Innate Immunity in acute coronary syndromes

Focus on complement

PhD Thesis

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Table of contents

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List of papers

Paper I: Acute heart failure following myocardial infarction: Complement activation correlates with the severity of heart failure in patients developing cardiogenic shock. Hilde L. Orrem, Per H. Nilsson, Søren E. Pischke, Guro Grindheim, Peter Garred, Ingebjørg Seljeflot, Trygve Husebye, Pål Aukrust, Arne Yndestad, Geir Ø. Andersen, Andreas Barratt-Due, Tom E. Mollnes. *ESC Heart Failure* 2018.

Paper II: IL-6 receptor inhibition by tocilizumab attenuated expression of C5a receptor 1 and 2 in non-ST-elevation myocardial infarction. Hilde L. Orrem, Per H. Nilsson, Søren E. Pischke, Ola Kleveland, Arne Yndestad, Karin Ekholt, Jan K. Damås, Terje Espevik, Bjørn Bendz, Bente Halvorsen, Ida Gregersen, Rune Wiseth, Geir Ø. Andersen, Thor Ueland, Lars Gullestad, Pål Aukrust, Andreas Barratt-Due, Tom E. Mollnes. *Frontiers in Immunol. 2018*;**9**(2035)

Paper III: Soluble IL-1 receptor 2 is associated with left ventricular remodelling in patients with ST-elevation myocardial infarction. Orrem HL, Shetelig C, Ueland T, Limalanathan S, Nilsson PH, Husebye T, Aukrust P, Seljeflot I, Hoffmann P, Eritsland J, Mollnes TE, Andersen GØ, Yndestad A. *Int J Cardiol. 2018*

Paper IV: 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. *Basic Res Cardiol. 2017;* **112**(3):20

Abbreviations

AAR	Area at risk
ACS	Acute coronary syndromes
CAD	Coronary artery disease
CMRI	Cardiac magnetic resonance imaging
CR	Complement receptor
CRP	C-reactive protein
DAMP	Danger associated molecular pattern
FCN	Ficolin
HF	Heart failure
HMGB 1	High mobility group box 1
I/R	Ischemia and reperfusion
IL	Interleukin
IL-1RAcP	Interleukin 1 receptor accessory protein
LV	Left ventricle
LVEDV	Left ventricle end diastolic volume
LVESV	Left ventricle end systolic volume
MASP	Membrane associated serine protease
MBL	Mannose binding lectin
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MVO	Microvascular obstruction
NSTE-ACS	Non-ST-elevation acute coronary syndrome
NSTEMI	Non-ST-elevation myocardial infarction
PAMP	Pathogen associated molecular pattern
PCI	Percutaneous coronary intervention
PRR	Pattern recognition receptor
R	Receptor
Ra	Receptor antagonist

SAP	Stable angina pectoris
STEMI	ST-elevation myocardial infarction
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UAP	Unstable angina pectoris

1 Background

"…inflammation is essentially an adaptive response that aims to restore homeostasis" Medzhitov 2010

1.1 Introduction

Inflammation is involved in every aspect of physiology and pathology and is essentially a protective response with the aim to restore homeostasis (1). However, exaggerated or sustained inflammation can cause considerable damage as illustrated by the auto-inflammatory and auto-immune diseases. Inflammation is mediated by the immune system where cellular and humoral components work together in concert to protect the host from infectious diseases, but also to clear the environment from dead or damaged cells and promote tissue repair. The immune system is dependent on regulatory mechanisms to protect healthy "self" and to keep inflammatory cascades from spiraling out of control and further to suppress inflammation when the stimulus is under control (2, 3).

Traditionally the immune system has been divided into two branches: the innate and the adaptive immune system. The innate immune system is important in the early detection of foreign intruders or damaged self and represents a first line of defense. The adaptive immune system shows an educated, antigen-specific reaction promoted by B- and T-lymphocytes (4). There is however, extensive overlap and interaction between the innate and the adaptive immune system.

The innate immune system is able to act rapidly against foreign intruders without previous exposure to the provocateur. It consists of cells like neutrophils, dendritic cells, monocytes, macrophages and natural killer cells and of plasma components like the complement system, cytokines and chemokines. Specific germ-line encoded receptors, called pattern recognition receptors (PRRs), bind highly conserved pathogen-associated molecular patterns (PAMPs) that are only present on microorganisms. Further, damaged or altered structures of the host itself, called damage-associated molecular patterns (DAMPs) can bind to and activate PRRs (5). These receptors can be circulating in the lymph or plasma, like

the recognition molecules of the complement system, or be bound to the surface of innate immune cells, endothelial cells or fibroblasts, like the interleukin (IL)-1-receptors (R) and the Toll-like receptors (TLRs). PRRs can also be localized in the cytosol, like the Nod- like receptors (NLRs) and some of the TLRs.

Binding of DAMPs or PAMPs to the PRRs activates extracellular cascade systems or intracellular signaling pathways inducing synthesis and release of inflammatory cytokines and chemokines (5). The innate immune system responds rather uniformly to activation by DAMPs and PAMPs (6). The inflammasome is an intracellular multiprotein complex dependent on PRR activation to assemble and activate the strongly inflammatory cytokine IL-1β, in a caspase-1-dependent process (7).

1.2 Atherosclerosis

Atherosclerosis is the basis of the acute coronary syndromes (ACS) and is considered a non-resolving chronic inflammatory condition in arterial vessel walls at the site of blood flow disturbances (8). Inflammatory cells like macrophages enter and retain in the intima. Scavenger receptors on the macrophages bind modified lipoproteins leading to engulfment of lipoproteins (9). These lipid-loaded macrophages called foam cells ultimately rupture or go into apoptosis. The soft core of the atheroma consists of foam cells together with apoptotic and necrotic cells, cell debris and cholesterol crystals (10). Cholesterol crystals induce inflammasome activation and cytokine release in a process that has been shown to be complement-dependent (11).

An atherosclerotic plaque typically evolves over years. Degradation of the extra-cellular matrix by matrix metalloproteinases (MMPs) leads to weakening of the plaque (12) and, even though regulatory T-cells and macrophages stimulate anti-inflammatory cytokine production in the plaque, inflammation will dominate leading to a larger and more vulnerable plaque (8).

The growing atherosclerotic plaque is covered by a fibrous cap, which protects the core from the circulation. The strength of the fibrous cap is dependent on the balance between synthesis and degradation of the extracellular matrix (13). If the fibrous cap ruptures, blood gets in direct contact with the inflammatory plaque core, leading to activation of complement and the coagulation cascades, clot formation and narrowing of the lumen of the coronary artery.

1.3 Acute coronary syndromes

1.3.1 Definition and epidemiology

An abrupt narrowing or occlusion of a coronary artery leads to imbalance between oxygen demand and oxygen supply in the myocardium distal to the occlusion with a subsequent death of cardiomyocytes and development of myocardial infarction (MI). These events are typically reflected clinically as the acute coronary syndromes (ACS) with chest pain as the cardinal clinical sign. The ACS are divided into two main groups based on the findings on the electrocardiogram (see Figure 1): 1) The ST-elevation myocardial infarction (STEMI), normally reflecting a total occlusion of the coronary artery, and 2) the non-ST-elevation acute coronary syndromes (NSTE-ACS), reflecting a subtotal occlusion of the coronary artery. This latter syndrome is further subdivided into two groups dependent on whether there are signs of myocardial necrosis with release of cardiac troponins or not. The syndrome is classified as non-ST-elevation myocardial infarction (NSTEMI) if there is presence of cardiac troponins while ischemia without release of troponins is classified as unstable angina pectoris (UAP) (14).

Coronary artery diseases (CAD) are the most frequent cause of death worldwide accounting for more than 7 million deaths per year (15). The incidence of STEMI declines whereas the incidence of NSTEMI increases. Even though hospital mortality rates are higher for STEMI patients, the 6-months mortality rates for the two groups are equal at about 12% (15). Introduction of percutaneous coronary

intervention (PCI) with rapid opening of the occluded coronary vessel and reperfusion of the ischemic myocardium has improved the prognosis of MI dramatically and is today the cornerstone in the treatment. Thirty-days mortality from STEMI has declined from 20% to less than 5% (16). For STEMI, reperfusion should be performed within 120 minutes from debut of symptoms (17) whereas in NSTE-ACS, the occlusion is normally not total and reperfusion is recommended dependent on risk stratification, to between 2 and 72 hours (14).

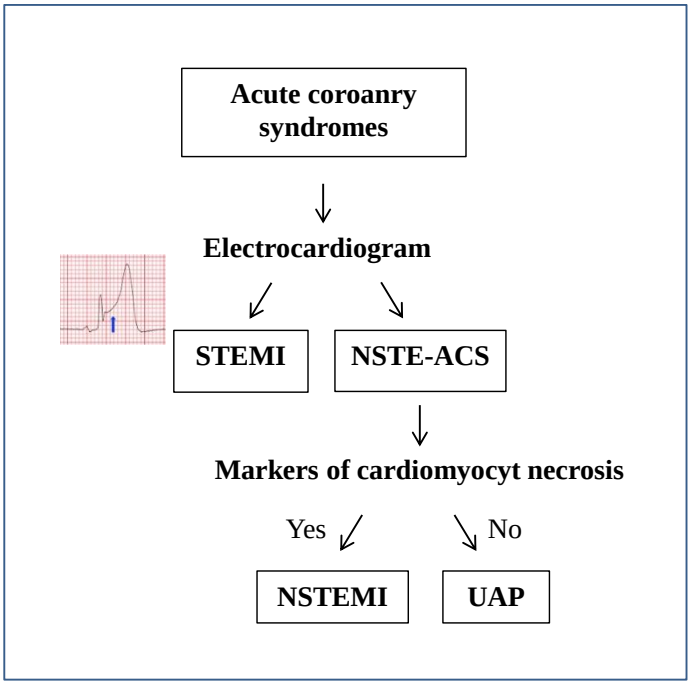

Figure 1: Acute coronary syndromes.

1.3.2 Cardiac remodeling and heart failure

Heart failure (HF) is a devastating complication to MI defined as an abnormal cardiac structure or function which leads to reduced cardiac output and/or increased intracardiac pressure (18). The prevalence of HF is high, affecting more than 10% of individuals above 70 years of age, and even if the incidence is slightly decreasing, the prevalence is increasing due to improved survival from MI and prolonged life span in the population (18). HF is the most common

condition leading to hospital admission among people above 65 years of age and with a five-year mortality rate of 45-60%, HF holds a prognosis more severe than many cancer conditions (19). Infarct size is an important determinant for one-year all-cause mortality and hospitalization for HF (20).

HF is classified as acute when there is rapid onset or worsening of symptoms and chronic when the symptoms have been stable for more than 1 month (18). Acute HF (AHF) is caused by ACS, mainly STEMI, in 42% of the cases and shows an in-hospital mortality rate of approximately 7% (21). The incidence of cardiogenic shock complicating STEMI is around 8% and holds a poor prognosis with an in-hospital mortality rate around 40% (22).

The New York Heart Association (NYHA) functional classification is widely used to describe severity of symptoms and exercise intolerance in patients with chronic HF. The NYHA classification correlates well with prognosis but poorly with measurements of LV function (18). In the AHF setting, the Killip classification system has proven beneficial. Patients with AHF are classified as Killip class I when they have no clinical signs of HF, while Killip class IV constitutes patients with cardiogenic shock (18).

Despite substantial improvement in the prognosis of MI, complications like cardiogenic shock or HF are still common (22). This leads to increased morbidity and mortality posing a tremendous burden to health-care systems (23, 24). There is therefore an urge for supplementary treatment strategies to improve outcome in patients suffering from ACS. Targeting various parts of the inflammatory cascade has been and still is considered a relevant strategy (25, 26).

1.4 Inflammation and acute coronary syndromes

Based on early observations from immunologists and cardiopathologists and strongly supported by recent findings, inflammation is considered an important contributor in all phases of the atherosclerotic disease from the formation of the fatty streak, to the mandatory reparatory process following a MI (8, 27).

Inflammatory markers and mediators like complement components, tumor necrosis factor (TNF), IL-1β and IL-6 are associated with the development of ACS (28, 29).

A MI induces release of DAMPs from dead and dying cardiomyocytes triggering an intense but transient inflammatory response that is mandatory for cardiac repair. However, exaggerated or enhanced inflammation can cause damage to the myocardium and have detrimental effects on cardiac function both in the short and long term (25, 30). Also reperfusion of the area deprived from blood supply, called the area at risk (AAR), can induce cellular damage to previously viable ischemic tissue (31). The pathophysiology behind the ischemia and reperfusion (I/R) injury is probably multifactorial. Recruitment of neutrophils, which can cause direct cytotoxic damage to viable cardiomyocytes, and compromised microcirculation caused by intra- and extracellular edema, rupture of capillaries and micro embolization are important contributors to the I/R injury (32). Microvascular obstruction (MVO) is the most severe form of compromised microcirculation and has proven to be an independent predictor for mortality following STEMI (33). Activation of inflammatory cascades is important for the development of I/R injury (34).

It has been known for decades that the inflammatory response following a MI goes through characteristic phases in the days and weeks following the acute occlusion of the coronary vessel (35). The response can be divided into three overlapping phases: the inflammatory phase, the proliferative phase and the maturation phase (25). A window of opportunity for intervention seems to exist where the detrimental inflammatory responses can be dampened. This can improve outcome for patients at risk of developing complications following MI (36).

1.4.1 Inflammatory phase

Resident cardiac mast cells and complement components are important early triggers of inflammation. Release of pre-formed cytokines and histamine facilitates the recruitment of inflammatory cells to the damaged area and also activates adjacent cells like endothelial cells and tissue resident macrophages (37). Dead and dying cardiomyocytes and matrix release DAMPs like high mobility group box 1 (HMGB1), nucleic acids, IL-1α, hyaluronic acid *etc.*, which bind to and activate membrane bound and fluid phase PRRs (see Figure 3). This activation induces a local as well as a systemic sterile inflammatory response. Inflammatory mediators like TNF, IL-1β, IL-6 and C5a, attract inflammatory cells like neutrophils and phagocytic monocytes and activate cardiac fibroblasts, which in the acute phase of inflammation produce degrading matrix metalloproteinases (MMPs) (38).

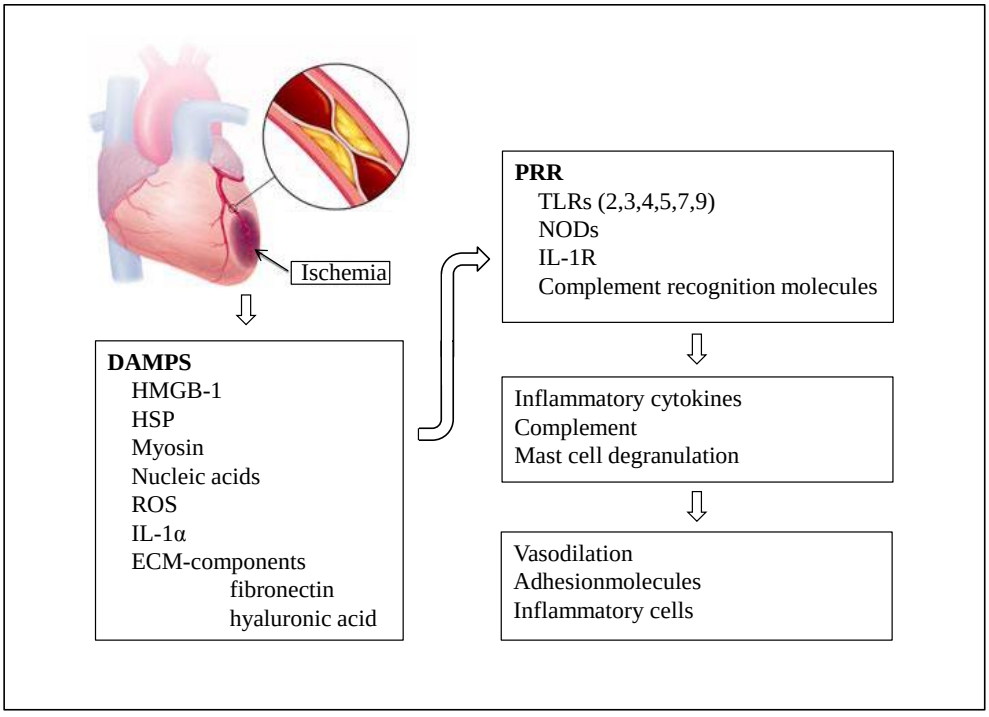

Figure 2. DAMPs from damaged myocardium induce cytokine production and inflammatory response. Modified from Epelman, Nat Rev Immunol 2015.

This inflammatory response is essential in clearing the infarcted area from dead cells and debris being important for the healing of the heart. However, an enhanced or exaggerated inflammatory response is detrimental for an adequate scar formation. Particularly neutrophils, which are critical in the response to microbial infections, appear first of all to be detrimental in the sterile inflammation following MI directing cytotoxic responses towards "self" (38, 39). Complement component C5a is a strong chemoattractant for neutrophils. Additionally, both C5a and sublytic amounts of C5b-9 can induce genes associated with apoptosis and complement has thus been suggested to enhance cardiomyocyte injury by stimulating apoptosis in viable cells following reperfusion (40).

1.4.2 Resolution of inflammation and scar formation

Just as important as the ability to respond rapidly to DAMPs and PAMPs, is the ability to resolve inflammation when the inflammatory trigger is under control (41). This is an active process involving regulatory T-cells, cleaved extracellular cytokine receptors (R) like the sIL-1Rs, and receptor antagonists (Ra) and production of anti-inflammatory cytokines like IL-10 and IL-37 (5, 25). The key cellular effector that drives inhibition of inflammation is unknown but phagocytosis of apoptotic neutrophils by phagocytic cells (efferocytosis) is probably involved in the this process where macrophages are transformed from inflammatory (M1) macrophages to pro-resolving (M2) macrophages and fibroblasts are transformed to myofibroblasts inducing scar formation (38).

M2 macrophages produce anti-inflammatory cytokines like IL-10 and Transforming growth factor-β (TGF-β) important for downregulation of inflammatory cytokines, chemokines and adhesion molecules and upregulation of tissue inhibitor of metalloproteinases (TIMPs) which promotes stabilization of the extracellular matrix. The differentiated cardiac fibroblasts express contractile proteins and secrete matrix proteins thereby preserving structural integrity of the infarcted heart (25).

The further maturation of the infarcted area is marked by collagen cross-linking and further apoptotic cell death. As the infarct heals, the ventricle dilates while the non-infarcted areas exhibit increased interstitial fibrosis.

1.4.3 The role of inflammation in cardiac remodeling and heart failure

Myocardial remodeling is defined as the structural and functional changes in the heart leading to the development of HF. This is a multifactorial process (23) where sustained or exaggerated inflammation following the MI, or defective suppression and resolution of the inflammatory response play fundamental roles (42, 43). The balance and quality of extracellular matrix degradation and renewal are important factors influencing the quality of the remodeling process.

Immune activation is well documented in patients with chronic HF recognized for the first time in 1990 when Levine and colleagues demonstrated elevated circulating levels of TNF in patients with severe HF (44). Since then inflammation has been linked to HF in a lot of publications (43, 45-47). Also inflammatory markers and mediators like CRP, TNF, IL-1β and IL-6 predict the development and prognosis of HF (28, 47). The immune activation in patients with AHF or cardiogenic shock has been more scarcely documented mainly because these patients are often excluded from randomized trials.

1.5 The complement cascade

The complement system is an ancient germ-line encoded part of the innate immune system first discovered by Paul Ehrich, Jules Bordet and George Nuttall in the late 19th century (48). It consists of more than 40 membrane bound or fluid phase components and include recognition molecules, soluble and membrane bound receptors, proteases, enzymatically active complexes and biologically active split products as well as soluble and membrane bound regulators. The fluid phase proteins circulate mainly as inactive pro-enzymes in the lymph and plasma with the ability to rapidly respond to foreign intruders or damaged self (49). Even

though these “sensors” circulate in the fluid phase, the activation of the complement cascade takes place on surfaces like cells, crystal structures and foreign material. This enables the system to act locally, even intracellularly, in addition to the fluid phase response (50).

The liver is the main source of complement proteins but also extrahepatic synthesis is evident (51). The complement system has got three key effector functions (48, 49):

1) Opsonisation and bridging innate and adaptive immune responses mainly mediated by C3 cleavage products C3b and iC3b. This is important in facilitating phagocytosis of pathogenic substances and in clearance of immune complexes and damaged cells.
2) Production of the anaphylatoxins C3a and C5a where the latter is the most important in generating the anaphylatoxin reaction characterized by vasodilation, capillary leakage and production of cytokines, chemokines and adhesion molecules promoting attraction of immune cells.
3) Cell lysis when the terminal product of the cascade, TCC also called C5b-9 or membrane attack complex (MAC), is inserted into cell membranes. Even sublytic concentrations of the complex can induce inflammation (52).

The complement system is under strict control by various regulators which is critical to avoid an inappropriate response with tissue damage as a result (53).

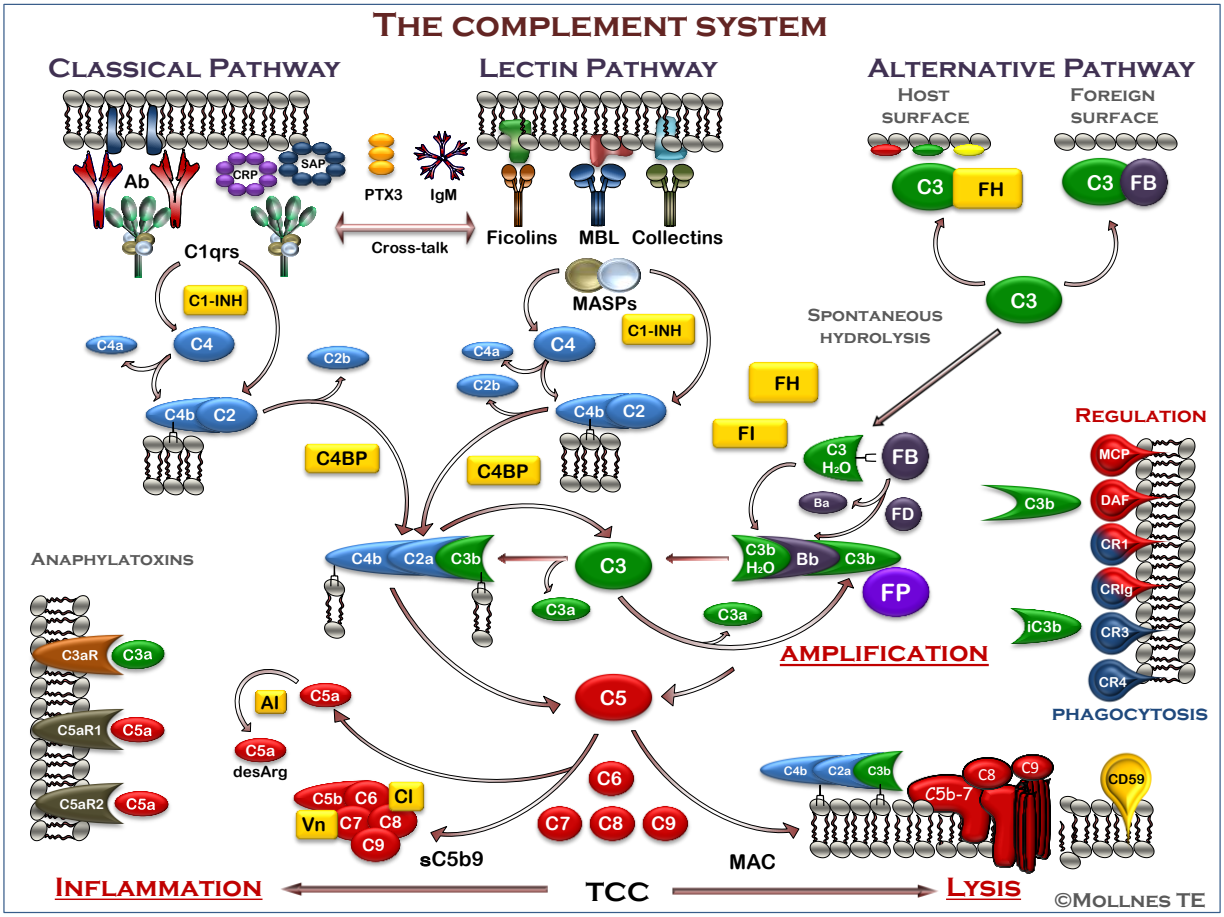

Figure 3: The complement system. Printed with permission from T.E Mollnes

1.5.1 Initiation pathways

The complement system is activated through three characterized pathways: the classical, the lectin and the alternative pathway.

The classical pathway is activated when the pattern recognition molecule C1q binds to antigen-bound IgM or clusters of antigen-bound IgG molecules (especially IgG1 and IgG3). Additionally, C1q can activate the complement cascade by binding other substances like the C-reactive protein (CRP) and structures on necrotic or apoptotic cells. Surface bound C1q recruits the proteases C1r and C1s from the fluid phase to form the C1qrs complex. This leads to cleavage of C4 into C4a and C4b the latter binds covalently to surfaces in the immediate vicinity. Surface bound C4b binds C2, which is further cleaved into

C2a and C2b. C2b leaves the complex resulting in the formation of the classical pathway C3 convertase, C4b2a (48, 49, 54).

