Universitätsklinikum Ulm

Zentrum für Chirurgie

Klinik für Unfall-, Hand-, Plastische und Wiederherstellungschirurgie

Ärztlicher Direktor: Univ. Prof. Dr. med. F. Gebhard

**DIVERGENT EFFECTS OF NEUTROPHILS ON FAS-INDUCED
PULMONARY INFLAMMATION, APOPTOSIS, AND LUNG DAMAGE**

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Bastian Sylvester Carl Wilhelm Bruns

aus Frankfurt a. M.

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Amtierender Dekan: Prof. Dr. T. Wirth
1. Berichterstatter: Prof. Dr. M. Perl
2. Berichterstatter: Prof. Dr. M. Weiß
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For my Family

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II. ABBREVIATIONS

ALI	Acute lung injury
AM	Alveolar macrophage
ANOVA	Analysis of variance
AP-1	Activating protein-1
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
Bw	Body weight
°C	Degree Celsius
CIP	Critical illness polyneuropathy
CLP	Cecal ligation und puncture
Casp-3	Caspase-3
CXC	Cysteins, separated by an arbitrary amino acid 'x'
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
d	Day
DED	Death effector domain
DISC	death inducing signalling complex
DPBS	Dulbecco's phosphate buffered saline
ELISA	Enzyme-linked immunosorbant assay
ERK1/2	Extracellular signal regulated kinase 1/2
FACS	Fluorescence activated cell sorting
FADD	Fas associated death domain
FasL	Fas ligand
FiO ₂	Fraction of inspired oxygen
FLICE	FADD-like interleukin-1 beta-converting enzyme
FLIP	FLICE inhibitory protein
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor

ABBREVIATIONS

h	Hour
HBP	Heparin binding protein
HRP	Streptavidin horseradish peroxidase
H&E	Hematoxylin-eosin
ICAM-1/2	intercellular adhesion molecule-1/2
ICU	Intensive care unit
IKK	I- κ B kinase komplex
IL	Interleukin
i.n.	intranasal
i.p.	intraperitoneal
ISS	Injury severity score
i.t.	intratracheal
KC	Keratinocyte-derived chemokine
LIS	Lung injury score
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
MPO	Myeloperoxidase
n	Total amount
NF- κ B	Nuclear factor-kappa B
NK	Natural killer cell
NOS	Nitric oxide synthase
PaO ₂	Partial pressure of arterial oxygen
PBS	Phosphate buffered saline
PMN	Polymorphonuclear neutrophil granulocyte
P	Niveau of significance
p38 MAPK	Mitogen-activated protein kinase p38
Raf-1	Rapidly accelerated fibrosarcoma kinase-1
RCT	Randomized control trial
RIP	Receptor interacting protein
RIPA-buffer	Radio immunoprecipitation assay-buffer

ABBREVIATIONS

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SIRS	Systemic inflammatory response syndrome
SNK	Student-Newman-Keuls test
Streptavidin-PE	Phycoerythrin-conjugated streptavidin
Tab.	Table
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TRADD	TNF-R (eceptor) associated death domain
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotide transferase dUTP nick end labeling
TxT	Chest trauma
U	Unit
VC	Vital capacity
VCAM-1	Vascular cell adhesion molecule-1

1. INTRODUCTION

1.1 Original description and definition of ALI

After observing twelve patients with acute respiratory distress that did not respond to respiratory therapy, Ashbaugh et al. first defined a form of lung dysfunction formerly known as the “respiratory distress syndrome” in 1967. The definition included acute onset of respiratory distress, oxygen refracted cyanosis, reduced lung compliance, and diffuse bilateral lung infiltrates on chest X-ray as early as 1 h, and as late as 96 h after divergent underlying conditions. The name was chosen in correlation to the well-known “infant respiratory distress syndrome” (IRDS) and postulated an association with intra-alveolar hyaline membranes, hence explaining the missing response to conventional respiratory therapy [13]. In 1994 the American-European Consensus Conference distinguished between “Acute Lung Injury” (ALI) and the “Acute Respiratory Distress Syndrome” (ARDS).

ALI is a clinical syndrome, characterized by acute and persistent inflammatory disease of the lungs featuring bilateral pulmonary infiltrates on chest X-ray, a Horovitz-index (ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen, $\text{PaO}_2/\text{FiO}_2$) of less than 300 mmHg, and absence of clinical evidence of left atrial hypertension (pulmonary capillary wedge pressure $\leq 18\text{mmHg}$). ARDS is defined correspondingly, but with increased hypoxemia of $\text{PaO}_2/\text{FiO}_2 \leq 200\text{ mmHg}$ [22]. In 2012 the Berlin Definition distinguished between mild ($200\text{ mmHg} \leq \text{PaO}_2/\text{FiO}_2 \leq 300\text{ mmHg}$), moderate ($100\text{ mmHg} \leq \text{PaO}_2/\text{FiO}_2 \leq 200\text{ mmHg}$), and severe ($\text{PaO}_2/\text{FiO}_2 \leq 100\text{ mmHg}$) ARDS with increased predictive validity for mortality [56]. Symptoms typically develop between 4 and 48h and persist for days or even weeks. Subacute or chronic lung diseases, such as sarcoidosis and idiopathic pulmonary fibrosis, are excluded per definition [22,194]. The distinction between ALI and ARDS is arbitrary, and the severity of hypoxia does neither correlate reliably with the extent of the underlying pathology [43], nor does it influence predictably clinical course or survival [172,174].

The clinical manifestations of ALI/ ARDS arise from divergent pathogenetic events, which collectively culminate in disruption of the alveolo-capillary barrier, leading to

interstitial and intra-alveolar hemorrhage and edema, hyaline membranes, and atelectasis [13,192].

1.2 Clinical relevance and epidemiology of ALI

ALI and ARDS present a worldwide medical challenge [165,192,194]. They have been associated with a high morbidity and mortality since publication of the syndrome definition in 1994 [157]. The incidence of ALI is 79:100,000 persons per year. With ARDS being the more severe form of the disease its incidence is reported to be around 59:100,000 persons per year [75,164,194]. About 7% of ICU patients in Europe and the U.S. will eventually develop ALI/ARDS [29,63]. Within the next 25 years Rubenfeld et al. estimate a duplication of incidence and consecutive deaths from these syndromes in the U.S. based upon projections of demographic characteristics [164]. Overall mortality risk estimates vary from 35-40% in randomized control trials (RCTs) to 40-45% based on observational studies [157]. Mortality greatly depends upon several factors including patient age, non-pulmonary organ dysfunction, and etiology [29,191,194]. Advanced age, shock, and hepatic failure are most predictive of death, whereas young trauma patients have the best outcomes [49]. Several observational studies conducted between the 1980s and mid-1990s postulated a decrease in mortality over time [2,126,135,162,178]. But the extent to which the results of these studies apply to the general ALI/ARDS-patient is questionable, due to the inclusion of many trauma patients, a subset of ALI/ARDS with a favourable prognosis [126,135,157,162,178]. Etiology may be categorized into direct, indirect, mixed, and idiopathic causes. Direct causes include pneumonia, aspiration, near-drowning, fat and amniotic-fluid embolism, smoke and toxic gas inhalation injury, pulmonary contusion, alveolar hemorrhage, and reperfusion pulmonary edema after lung transplantation or pulmonary embolectomy. Indirect causes comprise sepsis, trauma, shock, multiple transfusions, cardiopulmonary bypass, drug overdose, and acute pancreatitis [192,194]. Direct causes account for about 55%, indirect for 20% of ALI/ARDS cases [29]. In 21% there are mixed factors contributing, and in about 4% no underlying pathophysiology is found [29,153]. Furthermore it's important to emphasize the pathogenetic relevance of sepsis,

since about 25% of all ARDS cases stem from a severe sepsis [29]. Sepsis accounts for 46% of direct and 33% of indirect ALI cases [164]. It also presents the major risk factor for mortality, leading to death from ALI/ARDS in up to 50% of patients [29,191]. With respect to severe sepsis (comprising multiple organ failure (MOF)), mortality rates may be even higher [52]. Survivors of ALI/ARDS often suffer from persistent morbidity and sequelae associated with muscle-wasting and weakness (critical illness polyneuropathy (CIP)), as well as from an overall reduced physical condition and diminished vital capacity (VC) [74]. Also cognitive deficits may be present [74,77,78,182]. After 1 year, 45% of patients with ARDS showed cognitive impairment and 29% had mild to moderate symptoms of depression and anxiety, with the latter having a significant impact on the quality of life [77].

