanization, tissue samples were taken from the center of the Evans blue free area (AAR), at the border of the Evans blue free area (border zone) and in the Evans blue stained Cx region (control area) and snap-frozen in approximately 1 ml OCT™ (Sakura Finetek Europe, Zoeterwoude, the Netherlands) prior storage at -80^0C.

In press

Infarct size assessed by magnetic resonance imaging

After tissue sampling, air-filled balloons were placed in the left and right ventricle. MRI analysis was performed using a 3 Tesla scanner (Philips, the Netherlands). T1-weighted images (3D FFE, TR/TE = 5.4/2.3 ms, flip angle 35°, BW = 434 Hz, 125 slices and scan duration = 02:15) with a measured isotropic resolution of 0.8 mm covering the entire heart were acquired using a quadrature head coil. Additionally, T1 measurement sequence was performed (Look Locker sequence: T1w TFE with “shared” inversion pulse, TR/TE = 2.3/4.3 ms, flip angle =

3°, inversion delay = 38.4 ms, phase interval = 65.5 ms, BW = 853 Hz, SENSE factor 2, isotropic resolution of 1mm), and T1 maps were reconstructed using NordicIce (NordicNeuroLab, Bergen, Norway). The segmentation of the infarcted volumes was done in OsiriX [37]. T1map was used to discriminate infarcted areas with the 3D region-growing tool (threshold of 400). The used threshold lead to inclusion of pericardium and endocardium as well but as the amount is comparable and small in all groups and subjective manual processing would have been necessary, we did not subtract it from the total infarcted volume. Infarction size (ml) was determined in T1 weighted images and compared to the total left ventricular volume.

Infarct size assessed by histological staining

After MRI, the left ventricle was cut in 5 mm thick slices. The non-stained AAR was dissected and immersed in tetrazolium chloride (TTC, 1% in phosphate buffered saline, Sigma Aldrich, St. Louis, MO) at 38°C for 20 min. Slices were placed in 4% formaldehyde solution (Histolab Products AB, Gothenburg, Sweden) on ice for 30 min prior to digital scanning. Infarct size was determined as percentage of AAR as described previously [20] using Photoshop CS5 (Adobe Systems Software Ltd., Ireland).

Echocardiography

Systolic left ventricular function was assessed by echocardiography from a four-chamber view prior to ischemia and at the end of the reperfusion period (GE Vivid 7, Horton, Norway). Peak systolic velocity and systolic displacement of the mitral plane were obtained from pulse Doppler echocardiography and averaged from the septum and the lateral wall (Echopac PC Version 112, GE Vingmed Ultrasound, Horten, Norway).

In press

Immunofluorescence analysis

The snap-frozen tissues were cut into 5µm thick sections, air-dried for 60 minutes and fixed with cold acetone for 10 minutes. They were either processed immediately or stored at -80℃ until further analysis. Then, after hydration, the sections were stained using a two-step indirect immunofluorescence technique. For E-selectin, the following primary and secondary antibodies were used: mouse anti-human E-selectin (Sigma, St. Louis, MO) and goat anti-mouse IgG-Alexa546 (Molecular probes, Carlsbad, CA, USA). The antibodies used for Fibrinogen-like protein 2 (FGL-2) were rabbit anti-FGL2 (Aviva Systems Biology Corp, San Diego, CA) and sheep anti-rabbit IgG-Cy3 (Sigma, St. Louis, MO, USA). A nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO). A fluorescence microscope (DMI4000B; Leica, Wetzlar, Germany) was used to analyse the slides and the quantification of fluorescence intensity was performed using Image J software, version 1.50 (https://rsb.info.nih.gov/ij/) on TIFF images. All pictures were taken under the same conditions to allow for correct quantifications and comparison of fluorescence intensities.

In vitro assessment of complement inhibitory effects of coversin and eculizumab

Human and porcine whole blood samples anticoagulated with lepirudin (Celgene, Marburg, Germany) were pre-incubated with coversin or eculizumab (Alexion Pharmaceuticals, CT) in a twofold serial dilution (final concentrations of 1.6, 0.8, 0.4, 0.2 and 0.1 µM) or PBS for the uninhibited control in sterile polypropylene tubes for 5 min at 37°C. Subsequently, blood specimens were stimulated with zymosan at a final concentration of 50 µg/ml, or PBS for the negative control. After 30 min, the reaction was stopped by adding EDTA (final concentration 10 mM), samples centrifuged (3000 *g*, 15 min, 4°C). The resulting plasma was stored at -80℃ before analysis of C5b-9. Human and porcine serum samples were pre-incubated with coversin or eculizumab in a twofold serial dilution (final concentrations of 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1

In press

μM) or PBS for the uninhibited control in sterile polypropylene tubes for 5 min (room temperature) before analysis for functional complement activity.

Functional complement activity and C5b-9 (TCC)

Commercially available enzyme immune assay (Complement System Screen Wieslab; Euro Diagnostica, Malmö, Sweden) and murine anti-human C5b-9 antibody (clone aE11, Dako, Glosstrup, Denmark) were used according to manufacturer's instructions to detect functional complement activity and sC5b-9 production in plasma, respectively. Both methods detect the respective human and pig epitopes [41]. In tissue, the membrane form of C5b-9 was visualized in frozen sections from the AAR, border zone and control area. Tissue samples were incubated for 30 min at room temperature using the murine anti-human C5b-9 antibody (clone aE11, Dako, Glostrup, Denmark) diluted 1/25 in Dako antibody diluent (Dako K8006, Glostrup, Denmark), washed in phosphate buffered saline and stained by Ventana ultra View Universal DAB Detection Kit (Ventana Medical Systems, Inc., Tucson, AZ) according to the manufacturer's instructions. A Nikon Eclipse E1000M microscope was used and photos were obtained with original 40 X magnification.

Myocardial metabolism and inflammation

Microdialysis fluid from the AAR and control Cx region and EDTA plasma was assessed for inflammatory mediators interleukin (IL)-1β, IL-6, IL-8, IL-10, and TNF using a porcine multiplex cytokine assay on a Bio-Plex 100 system (Bio-Rad, Hercules, CA) as previously described [9]. LTB4 from plasma and myocardial tissue was measured using a competitive enzyme immunoassay according to the manufacturer's instructions (R&D systems, Minnesota, MN).

In press

Markers of cardiac injury

Serum troponin-T levels were determined at the institutional clinical laboratory (Modular E170, Roche Diagnostics, Switzerland). Plasma heart fatty acid binding protein H-FABP levels were measured by ELISA in accordance to manufacture's instruction (Hycult Biotech, Uden, The Netherlands).

Statistics

Investigators were blinded to the treatment during the experiments and all analyses.

Two animals died immediately after reperfusion due to ventricular fibrillation (one coversin and one placebo treated animal) and were excluded. Thus, functional complement activity was analysed in 16 animals subjected to LAD occlusion and three sham-operated animals. Complete inhibition of all three complement pathways by coversin treatment was confirmed in all animals, except for one, which was excluded after statistical confirmation of outlier behaviour (Grubbs' test, $p < 0.05$). Thus, 15 animals (seven coversin and eight control animals) were used in all further analyses if not stated otherwise.

Two animals (one coversin and one control animal) had significantly smaller AAR determined by Evans Blue staining due to anatomical variations of the LAD and were therefore excluded from MRI analysis. Microdialysis catheters ceased function before 120 min of reperfusion in two coversin and one control animal and statistical comparison was therefore done with five and seven animals, respectively.

If not stated otherwise, values are presented as mean ± standard deviation (SD). Values obtained for coversin treated and control animals were compared at defined time points using Mann-Whitney U test. 2-way ANOVA was used if more than two groups had to be compared. Linear

In press

mixed effect model (intervention as fixed effect and subject number as random effect) was used to compare groups throughout the whole study period. Multiple comparisons were *post-hoc* Bonferroni corrected. The Pearson correlation coefficient was calculated to compare infarct sizes determined by TTC and MRI. Statistical analyses were performed using SPSS 22 (IBM, Armonk, NY, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

In press

Results

Effect of coversin on myocardial infarction size

Evaluation by histological staining. Myocardial ischemia and reperfusion led to an average infarct size of 49.4% ± 14.2% (mean ± SD, necrotic tissue as % of the AAR) in the control group. Coversin treated animals showed an infarct size of 30.1% ± 14.0% of the AAR, representing a significant reduction of 39% as compared to controls (p=0.03, fig. 1a and b). The AAR was comparable between coversin treated and control animals as determined by Evans Blue staining (21.2 ± 6.4% and 25.5 ± 5.5% of left ventricular volume, respectively; p = 0.12, data not shown).

Evaluation by post mortem MRI. Infarcted volume in the left ventricle was decreased from 21.1 ± 2.4 % in placebo treated animals to 17.2 ± 2.7 % in coversin treated animals as determined by MRI (19% reduction, p=0.02, fig. 1c and d). Infarction determined by TTC staining and magnetic resonance imaging were highly correlated (R=0.92, p < 0.01, Online Resource 2).

Sham operated animals, in which the LAD was not ligated, did not reveal any signs of myocardial ischemia nor infarction evaluated by histological staining and MRI. Also in all other analysis reported in this study, sham treated animals were consistently stable at baseline levels throughout the study period and are therefore not reported in further results.

Effect of coversin on myocardial function

Myocardial function was measured by tissue Doppler echocardiography, whereas cardiac output and stroke volume were measured by thermal dilution at start and end of the experiment (fig 2). Peak systolic velocity was 29% higher in the coversin treated animals than in the controls (4.6 ± 1.1 cm sec^{-1} and 3.3 ± 0.7 cm sec^{-1}, respectively; p = 0.01, fig. 2a). Likewise,

In press

systolic displacement was 31% higher in coversin treated animals than in controls (7.4 ± 1.3 mm and 5.1 ± 0.7 mm, respectively; $p < 0.01$, fig. 2b). Stroke volume was 16% higher in the coversin treated animals than in the controls (23.4 ± 3.4 ml and 19.5 ± 2.4 ml, respectively; $p = 0.01$, fig. 2c). Cardiac output showed a non-significant trend to higher values in coversin treated animals compared to the controls (2.7 ± 0.4 L/min and 2.3 ± 0.2 L/min, respectively; $p = 0.09$, fig. 2d).

Effect of coversin on local myocardial inflammation

Microdialysis. The inflammasome-related IL-1β was increased at the end of reperfusion in the AAR only and this increase was significantly blunted by coversin treatment (fig. 3). IL-6 and IL-8 increased during reperfusion, both without significant effect of coversin treatment, while IL-10 and TNF did not increase form baseline levels (data not shown).

