C1 esterase inhibitor mediated protection from acute lung injury

Inaugural Dissertation
Submitted to the
Faculty of Medicine
of the Justus Liebig University Giessen

by
Hess, Rosanna Marie
from Frankfurt am Main, Germany

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I. Table of contents

I. Table of contents……...I
1. Introduction……1
1.1 Hemostasis……1
1.1.1 The pathways of coagulation……...1
1.1.2 Contact activation pathway……..…2
1.1.2.1 Factor XII……..... 2
1.1.2.1.1 FXII structure……..3
1.1.2.1.2 FXII activities…….....4
1.1.2.1.3 Activation and inhibition of FXII……...4
1.1.2.2 Factor XI……..5
1.1.2.3 Kallikrein…….. 6
1.1.2.4 High molecular weight kininogen…… 6
1.1.2.4.1 Bradykinin…….……... 7
1.1.3 Cellular interaction of the contact activation system…… 7
1.1.4 The role of contact activation in hemostasis and thrombosis……...8
1.1.5 The role of contact activation in inflammation…..... 9
1.2 C1 esterase inhibitor…… 9
1.3 Acute respiratory distress syndrome…….. 10
1.3.1 Definition and etiopathology of ARDS…… 11
1.3.2 Pathogenesis of ARDS…… 12
1.3.3 Alveolar coagulation in ARDS…… 13
1.3.3.1 The role of extrinsic coagulation factors in ARDS..... 13
1.3.3.2 The role of intrinsic coagulation factors in inflammation and fibrotic alteration of the lung…… 14
2. Material and methods……...17
2.1 Material……..... 17
2.1.1 Apparatuses and Equipments…… 17
2.1.2 Reagents……17
2.1.3 KITs……..... 20
2.2 Methods…… 21
2.2.1 Test samples…… 21
2.2.1.1 Study population……... 21
2.2.1.2 Murine samples…… 22
2.2.1.2.1 Bleomycin administration…… 22

2.2.1.2.2 C1 INH administration…….. 22
2.2.1.2.3 Histological staining…….. 22
2.2.1.2.4 Wet-dry-lung weight ratio…… 22
2.2.1.2.5 Flow cytometry…… 23
2.2.2 Western Blot…….. 23
2.2.2.1 Preparation of the samples for gel electrophoresis…….. 23
2.2.2.2 Sodium dodecyl sulfate polyacrylamid gel electrophoresis……..... 24
2.2.2.3 Immunoblotting…….. 24
2.2.3 Enzyme linked immunosorbent assay (ELISA)…… 25
2.2.3.1 FXII detection in BALF……..25
2.2.3.2 FXII detection in plasma…….. 25
2.2.3.3 BK detection in BALF and plasma……..26
2.2.4 Isolation of leukocyte……26
2.2.5 Molecular Analysis……27
2.2.5.1 RNA Isolation…….. 27
2.2.5.2 Reverse Transcriptase (RT) reaction…… 27
2.2.5.3 Real-time PCR (qPCR)……..... 28
2.2.6 Statistics……..29
3. Results……..... 30
3.1 Contact activation factors are elevated in BALF and lung homogenate of ARDS patients……30
3.2 FXII expression is increased in blood cells of ARDS patients……36
3.3 The protein level of FXII and BK is elevated in lungs of bleomycin treated mice……37
3.4 C1 esterase inhibitor (C1 INH) decreases inflammatory response in bleomycin challenged murine lungs……..40
3.4.1 C1 INH alleviates the inflammatory response induced by bleomycin application……... 40
3.4.2 Expression of proinflammatory mediators in bleomycin injured lungs is reduced after C1 INH application…… 43
3.4.3 C1 INH administration has no impact on coagulation in bleomycin challenged mice……..45
4. Discussion……...47
4.1 The contact activation factors are increased in ARDS lungs……. 47
4.2 C1 INH administration moderates the inflammatory response in acute lung injury……51
5. Conclusion…….. 56

6. Summary……..57
7. Zusammenfassung …….. 59
8. List of abbreviations…….. 62
9. List of figures and tables……65
9.1 List of figures…….... 65
9.2 List of tables……..... 66
10. References…….. 67
11. Declaration…….. 80
12. Acknowledgements…….. 81
13. Curriculum vitae……82

1. Introduction

1.1 Hemostasis

Hemostasis is an essential feature of the human defense system and the physiological response to blood vessel damage and injury of endothelial cells. Initially, the blood flow is reduced by local vasoconstriction, accompanied with aggregation of activated platelets to instantly cover the lesion of the endothelium and consequently prevent further extravasation of the blood. This initial process is also referred to as primary hemostasis. The concurrent activation of the coagulation proteases induces extensive fibrin generation, finally resulting in a stable fibrin-clot, which seals the surface of the blood vessel. Correspondingly, this process is described as secondary hemostasis. Importantly, this hemostatic response is strictly limited to the site of vessel injury, physiologically not affecting the systemic blood flow. Thus, an accurate, locally limited interaction of thrombocytes and damaged endothelium as well as a precise regulation of procoagulant and anticoagulant mediators is required. In the case of a pathological predominance of either condition, thrombotic formation or bleeding disorder, respectively, will result.

1.1.1 The pathways of coagulation

The classic model of the coagulation system distinguishes two different pathways of coagulation, the extrinsic and the intrinsic pathway, each characterized by a cascade of reactions of proteases and cofactors. Primordially, both coagulation pathways were thought to independently contribute to fibrin formation during hemostasis. However, presently a more complex scheme of coagulation emerges and research up to date indicates a preponderant importance of the extrinsic pathway as the main activator of coagulation *in vivo (1-4)*. The intrinsic pathway, also referred to as contact activation pathway, is initiated by surface contact (*5-7*) and includes the Hageman factor (FXII), factor XI (FXI), kallikrein (KLK) and high molecular weight kininogen (HK). While its contribution to fibrin formation in hemostasis is presumed to be of minor importance, the contact activation factors were found to be involved in pathological thrombosis and in other activities related to modulation of the complement- and kinin-system.

1.1.2 Contact activation pathway

1.1.2.1 Factor XII

The gene encoding for FXII is located on chromosome 5 (*8*). FXII zymogen is synthesized in the liver (*9*) and secreted as a single chain glycoprotein with a molecular weight of 80kDa (*10*). *In vitro* FXII has been found to have the special property of autoactivation when bound to activating surfaces. Activation of FXII can either result in generation of activated α-FXII (α-FXIIa) or by further cleavages in formation of activated β-FXII (β-FXIIa), also referred to as Hageman fragment (HFf), both possessing serine protease activity (*11*). Activated α-FXII consists of an amino-terminal heavy chain of 50 kDa and a carboxyl terminal light chain of 28 kDa, linked by a disulfide bond (*12, 13*). β-FXIIa is also composed of two chains, the 28 kDa chain identical with the light chain of α-FXIIa and a 2 kDa chain, which originates from the heavy chain, equally held together by a disulfide bond (*11, 12, 14*).

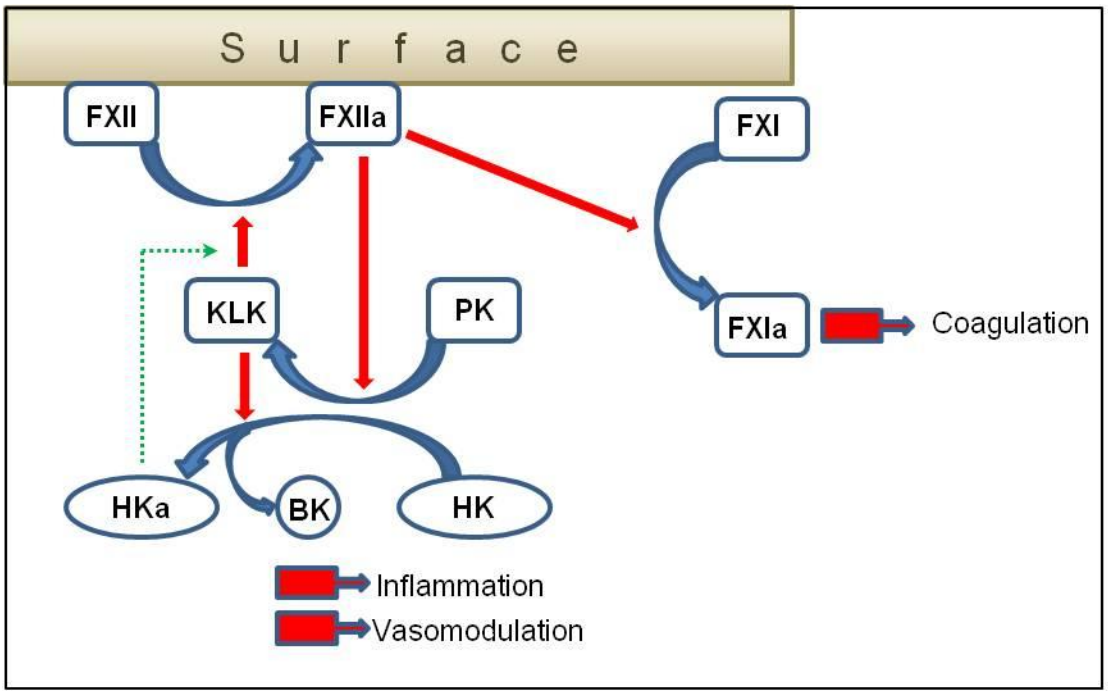

Figure 1.1 Contact activation pathway.

FXII is converted when bound to negatively charged surface into activated FXII (FXIIa). FXIIa activates prekallikrein (PK) into kallikrein (KLK), which reciprocally activates FXII. Additionally KLK cleaves high molecular weigh kininogen (HK) into HKa under release of vasoactive and proinflammatory bradykinin (BK). HK has an accelerating impact on KLK triggered FXII activation. FXIIa further activates FXI, FXIa cleaves FIX, a protease known to essentially contribute to coagulation.

1.1.2.1.1 FXII structure

Structurally, FXII is composed of various functional domains. Starting at the amino-terminus following regions were immuno-localized: The fibronectin domain type II, followed by an epidermal growth factor (EGF)-like domain, a fibronectin domain type I, a second EGF-like domain, a kringle-domain, a proline-rich domain and a carboxy-terminal located catalytic domain (*14*). Although the different domains of FXII have been identified, not all functional properties of the respective regions are yet determined. Surface binding sites have been detected in the fibronectin domain type II (residues 1-28) (*15*) and type I region (residues 134-153) (*16*). An additional surface binding site is surmised in either the kringle domain or the second EGF-like domain (*10*). The first EGF-like as well as the fibronectin type II region were identified to contain binding sequences for zinc, a putative promoter of FXII surface binding and activation (*17, 18*). Previous studies further provided evidence of a critical role of the fibronectin type II domain (residues 3-19) in FXI activation and interaction with endothelial cells and neutrophils (*19-21*). The precise function of the EGF-like domains of FXII remains to be examined; however, a mitogenic effect of this region has been demonstrated (*22*). Containing the catalytic domain, the light chain represents the site of proteolytical activity. This section includes the catalytic triad, composed of the amino acid residues His^{393}-Asp^{442}-Ser^{544}, which is specific for serine proteases and essential for its enzymatic activity (*23*). The amino acid sequence of the catalytic domain has been found to share homologies with those of other proteases, notably plasmin, tissue plasminogen activator (t-PA) and urokinase (*12*). The proline-rich domain, however, appears to be unique for FXII, its significance is not yet understood. It is worthwhile noting that the structure of FXII has a particularly striking similarity with the t-PA, only differing in one domain; whereas FXII consists of the proline-rich domain, t-PA contains a second kringle region (*14*).

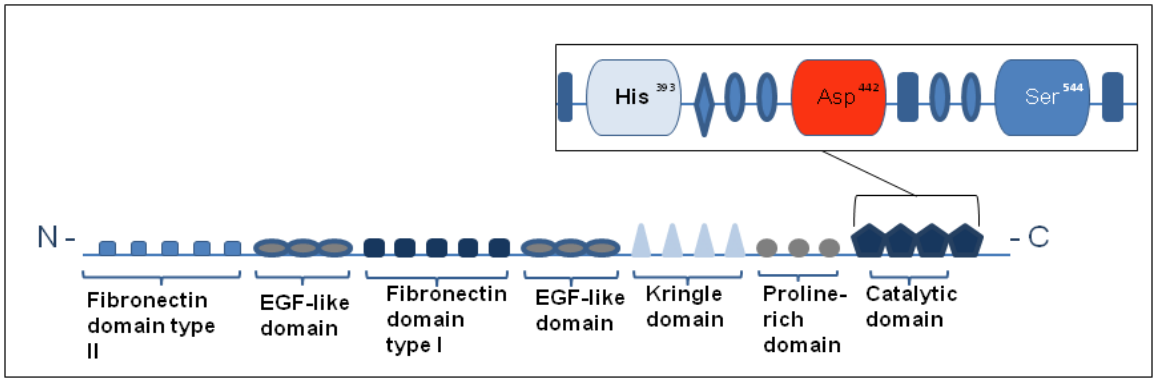

Figure 1.2 Model of FXII structure with detail view on catalytic domain.

1.1.2.1.2 FXII activities

Containing both the catalytic domain, α-FXIIa and β-FXIIa possess the ability of proteolytically activating prekallikrein (PK) to kallikrein (KLK), which launches the kinin-kallikrein system and reciprocally activates FXII, a reaction found to be enhanced by the cofactor high molecular weight kininogen (HK) (*13, 24*). Furthermore β-FXIIa possesses the property of promoting immunological defense by activating the first component (C1) of the classical complement pathway (*25-27*). However, lacking the particular domains, β-FXIIa does not bind to negatively charged surfaces (*26*). Also the ability to initiate the clotting activity of the intrinsic pathway by proteolytical activation of coagulation FXI is exclusively reserved to α-FXIIa (*27*).

Besides its procoagulant activity, in purified systems FXII has also been demonstrated to contain fibrinolytic properties at physiological concentration by direct activation of plasminogen (*28, 29*). This property has been reported to be enhanced by negatively charged surfaces, dextran sulfate (*30*) and in the presence of zinc ions (*29*). Furthermore, indirect fibrinolytic effects have been attributed to FXII firstly by cleavage of plasminogen proactivator (*31*) and secondly by complex formation with plasminogen activator inhibitor type-1 and its subsequent inactivation (*32*). In line with these findings are the structural homologies of FXII with t-PA (*12, 14*). A mitogenic effect of FXII has been demonstrated in HepG2 cells (*22*), fetal alveolar cells, endothelial cells, epithelioid carcinoma cells and aortic smooth muscle cells (*33*), most likely involving the EGF-like domains.

1.1.2.1.3 Activation and inhibition of FXII

FXII zymogen is activated by cleavage of the Arg^{353}-Val^{354} bound either during autoactivation or by KLK. Numerous substrates have been found to be *in vitro* capable of triggering autoactivation of FXII as kaolin (*34*), dextran sulfates (*35*), sulfatides (*36-38*), bacterial lipopolysaccarides (*39*), heparin, chondroit sulfate (*40*), articular cartilage and calcium pyrophosphat (*13, 41*). Although the process of FXII activation under physiological conditions remains unsettled, potential activators have been identified as RNA (*42*), collagen (*43*) and inorganic polyphosphates (PolyP) (*44*). The latter has been observed to be released by activated platelets *in vivo* and induce the contact activation system (*45*). The reciprocal KLK-driven activation of FXII with HK as cofactor amplifies FXIIa generation and is required for a sufficient rate to initiate the intrinsic clotting pathway and the kinin-kallikrein system (*46, 47*). The significant role of C1 esterase inhibitor (C1 INH) as irreversible inhibitor of FXII is generally acknowledged (*48, 49*). This serine protease inhibitor was first described in connection

with the first component of complement, as it was detected to inactivate the subcomponents C1r and C1s (*50*). Since then a wide range of additional proteases have been identified to be target of C1 INH inactivation, among them α-FXIIa and β-FXIIa (*51*). The inhibition rate is attenuated in the presence of kaolin, sulfatides and other negatively charged surfaces, thus α-FXIIa appears to be protected from inhibition when bound to a surface. Accordantly, this effect does not apply for β-FXIIa due to its lack of a surface binding site (*52*). Anti-thrombin III (AT III), α-2-macroglobulin and α-2-antiplasmin were additionally demonstrated to have inhibiting effects on FXIIa, though to a much lower degree (*48, 53*). However, the predominant role of C1 INH is challenged by a study, demonstrating a preponderant complex formation of FXII-AT III, when FXII activation is triggered by activated platelets. This might draw new attention to the *in vivo* role of AT III as inhibitor of FXII (*54*).

1.1.2.2 Factor XI

The liver represents the main source of FXI synthesis. However, human FXI RNA has additionally been located in the kidney and pancreas, though their contribution to the FXI plasma level is presumed to be negligible (*55*).

FXI is a zymogen of 160 kDa molecular weight, with the unique feature among coagulation proteases of a homodimeric structure (*55-57*). This homodimer contains two identical polypeptide chains, linked by a disulfide bond (*58, 59*). Each of the two chains is composed of a heavy and a light chain, the latter containing the catalytic portion with the classic triad of His, Asp and Ser as found in FXII (*60*). Each heavy chain is composed of four tandem repeats, containing 90-91 amino acid residues respectively, referred to as Apple domains (A1-4) (*57, 60, 61*). Remarkably, the amino acid sequence of FXI is to 58% homologous to prekallikrein (PK) (*57*), a discovery elucidated by a common evolutionary ancestor (*62, 63*). Both proteins share the feature of being substrates of FXII and are each by majority found associated in a non-covalent complex with HK (*63, 64*). The particular dimeric formation of FXI is presumed to be essential for the activation by α-FXIIa and thrombin (*56, 65*). FXIIa triggered FXI activation is performed by cleavage of the Arg ^{369}- Ile ^{370} bond (*57*), an interaction found to involve HK as important cofactor (*61, 66*). FXIa possesses serine protease properties and activates FIX, a crucial contributor to thrombin generation (*3, 67*).

1.1.2.3 Kallikrein

Contrary to FXI the precursor of plasma kallikrein, prekallikrein (PKLK), is of monomeric structure, containing 619 amino acids (*68*). Activation of PK is initiated by internal cleavage of the Arg^{371}- Ile^{372} bond, resulting in formation of a heavy chain of 52 kDa and a light chain, linked together by a disulfide bond (*60, 69*). Two different variations of the light chain were detected at 33 kDa and 36 kDa, respectively (*70*). Analogous to FXI, the heavy chain of KLK contains the four Apple domains (A1-4) of repeat sequences in the amino-terminal part (*68*). The light chain contains the carboxyl-terminal located catalytic domain and has similar amino acid sequences as the trypsin family of serine proteases (*68, 70*). Further cleavage of the heavy chain of KLK, a process determined to be of autolytic nature, induces β-KLK generation (*70, 71*). The heavy chain has been reported to be the essential site for interactions with HK and FXII (*72, 73*). Thus, regions participating in HK binding have been localized within the A2 domain (*74*) as well as in domain A1 and A4 (*75*) of the heavy chain. The non-covalent complex formation with HK not only facilitates surface binding with subsequent enhanced activation by FXII, but further protects KLK from inhibition by its major inhibitors C1 IHN and α-2-macroglobulin in plasma (*76, 77*). Activated KLK cleaves HK into HKa, liberating bradykinin (BK). HKa is found to have a more accelerating impact as cofactor on FXII activation than its precursor (*78*). Additionally, fibrinolytic activity is attributed to KLK; directly by activation of plasminogen and indirectly by cleavage of pro-urokinase, a plasminogen activator (*79, 80*).

1.1.2.4 High molecular weight kininogen (HK)

Despite having no enzymatic activity of its own, the pathophysiological functions of HK are manifold and of indispensable relevance in contact activation. Firstly, HK is the precursor of BK, a highly significant modulator of vasodilatation and inflammatory response. Secondly, HK has an accelerating effect as a cofactor on contact activation. HK is a glycoprotein with a molecular weight of 120 kDa. It is cleaved by KLK into a heavy chain of 62 kDa and a light chain of 56kDa, joined together by a disulfide bond (*60, 81-83*). HK consists of six different regions, referred to as D1-D6. Whereas the domains D1-D3 account for the heavy chain, D4 constitutes BK, and the light chain is composed of the domain D5-6 (*60, 81, 84*). Remarkably, all contact activation pathway activity is linked to the light chain (*76*). The binding site for anionic surfaces *in vitro* as well as for endothelial cells, neutrophils and platelets, is localized on D5 of the light chain (*84-87*). D6 has been identified to contain the binding site for FXI and PK (*84*). The presence of HK during contact activation is of essential importance; besides its

function as an associate for FXI and KLK in plasma, it also serves as an anchor, linking the proteases to the surface and consequently providing ideal conditions for FXII triggered activation (*66*).

1.1.2.4.1 Bradykinin

Liberated BK, a nanopeptid of nine amino acids, is involved in a wide range of vascular and inflammatory processes (*88, 89*). The half-life of BK is briefly limited due to a rapid degradation by different enzymes, particularly the angiotensin-converting enzyme (ACE) (*90, 91*). Vascular modulative effects include the release of superoxide anion (*92*), prostacyclin (*93*), nitric oxide (NO) (*94*) and promotion of hyperpolarization and relaxation of arterial smooth muscle cells (*95*). Furthermore, this kinin has been found to stimulate the release of t-PA, thus contributing to the regulation of fibrinolysis (*96, 97*). The inflammatory impact of BK is mediated through the bradykinin-1-receptor (B_1R) and bradykinin-2-receptor (B_2R). The former is exclusively expressed in response to inflammatory stimuli such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (*91*). By contrast, B_2R is constitutively produced (*90, 91*) and its expression is recognized to be reinforced by BK itself (*98, 99*). Both receptors are identified to be involved in BK induced recruitment of lymphocytic cells during inflammation (*100, 101*). BK stimulates the release of interleukin-8 (IL-8), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1) and TNF-β in lung fibroblasts (*101*). Particularly IL-8 is an essential inflammatory mediator and a highly effective neutrophil chemoattractant. BK further effectuates the release of the proinflammatory cytokines interleukin-6 (IL-6) (*102*) and IL-1β (*88*) in airway smooth muscle cells and human fibroblasts via B_2R.

1.1.3 Cellular interaction of the contact activation system

The finding of intrinsic components on human cells has given rise to the question whether these cells might serve as a platform for the proteins to assemble and represent the site of contact phase activation *in vivo*. Furthermore, a potential effect of contact proteins on cellular processes through receptor binding has been put into consideration. On the surface of human neutrophils FXII, FXI, PK and HK have been immune-localized (*21, 103*). Whereas FXII and HK bind directly to neutrophils, FXI and PK are attached to the cell surface through HK. Apparently, both chains of HK contain regions responsible for the leukocytes binding. An essential receptor for HK was determined to be Mac-1 (CD11b/CD18) (*85*), a leukocyte integrin known to be also

involved in neutrophil adhesion and transendothelial migration (*104*). The assembly of the contact proteins presents a potential source of BK generation attached to the immune cells. Considering its effect on the permeability of vessels, liberated BK might thereby locally promote neutrophils diathesis (*21*).

The presence of the intrinsic components has also been recognized on human endothelial cells. Up to this day, three different receptors, namely urokinase plasminogen activator receptor (u-PAR), gC1qR and cytokeratin-1, have been identified to be targets of zinc dependent FXII and HK binding. The interaction of FXII with u-PAR, in complex with β1 integrin and EGFR, has been observed to stimulate proliferation of endothelial cells and angiogenesis (*105*). Of great interest is further the binding process of the intrinsic factors to the receptor for the C1 subcomponent of the first component of complement gC1qR (*18, 19, 106*). The binding site of the contact proteins within the receptor appears to differ from that of C1q, as no mutual interference or inhibition is observed (*18*). Cytokeratin-1 constitutes the third identified receptor and exists partly as a bimolecular complex in association with u-PAR (*90, 107*) or gC1qR (*19, 90*). Lastly, contact activation factors have been localized on activated platelets, and their enzymatic activity while being bound to thrombocytes has been demonstrated (*44, 54*). Thus, platelets may also provide a platform for the activation and activity of contact pathway proteases.

1.1.4 The role of contact activation in hemostasis and thrombosis

The negligible contribution of intrinsic pathway factors to thrombin generation during hemostasis has been acknowledged in the past decades of research, mainly based on the objective fact that FXII deficient patients do not suffer of a bleeding disorder. More recently, data have been published which are consistent with this perception, but indicate an essential contribution of the proteases in pathological thrombus formation. This hypothesis was supported by the finding that FXII deficient ($FXII^{-/-}$) mice were protected from thrombus formation after artificially induced vessel injury. This protective effect was rescinded after FXII infusion, confirming the favorable effect of FXII deficiency (*108, 109*). Defective thrombus formation in $FXII^{-/-}$ mice was also noticed for cerebral ischemia models, further highlighting a predominant role of FXII in thrombosis (*110*), (*109, 111*). These antithrombotic effects in $FXII^{-/-}$ mice were observed not to be limited to FXII. Similar results were found in FXI deficient ($FXI^{-/-}$) mice, expanding a potential role of pathological thrombus formation to the intrinsic cascade (*112, 113*). Considering their lack of interference with physiological hemostasis, the intrinsic factors

might provide optimal conditions for thrombosis prevention. However, to date this antithrombotic effect of FXII depletion has not been established for humans.