In spite of these unfortunate statistics, therapeutic strategies remain limited, and are confined to protective ventilation strategies, intermittent prone positioning, and early intervention to prevent further complications [1,180,186,194]. The absence of pathogenetically oriented treatment options [153] prompts the necessity for further research in order to unravel the underlying pathophysiology of ALI.

1.3 Pathophysiology of ALI

The initial pathogenic triggers may be very divergent and the structure of early events remains largely unclear. However, the common final trunk leads to disruption of the alveolo-capillary barrier with interstitial and intra-alveolar edema, compromised gas exchange with respiratory failure, hypoxemia and organ dysfunction [129,153,182,192,194]. Several pathogenetic mechanisms may contribute to this tragic finale:

1.3.1 The Neutrophil Hypothesis of ALI

According to the Neutrophil Hypothesis, alveolo-capillary barrier disruption is primarily a result of activated PMN infiltrating the lungs. The blood of the pulmonary capillary network contains about 50 times more PMN when compared to other vascular beds [30].

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PMN activation and recruitment to the lung can be observed early within lung tissue and bronchoalveolar lavage fluids (BALF) of patients with ALI/ARDS, resulting in a diffuse neutrophilic alveolar infiltrate [16,17,158,194]. This effect is initiated and perpetuated by a plethora of local and systemic cytokines, chemokines, complement, and coagulation factors, thus establishing pulmonary inflammation [8,9,44,45,48,55,82-84,170,194]. Apoptosis of PMN is decreased, accompanied by an increased apoptosis of pneumocytes [111].

Besides exerting beneficial functions of host defense, PMN are also known to have the ability to damage host structures via production and release of reactive oxygen species (ROS), nitric oxide species (NOS), elastases, proteases, and other lytic enzymes [18,35,153,193]. Furthermore oxidative stress activates redox-sensitive transcription factors (Nuclear factor-kappa B (NF- κ B), Activating protein-1 (AP-1)), resulting in a rapid generation of pro-inflammatory cytokines and chemokines [70]. Usually mature neutrophils circulate for 6 to 12 h before they undergo constitutive apoptosis [153]. However, several inflammatory agents (LPS, TNF- α , IL-1 β , IL-6, IL-8, G-CSF, GM-CSF, etc.), brought upon by previously discussed mechanisms, can inhibit PMN apoptosis, thus allowing a longer life span [46,47,50,87,102]. A diminished apoptotic response of PMN during sepsis was shown to be associated with a decreased activity of caspase-3 and caspase-9 [188]. During sepsis, as well as major trauma [26], burn injury [33], and ALI/ARDS [53] a prolonged survival of PMN can be observed. In this regard, plasma from patients with ARDS exerted an anti-apoptotic effect on PMN, apparently signalling through the GM-CSF receptor [62]. Nevertheless, GM-CSF in air-spaces was also linked to an increased survival in ARDS patients [116]. The presence of PMN has been shown to be required for development of full-blown ALI/ARDS in a clinically relevant animal model of shock-induced septic lung damage. Inhibition of the chemokine receptors CXCR 1 and 2, antagonizing macrophage inflammatory protein-2 (MIP-2) or keratinocyte chemoattractant (KC), as well as PMN depletion markedly reduced lung inflammation, lung damage, and severity of ALI [103-105]. In various other models of ALI (transfusion, reperfusion, endotoxemia), PMN depletion also ameliorated lung damage, further strengthening the role of PMN as a hostile player [3,36,106]. Transfer of the neutrophil inhibitory factor (NIF)-gene from *Ancylostoma caninum* into the mouse lung reduced PMN influx as well as lung damage induced by lipopolysaccharide (LPS) i.t. or i.v. [205]. Clinical

evidence also points to an important role of PMN in development of posttraumatic MOF [28,131].

Interestingly, ALI/ARDS may also occur during neutropenia [99,160], but it should be noted that the disease tends to progress rapidly once PMN counts are restored [15]. In contrast, in clinical studies septic patients received granulocyte-macrophage colony stimulating factor (GM-CSF) without an increased incidence of ALI [137]. Also, in patients with bacterial infections decreased mortality was observed following GM-CSF treatment [136]. Thus, the role of neutrophils seems to depend strongly on the underlying pathophysiology. Hyperoxia-, or oleic acid-induced ALI have been found to be independent of neutrophils, whereas the presence of neutrophils was crucial in intravenous or intraalveolar LPS-, or gram-negative sepsis-induced ALI [67]. Prolonged presence of PMNs worsened ALI in a murine model of SIRS, whereas depletion was protective with previous hemorrhagic shock and consecutive sepsis [151].

1.3.2 The Epithelial Cell Hypothesis

The Epithelial Cell Hypothesis advocates a primal role of lung epithelial cell apoptosis in destruction of the alveolo-capillary barrier, which is a key event in the pathogenesis of ALI. The alveolo-capillary barrier consists of 1) capillary endothelial cells, 2) basal laminae, and 3) lung alveolar epithelial cells (pneumocytes, AT) of type 1 and 2 [192]. Under physiological conditions the epithelial cell barrier is less permeable than the endothelial barrier, which emphasizes its importance in the development of protein rich alveolar edema [192,195]. The epithelial cell barrier implements trans-epithelial fluid transport, including active clearance of alveolar edema into the interstitium [130,185]. Therefore, isolated endothelial injury may not feature alveolar edema, whereas epithelial injury rapidly disturbs fluid balance [110,111]. Alveoli are lined mostly with flat and fragile type 1 pneumocytes (90%), and to a lesser extent with the cuboidal, more resilient type 2 pneumocytes (10%). Type 2 cells are of utter importance due to their ability to produce surfactant, as well as proliferate and differentiate into type 1 cells. The latter is part of the process towards restitutio ad integrum after injury [192]. Loss of type 2 cells leads to reduced surfactant production and turnover in humans [65], which is associated with atelectasis,

inhomogeneities regarding ventilation, and consecutive hypoxic vasoconstriction with intrapulmonary shunt flow, culminating in impaired gas exchange and hypoxemia [66,69,101]. Altogether, it is hardly surprising that the extent of epithelial cell damage and hindered edema clearance was found to correlate clinically with outcome in ALI patients [114]. As discussed earlier, ARDS features decreased PMN apoptosis. This is contrasted by an increased apoptosis of pneumocytes [111], which was linked to mediators of the Fas system within bronchioalveolar lavage fluid (BALF) of patients suffering from ALI [118].

1.3.3 The Role of Fas in ALI/ARDS

BALF and lung tissue from patients with ALI or ARDS showed increased levels of Fas and FasL as compared to corresponding plasma levels and control patients, indicating a local release in the lung [7]. The deficiency of Fas or FasL, and the inhibition of downstream effectors like caspase-3 showed the capacity to reduce ALI experimentally [152,154]. The Fas/FasL system, which has traditionally been regarded as an apoptotic pathway, has also been shown to be of pro-inflammatory capacity:

Fas (CD95) is a 45-kd type I membrane receptor and part of the tumor necrosis factor family of cell surface receptors [85]. FasL (CD95L) is a type II membrane glycoprotein belonging to the tumor necrosis factor family of cytokines [181]. Fas and FasL both exist in membrane bound and soluble variants (sFas/sFasL). FasL is cleaved from cell membranes by metalloproteinases to produce sFasL, while creation of sFas requires alternative splicing [32]. It's speculated that the effects of the Fas system are lost if sFas is bound by FasL/sFasL, e.g. providing an inhibitory mechanism to Fas-mediated apoptosis [32]. Interestingly, the amount of sFas surpasses sFasL in BALF of patients suffering from ARDS [7].