The lectin pathway: Mannose-binding lectin (MBL), ficolin-1 (FCN-1), ficolin-2 (FCN-2) and ficolin-3 (FCN-3) and Collectin 10 and 11 are recognition molecules of the lectin pathway. These molecules recognize carbohydrate structures on the surface of bacteria, viruses and fungi and also on the surface of damaged "self". FCN-2 can bind to DNA and is involved in clearance of dying cells through recognition of exposed DNA (55). Neoepitopes revealed following endothelial damage can bind naturally occurring IgM and in this way induce complement dependent damage to the myocardium (56). The lectin pathway has thus been proposed to play a key role in the inflammatory response following ischemia and reperfusion myocardial injury (57). After binding of the recognition molecules, serine proteases called MBL-associated serine proteases (MASPs) are activated. Their effector functions are the same as C1r and C1s with cleavage of C4 and C2 and hence formation of the classical and lectin pathway C3 convertase (C4b2a).

The alternative pathway is also called the amplification pathway. There is a constant hydrolysis of some of the abundantly circulating C3 to form $C3H_2O$, which has a functional spectrum similar to C3b (see section 1.5.2). Due to this constant "tick-over", the complement system is always turned "on". The membrane bound $C3H_2O$ recruits Factor B which subsequently is activated by Factor D to Ba and Bb. Bb together with C3b forms the alternative pathway convertase, C3bBb with the ability to further cleaving C3 and in this way amplifying the reaction (58). Binding of properdin stabilizes and increases the half-life of the convertase substantially (59). Activation of the alternative amplification pathway is responsible for a major part of the complement activity initiated by the alternative and lectin pathway (60, 61).

1.5.2 The central and terminal part of the complement cascade

All three pathways merge at the central component C3, which together with C5 compose the central components of the complement cascade. C3 can be cleaved by the convertases, C4b2a or C3bBbP, to C3a and C3b. This cleavage induces a conformational change in C3b exposing a short-lived thioester facilitating a covalent binding of C3b to the membrane of adjacent cells thereby opsonizing the cell. Binding of C3b or its degradation products to complement receptors on immune cells leads to removal of immune complexes, phagocytosis of the opsonized cells and represents a bridge between innate and adaptive immunity (58).

C3b is essential for further activation of the complement cascade as C5 binds immobilized C3b and undergoes a conformational change that renders it susceptible for cleavage by the C3 convertase (62). C5 is cleaved into C5a and C5b (58). C5b associates with C6 and C7 to a complex that can insert into cell membranes. Further binding of C8 and multiple C9 molecules generates the membrane attack complex (MAC) also known as C5b-9 complex that can form lytic pores which can lyse susceptible cells. Even sublytic levels of C5b-9 can induce inflammation by activating various intracellular signaling pathways including activation of the inflammasome (52, 63). The complex also exists in a soluble form, the sC5b-9 which does not attack membranes but serves as an important fluid phase indicator of complement activation.

1.5.3 The complement anaphylatoxins and their receptors

C3a and C5a are small polypeptides called anaphylatoxins with potent inflammatory capacity capable of modulating the inflammatory environment in several ways. The anaphylatoxins translate danger sensed in the fluid phase to distinct cellular responses by binding to the transmembrane G-protein-coupled receptors (GPCRs), the C5aR1, C5aR2 and C3aR (64). C5aR1 and C3aR are classical GPCRs, activating intracellular signaling pathways through their

association with G-proteins. C5aR2 is unable to couple to G-proteins due to the lack of certain essential amino acids in one of the transmembrane sequences (64). Both C3a and C5a are rapidly degraded by serum and tissue carboxypeptidases to C5a-desArg and C3a-desArg.

C3a binds with high affinity to C3aR, which does not bind the desarginated form, C3a-desArg (65). C3aR is expressed by leukocytes of myeloid origin. The effects of C3aR activation is rather complex dependent on cell type and phase of inflammation and the complete effects of C3aR stimulation are still not completely understood. In the acute phase of inflammation, C3a has been shown to have anti-inflammatory effects by confining unmobilized bone-marrow neutrophils to the reservoir, resulting in less neutrophil migration to ischemic tissue (66). In a model of intestinal ischemia and reperfusion (I/R) injury, the effect of C3a was shown to be anti-inflammatory mainly due to this effect (67). In circulating PBMCs, C3a exerts anti-inflammatory properties by reducing cytokine release. However in adherent PBMCs, C3a induces enhanced production of cytokines (66). In this way C3a exerts anti-inflammatory properties in the acute phase when neutrophils dominate the picture, while in the more chronic phase of inflammation, when monocytes and macrophages dominate, C3a exposes an inflammatory profile. C3a is also involved in tissue regeneration by C3-induced mobilization of stem cells with possible positive modulatory effects on the myocardium (68).

C5a is a strong inflammatory signaling peptide which binds to both C5aR1 (CD88) and C5aR2 (GPR77, C5L2) with high affinity. The two receptors are widely distributed and most often co-expressed, C5aR1 in general at a higher level than C5aR2 (65, 69, 70). The binding of C5a to C5aR1 induces inflammatory effects like recruitment and activation of inflammatory cells and enhanced cytokine and chemokine production (71). C5aR activation also leads to upregulation of complement receptor (CR)3, which induce phagocytosis, thereby being an important mediator of inflammation.

The effect of C5a binding to its other receptor, the C5aR2 is still controversial and not fully elucidated. It was for long considered a non-signaling decoy receptor, mainly due to the lack of G-protein coupling but both inflammatory and anti-inflammatory effects of C5aR2 activation has been demonstrated. Due to the lack of G-protein interaction, C5aR2 has been regarded a decoy receptor for C5a (72). Additionally, an anti-inflammatory role through intracellular interaction with β-arrestin has been shown (73). In line with this, C5aR2-inhibition increased IL-6-release in a rodent CLP-sepsis model both *in vivo* and *in vitro* (74).

However, several experimental studies have shown inflammatory effects mediated by C5aR2. C5aR2 knock-out (KO)-mice showed improved survival and attenuated levels of inflammatory cytokines in a rodent sepsis model (75). C5aR2 may be important in the C5a-induced upregulation of inflammatory mediators like IL-6, TNF and the phagocytic CR3 (76). In cardiovascular diseases, C5aR2-level has been shown to correlate with disease severity and levels of inflammatory cytokines like IL-6 and TNF in human atherosclerotic plaque (77). Further, release of the alarmin HMGB1 was shown to be C5aR2-dependent (75). The effect of activating C5aR2 is still not fully clarified and further studies to explore the role of C5aR2 in pathophysiological conditions are warranted (65).

C4a is a similar product released after C4 activation. Until recently, no C4a receptor had been identified but recent works suggests that C4a binds the protease-activated receptor 1 (PAR 1) on endothelial cells inducing cell retraction and capillary leakage (78).

1.5.4 Regulators and receptors of the complement system

Due to its huge inflammatory potential, the complement system has to be tightly regulated by several soluble and membrane bound inhibitors that modify and control the response to damage and danger. By expressing complement regulators, healthy host cells are protected from complement attack. Diseases like atypical haemolytic uremic syndrome (aHUS), C3 glomerulopathies and paroxysmal

nocturnal haemoglobinuria (PNH) are caused by defects or dysfunction of the regulators (79).

Soluble regulators like C1-inhibitor (C1INH) inactivate C1r, C1s and the MASP proteases and counteract the activity of the lectin and classical pathway. C1INH also inactivates serine proteases of other cascade systems like the contact system, and defects in C1INH can cause hereditary angioedema where the angioedema is mediated by bradykinin (80). Factor I and factor H are the major fluid phase regulators of the alternative pathway. Factor I cleaves C3b and C4b and generates inactivated split products in a cofactor-dependent manner. Factor H is a cofactor for factor I, additionally acting as a membrane bound regulator by binding to sialic acid expressed on the surface of healthy host cells protecting them from complement attack. C4b binding protein (C4BP) acts as cofactor for factor I, stimulating cleavage of C4b in the C4b2a convertase and, to a lesser extent, inactivation of C3b (49). Clusterin and vitronectin regulate complement activity by inhibiting polymerization of the C5b-9 complex and act as scavengers for partially formed sC5b-9 complexes in the circulation. Carboxypeptidases ensure rapid degradation of the anaphylatoxin in the fluid phase, hence dampening the effector functions of complement.

Membrane bound regulators protects the cells on which they are expressed. Membrane cofactor protein (MCP, CD46) is a cofactor for factor I. Decay accelerating factor (DAF, CD55) accelerates the dissociation of both C3 convertases. CD59 is a glycophosphatidylinositol (GPI)-anchored, membrane bound regulator that prevents the final assembly of the C5b-9 complex on cell membranes (49).

Five distinct complement receptors that bind C3b or its degradation products iC3b, C3c or C3dg have been identified. CR1 and CR2 belong to the short consensus repeat (SCR) family, with domains shared by several complement regulatory proteins (81). CR1 (CD35) has cofactor activity for factor I in addition to dissociate the C3 convertases. Furthermore CR1 acts as an adherence receptor expressed on erythrocytes where it binds C3b-tagged immune complexes

transporting them to the liver and spleen where they are processed by tissue-resident macrophages (79). CR2 (CD21) is expressed on B-cells lowering the activation threshold of the B-cells and serves as a bridge between innate and adaptive immunity (82).

CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are members of the integrin receptor family. They are present on phagocytic cells and contribute to phagocytosis by binding C3b and the degradation products iC3b, C3c and C3dg, which cluster on and opsonize microbial particles (81). CRIg belongs to the immunoglobulin super family. It is expressed on Kupfer cells and is essential in removal of iC3b-opsonized particles from the circulation (83). CRIg further acts as a negative regulator of complement activation by blocking the C3b-interaction in the alternative pathway convertase (81).

1.5.5 Complement in ACS

Ever since Hill and Ward discovered C3 in the infarcted myocardium almost 50 years ago (84), complement has been associated with the development of atherosclerotic diseases, ACS and HF (85). Modified LDL can activate complement (86) and complement components have been found upregulated in the intima of atherosclerotic lesions (87, 88). Further, complement is prerequisite for cholesterol crystal-induced IL-1β formation (11). Complement hence is a central player in the inflammatory induced atherosclerotic disease. Complement components are also present in the failing myocardium (89) and is upregulated in chronic HF (90).

DAMPs released by necrotic cardiac cells activate the complement cascade. Tissue resident mast cells are stimulated to release histamine and inflammatory cytokines. Neutrophils are attracted to the site of inflammation by chemotactic activity induced by complement, inflammatory cytokines, reactive oxygen species (ROS) and by complement-induced upregulation of adhesion molecules on the endothelium (39, 91). Neutrophils secrete proteolytic enzymes like MMPs,

generate reactive oxygen species (ROS), stimulate cytokine release and produce inflammatory lipid mediators like leukotriene B4 (LTB4) and are therefore believed to be centrally involved in the I/R injury (39). C5a induce synthesis of inflammatory cytokines such as IL-1β, TNF and IL-6 (92) and C5b-9 can induce apoptosis thereby contributing to increased myocyte death (93). Sublytic C5b-9 can cause damage to the myocardium by enhancing inflammation (94).

Complement is also involved in the reparative process following tissue injury by clearing the infarcted area for dead cells and debris (85). Further, tissue regeneration and recruitment of progenitor cells has proven to involve complement (68, 95).

A huge number of animal studies support a role for complement in myocardial I/R injury (96-99). The concept of complement inhibition in MI has been tested out in clinical trials, unfortunately without the same convincing effects. This will be discussed later (see section 1.9).

1.6 Interleukin-1 and its soluble receptors

In paper III the soluble receptors of IL-1 were studied. The interest for IL-1β as a major player in cardiovascular diseases has increased as clinical trials have shown promising results when the biological effects of IL-1β have been blocked (100-103). Levels of soluble IL-1 regulators in disease states have been reported in a few studies (45, 104). However, little is known about the regulators of IL-1 in patients with ACS.

1.6.1 IL-1

The members of the IL-1 family are of the most potent inflammatory mediators of the innate immune system with the ability to stimulate both innate immune cells and cells from the adaptive immune system (105). IL-1 constitutes two cytokines, IL-1α and IL-1β, which are strong inflammatory cytokines primarily produced by

stimulated monocytes and macrophages (106). IL-1α is also constitutively present in epithelial cells (107). Both are synthesized as pro-peptides and pro- IL-1β needs to be cleaved to the active IL-1β. To avoid an excessive inflammatory response, their biological activity is tightly regulated at receptor level and dysregulation leads to auto-inflammatory diseases (108).

IL-1α is often classified as an alarmin released upon necrosis activating inflammatory cascades in the early phase of inflammation (7). It is an intracellular or membrane associated cytokine rarely detected in the circulation except from in severe disease (106). During hypoxia, pro-IL-1α is released from dying cells and can give rise to local IL-1β production (108).

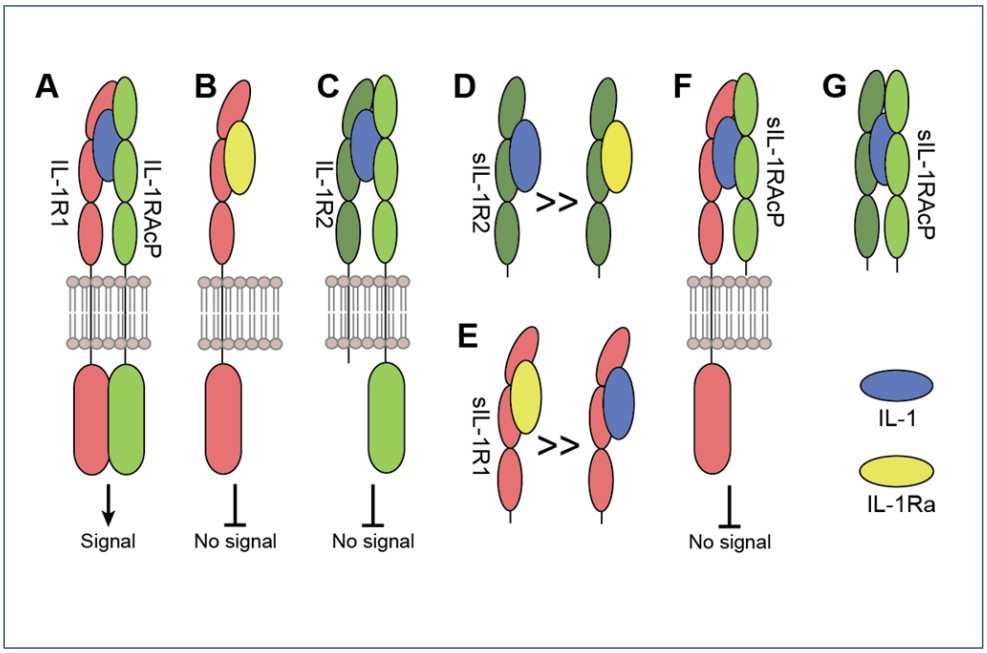

Figure 4. Regulators of IL-1 signaling. Printed with permission from A. Yndestad.

IL-1β is produced as an inactive precursor by hematopoietic cells in response to activation of IL-1R1, TLR, complement components, various cytokines and by IL-1 itself. Pro-IL-1β is activated and released from cells in a process involving caspase-1, which is regulated by the inflammasome (107). Pro-IL-1β can also be

activated by extracellular cleavage by neutrophil enzymes (108). IL-1β is a central pathogenic mediator of autoimmune, infectious, auto-inflammatory and degenerative diseases and is the most studied IL due to its strong mediating role in auto-inflammatory diseases (7).

1.6.2 The receptors

The biological activity of both IL-1α and IL-1β is mediated through binding to the IL-1 receptor 1 (IL-1R1), which is the only signaling receptor for IL-1. IL-1R1 is expressed on the surface of a wide variety of cells. It contains three extracellular immunoglobulin domains and one intracellular domain called Toll/IL-1 receptor (TIR) domain, highly homologous to other TLRs and mandatory for intracellular signaling (106). Binding by IL-1 to the extracellular domain of IL-1R1 leads to recruitment of the co-receptor, the IL-1 receptor accessory protein (IL-1RAcP), which is required for signal transduction (109). The IL-1β-IL-1R1-IL-1RAcP complex induces recruitment of adaptor molecules, like myeloid differentiation factor 88 (MyD88). This further promotes phosphorylation of kinases and translocation of nuclear factor (NF)-κB to the nucleus inducing the expression of a wide range of inflammatory cytokines (106).

The biological activity of IL-1 is negatively regulated by two receptors, the IL-1R2 and IL-1 receptor antagonist (IL-1Ra) (104). IL-1R2 lacks the intracellular TIR domain mandatory for signalling and binding of IL-1 to IL-1R2 hence does not generate any bioactivity. IL-1R2 further recruits and binds IL-1RAcP thereby sequestering both the ligand and the accessory protein required for signal transduction. IL-1R2 is mainly expressed by neutrophils, B-cells, monocytes and macrophages, thereby attenuating IL-1-induced inflammatory responses in these cells (104). Expression and release of IL-1R2 by neutrophils is, at least partly, involved in the mandatory resolution of inflammation (110). Anti-inflammatory stimuli (*e.g.* glucocorticoids and IL-4) increase neutrophil expression of IL-1R2 (111), whereas inflammatory molecules induce shedding of IL-1R2 from the membrane thus generating a soluble form of the Il-1R2, the sIL-1R2 (104). Both

membrane bound and sIL-1R2 bind IL-1β almost irreversibly. Binding of sIL-1R2 to the soluble IL-1RAcP (sIL-1RAcP), increase the affinity of sIL-1R2 to pro-IL-1β more than 100-fold. Thus, sIL-1RAcp serves as a negative regulator of IL-1 signalling (112).

IL-1Ra is produced by hepatocytes as a classical acute phase reactant and the expression is enhanced by inflammatory cytokines (113). IL-1Ra binds IL-1R1 with high affinity without inducing any cellular responses and thereby counteracts the inflammatory effects of IL-1. Both IL-1R2 and IL-1Ra are increased in various inflammatory conditions and their levels are believed to reflect disease severity (104, 113).

1.6.3 The role of IL-1 in ACS

IL-1β is centrally involved in plaque evolvement and in the detrimental inflammatory response following MI. IL-1 activation promotes inflammatory effects mainly indirectly by inducing gene expression of adhesion molecules, cytokines, chemokines and a vast number of inflammatory components like cyclooxygenase type 2, prostaglandin E2, phospholipase A2, nitric oxide and platelet activation factor (107). IL-1β is upregulated in atherosclerotic plaques and is activated following myocardial I/R injury (114). Polymorphism in the IL-1Ra gene correlates with restenosis and local atherosclerotic progression (26). IL-1Ra has shown correlation with myocardial damage and LV function (115). Saxena et al. characterized the receptors of IL-1 in mouse myocardium following I/R injury and found that IL-1R1 is responsible for a global inflammatory response and recruitment of both inflammatory and reparative monocytes. IL-1R1 dominates the early inflammatory phase, while the IL-1R2 dominates in the later, reparative phase of inflammation (116).

IL-1β is not reliably measured in plasma. IL-6 and CRP are downstream surrogate markers of IL-1 activity as IL-1 is a strong and important inducer of IL-6 production and IL-6 is the main inducer of hepatic CRP production. CRP has

shown a strong and consistent association with cardiovascular risk factors (26). Recent clinical trials have shown promising results when the IL-1 system is blocked (See section 1.9).

1.7 Interleukin-6

IL-6 is produced by monocytes, lymphocytes, endothelial cells, vascular smooth muscle cells and fibroblasts. It is part of the IL-6 family consisting of several cytokines signaling through the same signaling pathways. Binding of IL-6 to its receptor, IL-6R, with further binding to the glycoprotein 130 receptor, activates intracellular signaling pathways, which also involve negative feedback loops (117). IL-6R activation stimulates hepatic CRP and fibrinogen production, stimulates the expression of tissue factor (TF) and platelet aggregation and regulates the expression of adhesion molecules in endothelial cells (118).

IL-6 is involved in a broad range of inflammatory diseases and inhibiting its receptor with the humanized antibody tocilizumab has proven effective in auto-immune diseases like rheumatoid arthritis (117). It probably has pleiotropic effects in the myocardium and experimental data from IL-6 interventions varies. In a murine model of myocardial I/R injury, the effects of IL-6 inhibition on LV function was actually unfavorable (119).

Clinical data support a central role for IL-6 in human coronary artery diseases. IL-6 is highly upregulated at the site of coronary occlusion in patients with STEMI (120) and elevated circulating level of IL-6 in STEMI patients is associated with myocardial necrosis and future cardiovascular events and mortality (121, 122). Increased level of IL-6 in apparently healthy individuals is associated with increased risk of future cardiovascular events (123). Kleveland and colleagues showed in 2016, that inhibiting the IL-6R in patients with NSTEMI leads to reduced CRP and further to reduced level of troponin T (TnT) in PCI-treated NSTEMI patients (124). In my work, we have studied the effects of inhibiting IL-

6R on the expression of the three anaphylatoxin receptors in patients from the Kleveland trial.

1.8 Cross-talk and redundancy

Tissue damage with the release of DAMPs affects innate immunity in several ways and there is increasing evidence for a bi-directional cooperation between the different sensors that shape the inflammatory response (38). Being an up-stream, early sensor of danger and damage, complement can induce expression of various downstream inflammatory cytokines like IL-1β, TNF and IL-6 (125-128).

The crosstalk between IL-6 and complement has been described in several studies where modulation of the C5a/C5aR-axis has demonstrated to influence IL-6 production (52, 129, 130). The other way around, IL-6 has been shown to influence on complement. In rats, IL-6 infusion led to enhanced C5aR1 production in rat hepatocytes (131). Blocking IL-6 with a monoclonal anti-IL6 antibody in a sepsis model in mice showed improved survival and decreased expression of C5aR1 in liver, lung kidneys and heart (132).

The cross-talk between complement and IL-1β has recently gained increased attention. Triantafilou and colleagues showed in 2013 that sublytic C5b-9 can induce NLRP-3 inflammasome activation and IL-1β release by a calcium-dependent mechanism, which also enhance apoptosis (94). Samstad and colleagues demonstrated that cholesterol crystals in a whole blood model induced inflammasome activation and cytokine release, including IL-6 and IL-1β, in a complement dependent manner (11). Urate crystals can also induce complement-dependent IL-1β production (133). Thus, an extensive crosstalk between these inflammatory effector molecules exists.

A considerable crosstalk between complement and TLRs is recognized. TLR-dependent cytokine expression has been shown to be complement-dependent and mediated by anaphylatoxin receptor signaling (134). Redundancy has further been

described, since inhibiting both complement and TLR was more efficient than inhibiting only one component separately (135).

There is an extensive crosstalk between the complement system and the coagulation cascade. Plasmin, thrombin, Factor Xa and Factor XIa are claimed to cleave C3 and C5 to the biologically active C3a and C5a. Further, C5a can induce tissue factor activity on endothelial cells, macrophages, monocytes and platelets leading to activation of the tissue factor pathway of the coagulation cascade. Additionally, C5b-9 activates platelets thereby enhancing the pro-coagulant activity in blood (136).

1.9 Targeting innate immunity in MI

1.9.1 The complement system

Several experimental trials targeting various parts of the inflammatory cascade in MI have shown a reduction in infarct size. However, translation into clinical trials has been unsuccessful. Following some positive clinical trials in patients treated with coronary artery bypass surgery (137-139), the anti-C5 monoclonal antibody pexelizumab was further evaluated in three clinical trials with STEMI-patients (140-142). None of these studies met their predefined primary end-point of infarct size measured as area under the curve (AUC) of creatinine kinase MB (CK-MB). However, the COMMA-trial with 960 PCI-treated patients showed a reduction in 90 days mortality and a non-significant reduction in cardiogenic shock (141). This trial paved the way for a large multi-center randomized controlled trial (RCT) with 5745 patients, the APEX-AMI trial where the primary end-point was all cause 30-days mortality and secondary endpoints were cardiogenic shock and HF (140). This trial failed to meet any of its end-points and led to a lasting skepticism to anti-inflammatory strategies in MI. Sub-group analysis of the APEX-AMI-trial has demonstrated terminal pathway activation even in the pexilizumab-treated patients (143). Another sub-group analysis showed reduced mortality from shock

and HF in patient with inherited MBL-deficiency in the placebo group, indicating a role for the lectin pathway of complement in I/R-injury (144).

1.9.2 The IL-1 system

Anti-inflammatory effects of targeting IL-1 in acute coronary syndromes by recombinant IL-1Ra (anakinra) has been shown (101). In a larger phase II trial, anakinra showed attenuated inflammation but increase in major adverse cardiac events (MACE) after one year (102). Recently, treatment with canakinumab, a monoclonal antibody neutralizing IL-1β, led to a lower rate of recurrent cardiovascular events in patients with previous MI and elevated hsCRP (103).

1.9.3 IL-6R inhibition

Recently tocilizumab, an IL-6R antagonist, showed to reduce hsCRP and PCI-induced TnT release in NSTEMI patients (124).

2. Aims of the Study

The main aim of this thesis was to evaluate the innate immune system with focus on the complement cascade and the IL-1- signaling system, in acute coronary syndromes, particularly with respect to disease severity and adverse cardiac remodeling.

Specific aims

1) **To study temporal changes of complement activation and responsible activation pathways** in patients with severe HF and cardiogenic shock following STEMI and further explore if there was an association between the present complement activation and disease severity and left ventricle (LV) performance.