1.1.5 The role of contact activation in inflammation

Research of the recent decades indicates an essential importance of the contact pathway as modulator of inflammatory processes. In this context, FXII and KLK have been found to be capable of attracting neutrophils and inducing their aggregation (*114-116*). Moreover, both proteases were demonstrated to promote the degranulation of neutrophils and the subsequent release of neutrophil elastase (*116-119*). The pro-inflammatory attribute is further emphasized in a study showing that inhibition of FXII in septic baboons resulted in declining activation of the complement system and reduced release of neutrophil elastase and IL-8 (*118*). Additionally, FXII has been reported to have a reinforcing effect on cytokine expression, stimulating the synthesis of IL1-β in monocytes (*115*). A potential impact on septic conditions has been also described for FXI, thus inhibition of this protease was noted to attenuate inflammation and improve survival of affected mice (*120*). However, the crucial contribution of contact activation to inflammation emerges to be the HK-derived liberation and rapid provision of BK, the key participant in a broad diversity of inflammatory disorders (*121*).

1.2 C1 esterase inhibitor

C 1 esterase inhibitor (C1 INH) is a single chain glycoprotein, belonging to the family of serine protease inhibitor (serpins). The inhibitor has a molecular weight of 105 kDa and is exceptionally heavily glycosylated. Two domains can be distinguished; the carboxyl terminal located serpin domain represents the site of its inhibitory activity, containing the protease recognizing compartment, also called the reactive center loop (*122, 123*). The interaction of the active serine of the target protease with the serpin domain of C1 INH results in an irreversible complex formation with disruption of the molecular structure of the protease (*124*). The second domain of C1 INH is the particularly richly glycosylated amino terminal domain, which is unique to the inhibitor and has been acknowledged to be not involved in its protease inhibitory activity (*123*). Having an extended range of substrates within the coagulation, fibrinolytic and complement activation system, C1 INH represents a serpin of high biological relevance. A particularly critical role is assigned to the inhibitor as important regulator of inflammation as C1 INH is the exclusive inhibitor of the classic pathway of complement and manifested to be the main inhibitor of FXII and KLK of the contact system.

Proteolytical cleavage of C1 INH in the reactive center causes the inactivation of its protease inhibitory activity. An enzyme identified among others to be capable of C1 INH inactivation is the neutrophil elastase, an important participant in a wide range of inflammatory diseases, including the acute respiratory distress syndrome (*125*). Aside from its extended significance as inhibitor of plasma proteases, C1 INH has been recognized to directly interact with endothelial and immune cells and may therefore contain additional anti-inflammatory attributes independent from its activities as plasma protease inhibitor (*122, 126, 127*). Heterozygous C1 INH deficiency results in hereditary angioedema (HAE), a disease with the clinical pattern of sudden phases of increased vasopermeability with severe edema formation mediated by unrestricted BK generation (*122*).

1.3 Acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) is a lung disease of inflammatory nature, commonly culminating in fibroproliferative tissue remodeling and fibrotic alteration of the lung. Over the past decades, a high discrepancy has been reported in trials investigating the incidence of ARDS. These controversial observations may partly be explained by a deviation of the definition of the disease, recent improvements in medical treatment and regional differences. An incidence of 58.7/100,000/year was stated in an extensive investigation including 21 hospitals in the United States. Milder variations of lung injury were estimated to account for 78.9/100,000/year. Furthermore the incidence and mortality was found to be highly dependent on the age of the patients (*128*). A more limited incidence has been reported in European trials (*129-131*), for example a rate of 7.2/100,000/year was acknowledged in Spain (*131*). In line with these data, a recent retrospective evaluation in Iceland stated the incidence of ARDS at a similarly low level (*130*). Additionally, this study revealed a steady increase of 0.2 cases/year from 1988 to 2010 (*130*). Though various approaches concerning the treatment of ARDS have been made, only lung protective ventilation has proven to have a beneficial effect on survival (*132*). Thus, despite a declining trend (*130, 133*) mortality still remains high, varying between 33% (*130*) and 47.8% (*131*).

1.3.1 Definition and etiopathology of ARDS

The definition of ARDS has undergone multiple alterations and concisions since first described in 1967 by Ashbaugh. This first definition included a severe respiratory distress associated with hypoxemia refectory to oxygen therapy, a decline of lung compliance and diffuse lung infiltration appearing and rapidly spreading in the chest

radiograph (*134*). Intending to specify and internationally standardize the ARDS term, the American-European-Consensus Conference Committee (AECC) redefined the criteria of the disease in 1994. This recommendation is the currently prevalent utilized definition of ARDS, differentiating according to the degree of hypoxemia: A mild type, named acute lung injury (ALI) and a severe type called the acute respiratory distress syndrome (ARDS), using the ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (Pa_{O2} / Fi_{O2}) as criterion for the degree of hypoxemia. Thus, hypoxemia with a Pa_{O2}/ Fi_{O2} < 300mmHg is classified as ALI, a Pa_{O2}/ Fi_{O2} <200 mmHg as ARDS (*135*). Additionally, diseases with similar symptoms, but chronic course, were excluded from the definition. The pulmonary artery wedge pressure was determined to be less than 18 mmHg or absence of clinical signs of left atrial hypertension must be verified, excluding cardiac failure as origin of the symptoms. Radiologically, bilateral infiltration in the front chest radiograph was advocated to be a dispositive criterion for ALI/ ARDS (*135*).

The latest definition published, referred to as the Berlin Definition further specifies these criteria. Accordingly, it is suggested to differentiate three different categories, mild (Pa_{O2}/Fi_{O2}= 200-300 mmHg), moderate (Pa_{O2}/Fi_{O2} =100-200mmHg) and severe (Pa_{O2}/Fi_{O2} <100 mmHg) ARDS, based on the degree of hypoxemia, also stating the Positive Expiratory End Pressure (PEEP) at a minimum of 5 cmH_2O. Furthermore, the acute onset was concretized, determining a period of less than one week (*136*). The pulmonary wedge pressure was eliminated from the definition to avoid exclusion of ARDS patients with coexisting heart failure. Instead, patients with a diseased heart and clinical signs respective to the criteria of ARDS are included into the definition, when the extent of respiratory distress is not to be explained by cardiac failure only. The report proposes in such cases further clinical efforts to clearly identify the origin of the edema causing the respiratory distress (*136*). Etiologically, two different kinds of ARDS are to be distinguished. Direct ARDS is based on pulmonary genesis, most commonly as a result of a pulmonary infection, but also after aspiration, nearly-drowning or inhalation of toxic gas (*130, 131*). By contrast indirect ARDS has an extrapulmonary source and may occur as accompanying complication during sepsis, acute pancreatitis and blood transfusion (*132, 137*).

1.3.2 Pathogenesis of ARDS

The pathogenesis of ARDS is a complex and not entirely explored process, involving a high diversity of biochemical components contributing to the alterations of the lung tissue. Initially, the pathological characteristic is acute inflammation, accompanied by injury of the endothelial and epithelial alveolar membrane (*132, 138*). The permeability of the alveolar barrier increases, leading to influx of protein-rich fluids, which results in interstitial and alveolar edema (*132, 137*). In this period, a high amount of proinflammatory mediators notably secreted by alveolar macrophages are found in bronchoalveolar lavage fluids (BALF), as IL-1β, IL-6, IL-8 and TNF- α (*132, 139*). Elevated plasma levels of IL-1β and IL-6 have been found to be predictors of poor outcome, correlating with a high mortality rate (*140-142*). The particular significance of IL-8 as chemotactic stimulant has thoroughly been explored (*139, 140*). Along with the keratinocyte-derived chemokine (CXCL1) and macrophage inflammatory protein 2 (MIP-2) IL-8 is part of a complex network leading to excessive neutrophil activation and abundant neutrophil recruitment from capillaries via interstitium into the alveolar space (*140, 143*). Under these pathological conditions the infiltrating neutrophils release various proinflammatory mediators (*132, 140*). In particular, the neutrophil elastase has been focus of several investigations revealing a critical role of the enzyme in the pathology of ARDS (*140, 144, 145*). Besides, also other neutrophil-derived mediators as matrix metalloproteinases, cationic peptides and oxidants are identified to contribute to the pathogenesis of ARDS (*140, 144*). Accordingly, α-defensin, a cationic peptide synthesized by neutrophils and stored in granules, increases the alveolar endothelial and epithelial permeability and boosts neutrophil activation when set into extracellular space (*146*).

At this stage, the main histological feature is diffuse alveolar damage and a denuded basement membrane with formations of hyaline membranes, consisting of fibrin deposits (*132, 147, 148*). The destruction of alveolar cells, causing a decline of the normal epithelial fluid transport, aggravates the alveolar edema and prohibits its resolution (*132*). Physiologically, type I cells constitute 80% of the alveolar surface. Their loss within the inflammatory destruction is compensated by type II cells, which differentiate into type I to cover the denuded membrane. In consequence surfactant production, an ability mainly inherent to type II cells, is reduced. Deficiency of surfactant leads to an increased surface tension, precipitating atelectasis (*132, 140, 149*). This effect is reinforced by a decrease of functional surfactant due to its contamination with protein-rich fluids (*150*). The acute, exsudative phase merges into a fibroproliferative process hallmarked by alveolar type II and fibroblast proliferation with excessive deposition of fibrin, fibronectin and collagen (*151-153*). The ancient

perception that this proliferative phase is a late feature of ARDS has been challenged by investigations, demonstrating an elevation of procollagen peptide III, an early marker of collagen synthesis, from the third day onwards (*154*). This finding makes it reasonable to conclude that fibroproliferative alteration occurs simultaneously with the inflammatory response, rather than subsequently (*153*). Activated fibroblasts are found to migrate into the interstitium as well as into the intra-alveolar space and excessively release collagen and other extracellular matrix proteins. This contributes to the development of diffuse fibrosis, resulting in alveolar obliteration and diminution of pulmonary compliance (*148, 151, 153*). Interestingly, the individual course of ARDS appears heterogeneous and complete resolution subsequent to the inflammatory phase occurs in other patients (*132*). In this regard, genetic disposition is suspected to have essential influence on the susceptibility and course of ARDS (*155*). Some uncertainties remain concerning the pathogenesis of ARDS. For instance, only some mediators contributing to the development of fibrosing alveolitis are yet identified. Transforming growth factor-β1 (TGF-β1) is well recognized to be an essential factor to initiate collagen synthesis and deposition as well as fibroproliferation in the lung (*151, 156-158*) and found to be elevated in BALF of ARDS patients (*157*).
Also mediators affiliated to the inflammatory response, such as TNF-α and IL-1β, have been identified as contributive to the genesis of fibrosis (*159-161*), most likely by inducing TGF-β1 (*161*). Furthermore, another significant factor promoting fibrin deposition and fibrosing alteration of the lung is the unbalanced state of hemostasis in favor of procoagulation and antifibrinolysis (*152, 162-167*).

1.3.3 Alveolar coagulation in ARDS

1.3.3.1 The role of extrinsic coagulation factors in ARDS

A striking imbalance in alveolar hemostasis is detected in lungs of ARDS patients. Within the scope of animal studies, a significant increase of procoagulant activity is observed, while fibrinolytic efficiency has been noted to be reduced (*162, 163*). Correspondingly, extrinsic pathway activity has been found to be elevated in BALF of ARDS patients with concurrent inhibition of fibrinolytic activity by diminishment of urokinase-type plasminogen activator and increased plasminogen-activator inhibitor-1 levels. These conditions are observed, regardless of whether the acute respiratory distress syndrome is due to pulmonary or non-pulmonary causes (*162*). It is worthwhile noting that a comparable hemostatic status also applies for patients with severe pneumonia, who are spontaneously breathing. Thus, the assumption of the hemostatic

imbalance being only due to ventilator treatment can be dismissed, eliciting the idea of a pathological role of the elevated coagulant factors in lung injury (*162*). In line with this conclusion, attenuation of lung injury is observed in animal studies, when the procoagulant state is restrained by competitive inhibition of the tissue factor, the initiator of the extrinsic pathway. Furthermore, the blockade precipitates a decrease of local pro-inflammatory cytokines as IL-1β and IL-6 (*168*), underscoring the hypothesis of an inflammatory impact of coagulation factors and a pathological accountability beyond fibrin deposition in ARDS (*165, 169*). This assumption is supported by further research, confirming pro-inflammatory effects of coagulation factor Xa and thrombin (*169-171*), mainly linked to interactions with the protease-activated receptor (PAR)-1 (*167, 169, 172, 173*). This reinforcing effect between coagulation and inflammation mediators appears to be reciprocal as, for example, the tissue factor activation is stimulated by cytokines as TNF-α and IL-6 (*174*).

The preponderance of procoagulant activity results in abundant fibrin deposition, potentially culminating in interstitial and intra-alveolar fibrosis (*152, 153, 162, 163, 167*). Fibrin and its degrading products were found not only to have inflammatory attributes by inducing neutrophil recruitment (*175, 176*), but additionally they were reported to stimulate fibroblast migration and proliferation (*177*). Besides the fibrin deposition as consequence of boosted extrinsic pathway activity, factor Xa (FXa) and thrombin further contribute to the fibrotic alteration to the lung. Thus, both proteins are identified to stimulate procollagen synthesis in fibroblasts putatively via PAR-1 (*178, 179*) and to contain mitogenic effects on fibroblasts (*180, 181*).

1.3.3.2 The role of intrinsic factors in inflammation and fibrotic alteration of the lung

The focus of previous studies regarding the contribution of coagulation factors to lung inflammation and remodeling has commonly been on extrinsic factors rather than on proteins of the intrinsic pathway. Nevertheless, there is growing evidence of an underestimated relevance of intrinsic coagulation factors in the pathogenesis of lung injury. Thus, recent studies investigating a potential pathological contribution of intrinsic factors on Idiopathic Pulmonary Fibrosis (IPF) and murine bleomycin-induced lung injury, revealed a profibrotic activity of FXII related to its mitogenic activities towards human and mice lung fibroblasts (*182*). Within the scope of this investigation, a protection from bleomycin-induced lung fibrosis was noted in FXII deficient mice as well as in mice with inhibition of FXII by corn trypsin inhibitor (CTI). This profibrotic effect of FXII was independent of fibrin generation as no difference in fibrin disposition was

found compared to wild type (WT) mice (*182, 183*). Thus, a contribution of contact activation rather based on its pro-inflammatory and mitogenic properties during the pathogenesis of fibrotic lung diseases seems reasonable.

Pro-inflammatory attributes of FXII and KLK have been demonstrated in cultured cells and animal studies (*115, 117-119*). In line with these findings, a preventive effect in lung injury caused by Salmonella was observed when FXII and KLK were inhibited (*184*), illustrating a putative role of these factors not only in sepsis, but also in lung injury. Worthwhile mentioning is further the discovery of an inducing effect of TGF-β on FXII gene transcription in fibroblasts. Considering the elevated level of TGF-β in ARDS patients (*157*) and its established role in inflammatory and fibrotic processes in lung injury (*156, 158, 185*), the hypothesis of a contributing effect of FXII on ARDS pathogenesis gains further support. A possible stimulus for FXII activation in ARDS might be extracellular RNA, released from dying cells (*42*).

Given the extended involvement of BK in inflammation, great interest arises in the potential role of this contact activation product in the pathogenesis of ARDS. BK is well acknowledged to contain chemotactic features and might therefore promote neutrophil migration into the lung as found during the course of the disease. Particularly the stimulating effect of BK on human lung fibroblasts and airway epithelial cells to release chemokines has been demonstrated (*99, 101*). Furthermore, administration of inhibitors of either receptor of BK has led to attenuation of pulmonary leukocyte accumulation (*100*). Thus, BK is a pivotal intermediary during different inflammatory processes in the lung and it appears imperative to determine its putative role in the pathology of ARDS.

It seems reasonable to conclude that the perception of a functional role of coagulation factors limited on blood hemostasis is obsolete. Numerous cellular effects beyond coagulation have been found to be induced by coagulation proteases and a considerable impact on the development of inflammation and proliferation up to fibrotic tissue remodeling has been observed. Therefore the consideration of a critical role of coagulation factors in lung injury in general, and ARDS in particular, appears indispensable. Previous investigations support the idea of a pathological contribution of extrinsic pathway proteins on the pathological process in chronic and acute lung diseases, including ARDS. However, the research regarding a potential role of the contact pathway in severe lung injury and subsequent lung tissue remodeling is as yet in the initial stage.

The objective of the present study is to investigate the occurrence and properties of the contact pathway components in the pathogenesis of ARDS and in bleomycin-induced lung injury in mice. The incidence of FXII, KLK, HK, BK and FXI, respectively, was

examined in clinical as well as in animal samples. The possible therapeutic consequences of the inhibition of contact phase proteins were studied in a murine model of lung injury. The main emphasis of this study is on FXII, the initiator of intrinsic coagulation and the kinin-kallikrein system, and its potential contribution to the pathogenesis of ARDS.

2. Materials and methods

2.1 Materials

2.1.1 Apparatuses and Equipments

Centrifuge	Mikro20, Hettich GmbH, Tuttlingen, Germany
Centrifuge II	Heraeus Labofuge 400R, Functional line, ThermoFisher Scientific, Waltham, MA
Electrophoresis chambers	Biometra, Göttingen, Germany
Falcon tubes	Greiner Bio-One, Frickenhausen, Germany
Film cassette	Kodak, Rochester, NY
Filter tips	Eppendorf, Hamburg, Germany
Pipetboy	Eppendorf, Hamburg, Germany
Pipets	Eppendorf, Hamburg,Germany
Power Pac 1000	BIORAD, Hercules, CA
PVDF membrane	Amersham Biosciences, Little Chalfont, UK
Radiographic film	Amersham Biosciences, Little Chalfont, UK
SpectraMax 190	Molecular Devices, Silicon Valley, CA
StepOne Real time PCR System	Life Technologies, Carlsbad, CA
Thriller Thermoshaker-incubator	PeQlab, Erlangen, Germany
Vortex machine	VWR, Darmstadt Germany
Western blot chambers	Biometra, Göttingen, Germany

2.1.2 Reagents

Acrylamide solution	Roth, Karlsruhe, Germany
Agarose	Roth, Karlsruhe, Germany
Ammonium persulfate	Sigma-Aldrich, St-Louis, MO
Albumine, bovine serum	Sigma-Aldrich, St-Louis, MO
Biocoll	Biochrom, Cambrige, UK
β-mercaptoethanol	Sigma-Aldrich, St-Louis, MO
EDTA	Sigma-Aldrich, St-Louis, MO
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Sigma-Aldrich, St-Louis, MO
Glycerol	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
Hematoxylin	Roth, Karlsruhe, Germany
HEPES	Roth, Karlsruhe, Germany
Hydrochloric acid	Roth, Karlsruhe, Germany
Methanol	Roth, Karlsruhe, Germany
Milk powder	Roth, Karlsruhe Germany
Phenylmethylsulfonylflourid	Sigma-Aldrich, St-Louis, MO
Potassium chloride	Roth, Karlsruhe, Germany
Potassium phosphate monobasic	Sigma-Aldrich, St-Louis, MO
Skim Milk powder	Sigma-Aldrich, St-Louis, MO
Sodium bicarbonate	Sigma-Aldrich, St-Louis, MO
Sodium chloride	Sigma-Aldrich, St-Louis, MO
Sodium carbonate	Roth, Karlsruhe, Germany

Sodium deoxycholate	Sigma-Aldrich, St-Louis, MO
Sodium dodecyl sulfat (SDS)	Sigma-Aldrich, St-Louis, MO
Sodium hydroxide	Sigma-Aldrich, St-Louis, MO
Sodium phosphate dibasic	Sigma-Aldrich, St-Louis, MO
TEMED	Sigma-Aldrich, St-Louis, MO
Tris	Roth, Karlsruhe, Germany
Triton-X-100	Sigma-Aldrich, St-Louis, MO
Tween 20	Sigma-Aldrich, St-Louis, MO
Xylene	Roth, Karlsruhe, Germany

2.1.3 KITs

AssayMax FXII ELISA KIT	Assaypro, Saint Charles, MO
Matched Pair Antibody Set for FXII	Affinity Biologicals, Ancaster, Canada
Human Bradykinin ELISA KIT	Cusabio Biotech Co., Wuhan, China
PeqGOLD Total RNA Kit	PeqLab, Erlangen, Germany
Permanent AP Red Kit	Zytomed Systems GmbH, Berlin, Germany
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, MA
Pierce ECL Plus WB Substrate	Thermo Fisher Scientific, Waltham, MA
Zyto-Chem-Plus AP Polymer-Kit	Zytomed Systems GmbH, Berlin, Germany

2.2 Methods

2.2.1 Test samples

2.2.1.1 Study Population

The human BAL

F samples were obtained from 46 consecutive mechanically ventilated ARDS patients admitted to the intensive care unit of Department of Internal Medicine, Pneumology and Intensive Care Medicine at the University of Gießen. All patients met the criteria of the American-European Consensus Conference on ARDS (*135*), 28 were suffering from direct ARDS (Pneumonia, n=26; gastritic aspiraton, n=2) and 18 of indirect ARDS (sepsis, n=13; trauma/surgery, n=3; pancreatitis, n=1; polytransfusion, n=1). From the first day after diagnosis onward to day 8 BALF was obtained by flexible fiberoptic bronchoscopy at several different times. The control group was composed of 20 healthy, spontaneously breathing volunteers and ten mechanically-ventilated patients with cardiogenic pulmonary edema (CLE).

	ARDS (n=46)	**Healthy Controls (n=20)**	**CLE [a] (n=10)**
Age	49±13	41±17	57±9
Male/female	28/18	13/7	7/3
Pa_{O2}/Fi_{O2} [b]	127±35.7	422±17.3	156±37.1

[a] Cardiogenic pulmonary edema; [b] Pa_{O2} : Partial pressure of oxygen in arterial blood; Fi_{O2}: Fraction of inspired oxygen

Table 2.1 Study population for BALF samples.

The plasma samples were collected from ARDS patients (n=26) hospitalized at the University Clinic of Gießen and from healthy volunteers serving as controls (n=19) Blood leukocytes were isolated from 6 ARDS patients and 10 healthy subjects. Furthermore, the lung homogenate of 7 ARDS patients was obtained by autopsy. As the control the lung specimens of 6 patients were utilized of whom 5 had died of myocardial infarction and 1 of drug intoxication. Institutional approval was provided for all experiments involving human specimens by the Ethics Committee of the Faculty of Medicine of the University of Gießen.

2.2.1.2 Murine samples

2.2.1.2.1 Bleomycin administration

In all experiments 8 to 10 week old mice (C57BL/6NJ) were used. Bleomycin (Hexal, Holzkirchen, Germany) was applied by microsprayer (Penn-Century Inc., Philadelphia, PA) as a single dose of 5 U/kg bw. Age and sex-matched controls received saline. After 5 and 10 days, respectively, of bleomycin application, the mice were sacrificed with a lethal dose of pentobarbital. Bronchoalveolar lavage fluid (BALF) was collected and the lung tissue was either prepared for the histological examination or shock frozen for RNA or protein extraction. The animals were kept according to NIH guidelines and the experiments were undertaken with the permission of the local authorities.

2.2.1.2.2 C1 INH administration

Starting on day 1 post bleomycin or saline administration, respectively, the animals were daily treated with intraperitoneal C1 INH (500 µg/ mouse; CSL, Behring GmbH, Marburg, Germany) administration until they were sacrified on day 5. For the control group 0.9% NaCl was used as vehicle. Subsequently, murine BALF was obtained and lung homogenate prepared for histological assessment and RNA extraction was performed for real-time PCR.

2.2.1.2.3 Histological staining

Murine lung tissue was formalin fixed and embedded in paraffin blocks, which were sectioned into 5 micrometer and transferred onto microscope slides. Prior to the tissue staining the samples were deparaffinized and rehydrated using xylene and alcohol of descending concentrations (100%, 96%, 70%, 50%). Hematoxylin-eosin staining was performed to visualize the histological structure and inflammatory alteration of the tissue. The slides were scanned with Mirax Desk Digital Slide Scanner (Zeiss, Göttingen, Germany) and assessed using the Mirax Viewer (Zeiss).

2.2.1.2.4 Wet-to-dry lung weight ratio

The wet-to-dry weight ratio was evaluated by the gravimetric analysis to assess the degree of the alveolar oedema. Initially the weight of the wet left lobe was measured and then incubated at 60°C for 72h. The dried lung was then again weighted and the ratio between the wet and the dry left lobe was determined.

2.2.1.2.5 Flow cytometry

For identification of different cell phenotypes in murine BALF multicolour flow cytometry analysis was performed. To detect the neutrophils and other cell populations the following fluorochrome-conjugated surface antibodies were applied: CD11c, siglec-F, F4/80, GR1, CD 11b and CD11c. Following to the addition of the antibodies, the samples, after pretreatment with 10% (v/v) mouse serum were then incubated for 45 minutes in darkness on ice. Prior to flow cytometry the samples were washed using compositions of PBS. The analysis was then conducted using the FACS Canto II flow cytometer (Becton Dickinson, San Joe, CA).

2.2.2 Western Blot

For determination of the contact activation proteins in lung tissue, BALF and plasma, Western blotting was performed for murine and human samples.