Human lung epithelial cells express Fas and are responsive to FasL induced apoptosis especially in the distal airways [134]. FasL is expressed on lymphocytes [11,132,163], monocytes [143], neutrophils [102,167], eosinophils [132], and platelets [5]. In plasma sFasL is elevated in a variety of pathological settings, including autoimmune disease [108], malignancy [166,187], and congestive heart failure [199]. In BALF, sFasL is found to be increased in patients with diverse

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pulmonary conditions, like pulmonary fibrosis [96,98], hypersensitivity pneumonitis [97], bronchiolitis obliterans [98], as well as in ALI/ARDS [7,115].

In this regard, higher concentrations of pulmonary Fas and FasL were shown to be associated with increased syndrome mortality in ALI [7,115]. In an animal model for direct ALI, featuring pulmonary instillation of LPS, inhibition of the Fas pathway lead to decreased severity of ALI [91]. Even in models of indirect ALI, inhibition of Fas lead to decreased pulmonary apoptosis, inflammation, and neutrophil accumulation [148,152].

It is necessary to emphasize that Fas-triggered lung injury relies upon Fas-expression on pneumocytes, and not on myeloid cells like macrophages or PMN. Matute Bello et al. generated chimeric mice lacking Fas in either myeloid or non-myeloid cells by transplanting marrow cells from *lpr* mutant mice (lacking Fas) into lethally irradiated C57BL/6 mice or vice versa. Additional mice transplanted with marrow cells from their same strain served as controls. Both groups received intratracheal instillations of the Fas-activating monoclonal antibody (mAb) Jo2 or an isotype control antibody (Ab). Only animals expressing Fas in non-myeloid cells displayed significant increases in lung neutrophil content and in alveolar permeability. These mice also showed tissue evidence of lung injury and caspase-3 activation in cells of the alveolar walls [120]. This observation consolidates the role of Fas as an initiator of alveolar-capillary barrier disruption by induction of lung epithelial cell apoptosis. Fas activation has also been shown to cause apoptosis of alveolar endothelial cells, but this effect was less pronounced than the effect on the alveolar epithelium [73].

Furthermore Fas-deficiency was associated with an ameliorated severity of ALI in pulmonary sepsis (*Escheria coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Legionella pneumophila*) [117,189]. Contrary to these findings, in a model of *Pseudomonas aeruginosa* associated pulmonary sepsis, reduced pneumocyte apoptosis by inhibition of the Fas system lead to an increased animal mortality [64]. The accumulated data concerning the association between the Fas system and apoptosis in ALI/ARDS is divergent in many aspects and would therefore benefit from further illumination.

1.3.3.1 Connecting Neutrophil and Epithelial Cell Hypothesis

As mentioned earlier, activation of Fas may also trigger inflammation as opposed to apoptosis by inducing the release of different cytokines and chemokines from a variety of cells [10,153]. This may act as a bridge connecting the neutrophil and epithelial cell hypothesis of ALI by recruitment of activated PMN to the lung [118]. Pulmonary activation of Fas was shown to facilitate an increase in TNF- α , IL-6, MIP-1 α , MIP-2, MCP-1, and KC, as well as a recruitment of PMN [117,196]. Inflammation could be reduced by antagonizing FasL [196]. Neither sole myeloid, nor sole non-myeloid activation of Fas resulted in significant inflammation, hinting at a required collaboration of lung epithelial cells and leukocytes [120]. According to Perl et al., stimulation of Fas on pneumocytes lead to secretion of MIP-2, KC, and MCP-1 through mechanisms involving ERK and, potentially FLIP [152]. Previously, Hagimoto et al. described an NF- κ B dependent pathway [72]. Fas activation is known to induce inflammation by a variety of cell types, including monocytes, macrophages [145], peritoneal macrophages [76], and endothelial cells [200]. Pulmonary inflammation induced by bacterial LPS seems to involve the Fas pathway, since Fas (CD95)- and FasL (CD178)-deficient mice were protected from neutrophilic inflammation [119]. Also, Fas/FasL mutant mice lungs showed markedly reduced inflammation in response to hemorrhage-induced septic ALI compared to control animals [152]. Therefore, an alternative pathway seems to be pursued somewhere along the road from Fas-activation to apoptosis. Among the discussed candidates are NF- κ B [145], the extracellular signal regulated kinase 1/2 (ERK 1/2), and the mitogen-activated protein kinase p38 (p38 MAPK) [141].

1.3.3.2 The Role of c-FLIP in Fas-Signalling

The cellular FLICE inhibitory protein (c-FLIP) plays an important role in non-apoptotic Fas-signalling. FLIP comes in three variants: c-FLIP_{L(long)}, c-FLIP_{S(short)}, c-FLIP_{R(raji)} [59,94,95,169]. c-FLIP molecules contain two serial amino-terminal death effector domains (DED) followed by a carboxy-terminal extension comprising a caspase-like domain similar to caspase-8 and caspase-10. However, due to the substitution of several amino acids, c-FLIP is devoid of proteolytic activity [138,190]. c-FLIP_S and c-FLIP_R are regarded as anti-apoptotic [59,94],

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whereas c-FLIP_L, when over expressed has been reported to be pro-, or anti-apoptotic [39,61,203]. The discovery that c-FLIP expression was up-regulated by activation of NF- κ B, provided a first molecular explanation to the well-known TNF-induced, NF- κ B-dependent survival pathway [93,124,198]. Moreover, recent studies suggest that c-FLIP_L is implicated in activation of NF- κ B and MAPK pathways, including ERK 1/2, p38, and JNK in Fas-stimulated cells [31,60,81,90]. To be precise, within B-cells, T-cells, and dendritic cells the NH₂-terminal of c-FLIP interfered with the γ -subunit of the I- κ B kinase complex (IKK), thus allowing nuclear translocation of NF- κ B [60]. Interestingly, c-FLIP_L has been shown to interact with additional signalling molecules, such as the receptor interacting protein (RIP) and rapidly accelerated fibrosarcoma kinase-1 (Raf-1) [90,146], TRAF-1 and -2 [90,146,175], or caspase-10 [61,80,177]. c-FLIP binds to the DED of the adapter protein FADD, acting as a dominant-negative inhibitor of the processing and release of active caspase-8 or -10 into cytosol [125].

In summary, c-FLIP_L may act as an adapter-like molecule for the recruitment of proteins involved in cell proliferation to the Fas pathway, replacing procaspase-8 and FADD within the death inducing signalling complex (DISC), thus interfering with death receptor associated cell death upstream of mitochondrial events [190].

1.4 Hypothesis and Goal

In 2008, it was shown by Bem et al. that pulmonary inflammation, apoptosis, PMN influx, and alveolo-capillary barrier disruption were not reduced by previous depletion of resident alveolar macrophages (AM) in a murine model of Fas-induced lung injury [21]. So far it remains unclear whether lung damage after Fas activation is dependent on PMN lung immigration. The goal of this study was to dissect the role of Fas and PMN by neutrophil depletion and subsequent pulmonary Fas activation. We investigated the role of PMN with respect to chemokines, cytokines, apoptosis, histological lung damage, and alveolo-capillary barrier permeability in response to Fas. The hypothesis was:

The presence of neutrophils is a necessary premise for Fas-induced pulmonary inflammation, apoptosis, and lung damage.