Immunofluorescence. In control animals, myocardial ischemia and reperfusion led to increased expression of E-selectin in the border zone of the AAR, while E-selectin in both the infarcted center of the AAR and Cx control region was not changed (fig. 4, left panels). Coversin significantly reduced the E-Selectin expression in the border zone (fig. 4 middle and right panels). FGL-2 was increased in the infarcted center of the AAR and the Cx control region in comparison to sham treated animals without a significant effect of coversin (data not shown).

Systemic and local myocardial effect of coversin on complement and LTB4

Complement activity was measured at all time points throughout the experiment. Coversin completely ablated complement activity measured via all the three complement activation pathways throughout the reperfusion period, whereas the activity remained unchanged in the placebo group (fig. 5a-c). Coversin treatment significantly reduced sC5b-9 to levels below

In press

baseline, in contrast to the placebo group and consistent with complete inhibition of terminal complement ($p<0.01$, fig. 5d). Dense deposition of the C5b-9 complex in placebo treated animals was observed in the AAR, in the border zone, and to a lesser extent in the non-ischemic control region (fig. 5e, left panels). Coversin treatment almost completely prevented C5b-9 deposition in AAR, the border zone, and non-ischemic control region (fig. 5e, right panels).

Plasma LTB4 concentrations during reperfusion were lower in coversin treated animals but not significantly different from placebo ($p=0.07$, fig. 6a). Myocardial LTB4 concentration was not affected by treatment in AAR, border zone, nor non-ischemic control region (fig. 6b).

Systemic effect of coversin as assessed by plasma analyses

Plasma troponin T and H-FABP increased in both the placebo and coversin groups during the reperfusion period confirming myocardial cell damage during the ischemic event (fig. 7). Lower troponin T and H-FABP values were obtained in coversin treated animals in comparison to control animals throughout the reperfusion period without reaching significance, though a trend for lower values was observed for H-FABP ($p=0.07$, fig. 6b).

Plasma concentrations of IL-1β, IL-6, IL-8, IL-10 and TNF remained at baseline levels throughout the study period (data not shown).

Comparison of coversin and eculizumab on complement activation

Coversin, but not eculizumab, effectively inhibited functional complement activity in porcine serum (fig. 8a-c), while both were equally effective in human serum (fig. 8e-g). Similarly, formation of the fluid phase sC5b-9 by the complement activator zymosan in porcine whole

In press

blood was efficiently inhibited by coversin, but not eculizumab (fig. 8d). Both inhibitors were again equally effective in human whole blood where they completely prevented zymosan-induced sC5b-9 formation (fig. 8h).

In press

Discussion

In this porcine study of myocardial IRI, C5 inhibition by coversin prior and during reperfusion significantly reduced infarct size and improved ventricular function. Complete blockade of terminal complement pathway by coversin was revealed by lack of systemic complement activity in plasma and abolished deposition of C5b-9, which was extensive in the AAR in the control group. Finally, IL-1β and E-Selectin expression in the AAR were significantly reduced by coversin.

Targeting the complement system at the terminal stage preventing C5 cleavage is a reasonable approach as proximal complement activity is left unaffected and thus important immunoprotective and immunoregulatory functions exerted particularly by C3 are preserved [12]. End products of complement activation are C5a and C5b-9. Membrane bound C5b-9 induces inflammatory responses in the course of IRI by platelet and endothelial cell activation accompanied by leukocyte infiltration [11]. The potent anaphylatoxin C5a is regarded as a crucial factor in myocardial IRI [4, 24]. In our study, the detrimental effects of C5 cleavage were prevented resulting in protective effect on both infarct size and myocardial function. It is noteworthy that comparable porcine studies where C5a effect was diminished by C5a receptor antagonism [45] or a C5a monoclonal antibody [1] showed less protection of the AAR and no effect on ventricular function. This highlights the importance of C5b-9 in myocardial reperfusion injury, while improvement of ventricular function confirms the physiological relevance of our findings. However, specific effects of coversin on myocardial function need to be investigated in studies observing long-term effects after myocardial IRI.

Leukotrienes are important multifunctional mediators of inflammation and promote neutrophil chemotaxis and adherence to capillary walls [48]. LTB4 is expressed on leucocytes after

In press

myocardial IRI [36], gets elevated in plasma in the course of myocardial infarction [42] and has been shown to be able to discriminate between cardiac and non-cardiac chest pain [26]. Coversin has an internal binding pocket capturing LTB4 and C5-inhibition prevents LTB4 formation [5]. In the present study, LTB4 in plasma did not significantly increase in the course of ischemia nor during reperfusion in placebo treated animals. This may be related to the short reperfusion time of 4 hours in this study, as a doubling of LTB4 in humans appears during the first 24 hours after acute myocardial infarction, probably in the course of endothelial cell activation [42]. However, neither plasma nor myocardial LTB4 concentrations were affected by coversin treatment indicating a negligible effect of coversin on LTB4 in this model. Furthermore, selective LTB4 blockade has only exhibited minor effects on myocardial IRI in rodents [8]. These findings indicate that the main coversin related effects observed in this study could be attributed to C5 inhibition, while LTB4 inhibition might add to the effect of C5 inhibition in long-term studies.

Large clinical studies have explored the efficacy of C5 inhibition using pexelizumab, a monoclonal antibody blocking C5 cleavage, on the outcome of myocardial infarction treated with thrombolysis [30] and percutaneous coronary intervention [15, 23]. These studies did not demonstrate convincing beneficial effects and several questions have arisen in the aftermath. Firstly, the dosing regimen of pexelizumab was only tested once, yet this has been decisive for dosages in subsequent studies [15]. Secondly, complement activity was insufficiently inhibited in both studies, and blood samples from the last trial revealed a similar increase in the formation of sC5b-9 in both placebo and treatment group [31]. This supports the notion that full inhibition of C5 is necessary to effectively reduce the harmful effects of complement activity in the heart. Ideally, coversin should have been compared to the formerly used pexelizumab or todays clinically used eculizumab, which all inhibit cleavage of C5 at different binding sites [27].

In press

However, pexelizumab and eculizumab are monoclonal antibodies with specificity for human C5 only [10] and we have shown that they do not interact with porcine C5. In this study, 0.85 μM coversin was used. In the clinical trials, 1.2 μM pexelizumab was used [14], which is equivalent to 0.6 μM eculizumab because of the double-binding property of the antibody eculizumab in contrast to the single-chain variant pexelizumab [38]. Thus, slightly higher doses of inhibitors were used in this study compared to the clinical studies, which may explain the successful prevention of reperfusion injury in this study but more importantly add evidence to the assumption that the pexelizumab dose may have been too low in order to achieve full C5 inhibition. Thirdly, administration of the C5 inhibitor in the clinical studies was probably given too late, only minutes prior to reperfusion in the hospital [23]. Therapy aiming at reduction of myocardial reperfusion injury should be initiated as early as possible after diagnosis of ischemia [22]. In this study, we aimed to mimic the clinical situation and initiated coversin treatment with a considerable time-gap prior to reperfusion. This is comparable to the clinical situation when medical treatment is started at the time of diagnosis in the prehospital setting with a time-gap prior to interventional reperfusion therapy. This approach should be easily transferrable to clinical trials.

Coversin treatment abolished IL-1β induction, which is cleaved in the inflammasome from inactive proIL-1β and is regarded as an inducer of sterile inflammation in myocardial IRI [46]. Interestingly, C5 activation and membrane bound C5b-9 have been shown to directly activate the inflammasome [29, 33], suggesting that reduced cell death and significant reduction of IL-1β observed in the present study is related to C5-inhibition. E-Selectin is essential for leukocyte recruitment, is a good marker of endothelial cell activation and the expression is IL-1β dependent [33]. Thus, the observed reduction in E-selectin expression in the border zone of the

In press

AAR in the present study might be caused by C5 inhibition through IL-1β, explaining the reduced reperfusion injury. The lack of significant increase in the rest of the cytokines might be explained by the short reperfusion time, as generation of cytokines is time-dependent and additionally affected by the limited recovery in microdialysis [28].

Pigs do not possess coronary collaterals, while humans experiencing myocardial ischemia often do. To compensate for this limitation, we therefore adopted the length of the occlusion period in this study (40 min) to a comparable length of four hours of infarction in man [17]. Isoflurane was used as anaesthetic agent in this study, although the cardioprotective properties of isoflurane are known. We chose this gas as it confers myocardial stability. Both groups received identical amounts of isoflurane and the infarction size in the positive control group was considerable and comparable to similar studies in pigs [7]. Thus, the results obtained by coversin treatment appear coversin and not isoflurane mediated. Duration of treatment was relatively short with 4 hours of reperfusion and conclusions about long-term myocardial complement activation, function and effect of coversin on LTB4 can therefore not be made. Thus, a pig closed-chest study with longer periods of treatment, reperfusion and observation should be performed prior to clinical trials investigating coversin in myocardial IRI [13, 40]. The trend to lower troponin-T and H-FABP levels during reperfusion in combination with reduced infarct size in coversin treated animals indicate that indeed myocardial IRI was reduced by coversin.

Pigs are regarded as one of the most translatable animal models in myocardial IRI research. Additionally, coversin has the same C5 binding characteristics in humans and pigs and coversin is already in clinical use in one eculizumab resistant patient as well as in phase Ib and II clinical trials (Clinicaltrials.gov NCT02591862 as well as producer's webpage akaritx.com). Thus, the

In press

approach outlined in this study including the dosing regimen might be directly transferable to a clinical study investigating myocardial IRI when the long-term effects of coversin on myocardial cell survival and function as discussed above have been elucidated, complying with the proposed outline of future clinical studies targeting reperfusion injury in patients with myocardial infarction [16, 19].

In conclusion, we show in this clinically relevant model of myocardial IRI that complement inhibition of C5 reduces infarction size, possibly through reduction of IL-1β and E-selectin, and improves ventricular function. Accordingly, on the basis of concerns with previous studies and the results of this study we reason that there is a need to reconsider the use of complement inhibition especially at the level of C5 in clinical myocardial infarction.

Conflict of Interest

Dr. Nunn has a patent WO 2004/106369 Complement Inhibitors licensed and is an employee of Akari Therapeutics Plc who is developing coversin (OmCI) as a drug. The other authors declare that they have no conflict of interest.

In press

References

1. Amsterdam EA, Stahl GL, Pan HL, Rendig SV, Fletcher MP, Longhurst JC (1995) Limitation of reperfusion injury by a monoclonal antibody to C5a during myocardial infarction in pigs. Am J Physiol 268:H448-457 doi:n.a.