2) **To study cross-talk between complement and IL-6** by evaluating the effects of blocking IL-6R on the expression of C5aR1, C5aR2 and C3aR in blood from NSTEMI patients and further investigate the anaphylatoxin receptor expression in PBMCs from patients with different entities of CAD.

3) **To study temporal changes of the negative regulators of IL-1-signaling** in a patient population with STEMI and further investigate if there was any correlation with markers of myocardial injury and LV remodeling.

4) **To evaluate the effect of complement inhibition on the infarct size and LV function** in a model of porcine myocardial ischemia and reperfusion and further investigate local and systemic inflammation.

3. Methodological considerations

3.1 Study populations

In paper I-III, we analyzed biobank material from four different patient populations. As paper I-III represent sub-studies of already conducted trials, the results have to be considered exploratory. All studies had a randomized, double blind, placebo controlled design, which strengthens the results, also for the sub-studies.

Healthy controls were included for comparison in all papers. In paper I-III a total of 573 patients and 153 healthy controls were included and a total of 8278 laboratory analysis were performed. The large amount of studies and analysis handled in my work makes good laboratory practice important with proper labeling, sorting and registration in a spreadsheet.

The studies are described in details in the material and method section in the respective paper. All studies included in paper I-III were approved by The Regional Committee for Medical and Health Research Ethics of South-Eastern Norway and the Norwegian Data Protection Authority or the Data protection representative at the health authority. The study in paper I and II was additionally approved by The Norwegian Medicine Agency. All participants, patients as well as healthy controls, provided written informed consent.

In paper IV, the animal experiments were conducted in accordance with current European guidelines and approved by the ethics committee of the Norwegian Food and Safety Authority.

3.1.1 The LEAF trail

In paper I, plasma from all 61 patients recruited for the LEvosimendan in Acute heart Failure following myocardial infarction (LEAF) trail (NCT00324766) (145) was analyzed with respect to complement activation. The patients included in the trial developed acute heart failure within 48 hours following successful

revascularization of a STEMI and were recruited from an intensive care setting. Nine of the 61 patients fulfilled criteria for cardiogenic shock. Products from all three activation pathways were analyzed: C4bc from the lectin and classical pathway, C3bBbP from the alternative pathway, C3bc from the common pathway and sC5b-9 from the terminal pathway. Recognition molecules from the lectin pathway were further analyzed. LV function was evaluated with echocardiography at inclusion and at day 2 and after 4 months.

The weakness of this trial is first of all the small sample size and lack of measurement data before reperfusion. The shock group consisted of only 9 subjects and due to the severity of the disease, there were also missing data in both the AHF group and the shock group. The consistent findings with elevated levels of all activation products and their relationship with disease severity, strengthens the results. Also the strong and consistent correlation documented between LV function and complement activation in the shock group strengthens the results. Patients with AHF or cardiogenic shock are normally excluded from clinical trials. Due to this, very few studies have been performed on this sub-group of patients making the results presented in paper I interesting even though the sample size was small.

3.1.2 The interleukin-6 receptor inhibitor study

A total of 120 patients with NSTEMI scheduled for coronary angiography, were randomized to treatment with the IL-6R inhibitory monoclonal antibody tocilizumab (n=58) or placebo (n=59) in a double blind, two-center trial (NCT 01491074) (124). Tocilizumab was administrated as a single dose of 280 mg before coronary angiography providing IL-6-blockade for about two weeks. Samples included in paper III were drawn at baseline, before administration of study medicine and PCI, at day 1, day 2 and after 6 months. Plasma samples were analyzed for complement activation. In blood, the expression level of the three anaphylatoxin receptors, C5aR1, C5aR2 and C3aR was analyzed with qPCR. Since qPCR is a resource demanding method, only patients from one of the study

hospitals (n=60) (St Olavs Hospital, Trondheim, Norway) were included for the qPCR analysis. 28 of these patients had received tocilizumab and 32 had received placebo.

When dividing this group into early versus late inclusion and treatment with reperfusion or not, the numbers in each group were small, which is a limitation of this study. Protein data to evaluate the transcription of the genes was unfortunately not available. Further, PCR was performed on whole blood samples instead of isolated cells which made it impossible to identify which cells expressed the receptors. The advantage of using whole blood is that the cells are less manipulated with less risk of in vitro changes. Further a whole blood model better reflects an *in vivo* situation.

Cross-talk between the various parts of inflammation is known from basic research and animal experimental studies. It therefore seems logic to search for such interactions also in clinical trials where one component is inhibited. A more complete and complex understanding of the individual players and the interactions between the various players in the complex inflammatory cascades is essential in finding new possible therapeutic targets. The results presented in paper II contributes to improved understanding of the effects of IL-6R inhibition.

3.1.3 The POSTEMI-trial population

In paper III, samples from patients enrolled in the POstconditioning in ST-Elevation Myocardial Infarction (POSTEMI) trial were analysed with respect to sIL-1Rs (NCT00922675) (146). Patients admitted to hospital with first-time STEMI (n=272) were included. Cardiac magnetic resonance imaging (CMRI) was performed in the acute phase and after four months for evaluation of infarct size, MVO, LV ejection fraction (EF) and changes in LV performance measured as left ventricle end systolic volume (LVESV) and left ventricle end diastolic volume (LVEDV). Plasma samples were analysed for sIL-1R1, sIL-1R2, IL-1Ra and sIL-1RAcP.

The correlation between sIL-1R2 and markers of LV adverse remodeling was rather weak. However, there were consistent findings with correlation between sIL-1R2 and several markers of myocardial damage at several measurement points. The correlation remained significant even after correction for confounders in regression analysis. sIL-1R2 further increased the explained variation of change from baseline to 4 months (Δ) in indexed (i) LVEDV and ΔLVESVi compared to CRP alone, indicating that sIL-1R2 is involved in the chronic non-resolving inflammatory response associated with adverse LV remodeling.

Patients with AHF or shock were excluded from this trial. Since the majority of STEMI are not complicated by AHF or shock, the patients included here are more representative for the whole STEMI population. Since IL-1β-inhibition has been tested out in clinical trials lately (previously discussed), it is of particular interest to evaluate the regulators in this system. The findings in paper III are, however explorative and further investigations are needed.

3.1.4 Three patient groups with various entities of CAD

In paper II, three patient groups with different entities of CAD, previously described (147), were examined with respect to anaphylatoxin receptor expression in PBMCs isolated from blood sampled at admission to hospital, before any treatment was given. The three patient entities were defined as: i) stable angina pectoris (SAP) (n=22), ii) NSTE-ACS (n=21) that included UAP (n=14) and NSTEMI patients (n=7), and iii) STEMI patients (n=20). By including this patient cohort, we could explore the anaphylatoxin receptor expression in another CAD population and also in specific cells. The lack of follow up samples is a weakness in this population.

3.1.5 The Control groups

In paper I-III healthy controls were included for comparison. The controls were healthy individuals mainly recruited from blood donors. All samples from

controls were treated in the same way as the patient material. When a group of controls is included, it is a relevant question whether the controls are representative for the healthy population. There were few differences between the controls groups and the baseline characteristic in the patient population, except from in paper III where age, sex, smoking and BMI were significantly different in the controls. This had to be corrected for statistically by using analysis of covariance (ANCOVA).

3.1.6 *In vivo* porcine model with complement inhibition in myocardial infarction

In paper IV we explored the effects of targeting complement in an experimental model of I/R injury. Sixteen pigs of 20 kg were randomized to treatment with the tick derived C5-inhibitor *Ornithodoros moubata* Complement Inhibitor (OmCI), known as coversin, or placebo. Coversin effectively blocks C5 in humans and pigs and additionally inhibits LTB4 (148). The left anterior descending artery (LAD) was totally occluded for 40 minutes, which is equivalent with approximately 240 minutes of ischemia in man (149). A reperfusion period of 240 minutes was chosen to ensure adequate time for wash out of dehydrogenases from infarcted cardiomyocytes. The AAR was delineated by injecting Evans blue through a central venous catheter.

EDTA plasma and serum samples, blood gas samples and microdialysis samples were collected at specific time points throughout the experiment. Microdialysis catheters were inserted into the AAR and to a control region for sampling of several biomarkers which could reflect the inflammatory process at the site of injury. Tissue was sampled from the same areas and analyzed for markers of endothelial cell activation (E-selectin and Fibrinogen-like protein 2) and C5b-9 deposition by immunofluorescence staining of the tissue.

The AAR was isolated, cut into 5 mm slices and immersed in triphenyl tetrazolium chloride (TTC). TTC stains dehydrogenasis in viable cells red, while

infarcted tissue remains white when adequate washout is ensured and there is no new production of dehydrogenasis. Infarct size was determined as a percentage of non-viable white tissue of the total AAR using pixel count in Photoshop CS5. LV performance was evaluated by echocardiography of the left ventricle before induction of ischemia and after reperfusion, prior to euthanization. Infarct size was measured by MRI of the excised heart and by histological staining.

Animal studies are important tools for understanding molecular mechanisms in various pathophysiological conditions. However, the translation to clinical outcome is less certain. The strength of using pigs is that they to a large extent share anatomical, physiological and immunological properties with humans (150, 151). However, huge differences between experimental conditions and clinical settings exist: The mechanism behind coronary occlusion differs, animals included are young, healthy and "standardized" without comorbidity and coronary collaterals that often characterize patients suffering from MI.

The consistent and robust findings with reduced local inflammation in the AAR, reduced infarct size measured both by TTC staining and CMRI and the improved LV function in the coversin-treated animals strengthen this study together with the considerable differences between the groups. The lack of differences between the groups considering markers of myocardial necrosis is a weakness. The long-term effects could not be measured since the animals were sacrificed following reperfusion. The sample size was small, but this is important with respect to ethical considerations related to animal studies.

We have later tried to test other inhibitors in a similar model, but the model showed large variations in infarct size measured by TTC staining in all groups, also the control group. The reason for this is uncertain but a successful reperfusion with sufficient wash-out of tissue dehydrogenases is mandatory to evaluate infarct size by TTC. An insufficient and variable reperfusion could explain the large variations in infarct size and could be caused by damaged vasculature. Such damage would probably also affect other read-outs like

echocardiographic measurements since edema would preclude the results and make them difficult to interpret.

3.2 Blood sampling

Complement is constantly activated at a low level *in vivo* and is rapidly activated *in vitro*. To measure reliable complement activation, the samples must be properly collected and stored so that *in vitro* activation and degradation is avoided (152). In all four studies, blood was collected, handled and stored in accordance with current recommendations. Blood for plasma preparation was drawn into EDTA vacutainer tubes and immediately placed on crushed ice and centrifuged at 4 °C to separate plasma. Blood for serum preparation was allowed to clot for 60 minutes in room temperature and thereafter centrifuged at 2500*g* for 10 minutes for isolation of serum. All samples were stored at -80°C until analyzed and thawed only once.

In the IL-6 intervention study (paper II), venous blood was drawn directly into blood sampling tubes containing a stabilizing reagent which ensures lysis of whole blood cells and stabilization of RNA in the sample. In the same study, three different CAD patient entities were included. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood and stored as cell pellets. All samples were stored at -80°C until RNA isolation was performed.

3.3 Enzyme- linked immunoassays (ELISA) and multiplex technology

ELISAs are easy to use and is considered to have high sensitivity and specificity. To avoid *in vitro* cytokine release by leukocytes and platelets after sampling, EDTA plasma should be used (153). A challenge with both ELISA and multiplex analysis is variability between plates even though the use of a standard curve with known concentration will limit measurement differences. To avoid plate-to-plate differences to influence the results, we have mixed patient groups (shock and non-

shock) and controls on each plate but analyzed all samples from the same patient on the same plate. This strategy was used also for the qPCR analyzes.

ELISAs are either in-house made or commercially available but the principles are the same. A microtiter plate is coated with a primary antibody with binding capacity for the molecule at interest. A secondary antibody which binds to the molecule of interest is added. This antibody conjugates with an enzyme which after adding a substrate solution, catalyze a change of color proportional to the concentration of the molecule at interest. A standard solution with a known concentration of the molecule of interest is added to separate wells. The color intensity is read by a spectrophotometer and the concentration of the molecule of interest is calculated from color intensity and dilution factors.

In-house made ELISAs with monoclonal antibodies detecting neoepitopes present only after activation (C4bc, C3bc, sC5b-9) or pairs of antibodies binding complexes formed upon activation of the complement cascade (C3bBbP), were used to measure the complement activation products as described previously (154). The recognition molecules of the lectin pathway (FCN-1, FCN-2, FCN-3 and MBL) were measured by in-house ELISAs using monoclonal antibodies both for detection and capture. Commercially available ELISA kits were used to measure level of sIL-1R1, sIL-1R2, sIL-1RAcP and IL-1Ra in paper III, and heart fatty acid binding protein (H-FABP) and LTB4 from plasma and tissue in paper IV. To evaluate the functional activity of all three complement activation pathways in paper IV, commercially available ELISA kits were used (Complement System Screen Wieslab). All ELISAs were performed in accordance to the manufacture's instruction. In paper III, we tried to quantify IL-1β, but it proved to be difficult to detect. This is well known, even if IL-1β has been measured following MI both by our group and by other groups previously. Since IL-1β activation following MI has been shown before, we did not move on with further efforts to try to quantify IL-1β.

Multiplex technology was used in paper IV to measure IL-1β, IL-6, IL-8, IL-10, and TNF from EDTA-plasma and microdialysis fluid from the AAR and control

region. In multiplex technology several different biomarkers can be measured simultaneously using uniquely coloured beads each with a specific cytokine detection antibody attached to it. The samples are added to the beads followed by a biotinylated secondary antibody and a fluorescent reporter molecule that binds biotin. In a modified flow cytometer the beads and the reporter molecule are separated and the amount of each cytokine is measured by the fluorophore intensity which reflects the amount of each cytokine. A standard is added and enables a quantification of each cytokine.

3.4 Reverse transcription polymerase chain reaction (RT- PCR)

RT- PCR is a sensitive and reliable method for detection and quantification of nucleic acids based on fluorescence emitted from a reporter molecule at real time. The amount of cDNA is measured using oligonucleotides and a fluorescent probe which emits light whenever a small region of mRNA is amplified. When the amount of PCR product is large enough to be detected by the PCR machine and is stable in the linear phase of the amplification curve, the Ct-level is determined. The Ct- level reflects the original amount of the gene. The higher the Ct- level, the smaller the amount of product (more cycles needed to produce detectable amounts of the product).

We quantified the gene expression using the relative Ct quantification method where the expression of the gene of interest is related to the expression of a housekeeping gene or reference gene. An ideal reference gene should have a stable transcript in the specific experiment and also have an abundance of transcription similar to that of the gene of interest (155). Genes involved in processes essential for survival of cells are often expressed in a stable, non-regulated way and is often used as reference genes.

We tested different reference genes both in various biological contexts and in the patient samples. B2M was the most stably expressed of the different reference genes we tested (glyceraldehyde-3-phosphate dehydrogenase (GAPDH),

ribosomal protein, large, P0 (RPLP0), beta-2-microglobulin (B2M), TATA sequence binding protein (TBP) and ribosomal protein 18 (RSP18)). In addition to choosing a stably expressed reference gene, all samples from the same patient were analyzed on the same plate. They were analyzed according to inclusion number ensuring a randomized mix of placebo and tocilizumab-treated patients on each plate. Controls were spread on several plates and mixed with samples from patients. These efforts should reduce the probability for bias.

In paper II we analyzed the expression of C5aR1, C5aR2 and C3aR in blood from 60 patients from the IL-6R-inhibition study. In the CAD population, RNA was isolated from peripheral blood mononuclear cells (PBMC) and investigated with respect to expression of the same receptors. In this material, GAPDH was used as reference gene.

3.5. Measurement of left ventricles performance

Echocardiography is the most commonly preferred imaging modality used to evaluate LV systolic function due to its widespread availability, low cost and negligible risk (18). Established indices like LV ejection fraction (EF) or wall motion score index (WMSI) are validated methods but they rely on image quality and experience of the examiner. Experienced cardiologists performed echocardiography for evaluation of LV function in paper I and IV.

CMRI is considered the gold standard due to its accuracy, reproducibility and low risk. It is however less available and also dependent on a patient that is stable enough to perform a MRI investigation. CMRI was performed in patients included for the POSTEMI-trial (paper III) in the acute phase and after 4 months and in the porcine heart *ex vivo* (paper IV). The patients included in the LEAF trial (paper I), were critically ill and not stable enough to perform CMRI investigation.

3.6 Statistical considerations

The impact of statistical analysis has grown enormously since they were introduced in medicine and now governs our opinion on what is good medical practice. Results from RCTs and meta-analysis form the basis for the evidence based medicine.

Statistics is a tool to test for the relationship between two data sets, ideally being representative samples from a population. The null-hypothesis that is to be tested claims that there is no difference between the groups. The p-value helps determine whether to reject or not to reject the null hypothesis. It indicates the chance of getting this result or an even more extreme result if the null hypothesis is true and a typically accepted value is 5%. A significant p-value says nothing about biological relevance. Consistency of the findings and focus on what is biologically relevant is therefore always important. When comparing more than two groups or measurements, post-hoc analysis has to be performed to reduce the risk of rejecting the null hypothesis when it is true and in this way gain false positive results. This is called Type I error. If the null hypothesis is kept even if it is false, false negative results are produced. This is called Type II error.

In the work included in my thesis, continuous data have been tested for normality and parametric tests have been chosen when data were normally distributed. However, in my work, non-normally distribution has been common and non-parametric tests are therefore most widespread. Non-parametric tests generally have less power than parametric analysis but are recommended if the data are not normally distributed and when the sample size is small. In paper III, regression analyzes required normalization of not normally distributed data. This was done by logarithmically transforming the data with the natural logarithm (ln). To compare categorical variables, the chi square test was used in all papers.

In my work I have used statistical tests to:

1) **Compare differences between groups or time points**:

When comparing samples from two different groups we used the Mann-Whitney test and when comparing two measurements from the same group we used Wilcoxon test (paper II, within group differences compared to baseline measurement). When comparing three or more groups or time-points, we used ANOVA for normally distributed data and Kruskal-Wallis test for non-normally distributed data.

In paper III the sample size was large enough for the repeated measurement ANOVA to be used for assessing change of protein level over time. In paper II the sample size was smaller and not normally distributed and therefore the Friedmans test was used to compare repeated measurements. This is the non-parametric alternative for repeated measurement ANOVA. In paper I and IV, the sample size was small and in paper I there were several data points missing (n=4 from day 0-5 in the non-shock group and n=3 day 0-5 in the shock group). The repeated measurement ANOVA, including the Friedmans test cannot handle missing data without discarding all data from a subject if one data point is missing. This called for a mixed model analysis in the materials presented in paper I and IV. This method handles small sample sizes and incomplete and unbalanced data (156). A mixed model analysis takes into account that each individual has its own intercept and slope and models the mean response as a combination of population characteristics assumed to be shared by all individuals in a group, the fixed effects, and individual characteristics (effects that are unique for one particular individual in the group, the random effects). However, it claims a correct specification of the model and is more complex to build and interpret.

2) To evaluate associations between variables:

Spearman rank test was used for correlation analysis in paper I and III while Pearson Rho was used in paper IV. The magnitude of the correlation coefficient indicates the strength of the association. The p-value tells how much confidence we can have in the obtained results. A correlation coefficient range from -1 to +1

and the closer to -1 or +1 the coefficient value is the stronger is the relationship between the variables. A correlation coefficient of 0.3 or lower is considered a weak correlation, a correlation coefficient of 0.5 or above is considered moderately strong and a correlation coefficient above 0.7 is considered strong (157). The correlation coefficient says to which extent two variables move in the same direction but does not imply causation and the p-value only describes the reliability in the results. In samples collected following an acute coronary event, there will be myriads of substances secreted that influence each other in different ways which will influence the correlation.

To further explore associations, multiple regression analysis is desirable. In paper I, regression analysis was not performed due to the small sample size, which would make it difficult to draw conclusions. In paper IV, regression analysis was not relevant since what we wanted to evaluate were differences between the coversin-treated and placebo-treated animals. We were looking for differences large enough to be clinically relevant. In paper III, however, the sample size was adequate for further analysis. We therefore explored an eventual association between sIL-1R2 and markers of LV remodeling by doing multiple regression analysis.

4. Summary of Results

Paper 1: Acute heart failure following myocardial infarction: complement activation correlates with the severity of heart failure in patients developing cardiogenic shock

In this study complement activation products from all activation pathways were investigated in a group of patients developing AHF (n=52) or cardiogenic shock (n=9) following STEMI. C4bc (classical and lectin pathway), C3bBbP (alternative pathway), C3bc (common pathway) and sC5b-9 (terminal pathway) were all higher in the patients at inclusion compared to a group of healthy controls (n=44). The cardiogenic shock group had higher levels of complement activation products compared to the HF group throughout the first 5 days from inclusion. This difference persisted even at day 42. Complement activation measured as sC5b-9 further correlated significantly with LV performance measured as WMSI. A significant but weaker correlation was found between complement activation and markers of endothelial cell activation. Recognition molecules from the lectin pathway were analyzed and a decreased level of FCN-2 was found in the patients compared to healthy controls at inclusion. At day 42, FCN-2 was higher in the shock group compared to the non-shock group. No other differences in lectin pathway recognition molecules were found.

Conclusion: Complement was activated throughout the whole cascade in patients developing HF within 48 hours following STEMI with the highest levels in patients developing cardiogenic shock, a difference that persisted even at day 42. Complement activation correlated with left ventricular function in patients with cardiogenic shock. We could not conclude on any particular activation pathway in this patient population.

Paper II: IL-6 receptor inhibition by tocilizumab attenuated expression of C5a receptor 1 and 2 in non-ST-elevation myocardial infarction

In this study, the expression of C5aR1, C5aR2 and C3aR in 60 patients with NSTEMI randomized to treatment with the IL-6R-inhibitor tocilizumab (n=28) or placebo (n=32) were analyzed and compared to a group of healthy controls (n=15). The patients constituted all patients included at one of two hospitals recruiting patients for the IL-6R-inhibition trial (see section 3.1.2). Additionally, complement activation measured as sC5b-9 was analyzed in the whole study population included in the trial (n=117). Further, correlation between the anapylatoxin receptors and markers of inflammation (CRP) and myocardial necrosis (TnT) were studied. C5aR1, C5aR2 and C3aR expression were further analyzed in PBMCs from patients with SAP (n=22), NSTE-ACS (n=21) and STEMI (n=20) and in a group of healthy controls (n=29).

Tocilizumab attenuated the expression of C5aR1 and C5aR2 but not C3aR in NSTEMI patients. This effect was independent of treatment with PCI or time from onset of symptoms to inclusion. Expression of C5aR2 was decreased throughout the whole study period in both patient groups compared to the healthy controls. C5aR1 correlated significantly with change in TnT during the first days following NSTEMI. Tocilizumab did not affect the level of sC5b-9 during the period of hospitalization. The expression of C5aR1 was elevated in PBMCs from patients with different entities of CAD. The expression reflected disease severity as the highest expression was found in the STEMI population and the lowest expression in the SAP patients. C5aR2 was increased in STEMI-patients only.

Conclusion: Inhibiting the IL-6 receptor attenuated the expression of C5aR1 and C5aR2, which might contribute to the anti-inflammatory effects seen by such treatment. All three receptors were expressed in PBMCs in CAD patients. The level of C5aR1 reflected disease severity, suggesting a role for complement in plaque progression and destabilization.

Paper III: Soluble IL-1 receptor 2 is associated with left ventricular remodeling in patients with ST-elevation myocardial infarction

Levels and temporal changes of the soluble regulators of IL-1 signaling were analyzed in 272 STEMI patients and compared to a group of healthy controls (n=65). Further, the associations between the receptors and parameters of cardiac injury and ventricular remodeling were investigated.

Compared to the control group, IL-1Ra, sIL-1R1 and sIL-1R2 were significantly elevated following STEMI, while the level of sIL-1RAcP was decreased. Level of sIL-1R2 correlated positively with CRP, myocardial infarct size and markers of LV remodeling (changes in LVEDVi and LVESVi from baseline to four months). Patients with the highest level of sIL-1R2 in the acute phase were more likely to have increased change in LVEDVi and LVESVi. Importantly, sIL-1R2 remained significantly associated with change in LVEDVi and LVESVi after adjustment for clinical covariates.

Conclusion: Levels of the negative regulator of IL-1 signalling, sIL-1R2, is independently associated with parameters of LV adverse remodeling following STEMI even after adjustment for relevant covariates. Whether inhibition of IL-1 signalling is associated with the development of LV remodeling needs further investigation.

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

In this study we evaluated the effects of inhibiting complement at the level of C5 in a model of porcine myocardial I/R injury. The C5-inhibitor coversin was administrated before induction of ischemia and the effect on infarct size and LV function were evaluated in a double blind, randomized porcine trial.

Infarct size was significantly reduced by coversin treatment measured both by CMRI and TTC staining. LV function measured by echocardiography showed

improved ventricular function in the coversin-treated animals. Further, C5b-9 deposition in the myocardium was abolished by coversin and IL-1β in the AAR was significantly reduced. Also E-selectin in the borderzone was reduced by complement inhibition. We documented that the terminal pathway complement activity in plasma was blocked by coversin.

Conclusion: A total complement blockade by coversin reduced infarct size, improved LV function and attenuated local inflammatory markers in an experimental model of myocardial I/R- injury.