2. MATERIALS AND METHODS

2.1 Animals

This study was conducted with 48 male C57Bl/6 mice (Charles River, Sulzfeld, Germany) at an average age of 13 weeks. 10 mice per cage were kept at a 12 h day-and-night-rhythm at 21 °C and a 50-60% humidity. Animals were given access to water and standardized experimental animal diet (Altromin 1314 Forti, Altromin GmbH, Lage-Lippe, Germany) ad libitum in the course of the procedures. The experiments were realised at the research laboratory of the Department of Trauma, Hand, Plastic and Reconstructive Surgery of the Hospital of Ulm University (building IV, Safranberg, Ulm, Germany). All conducted procedures and preparations were approved by the institutional review board/ethics committee of the University of Ulm, as well as by the Federal Animal Care and Use Committee (permit no. 937, Regierungspräsidium Tübingen, Germany).

2.2 Reagents

In order to achieve previous neutrophil depletion, purified no azide and low endotoxin (NA/LE) rat anti-mouse Ly-6G/Ly-6C monoclonal antibody (MAb) Gr-1 (RB6-8C5) was injected intravenously. As an isotype control antibody, purified NA/LE rat anti-mouse IgG_{2b} MAb A95-1 was injected. For pulmonary Fas-activation via intratracheal (i.t.) instillation, purified NA/LE hamster anti-mouse CD95 (Fas/APO-1) MAb Jo2 was used. Purified NA/LE hamster anti-keyhole limpet hemocyanin IgG₂ MAb Ha4/8 was instilled as the corresponding isotype control. All antibodies were purchased from BD Biosciences, Heidelberg, Germany.

2.3 Experimental Design

Animals were fixed on a board and were anesthetized using an inhaled volatile mask narcosis. 2.5 vol% sevoflurane (Abbott, Wiesbaden, Germany) and 97.5 vol% oxygen (Westfalen AG, Münster, Germany) were applied at a continuous flow of 0.8 l/min. Animals received i.v. treatment with 20 µg/g b.w. MAb Gr-1 (=PMN-), or the corresponding isotype control A95-1 (=PMN+), diluted in 250 µl phosphate buffer (PBS Dulbecco's, Invitrogen, Karlsruhe, Germany). A 48 h incubation period was conducted for all groups. Afterwards test subjects were again anesthetized and fixed onto a 30° inclined perspex board, where the animal's mouth was kept open by a thread in order to achieve oropharyngeal application of either 2 µg/g bw Jo2 (=FAS), or Ha4/8 (=ISO) MAb, diluted in 100 µl PBS. An incubation period of 6 or 18 h followed, concluded by sevoflurane overdose (Figure 1).

Thoracotomy was performed immediately followed by cardiac puncture of the right atrium with a 30 G needle through which blood was drawn into a 1.0 ml syringe with a heparin-filled cone (Heparin-Natrium, Braun, Melsungen, Germany). The received blood was then filled into a 1.5 ml tube (Eppendorf, Hamburg, Germany) and stored on ice for the residual time of the experimental procedure. Next the trachea was incised, cannulated (22 G cannula on a 0.58 x 0.2 mm catheter, VWR, Darmstadt, Germany), and secured by suture. The right hilum was clamped, the right lung removed and divided into three parts (cytokines/chemokines, myeloperoxidase, western blot analysis), each of which was stored in a separate 2 ml tube containing sterile H₂O and a protease inhibitor cocktail (P8340 (Ileupeptin 5 µg/ml, aprotinin 2 µg/ml, pepstatin 1 µg/ml, EDTA 0.2 mg/ml, and N-ethylmaleinide 125 µg/ml), Sigma, Taufkirchen, Germany).

All tubes were stored on liquid nitrogen for the residual time of the experimental procedure. Bronchoalveolar lavage was performed by 3 x repeated injection and consecutive aspiration of 0.5 ml of cold PBS into and from the left lung. The aspirated aliquots (about 0.35 ml each) were then stored on ice in 2 ml tubes that had previously been filled with 5 µl of the aforementioned protease inhibitor cocktail. Afterwards the left lung was fixed with 10 % paraformaldehyde (J.T.

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Baker, Mallinckrodt Baker B.V. Deventer, Netherlands) at 15 cmH₂O for 15 minutes through the placed catheter. The left lung was exzised and stored in a tube (Poly Q-Vial 18 ml, Beckmann Coulter GmbH, Krefeld, Germany), filled with 10 % paraformaldehyde for further histological analysis.

Figure 1: Experimental design - treatment with intravenous Gr-1 (PMN-) or its isotype (PMN+), followed by a 48 hour incubation period, as well as intratracheal Jo2 (FAS) or its isotype (ISO), followed by a 6 or 18 hour incubation period. The mice were then sacrificed by sevoflurane overdose and samples were collected.

2.4 Bronchoalveolar Lavage (BAL)

Received samples were spun at 500 g at 4°C for 15 minutes and supernatants were stored in 120 µl aliquots at -80 °C for later analysis and one 10 µl aliquot for immediate total leukocyte counts. 1 µl crystal violet was added to the 10 µl aliquot and loaded into a Neubauer counting chamber for leukocyte counts from one of four quadrants. The results were extrapolated according to the 10:11 dilution and the number of quadrants. After centrifugation the remaining cell pellet was diluted in PBS to the equivalent amount of received bronchoalveolar lavage fluid for each corresponding sample. The probe was then mixed and 150 µl were used for cytopspin preparations. Samples were filled into the cylinders for the Zytospin 3-Zytozentrifuge (Shandon, Frankfurt a.M., Germany) and spun for 3 minutes at 1000 rpm. Afterwards the slides were air-dried for 10 min, fixed with 100 %

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methanol (Fluka, Deisenhofen, Germany), and then subjected to a modified Pappenheim's stain (Merck, Darmstadt, Germany). A cell count of 200 leukocytes was conducted via light microscopy at 100 x magnification by two members of our team who were not aware of the sample's group affiliation. They distinguished between lymphocytes, alveolar macrophages, and neutrophils. All results were stored in a Microsoft Excel file and multiplied by the total number of leukocytes in order to retrieve the number of PMN per 1 ml for each test subject.

2.5 Lung Tissue Homogenization

For cytokine measurement, a protease inhibitor cocktail (P8340, Sigma, Germany) and PBS were mixed at an 1:20 ratio in order to add 250 µl of the resulting solution to each sample. The probes rested on ice during the whole procedure. Tissue homogenization was achieved using the Ultra-Turrax T 25 (Janke und Kunkel, Staufen, Germany). Probes were treated until no more pieces of tissue were visible within the tube. In between homogenization of different samples the homogeniser's agitator was cleaned in multiple steps including manual removal of tissue pieces with a pair of tweezers as well as washing with distilled H₂O, PBS, and a disinfectant. After this first raw homogenization step probes were crushed further by repeated ultrasonic treatment (Sonifier, Branson, Hannover, Germany). In between different samples the tip of the sonifier was cleaned with PBS and disinfectant which was wiped off with a tissue. The samples were then centrifuged at 16,000 G at 4 °C for 15 min. From the supernatants 10 µl were used for determination of protein concentration and the rest was divided into aliquots and stored for cytokine analysis at -80 °C.

For western blot analysis we generated a radio immunoprecipitation assay-buffer (RIPA-buffer) and from that a RIPA-buffer protease inhibitor solution. For 10 ml RIPA-buffer we diluted 0.1 ml Igepal CA 630 (Sigma J7771, Germany) (1 %), 0.05 g sodium deoxycholate (Sigma D6750, Germany) (0.5 %), and 0.01 g of sodium dodecyl sulphate (SDS, Fluka 71729, Germany) (0.1 %) in 10 ml of DPBS. One ml of RIPA-protease inhibitor solution consisted of 1 ml RIPA-buffer, 10 mg phenylmethanesulfonyl (PMSF, Sigma P7626, Germany) in 1 ml isopropanol, 100 mM sodium orthovanadate (Sigma S6508-10G, Germany), and 50 µl protease

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inhibitor cocktail (Sigma P8340, Germany). Finally 200 µl RIPA-buffer protease inhibitor solution were added to each probe. All processed probes were resting on ice for the entire procedure. They were homogenized using the Ultra-Turrax T 25 (Janke und Kunkel, Staufen, Germany) until no more tissue structures were visible. In between different animal lungs the agitator was cleaned repeatedly by removal of tissue pieces as well as washing with distilled H₂O, PBS, and a disinfectant that was wiped off. Probes were then centrifuged at 16,000 G at 4 °C for 15 min. Subsequently supernatants were each pipetted into 1 x 10 µl aliquot for a protein concentration assay and about 3 x 50 µl aliquots for western blotting. Finally the samples were stored at -80 °C.