2. Banz Y, Hess OM, Robson SC, Csizmadia E, Mettler D, Meier P, Haeberli A, Shaw S, Smith RA, Rieben R (2007) Attenuation of myocardial reperfusion injury in pigs by Mirococept, a membrane-targeted complement inhibitor derived from human CR1. Cardiovasc Res 76:482-493 doi:10.1016/j.cardiores.2007.07.016

3. Banz Y, Hess OM, Robson SC, Mettler D, Meier P, Haeberli A, Csizmadia E, Korchagina EY, Bovin NV, Rieben R (2005) Locally targeted cytoprotection with dextran sulfate attenuates experimental porcine myocardial ischaemia/reperfusion injury. Eur Heart J 26:2334-2343 doi:10.1093/eurheartj/ehi421

4. Banz Y, Rieben R (2012) Role of complement and perspectives for intervention in ischemia-reperfusion damage. Ann Med 44:205-217 doi:10.3109/07853890.2010.535556

5. Barratt-Due A, Thorgersen EB, Egge K, Pischke S, Sokolov A, Hellerud BC, Lindstad JK, Pharo A, Bongoni AK, Rieben R, Nunn M, Scott H, Mollnes TE (2013) Combined inhibition of complement C5 and CD14 markedly attenuates inflammation, thrombogenicity, and hemodynamic changes in porcine sepsis. J Immunol 191:819-827 doi:10.4049/jimmunol.1201909

6. Barratt-Due A, Thorgersen EB, Lindstad JK, Pharo A, Lissina O, Lambris JD, Nunn MA, Mollnes TE (2011) Ornithodoros moubata Complement Inhibitor Is an Equally Effective C5 Inhibitor in Pigs and Humans. J Immunol 187:4913-4919 doi:10.4049/jimmunol.1101000

In press

7. Baumert JH, Hein M, Gerets C, Baltus T, Hecker KE, Rossaint R (2009) The Effect of Xenon on Isoflurane Protection Against Experimental Myocardial Infarction. J Cardiothorac Vasc Anesth 23:614-618 doi:10.1053/j.jvca.2009.01.028

8. Bitencourt CS, Bessi VL, Huynh DN, Menard L, Lefebvre JS, Levesque T, Hamdan L, Sohouhenou F, Faccioli LH, Borgeat P, Marleau S (2013) Cooperative role of endogenous leucotrienes and platelet-activating factor in ischaemia-reperfusion-mediated tissue injury. J Cell Mol Med 17:1554-1565 doi:10.1111/jcmm.12118

9. Bongoni AK, Lanz J, Rieben R, Banz Y (2013) Development of a bead-based multiplex assay for the simultaneous detection of porcine inflammation markers using xMAP technology. Cytometry A 83:636-647 doi:10.1002/cyto.a.22287

10. Brachet G, Bourquard T, Gallay N, Reiter E, Gouilleux-Gruart V, Poupon A, Watier H (2016) Eculizumab epitope on complement C5: Progress towards a better understanding of the mechanism of action. Mol Immunol 77:126-131 doi:10.1016/j.molimm.2016.07.016

11. Collard CD, Agah A, Reenstra W, Buras J, Stahl GL (1999) Endothelial nuclear factor-kappaB translocation and vascular cell adhesion molecule-1 induction by complement: inhibition with anti-human C5 therapy or cGMP analogues. Arterioscler Thromb Vasc Biol 19:2623-2629 doi:10.1161/01.ATV.19.11.2623

12. Coulthard LG, Woodruff TM (2015) Is the complement activation product C3a a proinflammatory molecule? Re-evaluating the evidence and the myth. J Immunol 194:3542-3548 doi:10.4049/jimmunol.1403068

13. Emmens RW, Wouters D, Zeerleder S, van Ham SM, Niessen HW, Krijnen PA (2016) On the value of therapeutic interventions targeting the complement system in acute myocardial infarction. Transl Res doi:10.1016/j.trsl.2016.10.005

In press

14. Fitch JC, Rollins S, Matis L, Alford B, Aranki S, Collard CD, Dewar M, Elefteriades J, Hines R, Kopf G, Kraker P, Li L, O'Hara R, Rinder C, Rinder H, Shaw R, Smith B, Stahl G, Shernan SK (1999) Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass. Circulation 100:2499-2506 doi:10.1161/01.CIR.100.25.2499
15. Granger CB, Mahaffey KW, Weaver WD, Theroux P, Hochman JS, Filloon TG, Rollins S, Todaro TG, Nicolau JC, Ruzyllo W, Armstrong PW, Investigators C (2003) Pexelizumab, an anti-C5 complement antibody, as adjunctive therapy to primary percutaneous coronary intervention in acute myocardial infarction: the COMplement inhibition in Myocardial infarction treated with Angioplasty (COMMA) trial. Circulation 108:1184-1190 doi:10.1161/01.CIR.0000087447.12918.85
16. Hausenloy DJ, Botker HE, Engstrom T, Erlinge D, Heusch G, Ibanez B, Kloner RA, Ovize M, Yellon DM, Garcia-Dorado D (2016) Targeting reperfusion injury in patients with ST-segment elevation myocardial infarction: trials and tribulations. Eur Heart J doi:10.1093/eurheartj/ehw145
17. Hedstrom E, Engblom H, Frogner F, Astrom-Olsson K, Ohlin H, Jovinge S, Arheden H (2009) Infarct evolution in man studied in patients with first-time coronary occlusion in comparison to different species - implications for assessment of myocardial salvage. J Cardiovasc Magn Reson 11:38 doi:10.1186/1532-429X-11-38
18. Hepburn NJ, Williams AS, Nunn MA, Chamberlain-Banoub JC, Hamer J, Morgan BP, Harris CL (2007) In vivo characterization and therapeutic efficacy of a C5-specific inhibitor from the soft tick Ornithodoros moubata. J Biol Chem 282:8292-8299 doi:10.1074/jbc.M609858200

In press

19. Heusch G, Gersh BJ (2016) The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: a continual challenge. Eur Heart J doi:10.1093/eurheartj/ehw224

20. Heusch G, Skyschally A, Schulz R (2011) The in-situ pig heart with regional ischemia/reperfusion - ready for translation. J Mol Cell Cardiol 50:951-963 doi:10.1016/j.yjmcc.2011.02.016

21. Horstick G, Heimann A, Gotze O, Hafner G, Berg O, Bohmer P, Becker P, Darius H, Rupprecht HJ, Loos M, Bhakdi S, Meyer J, Kempski O (1997) Intracoronary application of C1 esterase inhibitor improves cardiac function and reduces myocardial necrosis in an experimental model of ischemia and reperfusion. Circulation 95:701-708 doi:10.1161/01.CIR.95.3.701

22. Ibanez B, Heusch G, Ovize M, Van de Werf F (2015) Evolving therapies for myocardial ischemia/reperfusion injury. J Am Coll Cardiol 65:1454-1471 doi:10.1016/j.jacc.2015.02.032

23. Investigators AA, Armstrong PW, Granger CB, Adams PX, Hamm C, Holmes D, Jr., O'Neill WW, Todaro TG, Vahanian A, Van de Werf F (2007) Pexelizumab for acute ST-elevation myocardial infarction in patients undergoing primary percutaneous coronary intervention: a randomized controlled trial. JAMA 297:43-51 doi:10.1001/jama.297.1.43

24. Ito BR, Roth DM, Engler RL (1990) Thromboxane A2 and peptidoleukotrienes contribute to the myocardial ischemia and contractile dysfunction in response to intracoronary infusion of complement C5a in pigs. Circ Res 66:596-607 doi:10.1161/01.RES.66.3.596

25. Jenkins CM, Cedars A, Gross RW (2009) Eicosanoid signalling pathways in the heart. Cardiovasc Res 82:240-249 doi:10.1093/cvr/cvn346

In press

26. John F, Kavitha S, Panicker S, Nair T, Indira M (2013) Elevated levels of leukotriene B4 and thromboxane B2 distinguish chest pain of cardiac and non cardiac origin. Indian Heart J 65:295-299 doi:10.1016/j.ihj.2013.04.012

27. Jore MM, Johnson S, Sheppard D, Barber NM, Li YI, Nunn MA, Elmlund H, Lea SM (2016) Structural basis for therapeutic inhibition of complement C5. Nat Struct Mol Biol 23:378-386 doi:10.1038/nsmb.3196

28. Khan F, Pharo A, Lindstad JK, Mollnes TE, Tonnessen TI, Pischke SE (2015) Effect of Perfusion Fluids on Recovery of Inflammatory Mediators in Microdialysis. Scand J Immunol 82:467-475 doi:10.1111/sji.12332

29. Laudisi F, Spreafico R, Evrard M, Hughes TR, Mandriani B, Kandasamy M, Morgan BP, Sivasankar B, Mortellaro A (2013) Cutting edge: the NLRP3 inflammasome links complement-mediated inflammation and IL-1beta release. J Immunol 191:1006-1010 doi:10.4049/jimmunol.1300489

30. Mahaffey KW, Granger CB, Nicolau JC, Ruzyllo W, Weaver WD, Theroux P, Hochman JS, Filloon TG, Mojcik CF, Todaro TG, Armstrong PW, Investigators C (2003) Effect of pexelizumab, an anti-C5 complement antibody, as adjunctive therapy to fibrinolysis in acute myocardial infarction: the COMPlement inhibition in myocardial infarction treated with thromboLYtics (COMPLY) trial. Circulation 108:1176-1183 doi:10.1161/01.CIR.0000087404.53661.F8

31. Martel C, Granger CB, Ghitescu M, Stebbins A, Fortier A, Armstrong PW, Bonnefoy A, Theroux P (2012) Pexelizumab fails to inhibit assembly of the terminal complement complex in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention. Insight from a substudy of the Assessment of Pexelizumab in Acute Myocardial Infarction (APEX-AMI) trial. Am Heart J 164:43-51 doi:10.1016/j.ahj.2012.04.007

In press

32. Nunn MA, Sharma A, Paesen GC, Adamson S, Lissina O, Willis AC, Nuttall PA (2005) Complement inhibitor of C5 activation from the soft tick Ornithodoros moubata. J Immunol 174:2084-2091 doi:10.4049/jimmunol.174.4.2084

33. Nymo S, Gustavsen A, Nilsson PH, Lau C, Espevik T, Mollnes TE (2016) Human Endothelial Cell Activation by Escherichia coli and Staphylococcus aureus Is Mediated by TNF and IL-1beta Secondarily to Activation of C5 and CD14 in Whole Blood. J Immunol 196:2293-2299 doi:10.4049/jimmunol.1502220

34. Oshinski JN, Yang Z, Jones JR, Mata JF, French BA (2001) Imaging time after Gd-DTPA injection is critical in using delayed enhancement to determine infarct size accurately with magnetic resonance imaging. Circulation 104:2838-2842 doi:10.1161/hc4801.100351