5. General discussion

Inflammation is critically involved in both tissue injury and repair following a MI. Enhanced inflammation in the acute phase following a MI is associated with dilative remodeling with reduced LV function as a result, while such a response in the proliferative phase may lead to enhanced fibrotic remodeling and development of HF with preserved ejection fraction. Both conditions hold poor prognosis. Treatment strategies that attenuate the inflammatory response following MI may improve outcome. However, dampening inflammation holds a risk for interrupting the mandatory healing process of the heart with devastating consequences for the structural and functional result. Individual variation within the group of MI patients like age, gender, genetic phenotype, comorbidities and premorbid medication makes some patients more prone to HF development than other (23).

There is probably a window of opportunity for safe and efficient targeting of inflammation. In order to find appropriate targets for therapy, a thorough understanding of the action and interactions of relevant players in the inflammatory cascade is mandatory. Key questions in targeting inflammation in MI are therefore: which part of the inflammatory system is best to target? When is the best time point to target? Who will benefit from targeting inflammation? For how long time should inflammation be inhibited?

5.1. Inflammation in ACS

In my work I have found support for an activated inflammatory system in patients with ACS that is also related to disease severity.

5.1.1 Complement activation products in ACS

Complement activation in STEMI patients has previously been acknowledged (158, 159) and a SIRS-like clinical picture with elevated cytokine level in the

most severely affected patients has been described (160). However, since acute HF and shock are often exclusion criteria in clinical trials, pathophysiological processes in these patients are incompletely studied and understood. In paper I the temporal changes of complement in a population with AHF or cardiogenic shock following STEMI was studied. A significantly higher level of complement activation in these patients compared to healthy individuals was documented together with an association to disease severity. The level of complement activation correlated with LV function both in the acute phase and after 6 weeks indicating a role for complement in the AHF development as well as in the adverse remodeling process particularly seen in the most severely affected patients. Evaluating samples from HF and shock patients adds new information about patients normally excluded from clinical trials. This is further a group of patient where supplementary treatment is strongly needed.

All three activation pathways have been associated with complement activation in I/R injury (161). The lectin pathway, shown to be involved in the clearance of dying host cells, is currently regarded as the most relevant activation pathway following I/R injury (57, 162). In order to further elucidate the role of the lectin pathway in patients with AHF and cardiogenic shock following STEMI, the temporal profile of the recognition molecules FCN-1, FCN-2, FCN-3 and MBL were studied in paper I. FCN-2 was lower at inclusion and higher at day 42 compared to healthy controls and was also higher in the shock group compared to the HF group at this measurement point. In a study of STEMI patients without HF, Schoos et al. found a similar pattern for FCN-2 with an increase from baseline and lower levels compared to controls. In Schoos material, FCN-2 remained lower than the control group even at day 31 (163). Our findings might reflect an initial consumption and further upregulation of FCN-2 and indicate a role for this recognition molecule in damage sensing and inflammatory triggering in MI. The elevated level of FCN-2 might indicate that FCN-2 is involved in the chronic inflammatory process seen in patients with adverse cardiac remodeling and HF development following MI. This however, remains speculative and needs further evaluation.

Other activation pathways might also be involved in danger sensing and complement activation following a MI. The recognition molecule of the classical pathway, C1q, is known to recognize cell wall components of infectious agents as well as subcellular membranes and modified host proteins and could be responsible for complement activation following tissue injury (164). CRP is known to be co-localized with complement components in the heart following ischemia and to activate classical pathway of complement (165). Under all circumstances the alternative pathway is a strong amplifier of the response, no matter which pathway triggered the activation (60, 61).

In *Paper IV* we found support for a role for complement in the ischemia and reperfusion damage of the myocardium. Complement targeted in the APEX-AMI trial failed to reduce mortality, shock or HF (140). However, retrospective investigations have shown similar increase of sC5b-9 in the pexelizumab and the placebo-treated patients and thus questioning the conclusions of the trial (143). sC5b-9 has shown to enhance inflammation by activating the inflammasome and could therefore account for inflammatory induced myocardial damage (94). We showed in the porcine model that a terminal pathway inhibition at the level of C5 led to reduced infarct size and improved LV function. Additionally, IL-1β and E-selectin expression was reduced in the AAR of the coversin-treated animals supporting an upstream and amplifying role for complement in myocardial inflammatory I/R-injury.

Coversin additionally binds the arachidone acid metabolite LTB4. LTB4 attracts neutrophils to the site of damage, which might amplify the cytotoxic damage to the myocardium (166). LTB4 in the myocardium was unaffected by coversin treatment while the plasma concentration showed a non-significant reduction in the coversin treated animals. Thus the role of LTB4 in the myocardial inflammatory response following I/R-injury in our model is uncertain.

5.1.2 The anaphylatoxin receptors in ACS

In paper II we found the highest expression of C5aR1 in the STEMI patients while the SAP patients showed the lowest expression of C5aR1 indicating a relationship with disease severity for C5aR1. Increased expression of the C5aR1 in patients admitted to hospital with ACS fits with the observation that elevated levels of C5a in patients with atherosclerotic disease is associated with increased risk of MACE (29). C5aR1 on bone marrow-derived cells has shown to influence on infarct size and LV function in experimental rodent trials with myocardial I/R (167). In paper II, correlation between TnT and C5aR1 was documented. However, it is not clear to what extent the reduced C5aR expression contributed to a beneficial effect on myocyte survival in the tocilizumab-trial.

In the NSTEMI population treated with IL-6R inhibitor or placebo, we found decreased expression of C5aR2 in both groups throughout the whole study period and a marked attenuation of the expression in the tocilizumab-treated patients. The effect of attenuating C5aR2 expression is unclear as both inflammatory and anti-inflammatory roles for this receptor have been described. In a model of renal I/R injury, C5aR2 had unfavorable effects on renal function (168). A sepsis study documented reduced inflammation and improved outcome when C5aR2 was blocked (75). This makes it tempting to speculate in a beneficial effect of C5aR2 downregulation. In the group of ACS-patients, a modest elevation of C5aR2 was documented in the STEMI group only, which might be a result of disease severity. The divergent results for C5aR2 between PBMCs in STEMI patients and whole blood in NSTEMI patients might be explained by different cell types or different time points of sampling related to the MI.

C3aR was not affected by the treatment with tocilizumab but increased slightly the first two days following inclusion. The role of C3aR seems to be dependent on cell type and disease context (66). In the acute phase of inflammation, C3aR-activation induces net anti-inflammatory effects by rejecting neutrophil recruitment from the bone marrow. Thus the slightly elevated expression of C3aR in the first days following MI might be protective.

The effect of IL-6R inhibition on the anaphylatoxin receptors in a human material has never previously been demonstrated. The data presented in paper II contributes to the understanding of the mechanisms behind the anti-inflammatory effects of inhibiting IL-6.

5.1.3 Regulators of IL-1 signaling in STEMI patients

IL-1β is counteracted by two soluble negative regulators: sIL-1R2 and IL-1Ra. Elevated level of both these IL-1 antagonists was documented in our work presented in paper III. A similar pattern has been shown for patients with sepsis (169). IL-1Ra is known to be elevated following MI, but little is known about the other regulators of the IL-1 signaling system. However, macrophages from patients with high lipid levels have shown reduced expression of IL-1R2 indicating a poor regulation of inflammatory signaling in these cells in patients with atherosclerosis (112).

The negative regulators of IL-1 signaling, sIL-1R2, sIL-1R1 and IL-1Ra were all elevated in the acute phase of STEMI, while sIL-1RAcP was decreased. The change in the regulators of IL-1 signaling probably reflects activated inflammatory cascades following STEMI.

sIL-1RAcP binds sIL-1R2 and increases its affinity for IL-1 (112). Further sIL-1RAcP can bind IL-1R1 but has no signaling capacity. Thus, in its soluble form, IL-1RAcP acts as a negative regulator of IL-1 signaling. Whether the lower level of sIL-1RAcP observed in our study is caused by consumption or downregulation is not clear. Neither is the net effect of the observed lowered level of sIL-1RAcP clear. sIL-1R1 and sIL-1R2 bind IL-1 but has no signaling capacity thus acting as negative regulators of IL-1. sIL-1R1 was elevated immediately following STEMI with a rapid normalization. sIL-1RAcP and sIL-1R2 were both changed throughout the whole study period.

IL-1R2 is rapidly upregulated following ischemia and is rapidly shed from the membrane following inflammatory signals like TNF, ROS and LPS (112).

Aspirin, which is given to MI patients as part of the treatment, promotes shedding of the IL-1R2 from cell membranes (170). However, this cannot explain the association with disease severity documented in paper III as aspirin is given in the same dose independent of disease severity.

Previous studies have shown associations between IL-1Ra and myocardial necrosis (171, 172). In our material, no such association was found. IL-1Ra acted like an acute phase molecule with rapid increase immediately after the MI. It remained elevated even at 4 and 6 months and correlated with CRP and was thus associated with inflammation but not with myocardial necrosis or LV function in our material. sIL-1R2 acted as a better predictor of local tissue injury in our study.

5.2 Inflammation and adverse cardiac remodeling

The role of inflammation in adverse cardiac remodeling and HF development has been suggested and a support for this notion was found in the work included in my thesis.

5.2.1 Complement and adverse remodeling

In paper I we showed a significant correlation between sC5b-9 and LV function both measured in the acute phase and after six weeks indicating a role for complement in adverse cardiac remodeling and HF development. Due to the small sample size, we refrained from more explorative statistical methods to control for confounding variables. Elevated complement activation has also previously been associated with congestive HF and reduced ejection fraction in patients with STEMI without reperfusion (173). Ørn et al. did not find any correlation between complement activation and LV function measured by CMRI two months following PCI-treated STEMI (158). This may fit with the results in our study where the correlation between complement and WMSI was seen only in the shock group.

All complement activation products showed higher levels in the shock group after six weeks indicating that complement is a player in the chronic non-resolving inflammation that characterize some patients with coronary artery diseases and which makes them vulnerable for new cardiovascular events. Whether complement is a marker or a mediator in these patients is not clear but in the various clinical trials where complement has been targeted, the effect of complement inhibition has been seen in high-risk patients (PRIMO-trials). In the COMMA-trial, a non-significant reduction in cardiogenic shock was seen supporting a role for complement as a mediator in the most severely affected patients. This has to be interpreted with caution as all findings are from sub-group analysis and therefore has to be considered explorative.

5.2.2 IL-1 and adverse remodeling

In Paper III we addressed the role of sIL-1R2 and markers of myocardial damage and adverse remodeling. We found an association between sIL-1R2 and infarct size, LVEF, AAR and MVO in the acute phase of STEMI. Patients with high levels of sIL-1R2 (> median) had larger change in LVEDV and LVESV. When analyzing this further, we recognized an association between sIL-1R2 and change in LVEDVi and LVESVi form the acute phase to four months at several sample points. These associations remained significant even after adjusting for various confounders in multiple regression analysis. Even though no protective role for IL-1 in infarct healing has been reported (25) our findings may indicate a possible harmful effect by inhibiting IL-1 signaling even though this is at present not clear. The recently published CANTOS trial showed beneficial effects when IL-1β was blocked in patients (n=10 061) with previous MI and elevated CRP (103). However, Morton et al found increased incidence of MACE one year after IL-1Ra-treatment for NSTE-ACS (102). These results may indicate that IL-1-inhibition is favourable in the chronic phase of CAD while it might be detrimental to block IL-1 in the acute inflammatory phase following a MI.

5.2.3 Other findings related to adverse remodeling

Papers II and IV were not designed to evaluate long term effects on heart function. The animal experiments were terminated following 4 hours of reperfusion. However, infarct size is an important predictor for mortality and development of HF (174) and in this perspective, the animal study supports a role for complement inhibition in improving long term outcome following MI.

The work presented in Paper II did not evaluate LV performance. There were only a few difference observed between the placebo- and the tocilizumab-treated patients after six months. However, a slightly lower expression level of C5a-R1 was seen in the tocilizumab-group compared to placebo group indicating a possible long-term, anti-inflammatory effect of tocilizumab-treatment. Opposed to this finding, was a small but significant increase in sC5b-9 level in the tocilizumab-treated patients after six months. The difference is small and could be without clinical relevance, but it cannot be excluded that IL-6-inhibition can induce unintended long-term effects as IL-6 is a pleiotropic cytokine with both inflammatory and anti-inflammatory effects with a possible role in scar formation and wound healing (175). Further, treatment with antibodies may give rise to complement activation, even though this probably would have been reflected also in the acute phase when tocilizumab was still present in plasma.

5.2.4 Cross-talk and redundancy

We examined an eventual cross-talk between IL-6 and complement anaphylatoxin receptors in Paper II. During the 1990s Riedemann et al. performed sepsis studies in rodents and demonstrated cross-talk between complement and IL-6, (126, 132, 176). In paper II, we evaluated for the first time in human material the effect of IL-6R-inhibition on all three anaphylatoxin receptors and found that tocilizumab clearly reduced the expression of C5aR1 and C5aR2 while the C3aR was unaffected by the treatment. Whether this cross-talk contributed to the attenuated inflammation and TnT release seen in the NSTEMI patients (124) remains

speculative. However, proving this cross-talk shows the importance of investigating potential interactions between the various participants in the inflammatory cascade when one of the components is targeted.

In paper IV we found that complement inhibition at the level of C5 led to decreased level of IL-1β and E-selectin in the AAR showing that complement inhibition attenuates inflammation at the site of injury, even though a contribution from LTB4 inhibition cannot be excluded. It has been thoroughly described that complement activation, at the level of C3 and C5 as well as sC5b-9, gives rise to IL-1β production (63). Blocking complement would thus further attenuate IL-1-mediated inflammation at least locally in the myocardium.

A crosstalk between complement and the coagulation system may also be of importance in the acute thrombo-inflammatory response with development of MVO following MI. MVO has greater prognostic significance than the infarct size *per se* (33). Cross-talk with the coagulation cascade has not been addressed in the work included in my thesis, but complement is a well-known and important player in several thrombo-inflammatory diseases and is known to activate the coagulation cascade in several ways, including the ability of C5a and sublytic C5b-9 to upregulate TF on endothelial cells (136). It would therefore be of interest to evaluate this crosstalk in myocardial I/R injury (177).

5.3 Future perspectives and concluding remarks

Targeting the inflammatory cascade is considered interesting in order to improve outcome following MI. Questions like what and where to target, who will benefit from targeted treatment , when to target and for how long are still unanswered. In the future improved and new diagnostic tools, both considering biochemical mediators and markers and also imaging tools, together with the implementation of artificial intelligence will probably pave the way for personalized treatment strategies making it easier to handle these challenges. However, a thorough understanding of the temporal profiles and interaction between the various players

in the inflammatory cascade is of fundamental importance in order to clarify these questions.

In the window of opportunity in the immediate phase following a MI, it seems reasonable to target early and upstream activators of the inflammatory cascade to avoid excessive inflammation. The complement cascade is a highly relevant target in this respect. It might also be necessary to target more than one component as extensive cross-talk between the various sensors and effectors of inflammation exists. Our group has previously reported improved anti-inflammatory effects of combined complement and CD14-dependent TLR inhibition (178-180). CD14 is a co-receptor for several of the TLRs relevant also in myocardial ischemia and reperfusion damage.

At what level complement should be targeted is still unclear and inhibition of the lectin pathway has shown some promising results in I/R injury trials (181). However, inhibiting complement above the level of C3 might lead to reduced clearance of cells and debris due to reduced opsonization by C3 cleavage products. Further, complement at the level of C3 has shown protective effects on the myocardium in a rodent model of chronic myocardial infarction (68).

The large and negative APEX-AMI trial had an extremely cooling effect on the scientific community when it was launched in 2007. It was concluded that complement inhibition did not improve outcome in PCI-treated STEMI patients. Despite the fact that the level of sC5b-9 was the same in the placebo-group as in the group receiving pexilzumab and complement thus was not inhibited at the terminal pathway (143), neither scientists nor the pharmaceutical industry were encouraged to perform further clinical trials. It is known that terminal pathway activation can occur in situations with high complement activation due to high density of C3b on surfaces competing with the inhibitors about C5-binding. Adding other inhibitors can reduce the activation (182). sC5b-9 is known to activate inflammation and hence might contribute to enhanced inflammatory damage to the myocardium (94).

Early administration of inhibitors is more efficient since complement activity is reduced before it can trigger further inflammatory activity. One could speculate that the patients in the APEX-AMI-trial received too little complement inhibition too late. Our findings in the porcine in vivo trial support early administration of complement inhibition at the level of C5 ensuring inhibition even of the terminal pathway. This is possible in a clinical setting where C5-inhibitor can be administrated in the ambulance before the patient reaches the hospital. In our opinion a clinical trial with early administration of C5-inhibitor that ensures even terminal complement blockade should be conducted.

Defining the most suited end-point in clinical trials is highly relevant. Differences in a continuous variable like CRP, which is closely linked to complications following MI, can be shown with fewer participants than a binomical endpoint like death or HF (183). What is a good marker of myocardial damage in a clinical trial is also a relevant question. MVO has been shown to be more relevant in predicting HF-development than infarct size and should probably be preferred when evaluating treatment effects.

There is still a lot of uncertainty considering what to inhibit, when to inhibit, who will benefit from anti-inflammatory treatment and for how long inhibition is convenient. Our findings related to the IL-1 signaling system renders IL-1 inhibition in the acute phase of MI uncertain. Recent clinical trials may indicate a protective role of IL-1-inhibition in the more chronic phase of cardiovascular diseases. However, both complement, IL-1β and IL-6 are central and important players in the inflammatory response following MI and in the chronic non-resolving inflammation that is believed to contribute to the adverse remodeling of the heart. Our work has found support for a role for complement both in the acute setting of complicated MI and also in the detrimental remodeling process leading to HF development. *In vivo* cross-talk between complement and IL-6 and IL-1β has further been documented and future clinical trials should include complement when analyzing the effects of targeting any part of the inflammatory cascade.

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ORIGINAL RESEARCH ARTICLE

Acute heart failure following myocardial infarction: complement activation correlates with the severity of heart failure in patients developing cardiogenic shock

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Abstract

Aims Heart failure (HF) is an impending complication to myocardial infarction. We hypothesized that the degree of complement activation reflects severity of HF following acute myocardial infarction.

Methods and results The LEAF trial (LEvosimendan in Acute heart Failure following myocardial infarction) evaluating 61 patients developing HF within 48 h after percutaneous coronary intervention-treated ST-elevation myocardial infarction herein underwent a *post hoc* analysis. Blood samples were drawn from inclusion to Day 5 and at 42 day follow-up, and biomarkers were measured with enzyme immunoassays. Regional myocardial contractility was measured by echocardiography as wall motion score index (WMSI). The cardiogenic shock group (n = 9) was compared with the non-shock group (n = 52). Controls (n = 44) were age-matched and sex-matched healthy individuals. C4bc, C3bc, C3bBbP, and sC5b-9 were elevated in patients at inclusion compared with controls ($P < 0.01$). The shock group had higher levels compared with the non-shock group for all activation products except C3bBbP ($P < 0.05$). At Day 42, all products were higher in the shock group ($P < 0.05$). In the shock group, sC5b-9 correlated significantly with WMSI at baseline (r = 0.68; P = 0.045) and at Day 42 (r = 0.84; P = 0.036). Peak sC5b-9 level correlated strongly with WMSI at Day 42 (r = 0.98; P = 0.005). Circulating endothelial cell activation markers sICAM-1 and sVCAM-1 were higher in the shock group during the acute phase ($P < 0.01$), and their peak levels correlated with sC5b-9 peak level in the whole HF population (r = 0.32; P = 0.014 and r = 0.30; P = 0.022, respectively).

Conclusions Complement activation discriminated cardiogenic shock from non-shock in acute ST-elevation myocardial infarction complicated by HF and correlated with regional contractility and endothelial cell activation, suggesting a pathogenic role of complement in this condition.

Keywords Complement activation; Inflammation; Myocardial infarction; Acute heart failure; Cardiogenic shock; Wall motion score index

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Introduction

The current therapeutic strategy with rapid restoration of blood flow to the ischaemic myocardium by percutaneous coronary intervention (PCI) has markedly reduced the short-term and long-term morbidity and mortality in acute ST-elevation myocardial infarction (STEMI).[1,2] However, acute heart failure (HF) and cardiogenic shock are still important clinical complications of STEMI and remains the leading cause of death in patients with acute myocardial infarction (MI).[3–5] Cardiogenic shock is

defined as a state of mismatch between oxygen delivery and oxygen demand caused by critical tissue hypoperfusion due to reduced cardiac output, and the diagnosis is based on haemodynamic (e.g. hypotension), clinical (e.g. cold extremities), and biochemical (e.g. increased lactate) criteria.[6]

Acute coronary syndromes and MI are associated with inflammation,[7] and activation of the innate immune system such as Toll-like receptors and the complement system are implicated in mediating both adaptive (e.g. tissue repair) and maladaptive (e.g. cardiomyocyte necrosis and apoptosis) responses.[8–10] Cardiogenic shock following MI would exaggerate the inflammatory responses by tissue hypoperfusion and potentially induce a vicious circle.[11] Current management of cardiogenic shock involves strategies to increase cardiac output and antithrombotic treatment but do not target the inflammatory response *per se*.[3]

The complement system, for long appreciated only as a first line of defence against microbes, is today acclaimed for immune surveillance by much broader means. Damage-associated molecular patterns can trigger complement activation through three characterized pathways: the classical, the lectin, and the alternative pathway. They all merge at the central complement component C3 and continue into a common terminal pathway with cleavage of C5 and formation of the terminal C5b-9 complement complex, which, when inserted into membranes as the membrane attack complex, can lyse bacteria and activate host cells. The soluble form of C5b-9 (sC5b-9) is a fluid-phase marker indicating that the terminal pathway has been activated to its very end.[12]

Whereas a balanced activation of the complement system is regarded as beneficial for the host, an overwhelming activation could promote sustained inflammation and tissue damage, as seen during MI and the following ischaemia/reperfusion injury,[13] but its relation to acute HF development following MI is not clear. However, complement is activated in patients with chronic HF, regardless of aetiology, potentially associated with unfavourable outcome,[14–16] and recent studies have highlighted the activation of the lectin pathway as central in ischaemic heart disease and chronic HF.[17,18]

The present study is a *post hoc* study of the LEAF (LEvosimendan in Acute heart Failure following myocardial infarction) trial,[19] an interventional study on patients developing HF within 48 h following PCI-treated STEMI. We hypothesized that enhanced complement activation could be a hallmark of acute HF in this patient group and may discriminate between HF with or without cardiogenic shock.

Materials and methods

Study design and population

The patient population and study design in the LEAF trial have previously been described in detail.[19] Briefly, 61 patients with PCI-treated STEMI who (i) had successful opening of the occluded coronary artery, (ii) had decreased wall motion in at least 3 of 16 segments of the left ventricle evaluated by echocardiography, and (iii) developed clinical signs of HF within 48 h (range: 14–33 h) following PCI were randomized to treatment with the calcium sensitizer levosimendan or placebo.[19] HF was defined as dyspnoea at rest and the presence of at least one of the following symptoms: pulmonary oedema, signs of pulmonary congestion on X-ray, need for continuous positive airway pressure or mechanical ventilation, or need for intravenous diuretics due to symptoms of congestion or persistent oliguria (urine output $<$0.5 mL/kg/h) after volume therapy. Criteria for subgrouping patients into cardiogenic shock included both of the following: (i) systolic blood pressure $<$ 90 mmHg after 60 min of volume therapy or systolic blood pressure 90–100 mmHg despite vasoactive support and (ii) signs of organ hypoperfusion such as cold and clammy extremities, oliguria, or reduced consciousness. Exclusion criteria were septic shock, acute respiratory distress syndrome, creatinine $>$ 450 μmol/L, severe hepatic failure, age $<$ 20 years, heart rate $>$ 120 b.p.m., pregnancy, significant mechanical outflow obstruction, haemoglobin $<$ 8 g/dL, or allergy to the study medication or any of its components.

In the present study, the STEMI patients who developed cardiogenic shock (n = 9) were compared with patients with HF without any signs of cardiogenic shock (n = 52) in order to investigate differences in complement activation between severe and less severe degree of HF. For comparison, blood samples were obtained from 44 age-matched and sex-matched healthy controls. Importantly, to ensure that treatment with levosimendan did not affect the degree of complement activation, we compared the two treatment groups with respect to sC5b-9 over the whole study period. There was no significant difference between the groups (P = 0.72), and they were thereafter handled as one population.

Blood sampling protocol

Blood samples were collected from patients at the time of inclusion (Day 0), that is at time of HF diagnosis (median 24 h following PCI) and at Days 1, 2, 5 (acute phase of the disease), and 42 following inclusion (follow-up sample) as previously described.[19] Briefly, blood samples were collected in ethylendiaminetetraacetic acid (EDTA), citrate, and serum vacutainer tubes (BD, Plymouth, UK). EDTA and citrated plasma samples were stored on crushed ice immediately after sampling and centrifuged within 30 min at 3000 *g* for 20 min at 4°C to obtained platelet-poor plasma. Blood for serum preparation was allowed to clot for 60 min in room temperature and thereafter centrifuged at 2500 *g* for 10 min for

isolation of serum. All samples were stored at −80°C until analysed and thawed only once.