2.6 Protein Concentration

In order to measure protein concentration we used the BCA Protein Assay Kit (Art. no. 23225, Pierce, USA). Protein standard solutions containing bovine serum albumin (BSA) with different concentrations - ranging from 2.5 µg to 17.5 µg - were diluted to 50 µl by distilled H₂O. Animal samples were diluted likewise according to their sample type. Previously homogenized lung tissue samples were diluted 1:40 and bronchoalveolar lavage fluid was diluted 1:10. Afterwards 3 x 10 µl of each standard or sample were filled into a 96 well microtiter plate. We then received the working solution by mixing the delivered BCA reagents A and B at a 50:1 ratio. 200 µl of our working solution were pipetted into each well so that the microtiter plate could then be sealed and stored at 37 °C for 30 min. Finally we measured the extinction rate at a wave length of 562 nm with the Tecan Sunrise Plate Reader (Tecan Austria, Gröding, Austria). The protein concentrations were calculated according to the standard curve, which was derived from the known concentration of protein standard samples.

2.7 Whole Blood Analysis by Fluorescence Activated Cell Sorting (FACS)

Whole-blood samples extracted in the experimental procedure had been stored on ice in a 1.5 ml tube. 150 µl were deducted and used for FACS analysis. The rest were spun 10,000 x g at 4°C for 10 minutes. Afterwards the supernatants (plasma) were stored at -80 °C. Flow cytometry was performed using a CD45 antibody (anti-mouse MAb CD45-PE, Immunotech, Krefeld, Germany) for leukocyte detection, and a CD3e antibody to indicate T-cells. In order to evaluate depletion effectiveness we carefully mixed 50 µl of whole-blood with 2.5 µl CD45 and 2.5 µl CD3e MAb in a 5 ml test tube (Falcon 5 ml Rundbodenröhrchen, Polystyrol, BD Biosciences, Heidelberg, Germany). Afterwards the mixture was incubated for 20 min at 4 °C protected from light, and 1 ml VersaLyse solution (Beckman Coulter, France) was added. The samples were vortexed and again incubated protected from light for 10 min at 4 °C. Now we centrifuged our probes for 5 min at 300 g and 4 °C, disposed of the supernatants and washed the remaining pellet 2 x with 2 ml of cold PBS, again centrifuging and disposing of the supernatants each time. Finally 200 µl PBS were added and the samples were measured by flow cytometry (EPICS XL, Beckman Coulter, Krefeld, Germany). PMN in whole-blood were differentiated from other cells by appearing CD45 positive, CD3e negative, and characteristic forward- (FSC) and sideward scatter (SSC) behaviour.

2.8 Myeloperoxidase Activity (MPO)

We measured myeloperoxidase activity as a representative value for accumulation and activation of PMN by using a modified method from Suzuki et al. [184], as previously described by Perl et al. [149].

Each lung tissue sample was homogenized on ice within 1.5 ml of buffer solution in separate glass tubes (Art. no. 212K1513, VWR, Darmstadt, Germany). The buffer was composed of 6.12 g KH_2PO_4 (Art. no. 4873, Merck, Darmstadt, Germany) diluted in 900 ml of distilled water, which was alkalised to a pH of 6.0 by

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titration with 0.91 g (buffer no. 1), and subsequently 1.14 g K_2HPO_4 (Art. No. 105104, Merck, Darmstadt, Germany) in 100 ml distilled water (buffer no. 2). Additionally 0.5 g of hexadecyltrimethylammoniumbromide (HTAB, Sigma, Deisenhofen, Germany) (0.5 %) were mixed with 100 ml of buffer no. 2 resulting in buffer no. 3, which was used for sample homogenization. The glass tubes were then sealed by a cap (Art. no. 217Q8934, VWR, Darmstadt, Germany) and incubated at 60 °C for 2h. Thereafter the sealed probes rested at 4 °C until the next day. On the second day samples were centrifuged at 3950 x g at RT for 15 min. Twenty-five μ l of supernatant or standard dilution (1000 mU/ml, Art. no. 475911 NovaBioChem, Schwalbach, Germany) were mixed with 25 μ l of a tetramethylbenzidine-dimethylsulfoxide solution (10 mg TMB per 10 ml DMSO, both Sigma, Deisenhofen, Germany) and 200 μ l of 30 % H_2O_2 (Fluka, Deisenhofen, Germany) and incubated at 37 °C for 5 mins. The reaction was stopped with 50 μ l 2 M H_2SO_4 (Sigma, Deisenhofen, Germany) and the extinction rate measured at 450 nm.

In order to determine the protein concentration we resuspended the pellet of each sample with 100 μ l perchloric acid $HClO_4$, then vortexed, and immediately centrifuged the samples at 3950 U for 10 min. Supernatants were discarded and the probes washed twice by adding 3 ml distilled water with subsequent centrifugation at 3950 U for 10 min. Again supernatants were poured away and 2 ml N NaOH added to the sediment. The samples were vortexed and incubated at 56 °C for 30 min until the pellet was completely dissolved. We then added 4 % copper sulphate solution (Art. no. 1.02791.0250, Merck, Germany) to the BCA-reagent (Art. no. B9643, Sigma, Germany) at an 1:51 ratio (reagent C). With the protein standard BSA (bovine serum albumin, Art. No. A3803, Sigma, Germany) and distilled H_2O a protein standard dilution series of 2000-1000-500-250-125-62,5-31,25-0 μ g/ml was prepared. Finally we gave 15 μ l sample or protein standard into a microtiter plate and added 300 μ l reagent C to each well. After a 30 min incubation period at 37 °C we measured the protein concentration at 562 nm by photometry. Eventually we calculated the myeloperoxidase activity in U/mg according to the protein concentration of the corresponding sample.

2.9 Chemokines and Cytokines

The concentrations of MCP-1, MIP-2, and KC were determined within plasma, BALF, and lung tissue homogenate by sandwich-enzyme-linked immunosorbent assay technique (ELISA). The experiments were conducted according to the manufacturer's recommendations for evaluation of KC, MIP-2 (Duo Set Elisa Maus, R&D Systems, Minneapolis, USA), and MCP-1 (BD OptEIA™ ELISA Set, BD Pharmingen, San Diego, USA). Multiplex analysis was performed using the Bio-Plex Cytokine Assay (BioRad, Munich). Preparations were conducted according to the manufacturer's instructions and data acquisition followed by using the Bio-Plex 200 System (Art. no. 171-000201, Bio-Rad, Munich) with Bio-Plex Manager 5.0 software and a personal computer. Levels below the detection limit of the assays were set to zero for statistical purposes.

2.9.1 ELISA

We used a sandwich ELISA technique to quantify keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-2 (MIP-2) from lung homogenate, plasma, and BAL. The procedure is termed sandwich ELISA due to the binding of antibodies on two locations of the desired protein. The so-called capture-antibody is bound to the ELISA plate by its Fc-fragment and captures the target protein, whereas a detection-antibody binds a different epitope on the target. Since the detection-antibody is linked to streptavidin horseradish peroxidase (HRP) a quantifiable colour change is induced by addition of tetramethylbenzidine (TMB).