2.2.2.1 Preparation of the samples for gel electrophoresis

Up till then stored at – 80°C, the lung tissue samples were initially lysed in Radio Immuno Precipitation Assay (RIPA) buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton-X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS)], supplemented with protease inhibitors [1 mM PMSF, 1 µg/ml complete protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany)] and 1 mM Na_3VO_4. Afterwards the samples were incubated for 30 minutes on ice and then centrifuged for 10 minutes at 10 000 rpm at 4°C. Supernatants were collected and protein concentration was measured using the Assay BCA Protein Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacture´s instruction. The measurements were executed with the microplate photometer (SpectraMax 190, Molecular Devices, Silicon Valley, CA). To each sample of 20 µg proteins 5 µl of 5x SDS-loading buffer [0.25 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) β-mercaptoethanol] was added. The mixture was then heated at 98 °C for 10 minutes (Thriller Thermoshaker-incubator, peQlab, Erlangen, Germany) followed by centrifugation. Plasma and BALF samples had also been stored at -80°C and were slowly thawed on ice before 20 µl of each samples was mixed with 5 µl of 5x SDS-loading buffer.

2.2.2.2 Sodium dodecyl sulfate polyacrylamid gel electrophoresis

Sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS PAGE) was utilized to separate the proteins according to their molecular weight. Firstly, a discontinuous SDS Polyacrylamid gel was prepared, consistent of a lower separating gel [resolving gel (10%): 10% acrylamide: bisacrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS), 0.1% (v/v) tetramethylethylenediamine (TEMED)] and a laminating stacking gel [stacking gel (4%): 4% acrylamide: bisacrylamide, 125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED]. After polymerization the prepared samples and 5 µl of protein marker (Page Ruler, Prestained Protein Ladder, ThermoFisher Scientific, Waltham, MA) were applied upon the gel.

The proteins were then separated in SDS-running buffer [25 mM Tris, 250 mM glycine, 0,1 % (w/v) SDS] via electrophoresis at a voltage of 100 V (Power Pac 1000, Biorad, Hercules, CA).

2.2.2.3 Immunblotting

The separated proteins were electrotransferred from the gel onto a PVDF membrane (Amersham Biosience, Little Chalfont, UK). This transfer was performed in blotting buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol] at a voltage of 100 V for 1 hour and was proceeded on ice. Blockage of non specific binding sites was achieved by incubation of the membrane with 5% (w/v) Skim Milk Powder in Tris-Buffered Saline containing Tween 20 [TBS-T (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20)]. Afterwards the membrane was washed with TBS-T and then incubated overnight at 4°C with one of the following primary antibodies: goat anti-FXII (Zytomed Systems, Berlin, Germany, dilution 1:1000); goat anti-FXI (Abcam, Cambridge, UK, dilution 1:1000); rabbit anti-KLK (Zytomed Systems,dilution 1:1000); rabbit anti-HK (Abcam, dilution 1:1000). All primary antibodies were diluted in 1% (w/v) bovine serum albumin (BSA) in TBS-T. After washing with TBS-T, the membrane was incubated with the peroxidase-labeled secondary antibody (all from Dako, Gostrup, Denmark, dilution 1:3000 in 5% (w/v) Skim Milk Powder in TBS-T) for 1 hour at room temperature. Final detection of proteins was performed using the ECL Plus Kit (Amersham Bioscience). To determine the amount of protein loaded on the gel, the membrane was stripped in stripping buffer (100 mM glycine, 0.32% (v/v) HCl) and reprobed with mouse β-actin antibody (Sigma- Aldrich, St. Louis, MO, dilution 1:10000).

2.2.3 Enzyme linked immunsorbent assay (ELISA)

Further quantitative analysis targeting Factor XII and BK was performed by an Enzyme Linked Immunsorbent Assay (ELISA). For FXII detection in human BALF Matched Pair Antibody Set for FXII (Affinity Biologicals, Ancaster, Canada) was utilized and AssayMax Human Factor FXII (Assay Pro, Saint Charles, MO) for human plasma samples. BK determination was implemented with the Human Bradykinin (BK) ELISA Kit (Cusabio Biotech, Wuhan, China). Each assay was executed precisely in accordance with the respective manual instruction.

2.2.3.1 FXII detection in BALF

The microtiter plates were coated with FXII capture antibody diluted in coating buffer (1:100) over night at 4°C. The content of each well was decanted before they were filled with 150 µl of blocking buffer to prevent unspecific binding. Blockage was executed for 90 minutes at room temperature. This was followed by a diligent washing procedure with washing buffer, repeated 3 times. The thorough removal of fluids after every washing step was realized by subsequent tapping of the plate on absorbent paper towels. The standard´s were prepared as recommended by the manufacture with 0.9% NaCl serving as diluent. Into each well 50 µl of the standards and samples were pipetted. Incubation was performed for 1 hour at room temperature with a following washing procedure. The peroxidase conjugated detecting antibody was then administered at a dilution of 1:100. After 1 hour of incubation, another washing step was executed. For visualization of the peroxidase activity 3,3′,5,5′-tetramethylbenzidine (TMB) Substrate (Thermo Scientific) was added. The color generating reaction was interrupted by addition of 2 M H_2SO_4 after 5 minutes. The concentration of the target protein was then determined by quantification of the optical density in the microplate reader (SpectraMAX 190) at 490 nm.

2.2.3.2 FXII detection in plasma

The AssayPro Kit provided microplates precoated with murine FXII antibody. The standards and samples were therefore directly administered at a volume of 50µl into each well. Prior to the performance of the assay the optimal dilution factor for the plasma samples had been determined in a test run to be 1:2000. The EIA Diluent of the Kit served as diluting agent. The incubation was then executed at room temperature for 2 hours. This was followed by a washing sequence, which was repeated 5 times as prescribed in the manual with subsequent thorough removal of residual liquids. Then 50 µl of biotinylated FXII antibody diluted in EIA Diluent (1:100) was added and

incubated for 1 hour. After another washing step, 50 µl of peroxidase conjugated Streptavidin was pipetted into each well. Possessing a particularly high affinity to biotin, Streptavidin binds to the biotinylated FXII antibody. Unbound material was eliminated after 30 minutes by another washing procedure. Peroxidase activity was then expressed by addition of the chromogen substrate TMB. After 10 minutes, a 0.5 N HCl stopped the chromogen substrate reaction. The absorbance was determined at a wavelength of 450 nm.

2.2.3.3 BK detection in BALF and plasma

The microtiter plates for BK detection were delivered pre-coated. Initially, the optimal dilution of the plasma samples was determined in a test run to be 1:400. Corresponding to the FXII assay, BALF samples were not diluted. The standards were prepared as advised by the manual. In each well 100 µl of standard and samples were administered and incubated for 2 hours at 37°C (Hereaus Function Line Incubator, ThermoFisher Scientific). The fluids were then decanted. Biotinlyated antibody was added and incubated at 37°C for 2 hours. After removal of the antibody working solution, a volume of 200 µl washing buffer was filled into each well. After 2 minutes the washing solution was completely removed by aspiration of the fluids and subsequent patting of the plate on a paper towel. This process was repeated 3 times. Then 100 µl of HRP-avidin was applied into each well and incubated for 1 hour at 37°C. A washing step followed, this time repeated 5 times in a row. TMB Substrate was added and after 20 minutes the color forming reaction was interrupted by 2 M H_2So_4. The color generated was quantified at a wavelength of 450 nm.

2.2.4 Isolation of leukocytes

Blood samples were collected in 7.5 ml EDTA-tubes and mixed with the same amount of PBS. This mixture was prudently placed upon 14 ml Biocoll 2 400 rpm (Biochrom AG, Berlin, Germany), followed by 30 minutes of centrifugation at room temperature separating the blood components into different phases. Forming a thin layer above the plasma phase, leukocyte phase was extracted and collected in a tube. 45 ml PBS was added at a subsequent speed of 1 500 rpm for 10 minutes with brake. Supernatant liquids were removed. In case of contamination with erythrocytes, 1 ml of ultra pure water (Mini Plasco connect, B.Braun, Melsungen, Germany) was added into the tube and swiveled for a few seconds. Subsequently, 9 ml of PBS was added. This was followed by 10 minutes of centrifugation at 1 500 rpm at room temperatur. Supernatant

fluids were discarded from the leukocyte pellet. Isolated leucocytes were used for RNA isolation.

2.2.5 Molecular Analysis

2.2.5.1 RNA Isolation

The pellet of isolated leucocytes was thoroughly mixed with 400 µl of RNA lysis buffer (PeqGOLD Total RNA Kit, PeqLab, Erlangen, Germany). After incubation the fluids were run through a DNA column for 1 minute at 12 000 rpm (Mikro 20, Hettich GmbH, Tuttlingen, Germany). 400 µl of Ethanol was added to the flow through and vortexed. The fluids were then placed on a RNA column and centrifuged for 1 minute at 12 000 rpm. This was followed by a washing treatment including 3 steps with the solutions provided by the Kit with subsequent centrifugation for 15 seconds, respectively. The column was dried in a following spin and then transferred to a sterile collection tube and RNA free water was applied to the column and incubated for 2 minutes. Then at 5 000 rpm the RNA was eluted for 1 minute. The RNA concentration was determined with a biophotometer (Eppendorf, Hamburg, Germany)

2.2.5.2 Reverse transcriptase (RT) reaction

The ingredients for cDNA synthesis are illustrated in the table (Table 2.2).To this mixture 1 µg of RNA was then added with a maximum volume of 10µl. The reaction was then performed in the thermocycler (TGradient Thermocycler, Biometra, Göttingen, Germany) at 25 °C for 10 minutes, 37°C for 2 hours and 85°C for 5 minutes.

Master Mix Ingredients	Volume (µl)
MultiScribe™ Reverse Transcriptase (200 U/µl, Applied Biosystems, Carlsbad, CA)	1.0
10x Random Primers (25 µM, Applied Biosystems, Carlsbad, CA)	2.0
Rnase Inhibitor (20 U/µl , Applied Biosystems, Carlsbad, CA)	1.0
dNTP (100 mM, Fermentas, St.Leon-Roth, Germany)	0.8
10x Reverse Transcriptase Buffer (Fermentas, St.Leon-Roth, Germany)	2.0
RNase free water (Ampuwa®)	3.2
TOTAL	**10**

Table 2.2 Ingredients of the reverse transcriptase reaction.

2.2.5.3 Real-time PCR (qPCR)

The real time polymerase chain reaction was executed utilizing Platinum® SYBR Green qPCR Super Mix (Invitrogen, Karlsruhe, Germany). The primers used in qPCR experiments are listed in the following table (Table 2.3).

Gene	Accession number	Nucleotide sequence (5´→ 3´)	T_m [a] (°C)	Amplicon size (nt) [b]
Human FXII	NM_000505.3	F [c]: CCA AGG AGC ATA AGT ACA AAG C R [d]: GTA CAG CTG CCG GTG GTA CT	60	97
Murine IL-1β	NM_008361.3	F: CTG AAA GCT CTC CAC CTC CA R: CCA AGG CCA CAG GTA TTT TG	60	105
Murine MIP-2	NM_011339.2	F: TCG AGA CCA TTT ACT GCA ACA R: GGC CAA CAG TAG CCT TCA CC	60	104
Murine TNF-α	NM_013693.3	F: CGA GTG ACA AGC CTG TAG CC R: CTT TGA GAT CCA TGC CGT TG	60	110
Human β-actin	NM_001101.3	F: ATT GCC GAC AGG ATG CAG GAA R: GCT GAT CCA CAT CTG CTG GAA	60	149
Murine ß-actin	NM_007393.3	F: AGA GGG AAA TCG TGC GTG R: CAA TAG TGA TGA CCT GGC	60	137

[a] T_m, Melting temperature; [b] nt, nucleotides [c] F, forward; [d] R, reverse

Table 2.3 Sequences of the primers used in the study.

In table 2.4 all components of the reaction are represented. The ingredients were pipetted into a microtitre plate (Thermo-Fast 96 PCR Detection Plate, ABGene ThermoFisher Scientific) and the volume of 1 µl of cDNA was added. Subsequently,

the plate was sealed with a foil (Absolute Q-PCR Seal, ABGene- Thermo Fisher Scientific) and the reaction was performed by the StepOnePlus™ Real time PCR System (Life Technologies, Carlsbad, CA).

Ingredients of qPCR	Volume (µl)
Reverse Primer (10 pmol/µl)	0.5
Forward primer (10 pmol/µl)	0.5
Sybr mix	12.5
RNase free water (Ampuwa®)	10.0
cDNA	1.0
TOTAL	**24.5**

Table 2.4 Ingredients of the qPCR reaction.

The cycling conditions were comprised of initial 10 min at 95°C, followed by 40 cycles of the following temperature-time profile: 95°C for 15 seconds and 1 minute at 60°C. The data were evaluated with StepOne™ Software (Life Technologies). Exclusion of non specific amplification or contamination was verified in melt curve analysis and agarose gel electrophoresis. All changes in the target gene mRNA levels are presented as delta ct (Δct), which was calculated by subtracting the ct value of the target gene from the ct value of the reference gene.

2.2.6 Statistics

For statistic assessment of the results the GraphPad Prism software (GraphPad Software,Inc., San Diego, CA) was used. All data of patient and animal experiments are presented in form of box-whisker-plots. The upper whiskers indicate the maximal, the lower the minimal value, while the box constitutes the interquartile range. The median value is marked as a horizontal line within the box. In the experiments comparing two different groups, the statistic significance was determined by means of the Mann-Whitney-U test. In the case of three groups, ANOVA was utilized, followed by Turkey´s post noc test. The p value less than 0.05 was considered as statistically significant.

3. Results

3.1 Contact activation factors are elevated in BALF and lung homogenate of ARDS patients

In order to investigate the characteristics of contact activation proteins in the pathogenesis of ARDS, their occurrence in lungs of affected patients was determined. As illustrated in Figure 4.1 Western blot analysis revealed increased protein levels of FXII, FXI, HK and KLK in BALF of ARDS patients compared to the healthy control group. Remarkably, two signals appeared for FXII, one 80 kDa band representing the FXII zymogen and a 50 kDa band corresponding to the heavy chain of FXIIa, indicating that FXII conversion into activated FXII is taking place. The nonappearance of the light chain of FXII at 30 kDa is justified by the specific antibody targeting an epitope only presented on the heavy chain of FXII.

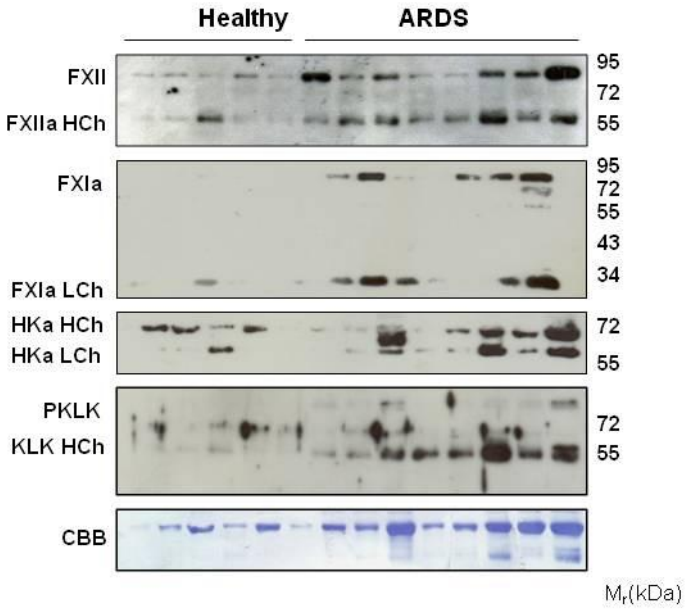

Figure 4.1 Elevated levels of contact activation factors in BALF of ARDS patients.
FXII, FXI, HK and KLK protein levels in BALF of ARDS patients in comparison to the healthy control group assessed by Western blot. Here, 8 out of 46 ARDS patients and 5 out of 20 healthy controls are demonstrated. The different bands are labeled. HCh, heavy chain; LCh, light chain. Coomassie Brilliant Blue (CBB) was applied as loading control for visualization of total protein content.

Concurrent with indication of FXII conversion, activation of KLK was implied, as virtually no inactive zymogen at 88 kDa was detectable, while a strong signal for cleaved KLK heavy chain was apparent at 52 kDa in the BALF of ARDS patients. Accordingly, KLK substrate HK was identified in its activated two chain form, represented by its heavy chain at 62 kDa and the light chain at 56 kDa. Similar

observations were made for FXI, which was represented in its cleaved two chain active form at 80 kDa.

In line with the Western blot results, the quantification of FXII concentration assessed by ELISA showed a significantly higher amount of the protein in lavage specimens of ARDS patients. In the patients group the interquartile range of FXII level was found to extend from 140 to 390 ng/ml with a total average of 274 ng/ml. The determination of FXII in the control group however revealed a total average of 130 ng/ml with an interquartile range stretching from 55 to 195 ng/ml (Figure 4.2 A). Interestingly, no significant increased of the FXII level was observed in patients suffering from cardiac lung edema compared to the control group, excluding the possibility of the FXII increase being due to the mechanical ventilation. In respect of the two different etiopathogenic forms of ARDS, FXII concentration was observed to be at a similarly high level in direct as in indirect ARDS samples (Figure 4.2 B). Further distinction revealed a variation of the FXII concentration according to the temporal course of the disease. Within the first 24 hours of the diagnosis the amount of FXII is found to be increased, remaining high for four days and subsequently decreasing on day 6-8 to values comparable to those of the control group (Figure 4.2 C). In contrast to the observations made for BALF samples, the determination of FXII in blood plasma revealed decreased levels of the protein in ARDS patients compared to the healthy control group. Thus, in the specimens of the patients the FXII concentration showed a total average of 32 µg/ml with an interquartile range between 20 µg/ml to 47 µg/ml, whereas the healthy group showed significantly higher values with a total average of 53 µg/ml and an interquartile range from 36 µg/ml to 68 µg/ml (Figure 4.2 D).

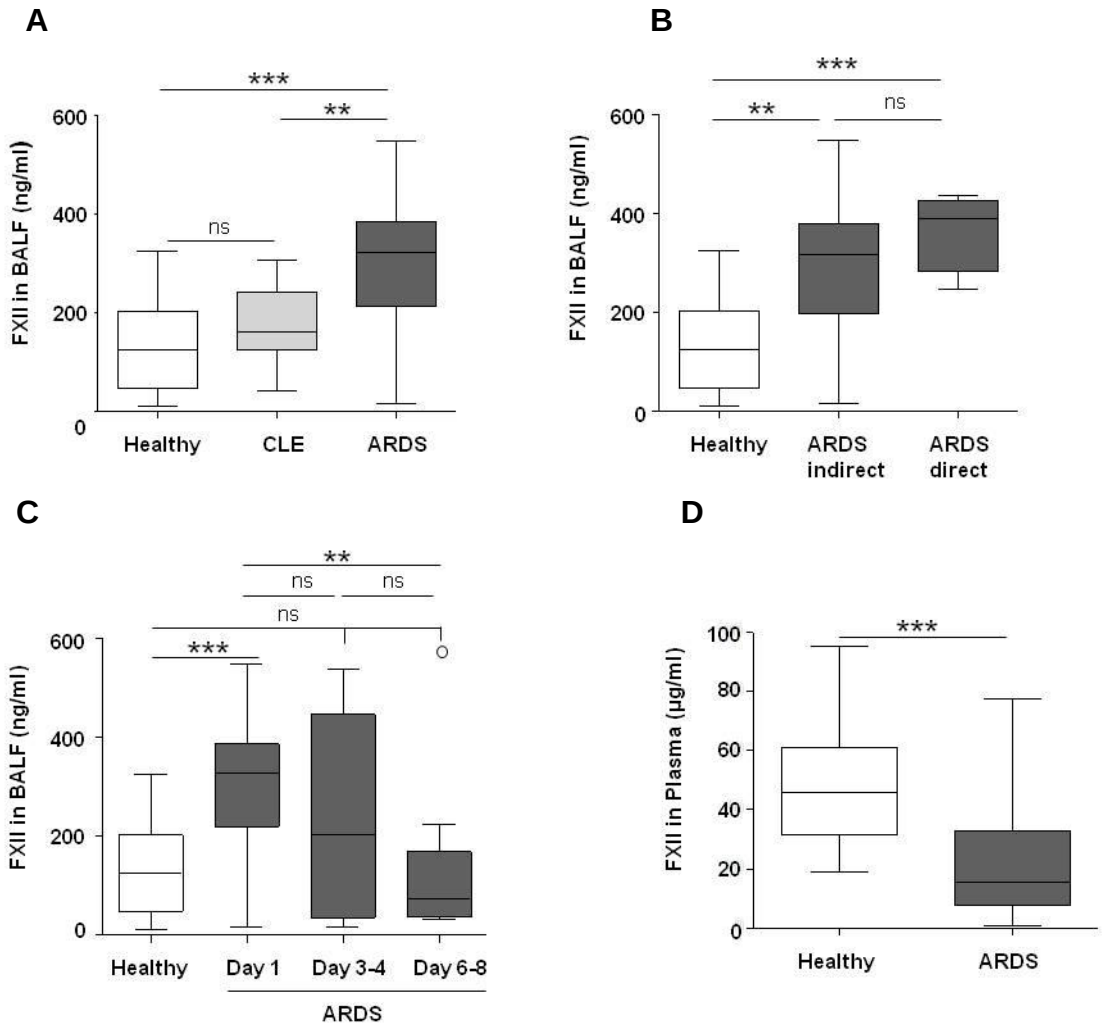

Figure 4.2 Increased levels of FXII in the BALF of ARDS patients.

(A) FXII levels were determined in BALF obtained from ARDS patients (n=46), patients with cardiogenic lung edema (CLE) (n=10) and healthy controls (n=20). **(B)** Differentiation of FXII levels in BALF of direct (n=28) and indirect (n=18) ARDS patients, all versus healthy control group subjects. **(C)** FXII levels in BALF from patients in the course of ARDS on day 1 (n= 46) , day 3-4 (n= 28) and 6-8 (n=27) opposed to healthy controls. **(D)** Determination of FXII level in blood plasma of ARDS patients (n=26) in comparison with control group (n=19). All data assessed by ELISA. $^{**}p < 0.01$; $^{***}p < 0.001$; ns, non significant.

Representing an indirect product of the FXII activation with a major involvement in inflammatory processes, BK was another component of interest for the investigation. Particularly the observation of elevated occurrence of cleaved HK in the immunoblot of the patients gave rise to the assumption of a correspondingly altered BK level.

Therefore determination of BK concentration in BALF was assessed by ELISA, showing significantly higher amounts of the protein in specimens of the patients group (Figure 4.3).

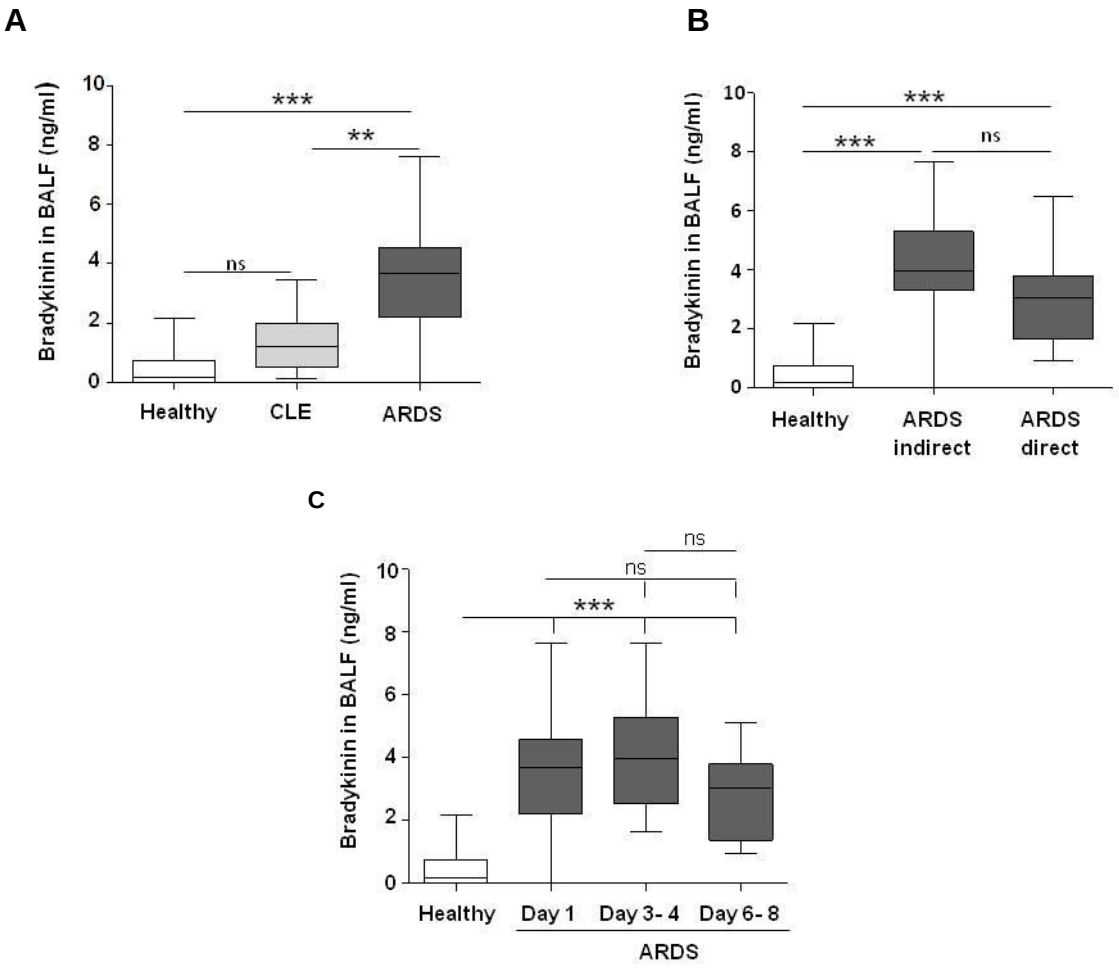

Figure 4.3 BK is elevated in BALF of ARDS patients.