35. Ricklin D, Hajishengallis G, Yang K, Lambris JD (2010) Complement: a key system for immune surveillance and homeostasis. Nat Immunol 11:785-797 doi:10.1038/ni.1923

36. Riou LM, Ruiz M, Sullivan GW, Linden J, Leong-Poi H, Lindner JR, Harris TD, Beller GA, Glover DK (2002) Assessment of myocardial inflammation produced by experimental coronary occlusion and reperfusion with 99mTc-RP517, a new leukotriene B4 receptor antagonist that preferentially labels neutrophils in vivo. Circulation 106:592-598 doi:10.1161/01.CIR.0000023878.04716.6D

37. Rosset A, Spadola L, Ratib O (2004) OsiriX: an open-source software for navigating in multidimensional DICOM images. J Digit Imaging 17:205-216 doi:10.1007/s10278-004-1014-6

38. Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L (2007) Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. Nat Biotech 25:1256-1264 doi:10.1038/nbt1344

In press

39. Roversi P, Ryffel B, Togbe D, Maillet I, Teixeira M, Ahmat N, Paesen GC, Lissina O, Boland W, Ploss K, Caesar JJ, Leonhartsberger S, Lea SM, Nunn MA (2013) Bifunctional lipocalin ameliorates murine immune complex-induced acute lung injury. J Biol Chem 288:18789-18802 doi:10.1074/jbc.M112.420331

40. Santos-Gallego CG, Vahl TP, Goliasch G, Picatoste B, Arias T, Ishikawa K, Njerve IU, Sanz J, Narula J, Sengupta PP, Hajjar RJ, Fuster V, Badimon JJ (2016) Sphingosine-1-Phosphate Receptor Agonist Fingolimod Increases Myocardial Salvage and Decreases Adverse Postinfarction Left Ventricular Remodeling in a Porcine Model of Ischemia/Reperfusion. Circulation 133:954-966 doi:10.1161/circulationaha.115.012427

41. Skjeflo EW, Sagatun C, Dybwik K, Aam S, Urving SH, Nunn MA, Fure H, Lau C, Brekke OL, Huber-Lang M, Espevik T, Barratt-Due A, Nielsen EW, Mollnes TE (2015) Combined inhibition of complement and CD14 improved outcome in porcine polymicrobial sepsis. Crit Care 19:415 doi:10.1186/s13054-015-1129-9

42. Takase B, Maruyama T, Kurita A, Uehata A, Nishioka T, Mizuno K, Nakamura H, Katsura K, Kanda Y (1996) Arachidonic acid metabolites in acute myocardial infarction. Angiology 47:649-661 doi:10.1177/000331979604700703

43. Thomas TC, Rollins SA, Rother RP, Giannoni MA, Hartman SL, Elliott EA, Nye SH, Matis LA, Squinto SP, Evans MJ (1996) Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv. Mol Immunol 33:1389-1401 doi:10.1016/S0161-5890(96)00078-8

44. Vakeva AP, Agah A, Rollins SA, Matis LA, Li L, Stahl GL (1998) Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy. Circulation 97:2259-2267 doi:10.1161/01.CIR.97.22.2259

In press

45. van der Pals J, Koul S, Andersson P, Gotberg M, Ubachs JF, Kanski M, Arheden H, Olivecrona GK, Larsson B, Erlinge D (2010) Treatment with the C5a receptor antagonist ADC-1004 reduces myocardial infarction in a porcine ischemia-reperfusion model. BMC Cardiovasc Disord 10:45 doi:10.1186/1471-2261-10-45
46. Van Tassell BW, Toldo S, Mezzaroma E, Abbate A (2013) Targeting interleukin-1 in heart disease. Circulation 128:1910-1923 doi:10.1161/CIRCULATIONAHA.113.003199
47. Weisman HF, Bartow T, Leppo MK, Marsh HC, Jr., Carson GR, Concino MF, Boyle MP, Roux KH, Weisfeldt ML, Fearon DT (1990) Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. Science 249:146-151 doi:10.1126/science.2371562
48. Yoshikai Y (2001) Roles of prostaglandins and leukotrienes in acute inflammation caused by bacterial infection. Curr Opin Infect Dis 14:257-263 doi:00001432-200106000-00003

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Figure legends

Fig. 1 – Coversin reduced infarction size

(a) Coversin (C5 inhibitor) reduced infarction in the area at risk (AAR) by 39%, p=0.03 determined by TTC staining. (b) TTC staining of the AAR (example slices from one animal in each group) shows infarcted areas in white and non-infarcted areas in red. (c) Coversin reduced infarction in the left ventricle by 19%, p=0.02 determined by gadolinium stained magnetic resonance imaging (MRI). (d) Transversal (first row) and frontal (second row) T1-weighted MRI images of the same animals shown in panel B with shaded right ventricle as only the left ventricle was analyzed. White area and black area within white area depict infarction and non-perfused infarction, respectively. Horizontal line denotes mean (n = 8 (placebo) and n = 7 (coversin)). Mann-Whitney U-test. LV; left ventricle

Fig. 2 – Coversin improved myocardial function

Tissue Doppler echocardiography was evaluated from the mitral plane and averaged from septal and lateral wall movements. Open bars represent control and filled bars coversin treated animals. Systolic velocity was reduced at four hours after reperfusion in both groups but was 29%, p = 0.01 higher in coversin compared to control animals (a). Likewise, systolic displacement was 31%, p < 0.01 higher in coversin treated animals in comparison to placebo treated animals (b). Thermal dilution derived stroke volume (c) was 14%, p = 0.01 higher, while cardiac output (d) showed a trend of 16%, p = 0.09 increase in coversin treated animals. Values presented as mean ± SD (n = 8 (placebo) and n = 7 (coversin)). Mann-Whitney U-test

Fig. 3 – Coversin reduced local myocardial IL-1β production

IL-1β obtained by microdialysis was induced in the area at risk (AAR) and not the control region after four hours of reperfusion. Coversin treatment (filled bars) significantly reduced IL-

In press

1β in the AAR by 80% in comparison to placebo treated animals (open bars). Values presented as mean ± SEM (*n* = 7 (placebo) and *n* = 5 (coversin)). Two-way ANOVA with *post-hoc* Bonferroni correction for multiple testing

Fig. 4 – Coversin reduced E-selectin expression

Myocardium was stained with antibody against E-selectin. E-selectin expression was increased in placebo treated animals in the border zone of the AAR and unchanged in the center of the AAR and non-ischemic Cx control region (left panels). Coversin treatment led to significant decrease of E-selectin, expressed by reduced density of staining (middle and right panels). Horizontal line denotes mean (*n* = 8 (placebo) and *n* = 7 (coversin)). Mann-Whitney U-test

Fig. 5 – Coversin eliminated complement activity

Complement activity was assessed in plasma and the classical (a), lectin (b) and alternative pathway (c) were monitored using C5b-9 deposition as common readout. Coversin bolus treatment during coronary ischemia led to significantly reduced complement activity in all pathways (filled circles) and was not affected in control animals (open boxes). Complement activity remained low in all three pathways throughout the reperfusion period until the end of the experiment. Consequently, the plasma soluble complement activation product sC5b-9 was significantly reduced in plasma of coversin treated animals in comparison to controls (d). Myocardium was stained with an antibody against C5b-9 (e). Visually, deposition of C5b-9 (brown) was markedly decreased in the area at risk, the border zone and the non-ischemic control region in coversin treated animals in comparison to placebo treated animals. a-d; Values presented as mean ± SD (*n* = 8 (placebo) and *n* = 7 (coversin)). Linear mixed effect model. CAU; Complement Arbitrary Units. e; Results of two representative animals are shown

In press

Fig. 6 – Coversin did not reduce LTB4

(a) LTB4 was assessed in plasma throughout the study period. LTB4 showed a non-significant trend to lower values during reperfusion in coversin treated animals compared to placebo. LTB4 in myocardial tissue from three different regions at the end of the experiment was not affected by Coversin treatment (filled bars) in comparison to control (open bars) (all $p>0.1$). Values presented as mean ± SD. (a); linear mixed effect model, (b); Two-way ANOVA with *post-hoc* Bonferroni correction for multiple testing

Fig. 7 – Coversin did not significantly decrease plasma markers of myocardial ischemia

Troponin T (a) and H-FABP (b) were detected in plasma throughout the study period. Myocardial ischemia lead to an increase in troponin T and H-FABP in both control (open boxes) and coversin treated (filled circles). Coversin treated animals showed a trend towards lower H-FABP levels throughout the whole reperfusion period without reaching significance in comparison to control animals (troponin T: $p = 0.39$; H-FABP: $p=0.07$). Values presented as mean ± SEM ($n = 8$ (placebo) and $n = 7$ (coversin)). Linear mixed effect model

Fig. 8 – Coversin, but not eculizumab, inhibits porcine complement activation

Complement inhibitory effect of coversin (filled circles) and eculizumab (open circles) were assessed in the functional classical (a, e), lectin (b, f), and alternative pathway (c, g) assays in porcine (a-c) and human (e-g) serum using percentage of solid phase C5b-9 deposition as readout. Porcine (d) and human (h) whole blood was incubated with the complement activator zymosan and the effect of the inhibitors was examined using the soluble sC5b-9 complex as readout. Coversin, but not eculizumab, effectively inhibited porcine complement activity in a dose dependent manner, and was effective at the calculated *in vivo* concentration of 0.8 µM used in this study. Human complement activity was effectively inhibited by both inhibitors in a dose dependent manner. Complement activity of all three pathways was analyzed in duplicates

In press

and plasma from zymosan activated whole blood samples was analyzed in triplicates. CAU; complement arbitrary units, neg ctr; negative control, sC5b-9; soluble C5b-9