At first our capture antibody (Duo Set Elisa Mouse MCP-1, R&D Systems) was diluted with PBS (Art. no. 14190-094, Gibco) at 2 µg/ml. Then we pipetted 100 µl into the wells of a microtiter plate (C96 Maxisorp Cert. NUNC-Immuno Plate, Art. no. 446612, NUNC, Wiesbaden), sealed it by foil (Art. no. 236366, NUNC, Wiesbaden), and incubated it at 4 °C over night. The next morning we disposed of the remaining fluid in the wells and washed the plate three times with a washing

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buffer (8.0 g NaCl, 0.2 g KCL, 1.5 g $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, 0.4 g KH_2PO_4 , 0.5 g Tween 20, 1000 ml dd H_2O , pH 7.0). Then we added 200 μl of assay diluent, containing 1 g bovine serum albumin (BSA, Art. no. A-8022, Sigma) diluted in 100 ml PBS, into each well and sealed the plate. In order to block non-specific binding incubation went on for 1.5 h at RT. Again, three washing steps were conducted whereupon 100 μl of diluted standard (1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.6 pg/ml, and neutral value, BD Biosciences, Heidelberg) or sample were added to the wells according to the previously designed allocation table. Dilution was achieved by using assay diluent. Now the plate was sealed and incubated over night at 4 °C.

The next day we washed the plate three times and continued by adding 100 μl of detection-antibody (BD Biosciences, Heidelberg), again diluted by assay diluent to each well. After an incubation period of 2 h at RT we pursued multiple washing steps and finally added 100 μl of Streptavidin-HRP (Duo Set ELISA, diluted 1:200 with assay diluent) to each well. After sealing the plate another incubation period of 20 min at RT followed. Then we washed the plate three times before adding 100 μl of TMB (TMB-Substrat, Art. no. 2642 KK, Pharmingen) into each well with a subsequent incubation period of 30 min at RT in the dark. Finally 50 μl of 2 N H_2SO_4 were added in order to stop the reaction. Optical density was quantified by photometry at 450 nm and protein concentration calculated based upon the known concentrations of our protein standard curve.

2.9.2 Cytokine multiplex

In order to quantify tumor necrosis factor- α (TNF- α), Interleukin-1 (IL-1 β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), macrophage inflammatory protein-1 α (MIP-1 α), granulocyte-colony stimulating factor (G-CSF), interferon- γ (IFN- γ), and RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) (CCL5 – chemokine ligand-5) in lung tissue homogenate, plasma, and bronchoalveolar lavage fluid (BALF) we used the Bio-Plex Cytokine Assay (BioRad, Munich). This multiplex assay allows the simultaneous detection of up to 100 cytokines within a single well of a 96-well microplate by the use of antibody-coupled, colour-coded polystyrene beads which can be conjugated with a specific

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reactant for the target molecule. Each analyte is represented by a specific bead colour. The unknown cytokine concentration of measured probes is derived from measurement of a standard solution containing a known amount of cytokine. A series of washes is performed in order to remove unbound protein, followed by treatment with a biotinylated detection antibody which also binds the desired cytokine but at a different epitope. Since two antibodies bind the target molecule at different sites this method is designated a sandwich immunoassay format. The final reaction mixture is retrieved by adding streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies. The content of each well is drawn up into the flow-based Bio-plex suspension array system of the Bio-Plex Assay Reader (Bio-Rad, Munich) and subsequently identified and quantified according to bead colour and fluorescence by the use of a laser system. The first laser acquires stimulation of the bead's fluorophores, thus establishing analyte identity. The second laser stimulates the bound PE therefore quantifying the analyte. Emission is evaluated by a photoelectric cell. Unknown cytokine concentrations are automatically calculated by the Bio-Plex Manager software using a standard curve derived from the aforementioned cytokine standard.

Data acquisition was achieved according to the manufacturer's instructions. The experiment was conducted in a sterile 96-well filter plate included in the assay kit. At first the plate was prewet adding 100 µl the provided assay buffer to each well. Then we combined all beads into a tube, added assay buffer up to a volume of 5.5 ml and vortexed it. 50 µl were then pipetted into each well. Afterwards we washed the plate twice using 100 µl of the provided washing buffer and a vacuum system. After careful preparation of our probes and the standards, each standard being a 4-fold dilution of the preceding, starting at 32,000 pg/ml, we then added them into the designated wells. The plate was sealed by foil and incubated on a shaker set on 300 rpm for 30 min at RT. Meanwhile the detection antibodies were prepared according to the manufacturer's protocol. After washing the plate thrice we added 25 µl detection antibody dilution to each well and another 30 min incubation period on a shaker followed. Again, preparation of streptavidin-PE was conducted as explained in the manual and successive to three washing steps we added 50 µl to each well. We incubated the sealed plate with streptavidin-PE for 10 min at RT on a shaker, then washed it three times, and finally added 125 µl of the provided

assay buffer to each well in order to resuspend the beads. After a brief period of shaking we were then able to read the plate by using the Bio-Plex 200 System (Art. no. 171-000201, Bio-Rad, Munich) with Bio-Plex Manager 5.0 software and a personal computer.

2.10 Apoptosis

2.10.1 Activated Caspase-3 Western Blotting

The first step of the western blot analysis was sodium dodecyl sulfate (SDS, Art. no. 71729, Fluka) polyacrylamide gel (18%) electrophoresis (SDS-PAGE). All gels consisted of a lower gel (6 ml Rotiphorese, 2.5 ml lower buffer (4 x Tris-HCL (Art. no. 75825, USB)/SDS, pH 8.8), 1.5 ml distilled water, 30 µl of 25 % ammonium persulfate (2.5 g APS in 10 ml H₂O) (APS, Art. no. A-3678, Sigma), and 5 µl Temed (Art. no. T-9281, Sigma)) for protein distribution, and an upper gel (0.8 ml Rotiphorese, 1.25 ml upper buffer (4 x Tris-HCL/SDS, pH 6.8), 2.95 ml aqua dest., 40 µl 25 % APS, and 10 µl Temed), used to hold the samples. We inserted a ridge into the upper gel for 15 min in order to form 10, or 15 pockets respectively. Afterwards the pockets were flushed out with running buffer to ensure stable and equal conditions for all samples. Western blot probes were diluted with aqua dest. to an equal protein concentration of 60 µg, and subsequently 15 µl sample buffer (Laemmli Sample Buffer (Art. no. 161-0737, BioRad) / 2-Mercaptoethanol (Art. no. M3148, Sigma)) were added to each probe which were then stored at 95 °C for 7 min in order to achieve protein denaturation. The samples or the molecular weight marker (Rainbow Marker, RPN 755, Amersham) were pipetted into the preformed pockets and with a running buffer of 0.25 M Tris-HCl, 0.96 M Glycin, and 1 % SDS electrophoresis was performed at 125 V for 2 h and 45 min. We used the Semi-DRY-Transfer Cell (BioRad, Germany) for 2 h at 0.18 A in order to transfer proteins onto a Hybond P-PVDS membrane (Amersham Biosciences, Germany). Unspecific binding was corrupted by washing the dedicated membranes in a Tris Base (Trizma Base, Art. no. T6066, Sigma) buffer, containing 5% of instant milk powder (Roth, Germany) (TBS), and 0.1% Tween-20 (Sigma, Germany) (TBST). Membranes were incubated with the primary antibody (1:500, Cleaved Caspase-3,

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Art. no. 9661, Cell Signalling) for 12 h at 4 °C. The secondary antibody (Anti-rabbit IgG, HRP-linked Antibody, Cell Signalling) was applied by incubation for 1 h in a 1:1500 dilution ratio. In order to dispose of redundant secondary antibody the membranes were again washed in TBST and TBS. Results were visualized by adding a layer of ChemiGlow (CGW-8000, Alpha Innotech, Germany) onto the membranes and transferring the results to ECL-Hyperfilm (Amersham Biosciences, Germany) via incubation in a film magazine (Filmkassette, Amersham, Germany) for varying time intervals. Photographic processing was achieved using a film processor (Kodak Entwicklungsmaschine, Kodak New York, USA). After processing, the films were labelled and scanned. In order to quantify the results integrated density analysis was conducted using ImageJ 1.43u (Wayne Rasband, National Institutes of Health, USA).