(A) Quantification of BK level determined in BALF specimens from ARDS patients (n= 46), patients with cardiogenic lung edema (n=10) and the healthy controls (n=20) assessed by ELISA. **(B)** Comparison of BK levels in BALF of ARDS patients of pulmonary (n=28) versus non pulmonary (n=18) source. **(C)** Analysis of the BK concentration in BALF obtained from ARDS patients at day 1 (n=46), day 3-4 (n=28) and day 6-8 (n= 27) during the temporal progression of the disease. ** $p < 0.01$, *** $p < 0.001$; ns, non significant.

While the BALF of ARDS patients contained an average concentration of 3.47 ng/ml BK with an interquartile range between 2.10 - 4.00 ng/ml, the average control group was stated at a significantly lower level of 0.45 ng/ml and an interquartile range of 0-0.50 ng/ml. By contrast, in patients with a lung edema due to cardiac failure, no significant alteration of BK levels was detected in comparison to the healthy donors (Figure 4.3 A). In view of the etiopathologic differentiation, elevated BK levels were observed in direct as in indirect ARDS, showing no significant difference between the two groups (Figure 4.3 B). Analysis of the temporal evolvement reveals an initial high level on day 1 after the diagnosis, remaining elevated for eight days with declining tendency from day six on (Figure 4.3 C).

Additional experiments were performed to examine the expression of intrinsic blood coagulation factors in human lung homogenate. Western blot analysis revealed a steady high protein level in ARDS affected lung tissue in comparison to the control group (Figure 4.4). With regard to the previous observations in BALF specimens; indicators of FXII conversion were equally present in the lung tissue.

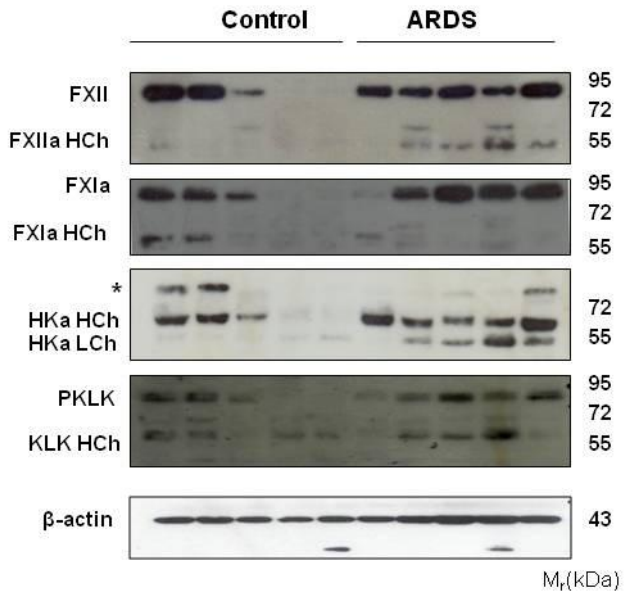

Figure 4.4 Altered expression of contact activation factors in ARDS lung tissue.
Determination of protein level of FXII, FXI, HK and KLK in lung homogenate of ARDS patients and healthy controls. 5 out of 7 ARDS patients and 5 out of 6 controls are demonstrated. As a loading control β-actin was used. * unspecific band.

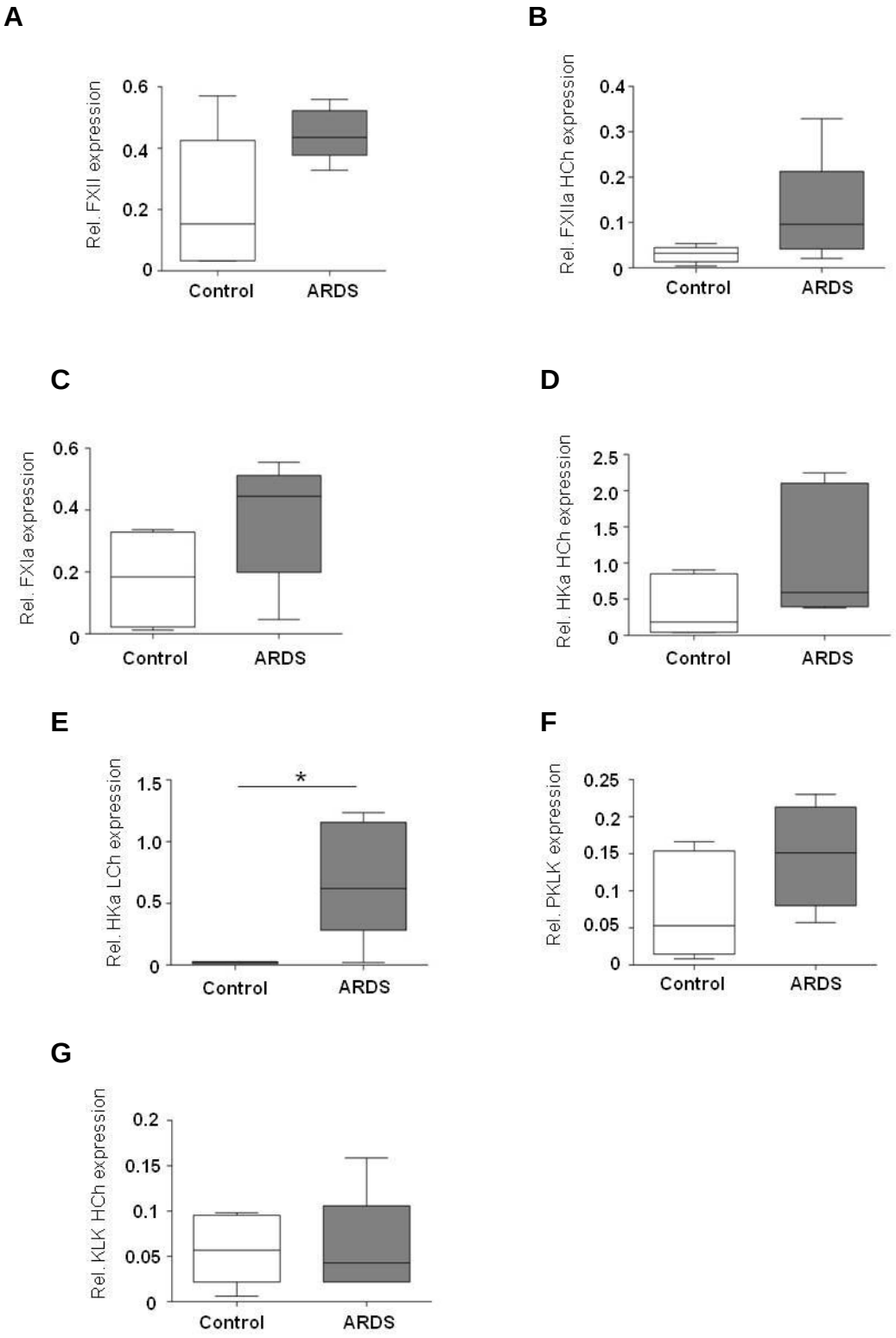

Figure 4.5 Altered expression of contact activation factors in ARDS lung tissue

Densitometry analysis of the immunoblot of Figure 4.4 for FXII zymogen **(A)** and FXIIa HCh **(B)**, FXIa **(C)**, HKa HCh **(D)** and HKa LCh**(E)**, PKLK **(F)** and KLK HCh **(G)**. ARDS (n=7), Control (n=6). Rel., relative; HCh, heavy chain; LCh, light chain. *$p < 0.05$

Quantification of the Western blot results performed by densitometry analysis confirmed higher protein levels in ARDS lung tissue. Accordingly the relative protein expression of FXII was detected in ARDS lungs to shift between 0.33 - 0.56 with an average of 0.45, whereas the control group showed FXII rates of 0.03 - 0.57 with an average of 0.21. The protein expression of FXIIa heavy chain revealed an average of 0.12, in comparison of 0.03 to the control group. The relative protein rate of FXIa in ARDS lungs was detected to vary among 0.04 and 0.45 with an average of 0.38, while in control lungs FXIa protein expression was determined at 0.012 to 0.34 with an average rate of 0.17. The expression of HKa heavy chain was detected in ARDS lungs to shift from 1.12 to 0.39, in the control group from 0.03 to 0.9. HKa light chain was found at a significantly higher protein rate in ARDS lungs compared to healthy controls, with rates of 1.2 to 0.02, whereas in lung tissue of the control group the protein expression shifted from 0.01 to 0.03. Minor variation in protein expression was determined for PKLK, with an average in ARDS patients of 0.15 and in the control group of 0.08. For KLK protein expression no difference was detectable in the ARDS group compared to the healthy controls.

In summary, FXII, FXIIa, FXI and HKa were found in higher protein rates in ARDS lungs compared to the control group, however not reaching statistical significance (Figure 4.5 A-G).

3.2 FXII expression is increased in blood cells of ARDS patients

The role of hepatocytes as main producer of FXII is generally acknowledged, however evidence of extrahepatic synthesis has been obtained in previous research (*183*). Consequently, the interest arose whether within lung injury supplementary cells might play a role as a potential source of FXII protease. To this end, mRNA of FXII was evaluated in human lung homogenate and leukocytes by means of qPCR. Interestingly, the FXII expression was significantly higher in lung homogenate of ARDS patients in comparison to control subjects (Figure 4.6 A). Furthermore, in leukocytes isolated from the blood of healthy donors FXII mRNA was not detectable, whereas in leukocytes of ARDS patients FXII was distinctly expressed (Figure 4.6 B).

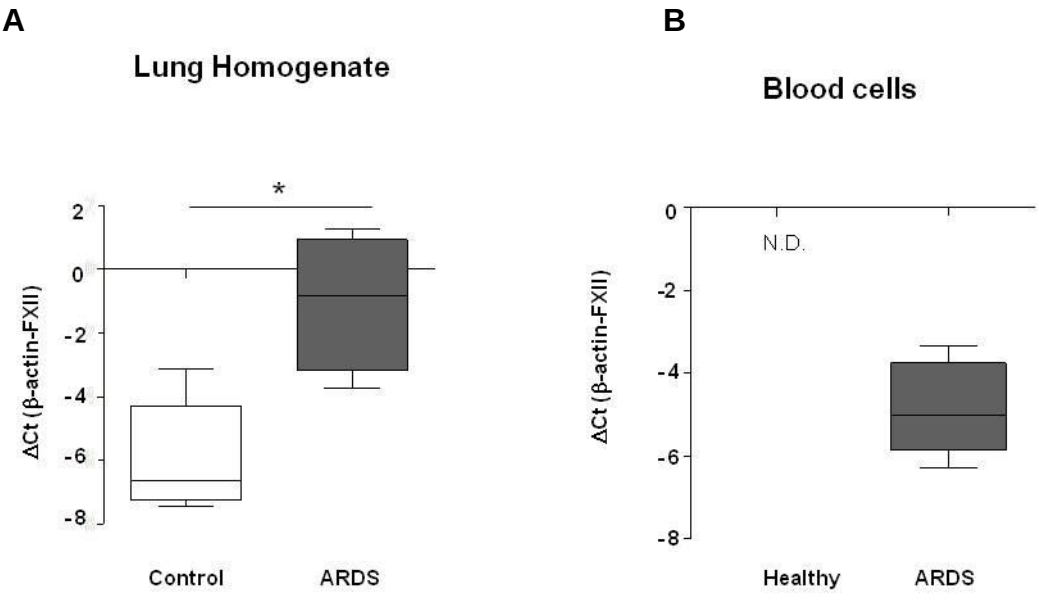

Figure 4.6 Expression of FXII is altered in lung tissue and blood cells of ARDS patients. Expression of FXII in **(A)** lung homogenates obtained from control subjects (n=5) and ARDS (n=5) patients and in **(B)** leucocytes of healthy donors (n=10) and ARDS patients (n=6) assessed by qPCR. All data are expressed as Δc_t using β-actin as a reference gene. *$p < 0.05$; N.D., non-detectable.

3.3 The protein level of FXII and BK is elevated in lungs of bleomycin-treated mice

To ascertain whether comparable conditions apply for the animal model of lung injury, Western blots of FXII were performed for BALF of bleomycin-treated mice. To reproduce the feature of human lung injury most realistically, the lavage samples used were of mice 5 days after bleomycin application, thus representing the acute inflammatory response. FXII concentrations were found to be significantly elevated in murine BALF of bleomycin challenged mice compared to the saline treated mice in Western blot analysis (Figure 4.7).

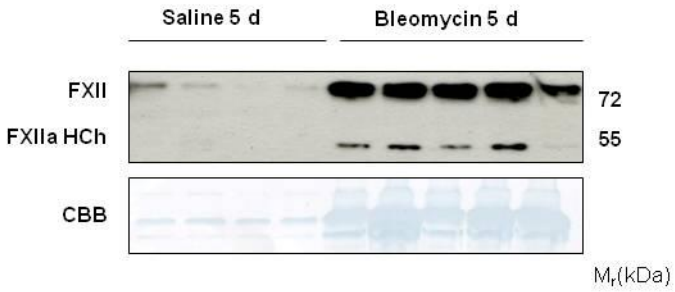

Figure 4.7 Levels of FXII and FXIIa is elevated in BALF of bleomycin treated mice.

Levels of FXII and FXIIa in BALF of mice treated with either saline or bleomycin 5 days post application. Representative Western blot is demonstrated. Here 4 Saline and 5 out of 9 bleomycin challenged mice are shown.

FXII conversion in BALF of bleomycin treated mice was indicated by a signal for FXII zymogen with concurrent appearance of FXII heavy, leading to the assumption of ongoing FXII activation. Moreover, detection of FXII was executed for murine lung tissue samples at day 5 and day 10 post bleomycin application. Illustrated in Figure 4.8, a strong signal was observed in bleomycin challenged murine lung homogenate, whereas saline treated mice showed markedly lower expression of FXII. Quantification of the protein band by densitometry analysis confirmed significantly higher FXII/FXIIa levels in samples of bleomycin injured lungs (Figure 4.8 B, C). In view of the temporal course, FXII concentration is found to be higher at day 5 post bleomycin instillation with subsequent reduction.

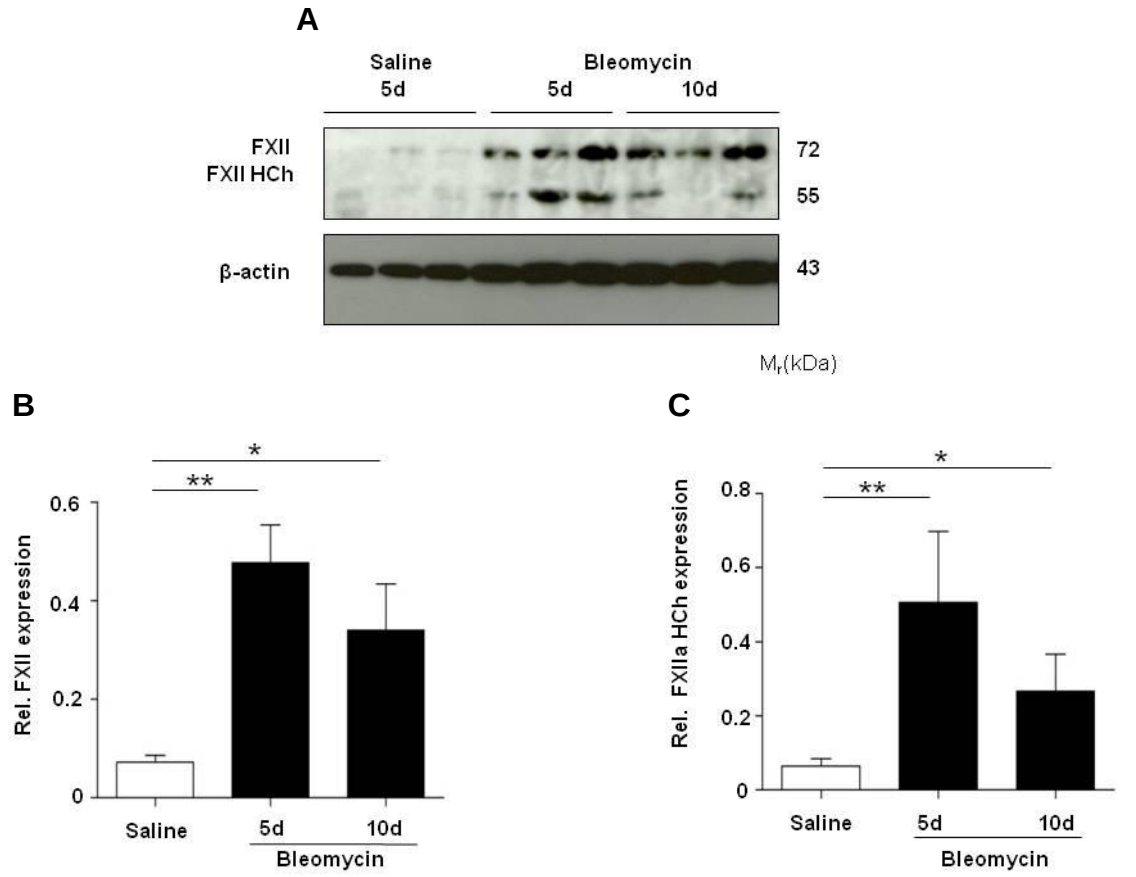

Figure 4.8 Increased protein level of FXII/FXIIa in lung homogenate of mice at day 5 and day 10 after bleomycin instillation.
(A) Levels of FXII/XIIa in lung tissue of mice treated with bleomycin on day 5 and day 10 post application. Representative Western blot is demonstrated. As a loading control β-actin was applied. Quantification of Western blot results for **(B)** FXII zymogen and **(C)** FXIIa HCh was assessed by densitometric analysis. Saline (n=5), bleomycin day 5 (n=9) and bleomycin day 10 (n=9) mice are shown. HCh, heavy chain; Rel., relative. *$p < 0.05$, **$p < 0.01$.

Determination of BK protein level in murine lungs was further assessed by ELISA, revealing significantly higher BK levels in the BALF of bleomycin injured lungs. Whereas the average concentration of BK in BALF of the saline treated control group was 15.27 ng/ml, the mean value of BK after bleomycin administration was 43.3 ng/ml at day 5 and 34.94 ng/ml at day 10 post application.

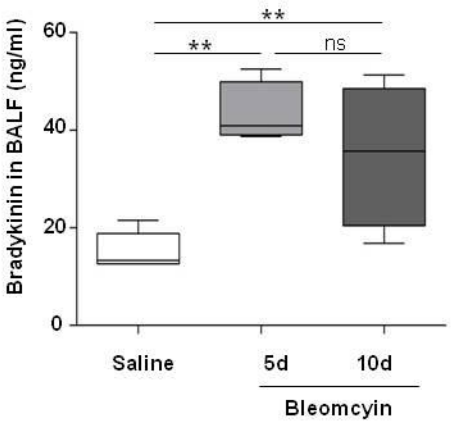

Figure 4.9 Increased level of BK in BALF of mice at day 5 and day 10 after bleomycin application Determination of BK level in BALF of mice at day 5 (n=9) and day 10 (n=9) after bleomycin application compared to saline control group (n=5) assessed by ELISA. ***p* < 0.01; ns, non significant

3.4 C1 esterase inhibitor (C1 INH) decreases inflammatory response in bleomycin challenged murine lungs

3.4.1 C1 INH alleviates the inflammatory response induced by bleomycin application

To further define the role of contact activation, effects of its inhibition on acute lung injury were examined in murine lungs. The animals were divided into three different experimental groups. While the first group received saline application, the second and third group were challenged with bleomycin. Subsequently, C1 INH, acknowledged to be a main inhibitor of the contact system, was administered to the former (Saline+C1 INH) and the second group (Bleo+C1 INH), whereas the third group received a vehicle only (Bleo+Vehicle). All animals of the investigation were sacrified on day 5 after bleomycin or saline application, respectively.

Morphological evaluation of the lung tissue was then performed. In the lungs of saline treated mice that received C1 IHN no alterations of the lung tissue were found and the physiological alveolar architecture was maintained (Figure 4.10). In the bleomycin group, the mice with vehicle administration only showed the typical feature of bleomycin induced lung injury including infiltration of leukocytes into the interstitial space and swelling of the alveolar wall. By contrast, in lungs of mice treated with C1 INH a striking diminution of the bleomycin caused inflammation was observed. Accordingly, a considerable reduction of inflammatory cells in the interstitial and alveolar compartment was apparent (Figure 4.10)

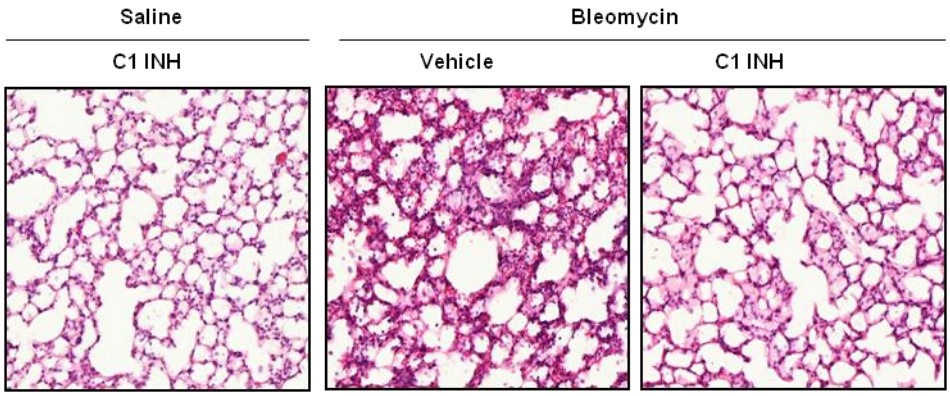

Figure 4.10 Application of C1 INH reduces the inflammatory response in bleomycin treated mice. Hematoxylin-eosin stained paraffin embedded lungs obtained from animals either treated with saline+C1 INH, bleomycin+vehicle or bleomycin+C1 INH, n=5-8/group. Magnification 20x.

These observations were confirmed by the analysis of the BALF, revealing an increase of leukocytes in specimen of mice after bleomycin challenge and a significant lower number of leukocytes after C1 INH administration compared to those after vehicle treatment (Figure 4.11 A).

Accordingly, neutrophils, a subgroup of leukocytes known to be of pivotal importance in the pathogenesis of ARDS were significantly reduced in animals receiving C1 INH (Figure 4.11 B), the relative proportion of neutrophils among total leukocytes, however, remained the same (Figure 4.11 C).