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Figure 1

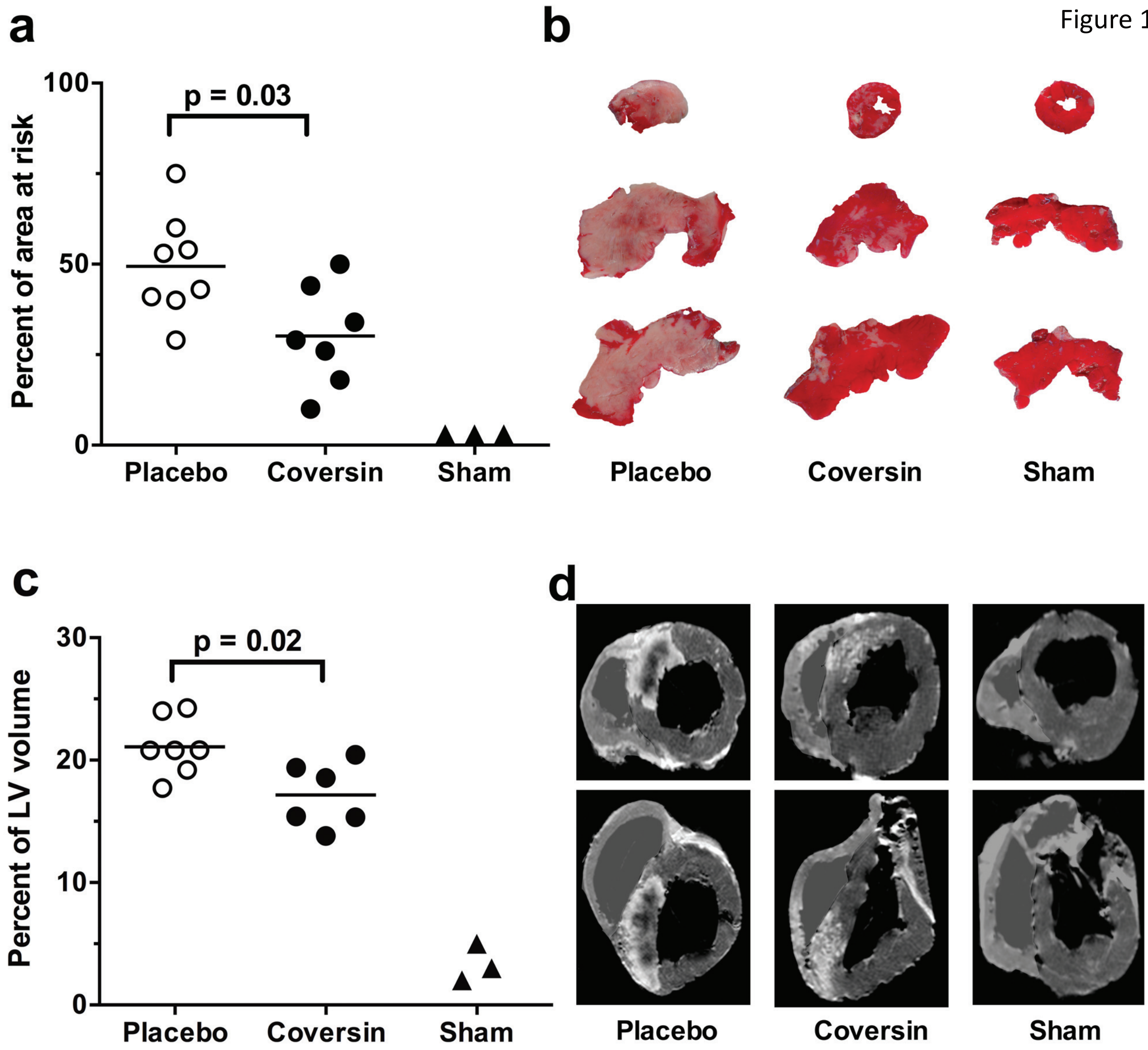
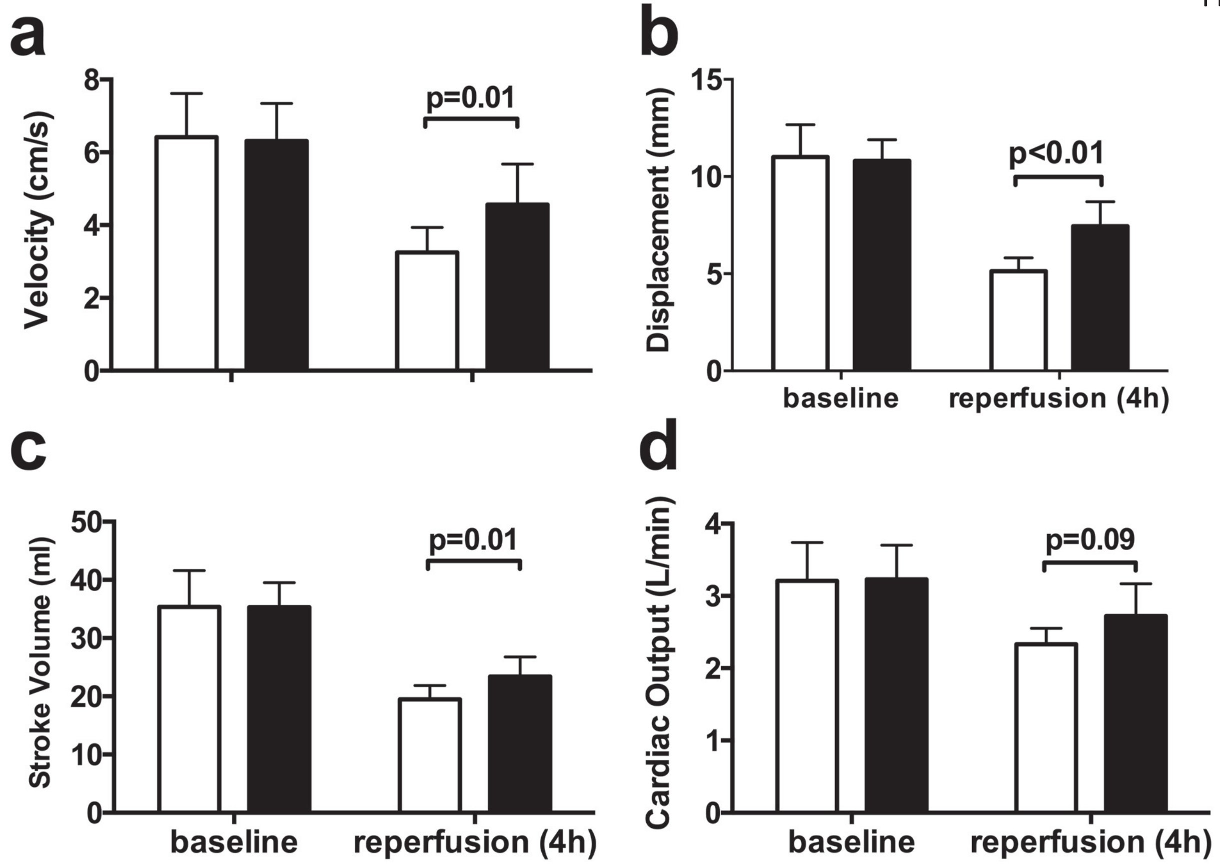


Figure 2



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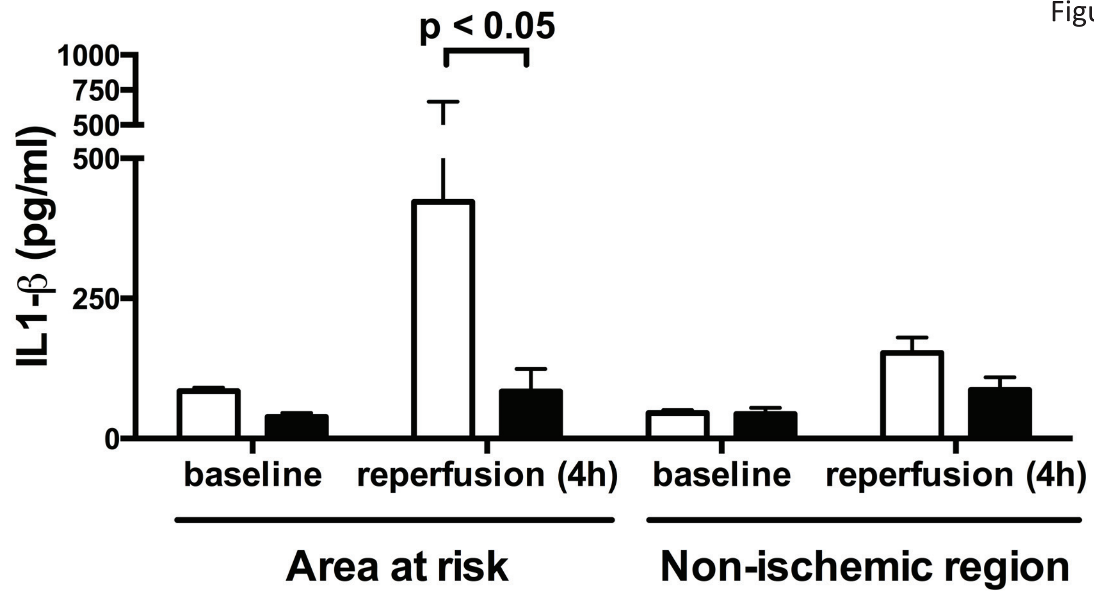
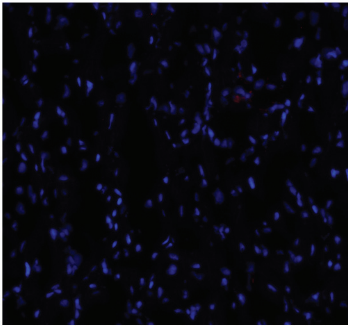
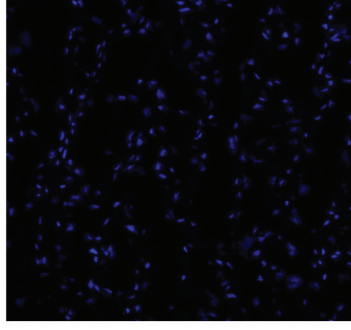