Assays for complement activation markers

The complement activation products C4bc (classical and lectin pathway), C3bc (common pathway), C3bBbP (alternative pathway), and sC5b-9 (terminal pathway) were measured in EDTA-plasma samples from patients and controls by in-house enzyme-linked immunosorbent assays. All assays are based on either monoclonal antibodies detecting activation-specific neoepitopes (C4bc, C3bc, and C5b-9) or pairs of antibodies detecting complexes formed between single components upon activation (C3bBbP) as previously described in detail.[20] The level of the respective marker was related to the International Complement Standard #2, defined to contain 1000 complement arbitrary units per millilitre.[20]

Lectin pathway recognition molecules

Plasma concentrations of mannose-binding lectin (MBL), ficolin-1 (FCN1), ficolin-2 (FCN2), and ficolin-3 (FCN3) were determined by sandwich enzyme-linked immunosorbent assays using specific in-house produced monoclonal antibodies as previously described.[21–24]

Markers of endothelial activation

Levels of soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) of the current material have previously been analysed in serum and published.[25] In the present study, we extended the data analyses by comparing these markers between patients with and without cardiogenic shock, to explore whether they corresponded with the degree of HF and whether there were any correlations between these markers and markers of complement activation.

Echocardiography

Left ventricular function was measured as wall motion score index (WMSI) by echocardiography as previously described.[19] A 16-segment model was used where a normally contracting or hyperkinetic segment was given a score of 1, a hypokinetic segment scored 2, akinesia gave a score of 3, and a dyskinetic segment scored 4 points. WMSI was calculated by dividing the sum of scores by the number of segments scored. All examinations were performed by two experienced echocardiographers on Days 0, 1, and 42, and the analyses were performed by one observer. An ultrasonic device system (Vivid i or Vivid 7, GE Vingmed Ultrasound, Horten, Norway) was used for the examinations, and the analyses were performed with dedicated software (Echopac GE Vingmed Ultrasound).

Infectious complications

In order to test whether infectious complications contributed to activation of the complement system, levels of activation makers were compared in patients with documented or suspected infection, based on positive culture testing, X-rays, and clinical evaluation (n = 14), to patients without infection (n = 38). This comparison was only performed in the non-shock group because the cardiogenic shock group did not include enough patients to ensure statistical testing. Statistical tests for correlation between complement activation and biochemical markers of infection [C-reactive protein, white blood cell (WBC) count, or interleukin (IL)-6] were also performed.

Data presentation and statistics

In addition to the patient cohort, a control group comprising 44 age-matched and sex-matched healthy individuals was included. The patient cohort was divided into two groups: one group consisting of patients who developed HF without any signs of cardiogenic shock, the non-shock group (n = 52), and one group consisting of patients who developed cardiogenic shock, referred to as the shock group (n = 9).[19] Differences between these two groups during the first 5 days after inclusion (Days 0–5) were analysed with linear mixed model analyses. Differences between the two groups were tested with t-test or alternatively with the Mann–Whitney U-test when data were not normally distributed. To compare categorical data between groups, the χ^2 test or Fisher's exact test was used. Differences between more than two groups were tested with Kruskal–Wallis test using Dunn's *post hoc* test. Bonferroni correction was used to correct for multiple testing. Correlation analyses were measured by the Spearman correlation test. All results are given as mean and standard error of the mean. A P value of <0.05 was considered statistically significant. IBM SPSS Statistics version 21 (Armonk, NY) was used for analysis, while GraphPad Prism version 6 (San Diego, CA) was used for data presentation.

Ethics

The study was approved by The Regional Ethics Committee South-Eastern Norway Regional Health Authority, and the study was conducted in accordance with the principles of the Declaration of Helsinki (clinicaltrials.gov NCT00324766). All patients provided written informed consent. If a patient was unable to give informed consent, relatives were informed, and a written consent was acquired from the patient as soon as possible.

Results

Complement activation

Sixty-one patients were included in the study, and those who developed cardiogenic shock (n = 9) were compared with patients with HF without any signs of cardiogenic shock (n = 52) (*Table 1*). At the time of inclusion, C4bc, reflecting classical and lectin pathway activation, C3bc, reflecting C3 activation, C3bBbP, reflecting activation of the alternative pathway, and sC5b-9, reflecting the terminal pathway activation, were significantly elevated in the patient cohort (n = 61) compared with the healthy controls (n = 44) ($P < 0.05$ for all; *Figure 1A–D*). Patients developing shock had significantly higher levels of C4bc, C3bc, and sC5b-9 in the acute phase of the disease (Days 0–5), compared with patients without shock ($P < 0.05$ for all; *Figure 1A, B,* and *D*). Even at Day 42, there was an enhanced complement activation reflected by higher levels of all four activation markers (C4bc, C3bc, C3bBbP, and sC5b-9) in the shock group compared with the non-shock group ($P < 0.05$ for all; *Figure 1E–H*).

Lectin pathway recognition molecules

The level of FCN2 was at the time of inclusion lower among patients vs. controls ($P < 0.05$), whereas no significant differences were observed for MBL, FCN1, or FCN3 (*Figure 2A–D*). During the acute phase of the disease (Days 0–5), FCN2 increased significantly ($P < 0.05$) in the patient cohort as a whole, but there were no significant group differences between those with and without cardiogenic shock (*Figure 2*C). At Day 42, however, the shock group had a significantly higher level of FCN2 compared with the non-shock group ($P < 0.05$; *Figure 2G*). No significant group differences were found for MBL, FCN1, or FCN3 (*Figure 2E, F,* and *H*). Furthermore, there was no correlation between C4bc and MBL or the ficolins.

Markers of endothelial activation

We have previously published data on endothelial activation in these patients.[25] When now analysing their relation to cardiogenic shock, we found that sICAM-1 and sVCAM-1 were significantly higher in the shock group compared with the non-shock group during the acute phase of the disease (Days 0–5) ($P < 0.01$ for both; *Figure 3A,B*) with no significant differences at Day 42, (*Figure 3C,D*).

Correlation between complement activation and regional myocardial contractility

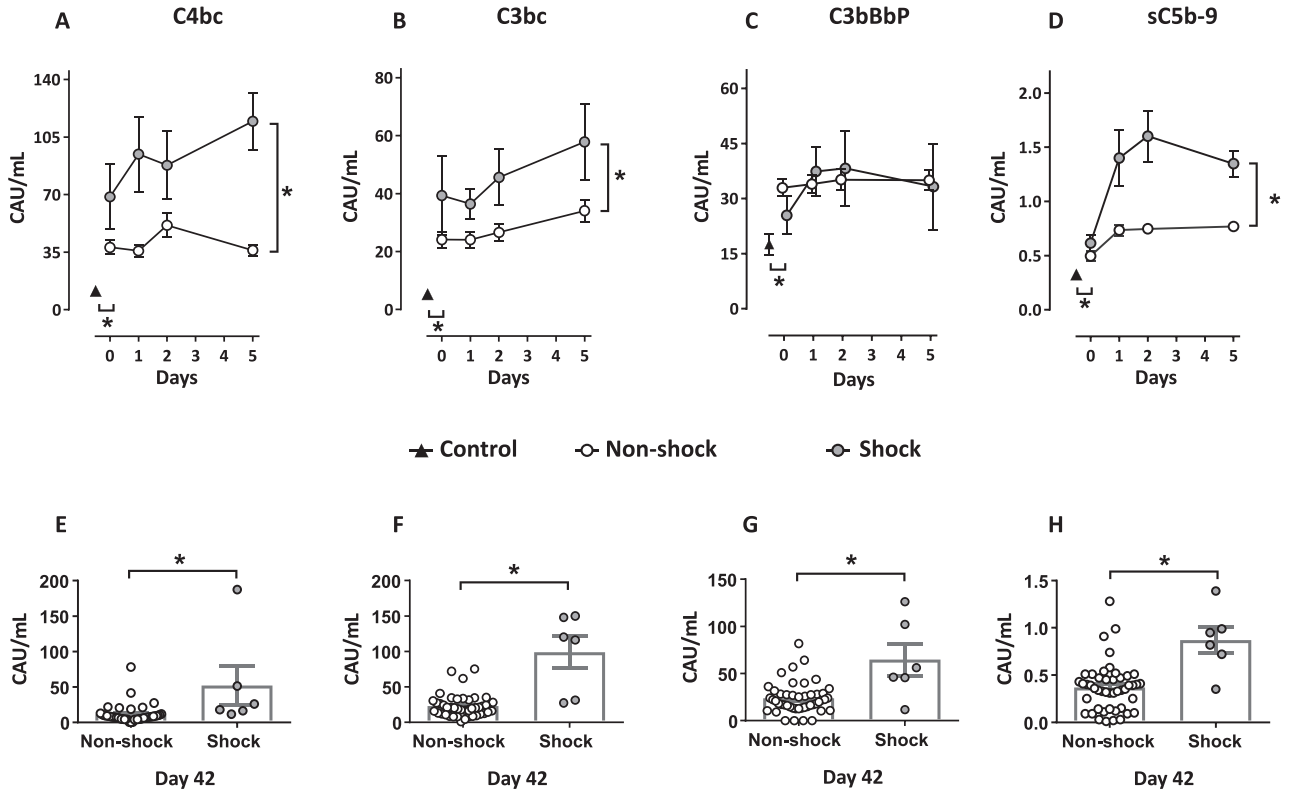
In the shock group, there was a significant correlation between complement activation as measured by sC5b-9 and

Table 1 Baseline characteristics of 61 patients with ST-elevation myocardial infarction developing acute heart failure with or without cardiogenic shock

	Shock	Non-shock	*P* value
Total number (female/male)	9 (3/6)	52 (15/37)	0.89
Age (years, mean, and range)	57 (49–68)	66 (56–74)	0.08
TnT[a] (ng/L)	14 640 (7580–20 925)	12 279 (7811–16 607)	0.43
Creatinine[a] (μmol/L)	81 (52–150)	82 (69–95)	0.91
eGFR[a] (mL/min/m^2)	60 (33–60)	60 (60–60)	0.24
NT-proBNP[a] (pmol/L)	315 (202–721)	463 (266–840)	0.52
C-reactive protein[a] (mg/L)	40 (24–100)	57 (35–97)	0.42
WBC count (×10^9/L)[a]	11 (8.9–17)	12 (10–15)	0.49
IL-6[a] (pg/mL)	29 (19–40)	27 (21–33)	0.54
Previous hypertension, *n* (%)	5 (56)	16 (31)	0.15
Previous dyslipidemia, *n* (%)	1 (11)	12 (23)	0.42
Current smoking, *n* (%)	6 (67)	16 (30)	<0.05
Previous diabetes mellitus, *n* (%)	1 (11)	5 (10)	0.89
Previous statin treatment, *n* (%)	2 (22)	13 (25)	0.86
Previous myocardial infarction, *n* (%)	3 (33)	8 (15)	0.20
Multi-vessel disease, *n* (%)	5 (56)	26 (44)	0.76
Atrial fibrillation[a], *n* (%)	1 (9)	1 (2)	0.16
Systolic blood pressure[a], mmHg	85 (72–94)	106 (96–117)	<0.001
Diastolic blood pressure[a], mmHg	55 (48–58)	67 (60–72)	<0.001
Hours from symptom start to PCI	3 (2–8)	3 (2–6)	0.80
Hours from PCI to baseline	17 (10–23)	23 (14–32)	0.07
LVEF[a], %	44 (34–49)	41 (38–47)	0.88
Antimicrobial treatment, *n* (%)	8 (89)	14 (27)	<0.001
Mortality within 6 months, *n* (%)	3 (33)	2 (4)	<0.05

GFR, glomerular filtration rate; IL-6, interleukin 6; LVEF, left ventricular ejection fraction; NT-proBNP, N terminal pro brain natriuretic peptide; PCI, percutaneous coronary intervention; TnT, troponin T; WBC, white blood cell.
Data are given as median (25th and 75th percentile) or number (%).
[a]At the time of inclusion, that is median 24 h following PCI.

Figure 1 Complement activation products during the first 5 days of the disease and at Day 42 after inclusion. Figures in the upper panel (A–D) show values at inclusion and throughout the acute phase of the disease (Days 0–5). (A) Plasma levels for C4bc (classical and lectin pathway activation), (B) C3bc (common activation of all initial pathways), (C) C3bBbP (alternative pathway activation), and (D) sC5b-9 (terminal pathway activation) are shown for patients with cardiogenic shock (n = 9, grey circles), patients with heart failure without cardiogenic shock (n = 52, open circles), and healthy controls (n = 44, black triangles). Statistical differences between the shock group and the non-shock group of patients from inclusion (Day 0) to Day 5 (the acute phase of the disease) are indicated with brackets and *($P < 0.05$) at the right-hand side of the graph. Statistical difference between patients and controls at the time of inclusion are indicated with *($P < 0.05$). Figures at the lower panel (E–H) show plasma levels at Day 42 for (E) C4bc, (F) C3bc, (G) C3bBbP, and (H) sC5b-9 for patients with cardiogenic shock (n = 7, grey columns) and patients without cardiogenic shock (n = 45, white columns). Data are given as mean ± standard error of the mean. CAU, complement arbitrary units.



WMSI at the day of inclusion (Day 0) (r = 0.678, P = 0.045) and at Day 42 (r = 0.841, P = 0.036; *Table 2*). At these two time points, both blood sampling and WMSI were performed, and thus, direct correlation tests could be performed. sC5b-9 reached its highest level at Day 2, where WMSI was not performed. Interestingly, this peak sC5b-9 level correlated significantly with WMSI at Day 42 in the shock group (r = 0.975, P = 0.005; *Table 2*). Weaker or no correlations were found for the other complement activation products: WMSI Day 1 correlated with C3bBbP measured at Days 2 (r = 0.943, P = 0.005) and 42 (r = 0.829, P = 0.042), and WMSI measured at Day 0 correlated with C4bc measured at Day 0 (r = 0.703, P = 0.035, data not shown). No correlations were found between WMSI and C3bc. In the non-shock group, the only significant correlation was found between C4bc measured at Day 1 and WMSI at Day 0 (data not shown).

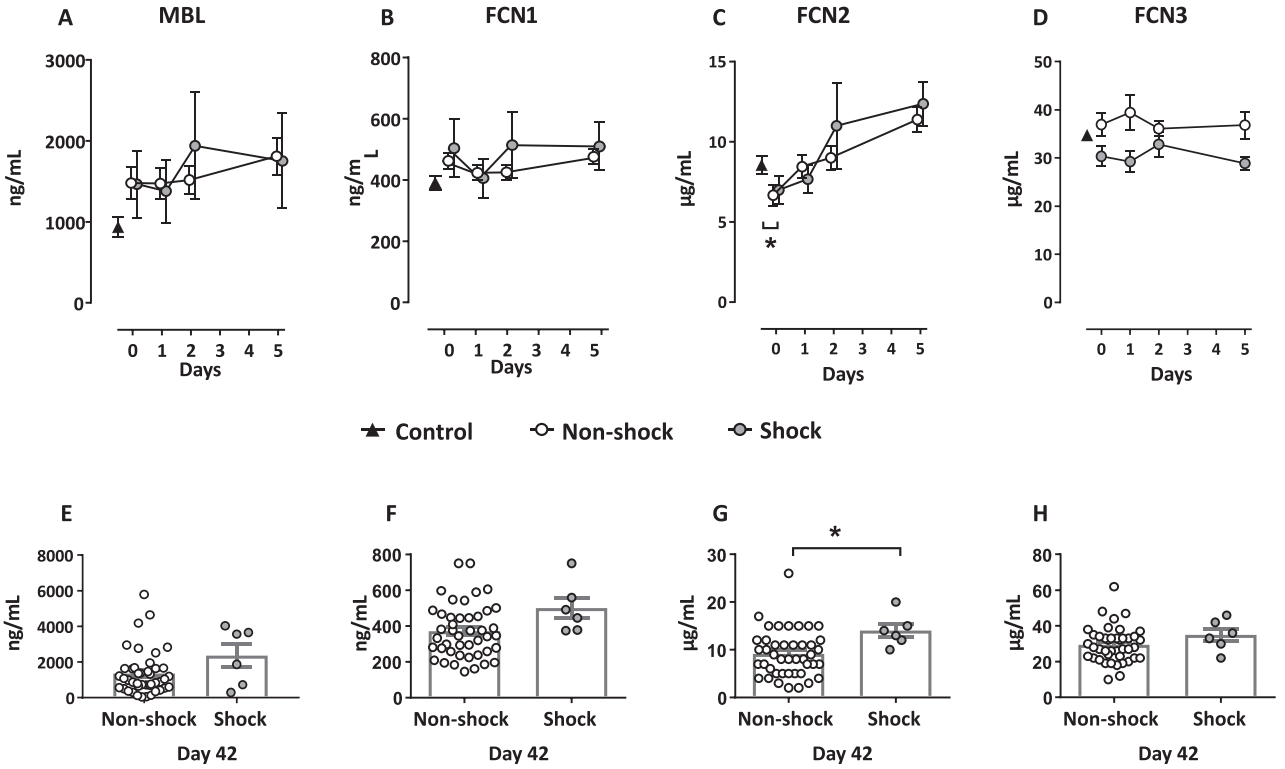
Correlation between complement activation and the markers of endothelial activation

There was a significant correlation between complement activation in the whole patient group (n = 61) at Day 2, when sC5b-9 peaked, and peak level of sVCAM-1 (r = 0.296, P = 0.022) and sICAM-1 (r = 0.317, P = 0.014), whereas no correlation was found when the shock and non-shock groups were analysed separately.

Complement activation following infection

There was no significant difference in complement activation, measured as sC5b-9, between patients with infection (n = 14) and patients without infection (n = 38) in the non-shock group during the acute phase of the disease (Days 0–5) (P = 0.44).

Figure 2 Lectin pathway proteins levels during the first 5 days of the disease and at Day 42. Figures at the upper panel (A–D) show plasma levels for the (A) mannose-binding lectin (MBL), (B) Ficolin-1 (FCN1), (C) Ficolin-2 (FCN2), and (D) Ficolin-3 (FCN3) for patients from inclusion (Day 0) to Day 5 (the acute phase of the disease). Figures in the lower panel (E–H) show plasma levels for the (E) MBL, (F) FCN1, (G) FCN2, and (H) FCN3 at Day 42. The figures are shown with the same patient populations and details as described in the *Figure 1* legend. $*P < 0.05$.



There was no difference between the non-shock and the shock group with respect to levels of C-reactive protein, WBC count, or IL-6 (*Table 1*). We found no association between peak values of sC5b-9 and markers of infection (C-reactive protein, WBC count, or IL-6, all measured as peak values) (*Table S1*).

Discussion

In the present study, we found increased complement activation in patients who developed acute HF following PCI-treated STEMI compared with healthy controls. Notably, the degree of complement activation discriminated those patients developing cardiogenic shock from those in the non-shock group. The increased activation persisted even 6 weeks after STEMI in the shock group. In these patients, there was also a strong correlation between complement activation and regional contractility measured as WMSI both at inclusion and at 6 weeks. Although complement activation has been shown to be involved in the progress of HF, this is, to the best of our knowledge, the first study to document that the degree of complement activation is directly related to the disease severity and impaired myocardial function in patients developing acute HF following STEMI.

The patient population in this study was characterized by large MIs determined by high levels of troponins and clinical and echocardiographic findings.[19] In the present study, we show that the complement activation products C4bc, C3bc, C3bBbP, and sC5b-9, representing complement activation from initiation to terminal activation, were increased at the time when the patients were diagnosed with HF (14–33 h following PCI treatment), compared with healthy individuals. Furthermore, there was stronger and more persistent complement activation in the most severely affected patients. This persistent activation indicates that complement might play an important role in the pathophysiological process of HF. In fact, the peak level of sC5b-9 during the acute phase correlated significantly with WMSI after 6 weeks, suggesting that complement-mediated mechanisms could promote myocardial damage with subsequent development of severe HF following STEMI.

Because of its amplification loop, the alternative pathway can contribute substantially to complement activation from the level of C3 and further downstream the activation cascade.[26,27] The lack of difference between the two patient groups with respect to the activation product C3bBbP is therefore somewhat surprising. The amplification loop is, however, under strict control by regulatory proteins like factor H, and complement activation triggered presuming via the lectin pathway with a tight

Figure 3 Serum levels of the endothelial cell activation markers sICAM-1 and sVCAM-1 during the first 5 days of the disease and at Day 42. Figures in the upper panel show serum levels of the (A) soluble intercellular adhesion molecule 1 (sICAM-1) and the (B) soluble vascular adhesion molecule 1 (sVCAM-1) for patients from inclusion (Day 0) to Day 5 (the acute phase of the disease). Figures in the lower panel show serum levels of (C) sICAM-1 and (D) sVCAM-1 at the control measurement at Day 42. The figures are shown with the same patient populations and details as described in the *Figure 1* legend. $^{*}P < 0.05$.

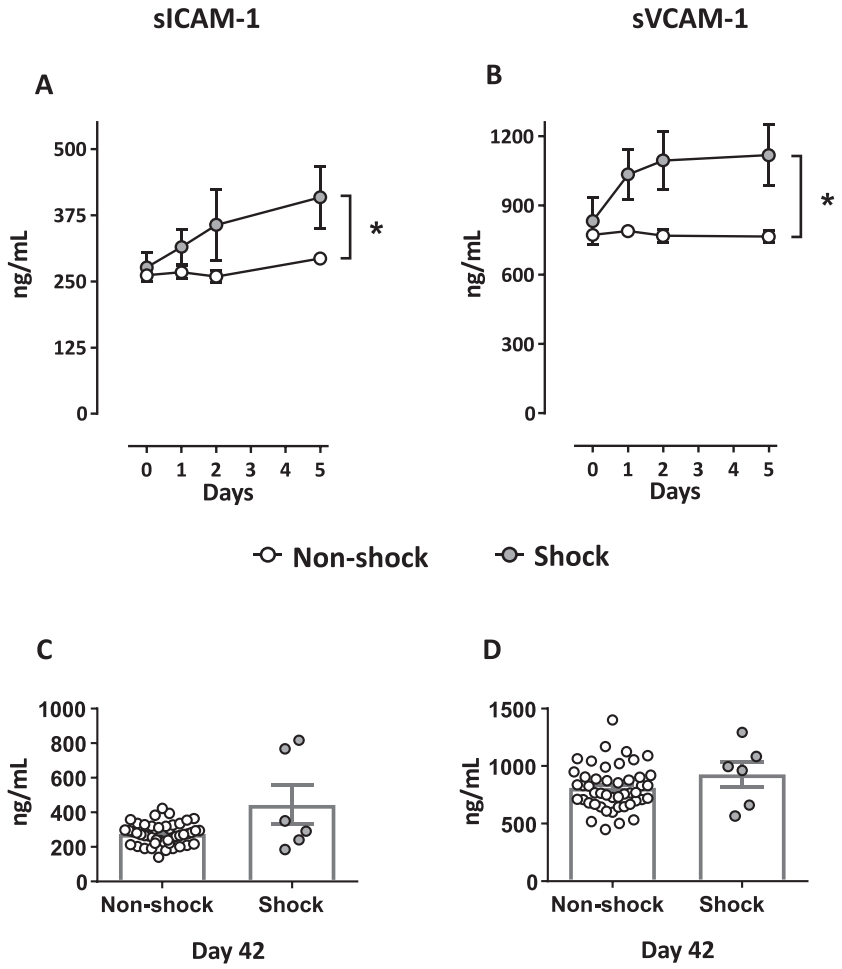


Table 2 Correlation between left ventricular regional contractility measured as wall motion score index and complement activation measured by sC5b-9 in patients with cardiogenic shock ($n = 9$) following percutaneous coronary intervention-treated ST-elevation myocardial infarction

		sC5b-9 Day 0	sC5b-9 Day 2	sC5b-9 Day 42
WMSI Day 0	*r*	0.678	0.206	0.522
	P	**0.045**	0.696	0.288
WMSI Day 1	*r*	0.311	0.812	0.488
	P	0.415	0.050	0.329
WMSI Day 42	*r*	0.551	0.975	0.841
	P	0.257	**0.005**	**0.036**

WMSI, wall motion score index. Statistical significance is shown in bold.

regulatory control of the alternative pathway in both groups in the early phase of disease may be a reasonable explanation for this finding. The regulatory balance may then have changed after the initial phase, explaining the significant difference in C3bBbP in the two groups at Day 42.

Several clinical and experimental studies have previously demonstrated increased complement activation in cardiovascular disease and HF.[14,15,28–32] Particularly, the lectin pathway has been linked to complement-mediated myocardial injury and HF,[18,33–35] and lectin pathway recognition molecules were therefore thoroughly investigated in the present study. MBL and the ficolins are circulating recognition molecules binding to molecular structures on damaged host cells further activating the mannose-binding serine proteases, MASP1 and MASP2.[36]

MBL is also an acute phase reactant,[37] and altered levels of FCN1–3 are reported in various pathological conditions, either due to consumption or changed expression.[38] The lower level of FCN2 in the patient population at inclusion compared with healthy controls is in line with a previous observation seen in STEMI patients[35] and is suggested to reflect consumption in the early phase of the disease. From the inclusion level, FCN2 increased significantly during the first 5 days of the disease and was at Day 42 significantly higher in the shock group. The other recognition molecules, MBL, FCN1, and FCN3, did not differ significantly from the healthy controls at inclusion. FCN1 was higher than the controls, although not significant, and showed no change during the course. The reason for the different patterns for FCN1 and FCN2 is uncertain but might be related to their different profiles for release and consumption, which makes it difficult to compare these two. FCN1 is synthesized by peripheral leukocytes. Upon cell activation, secretion of FCN1 increases, but the majority is tethered to the cell membrane of the activated cell.[39] This can explain the small, however non-significant, early increase of FCN1 in patients. FCN2 is synthesized in the liver as a soluble protein.[40] Increased secretion of FCN2 is, in relation to FCN1, delayed, which enables a consumption profile early after MI. Further on, FCN1 and FCN2 are highly homologous, but FCN2 has four carbohydrate-binding domains, whereas FCN1 has only one.[41] FCN2 might therefore bind its ligand more tightly as compared with FCN1, but without knowing the exact target, this remains speculative.