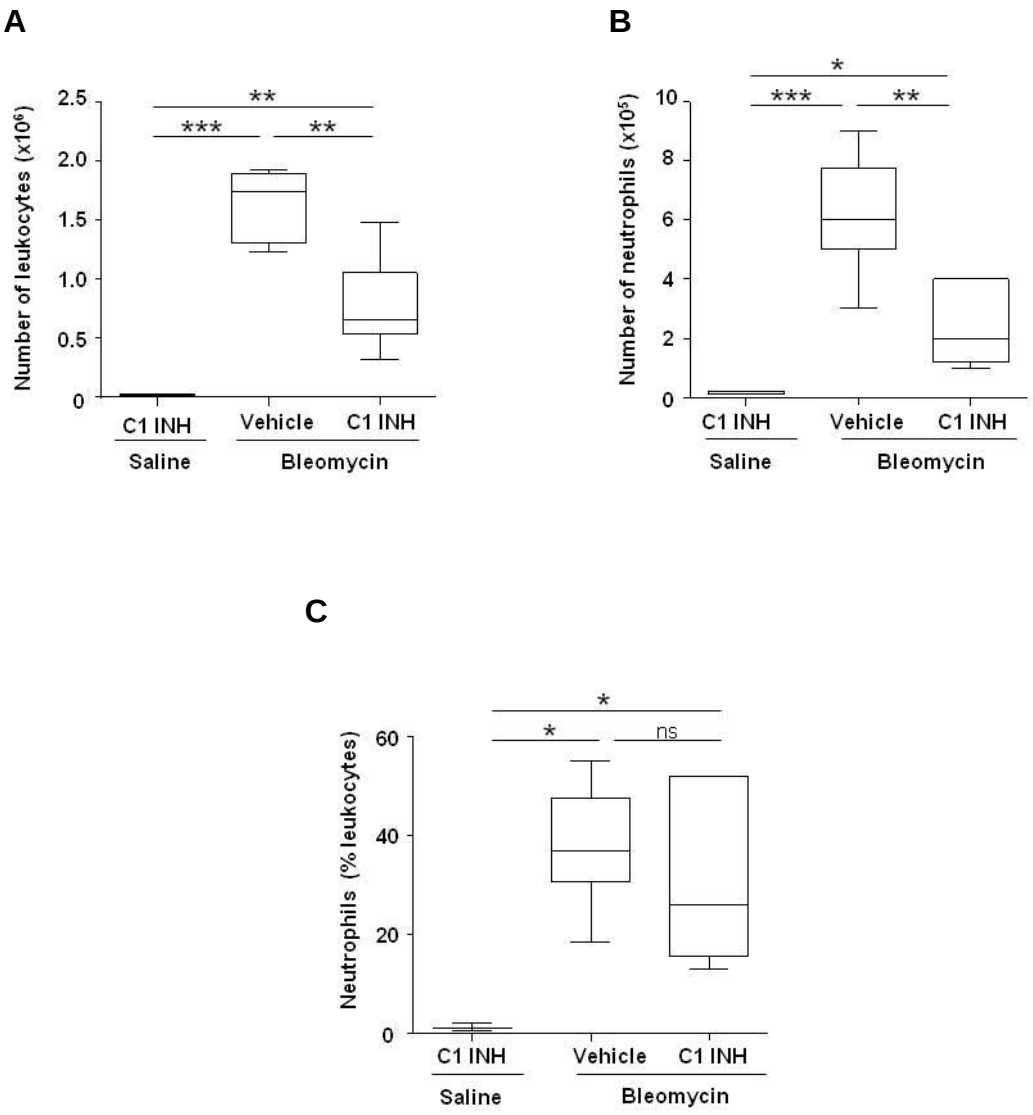

Figure 4.11 Application of C1 INH reduces number of inflammatory cells in bleomycin challenged mice. (A-C) Quantification of leukocytes and neutrophils in BALF of bleomycin challenged mice assessed by flow cytometry. n=5-8/group; *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ns, non significant.

Evaluation of the wet/dry ratio as indicator for edema formation in the lungs revealed a significantly higher rate after bleomycin application compared to the saline control group. The edema was found to be significantly reduced after C1 INH administration (Figure 4.12.)

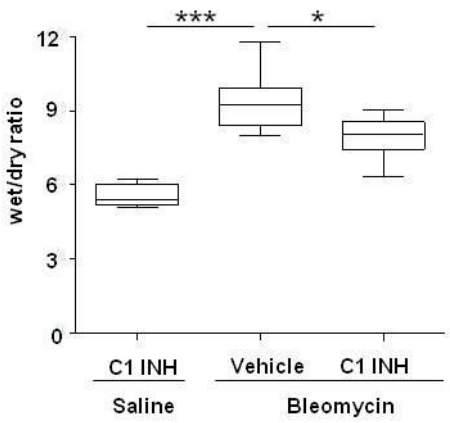

Figure 4.12 Decrease of edema formation after C1 INH administration in bleomycin treated mice. Determination of the wet/dry ratio in the lungs of either saline control group or bleomycin challenged lungs with vehicle or C1 INH application; n=5-8/group; *p< 0.05, ***p< 0.001.

3.4.2 Expression of proinflammatory mediators in bleomycin injured lungs is reduced after C1 INH application

To further scrutinize the putative effect of C1 INH administration on bleomycin injured lungs, mediators known to be involved in the inflammatory response were examined. The macrophage inflammatory protein 2 (MPI-2/CXCL2) is known to be a crucial participant in neutrophil recruitment in the course of ARDS. Determination of MIP-2 mRNA expression in lung homogenate revealed a remarkable increase of MIP-2 in bleomycin injured lungs with vehicle administration and a significantly decreased expression in mice treated with C1 INH (Figure 4.13 A). TNF-α, known to be a pro-inflammatory mediator and contributor to fibrotic alterations in the lung, was found to be significantly higher expressed in vehicle treated bleomycin mice, whereas C1 INH application induced a significant decline of the cytokine (Figure 4.13 B). By contrast, C1 INH failed to exhibit an impact on the expression of IL-1β. Thus, IL-1β expression was significantly increased in all bleomycin treated mice, independent of whether they had subsequently received C1 INH or vehicle only (Figure 4.13 C).

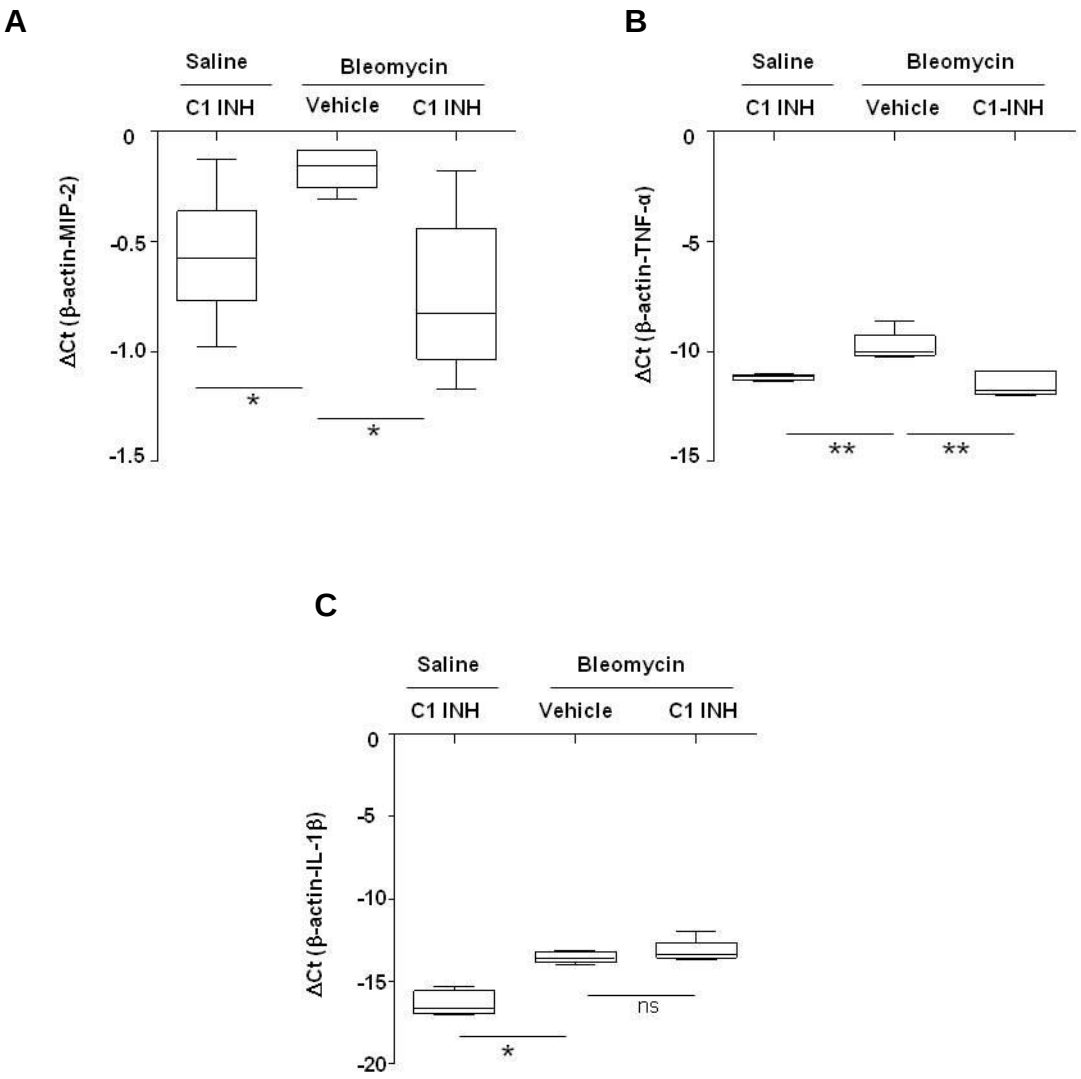

Figure 4.13 Expression of MIP-2 and TNF-α is reduced after C1 INH administration in bleomycin challenged mice.

Expression of MIP-2 **(A)**, TNF- α **(B)** and IL-1β **(C)** in lung homogenates of mice post saline instillation with C1 INH application (saline+C1 INH), in bleomycin mice with vehicle administration (bleomycin+vehicle) and bleomycin mice with C1 INH application (bleomycin+C1 INH) assessed by qPCR. Data are expressed as ∆ct using β-actin as a reference gene, n=5-8/group. *p< 0.05, **p< 0.01; ns, non significant.

3.4.3 C1 INH administration has no impact on coagulation in bleomycin challenged mice

It is well established that alveolar coagulation is subject to alterations in lungs of ARDS patients; therefore it was of particular interest to investigate possible effects of C1-INH administration on the hemostatic status. For this reason, measurement of the clotting time was performed for a saline control group and bleomycin challenged mice, the latter either treated with C1 INH or vehicle only. The evaluation revealed a highly significant acceleration of the clotting time in bleomycin challenged mice compared to the saline control group. Whereas the clotting time of the saline treated mice was observed to vary between 580 to 800 seconds, a striking diminution with an average of 100 seconds was noted in mice after bleomycin application. This observation is consistent with the current knowledge of a predominantly procoagulant setting during lung injury. Remarkably, the clotting time was not decelerated after C1 INH administration, thus no difference to the vehicle treated mice was observable (Figure 4.14 A).

Representing the final product of coagulation activity, fibrinogen was another object of the investigation. Determination of fibrin(ogen) in murine lung samples assessed by Western blot analysis revealed an explicit increase of the protein in samples of animals challenged with bleomycin. No difference was noted in the experimental groups, showing a comparably high amount of fibrinogen independent of whether they were treated with C1 INH or vehicle only (Figure 4.14 B). Further examination included determination of D-dimer, the decomposition product of cross-linked fibrin and therefore marker of fibrin formation and dissolution. The amount of D-dimer in the plasma of mice with lung injury was detected to be significantly higher than in the control group. No significant effects could be found in the experimental group after C1 INH treatment (Figure 4.14 C). In conclusion, after bleomycin application the mice developed the known features of acute lung injury with an increase of procoagulant activity, represented by the accelerated clotting time and increase of fibrin and fibrin degradation products. No significant distinction could be identified in the experimental group between animals which had received C1 INH treatment and those after vehicle application. Consequently, the results demonstrate the administration of C1 INH as having no effect on the hemostatic state.

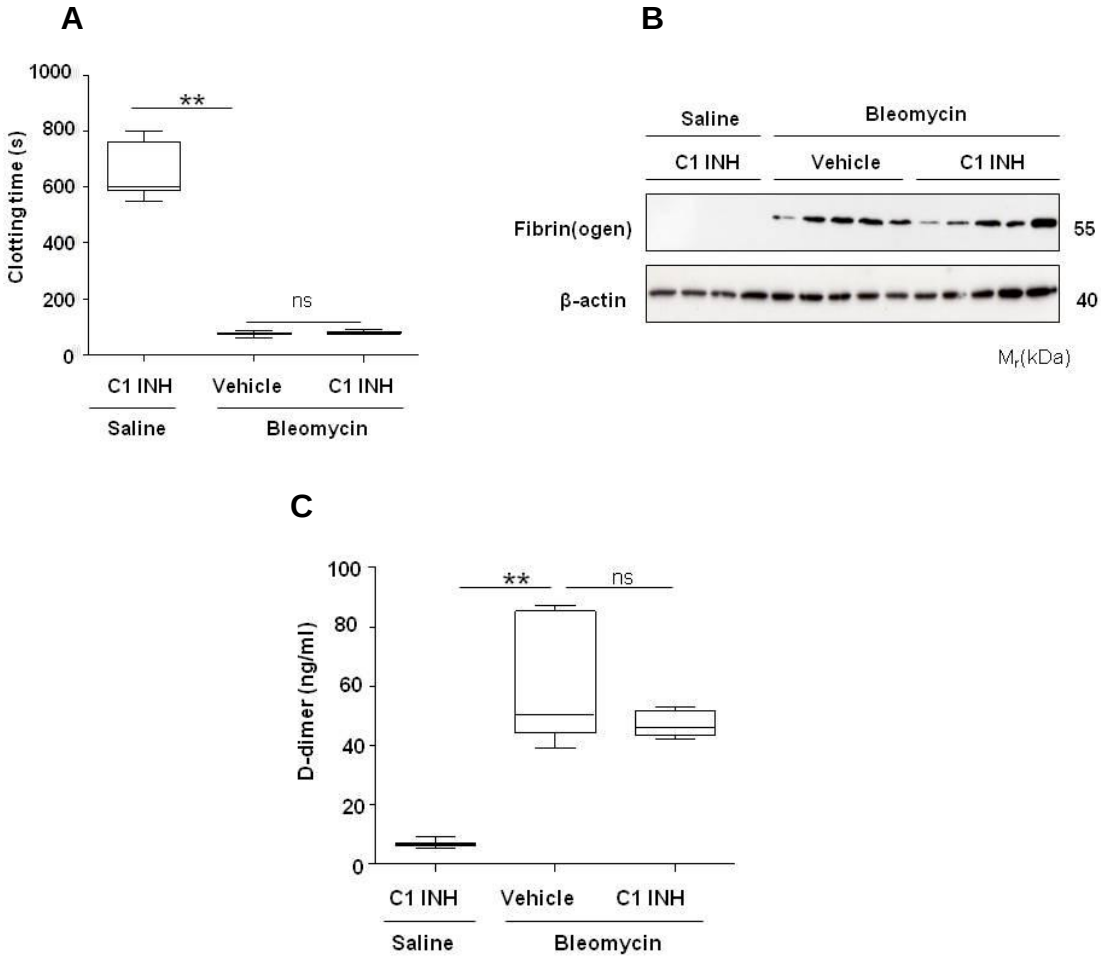

Figure 4.14 C1 INH application has no impact on altered coagulation in bleomycin induced lung injury.

(A) Procoagulant activity of BALF from animals subjected to saline or bleomycin and then treated with either C1 INH or vehicle. **(B)** Fibrin(ogen) deposition in the lungs of mice post saline instillation and C1 INH administration (Saline+C1 INH) and bleomycin mice treated either with C1 INH (bleomycin+C1 INH) or vehicle only (bleomycin+vehicle). Representative Western blot is shown. **(C)** Quantification of D-dimer antigen level in BALF samples from each group, Saline+C1 INH, bleomycin+C1 INH and bleomycin + vehicle. n= 5-8/group. ***p*<0.01; ns, non significant.

4. Discussion

4.1 The contact activation factors are increased in ARDS lungs

The altered alveolar hemostatic state is a condition generally recognized in inflammatory lung diseases and it has been an object of research to clarify the role of coagulation factors within inflammatory and fibrotic processes in this context. Extrinsic proteases of blood coagulation are found to be increased in the lungs of ARDS patients (*162*) and have been identified to not only promote fibrin formation via the coagulation cascade, but to additionally contain pro-inflammatory properties by inducing the expression of cytokines (*168, 173*). Furthermore, pro-fibrotic effects are attributed to the FXa protease, attaching further importance to the components of the clotting cascade in view of fibrotic remodeling of the lung (*186*). The present study examines the impact of the contact system on the pathogenesis of acute lung injury, particularly ARDS, exhibiting elevated protein levels of FXII, FXI, KLK and HK in BALF and lung tissue of ARDS patients. This surge of intrinsic blood coagulation factors in BALF and lung tissue is presumably primarily due to the impaired integrity of the alveolar barrier generally found in the lung of ARDS patients, leading to influx and accumulation of proteins in the alveolar and interstitial compartment (*132, 187*). However, it seems worthwhile considering whether besides the alveolar leakage; intrapulmonary generation may be another cause for the accrued amount of intrinsic factors at the site of lung injury. In this context, results of the current study draw particular attention to inflammatory cells as putative source of FXII, revealing FXII expression in leukocytes of ARDS patients whereas in the white blood cells of healthy subjects FXII was not detectable. Thus, the inflammatory cells may represent an extrahepatic origin of FXII production and leukocytes invading into the pulmonary tissue within the inflammation may contribute to elevated FXII levels in the lung tissue of ARDS patients. Moreover, the possibility of FXII generation by resident lung cells can neither be excluded from consideration. Evidence of intrapulmonary FXII production has been obtained in lungs of IPF patients as under fibrotic conditions human lung fibroblast have been detected to express FXII (*182*). Whether local synthesis by lung fibroblasts or epithelial alveolar cells may play a role in inflammatory lung diseases remains object of future studies.

Another important aspect of this study was the provision of evidence of intrinsic pathway activity in the lungs of ARDS patients. Accordingly, conversion of FXII into its activated form and complete activation of its substrates was detected in the presented Western blot results. However, it should not be neglected that FXII independent pathways have been described for KLK and FXI cleavage respectively, which

consequently also need to be taken into consideration as a potential mechanism for their activation in affected lungs (*188, 189*). Within the course of the disease a higher rate of FXII conversion is suspected to eventuate with local accumulation in the lung. Amplified degradation of the protein due to the boosted activation leads then to extended consumption of FXII, which is reflected in the decline of the protease in the plasma. This is particularly remarkable, as in septic patients decreased FXII plasma levels have been noted to signify an unfavorable prognosis (*190*). Consequently, the question arises of how FXII activation may occur during lung injury. A variety of approaches have been pursued in the past years concerning the mechanism of FXII activation under physiological conditions. In view of potential pro-inflammatory features of the protease, particular interest has been drawn to studies indicating that the activation of contact factors might occur on the surface of neutrophils. In line with this approach, previous investigations demonstrated binding of purified FXII and HK, respectively, to the surface of neutrophils, notably in immediate vicinity to each other, resulting in an increase of KLK activity (*191*). In this way, the activation of the contact system proteases in acute lung injury might occur on infiltrating neutrophils at the site of inflammation. Additionally, platelets have been suspected to be associated with the initiation of the contact activation system (*44*). A possible mechanism seems to include the release of inorganic polyphosphate (PolyP) from thrombocytes, which bind to FXII and thereby trigger its activation and subsequent BK generation (*45*). Another potential activator of FXII further represents extracellular RNA, contingently originating from dying cells within tissue damage or disintegrating pathogens (*42*). In ARDS of infectious origin bacterial mediated contact activation may further play an essential role. Accordingly, assembly and activation of the proteases belonging to the contact system have been observed on the surface of *Streptococcus pyrogens, Staphylococcus aureus* and *Escherichia coli* (*192-194*).

The current results further revealed an independence of the FXII augmentation from the etiopathologic origin of the disease. Thus, no considerable difference was detectable whether ARDS was based on pulmonary or non pulmonary genesis. This observation coincides with the findings for extrinsic coagulation factors, showing equally elevated activity of tissue factor and FVII in direct as in indirect ARDS (*162*). The differentiated analysis of the temporal course revealed an instant increase of FXII within the first 24 hours of the diagnosis. The protein level remains high for four days and then declines to rates comparable to the values of the healthy control group. In view of a potential role of contact system activation in ARDS, this discovery draws particular attention to the early stage of the disease, which is known to be

characterized by inflammatory alteration of the lung tissue with a surge of cytokines and infiltration of leukocytes (*187*). In this context it is noteworthy that pro-inflammatory attributes of contact activation proteases in general and in the lung in particular have been addressed in previous research (*115, 184*). Thus, ample evidence has been provided that KLK and FXIIa induce aggregation of neutrophils and stimulate the release of neutrophil derived elastase *in vitro* (*114, 117, 119*). Accordingly, in KLK and FXII deficient plasma a remarkable decline of neutrophil elastase liberation has been described (*119*). Therefore it is of special interest, that in BALF and plasma of ARDS patients the level of the neutrophil elastase has been detected to be significantly elevated (*195, 196*). Containing destructive effects on the endothelium of the lung, the neutrophil elastase crucially contributes to the alteration of the alveolar membrane, leading to a protein rich edema and extravasation of inflammatory mediators into the alveolar and interstitial space (*197*). The molecular mechanism of the interaction between the contact system factors and the leukocytes engendering the release of neutrophil elastase remains unknown. Earlier investigations suggest an involvement of the catalytic domain as well as the heavy chain, since β-FXIIa failed to unfold its impact on the immune cells (*117*), possibly due its inability to bind to the cells. Of further particular interest in this regard is the present finding of elevated amounts of BK in BALF of ARDS patients. Given the manifested role of the nanopeptide in inflammatory processes (*88, 89, 100, 102*), the suggestion arises as to whether a significant participation in the pathogenesis of acute lung injury might be assigned to BK. (*101*) Up to date, various pro-inflammatory effects of BK in the lung have been explored as inducing the release of cytokines with neutrophil chemotactic activity, particularly IL-8, in pulmonary epithelial cells (*101, 102*). Aside from FXII and KLK, BK represents another component, demonstrated to induce the release of neutrophil elastase and thereby promoting the leakage of the alveolar membrane (*198*). In addition to the inflammatory attributes of the contact system factors, a pro-fibrotic contribution in ARDS might also be worthwhile considering. According to the present state of knowledge, fibrotic alterations in affected lungs appear within the early phase of the disease (*153, 199*), thus concurrent with the elevated FXII levels observed in the present study. In a former animal study, limited fibrotic manifestation in FXII $^{(-/-)}$ mice after bleomycin challenge has been demonstrated (*182*), implying pro-fibrotic features of the serine protease. A fibrotic capability of FXII might be attributed to its activity as a component of blood coagulation, initiating via proteolytical cleavage the intrinsic coagulation cascade and thereby promoting fibrin generation. This is contradicted, however, by the restricted significance of the intrinsic pathway in hemostasis *in vivo*. Furthermore, despite reduced fibrosis in FXII $^{(-/-)}$ mice, fibrin generation was equally

detected in the experimental and control group, thus the involvement of the contact system protease in fibro-proliferative alteration seems to be found beyond its procoagulative action (*182*). Promising approaches in this context are attributed to the mitogenic features of FXII. Accordingly, FXII has been identified to contain mitogenic activity on hepatocytes, endothelial cells, alveolar cells and aortic smooth muscle cells (*22, 33*), most likely involving the EGF-like domains of the protease. In line with these findings, a FXII mediated proliferative effect under participation of u-PAR has also been observed in murine lung fibroblasts (*182*). Besides, pro-fibrotic attributes may also be assigned to BK as in lung fibroblasts the peptide has been described to provoke proliferation, increased expression of α-smooth muscle actin (α-SMA) and enhanced collagen synthesis (*200*). Thus, BK possibly represents another component involved in the fibrotic response within ARDS.

The observations in ARDS lungs of the current study corresponded to the findings in the animal model of lung injury. To reproduce the pattern of lung injury, mice were intratracheal bleomycin challenged. Evoking similar pathological changes in murine lungs by inducing the release of cytokines, infiltration of neutrophils and formation of a pulmonary edema, bleomycin application is a common method to provoke the pathological pattern observed in the lungs of ARDS patients (*201*). Besides, another analogy with ARDS is found as the bleomycin induced neutrophilic alveolitis merges into a reversible fibrotic alteration. The conditions 5-10 days post bleomycin application particularly meet the criteria of the acute inflammatory early fibrotic phase of lung injury (*201*). Therefore, the experimental animals used were sacrified on day 5 or 10 after intratracheal bleomycin instillation. However, the pathological processes within acute lung injury are multifarious and may not be fully reproduced in animal models, thus there are naturally limitations to these experiments. This study revealed an elevation of the FXII level in BALF of mice after bleomycin challenge with indicators for FXII conversion, leading to assume an ongoing activity of the contact system. Significantly increased levels of FXII zymogen and FXIIa were further detected in murine lung homogenate. The temporal comparison showed the protease at a higher level on day 5 then on day 10. These observations are in line with the findings for ARDS lungs, revealing the FXII concentration to be initially at its highest and then to decline, emphasizing the particular involvement of contact activation within the early events of lung injury.

As there are few measures identified to be of therapeutic benefit in ARDS, it is crucially important for future approaches to gain additional knowledge of the mediators involved in the pathological process. The current finding of elevated and activated contact

activation proteases in ARDS lungs with an elevated amount of the downstream product BK underscores the hypothesis of a contributive role of these factors within the progression of the disease. Not interfering with hemostasis, the inhibition of contact activation may represent an attractive therapeutic option. In the future, further research is required to focus on revealing more details of the pathological circumstances leading to pulmonary accumulation of the proteases and to identify the molecular processes contact activation may actuate within the development of ARDS.

4.2 C1 INH administration moderates the inflammatory response in acute lung injury

Another pivotal discovery of this study was the recognition of a mitigating effect of C1 INH on the inflammatory processes in bleomycin induced lung injury. This favorable impact includes a regression of the alveolitis with a marked reduction of inflammatory cells, especially neutrophils, just as a decline of the pulmonary edema. Remarkably both pulmonary edema formation as excessive neutrophil infiltration with subsequent degranulation, are key features of ARDS. Here the curiosity arises to determine the components involved in this protective effect of C1 INH, drawing paramount interest to the substrates possessing pro-inflammatory attributes. FXII, KLK and complement factor C1 are acknowledged to be inhibited by C1 INH (*48-51*). The inhibition of KLK further restricts the generation of BK, equally representing a mediator of inflammation. It is noteworthy that the complement system as well as the contact activation system are found activated in lung injury (*202-204*), thus the inhibition of either system might cause the beneficial effects of C1 INH administration.