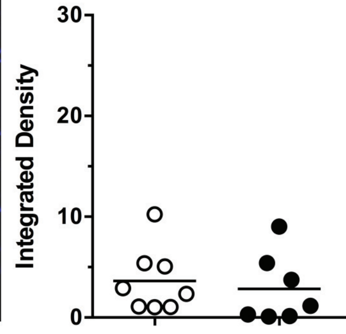
Placebo Coversin

Figure 4

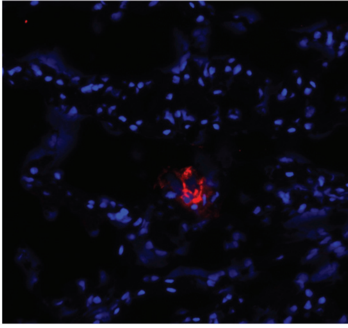
Area at risk

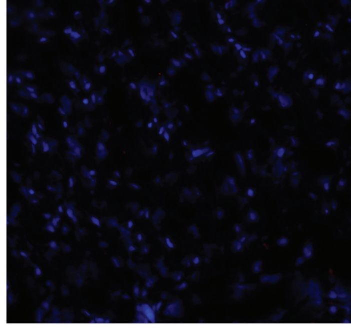


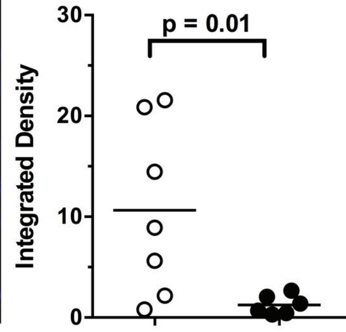




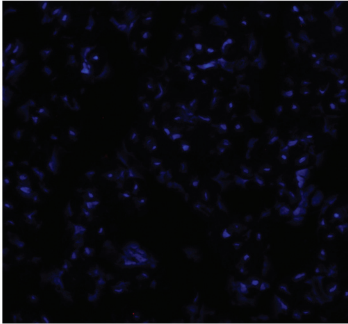
Border zone

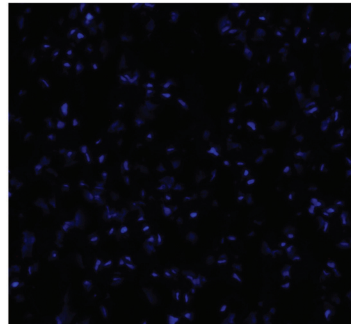






Non-ischemic control region





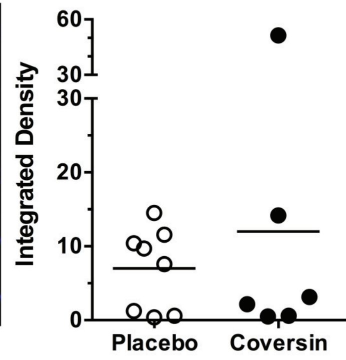
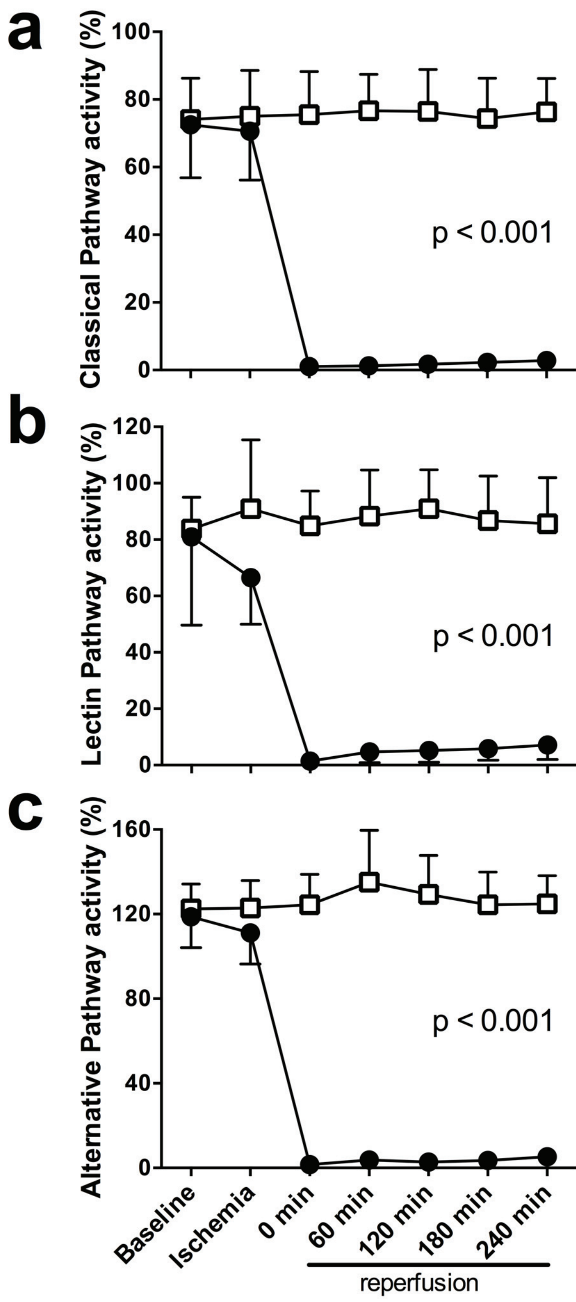
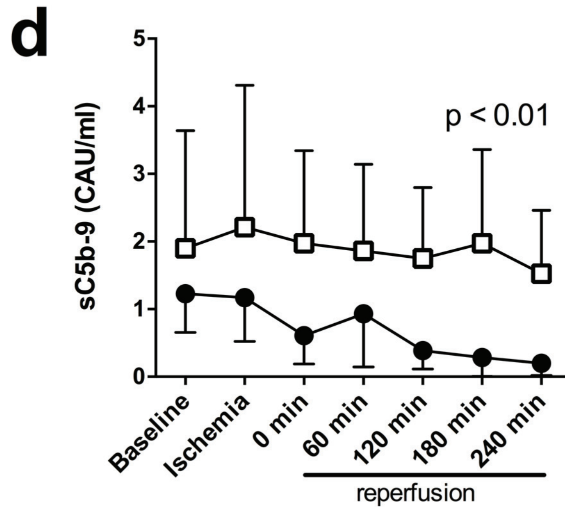


Figure 5





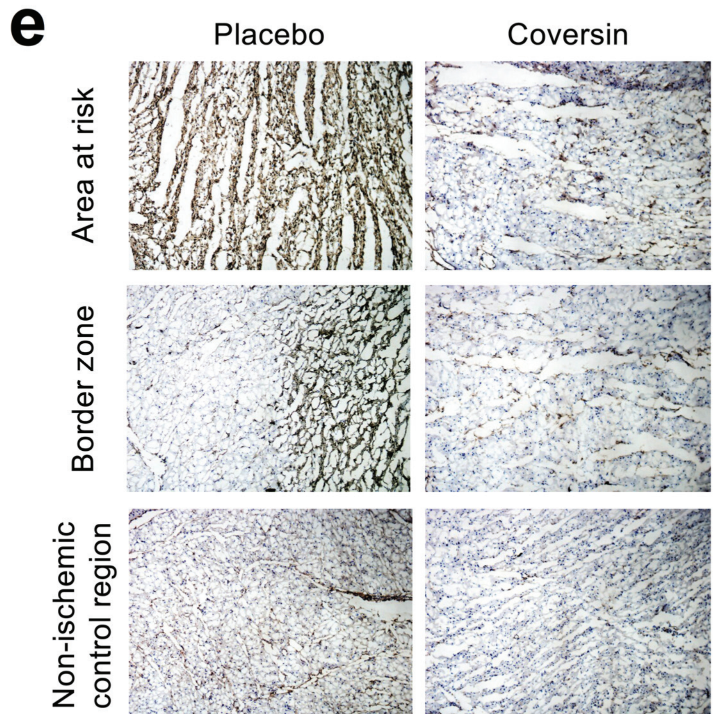
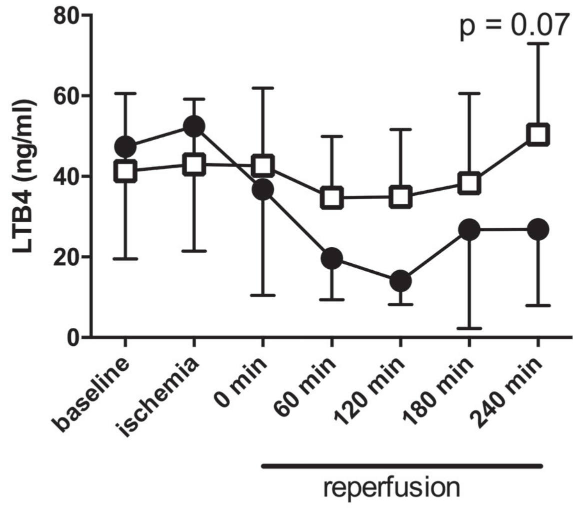


Figure 6



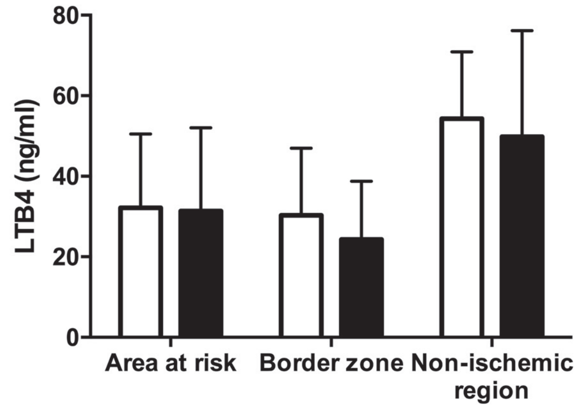


Figure 7

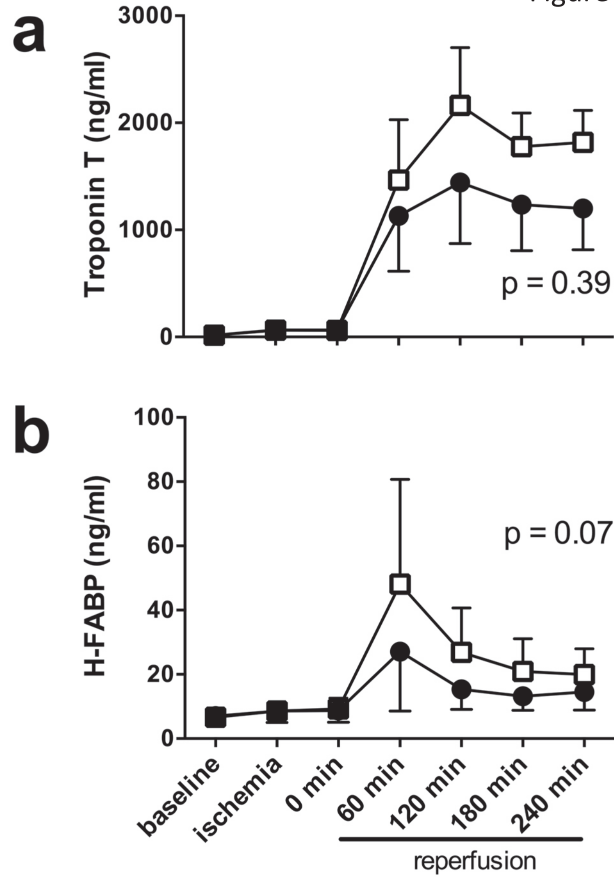
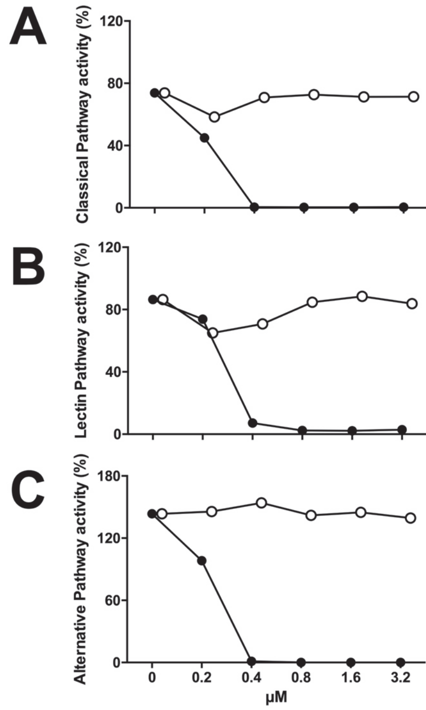
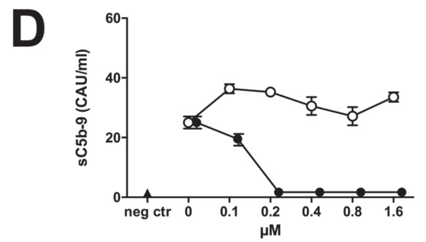