C4bc reflects both classical and lectin pathway activation. Although classical pathway activation cannot be excluded, our findings of increased C4bc is in accordance with lectin pathway activation during the acute phase,[33,42] although the role of the lectin pathway in post-MI HF is still elusive.

Microbial infections are well-known activators of the complement system.[43] We therefore compared complement activation in patients with or without signs of infection. The non-shock group contained a sufficient amount of patients treated for infections, documented or suspected, to enable statistical analysis regarding infectious complications and complement activation. Notably, there was no difference in complement activation in patients with or without infection in this group. Antibiotics were given mainly because of suspected aspiration, and septic patients were excluded from the trial. Furthermore, there were no correlations between peak levels of sC5b-9, C-reactive protein, IL-6, or WBC count. Thus, there is no evidence that the increased complement activation is caused by infections but rather by the cardiogenic shock *per se*.

The patients in the shock group were characterized by significantly increased levels of the soluble adhesion molecules sVCAM-1 and sICAM-1 as compared with the non-shock group reflecting enhanced endothelial cell activation in those with the most severe HF. Activated endothelial cells have been shown to secrete complement components and to express adhesion molecules ICAM-1 and VCAM-1 in response to sC5b-9 and are also targets for complement activation products.[44] Herein, we also found a significant correlation between sC5b-9 and the adhesion molecules in the whole HF group, further suggesting crosstalk between endothelial cells and terminal complement activation in patients with acute, severe HF following MI. With a positive correlation of sustained complement activation and development of cardiogenic shock, the critical question arises whether complement activation solely is the result of hypoperfusion caused by cardiogenic shock, or whether it also contributes to exacerbation of shock and, in extension, if these patients would benefit from complement inhibition. Increased systemic complement activation has previously been shown in patients with chronic HF consistent with tissue hypoperfusion, acidosis, and endothelial cell damage.[14] Neoantigens exposed in ischaemic tissue are linked to recognition by natural IgM and subsequent lectin pathway activation,[45] which would support sustained complement activation. If complement significantly aggravates the shock syndrome, there would be fear for a vicious circle. By being part of the innate immune system, complement is instantly activated upon 'danger' and has the potential for initiating a broad range of inflammatory responses. Specific complement inhibition may therefore be suitable in patients where attenuation of inflammation is desired, including patients with post-MI HF and particularly those with cardiogenic shock. Various clinical trials targeting different parts of the inflammatory response have failed to reach significance with regard to their primary endpoints.[46] However, in the COMplement inhibition in Myocardial infarction treated with Angioplasty trial,[47] where complement inhibition with the C5-inhibitor pexelizumab was given as a bolus dose and with continuous infusion for 20 h following MI, a significant reduction in 90 day mortality was seen. The incidence of cardiogenic shock was reduced with 45%, however, non-significantly. The Assessment of Pexelizumab in Acute Myocardial Infarction trial did not show any effect of pexelizumab,[48] but there is a remaining question whether C5 was appropriately inhibited.[49] In order to rule out if complement inhibition would be beneficial in patients with acute severe HF and cardiogenic shock due to MI, more clinical trials are needed.

The current study is of explorative character, however, on a well-defined cohort with close follow-up and careful plasma preparation, which is critical for accurate complement analysis. The low numbers of patients in the group of cardiogenic shock as well as the lack of blood samples before PCI are limitations of the present study. The major differences found between the groups, with statistical significance for all complement activation products and endothelial cell markers, however, increase the impact of the data because the risk of type I error can be regarded as small.

The patients included in this study represent a group of patients often excluded from clinical trials due to the severity of the disease. However, our results, consistently demonstrating an increased and persistent complement activation correlating to disease severity and endothelial cell activation, indicating that patients with advanced HF complicating large MI, may particularly benefit from therapy targeting complement activation. Our findings add new understanding to the inflammatory

profile in patients with acute severe HF, which can pave the way for new prognostic markers and targets for therapy.

Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1. Spearman correlation analysis and linear regression analyses between peak values of sC5b-9 and various relevant variables.

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Paper II

frontiers in Immunology

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IL-6 Receptor Inhibition by Tocilizumab Attenuated Expression of C5a Receptor 1 and 2 in Non-ST-Elevation Myocardial Infarction

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Background: Elevated interleukin-6 (IL-6) and complement activation are associated with detrimental effects of inflammation in coronary artery disease (CAD). The complement anaphylatoxins C5a and C3a interact with their receptors; the highly inflammatory C5aR1, and the C5aR2 and C3aR. We evaluated the effect of the IL-6 receptor (IL-6R)-antagonist tocilizumab on the expression of the anaphylatoxin receptors in whole blood from non-ST-elevation myocardial infarction (NSTEMI) patients. Separately, anaphylatoxin receptor expression in peripheral blood mononuclear cells (PBMC) from patients with different entities of CAD was investigated.

Materials and Methods: NSTEMI patients were randomized to one dose of tocilizumab ($n = 28$) or placebo ($n = 32$) and observed for 6 months. Whole blood samples drawn at inclusion, at day 2, 3 and after 6 months were used for mRNA isolation. Plasma was prepared for analysis of complement activation measured as sC5b-9 by ELISA. Furthermore, patients with different CAD entities comprising stable angina pectoris (SAP, $n = 22$), non-ST-elevation acute coronary syndrome (NSTE-ACS, $n = 21$) and ST-elevation myocardial infarction (STEMI, $n = 20$) were included. PBMC was isolated from blood samples obtained at admission to hospital and mRNA isolated. Anaphylatoxin-receptor-expression was analyzed with qPCR using mRNA from whole blood and PBMC, respectively.

Results: Our main findings were (i) Tocilizumab decreased C5aR1 and C5aR2 mRNA expression significantly ($p < 0.001$) and substantially (>50%) at day 2 and 3, whereas C3aR expression was unaffected. (ii) Tocilizumab did not affect complement activation. (iii) In analyzes of different CAD entities, C5aR1 expression was significantly increased in all CAD subgroups compared to controls with the highest level in the STEMI patients ($p < 0.001$). For C5aR2 and C3aR the expression compared to controls were more moderate with increased expression of C5aR2 in the STEMI group ($p < 0.05$) and C3aR in the NSTE-ACS group ($p < 0.05$).

Conclusion: Expression of C5aR1 and C5aR2 in whole blood was significantly attenuated by IL-6R-inhibition in NSTEMI patients. These receptors were significantly upregulated in PBMC CAD patients with particularly high levels of C5aR1 in STEMI patients.

Keywords: complement, C5a receptors, C3a receptor, IL-6, myocardial infarction, inflammation

INTRODUCTION

Inflammation plays a pivotal role in the pathophysiology of coronary artery disease (CAD) from the establishment of the atherosclerotic plaque through rupture or erosion of the plaque leading to partial or total occlusion of the coronary vessel. This might lead to myocardial necrosis and thereby a myocardial infarction (MI). A total occlusion typically leads to ST-elevation in the electrocardiogram whereas a partial occlusion or an occlusion with collateral circulation doses not show these changes and are classified as unstable coronary syndromes. Unstable coronary syndromes with elevated levels of Troponin T, a marker of myocardial necrosis, are classified as non-ST-elevation MI (non-STEMI) whereas without rice in TnT are classified as non-ST-elevation acute coronary syndromes (1). Rapid restoration of coronary blood flow by re-opening of the occluded coronary vessel with percutaneous coronary intervention (PCI), has considerably improved outcome following MI. However, CAD is still associated with considerable morbidity and mortality (2).

Both the myocardial necrosis and the reperfusion of the infarcted myocardium activate inflammatory mechanisms. Innate and adaptive immune mechanisms are involved in this process and act together to orchestrate a response to damage (3). A balanced inflammatory response is required for proper healing following myocardial infarction (MI), whereas excessive inflammation could give rise to collateral tissue damage with detrimental effects on the myocardium (4). The complement system is an important sensor and effector system of innate immunity and plays a role in all phases of CAD (5). The complement system exerts its main inflammatory functions through proteolytic activation of C3 and C5, which upon cleavage liberate the complement anaphylatoxins C5a and C3a. The anaphylatoxins bind to their respective receptors: the C5a receptor 1 and 2 (C5aR1, C5aR2) and the C3a receptor (C3aR) (6), and the C5a-C5aR1-axis seems to be involved in atherogenesis and CAD (7–9). C5aR inherits an inflammatory role following tissue injury stimulating the release of cytokines like tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, and chemokines, e.g., IL-8 (10), and induce thrombogenicity by upregulation of tissue factor (11). The effect of activating C5aR2 and C3aR are more diverse and the effect of activating these receptors in the context of acute coronary syndromes (ACS) is at present less clear.

IL-6 and complement may both contribute to the progression of cardiovascular diseases (5, 12, 13) but there are limited data on the interaction between these inflammatory proteins. In a mouse sepsis model, IL-6 inhibition reduced the expression of tissue C5aR (14), but to the best of our knowledge the effects of IL-6 inhibition on the anaphylatoxin receptor expression in human CAD have not been investigated. In a recent study, the IL-6 receptor (IL-6R) antagonist tocilizumab reduced C-reactive protein (CRP) and percutaneous coronary intervention (PCI)-related troponin T (TnT) release in patients with non-ST-elevation myocardial infarction (NSTEMI) (15). In the present study, we aimed to investigate the expression of the anaphylatoxin receptors in a sub-group of this patient cohort (15). Additionally, anaphylatoxin receptor expression was investigated in samples from patients with different entities of CAD before any intervention was initiated.

MATERIALS AND METHODS

In this study we included two different patient cohorts: one cohort consisting of NSTEMI-patients randomized to anti-inflammatory treatment with an IL-6R antagonist or placebo where blood was sampled from inclusion, before treatment and with repeated measurements, and another cohort consisting of patients with different entities of CAD where blood samples were drawn at hospital admission, before treatment was given.

NSTEMI Patients Treated With Tocilizumab

The present work is a sub-study of a previously published double-blind, placebo-controlled two-center study on patients ($n = 117$) admitted with NSTEMI randomized to treatment with the IL-6R inhibitory monoclonal antibody tocilizumab

($n = 58$) or placebo ($n = 59$) (ClinicalTrails.gov, NCT01491074) (15). Tocilizumab was administrated as a single dose of 280 mg immediately prior to coronary angiography. This dose provides a complete IL-6 blockade for approximately 2 weeks (15). Briefly, patients between 18 and 80 years of age with NSTEMI scheduled for coronary angiography were included. Exclusion criteria were clinically significant cardiac disease other than CAD, disease or medication affecting inflammation, contraindications to the treatment drug and clinically unstable patients. Patients were included at a median of 2 days after symptom onset. There were no significant between-group differences in baseline characteristics (15). Fifteen age and sex-matched healthy controls were included. A flow chart describing the whole patient population randomized to tocilizumab or placebo and the number of patients with or without PCI, and with early ($\leq$2 days) vs. late (>2 days) inclusion after symptoms onset is shown in **Figure 1**.

We evaluated the expression of anaphylatoxin receptors (C5aR1, C5aR2, and C3aR) in 60 of the patients treated with tocilizumab ($n = 28$) or placebo ($n = 32$). These patients represent all patients included at one of the two study centers (St. Olavs hospital). Due to lack of resources, we only investigated patients from half of the original study population. In this subgroup of patients, there was a significant difference in gender, but no other differences in baseline characteristics were found (**Table 1**). The whole study population ($n = 117$) was included for plasma complement activation analysis.

Patients With Various CAD Entities

Three patient groups with different entities of CAD, described in detail elsewhere (16), were examined with respect to anaphylatoxins receptor expression in blood samples obtained at admission to hospital. CAD was defined as coronary artery stenosis >50% verified by coronary angiography. The three patient entities were defined as: (i) stable angina pectoris (SAP) ($n = 22$), defined as episodes with reversible ischemic chest pain, referred to elective coronary angiography. (ii) Non-ST-elevation acute coronary syndromes (NSTE-ACS) that included unstable angina and NSTEMI patients ($n = 21$), defined as angina at rest or crescendo angina, referred to urgent coronary angiography within 48 h. (iii) STEMI ($n = 20$) defined as elevated plasma levels of Troponin T (TnT; at least one value above the 99th percentile) together with ischemic symptoms and ST-segment elevation or new left bundle branch block in the electrocardiogram referred to immediate coronary angiography and PCI if indicated (16). Patients that had malignant or chronic inflammatory diseases, intercurrent infections, or were treated with glucocorticosteroids were not included. Age and sex-matched healthy controls ($n = 29$) were also included.

Blood Sampling Protocol

NSTEMI Tocilizumab Study

Blood samples drawn at the time of inclusion, i.e., before study medicine was given and angiography performed, at day 2 and 3 following inclusion and after 6 months were included in this sub-study. Blood was collected in EDTA vacutainer tubes (BD Biosciences, Plymouth, UK), kept on crushed ice and centrifuged within 30 min at 2,500 *g* for 20 min at 4°C. Plasma was stored at −80°C until analyzed, and samples were thawed only once. Whole blood (3 mL) was collected in Tempus Blood RNA tubes (ThermoFischer, Paisley, UK) from patients and healthy controls ensuring immediate lysis of all blood cells and stabilization of RNA. Tempus Blood RNA tubes were stored at −80°C until RNA preparation.

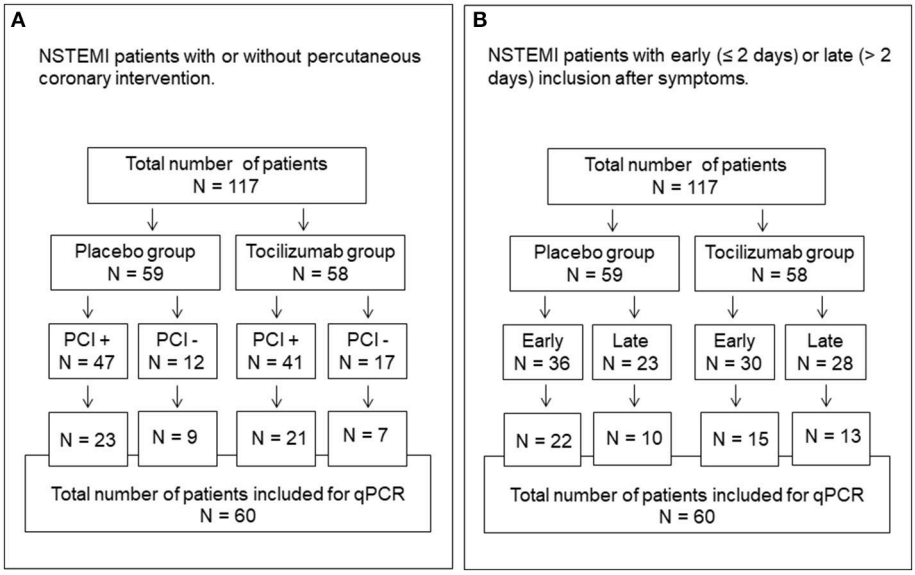

FIGURE 1 | Flow chart showing the total number of patients and randomization to tocilizumab and placebo groups. **(A)** Number in each group with or without PCI. **(B)** Number in each group included early ($\leq$2 days) or late (>2 days) from the onset of symptoms. NSTEMI, non-ST-elevated myocardial infarction.

TABLE 1 | Clinical and biochemical characteristics of the study population 1 ($n = 60$).

Characteristics	Placebo	Tocilizumab	*p*-value
Total number, *n*	32	28	
Male sex, *n* (%)	32 (100)	23 (82)	**0.02**
Age, years, mean (SD)	59 (9)	58 (6)	1.00
Body mass index, kg/m^2, median (IQR)	27 (25, 29)	28 (27, 30)	1.00
Hypertension, *n* (%)	10 (31)	15 (54)	0.12
Diabetes mellitus, *n* (%)	5 (16)	5 (18)	1.00
Previous myocardial infarction, *n* (%)	5 (16)	3 (11)	0.70
Current smoking, *n* (%)	13 (41)	9 (32)	0.60
Systolic blood pressure, BL, mmHg (SD)	135 (14)	138 (17)	1.00
Diastolic blood pressure, BL, mmHg (SD)	82 (11)	83 (10)	1.00
PCI, *n* (%)	23 (72)	21 (75)	1.00
LMWH BL, *n* (%)	29 (91)	26 (93)	1.00
CRP, BL, mg/L, median (IQR)	2.2 (0.7, 7.9)	3.3 (1.2, 6.7)	1.00
Troponin-T, BL, ng/L, median (IQR)	187 (87, 485)	107 (630)	1.00
Creatinine, BL, μmol/L, median (IQR)	77 (71, 90)	75 (63, 89)	1.00
Peak CRP, mg/L, median (IQR)	5.4 (1.2, 8.3)	5.4 (1.4, 18.7)	1.00
Peak Troponin-T, ng/L, median (IQR)	242 (92, 836)	128 (66, 937)	1.00
Peak Creatinine, μmol/L, median (IQR)	86 (74, 93)	81 (71, 95)	1.00

Data are given as mean with (standard deviation, SD) or median with (25 and 75th percentile, IQR) and number with (%). BL, baseline values; PCI, percutaneous coronary intervention; LMWH, low molecular heparin administered before baseline; CRP, C-reactive protein; R, receptor. Bold value indicate statistical significance.

Patients With Different CAD Entities and Healthy Controls

Venous blood was drawn from healthy controls and patients with SAP and NSTE-ACS before angiography. Arterial blood was drawn from the arterial cannula immediately before coronary angiography in patients with STEMI. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood in all three patient groups and the healthy controls by Isopaque-Ficoll (Lymphoprep, FreseniusKabi Norge AS, Oslo, Norway) gradient centrifugation within 1 h after sampling, stored at −80°C as cell pellets until RNA isolation was performed.

RNA Isolation and Quantitative PCR (qPCR)

NSTEMI Tocilizumab Study

Whole blood RNA purification was performed by Aaros Applied Biotechnology, Aarhus, Denmark. mRNA from the healthy controls was isolated using Tempus Spin RNA isolation Kit (ThermoFischer, Paisley, UK). cDNA was produced using the high capacity cDNA reverse transcriptase kit (Applied Biosystem, Foster City, CA). TaqMan qPCR primers (FAM-MGB dye-labeled) were purchased from Applied Biosystems for the following genes: C5aR1 (HS00704891), C5aR2 (Hs01933768) and C3aR (Hs0026963). Beta-2-microglobulin (HS 00187842) was stably expressed and used as endogenous control. Each sample was analyzed in triplicate and the reaction was run in 96 well-MicroAmp optical reaction plate on a StepOnePlus system (Applied Biosystems).

Patients With Different CAD Entities

RNA from PBMC was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using qScript cDNA SuperMix (Qantabio, Beverly MA). SybrGreen primers were used for qPCR (primer sequences can be given upon request) with GAPDH as endogenous control. Each sample was analyzed in duplicate in 384 well-optical reaction plate on a 7900 HT Fast Real-time PCR system.

Complement Activation

Plasma complement activation was evaluated by quantification of the terminal complement complex (TCC) in its soluble form (sC5b-9) using an enzyme-linked immunosorbent assay (ELISA) previously described in detail (17). Briefly, the mAb, aE11, which binds to a neoepitope exposed in C9 when incorporated into the C5b-9 complex, was used as capturing antibody and a biotinylated monoclonal anti-C6 (clone 9C4) was used for detection. The level was related to the International Complement Standard #2, defined to contain 1,000 complement arbitrary units (CAU) per mL (17).

Data Presentation and Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 24 (Armonk, NY) or Graph Pad Prism, version 7 (San Diego, CA). Differences between two groups were tested with *t*-test or Mann-Whitney *U* test when the data were not normally distributed. Differences between more than two groups were tested with ordinary one-way ANOVA or with Kruskal-Wallis test dependent on distribution. Change from baseline was calculated for each time point (e.g., time point-baseline). Longitudinal data were analyzed with Friedman test followed by Wilcoxon signed-rank test to compare the specific time point with baseline levels within each treatment group. To compare differences in categorical data between groups the Chi-square test was used. Correlation analysis was measured by the Spearman correlation test. Bonferroni correction was used to correct for multiple testing. Results are given as median with interquartile range or mean with 95% confidence interval (CI). All tests were two-sided and a p-level of <0.05 was regarded as statistically significant.

Ethics

Both studies were approved by the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway and the tocilizumab study also by The Norwegian Medicine Agency and both studies were conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent.

RESULTS

The Effect of IL-6R Inhibition on Anaphylatoxin Receptor Expression in Whole Blood From NSTEMI Patients

C5aR1

Expression of C5aR1 was significantly lower in the tocilizumab group compared to the placebo group at day 2 and 3 (**Figure 2A**). Compared to baseline and the healthy controls, the expression of C5aR1 at day 2 and 3 was significantly lower in the tocilizumab group, whereas no difference was observed for the placebo group. After 6 months the expression of C5aR1 in the tocilizumab group was still significantly lower compared to baseline, which was not the case for the placebo group. Compared to healthy controls there was no difference at baseline or after 6 months in any of the two patient groups (**Figure 2A**).

C5aR2

Expression of C5aR2 was significantly lower in the tocilizumab group compared to the placebo group at day 2 and 3 (**Figure 2B**). Compared to baseline levels, the expression of C5aR2 was significantly lower in the tocilizumab group at day 2 and 3, whereas no such difference was observed in the placebo group. There were no differences between the two patients groups at baseline or after 6 months. Compared to healthy controls, C5aR2 expression was significantly decreased in the tocilizumab group and the placebo group during the whole study period (**Figure 2B**).

C3aR

C3aR expression behaved strictly different from the C5a receptors. There were no differences in receptor expression between the tocilizumab group and the placebo group at any

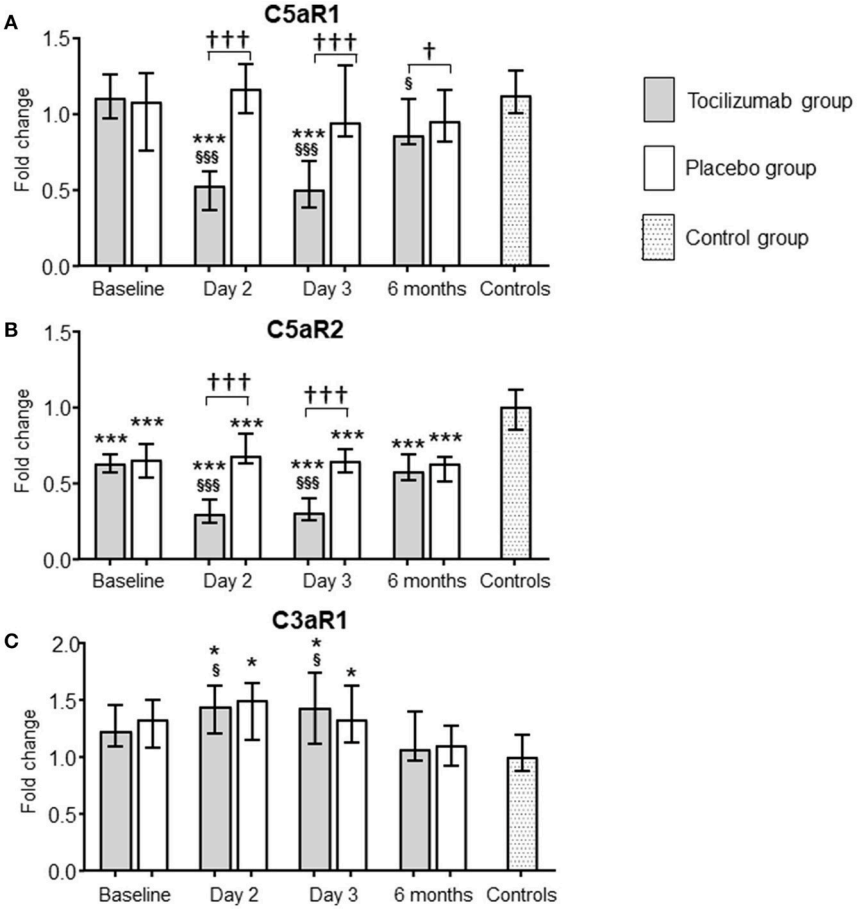

FIGURE 2 | Effect of tocilizumab on the expression of C5aR1, C5aR2, and C3aR in NSTEMI patients. The effect of tocilizumab, a monoclonal antibody inhibiting interleukin 6 receptor (IL-6R), on the expression of the three complement anaphylatoxin receptors [C5aR1 **(A)**, C5aR2 **(B)**, and C3aR **(C)**] was investigated in patients with non-ST-elevation myocardial infarction (NSTEMI). mRNA levels were quantified by qPCR and related to the reference gene beta-2-microglobulin. The tocilizumab group (gray bars, $n = 28$) and the placebo group (white bars, $n = 32$) are presented at four different time-points. Baseline levels show the receptor expression at inclusion, i.e., after hospital admission, before treatment was given. Follow-up time points were day 2 and 3, and 6 months. A group of healthy individuals ($n = 15$) were included as controls. The qPCR results were quantified using the $2^{-\Delta\Delta CT}$ method, normalized to reference genes and presented as fold change with the healthy controls as calibrator. Data are given as median and 95% CI. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. healthy controls. $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ differences in change from baseline between tocilizumab and placebo. $^{\S}p < 0.05$, $^{\S\S\S}p < 0.001$ vs. baseline.

of the time points (**Figure 2C**). In the tocilizumab group there was a significantly higher expression of C3aR at day 2 and 3 when compared to baseline, whereas both patients groups had significantly higher levels at day 2 and 3 compared to healthy controls (**Figure 2C**). At baseline and after 6 months, there were no differences in C3aR expression between the patient groups and health controls (**Figure 2C**).