In research to date, mounting evidence of an advantageous impact of C1 INH administration in inflammatory diseases has been gained. Accordingly, a beneficial effect of the inhibitor application has been observed in an *in vivo* study on septic patients, revealing a decline of PMN activation after C1 INH administration accompanied by a reduced expression of disease-relevant cytokines (*205*). Besides, the referenced report accentuates the significance of the complement system, as C1 INH substitution attenuated the activation of the classic pathway of complement, whereas no such effect was witnessed for FXII or KLK (*205*). A preventive impact on the pulmonary function was further demonstrated in endotoxin challenged animal lungs, significantly ameliorating the Pa_{O2} after C1 INH administration. The authors of the study suggest the beneficial effect to be mainly traced back to the inhibition of contact system factors (*203*). In line with this assumption, a reduced inflammatory

response was observed in experiments on septic baboons after C1 INH treatment with concomitantly decreased activity of contact system proteases (*206*). There are different conceivable scenarios of how contact system activation might participate in the pathogenesis of acute lung injury. Firstly, as pointed out above, contact system factors induce the aggregation of neutrophils and the release of the neutrophil elastase, an enzyme essentially contributing to the destruction of the alveolar membrane. Additionally the neutrophil elastase has been described to inactivate C1 INH under generation of cleaved C1 INH which lacks the ability to inhibiting plasma proteases. It may be therefore speculated that FXII induced release of the neutrophil elastase leads to enhanced C1 INH inactivation, which putatively may cause unrestricted activation of the contact and the complement activation system. Interestingly, in septic patients the level of cleaved, inactivated C1 INH is described to be elevated and to correlate with the clinical outcome (*207*). Similar conditions seem conceivable for ARDS and insufficient inhibition of the contact and complement activation system might reinforce the inflammatory response. Besides the neutrophil elastase, other tissue destroying components may be released due to contact system triggered neutrophil degranulation. Increased endothelial permeability in the lung may be augmented by HK derived-BK generation (*90*). As FXII, KLK and HK have been localized on endothelial cells, the alveolar endothelium might represent a potential site of activation of the contact system proteases where liberation of BK occurs. Released BK may effectuate in paracrine manner the increase of alveolar permeability, thus exacerbating the influx of protein rich fluids into the alveolar space.

Previous investigations on the impact of C1 INH in septic baboons have revealed a decline of the cytokines IL-6, IL-8, Il-10 and TNF-α after the administration of the inhibitor, an effect suspected by the authors to be attributable to the inhibition of the contact activation system (*206*). Supporting this assumption, inhibition of FXII by monoclonal antibody C6B7 in baboons similarly revealed a decrease of IL-6 release during sepsis (*118*). In consistency with this observation, the present study demonstrated a decrease of the pro-inflammatory mediators MIP-2 and TNF-α after C1 INH administration in bleomycin induced lung injury (*206*), whereas the expression of IL-1β remained unaffected. Finally, the activities of contact activation factors towards the complement system remain to be considered. Previous studies demonstrated that the first component of the complement system may also be activated by β-FXII, thus linking the pathways of the complement system and the contact activation cascade together (*25, 26*). The C1 of the complement represents the initiating component of the classic pathway, through proteolytic processing leading to the release of C5a, an

acknowledged chemo-attractant. C5a is recognized to bind to endothelial cells, induce neutrophil capture and increase vascular permeability under hypoxic conditions (*208*). The complement system as the central target of endogenous C1 IHN administration has been the focus in previous studies with controversial results. In septic patients, C1 IHN substitution was demonstrated to lead to inhibition of the complement system and concurrent decline of neutrophil degranulation (*205*). However, in bacterial induced lung injury in an animal model, the administration of the inhibitor failed to prevent complement activation (*202*). Similarly, an insufficient inhibition of the complement system via C1 IHN administration has also been described for septic baboons (*206*). A most likely explanation for this may be found in the fact that the C1 INH interferes at an early level of the classic pathway of the complement system, without affecting the alternative cascade or other proteolytic processes leading to generation of the downstream complement products. Therefore, it is of no surprise, that C5a was found to be barely lowered after C1 INH administration (*206*). Based on these observations, it seems reasonable to presume that the complement system solely is insufficient to induce severe lung injury, as found in ARDS lungs. Instead, it seems more reasonable to conclude, that both systems, contact system activation in concert with the complement pathway precipitate the infiltration of neutrophils into the lung and concurrent edema formation. Summarily, the present results sustain the critical role of the contact system without dismissing an additional participation of the complement pathway.

The imbalanced hemostatic state as generally found in inflammatory lung diseases was equally observed in the bleomycin-injured lungs. Accordingly, the clotting time was accelerated and D-dimer as indicator of boosted coagulation activity was significantly higher after bleomycin application. C1 INH substitution exhibited no impact on this procoagulative state, underlining the strongly limited significance of FXII in blood clotting. Determination of fibrin deposits revealed to be increased after bleomycin challenge. In line with previous investigations, the fibrin accumulation remained unaffected from C1 INH triggered-FXII blockage (*194*). These results indicate that the role of contact system within the pathogenesis of lung injury does not include an impact on the fibrin generation. Similar observations have been made in murine lungs with bleomycin induced fibrosis. Despite a significantly lower extent of fibrotic alteration in the lung of FXII $^{(-/-)}$ mice compared to the WT mice, no difference in fibrin generation was detectable between both groups (*182*).

The limitation of the investigation at hand is evidently based on the extended inhibiting activities of C1 INH, thus the present results do not exclude that the anti-inflammatory effect may be due to inhibition of factors other than the contact system proteases.

Furthermore, the protection from lung injury might also be directly mediated by C1 INH itself, as evidence of anti-inflammatory activities independent of its function as protease inhibitor has been provided. Thus, C1 INH at plasma concentrations generally found in inflammation is observed to directly interfere with leukocyte extravasation (*127*). By binding to P- and E- selectin adhesion molecules, C1 INH has been demonstrated to restrict leukocyte adhesion to endothelial cells in both *in vitro* and *in vivo* experiments (*126, 127*). Furthermore, in animal models of sepsis and endotoxin shock, C1 INH attaches directly to the bacterial lipopolysaccharids (LPS) and thereby inhibits LPS induced activation of macrophages and suppresses TNF-α synthesis (*209*). With regard to the results of the present study, the beneficial effect of C1 INH application in lung injury might be precipitated by direct C1 INH activity with the alveolar epithelial cells. It might therefore be of interest for future studies to examine whether the protective impact in lung injury is also observed when C1 INH is applied in its cleaved, inactivated form.
Further research is also required to prove the explicit involvement of the contact activation system. Thus, a subject for prospective investigation might be the assessment of contact system activity after C1 INH substitution in bleomycin-injured lungs. A conceivable approach may further be the examination of a potential protection of FXII $^{(-/-)}$ mice from inflammatory alteration of the lung. Following the same reasoning, investigations on BK receptor $^{(-/-)}$ mice may elucidate the particular involvement of the peptide in lung injury. An additional restriction of C1 INH may be due to its inability to inhibited surface bound contact proteases. Pursuing the idea of alveolar endothelial attached contact activation with local synthesis of BK, C1 INH administration may not achieve complete inhibition of the proteases. Another approach might therefore be to make use of a different inhibitor which also accesses to cell surface bound FXII, for instance the monoclonal Ab to FXII (C6B7) (*194*).

However, despite the stated limitations, the significance of C1 INH as a main inhibitor of the contact, kallikrein-kinin and complement system is up to date undisputed, justifying that the inhibitor was chosen in the presented experiments. The anti-inflammatory effect of C1 INH is substantiated by the current results, leading to the conclusion that the inhibitor might represent an effective way to interfere with contact activation triggered impairment of the lung. C1 INH for therapeutic application has

already been established in the treatment of hereditary angioedema with very few side effects; here an extended role of C1 INH as potential future medication in inflammatory lung diseases finds support.

5. Conclusion

In the past the biological relevance of the intrinsic proteases had been put to question, based on their dispensability in blood coagulation *in vivo.* Presently, the interest has been revived by the discovery of proinflammatory attributes of individual factors. This supplement role as intermediary in inflammation particular includes the FXII- and KLK-triggered induction of neutrophil aggregation and degranulation as well as the release of different cytokines. Of particular significance is further the KLK initiated liberation of BK. Being a well established mediator of vasodilatation and inflammatory processes, BK has been described to stimulate the release of various cytokines in lung fibroblasts and airway smooth muscle cells.

In line with this recognition the results of the present study imply an involvement of the contact activation proteases in inflammatory lung diseases such as ARDS. In BALF and lung homogenate of ARDS patients the protein level of FXII, FXI, KLK and HK were elevated with indications of enhanced proteolytic activity of the contact system. The protein levels of FXII and BK were simultaneously raised in the early, inflammatory phase of ARDS and declined from day 6 onwards. The increase of both proteins was detected to be independent of the etiopathological origin and specific for ARDS as no increase of FXII and BK was apparent in BALF of patients with pulmonary edema based on cardiac failure. These findings were in accordance with the results of the animal model of lung injury, showing FXII and BK protein levels significantly elevated. In the qPCR analysis FXII was detected to be expressed by leukocytes of ARDS patients, revealing a potential extrahepatic source of the contact system initiating factor during lung injury.

The administration of C1 INH prevented the inflammatory destruction of the alveolar tissue in bleomycin lungs. This beneficial effect of the inhibitor included a decline of immigrating immune cells, particularly of neutrophils and the decrease of the inflammatory cytokines TNF-α and MIP-2, both known to reinforce the inflammatory response in lung injury. Moreover, a significant regression of the alveolar edema was observed after C1 INH application. As expected the unbalanced hemostatic state remained unaffected by C1 INH treatment.

In conclusion, the present data demonstrate an anti-inflammatory effect of C1 INH administration on lung injury and provid founded reason to suggest an involvement of the contact system proteases.

6. Summary

The pathological features of the acute respiratory distress syndrome (ARDS) are hallmarked by inflammatory processes in the lung with an impairment of the alveolar membrane precipitating the formation of a pulmonary edema and commonly accompanied by massive interstitial fibrin disposition. Despite the increasing knowledge of the disease, protective ventilation with a low tidal volume is the only therapeutic measure recognized to substantially reduce the mortality. Accordingly, over the past decades mortality has remained at a high level, emphasizing the necessity for further scientific research and analysis. Research up to date has substantiated the finding of an altered alveolar hemostatic state, accrediting a potential role in lung injury to the factors of blood coagulation. Thus, in the lungs of ARDS patients boosted activity of procoagulant proteases has been observed with contemporary restriction of the fibrinolytic system, resulting in amplified generation and interstitial accumulation of fibrin. Investigations performed heretofore focused particularly on the extrinsic factors of blood coagulation due to their established role as a source of fibrin. On the contrary, the intrinsic proteases are acknowledged to be of limited importance for blood clotting *in vivo* however, accumulating evidence has been gained of an extended participation in fibrotic and inflammatory processes. The intrinsic pathway of blood coagulation is also referred to as the contact system and consists of the factor XII (FXII), factor XI (FXI), kallikrein (KLK) and high molecular weight kininogen (HK).

C1 esterase inhibitor (C1 INH) represents not only the sole inhibitor of the classic pathway of complement activation but also the main inhibitor of the contact system, irreversibly inactivating FXII and KLK by complex formation. The anti-inflammatory attributes of C1 INH have been detected to extend beyond its activity as serine protease inhibitor, including also the prevention of leukocyte migration by directly interacting with endothelial cells. This study contemplates the involvement of the intrinsic factors of blood coagulation in lung injury with particular focus on FXII, the initiator of the intrinsic cascade. The investigation further aims at determining whether C1 INH application might display an effect on the course of the disease. Elevated levels of FXII, FXI, KLK and HK in the bronchoalveolar lavage fluids (BALF) and lung tissue of ARDS patients were observed with evidence of ongoing contact system activity. The investigation revealed an instant increase of FXII concomitant with the onset of the disease, leading to assume that the impact of the contact system is to be found within the early phase of the disease. In consistency with these results for ARDS patients were the observations made in the animal model of lung injury; here correspondingly increased FXII levels in BALF and lung homogenate became evident

after bleomycin application. As unrestrained activation and infiltration of neutrophils represents a central feature of ARDS, one might speculate that the mechanism of how contact system proteases may aggravate lung injury is possibly related to the ability of FXII and KLK to trigger neutrophil aggregation. Another decisive discovery of the investigation was the increased BK level in the BALF of ARDS patients and the mouse model of lung injury, presumably due to enhanced contact system activity. In addition to its proinflammatory attributes, BK can be assumed to contribute to lung injury by increasing the alveolar capillary permeability and thereby aggravating the pulmonary edema. The study further indicated that under pathological conditions, cells other than hepatocytes play a role in FXII generation as FXII mRNA was detected in leukocytes of ARDS patients. Of pivotal interest for future therapeutic approaches is the here presented recognition of a favorable effect of C1 INH administration in lung injury. Thus, C1 INH application was shown to attenuate the inflammatory process in pulmonary tissue of bleomycin injured lungs, significantly reducing the number of inflammatory cells, particularly neutrophils and inducing a regression of the alveolar edema. Furthermore, administration of the inhibitor was followed by a significant decrease of tumor necrosis factor- α (TNF-α) and macrophage inflammatory protein-2 (MIP-2); both cytokines are well established as mediators of inflammation in lung injury.
In summary, the present study provides reason to assume an implication of the contact system factors in the inflammatory process of the ARDS, possibly by promoting the release of cytokines causing neutrophil derived lung tissue destruction. Furthermore, a protective effect of C1 INH in lung injury is described, pointing towards the anti-inflammatory properties of the serine protease inhibitor such as the inhibition of neutrophil recruitment to the sites of injury via binding of C1 INH to selectins. Therefore the investigation draws new attention to C1 INH as a potential future option in the therapy of inflammatory lung diseases such as ARDS.

7. Zusammenfassung

Der pathologische Prozess des acute respiratory distress syndrome (ARDS) ist gekennzeichnet durch eine entzündliche Veränderung der Lunge mit Beeinträchtigung der Alveolarmembran, in deren Folge es zur Ausbildung eines Lungenödems und massiver interstitieller Fibrinablagerung kommt. Trotz zunehmender Erkenntnisse über die Erkrankung bleibt die lungenprotektive Beatmung mit niedrigen Tidalvolumina die bis heute einzig anerkannte, therapeutisch wirksame Maßnahme. Gegenwärtig liegt die Mortalität des ARDS weiterhin auf einem hohen Niveau von bis zu 48%, woraus sich die dringende Notwendigkeit weiterer wissenschaftlicher Untersuchungen ergibt. Die Entdeckung eines veränderten alveolar-hämostatischen Status bei betroffenen Patienten wurde in verschiedenen Studien bestätigt und lässt einen möglichen Einfluss verschiedener Blutgerinnungsfaktoren auf die Lungenschädigung vermuten. In der bronchoalveolären Lavage (BALF) von ARDS Patienten ist die Gerinnungsaktivität erhöht mit gleichzeitiger Reduktion der Fibrinolyse, woraus eine verstärkte Produktion sowie interstitielle Ansammlung von Fibrin resultiert. Aufgrund ihrer etablierten Rolle in der Generierung von Fibrin konzentrierten sich bisherige Studien in diesem Zusammenhang insbesondere auf die Bedeutung extrinsischer Gerinnungsfaktoren. Die begrenzte Wirkung der Faktoren des intrinsischen Weges in der Blutgerinnung *in vivo* ist allgemein anerkannt, hingegen scheint ihre Beteiligung an verschiedenen entzündlichen und fibrotischen Prozessen von höherer Relevanz zu sein. Der intrinsische Weg der Blutgerinnung setzt sich zusammen aus den Faktoren XII (FXII), Faktor XI (FXI), Kallikrein (KLK) und high molecular weight kininogen (HK) und wird auch als Kontaktaktivierungssystem bezeichnet.

Der C1 Esterase Inhibitor (C1 INH) ist ein Serinprotease-Inhibitor und in dieser Funktion nicht nur der einzige Inhibitor des klassischen Wegs des Komplementsystems, sondern auch Hauptinhibitor des intrinsischen Systems. Die anti-entzündlichen Eigenschaften von C1 INH sind jedoch nicht allein auf seine Aktivität als Proteaseinhibitor begrenzt. Eine direkte Wechselwirkung von C1 INH mit Endothelialzellen wurde ebenfalls beschrieben, welche die Bindung von Leukozyten an das Endothel und somit die Diapedese verhindert.

Gegenstand dieser Studie ist die Untersuchung einer potentiellen Beteiligung der Faktoren des Kontaktaktivierungssystems in der Pathogenese akuter Lungenschädigung. Hierbei liegt der Schwerpunkt der Experimente vorwiegend auf dem FXII, welcher als Initiator der Kaskade von besonderem Interesse ist. Ein weiterer

Fokus ist die Untersuchung eines möglichen Effekts von C1 INH auf den Krankheitsverlauf. In den Experimenten zeigte sich ein erhöhter Proteinlevel der intrinsischen Faktoren FXII, FXI, KLK und HK in der Lungenlavage sowie im Lungengewebe von ARDS Patienten. Außerdem ließen die Beobachtungen auf eine fortlaufende Aktivität des Kontaktaktiverungssystems schließen. Der Anstieg des FXII Levels zeigte sich hierbei unmittelbar mit dem Beginn der Erkrankung. Diese Beobachtung lässt vermuten, dass der Einfluss der intrinsischen Faktoren auf den pathologischen Verlauf insbesondere in der frühen Phase der Erkrankung zu finden ist. Übereinstimmend mit diesen Erkenntnissen waren auch die Ergebnisse der Versuche an Tiermodellen der akuten Lungenschädigung. Auch hier zeigte sich ein Anstieg des FXII Levels in BALF und im Lungenhomogenat nach Bleomycin Applikation. Angesichts der massiven Infiltration von Leukozyten im Verlauf des ARDS, scheint ein möglicher Beitrag des Kontaktaktiverungssystems zur Lungenschädigung die von FXII und KLK induzierte Aktivierung von Neutrophilen zu sein. Eine weitere wichtige Erkenntnis war die Beobachtung eines erhöhten Bradykinin (BK) Levels bei ARDS Patienten ebenso wie im Tiermodel der Lungenschädigung, mutmaßlich ausgelöst durch die erhöhte Aktivität des Kontaktaktivierungungssystems. Zur Schädigung der Lunge trägt BK möglicherweise neben seiner proentzündlichen Wirkung auch durch die Erhöhung der alveolarkapillären Permeabilität bei, welche eine Zunahme des Lungenödems zur Folge hat. Desweiteren konnte der Nachweis von mRNA in Leukozyten von ARDS Patienten erbracht werden, eine extrahepatische FXII Synthese scheint folglich unter pathologischen Bedingungen eine zusätzliche Rolle zu spielen. Von besonderem Interesse für zukünftige therapeutische Forschungsansätze ist der hier erbrachte Nachweis eines positiven Effekts von C1 INH Applikation auf die Lungenschädigung. Die Verabreichung von C1 INH milderte den entzündlichen Prozess im Lungengewebe, reduzierte die Anzahl der inflammatorischen Zellen, insbesondere der Neutrophilen und bewirkte eine Reduktion des Lungenödems. Desweiteren zeigte sich ein signifikanter Rückgang der Zytokine Tumornekrose Faktor-α (TNF-α) und macrophage inflammatory protein-2 (MIP-2).

Zusammenfassend weisen die Ergebnisse dieser Studie auf eine Beteiligung der Faktoren des Komplementaktivierungssystems in der Pathogenese von ARDS hin. Möglicherweise induzieren die untersuchten Proteasen hierbei die Zytokinfreisetzung und unterstützen so die Lungengewebsschädigung durch Überstimulation von Neutrophilen. Die Gabe von C1 INH zeigte einen protektiven Effekt auf die Lungenschädigung und weist auf die antiinflammatorische Kapazität des Serine Protease Inhibitors hin. Diese beruht möglicherweise auf der Fähigkeit des C1 INH

mittels Blockade von Selektinen die endothelial Leukozyten Adhäsion zu stören und somit die Invasion der Entzündungszelle in das Lungengewebe zu hemmen. Die Untersuchung richtet somit ein neues Interesse auf C1 INH als eine zukünftige therapeutische Option in der Behandlung von entzündlichen Lungenerkrankungen wie ARDS.

8. List of abbreviations

AECC	American European Conference Committee
ALI	Acute lung injury
APS	Ammonium persulfate
α-SMA	α-smooth muscle actin
Asp	Aspartic acid
ARDS	Acute respiratory distress syndrome
AT III	Anti-thrombin III
BALF	Broncho-alveolar lavage fluid
BK	Bradykinin
B_1R	Bradykinin-1-receptor
B_2R	Bradykinin-2-receptor
BSA	Bovine serum albumin
C1q	First component of the complement system
C1 INH	C1 esterase inhibitor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLE	Cardiogenic pulmonary edema
CTI	Corn trypsin inhibitor
CXCL1	Chemokine (C-X-C motif) ligand 1
EDTA	Ethylendinitriol-N,N,N`,N`,-tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay

FX	Factor X
FXI	Factor XI
FXII	Factor XII
gC1qR	Complement component C1 subcomponent-binding receptor
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage-colony stimulating factor
HAE	Hereditary angioedema
His	Histidine
HK	High molecular weigh kininogen
IL	Interleukin
KLK	Kallikrein
LH	Lung homogenate
MCP-1	Monocyte chemoattractant protein-1
MIP-2	Macrophage inflammatory protein-2
PBS	Phosphate buffered saline
PAR	Protease-activated receptor
PEEP	Positive expiratory end pressure
PK	Prekallikrein
PolyP	Inorganic Polyphosphate
qPCR	Real-time PCR
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
Ser	Serine
TBS	Tris buffered saline buffer

TEMED	Tetramethylethylenediamine
TF	Tissue factor
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
TNF-β	Tumor necrosis factor-β
t-PA	Tissue-plasminogen activator
u-PAR	Urokinase-plasminogen activator receptor

9. List of figures and tables

9.1 List of figures

Figure 1.1 Contact activation pathway.

Figure 1.2 Model of FXII structure with detailed view of catalytic domain.

Figure 4.1 Elevated levels of contact activation factors in BALF of ARDS patients.

Figure 4.2 Increased levels of FXII in the BALF of ARDS patients.

Figure 4.3 BK is elevated in BALF of ARDS patients.

Figure 4.4 Altered expression of contact activation factors in ARDS lung tissue.

Figure 4.5 Altered expression of contact activation factors in ARDS lung tissue, quantification.

Figure 4.6 Expression of FXII is altered in lung tissue and blood cells of ARDS patients.

Figure 4.7 Levels of FXII and FXIIa are elevated in BALF of bleomycin treated mice.

Figure 4.8 Increased protein levels of FXII/FXIIa in lung homogenate of mice at day 5 and day 10 after bleomycin instillation.

Figure 4.9 Increased level of BK in BALF of mice at day 5 and day 10 after bleomycin application.

Figure 4.10 Application of C1 INH reduces the inflammatory response in bleomycin treated mice.

Figure 4.11 Application of C1 INH reduces number of inflammatory cells in bleomycin challenged mice.

Figure 4.12 Decrease of edema formation after C1 INH administration in bleomycin treated mice.

Figure 4.13 Expression of MIP-2 and TNF-α is reduced after C1 INH administration in bleomycin challenged mice.

Figure 4.14 C1 INH administration has no impact on altered coagulation in bleomycin induced lung injury.

9.2 List of tables

Table 2.1 Study population for BALF samples.

Table 2.2 Ingredients of the reverse transcriptase reaction.

Table 2.3 Sequences of the primers used in the study.

Table 2.4 Ingredients of the qPCR reaction.