2.10.2 TUNEL

This method detects DNA fragmentation of the apoptotic cell by labelling 3'-OH-groups via the terminal deoxynucleotidyl transferase (TdT) with fluorescence markers. It was therefore used in order to visualize and quantify apoptosis in lung tissue sections. The number of fluorescence-positive cells was counted in 10 high power fields (400x).

Lung tissue sections of 2 µm were cut from paraffin embedded probes using a microtome (Microm HM450, Microm). These were transferred onto slides and stored in xylene for 2 x 5 min. Subsequently slides were further dewaxed using a descending alcohol dilution series (100%-, 96%-, and 70% ethanol). The probes were washed in distilled H₂O and each slide was embedded in proteinase K-buffer (15 µg proteinase K recombinant (Roche, Germany) in 1 ml 10 mM Tris/HCl buffer (USB, Germany)) for 15 min at 37 °C. Afterwards slides were washed twice in PBS, dried, and 50 µl of a label-, and enzyme-solution mixture (In situ Cell Death Detection Kit TMR, Art. no. 1216792910, Roche) were added to each section. 100 µl of label-solution were deducted and treated slides served as a negative control. Samples were incubated at 37 °C for 1 h and then washed with PBS three times. For nuclear staining a bisbenzimidazole solution was applied (1 mg bisbenzimidazole in 500 ml PBST) to each slide for 15 min without light. Slides were washed again, embedded in Dako Cytomation Fluorescent Mounting Medium (Roche, Germany),

and stored at 4° C for up to one week before evaluation of 10 fields (400x) of each slide with the Axioskop (Carl Zeiss, Germany).

2.11 Histology

2.11.1 Sample Preparation

Since the left lung of each experimental animal had been reserved for histological analysis they were recovered from the 10% paraformaldehyde solution after 48 h. The samples were washed with H₂O for 5 h in order to remove paraformaldehyde residues. Afterwards we used an ascending alcohol dilution series for dehydration (70%-, 80%-, 90%-, 100% ethanol, methyl benzoate, toluene, xylene, and again 100% ethanol) with the Leica Asp200 (Leica Microsystems, Germany). After subsequent paraffin embedding and consecutive freezing to -4 °C, we received 1 µm, and 2 µm lung tissue sections (Microm, Germany). From each lung multiple sections were transferred onto slides for microscopy.

2.11.2 Hematoxylin and Eosin (H&E) Staining and Evaluation

Hematoxylin and eosin stains of our 1 µm lung tissue sections were prepared by the Department of Pathology of the University of Ulm. The stains were analyzed by light microscopy at 400x magnification (Axioskop, Carl Zeiss, Germany). The slides were evaluated according to the following criteria: Inflammatory cell influx, alveolar wall thickening, and disruption of the alveolar architecture. (Furthermore a lung injury score was calculated.) Different members of our team were instructed on how to analyze microscopical findings of Fas-induced lung injury. They reviewed the slides independently and were unaware of the experimental animal's group affiliation.

2.12 Statistical Analysis

All results were processed using Microsoft Excel and SigmaPlot 11.0 (Systat Software GmbH, Schimmelbuschstrasse 25, 40699 Erkrath, Germany). With the later, results were evaluated according to their statistical significance and displayed in plots with the bar representing the mean and error bars indicating the standard error of the mean (SEM). Statistical analysis included one- or two-factorial analysis of variance (One-Way-ANOVA, Two-Way-ANOVA). In the absence of normal distribution ANOVA-On-Ranks was performed. We conducted a Student-Newman-Keuls test (SNK) as a post hoc test for multiple comparisons. A $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Experimental Animals

This study was conducted with 48 male Charles River (Sulzfeld, Germany) C57Bl/6 mice at an average age of 13 weeks. Animal body weight (b.w.) ranged from 21.9 – 29.5 g with an overall mean of 24.9 g $\pm$ 1.8 g standard error of the mean (SEM). Neither were there significant differences regarding weight in between groups before ($p = 0.129$), nor after injection of the Gr-1/isotype antibody ($p = 0.186$).

3.2 PMN-Depletion and Fas-Activation

48 h after i.v. injection of 20 μ g/g b.w. Gr-1/isotype antibody, and an additional 6 or 18 h after i.t. instillation of 2 μ g/g b.w. Jo2/isotype antibody, whole blood flow cytometry and BALF cytospin were performed in order to examine depletion effectiveness and kinetics. Furthermore myeloperoxidase activity was assessed in lung tissue as a combined marker for neutrophil presence and activity.

Within the blood (Figure 2A), BALF (Figure 2C), and with respect to lung MPO activity (Figure 2D) the results of our analysis showed that pulmonary activation of the Fas system lead to a significant increase in the number of neutrophils in all three sample types. Regarding MPO activity and the number of PMN within BALF, an increase was observed at both points of time (6h and 18 h). However, within whole blood samples a significant raise in PMN occurred at 18 h only. The observed increases were significantly reduced in previously depleted animals at 6, and at 18 h respectively.

In summary these results indicate an induction of local and systemical neutrophil recruitment and pulmonary neutrophil activity by the Jo2 antibody, i.e. by Fas activation. This was markedly reduced in Gr-1-treated animals. Therefore, neutrophil depletion reduces Fas-induced influx and activation of PMN.

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Figure 2: PMN percentage of leukocytes in peripheral blood (A) as evaluated by flow cytometry (B1/B2). Also shown are the number of neutrophils per ml BALF (C), and lung myeloperoxidase activity per gram lung tissue (D). Mean $\pm$ SEM at 6 / 18 h after intratracheal instillation of Jo2 (FAS) or isotype (ISO) antibody with (PMN-) or without depletion (PMN+). * $p < 0.05$ vs. corresponding isotype, # vs. corresponding PMN+, @ vs. corresponding group at 18 h, Two Way ANOVA / ANOVA on Ranks, SNK, $n = 5-6$.

3.3 Chemokine and Cytokine Profile

3.3.1 BALF

Within BALF IL-1 β , IL-6, IL-10, G-CSF, MIP-1 α , MIP-2, MCP-1, and KC were quantified. In the presence of neutrophils, IL-6, IL-10, and G-CSF were significantly increased late (18 h) after Jo2 instillation (Figure 3B-D). MCP-1 responded early and late (6 h, 18 h) (Figure 3G). In the absence of neutrophils however, IL-6, IL-10, G-CSF, MIP-1 α , MIP-2, KC, and MCP-1 were raised markedly at 18 h in BALF without Fas activation. Additional Jo2 instillation did not show the capacity to enhance this response. On the contrary, regarding MIP-1 α , MIP-2, KC, and G-CSF Fas lead to a significant reduction in chemokine/cytokine

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response in depleted animals (Figure 3B-H). With respect to the IL-1 β (Figure 3A), a resemblance of aforementioned results was seen that did not reach significance. In summary, Fas activation lead to an inflammatory reaction measured within BALF. Interestingly, an even stronger effect was seen in depleted animals that did not receive Jo2. However, additional Fas stimulus led to rather declined chemokine/cytokine levels as compared to mere depletion. Therefore, depletion of PMN led to a chemokine and cytokine response in BALF not further enhanced by pulmonary Fas activation.