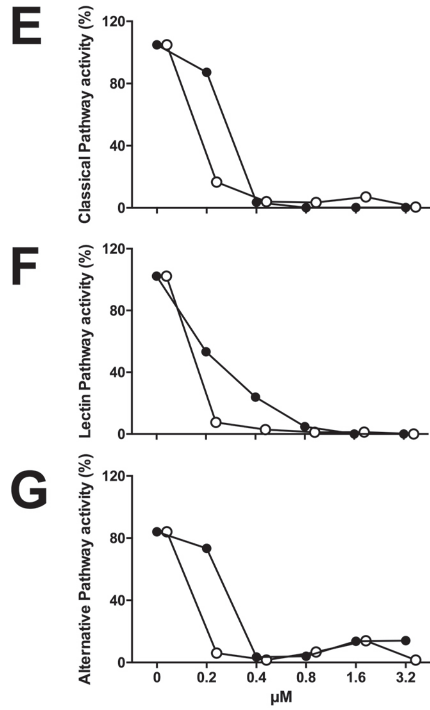
Figure 8

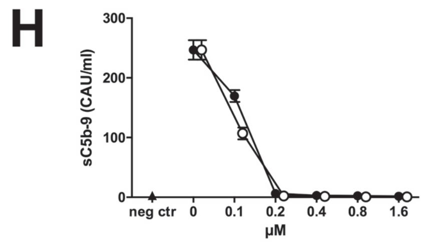
Porcine





Human





Paper IV

Effect on mother and child of eculizumab given before caesarean section in a patient with severe antiphospholipid syndrome: a case report

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Abbreviations: APS = antiphospholipid syndrome, CAPS = catastrophic antiphospholipid syndrome, E-C5-complex = eculizumab-C5 complex, EDTA = ethylenediaminetetraacetic acid, IgG = immunoglobulin G, IgG 2/4 = immunoglobulin G subclass 2/4, TCC = terminal complement complex, C5b-9.

Key words: antiphospholipid syndrome, pregnancy, complement, eculizumab.

In press

Abstract

Background: Antiphospholipid syndrome in pregnancy may trigger the life-threatening catastrophic antiphospholipid syndrome. Complement activation is implicated in the pathogenesis, and inhibition of complement factor C5 is suggested as an additional treatment option.

Methods: We present a pregnant patient treated with the C5-inhibitor eculizumab due to high risk of developing devastating antiphospholipid syndrome-related complications. The complement inhibitory effects of the treatment were examined both in the patient and the premature infant.

Results: Complement activity in the mother recovered considerably faster than anticipated, however no new thrombosis or catastrophic antiphospholipid syndrome developed during the last week of pregnancy or postpartum. Blood sampling from the umbilical vein and artery, and from the infant after delivery, showed low complement activity, however only 0.3% of the eculizumab concentration detected in the mother, consistent with low placental passage of eculizumab.

Conclusion: The data underscore the importance of close monitoring of complement inhibition and individualizing dosage regimens in pregnant patients receiving eculizumab. We document how traditional functional complement activity tests cannot assess the effect of eculizumab in premature infants due to the very low levels of complement factors detected in this infant born in gestational week 33. Only trace amounts of eculizumab passed the placenta. In conclusion, complement C5 inhibition might be a safe candidate treatment option for antiphospholipid syndrome during pregnancy and delivery, and additionally, enables prolongation of pregnancy with important weeks.

In press

Introduction

Antiphospholipid syndrome (APS) is characterized by arterial, venous or small-vessel thrombosis and/or pregnancy morbidity in the presence of persistent antiphospholipid antibodies (anti-cardiolipin antibodies, anti-beta2 glycoprotein 1 antibodies and lupus anticoagulant)[1]. Although the pathogenesis is not fully understood, the binding of antiphospholipid antibodies to β2 beta2 glycoprotein 1 promotes endothelial cell activation determined by upregulation of adhesion molecules, tissue factor and production and secretion of proinflammatory cytokines, which enhance the risk of thrombosis formation [2]. Complement appears to play a significant role in the pathophysiology based on both *in vitro* and *in vivo* studies [3-5]. Catastrophic APS (CAPS), although rare, is a devastating and life-threatening syndrome featured by multi-organ thrombosis. Infection, surgery, pregnancy and puerperium are identified triggers of CAPS [6,7]. Current treatment options in addition to anticoagulation are glucocorticoids, plasma exchange or intravenous immunoglobulins, however, case reports have reported that inhibition of complement may be lifesaving [8-10].

Case Report

A 22-year-old primigravida was admitted to hospital in the second trimester with painful ulcerations of ischemic origin in her right leg. Barely 14 years old, she developed her first episode of lower limb arterial thrombosis which was treated with bypass grafting and digital amputations. No arteriosclerosis or vasculitis was detected and she was diagnosed with antiphospholipid syndrome (APS), fulfilling the Sydney criteria [1] with persistent triple positive anti-phospholipid antibodies: anti-cardiolipin IgG 205 GPL-U/L (ref. < 10 GPL-U/L), anti-beta 2 glycoprotein 1 IgG 125 U/mL (ref. < 10 U/mL) and positive lupus anticoagulant 2.41 (ref. < 1.3 Silica Clotting time). Lifelong warfarin treatment was commenced. A recurrent episode of thrombosis was treated with percutaneous transluminal angioplasty and an episode of microemboli resolved with intensified anticoagulant treatment.

In conjunction with pregnancy, warfarin was substituted with low molecular weight heparin adjusted up to 10000 IU twice daily (anti factor Xa levels of 0.9 – 1.1 IU/mL) and low dose aspirin (75 mg daily). Ischemia was treated conservatively with analgesia in addition to anticoagulation therapy and pregnancy was monitored by regular ultrasounds following fetal growth and placental function.

In press

Based on her multiple previous arterial thromboses and ongoing ischemia during pregnancy, the risk of developing catastrophic APS (CAPS) in relation to pregnancy, delivery and puerperium was considered significant. Ruffatti et al. published data suggesting that addition of second line therapy increases live-birth rates in high risk pregnant patients with APS, although no guidelines are currently available on the ideal treatment strategy [11]. Previous experience with the efficacy of the complement C5 inhibitor eculizumab in treatment of CAPS and described safety in pregnancy [8,12,13] prompted the choice of eculizumab. Thus, 600 mg of eculizumab was administered eight days before delivery (day 0) in addition to prophylactic antibiotics. Serum (prepared by drawing whole blood into empty tubes, left for clotting 60 min followed by centrifugation 15 min, 3500 *g,* 4˚C) and ethylenediaminetetraacetic acid (EDTA) plasma (prepared by drawing blood into K2EDTA tubes, followed by immediate centrifugation 15 min, 3500 *g,* 4˚C) samples were obtained from the patient before and at several time points after eculizumab administration and analyzed directly or stored at -70˚C. Complement activity in plasma (Total Complement System Screen, WIESLAB®, Malmo, Sweden) decreased to zero after the first eculizumab infusion and remained low at day 2, however had returned to normal levels already by day 7 (Figure 1A). E-C5 complexes in serum (enzyme immunoassay as described in [12]) increased from zero to 67 after the first eculizumab dose (Figure 1A). Interestingly, the patient reported decreased ischemic pain following the first dose of eculizumab and opioid analgesia was successfully reduced.

A second dose of eculizumab 600 mg was infused on day 7 and a caesarean section was performed the following day (day 8) in gestational week 32+4, resulting in delivery of a healthy infant (Apgar 8/9/9) with a normal weight (1875 gram) for gestational age. Serum and EDTA plasma samples were obtained from the umbilical cord artery and vein by careful needle puncture to avoid contamination from mothers' blood and Wharton's jelly, directly after cord clamping during caesarean section. In addition, EDTA-plasma was obtained from the infant two hours after delivery. Samples were analyzed directly or stored at -70˚C. The infant's total complement activity was <1 %, however consistent with the measured low complement protein concentrations (standard routine immunochemical methods, Department of Clinical Immunology and Transfusion Medicine, Lund, Sweden) as expected at gestational week 32 (Table 1)[14]. Umbilical and infant E-C5 complexes were 0.3 µg/mL. Concomitant patient E-C5 complexes were 98 µg/mL consistent with a 0.3 % placental passage of eculizumab (Table 1). To further support the findings of low eculizumab passage, we added

In press

eculizumab (final concentration of 100 µg/ml) or purified C5 (50 µg/ml), respectively, to the infant serum *in vitro* and subsequently analyzed for E-C5 complexes. Eculizumab, but not C5, increased the E-C5 complex formation in infant serum substantially (Figure 1B), consistent with the presence of free C5 (Table 1) and negligible amounts of eculizumab.

Close monitoring of the patient's complement activity after the second dose revealed increased activity already after three days and again normal activity within one week (Figure 1A). The patient did not develop new thrombosis or CAPS during the last week of pregnancy or postpartum. The intervention given (eculizumab) was part of normal health care and thus ethical approval was neither obliged, nor sought. However, written consent was obtained from the patient in order to publish the case report.

Discussion

A history of thrombosis and triple positive antiphospholipid antibodies, as seen in the present patient, is associated with a higher risk of maternal and fetal complications in pregnant women with APS [11,15]. Our patient demonstrated multiple risk factors and showed clinical signs of thrombosis despite intensive anticoagulant treatment. Additionally, the risk of peri- and postoperative hemorrhage due to intense anticoagulant treatment was high, while on the other hand, surgery and cessation of anticoagulant treatment would increase the risk of triggering CAPS. Consequently, additional treatment was warranted. Eculizumab has been suggested in several recent reports as a second line treatment option in APS and CAPS [6,8,16]. Hence, a short term course of eculizumab was chosen before cesarean section to reduce the risk of CAPS triggered by delivery and puerperium. The ischemic symptoms declined following the first dose and no adverse effects were seen.