10. References

1. Mackman, N. (2004) Role of tissue factor in hemostasis, thrombosis, and vascular development, *Arterioscler Thromb Vasc Biol 24*, 1015-1022.
2. Mackman, N., Tilley, R. E., and Key, N. S. (2007) Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis, *Arterioscler Thromb Vasc Biol 27*, 1687-1693.
3. Hoffman, M. (2003) Remodeling the blood coagulation cascade, *J Thromb Thrombolysis 16*, 17-20.
4. Gailani, D., and Renne, T. (2007) Intrinsic pathway of coagulation and arterial thrombosis, *Arterioscler Thromb Vasc Biol 27*, 2507-2513.
5. Macfarlane, R. G. (1964) An Enzyme Cascade in the Blood Clotting Mechanism, and Its Function as a Biochemical Amplifier, *Nature 202*, 498-499.
6. Vogler, E. A., and Siedlecki, C. A. (2009) Contact activation of blood-plasma coagulation, *Biomaterials 30*, 1857-1869.
7. Davie, E. W., and Ratnoff, O. D. (1964) Waterfall Sequence for Intrinsic Blood Clotting, *Science 145*, 1310-1312.
8. Citarella, F., Tripodi, M., Fantoni, A., Bernardi, F., Romeo, G., and Rocchi, M. (1988) Assignment of human coagulation factor XII (fXII) to chromosome 5 by cDNA hybridization to DNA from somatic cell hybrids, *Hum Genet 80*, 397-398.
9. Gordon, E. M., Gallagher, C. A., Johnson, T. R., Blossey, B. K., and Ilan, J. (1990) Hepatocytes express blood coagulation factor XII (Hageman factor), *J Lab Clin Med 115*, 463-469.
10. Citarella, F., Ravon, D. M., Pascucci, B., Felici, A., Fantoni, A., and Hack, C. E. (1996) Structure/function analysis of human factor XII using recombinant deletion mutants. Evidence for an additional region involved in the binding to negatively charged surfaces, *Eur J Biochem 238*, 240-249.
11. Fujikawa, K., and McMullen, B. A. (1983) Amino acid sequence of human beta-factor XIIa, *J Biol Chem 258*, 10924-10933.
12. McMullen, B. A., and Fujikawa, K. (1985) Amino acid sequence of the heavy chain of human alpha-factor XIIa (activated Hageman factor), *J Biol Chem 260*, 5328-5341.
13. Stavrou, E., and Schmaier, A. H. Factor XII: what does it contribute to our understanding of the physiology and pathophysiology of hemostasis & thrombosis, *Thromb Res 125*, 210-215.
14. Cool, D. E., Edgell, C. J., Louie, G. V., Zoller, M. J., Brayer, G. D., and MacGillivray, R. T. (1985) Characterization of human blood coagulation factor XII cDNA. Prediction of the primary structure of factor XII and the tertiary structure of beta-factor XIIa, *J Biol Chem 260*, 13666-13676.
15. Clarke, B. J., Cote, H. C., Cool, D. E., Clark-Lewis, I., Saito, H., Pixley, R. A., Colman, R. W., and MacGillivray, R. T. (1989) Mapping of a putative surface-binding site of human coagulation factor XII, *J Biol Chem 264*, 11497-11502.
16. Pixley, R. A., Stumpo, L. G., Birkmeyer, K., Silver, L., and Colman, R. W. (1987) A monoclonal antibody recognizing an icosapeptide sequence in the heavy chain of human factor XII inhibits surface-catalyzed activation, *J Biol Chem 262*, 10140-10145.
17. Rojkaer, R., and Schousboe, I. (1997) Partial identification of the Zn2+-binding sites in factor XII and its activation derivatives, *Eur J Biochem 247*, 491-496.
18. Joseph, K., Ghebrehiwet, B., Peerschke, E. I., Reid, K. B., and Kaplan, A. P. (1996) Identification of the zinc-dependent endothelial cell binding protein for high molecular weight kininogen and factor XII: identity with the receptor that

binds to the globular "heads" of C1q (gC1q-R), *Proc Natl Acad Sci U S A 93*, 8552-8557.
19. Mahdi, F., Madar, Z. S., Figueroa, C. D., and Schmaier, A. H. (2002) Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes, *Blood 99*, 3585-3596.
20. Citarella, F., Fedele, G., Roem, D., Fantoni, A., and Hack, C. E. (1998) The second exon-encoded factor XII region is involved in the interaction of factor XII with factor XI and does not contribute to the binding site for negatively charged surfaces, *Blood 92*, 4198-4206.
21. Henderson, L. M., Figueroa, C. D., Muller-Esterl, W., and Bhoola, K. D. (1994) Assembly of contact-phase factors on the surface of the human neutrophil membrane, *Blood 84*, 474-482.
22. Schmeidler-Sapiro, K. T., Ratnoff, O. D., and Gordon, E. M. (1991) Mitogenic effects of coagulation factor XII and factor XIIa on HepG2 cells, *Proc Natl Acad Sci U S A 88*, 4382-4385.
23. Polgar, L. (2005) The catalytic triad of serine peptidases, *Cell Mol Life Sci 62*, 2161-2172.
24. Heimark, R. L., Kurachi, K., Fujikawa, K., and Davie, E. W. (1980) Surface activation of blood coagulation, fibrinolysis and kinin formation, *Nature 286*, 456-460.
25. Ghebrehiwet, B., Randazzo, B. P., Dunn, J. T., Silverberg, M., and Kaplan, A. P. (1983) Mechanisms of activation of the classical pathway of complement by Hageman factor fragment, *J Clin Invest 71*, 1450-1456.
26. Ghebrehiwet, B., Silverberg, M., and Kaplan, A. P. (1981) Activation of the classical pathway of complement by Hageman factor fragment, *J Exp Med 153*, 665-676.
27. Revak, S. D., Cochrane, C. G., Bouma, B. N., and Griffin, J. H. (1978) Surface and fluid phase activities of two forms of activated Hageman factor produced during contact activation of plasma, *J Exp Med 147*, 719-729.
28. Goldsmith, G. H., Jr., Saito, H., and Ratnoff, O. S. (1978) The activation of plasminogen by Hageman factor (Factor XII) and Hageman factor fragments, *J Clin Invest 62*, 54-60.
29. Schousboe, I. (1997) Factor XIIa activation of plasminogen is enhanced by contact activating surfaces and Zn2+, *Blood Coagul Fibrinolysis 8*, 97-104.
30. Braat, E. A., Dooijewaard, G., and Rijken, D. C. (1999) Fibrinolytic properties of activated FXII, *Eur J Biochem 263*, 904-911.
31. Kaplan, A. P., and Austen, K. F. (1972) The fibrinolytic pathway of human plasma. Isolation and characterization of the plasminogen proactivator, *J Exp Med 136*, 1378-1393.
32. Tanaka, A., Suzuki, Y., Sugihara, K., Kanayama, N., and Urano, T. (2009) Inactivation of plasminogen activator inhibitor type 1 by activated factor XII plays a role in the enhancement of fibrinolysis by contact factors in-vitro, *Life Sci 85*, 220-225.
33. Gordon, E. M., Venkatesan, N., Salazar, R., Tang, H., Schmeidler-Sapiro, K., Buckley, S., Warburton, D., and Hall, F. L. (1996) Factor XII-induced mitogenesis is mediated via a distinct signal transduction pathway that activates a mitogen-activated protein kinase, *Proc Natl Acad Sci U S A 93*, 2174-2179.
34. Kirby, E. P., and McDevitt, P. J. (1983) The binding of bovine factor XII to kaolin, *Blood 61*, 652-659.
35. van der Graaf, F., Keus, F. J., Vlooswijk, R. A., and Bouma, B. N. (1982) The contact activation mechanism in human plasma: activation induced by dextran sulfate, *Blood 59*, 1225-1233.

36. Fujikawa, K., Heimark, R. L., Kurachi, K., and Davie, E. W. (1980) Activation of bovine factor XII (Hageman factor) by plasma kallikrein, *Biochemistry 19*, 1322-1330.
37. Espana, F., and Ratnoff, O. D. (1983) Activation of Hageman factor (factor XII) by sulfatides and other agents in the absence of plasma proteases, *J Lab Clin Med 102*, 31-45.
38. Tans, G., Rosing, J., and Griffin, J. H. (1983) Sulfatide-dependent autoactivation of human blood coagulation Factor XII (Hageman Factor), *J Biol Chem 258*, 8215-8222.
39. Morrison, D. C., and Cochrane, C. G. (1974) Direct evidence for Hageman factor (factor XII) activation by bacterial lipopolysaccharides (endotoxins), *J Exp Med 140*, 797-811.
40. Hojima, Y., Cochrane, C. G., Wiggins, R. C., Austen, K. F., and Stevens, R. L. (1984) In vitro activation of the contact (Hageman factor) system of plasma by heparin and chondroitin sulfate E, *Blood 63*, 1453-1459.
41. Schmaier, A. H. (2008) The elusive physiologic role of Factor XII, *J Clin Invest 118*, 3006-3009.
42. Kannemeier, C., Shibamiya, A., Nakazawa, F., Trusheim, H., Ruppert, C., Markart, P., Song, Y., Tzima, E., Kennerknecht, E., Niepmann, M., von Bruehl, M. L., Sedding, D., Massberg, S., Gunther, A., Engelmann, B., and Preissner, K. T. (2007) Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation, *Proc Natl Acad Sci U S A 104*, 6388-6393.
43. Wilner, G. D., Nossel, H. L., and LeRoy, E. C. (1968) Activation of Hageman factor by collagen, *J Clin Invest 47*, 2608-2615.
44. Johne, J., Blume, C., Benz, P. M., Pozgajova, M., Ullrich, M., Schuh, K., Nieswandt, B., Walter, U., and Renne, T. (2006) Platelets promote coagulation factor XII-mediated proteolytic cascade systems in plasma, *Biol Chem 387*, 173-178.
45. Muller, F., Mutch, N. J., Schenk, W. A., Smith, S. A., Esterl, L., Spronk, H. M., Schmidbauer, S., Gahl, W. A., Morrissey, J. H., and Renne, T. (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo, *Cell 139*, 1143-1156.
46. Revak, S. D., Cochrane, C. G., and Griffin, J. H. (1977) The binding and cleavage characteristics of human Hageman factor during contact activation. A comparison of normal plasma with plasmas deficient in factor XI, prekallikrein, or high molecular weight kininogen, *J Clin Invest 59*, 1167-1175.
47. Colman, R. W. (1984) Surface-mediated defense reactions. The plasma contact activation system, *J Clin Invest 73*, 1249-1253.
48. de Agostini, A., Lijnen, H. R., Pixley, R. A., Colman, R. W., and Schapira, M. (1984) Inactivation of factor XII active fragment in normal plasma. Predominant role of C-1-inhibitor, *J Clin Invest 73*, 1542-1549.
49. Schreiber, A. D., Kaplan, A. P., and Austen, K. F. (1973) Inhibition by C1INH of Hagemann factor fragment activation of coagulation, fibrinolysis, and kinin generation, *J Clin Invest 52*, 1402-1409.
50. Ratnoff, O. D., Pensky, J., Ogston, D., and Naff, G. B. (1969) The inhibition of plasmin, plasma kallikrein, plasma permeability factor, and the C'1r subcomponent of the first component of complement by serum C'1 esterase inhibitor, *J Exp Med 129*, 315-331.
51. Weiss, R., Silverberg, M., and Kaplan, A. P. (1986) The effect of C1 inhibitor upon Hageman factor autoactivation, *Blood 68*, 239-243.
52. Pixley, R. A., Schmaier, A., and Colman, R. W. (1987) Effect of negatively charged activating compounds on inactivation of factor XIIa by Cl inhibitor, *Arch Biochem Biophys 256*, 490-498.
53. Schreiber, A. D., Kaplan, A. P., and Austen, K. F. (1973) Plasma inhibitors of the components of the fibrinolytic pathway in man, *J Clin Invest 52*, 1394-1401.

54. Back, J., Lang, M. H., Elgue, G., Kalbitz, M., Sanchez, J., Ekdahl, K. N., and Nilsson, B. (2009) Distinctive regulation of contact activation by antithrombin and C1-inhibitor on activated platelets and material surfaces, *Biomaterials 30*, 6573-6580.
55. Gailani, D., Sun, M. F., and Sun, Y. (1997) A comparison of murine and human factor XI, *Blood 90*, 1055-1064.
56. Geng, Y., Verhamme, I. M., Smith, S. B., Sun, M. F., Matafonov, A., Cheng, Q., Smith, S. A., Morrissey, J. H., and Gailani, D. The dimeric structure of factor XI and zymogen activation, *Blood 121*, 3962-3969.
57. Fujikawa, K., Chung, D. W., Hendrickson, L. E., and Davie, E. W. (1986) Amino acid sequence of human factor XI, a blood coagulation factor with four tandem repeats that are highly homologous with plasma prekallikrein, *Biochemistry 25*, 2417-2424.
58. Bouma, B. N., and Griffin, J. H. (1977) Human blood coagulation factor XI. Purification, properties, and mechanism of activation by activated factor XII, *J Biol Chem 252*, 6432-6437.
59. McMullen, B. A., Fujikawa, K., and Davie, E. W. (1991) Location of the disulfide bonds in human coagulation factor XI: the presence of tandem apple domains, *Biochemistry 30*, 2056-2060.
60. Yarovaya, G. A., Blokhina, T. B., and Neshkova, E. A. (2002) Contact system. New concepts on activation mechanisms and bioregulatory functions, *Biochemistry (Mosc) 67*, 13-24.
61. Emsley, J., McEwan, P. A., and Gailani, D. Structure and function of factor XI, *Blood 115*, 2569-2577.
62. Ponczek, M. B., Gailani, D., and Doolittle, R. F. (2008) Evolution of the contact phase of vertebrate blood coagulation, *J Thromb Haemost 6*, 1876-1883.
63. Mandle, R. J., Colman, R. W., and Kaplan, A. P. (1976) Identification of prekallikrein and high-molecular-weight kininogen as a complex in human plasma, *Proc Natl Acad Sci U S A 73*, 4179-4183.
64. Thompson, R. E., Mandle, R., Jr., and Kaplan, A. P. (1977) Association of factor XI and high molecular weight kininogen in human plasma, *J Clin Invest 60*, 1376-1380.
65. Wu, W., Sinha, D., Shikov, S., Yip, C. K., Walz, T., Billings, P. C., Lear, J. D., and Walsh, P. N. (2008) Factor XI homodimer structure is essential for normal proteolytic activation by factor XIIa, thrombin, and factor XIa, *J Biol Chem 283*, 18655-18664.
66. Wiggins, R. C., Bouma, B. N., Cochrane, C. G., and Griffin, J. H. (1977) Role of high-molecular-weight kininogen in surface-binding and activation of coagulation Factor XI and prekallikrein, *Proc Natl Acad Sci U S A 74*, 4636-4640.
67. Romney, G., and Glick, M. (2009) An updated concept of coagulation with clinical implications, *J Am Dent Assoc 140*, 567-574.
68. Chung, D. W., Fujikawa, K., McMullen, B. A., and Davie, E. W. (1986) Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats, *Biochemistry 25*, 2410-2417.
69. Colman, R. W., and Schmaier, A. H. (1997) Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes, *Blood 90*, 3819-3843.
70. Colman, R. W., Wachtfogel, Y. T., Kucich, U., Weinbaum, G., Hahn, S., Pixley, R. A., Scott, C. F., de Agostini, A., Burger, D., and Schapira, M. (1985) Effect of cleavage of the heavy chain of human plasma kallikrein on its functional properties, *Blood 65*, 311-318.
71. Burger, D., Schleuning, W. D., and Schapira, M. (1986) Human plasma prekallikrein. Immunoaffinity purification and activation to alpha- and beta-kallikrein, *J Biol Chem 261*, 324-327.

72. Page, J. D., and Colman, R. W. (1991) Localization of distinct functional domains on prekallikrein for interaction with both high molecular weight kininogen and activated factor XII in a 28-kDa fragment (amino acids 141-371), *J Biol Chem 266*, 8143-8148.
73. Herwald, H., Jahnen-Dechent, W., Alla, S. A., Hock, J., Bouma, B. N., and Muller-Esterl, W. (1993) Mapping of the high molecular weight kininogen binding site of prekallikrein. Evidence for a discontinuous epitope formed by distinct segments of the prekallikrein heavy chain, *J Biol Chem 268*, 14527-14535.
74. Renne, T., Sugiyama, A., Gailani, D., Jahnen-Dechent, W., Walter, U., and Muller-Esterl, W. (2002) Fine mapping of the H-kininogen binding site in plasma prekallikrein apple domain 2, *Int Immunopharmacol 2*, 1867-1873.
75. Page, J. D., You, J. L., Harris, R. B., and Colman, R. W. (1994) Localization of the binding site on plasma kallikrein for high-molecular-weight kininogen to both apple 1 and apple 4 domains of the heavy chain, *Arch Biochem Biophys 314*, 159-164.
76. Colman, R. W., Schapira, M., and Scott, C. F. (1981) Regulation of the formation and inhibition of human plasma kallikrein, *Ann N Y Acad Sci 370*, 261-270.
77. Schapira, M., Scott, C. F., and Colman, R. W. (1981) Protection of human plasma kallikrein from inactivation by C1 inhibitor and other protease inhibitors. The role of high molecular weight kininogen, *Biochemistry 20*, 2738-2743.
78. Scott, C. F., Silver, L. D., Schapira, M., and Colman, R. W. (1984) Cleavage of human high molecular weight kininogen markedly enhances its coagulant activity. Evidence that this molecule exists as a procofactor, *J Clin Invest 73*, 954-962.
79. Ichinose, A., Fujikawa, K., and Suyama, T. (1986) The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin, *J Biol Chem 261*, 3486-3489.
80. Colman, R. W. (1969) Activation of plasminogen by human plasma kallikrein, *Biochem Biophys Res Commun 35*, 273-279.
81. Kellermann, J., Lottspeich, F., Henschen, A., and Muller-Esterl, W. (1986) Completion of the primary structure of human high-molecular-mass kininogen. The amino acid sequence of the entire heavy chain and evidence for its evolution by gene triplication, *Eur J Biochem 154*, 471-478.
82. Lottspeich, F., Kellermann, J., Henschen, A., Foertsch, B., and Muller-Esterl, W. (1985) The amino acid sequence of the light chain of human high-molecular-mass kininogen, *Eur J Biochem 152*, 307-314.
83. Nishikawa, K., Shibayama, Y., Kuna, P., Calcaterra, E., Kaplan, A. P., and Reddigari, S. R. (1992) Generation of vasoactive peptide bradykinin from human umbilical vein endothelium-bound high molecular weight kininogen by plasma kallikrein, *Blood 80*, 1980-1988.
84. Kunapuli, S. P., DeLa Cadena, R. A., and Colman, R. W. (1993) Deletion mutagenesis of high molecular weight kininogen light chain. Identification of two anionic surface binding subdomains, *J Biol Chem 268*, 2486-2492.
85. Wachtfogel, Y. T., DeLa Cadena, R. A., Kunapuli, S. P., Rick, L., Miller, M., Schultze, R. L., Altieri, D. C., Edgington, T. S., and Colman, R. W. (1994) High molecular weight kininogen binds to Mac-1 on neutrophils by its heavy chain (domain 3) and its light chain (domain 5), *J Biol Chem 269*, 19307-19312.
86. Hasan, A. A., Cines, D. B., Herwald, H., Schmaier, A. H., and Muller-Esterl, W. (1995) Mapping the cell binding site on high molecular weight kininogen domain 5, *J Biol Chem 270*, 19256-19261.
87. Gustafson, E. J., Schutsky, D., Knight, L. C., and Schmaier, A. H. (1986) High molecular weight kininogen binds to unstimulated platelets, *J Clin Invest 78*, 310-318.

88. Pan, Z. K., Zuraw, B. L., Lung, C. C., Prossnitz, E. R., Browning, D. D., and Ye, R. D. (1996) Bradykinin stimulates NF-kappaB activation and interleukin 1beta gene expression in cultured human fibroblasts, *J Clin Invest 98*, 2042-2049.
89. Brunius, G., Domeij, H., Gustavsson, A., and Yucel-Lindberg, T. (2005) Bradykinin upregulates IL-8 production in human gingival fibroblasts stimulated by interleukin-1beta and tumor necrosis factor alpha, *Regul Pept 126*, 183-188.
90. Kaplan, A. P., and Ghebrehiwet, B. The plasma bradykinin-forming pathways and its interrelationships with complement, *Mol Immunol 47*, 2161-2169.
91. Duchene, J., and Ahluwalia, A. (2009) The kinin B(1) receptor and inflammation: new therapeutic target for cardiovascular disease, *Curr Opin Pharmacol 9*, 125-131.
92. Holland, J. A., Pritchard, K. A., Pappolla, M. A., Wolin, M. S., Rogers, N. J., and Stemerman, M. B. (1990) Bradykinin induces superoxide anion release from human endothelial cells, *J Cell Physiol 143*, 21-25.
93. Hong, S. L. (1980) Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein, *Thromb Res 18*, 787-795.
94. Palmer, R. M., Ferrige, A. G., and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, *Nature 327*, 524-526.
95. Nakashima, M., Mombouli, J. V., Taylor, A. A., and Vanhoutte, P. M. (1993) Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries, *J Clin Invest 92*, 2867-2871.
96. Balaguer, J. M., Yu, C., Byrne, J. G., Ball, S. K., Petracek, M. R., Brown, N. J., and Pretorius, M. Contribution of endogenous bradykinin to fibrinolysis, inflammation, and blood product transfusion following cardiac surgery: a randomized clinical trial, *Clin Pharmacol Ther 93*, 326-334.
97. Brown, N. J., Gainer, J. V., Stein, C. M., and Vaughan, D. E. (1999) Bradykinin stimulates tissue plasminogen activator release in human vasculature, *Hypertension 33*, 1431-1435.
98. Sabatini, F., Luppi, F., Petecchia, L., Stefano, A. D., Longo, A. M., Eva, A., Vanni, C., Hiemstra, P. S., Sterk, P. J., Sorbello, V., Fabbri, L. M., Rossi, G. A., and Ricciardolo, F. L. Bradykinin-induced asthmatic fibroblast/myofibroblast activities via bradykinin B2 receptor and different MAPK pathways, *Eur J Pharmacol 710*, 100-109.
99. Ricciardolo, F. L., Sorbello, V., Benedetto, S., Defilippi, I., Sabatini, F., Robotti, A., van Renswouw, D. C., Bucca, C., Folkerts, G., and De Rose, V. Bradykinin- and lipopolysaccharide-induced bradykinin B2 receptor expression, interleukin 8 release and "nitrosative stress" in bronchial epithelial cells BEAS-2B: role for neutrophils, *Eur J Pharmacol 694*, 30-38.
100. Perron, M. S., Gobeil, F., Jr., Pelletier, S., Regoli, D., and Sirois, P. (1999) Involvement of bradykinin B1 and B2 receptors in pulmonary leukocyte accumulation induced by Sephadex beads in guinea pigs, *Eur J Pharmacol 376*, 83-89.
101. Koyama, S., Sato, E., Numanami, H., Kubo, K., Nagai, S., and Izumi, T. (2000) Bradykinin stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activity, *Am J Respir Cell Mol Biol 22*, 75-84.
102. Huang, C. D., Tliba, O., Panettieri, R. A., Jr., and Amrani, Y. (2003) Bradykinin induces interleukin-6 production in human airway smooth muscle cells: modulation by Th2 cytokines and dexamethasone, *Am J Respir Cell Mol Biol 28*, 330-338.
103. Figueroa, C. D., Henderson, L. M., Kaufmann, J., De La Cadena, R. A., Colman, R. W., Muller-Esterl, W., and Bhoola, K. D. (1992) Immunovisualization of high (HK) and low (LK) molecular weight kininogens on isolated human neutrophils, *Blood 79*, 754-759.