Effects of Coronary Intervention and Time From Symptom Onset to Inclusion on the Expression of Anaphylatoxin Receptors

The effect of tocilizumab could potentially depend on whether the patients were treated with PCI or not, or whether they were included early (≤2 days) or late (>2 days) from the onset of symptoms. However, the pattern of the C5aR1, C5aR2 and C3aR expression was virtually identical in patients with or without PCI (**Figures 3A–C**) and in patients included early or late (**Figures 3D–F**). A flow chart of the patients is shown in **Figure 1**.

Systemic Complement Activation in NSTEMI Patients

To see if inhibition of IL-6R affected complement activation, sC5b-9 was evaluated in all patients in the tocilizumab study ($n = 117$) from baseline to day 3, and at 6 months follow up. Plasma concentration of sC5b-9 did not change over time in the NSTEMI patients (**Figure 4A**). Tocilizumab had no effect on the degree of systemic complement activation. The same pattern was seen regardless of PCI treatment or not (**Figure 4B**) and independent of early (≤2 days) or late (>2 days) inclusion from the onset of symptoms (**Figure 4C**).

Association Between the Expression of Anaphylatoxin Receptors and Key Biomarkers in the NSTEMI Patients During Hospitalization

The original tocilizumab study found a fall in leukocytes in the tocilizumab-group, primarily caused by a decrease in

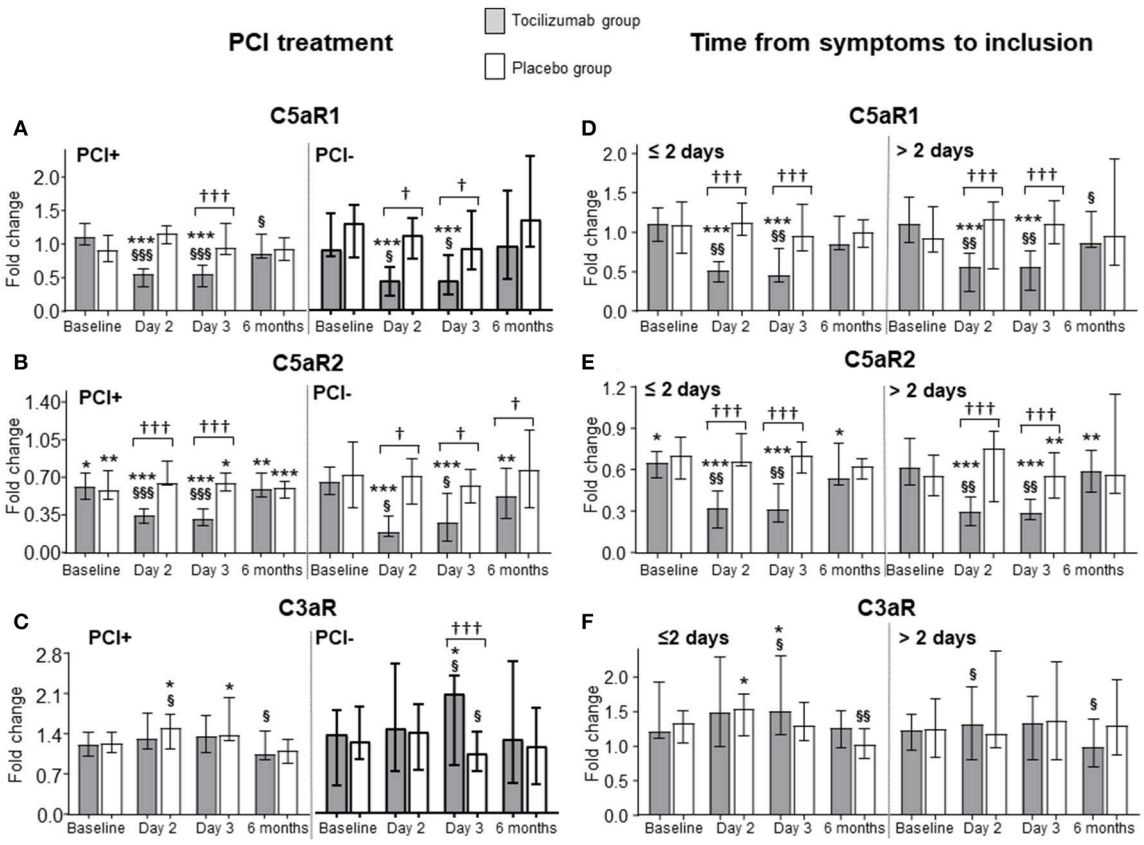

FIGURE 3 | Effect of coronary intervention and time of inclusion on the expression of C5aR1, C5aR2, and C3aR in NSTEMI patients. Expression level of complement anaphylatoxin receptors C5aR1 **(A,D)**, C5aR2 **(B,E)**, and C3aR **(C,F)** in patients with non-ST-elevation myocardial infarction (NSTEMI) receiving placebo ($n = 32$) or tocilizumab ($n = 28$) divided into two groups according to percutaneous coronary intervention (PCI) (23 placebo and 21 tocilizumab, gray bars) or not (7 placebo and 9 tocilizumab, white bars) **(A–C)**, and divided into two groups according to inclusion ≤2 days (22 placebo and 15 tocilizumab, gray bars) or >2 days (10 placebo and 13 tocilizumab, white bars) from symptom onset **(D–F)**. Baseline levels show the receptor expression at inclusion, i.e., after hospital admission, before treatment was given. Follow-up time points were day 2 and 3, and 6 months. A group of healthy individuals ($n = 15$) were included as controls. The qPCR results were quantified using the $2^{-\Delta\Delta CT}$ method, normalized to reference genes and presented as fold change with the healthy controls as calibrator. Data are given as median and 95% CI. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. healthy controls. $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ differences in change from baseline between tocilizumab and placebo. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ vs. baseline.

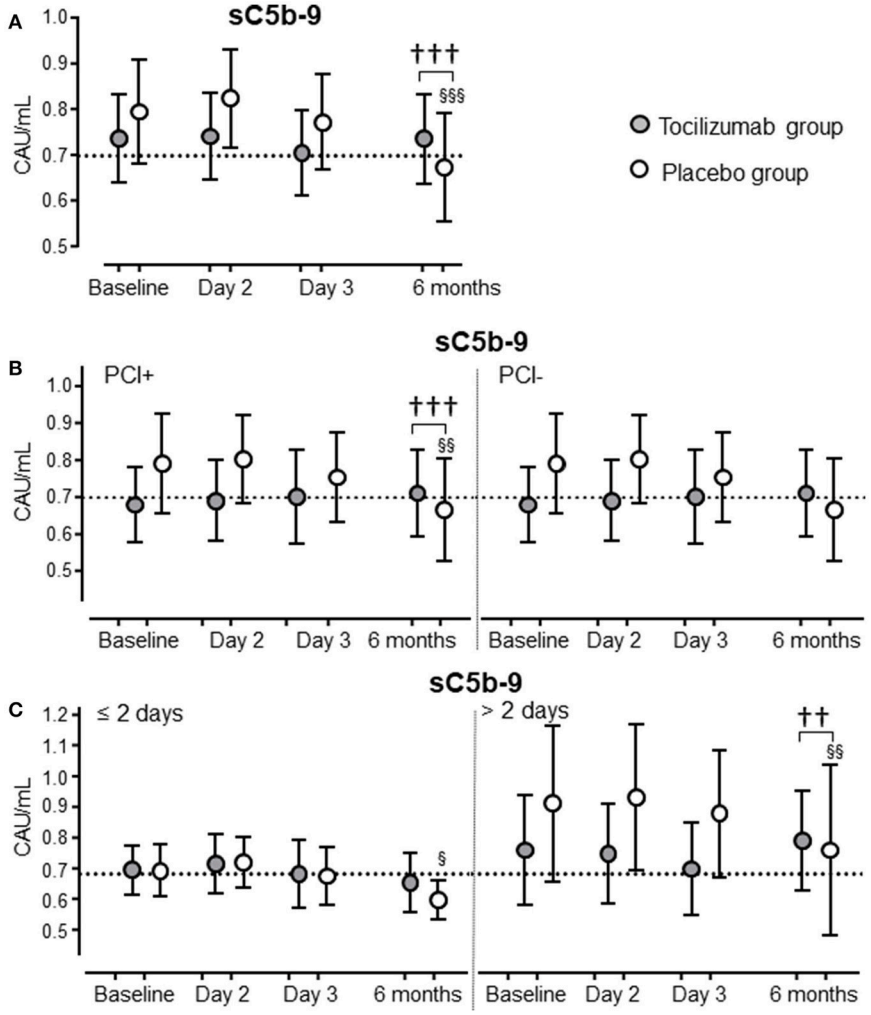

FIGURE 4 | Systemic complement activation in NSTEMI patients treated with tocilizumab or PCI. Complement activation, as evaluated by plasma sC5b-9, is shown for NSTEMI patients (total $n = 117$) treated with tocilizumab ($n = 58$; closed circles) or placebo ($n = 59$; open circles) **(A)**. Complement activation, as evaluated by plasma sC5b-9, is shown for the same population of NSTEMI patients further divided into two groups according to percutaneous coronary intervention (47 placebo and 41 tocilizumab) or not (12 placebo and 17 tocilizumab) **(B)**, and according to inclusion ≤2 days (36 placebo and 30 tocilizumab) or >2 days (23 placebo and 28 tocilizumab) from symptom onset to inclusion **(C)**. The dotted line represents normal upper level of sC5b-9 in healthy blood donors. Data are given as mean and 95% CI. $^{††}P < 0.01$, $^{†††}P < 0.001$ differences in change from baseline between tocilizumab and placebo. $^{§}p < 0.05$, $^{§§}p < 0.01$, $^{§§§}p < 0.001$ vs. baseline.

neutrophils from baseline to day 3 which led to a significant between-group difference in change from baseline (15). The same statistical differences were also found in the sub-group of patients studied here (**Table 2**). We found no correlation between change in neutrophils and change in expression level of any of the three anaphylatoxin receptors in the treatment group (**Supplementary Table 1**). However, in the placebo group there was a significant correlation between change in neutrophils and change in the expression level of C5aR1 and C5aR2 (**Supplementary Table 1**).

In the original tocilizumab study, IL-6 and sIL-6R increased significantly from baseline to day 3 in the tocilizumab-treated patients (15). A similar pattern was found in the sub-group of patients investigated in this study (**Table 2**). In the tocilizumab group no correlation was found between change in expression for any of the anaphylatoxin receptors and IL-6 in the tocilizumab. sIL-6R correlated with the expression of C3aR in the tocilizumab-treated patients (**Supplementary Table 1**). In the placebo group we found a significant correlation between all three anaphylatoxin receptors and IL6 whereas no correlation was found for sIL-6R (**Supplementary Table 1**).

Associations Between the Anaphylatoxin Receptors and CRP and TnT in the NSTEMI Patients During Hospitalization

We evaluated whether AUC for the three anaphylatoxin receptors showed any correlation with AUC for CRP and

TnT representing the primary and most important secondary endpoint, respectively, in the original tocilizumab study (15) (**Table 3**). There was a significant correlation between C5aR1 and C3aR, but not C5aR2, and CRP in the placebo group, whereas only C3aR was correlated with CRP in the tocilizumab group (**Table 3**; **Supplementary Figure 1**). TnT correlated significantly with all three receptors in the placebo group, whereas only C5aR1 correlated with TnT in the tocilizumab group (**Table 3**; **Supplementary Figure 1**). All correlations between receptor expression and CRP and TnT were positive. The regression plots, however, show negative regression lines since the statistics were calculated on delta-CT values and a decrease in the delta-CT value represents an increase in receptor expression.

Expression of Anaphylatoxin Receptors in PBMC From Patients With Different CAD Entities

In order to explore whether the expression of anaphylatoxin receptors is dependent on the severity of CAD, independent on any intervention, we investigated the expression of these receptors in PBMCs in samples obtained from patients admitted to hospital comprising three different entities of CAD: SAP ($n = 22$), NSTE-ACS ($n = 21$), and STEMI ($n = 20$).

Whereas C5aR1 expression was significantly increased in all CAD subgroups compared to healthy controls with the highest levels in the STEMI patients (**Figure 5A**), the increase in C5aR2 and C3aR were more moderate, showing significantly increased levels as compared with controls in the STEMI group (C5aR2) and NSTE-ACS group (C3aR) only (**Figures 5B,C**).

TABLE 2 | Baseline values and values at day 3 for different relevant biomarkers in the NSTEMI patients.

	Group	Baseline	Day 3	
IL-6 pg/mL	Placebo	3.0 (1.2–5.1)	3.2 (1.7–6.3)	†
	Tocilizumab	2.4 (1.3–4.5)	22(14.3–29)***	
sIL-6R ng/mL	Placebo	71 (13)	70 (11)	†††
	Tocilizumab	64 (11)	88 (8.4)	
Leukocytes (10^9/L)	Placebo	7.6 (1.7)	7.6 (1.3)	†††
	Tocilizumab	8.0 (2.4)	4.9 (1.6)	
Neutrophils (10^9/L)	Placebo	4.5 (3.3–5.7)	4.3 (3.7–5.2)	†††
	Tocilizumab	5.1 (3.2–6.0)	1.9 (1.2–2.7)	
Monocytes (10^9/L)	Placebo	0.7 (0.2)	0.7 (0.6–0.9)	
	Tocilizumab	0.7 (0.5–0.9)	0.6 (0.2)	
Lymphocytes (10^9/L)	Placebo	2.0 (0.5)	2.1 (0.6)	
	Tocilizumab	2.0 (0.7)	1.9 (1.6–2.5)	

Data are given as mean with (standard deviation, SD) or median with (25 and 75th percentile, IQR). IL, interleukin; R, receptor; s, soluble.
****$p < 0.001$ comparing differences within group from baseline.*
†$p < 0.05$, †††$p < 0.001$ comparing between-group differences in change from baseline.

TABLE 3 | Spearman Rho correlation between AUC during hospitalization for CRP and TnT and the three anaphylatoxin receptors in the NSTEMI patients.

		AUC C5aR1	AUC C5aR2	AUC C3aR
AUC CRP	Placebo	**0.431***	0.214	**0.506****
	Tocilizumab	0.264	0.082	0.338
AUC TnT	Placebo	**0.399***	**0.494****	**0.400***
	Tocilizumab	**0.534***	0.315	0.259

AUC, area under the curve; TnT, troponin T; CRP, C-reactive protein; R, receptor.
*Data: Spearman Rho correlation coefficient with *$p < 0.05$, **$p < 0.01$. Bold values indicate statistical significance.*

DISCUSSION

This study demonstrates for the first time that inhibiting IL-6R profoundly attenuated the expression of C5aR1 and C5aR2 in peripheral whole blood in NSTEMI patients. Treatment with PCI is known to cause a reperfusion injury, which in itself can enhance inflammation. However, the effect on the anaphylatoxin receptor expression seen in this study was independent of treatment with PCI or time between debut of symptoms and inclusion. In contrast, C3aR expression was not affected by the IL-6-inhibitory treatment. Moreover, changes in C5aR1 was significantly correlated with changes in TnT during tocilizumab treatment suggesting the beneficial effect of IL-6R inhibition at least partly could involve downregulation of the inflammatory C5aR1.

Inflammation plays a pivotal role in the wake of a MI being essential for cardiac repair (18). However, sustained and excessive inflammation may contribute to increased tissue damage and is associated with worse prognosis in ACS (19). Elevated levels of inflammatory markers like CRP, IL-6 and C5a are related to the detrimental effects of inflammation in CAD (5, 13, 19–22) and anti-inflammatory treatment is suggested to improve outcome after MI (23). Genetic studies suggest that inhibiting either IL-6 or complement could prove beneficial in patients with CAD (24–26), and it has recently been shown that a single dose of tocilizumab attenuates the increase in CRP and PCI-related TnT release in NSTEMI patients (15).

The activation of C5aR1 induces pro-inflammatory effects like recruitment and activation of inflammatory cells and enhanced cytokine and chemokine production. Experimental studies have shown reduction in infarct size and inflammation when the C5a/C5aR1-axis has been attenuated (27–30). Furthermore, lack of C5aR1 on circulating leukocytes led to reduced infarct size and improved clinical outcome in an *in vivo* mouse model of MI (31). IL-6 inhibition is previously shown to attenuate expression of anaphylatoxin receptors in an experimental model of sepsis (14). Herein, we show a similar pattern in NSTEMI patients with a significant downregulation of C5Ra1 by tocilizumab in the first days following NSTEMI. Notably, this downregulation was significantly correlated with TnT release in the tocilizumab group suggesting that downregulation of C5aR1 might contribute to the attenuated TnT release by tocilizumab seen in these patients (15). The gradual increase in C5aR1 expression in the different CAD subgroups from SAP through NSTE-ACS with the highest level in STEMI patients may further

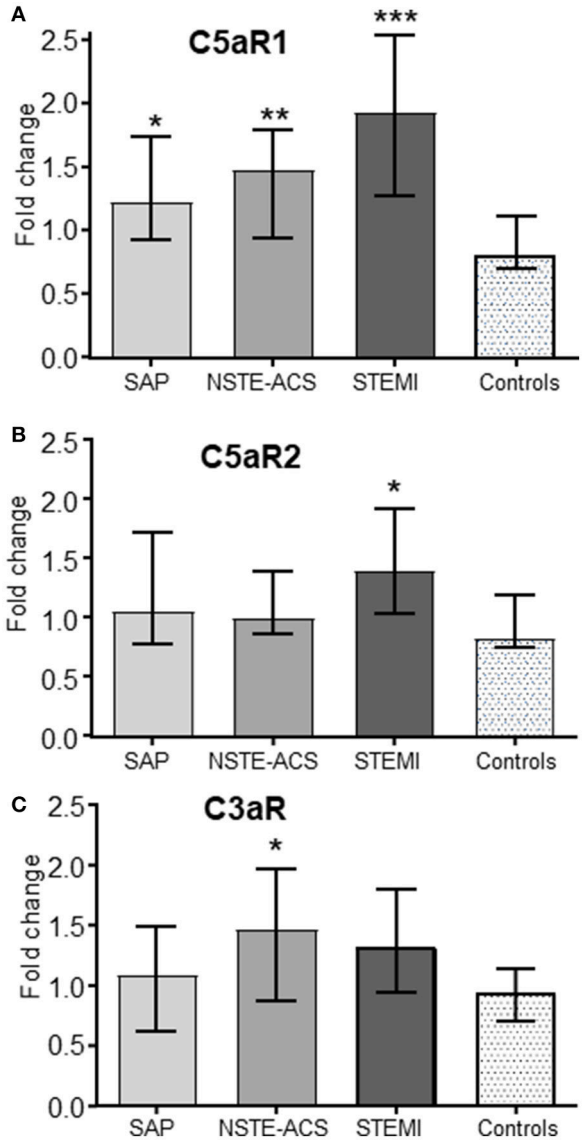

FIGURE 5 | Expression of C5aR1, C5aR2 and C3aR in patients with various entities of CAD. Expression of the C5aR1 **(A)**, C5aR2 **(B)**, and C3aR **(C)** in three different patients groups with verified coronary artery disease (CAD): stable angina pectoris (SAP, $n = 22$), non-ST-elevation acute coronary syndromes (NSTE-ACS, $n = 21$) and ST-elevation myocardial infarction (STEMI, $n = 20$). A group of healthy age and sex-matched individuals were included as controls (Ctrls, $n = 29$). mRNA levels were quantified by qPCR using the $2^{-\Delta\Delta CT}$ method, normalized to reference genes (GAPDH) and presented as fold change with the healthy controls as calibrator. Data are given as median and 95% CI. Statistical significant differences are indicated between the patient populations and the healthy controls. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. healthy controls.

support a role for this receptor in plaque progression and destabilization.

C5aR2, previously considered a non-signaling receptor, has been shown to have both pro- and anti-inflammatory effects and its function seems to be dependent on cell type, disease context and species (32). In experimental studies of CAD, there is some evidence that antagonizing C5aR2 might have beneficial effects (9). In the present study we showed a downregulation of C5aR2 by tocilizumab in NSTEMI patients. However, changes in C5aR2 were not correlated with changes in TnT during tocilizumab treatment, and in contrast to C5aR1 expression, the changes in C5aR2 expression in PBMC in the different CAD subgroups were rather modest.

In the NSTEMI patient group, a reduction in C5aR2 expression was observed both in the tocilizumab group and the placebo group, throughout the whole study period. The expression of C5aR2 is known to be attenuated in the context of inflammation (33) and the reduced level of C5aR2 in both the placebo group and the tocilizumab group even observed at inclusion might be due to the inflammatory response caused by the MI itself. We did not find the same reduction in C5aR2 expression compared to controls in the PBMC CAD group. The reason for this is unknown, but might be due to differences in time of sampling in relation to the myocardial injury, different expression level in different cell types or different methods. The effect on C5aR2 seen after 6 months might be related to the enhanced inflammation caused by the reperfusion injury caused by treatment with PCI. Also attenuating IL-6, which is a pleiotrop cytokine, might indirectly change the expression of C5aR2. Thus, the effects of C5aR2 in the setting of CAD and myocardial damage are still unclear and needs further investigations.

C3aR was previously regarded as a pro-inflammatory receptor but recent studies support a more complex effector function for this receptor with anti-inflammatory effects in the acute phase of inflammation by preventing neutrophil mobilization from the bone marrow (34). In an experimental study of intestinal ischemia and reperfusion injury, C3aR was shown to ameliorate ischemia-reperfusion injury in mice (35). Herein, we found a marked increase in C3aR expression in NSTEMI patients that was not modulated by tocilizumab. Moreover, C3aR, but not the two C5a receptors, correlated positively with changes in CRP during IL-6 receptor inhibition. Whatever the effect of C3aR, these findings suggest that IL-6 differently affect the expression of the C5a receptors and C3aR.

There was a reduction in the number of leukocytes and particularly neutrophils in the tocilizumab-treated NSTEMI patients as demented in the original study (15). This could, however, not explain the decreased C5aR1 and C5aR2 expression. First, there was no correlation between the change in receptor expression and change in neutrophil levels in the tocilizumab group. Second, the amount of mRNA in all samples was identical coming mainly from granulocytes, lymphocytes and monocytes, which constitute the main amount of nucleated cells in peripheral blood. Also lymphocytes and monocytes express anaphylatoxin receptors. Lymphocytes have previously been found to express C5aR1 (36–39) and the two C5a-anaphylatoxin receptors are typically co-expressed (33). Monocytes also express all three anaphylatoxin receptors shown for the CAD-population in this study. Third, the decrease was explicitly seen for the C5a receptors and not for the C3aR, indicating that the decrease was selective. Taken together this supports a real reduction in expression of C5aR1 and C5aR2.

In the present study, we used whole blood and PBMC, precluding us for detecting individual cell populations as would have been possible using cell sorting. There is, however, an advantage of using whole blood for this purpose, since the cells are less manipulated and *in vitro* changes in cell activity is reduced and the changes are to a greater extent reflecting the *in vivo* situation.

No correlation between IL-6 or sIL-6R and the three different anaphylatoxin receptors in the tocilizumab-treated patients, were observed. Tocilizumab was administrated in doses high enough to give a total IL-6 blockade for about 2 weeks (15) thus the level of IL-6 or sIL-6R is rather irrelevant since the effect of the cytokine is totally blocked in all patients during the hospital stay. We did find a correlation between the anaphylatoxin receptors and IL-6 and sIL-6R in the in the placebo group consistent with rather little change in both IL-6 and the anaphylatoxin receptors during the time course in this group.

sC5b-9 did not increase in the present study which most likely was due to the relatively small MIs in the NSTEMI patients. Complement is however constantly activated at a low level and acts in the circulation as a humoral alarm system ready to respond to any danger threatening the host (40). Importantly, the absence of significant systemic complement activation does not preclude the presence of local activation with the ability to act at the site of damage. Thus, downregulation of the receptors for C5a might have beneficial effects both locally and systemically.

The present study has some limitations. The number of patients was rather low. Also, the lack of protein data on the anaphylatoxin receptor expression may weaken our conclusions. Finally, it should be emphasized that correlations do not necessarily mean any causal relationship and more mechanistic studies are needed to further explore the role of anaphylatoxin receptors in CAD.

In conclusion, a substantial and statistically highly significant reduction of C5a receptors was observed in NSTEMI patients treated with tocilizumab, and as for C5aR1, the downregulation correlated with attenuated TnT release. C5aR1 expression in PBMC did also reflect disease severity in another separate CAD population. The cross-talk between complement C5aR1 and IL-6 might contribute to the attenuated TnT release during tocilizumab treatment in these NSTEMI patients.