Complement activity surprisingly increased to normal levels within seven days after both doses of eculizumab despite complete inhibition of complement activity after each infusion. Others have observed a similar requirement of an increased eculizumab dosage regimen as pregnancy proceeds [13]. Eculizumab is distributed mainly in the plasma volume and degraded by lysosomal enzymes [17]. However, pregnancy-induced changes of the normal physiology may influence the pharmacokinetics and pharmacodynamics of eculizumab, underscoring the importance of individual treatment monitoring.

In press

Eculizumab is a monoclonal antibody based on an IgG2/4 hybrid. Data from pregnancies in patients with paroxysmal nocturnal hemoglobinuria treated with eculizumab suggests that the treatment is safe [13]. We examined both arterial and venous blood samples from the umbilical cord, as well as a blood sample from the infant two hours after delivery. All three samples showed similar results, suggesting that virtually no eculizumab (approximately 0.3%) had passed the placenta. To our knowledge the amount of eculizumab has previously not been examined in preterm newborns at this stage of gestation. Collectively, these data indicate that the IgG2/4 chimeric structure of eculizumab largely prevents the molecule from passing the placental barrier.

The observed complement activity of less than 1% in the infant could have been misinterpreted as an eculizumab effect. Eculizumab blocks cleavage of C5 and thus prevents assembly of the terminal C5b-9 complement complex (TCC) detected in the complement function ELISA. However, a normal complement activity test requires adequate levels of complement proteins to produce sufficient amounts of TCC for assay detection. Premature infants have low levels of most complement proteins [14], as did the infant in this report. The detection of merely trace levels of E-C5 complexes in the infant samples and the results from the *in vitro* assay demonstrating that only the addition of eculizumab, but not purified C5, could increase the E-C5 complex formation in infant serum, indicate that the low complement activity resulted from the low levels of most of the complement proteins (e.g. C4, C3, C5, C6 and C8) and was not due to the presence of eculizumab. Consequently, abnormal results in complement activity assays, such as in premature infants, must be carefully interpreted to avoid incorrect conclusions.

Conclusion

In conclusion, these results add up to the increasing body of literature stating that eculizumab treatment can be considered safe in pregnancy as negligible amounts pass over the placental barrier. Our data suggests complement inhibition as a treatment option to safely prolong pregnancy and reduce the risk of CAPS triggered by pregnancy, surgery and puerperium without affecting the infant. In addition, we stress the importance of monitoring treatment effects of eculizumab using reliable methods to ensure individual adequate complement inhibition.

In press

Acknowledgements

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Disclosure of Conflicts of Interest

The authors have no conflicts of interest to disclose.

In press

References

1. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost.* 2006;4(2):295-306.
2. Allen KL, Fonseca FV, Betapudi V, Willard B, Zhang J, McCrae KR. A novel pathway for human endothelial cell activation by antiphospholipid/anti-beta2 glycoprotein I antibodies. *Blood.* 2012;119(3):884-893.
3. Holers VM, Girardi G, Mo L, et al. Complement C3 Activation Is Required for Antiphospholipid Antibody-induced Fetal Loss. *The Journal of Experimental Medicine.* 2002;195(2):211-220.
4. Romay-Penabad Z, Carrera Marin AL, Willis R, et al. Complement C5-inhibitor rEV576 (coversin) ameliorates in-vivo effects of antiphospholipid antibodies. *Lupus.* 2014;23(12):1324-1326.
5. Giannakopoulos B, Krilis SA. The pathogenesis of the antiphospholipid syndrome. *N Engl J Med.* 2013;368(11):1033-1044.
6. Gomez-Puerta JA, Espinosa G, Cervera R. Catastrophic antiphospholipid syndrome: diagnosis and management in pregnancy. *Clin Lab Med.* 2013;33(2):391-400.
7. Cervera R, Rodriguez-Pinto I. Catastrophic antiphospholipid syndrome: task force report summary. *Lupus.* 2014;23(12):1283-1285.
8. Barratt-Due A, Floisand Y, Orrem HL, et al. Complement activation is a crucial pathogenic factor in catastrophic antiphospholipid syndrome. *Rheumatology (Oxford).* 2016;55(7):1337-1339.
9. Kronbichler A, Frank R, Kirschfink M, et al. Efficacy of eculizumab in a patient with immunoadsorption-dependent catastrophic antiphospholipid syndrome: a case report. *Medicine (Baltimore).* 2014;93(26):e143.
10. Rodriguez-Pintó I, Espinosa G, Cervera R. Catastrophic antiphospholipid syndrome: The current management approach. *Best Practice & Research Clinical Rheumatology.* 2016;30(2):239-249.
11. Ruffatti A, Salvan E, Del Ross T, et al. Treatment strategies and pregnancy outcomes in antiphospholipid syndrome patients with thrombosis and triple antiphospholipid positivity. A European multicentre retrospective study. *Thromb Haemost.* 2014;112(4):727-735.

In press

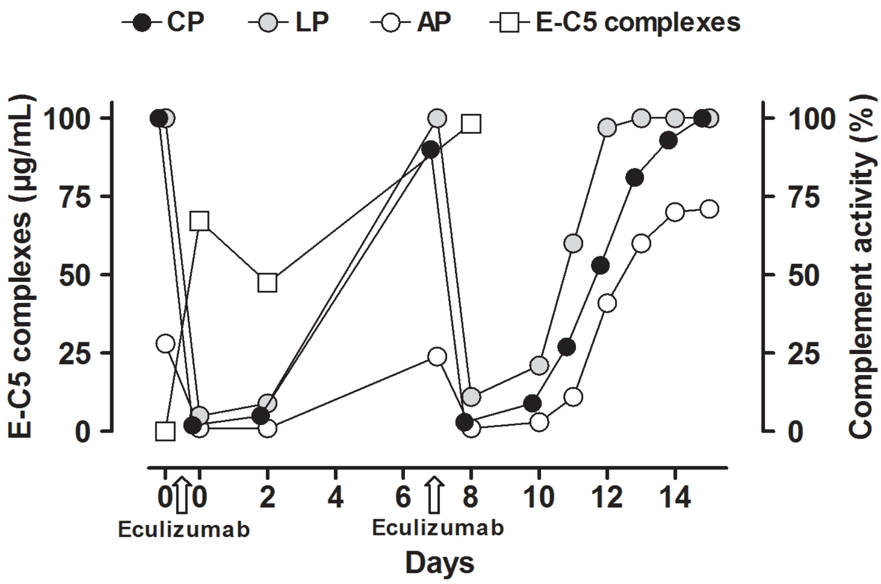
12. Hallstensen RF, Bergseth G, Foss S, et al. Eculizumab treatment during pregnancy does not affect the complement system activity of the newborn. *Immunobiology.* 2015;220(4):452-459.
13. Kelly RJ, Hochsmann B, Szer J, et al. Eculizumab in Pregnant Patients with Paroxysmal Nocturnal Hemoglobinuria. *N Engl J Med.* 2015;373(11):1032-1039.
14. McGreal EP, Hearne K, Spiller OB. Off to a slow start: Under-development of the complement system in term newborns is more substantial following premature birth. *Immunobiology.* 2012;217(2):176-186.
15. Bramham K, Hunt BJ, Germain S, et al. Pregnancy outcome in different clinical phenotypes of antiphospholipid syndrome. *Lupus.* 2010;19(1):58-64.
16. Erkan D, Aguiar CL, Andrade D, et al. 14th International Congress on Antiphospholipid Antibodies: task force report on antiphospholipid syndrome treatment trends. *Autoimmunity reviews.* 2014;13(6):685-696.
17. Dmytrijuk A, Robie-Suh K, Cohen MH, Rieves D, Weiss K, Pazdur R. FDA report: eculizumab (Soliris) for the treatment of patients with paroxysmal nocturnal hemoglobinuria. *Oncologist.* 2008;13(9):993-1000.

Figure legend

Figure 1. Complement activity and eculizumab-C5 (E-C5) complexes in a pregnant patient with APS and the newborn infant. A: The patient received eculizumab 600 mg day 0 and 7 and a caesarean section was performed on day 8. Effect of eculizumab on complement functional activity was measured as a common readout (C5b-9 formation) for the classical pathway (CP), lectin pathway (LP) and alternative pathway (AP), by ELISA in patient serum obtained before and repeatedly after the administration of eculizumab. E-C5 complexes were measured by ELISA at day 0, 2 and 8 in the patient serum before and after administration of eculizumab. Complement activity was completely abolished by eculizumab 600 mg, however normalized within seven days. The increased activity was revealed after three days following the second dose. Consistently, E-C5 complexes showed an inverse pattern with high levels following eculizumab administration. **B:** The infant's E-C5 complexes were measured by ELISA in infant serum (left column) and subsequently after *in vitro* challenge with purified complement protein C5 (50 µg/ml) (middle column) and eculizumab (100 µg/ml) (right column). The increased E-C5 complex formation following challenge with eculizumab, but not C5, is consistent with the presence of free C5 in infant serum and negligible levels of eculizumab.

In press

Figure 1



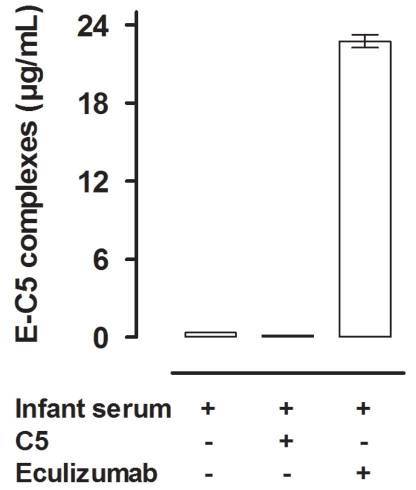


Table 1. Complement protein levels, complement activity and eculizumab-C5 complexes in samples from a pregnant eculizumab-treated patient with antiphospholipid syndrome before delivery and from the patient and newborn infant immediately after delivery.

	Reference range (adult)	Patient day 0[1]	Patient day 8[2]	Infant day 8[2]
C1q (%)	78-131	55	47	33
C4 (g/L)	0.1-0.5	0.20	0.18	0.06
C3 (g/L)	0.7-2.0	1.38	1.40	0.41
C5 (%)	72-171	159	272	43
C6 (%)	63-154	128	119	15
C7 (%)	64-154	112	129	47
C8 (%)	45-203	91	99	19
Classical pathway activity (%)	>40	100	3	0
Lectin pathway activity (%)	>10	100	11	0
Alternative pathway activity (%)	>10	28	1	0.5
Eculizumab-C5 complexes (μg/mL)		0	98	0.3

[1] Day 0: day of the first dose of eculizumab (sample obtained before treatment).

[2] Day 8: day of delivery by caesarean section (day after the second dose of eculizumab).