104. Futosi, K., Fodor, S., and Mocsai, A. Neutrophil cell surface receptors and their intracellular signal transduction pathways, *Int Immunopharmacol 17*, 638-650.
105. LaRusch, G. A., Mahdi, F., Shariat-Madar, Z., Adams, G., Sitrin, R. G., Zhang, W. M., McCrae, K. R., and Schmaier, A. H. Factor XII stimulates ERK1/2 and Akt through uPAR, integrins, and the EGFR to initiate angiogenesis, *Blood 115*, 5111-5120.
106. Herwald, H., Dedio, J., Kellner, R., Loos, M., and Muller-Esterl, W. (1996) Isolation and characterization of the kininogen-binding protein p33 from endothelial cells. Identity with the gC1q receptor, *J Biol Chem 271*, 13040-13047.
107. Hasan, A. A., Zisman, T., and Schmaier, A. H. (1998) Identification of cytokeratin 1 as a binding protein and presentation receptor for kininogens on endothelial cells, *Proc Natl Acad Sci U S A 95*, 3615-3620.
108. Renne, T., Pozgajova, M., Gruner, S., Schuh, K., Pauer, H. U., Burfeind, P., Gailani, D., and Nieswandt, B. (2005) Defective thrombus formation in mice lacking coagulation factor XII, *J Exp Med 202*, 271-281.
109. Kleinschnitz, C., Stoll, G., Bendszus, M., Schuh, K., Pauer, H. U., Burfeind, P., Renne, C., Gailani, D., Nieswandt, B., and Renne, T. (2006) Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis, *J Exp Med 203*, 513-518.
110. Konings, J., Govers-Riemslag, J. W., Philippou, H., Mutch, N. J., Borissoff, J. I., Allan, P., Mohan, S., Tans, G., Ten Cate, H., and Ariens, R. A. Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin, *Blood 118*, 3942-3951.
111. Pham, M., Kleinschnitz, C., Helluy, X., Bartsch, A. J., Austinat, M., Behr, V. C., Renne, T., Nieswandt, B., Stoll, G., and Bendszus, M. Enhanced cortical reperfusion protects coagulation factor XII-deficient mice from ischemic stroke as revealed by high-field MRI, *Neuroimage 49*, 2907-2914.
112. Wang, X., Cheng, Q., Xu, L., Feuerstein, G. Z., Hsu, M. Y., Smith, P. L., Seiffert, D. A., Schumacher, W. A., Ogletree, M. L., and Gailani, D. (2005) Effects of factor IX or factor XI deficiency on ferric chloride-induced carotid artery occlusion in mice, *J Thromb Haemost 3*, 695-702.
113. Revenko, A. S., Gao, D., Crosby, J. R., Bhattacharjee, G., Zhao, C., May, C., Gailani, D., Monia, B. P., and MacLeod, A. R. Selective depletion of plasma prekallikrein or coagulation factor XII inhibits thrombosis in mice without increased risk of bleeding, *Blood 118*, 5302-5311.
114. Kaplan, A. P., Kay, A. B., and Austen, K. F. (1972) A prealbumin activator of prekallikrein. 3. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein, *J Exp Med 135*, 81-97.
115. Toossi, Z., Sedor, J. R., Mettler, M. A., Everson, B., Young, T., and Ratnoff, O. D. (1992) Induction of expression of monocyte interleukin 1 by Hageman factor (factor XII), *Proc Natl Acad Sci U S A 89*, 11969-11972.
116. Schapira, M., Despland, E., Scott, C. F., Boxer, L. A., and Colman, R. W. (1982) Purified human plasma kallikrein aggregates human blood neutrophils, *J Clin Invest 69*, 1199-1202.
117. Wachtfogel, Y. T., Pixley, R. A., Kucich, U., Abrams, W., Weinbaum, G., Schapira, M., and Colman, R. W. (1986) Purified plasma factor XIIa aggregates human neutrophils and causes degranulation, *Blood 67*, 1731-1737.
118. Jansen, P. M., Pixley, R. A., Brouwer, M., de Jong, I. W., Chang, A. C., Hack, C. E., Taylor, F. B., Jr., and Colman, R. W. (1996) Inhibition of factor XII in septic baboons attenuates the activation of complement and fibrinolytic systems and reduces the release of interleukin-6 and neutrophil elastase, *Blood 87*, 2337-2344.
119. Wachtfogel, Y. T., Kucich, U., James, H. L., Scott, C. F., Schapira, M., Zimmerman, M., Cohen, A. B., and Colman, R. W. (1983) Human plasma

kallikrein releases neutrophil elastase during blood coagulation, *J Clin Invest 72*, 1672-1677.
120. Tucker, E. I., Verbout, N. G., Leung, P. Y., Hurst, S., McCarty, O. J., Gailani, D., and Gruber, A. Inhibition of factor XI activation attenuates inflammation and coagulopathy while improving the survival of mouse polymicrobial sepsis, *Blood 119*, 4762-4768.
121. Kaplan, A. P., Joseph, K., and Silverberg, M. (2002) Pathways for bradykinin formation and inflammatory disease, *J Allergy Clin Immunol 109*, 195-209.
122. Davis, A. E., 3rd, Lu, F., and Mejia, P. C1 inhibitor, a multi-functional serine protease inhibitor, *Thromb Haemost 104*, 886-893.
123. Coutinho, M., Aulak, K. S., and Davis, A. E., 3rd. (1994) Functional analysis of the serpin domain of C1 inhibitor, *J Immunol 153*, 3648-3654.
124. Huntington, J. A., Read, R. J., and Carrell, R. W. (2000) Structure of a serpin-protease complex shows inhibition by deformation, *Nature 407*, 923-926.
125. Pemberton, P. A., Harrison, R. A., Lachmann, P. J., and Carrell, R. W. (1989) The structural basis for neutrophil inactivation of C1 inhibitor, *Biochem J 258*, 193-198.
126. Cai, S., and Davis, A. E., 3rd. (2003) Complement regulatory protein C1 inhibitor binds to selectins and interferes with endothelial-leukocyte adhesion, *J Immunol 171*, 4786-4791.
127. Cai, S., Dole, V. S., Bergmeier, W., Scafidi, J., Feng, H., Wagner, D. D., and Davis, A. E., 3rd. (2005) A direct role for C1 inhibitor in regulation of leukocyte adhesion, *J Immunol 174*, 6462-6466.
128. Rubenfeld, G. D., Caldwell, E., Peabody, E., Weaver, J., Martin, D. P., Neff, M., Stern, E. J., and Hudson, L. D. (2005) Incidence and outcomes of acute lung injury, *N Engl J Med 353*, 1685-1693.
129. Linko, R., Okkonen, M., Pettila, V., Perttila, J., Parviainen, I., Ruokonen, E., Tenhunen, J., Ala-Kokko, T., and Varpula, T. (2009) Acute respiratory failure in intensive care units. FINNALI: a prospective cohort study, *Intensive Care Med 35*, 1352-1361.
130. Sigurdsson, M. I., Sigvaldason, K., Gunnarsson, T. S., Moller, A., and Sigurdsson, G. H. Acute respiratory distress syndrome: nationwide changes in incidence, treatment and mortality over 23 years, *Acta Anaesthesiol Scand 57*, 37-45.
131. Villar, J., Blanco, J., Anon, J. M., Santos-Bouza, A., Blanch, L., Ambros, A., Gandia, F., Carriedo, D., Mosteiro, F., Basaldua, S., Fernandez, R. L., and Kacmarek, R. M. The ALIEN study: incidence and outcome of acute respiratory distress syndrome in the era of lung protective ventilation, *Intensive Care Med 37*, 1932-1941.
132. Ware, L. B., and Matthay, A. (2000) The Acute Respiratory Distress Syndrome, *The New England Journal of Medicine*
342, 1334-1349.
133. Erickson, S. E., Martin, G. S., Davis, J. L., Matthay, M. A., and Eisner, M. D. (2009) Recent trends in acute lung injury mortality: 1996-2005, *Crit Care Med 37*, 1574-1579.
134. Ashbaugh, D. G., Bigelow, D. B., Petty, T. L., and Levine, B. E. (1967) Acute respiratory distress in adults, *Lancet 2*, 319-323.
135. Bernard, G. R., Artigas, A., Brigham, K. L., Carlet, J., Falke, K., Hudson, L., Lamy, M., LeGall, J. R., Morris, A., and Spragg, R. (1994) Report of the American-European Consensus conference on acute respiratory distress syndrome: definitions, mechanisms, relevant outcomes, and clinical trial coordination. Consensus Committee, *J Crit Care 9*, 72-81.
136. Force, T. A. D. T. (2012) Acute respiratory distress syndrome: the Berlin definition, *JAMA 307(23)*, 2526-2533.

137. Villar, J. What is the acute respiratory distress syndrome?, *Respir Care 56*, 1539-1545.
138. Tsushima, K., King, L. S., Aggarwal, N. R., De Gorordo, A., D'Alessio, F. R., and Kubo, K. (2009) Acute lung injury review, *Intern Med 48*, 621-630.
139. Goodman, R. B., Strieter, R. M., Martin, D. P., Steinberg, K. P., Milberg, J. A., Maunder, R. J., Kunkel, S. L., Walz, A., Hudson, L. D., and Martin, T. R. (1996) Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome, *Am J Respir Crit Care Med 154*, 602-611.
140. Grommes, J., and Soehnlein, O. Contribution of neutrophils to acute lung injury, *Mol Med 17*, 293-307.
141. Meduri, G. U., Headley, S., Kohler, G., Stentz, F., Tolley, E., Umberger, R., and Leeper, K. (1995) Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time, *Chest 107*, 1062-1073.
142. Meduri, G. U., Kohler, G., Headley, S., Tolley, E., Stentz, F., and Postlethwaite, A. (1995) Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome, *Chest 108*, 1303-1314.
143. Bhatia, M., Zemans, R. L., and Jeyaseelan, S. Role of chemokines in the pathogenesis of acute lung injury, *Am J Respir Cell Mol Biol 46*, 566-572.
144. Zemans, R. L., Colgan, S. P., and Downey, G. P. (2009) Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury, *Am J Respir Cell Mol Biol 40*, 519-535.
145. Ginzberg, H. H., Cherapanov, V., Dong, Q., Cantin, A., McCulloch, C. A., Shannon, P. T., and Downey, G. P. (2001) Neutrophil-mediated epithelial injury during transmigration: role of elastase, *Am J Physiol Gastrointest Liver Physiol 281*, G705-717.
146. Bdeir, K., Higazi, A. A., Kulikovskaya, I., Christofidou-Solomidou, M., Vinogradov, S. A., Allen, T. C., Idell, S., Linzmeier, R., Ganz, T., and Cines, D. B. Neutrophil alpha-defensins cause lung injury by disrupting the capillary-epithelial barrier, *Am J Respir Crit Care Med 181*, 935-946.
147. Morales, M. M., Pires-Neto, R. C., Inforsato, N., Lancas, T., da Silva, L. F., Saldiva, P. H., Mauad, T., Carvalho, C. R., Amato, M. B., and Dolhnikoff, M. Small airway remodeling in acute respiratory distress syndrome: a study in autopsy lung tissue, *Crit Care 15*, R4.
148. Fukuda, Y., Ishizaki, M., Masuda, Y., Kimura, G., Kawanami, O., and Masugi, Y. (1987) The role of intraalveolar fibrosis in the process of pulmonary structural remodeling in patients with diffuse alveolar damage, *Am J Pathol 126*, 171-182.
149. Matthay, M. A., and Zemans, R. L. The acute respiratory distress syndrome: pathogenesis and treatment, *Annu Rev Pathol 6*, 147-163.
150. Bellingan, G. J. (2002) The pulmonary physician in critical care * 6: The pathogenesis of ALI/ARDS, *Thorax 57*, 540-546.
151. Rocco, P. R., Dos Santos, C., and Pelosi, P. (2009) Lung parenchyma remodeling in acute respiratory distress syndrome, *Minerva Anestesiol 75*, 730-740.
152. Glas, G. J., Van Der Sluijs, K. F., Schultz, M. J., Hofstra, J. J., Van Der Poll, T., and Levi, M. Bronchoalveolar hemostasis in lung injury and acute respiratory distress syndrome, *J Thromb Haemost 11*, 17-25.
153. Marshall, R., Bellingan, G., and Laurent, G. (1998) The acute respiratory distress syndrome: fibrosis in the fast lane, *Thorax 53*, 815-817.
154. Clark, J. G., Milberg, J. A., Steinberg, K. P., and Hudson, L. D. (1995) Type III procollagen peptide in the adult respiratory distress syndrome. Association of increased peptide levels in bronchoalveolar lavage fluid with increased risk for death, *Ann Intern Med 122*, 17-23.
155. Flores, C., Pino-Yanes, M. M., Casula, M., and Villar, J. Genetics of acute lung injury: past, present and future, *Minerva Anestesiol 76*, 860-864.

156. Ignotz, R. A., and Massague, J. (1986) Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix, *J Biol Chem 261*, 4337-4345.
157. Fahy, R. J., Lichtenberger, F., McKeegan, C. B., Nuovo, G. J., Marsh, C. B., and Wewers, M. D. (2003) The acute respiratory distress syndrome: a role for transforming growth factor-beta 1, *Am J Respir Cell Mol Biol 28*, 499-503.
158. Degryse, A. L., Tanjore, H., Xu, X. C., Polosukhin, V. V., Jones, B. R., Boomershine, C. S., Ortiz, C., Sherrill, T. P., McMahon, F. B., Gleaves, L. A., Blackwell, T. S., and Lawson, W. E. TGFbeta signaling in lung epithelium regulates bleomycin-induced alveolar injury and fibroblast recruitment, *Am J Physiol Lung Cell Mol Physiol 300*, L887-897.
159. Phan, S. H., and Kunkel, S. L. (1992) Lung cytokine production in bleomycin-induced pulmonary fibrosis, *Exp Lung Res 18*, 29-43.
160. Kalayarasan, S., Sriram, N., and Sudhandiran, G. (2008) Diallyl sulfide attenuates bleomycin-induced pulmonary fibrosis: critical role of iNOS, NF-kappaB, TNF-alpha and IL-1beta, *Life Sci 82*, 1142-1153.
161. Kolb, M., Margetts, P. J., Anthony, D. C., Pitossi, F., and Gauldie, J. (2001) Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis, *J Clin Invest 107*, 1529-1536.
162. Gunther, A., Mosavi, P., Heinemann, S., Ruppert, C., Muth, H., Markart, P., Grimminger, F., Walmrath, D., Temmesfeld-Wollbruck, B., and Seeger, W. (2000) Alveolar fibrin formation caused by enhanced procoagulant and depressed fibrinolytic capacities in severe pneumonia. Comparison with the acute respiratory distress syndrome, *Am J Respir Crit Care Med 161*, 454-462.
163. Idell, S., James, K. K., Levin, E. G., Schwartz, B. S., Manchanda, N., Maunder, R. J., Martin, T. R., McLarty, J., and Fair, D. S. (1989) Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome, *J Clin Invest 84*, 695-705.
164. Idell, S., Koenig, K. B., Fair, D. S., Martin, T. R., McLarty, J., and Maunder, R. J. (1991) Serial abnormalities of fibrin turnover in evolving adult respiratory distress syndrome, *Am J Physiol 261*, L240-248.
165. Wygrecka, M., Jablonska, E., Guenther, A., Preissner, K. T., and Markart, P. (2008) Current view on alveolar coagulation and fibrinolysis in acute inflammatory and chronic interstitial lung diseases, *Thromb Haemost 99*, 494-501.
166. Ware, L. B., Matthay, M. A., Parsons, P. E., Thompson, B. T., Januzzi, J. L., and Eisner, M. D. (2007) Pathogenetic and prognostic significance of altered coagulation and fibrinolysis in acute lung injury/acute respiratory distress syndrome, *Crit Care Med 35*, 1821-1828.
167. Chambers, R. C., and Laurent, G. J. (2002) Coagulation cascade proteases and tissue fibrosis, *Biochem Soc Trans 30*, 194-200.
168. Miller, D. L., Welty-Wolf, K., Carraway, M. S., Ezban, M., Ghio, A., Suliman, H., and Piantadosi, C. A. (2002) Extrinsic coagulation blockade attenuates lung injury and proinflammatory cytokine release after intratracheal lipopolysaccharide, *Am J Respir Cell Mol Biol 26*, 650-658.
169. Chu, A. J. (2005) Tissue factor mediates inflammation, *Arch Biochem Biophys 440*, 123-132.
170. O'Brien, M. The reciprocal relationship between inflammation and coagulation, *Top Companion Anim Med 27*, 46-52.
171. Cirino, G., Cicala, C., Bucci, M., Sorrentino, L., Ambrosini, G., DeDominicis, G., and Altieri, D. C. (1997) Factor Xa as an interface between coagulation and inflammation. Molecular mimicry of factor Xa association with effector cell protease receptor-1 induces acute inflammation in vivo, *J Clin Invest 99*, 2446-2451.

172. Ma, L., and Dorling, A. The roles of thrombin and protease-activated receptors in inflammation, *Semin Immunopathol 34*, 63-72.
173. Ludwicka-Bradley, A., Tourkina, E., Suzuki, S., Tyson, E., Bonner, M., Fenton, J. W., 2nd, Hoffman, S., and Silver, R. M. (2000) Thrombin upregulates interleukin-8 in lung fibroblasts via cleavage of proteolytically activated receptor-I and protein kinase C-gamma activation, *Am J Respir Cell Mol Biol 22*, 235-243.
174. Szotowski, B., Antoniak, S., Poller, W., Schultheiss, H. P., and Rauch, U. (2005) Procoagulant soluble tissue factor is released from endothelial cells in response to inflammatory cytokines, *Circ Res 96*, 1233-1239.
175. Senior, R. M., Skogen, W. F., Griffin, G. L., and Wilner, G. D. (1986) Effects of fibrinogen derivatives upon the inflammatory response. Studies with human fibrinopeptide B, *J Clin Invest 77*, 1014-1019.
176. Leavell, K. J., Peterson, M. W., and Gross, T. J. (1996) The role of fibrin degradation products in neutrophil recruitment to the lung, *Am J Respir Cell Mol Biol 14*, 53-60.
177. Gray, A. J., Bishop, J. E., Reeves, J. T., Mecham, R. P., and Laurent, G. J. (1995) Partially degraded fibrin(ogen) stimulates fibroblast proliferation in vitro, *Am J Respir Cell Mol Biol 12*, 684-690.
178. Chambers, R. C., Dabbagh, K., McAnulty, R. J., Gray, A. J., Blanc-Brude, O. P., and Laurent, G. J. (1998) Thrombin stimulates fibroblast procollagen production via proteolytic activation of protease-activated receptor 1, *Biochem J 333 (Pt 1)*, 121-127.
179. Blanc-Brude, O. P., Archer, F., Leoni, P., Derian, C., Bolsover, S., Laurent, G. J., and Chambers, R. C. (2005) Factor Xa stimulates fibroblast procollagen production, proliferation, and calcium signaling via PAR1 activation, *Exp Cell Res 304*, 16-27.
180. Blanc-Brude, O. P., Chambers, R. C., Leoni, P., Dik, W. A., and Laurent, G. J. (2001) Factor Xa is a fibroblast mitogen via binding to effector-cell protease receptor-1 and autocrine release of PDGF, *Am J Physiol Cell Physiol 281*, C681-689.
181. Chen, L. B., and Buchanan, J. M. (1975) Mitogenic activity of blood components. I. Thrombin and prothrombin, *Proc Natl Acad Sci U S A 72*, 131-135.
182. Jabłońska, E., Markart, P., Preissner, K. T., Zakrzewicz, D., and Wygrecka, M. (2010) Role of intrinsic coagulation pathway in the pathogenesis of idiopathic pulmonary fibrosis, in *Department of Biochemistry*, p 90, University of Giessen, Giessen.
183. Jablonska, E., Markart, P., Zakrzewicz, D., Preissner, K. T., and Wygrecka, M. Transforming growth factor-beta1 induces expression of human coagulation factor XII via Smad3 and JNK signaling pathways in human lung fibroblasts, *J Biol Chem 285*, 11638-11651.
184. Persson, K., Morgelin, M., Lindbom, L., Alm, P., Bjorck, L., and Herwald, H. (2000) Severe lung lesions caused by Salmonella are prevented by inhibition of the contact system, *J Exp Med 192*, 1415-1424.
185. Pittet, J. F., Griffiths, M. J., Geiser, T., Kaminski, N., Dalton, S. L., Huang, X., Brown, L. A., Gotwals, P. J., Koteliansky, V. E., Matthay, M. A., and Sheppard, D. (2001) TGF-beta is a critical mediator of acute lung injury, *J Clin Invest 107*, 1537-1544.
186. Scotton, C. J., Krupiczojc, M. A., Konigshoff, M., Mercer, P. F., Lee, Y. C., Kaminski, N., Morser, J., Post, J. M., Maher, T. M., Nicholson, A. G., Moffatt, J. D., Laurent, G. J., Derian, C. K., Eickelberg, O., and Chambers, R. C. (2009) Increased local expression of coagulation factor X contributes to the fibrotic response in human and murine lung injury, *J Clin Invest 119*, 2550-2563.

187. Johnson, E. R., and Matthay, M. A. Acute lung injury: epidemiology, pathogenesis, and treatment, *J Aerosol Med Pulm Drug Deliv 23*, 243-252.
188. Motta, G., Rojkjaer, R., Hasan, A. A., Cines, D. B., and Schmaier, A. H. (1998) High molecular weight kininogen regulates prekallikrein assembly and activation on endothelial cells: a novel mechanism for contact activation, *Blood 91*, 516-528.
189. Gailani, D., and Broze, G. J., Jr. (1993) Factor XII-independent activation of factor XI in plasma: effects of sulfatides on tissue factor-induced coagulation, *Blood 82*, 813-819.
190. Pixley, R. A., Zellis, S., Bankes, P., DeLa Cadena, R. A., Page, J. D., Scott, C. F., Kappelmayer, J., Wyshock, E. G., Kelly, J. J., and Colman, R. W. (1995) Prognostic value of assessing contact system activation and factor V in systemic inflammatory response syndrome, *Crit Care Med 23*, 41-51.
191. Oehmcke, S., Morgelin, M., and Herwald, H. (2009) Activation of the human contact system on neutrophil extracellular traps, *J Innate Immun 1*, 225-230.
192. Bengtson, S. H., Sanden, C., Morgelin, M., Marx, P. F., Olin, A. I., Leeb-Lundberg, L. M., Meijers, J. C., and Herwald, H. (2009) Activation of TAFI on the surface of Streptococcus pyogenes evokes inflammatory reactions by modulating the kallikrein/kinin system, *J Innate Immun 1*, 18-28.
193. Mattsson, E., Herwald, H., Cramer, H., Persson, K., Sjobring, U., and Bjorck, L. (2001) Staphylococcus aureus induces release of bradykinin in human plasma, *Infect Immun 69*, 3877-3882.
194. Pixley, R. A., De La Cadena, R., Page, J. D., Kaufman, N., Wyshock, E. G., Chang, A., Taylor, F. B., Jr., and Colman, R. W. (1993) The contact system contributes to hypotension but not disseminated intravascular coagulation in lethal bacteremia. In vivo use of a monoclonal anti-factor XII antibody to block contact activation in baboons, *J Clin Invest 91*, 61-68.
195. Idell, S., Kucich, U., Fein, A., Kueppers, F., James, H. L., Walsh, P. N., Weinbaum, G., Colman, R. W., and Cohen, A. B. (1985) Neutrophil elastase-releasing factors in bronchoalveolar lavage from patients with adult respiratory distress syndrome, *Am Rev Respir Dis 132*, 1098-1105.
196. Donnelly, S. C., MacGregor, I., Zamani, A., Gordon, M. W., Robertson, C. E., Steedman, D. J., Little, K., and Haslett, C. (1995) Plasma elastase levels and the development of the adult respiratory distress syndrome, *Am J Respir Crit Care Med 151*, 1428-1433.
197. Carden, D., Xiao, F., Moak, C., Willis, B. H., Robinson-Jackson, S., and Alexander, S. (1998) Neutrophil elastase promotes lung microvascular injury and proteolysis of endothelial cadherins, *Am J Physiol 275*, H385-392.
198. Carl, V. S., Moore, E. E., Moore, F. A., and Whalley, E. T. (1996) Involvement of bradykinin B1 and B2 receptors in human PMN elastase release and increase in endothelial cell monolayer permeability, *Immunopharmacology 33*, 325-329.
199. Marshall, R. P., Bellingan, G., Webb, S., Puddicombe, A., Goldsack, N., McAnulty, R. J., and Laurent, G. J. (2000) Fibroproliferation occurs early in the acute respiratory distress syndrome and impacts on outcome, *Am J Respir Crit Care Med 162*, 1783-1788.
200. Vancheri, C., Gili, E., Failla, M., Mastruzzo, C., Salinaro, E. T., Lofurno, D., Pistorio, M. P., La Rosa, C., Caruso, M., and Crimi, N. (2005) Bradykinin differentiates human lung fibroblasts to a myofibroblast phenotype via the B2 receptor, *J Allergy Clin Immunol 116*, 1242-1248.
201. Matute-Bello, G., Frevert, C. W., and Martin, T. R. (2008) Animal models of acute lung injury, *Am J Physiol Lung Cell Mol Physiol 295*, L379-399.
202. de Beer, F. M., Aslami, H., Hoeksma, J., van Mierlo, G., Wouters, D., Zeerleder, S., Roelofs, J. J., Juffermans, N. P., Schultz, M. J., and Lagrand, W. K. Plasma-Derived Human C1-Esterase Inhibitor Does Not Prevent Mechanical

Ventilation-Induced Pulmonary Complement Activation in a Rat Model of Streptococcus pneumoniae Pneumonia, *Cell Biochem Biophys*.

203. Guerrero, R., Velasco, F., Rodriguez, M., Lopez, A., Rojas, R., Alvarez, M. A., Villalba, R., Rubio, V., Torres, A., and del Castillo, D. (1993) Endotoxin-induced pulmonary dysfunction is prevented by C1-esterase inhibitor, *J Clin Invest 91*, 2754-2760.
204. Carvalho, A. C., DeMarinis, S., Scott, C. F., Silver, L. D., Schmaier, A. H., and Colman, R. W. (1988) Activation of the contact system of plasma proteolysis in the adult respiratory distress syndrome, *J Lab Clin Med 112*, 270-277.
205. Zeerleder, S., Caliezi, C., van Mierlo, G., Eerenberg-Belmer, A., Sulzer, I., Hack, C. E., and Wuillemin, W. A. (2003) Administration of C1 inhibitor reduces neutrophil activation in patients with sepsis, *Clin Diagn Lab Immunol 10*, 529-535.
206. Jansen, P. M., Eisele, B., de Jong, I. W., Chang, A., Delvos, U., Taylor, F. B., Jr., and Hack, C. E. (1998) Effect of C1 inhibitor on inflammatory and physiologic response patterns in primates suffering from lethal septic shock, *J Immunol 160*, 475-484.
207. Nuijens, J. H., Eerenberg-Belmer, A. J., Huijbregts, C. C., Schreuder, W. O., Felt-Bersma, R. J., Abbink, J. J., Thijs, L. G., and Hack, C. E. (1989) Proteolytic inactivation of plasma C1- inhibitor in sepsis, *J Clin Invest 84*, 443-450.
208. Henson, P. M., Larsen, G. L., Webster, R. O., Mitchell, B. C., Goins, A. J., and Henson, J. E. (1982) Pulmonary microvascular alterations and injury induced by complement fragments: synergistic effect of complement activation, neutrophil sequestration, and prostaglandins, *Ann N Y Acad Sci 384*, 287-300.
209. Liu, D., Cai, S., Gu, X., Scafidi, J., Wu, X., and Davis, A. E., 3rd. (2003) C1 inhibitor prevents endotoxin shock via a direct interaction with lipopolysaccharide, *J Immunol 171*, 2594-2601.

11. Declaration

Hereby, I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Rosanna Marie Hess