AUTHOR CONTRIBUTIONS

HO, TM, PN, AB-D, PA, BH, OK, JD, BB, RW, LG, AY, TE, and SP contributed to conception and design; OK, LG, GA, BH, HO, IG, and KE contributed with acquisition of data; HO, TM, AB-D, PN, OK, GA, PA, BH, IG, KE, SP, AY, and TU contributed with analysis and interpretation of data; HO, TM, PN, AB-D, OK, and PA drafted the article; All authors critically revised the article and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02035/full#supplementary-material

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Paper III

Paper IV

Basic Res Cardiol (2017) 112:20
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ORIGINAL CONTRIBUTION

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

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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

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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 infarct size 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 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 [6, 32]. 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.

Materials 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, anesthesia, 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, anesthesia 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 40 min 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-life 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 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, USA) 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 °C. 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 °C.

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 1 mm), 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, USA) 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).

Immunofluorescence analysis

The snap-frozen tissues were cut into 5 µm thick sections, air-dried for 60 min and fixed with cold acetone for 10 min. They were either processed immediately or stored at −80 °C 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, USA) 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, USA) 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, USA). A fluorescence microscope (DMI4000B; Leica, Wetzlar, Germany) was used to analyze 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, USA) 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 °C 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 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, Glostrup, 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× 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, USA) 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, USA).

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 analyzed 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 behavior (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. Two-way ANOVA was used if more than two groups had to be compared. Linear 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).

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, 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, 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 and 3.3 ± 0.7 cm s^{-1}, respectively; $p = 0.01$, Fig. 2a). Likewise, systolic displacement was 31% higher in coversin treated animals than in controls (7.4 ± 1.3 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 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 and 2.3 ± 0.2 l/min, respectively; $p = 0.09$, Fig. 2d).

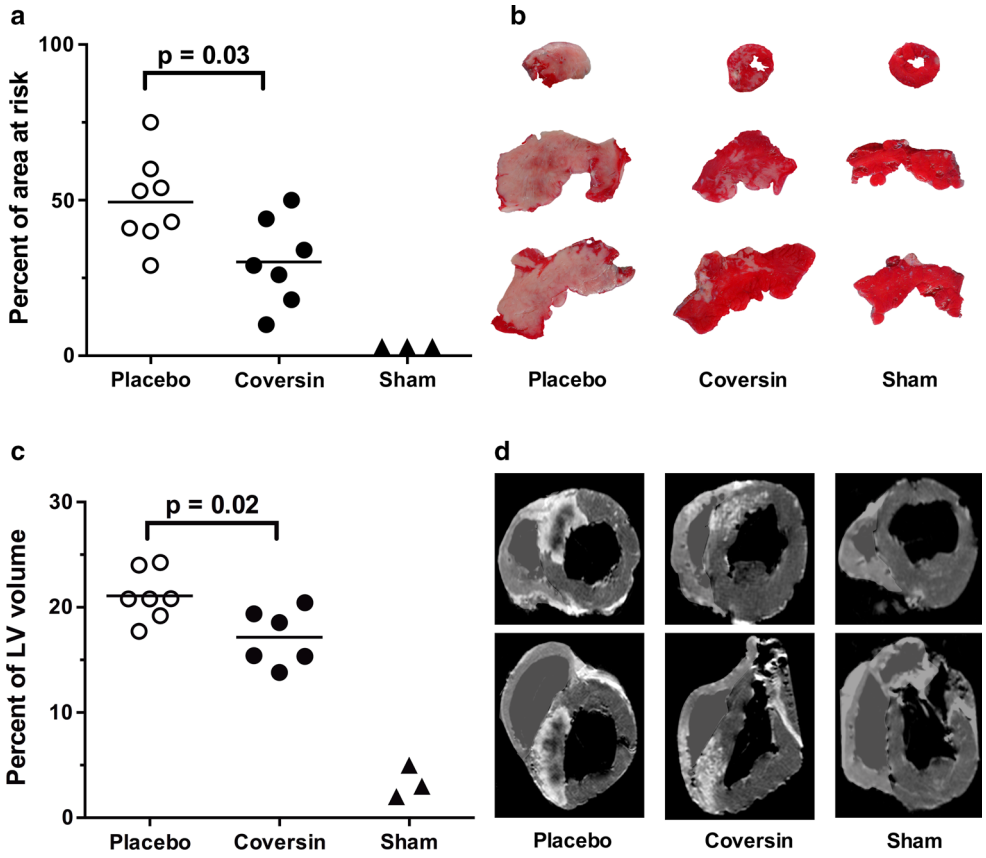

Fig. 1 Coversin reduced infarction size. **a** Coversin (C5 inhibitor) reduced infarction in the area at risk (AAR) by 39%, $p = 0.03$ determined by TTC staining. **b** TTC staining of the AAR (example slices from one animal in each group) shows infarcted areas in white and non-infarcted areas in *red*. **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

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 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).

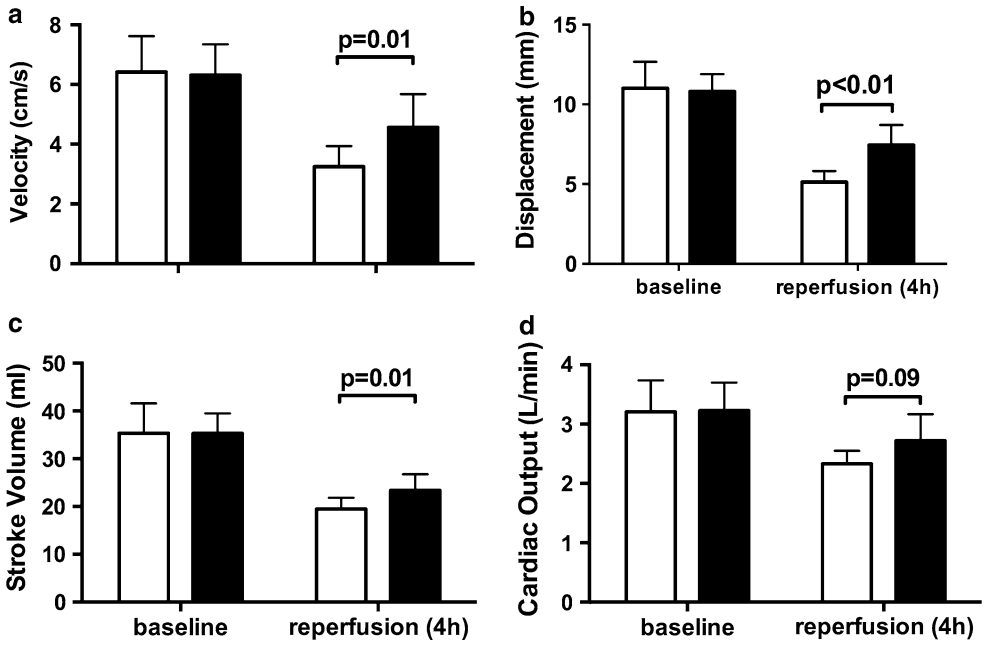

Fig. 2 Coversin improved myocardial function. Tissue Doppler echocardiography was evaluated from the mitral plane and averaged from septal and lateral wall movements. *Open bars* represent control and *filled bars* coversin treated animals. Systolic velocity was reduced at 4 h after reperfusion in both 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

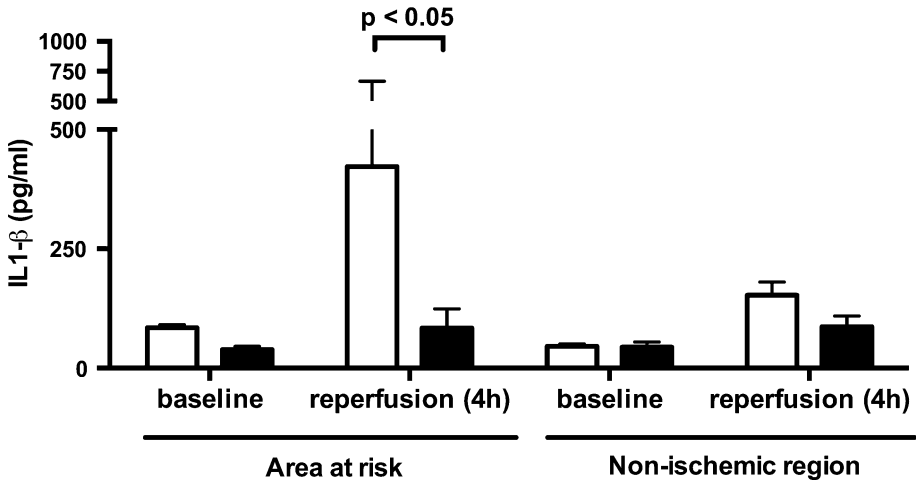

Fig. 3 Coversin reduced local myocardial IL-1β production. IL-1β obtained by microdialysis was induced in the area at risk (AAR) and not the control region after 4 h of reperfusion. Coversin treatment (*filled bars*) significantly reduced IL-1β in the AAR by 80% in comparison to placebo treated animals (*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

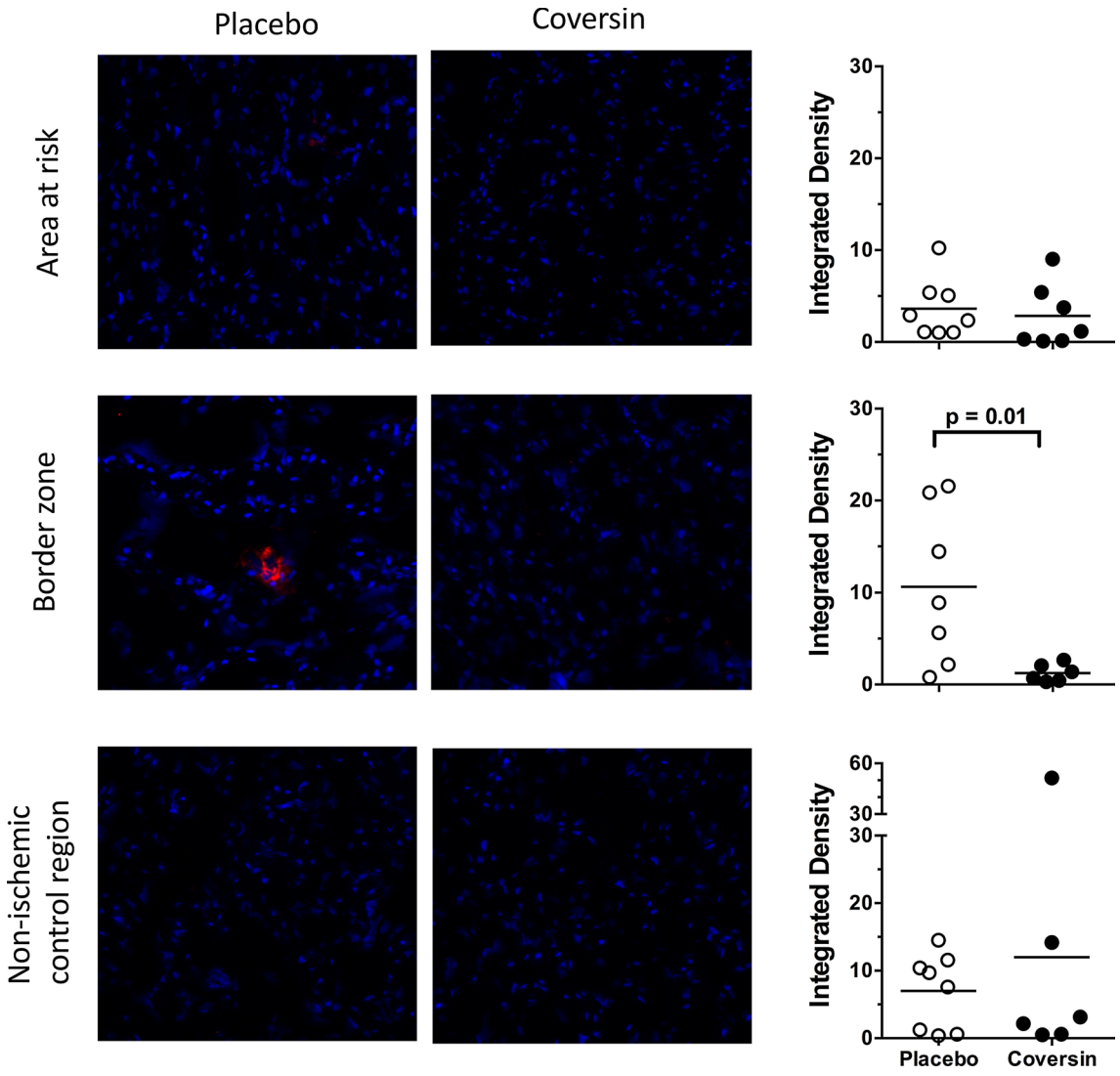

Fig. 4 Coversin reduced E-selectin expression. Myocardium was stained with antibody against E-selectin. E-selectin expression was increased in placebo treated animals in the border zone of the AAR and unchanged in the center of the AAR and non-ischemic Cx control region (*left panels*). Coversin treatment 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

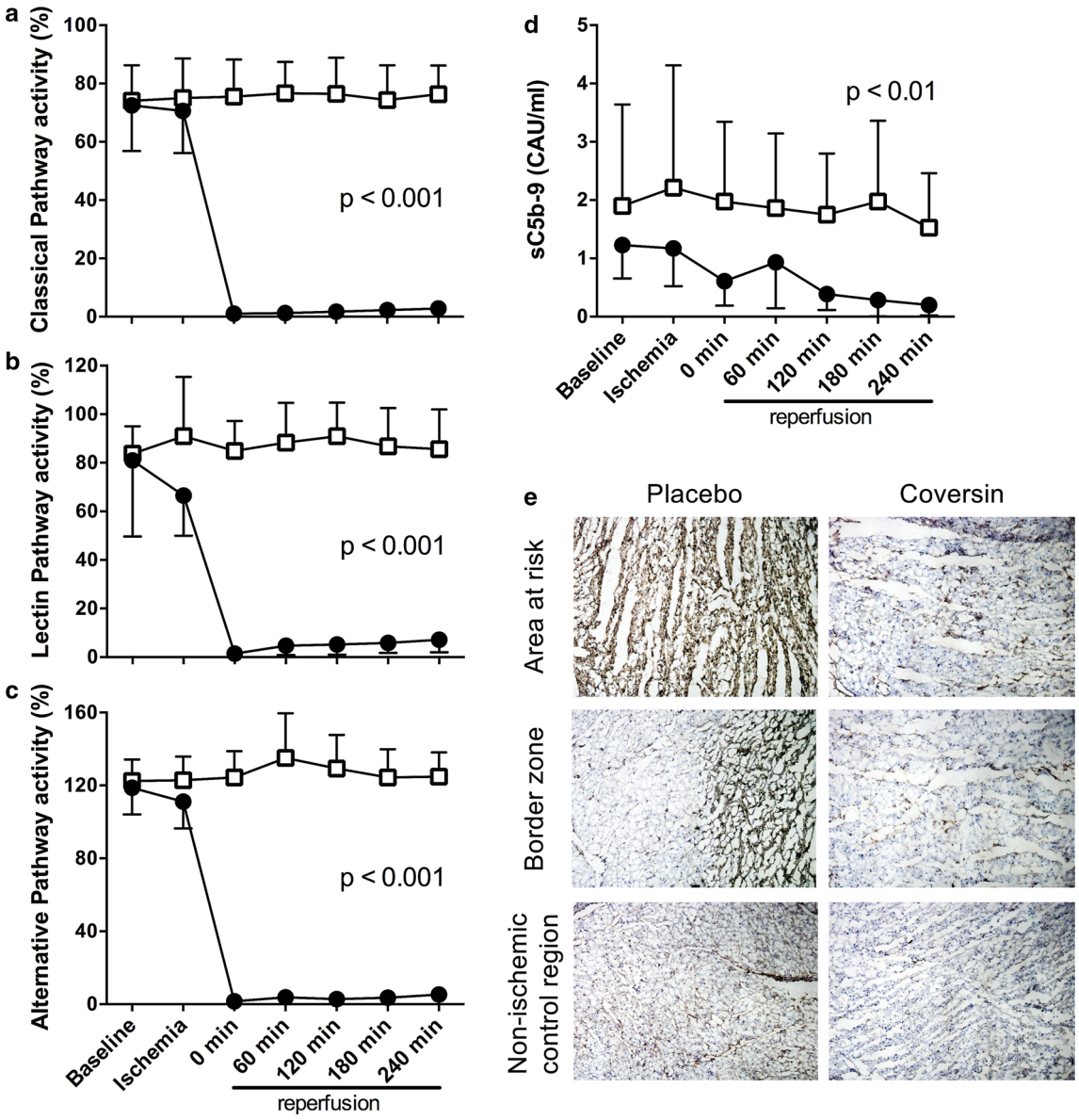

Fig. 5 Coversin eliminated complement activity. Complement activity was assessed in plasma and the classical (**a**), lectin (**b**) and alternative pathway (**c**) were monitored using C5b-9 deposition as common readout. Coversin bolus treatment during coronary ischemia led to significantly reduced 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

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).

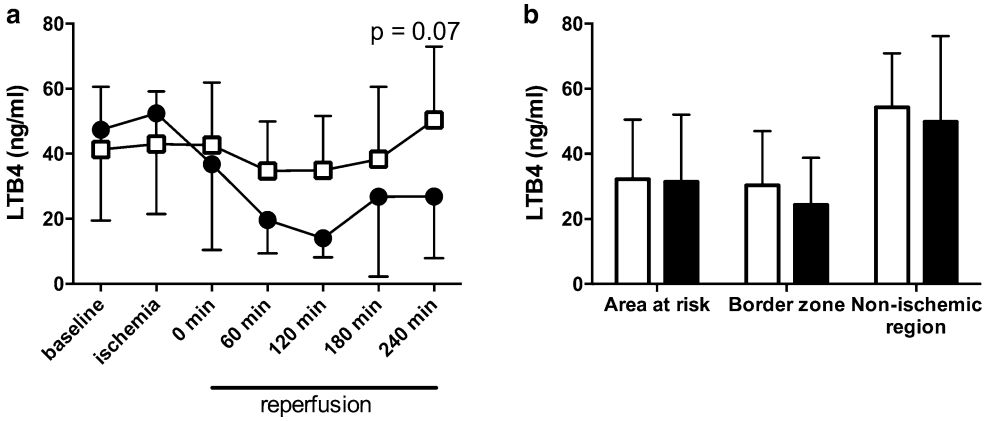

Fig. 6 Coversin did not reduce LTB4. **a** LTB4 was assessed in plasma throughout the study period. LTB4 showed a non-significant trend to lower values during reperfusion in coversin treated animals compared to placebo. LTB4 in myocardial tissue from three different regions at the end of the experiment 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

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 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).

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 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 h in this study, as a doubling of LTB4 in humans appears during the first 24 h 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

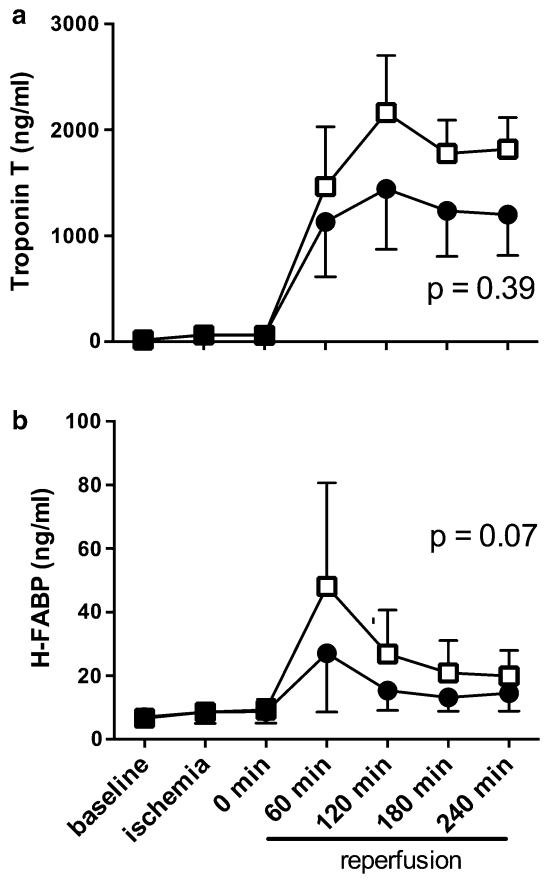

Fig. 7 Coversin did not significantly decrease plasma markers of myocardial ischemia. Troponin T (**a**) and H-FABP (**b**) were detected in plasma throughout the study period. Myocardial ischemia lead to an increase in troponin T and H-FABP in both control (*open boxes*) and coversin treated (*filled 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

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 today's clinically used eculizumab, which all inhibit cleavage of C5 at different binding sites [27]. 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 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 4 h of infarction in man [17]. Isoflurane was used

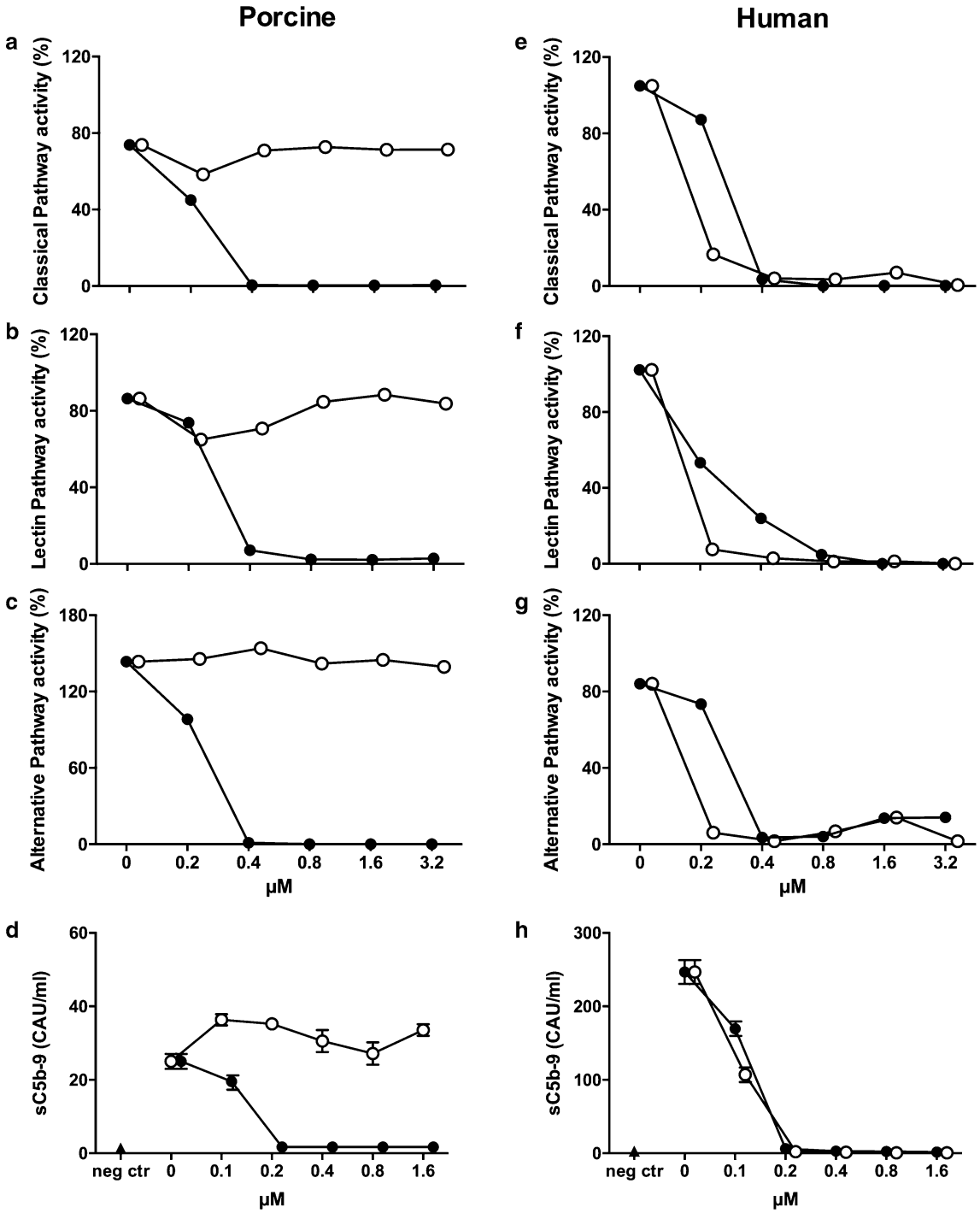

Fig. 8 Coversin, but not eculizumab, inhibits porcine complement activation. Complement inhibitory effect of coversin (*filled circles*) and eculizumab (*open circles*) were assessed in the functional classical (**a**, **e**), lectin (**b**, **f**), and alternative pathway (**c**, **g**) assays in porcine (**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 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

as anesthetic 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 h 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 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.

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Compliance with ethical standards

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.

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Errata list

Name of candidate: Hilde Lang Orrem

Abbreviation for different types of corrections:

- Cor- correction of language
- Cpltf- change of page layout or text format

Side	Line	Original text	Type of correction	Corrected text
9	3	*Medhitov*	Cor	*Medzhitov*
16	14	DAMPs ad PAMPs	Cor	DAMPs and PAMPs
16	18	… cytokines like	Cor	…cytokines like IL-10 and IL-37
22	1	C5aR2 are	Cor	C5aR2 is
25	18	…complement components has been found…	Cor	…complement components have been found…
30	20	Clinical data supports	Cor	Clinical data support
37	23	admitted to hospital	Cpltf	admitted to hospital
55	23	...different cells types…	Cor	...different cell types…