3.3.2 Lung Tissue Homogenate

Within lung tissue IL-1 β , IL-6, IL-10, G-CSF, MIP-1 α , MIP-2, MCP-1, and KC were quantified. In the presence of neutrophils, IL-6, G-CSF, and MCP-1 were increased within lung homogenate in response to i.t. Jo2 instillation after 18 h (Figure 4B, D, G). KC showed an increase at both time points, and there was a significant raise from 6 to 18 h (Figure 4H). With the exception of IL-6 (Figure 4B) the aforementioned mediators, as well as MIP-1 α and MIP-2 (Figure 4E-F), again showed a strong response to mere neutrophil depletion. This effect was not enhanced by additional Fas activation via i.t. Jo2. With respect to KC, there was even a significant reduction in depleted animals, receiving additional Fas stimulation at 6 and at 18 h, as compared to depleted mice, incubated with the Gr-1 isotype antibody (Figure 4B, 4D-H). As in BALF cytokine analysis, the results for IL-1 β showed a structural affinity to aforementioned analytes but did not reach significance (Figure 4A). Regarding IL-10, the highest levels within lung homogenate were seen in depleted animals. At 6 h Jo2 induced an increase in spite of depletion, at 18 h however, the strongest response was seen in Gr-1-treated animals without additional Fas stimulation (Figure 4C). In short, with the exception of few analytes, depletion led to a chemokine and cytokine response in lung homogenate that was not further enhanced by pulmonary Fas activation.

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Figure 3: Cytokine concentrations in bronchoalveolar lavage fluid from mice treated with intravenous Gr-1 (PMN-) or its isotype (PMN+) 48 h prior to intratracheal application of Jo2 or its isotype and a 6 or 18 hour incubation period, including IL-1 β (A), IL-6 (B), IL-10 (C), MIP-1 α (E), MIP-2 (F), KC (H), MCP-1 (G), and G-CSF (D). * $p < 0.05$ vs. corresponding isotype, # vs. corresponding PMN+, @ vs. corresponding group at 18 h, Two Way ANOVA / ANOVA on Ranks, SNK, $n = 4-6$.

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Figure 4: Cytokine concentrations in lung homogenate from mice treated with intravenous Gr-1 (PMN-) or its isotype (PMN+) as well as intratracheal Jo2 or its isotype and a 6 or 18 hour incubation period, including IL-1 β (A), IL-6 (B), IL-10 (C), MIP-1 α (E), MIP-2 (F), KC (H), MCP-1 (G), and G-CSF (D). * $p < 0.05$ vs. corresponding isotype, # vs. corresponding PMN+, @ vs. corresponding group at 18 h, Two Way ANOVA / ANOVA on Ranks, SNK, $n = 4-6$.

3.3.3 Plasma

Within plasma IL-1 β , IL-6, IL-10, G-CSF, MIP-1 α , MIP-2, MCP-1, and KC were quantified. In non-depleted animals, IL-6 plasma cytokine levels were markedly increased late after Fas instillation. Depletion alone induced a significant increase in IL-6. This effect was significantly reduced by additional Jo2 stimulus (Figure 5B). Cytokine results for G-CSF showed a similar pattern, but did not reach significance (Figure 5D). Plasma levels of MCP-1 and KC were significantly increased by Fas early and late in the presence of PMN. In the absence of neutrophils a marked raise was observed that was not further increased by additional instillation of Jo2 (Figure 5F-G). MIP-2 showed a late increase in depleted animals with significantly lower levels in Jo2-treated mice. However, stimulation with Jo2 in the presence of PMN did not result in significant changes (Figure 5E). The results for IL-10 presented a similar pattern but only were significant at 6 h for depleted mice without Fas activation (Figure 5C). Taken together local i.t. application of Jo2 lead to an increase in systemic plasma levels of several chemokines/cytokines. Furthermore, systemic depletion of PMN lead to a chemokine and cytokine response in plasma not further enhanced by local pulmonary Fas activation. In some analytes the additional activation of Fas even resulted in a decrease of mediator levels, reaching significance in IL-6 and MIP-2 (Figure 5B, 5E). A corresponding trend was seen for IL-1 β , G-CSF, and KC (Figure 5A, 5D, 5G).

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Figure 5: Cytokine concentrations in plasma from mice treated with intravenous Gr-1 (PMN-) or its isotype (PMN+) as well as intratracheal Jo2 or its isotype and a 6 or 18 hour incubation period, including IL-1 β (A), IL-6 (B), IL-10 (C), MIP-2 (E), MCP-1 (F), KC (G), and G-CSF (D). * $p < 0.05$ vs. corresponding isotype, # vs. corresponding PMN+, @ vs. corresponding group at 18 h, Two Way ANOVA / ANOVA on Ranks, SNK, $n = 4-6$.

3.4 Apoptosis

Western blot and consecutive integrated density analysis of cleaved caspase-3 in lung tissue were conducted in order to investigate pulmonary apoptosis. After 6 h and 18 h of intratracheal deposition of the Fas-inducing antibody Jo2 an increased rate of apoptosis as compared to the control group was observed. Significance was reached at 18 h (Figure 6 A2, B2). PMN-depleted mice did not show a reduction of this pro-apoptotic effect of Fas (Figure 6 A1-B2). In conclusion, Fas induced an increase of the pulmonary apoptotic rate that persisted in the absence of neutrophils. Therefore our results suggest that the presence of PMN is not a necessary premise for Fas-induced pulmonary apoptosis, indicating apoptosis of other cell types.

Figure 6: Exemplary cleaved caspase-3 western blot analysis (A1-2) and integrated density values (IDT) of the corresponding groups (B1-2) of pulmonary lung homogenate from mice treated with intravenous Gr-1 (PMN-) or its isotype (PMN+) 48 h prior to intratracheal application of Jo2 (FAS) or its isotype (ISO), following a 6 or 18 hour incubation period. * $p < 0.05$ vs. PMN+/ISO/6h, ANOVA On Ranks, SNK, $n = 5-6$.

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Furthermore, terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) and bisbenzimidazole nuclear counterstaining were conducted to evaluate apoptotic behaviour of lung epithelial cells (Figure 7B-C). Six h after Fas activation increased apoptosis was observed with a declining trend after depletion. At 18 h of incubation with Jo2 there was a statistically significant raise in TUNEL-positive cells. Depleted mice also presented with a significant increase in TUNEL-positive cells at 18 h independent of i.t. Fas (Figure 7A).

Figure 7: Terminal deoxynucleotidyltransferase biotin-dUTP nick end labeling (TUNEL)-positive cells per 10 high-power fields, displayed as mean $\pm$ SEM (A). Mice treated with intravenous Gr-1 (PMN-) or its isotype (PMN+) as well as intratracheal Jo2 (FAS) or its isotype (ISO) and a 6 or 18 h incubation period. Representative pulmonary TUNEL stains of a non-depleted (B), and a depleted (C) animal treated with intratracheal Jo2 antibody and an 18 h incubation period. * $p < 0.05$ vs. corresponding isotype, # vs. corresponding PMN+, @ vs. corresponding group at 18 h, Two Way ANOVA, SNK, $n=5-6$.

3.5 Lung Damage

3.5.1 BALF Protein Concentration

With respect to alveolo-capillary barrier dysfunction, there was an early increase in BALF protein concentration 6 h after i.t. Jo2 in the presence of neutrophils. In the absence of PMN no such increase was observed, suggesting a protective effect of neutrophil depletion (Figure 8A). At 18 h a similar reduction of BALF protein concentration was shown in depleted animals receiving Jo2 (Figure 8A).

3.5.2 Histological Evaluation

Microscopic evaluation of lung tissue sections of mice receiving Fas isotype treatment showed few inflammatory cells, intact alveolar architecture, and regular alveolar wall constitution (Figure 8B, D). However, in the presence of neutrophils (PMN+), sections of Fas-treated animals showed inflammatory cell influx, alveolar architecture disruption, and alveolar wall thickening, indicating lung damage (Figure 8C). Previously depleted animals did show these pathological findings to a much lesser degree (Figure 8E), supporting the previous results of PMN-dependent Fas-induced lung damage.

Taken together these results suggest that depletion of PMN blunts Fas-induced lung damage.

RESULTS

Figure 8: BAL fluid (BALF) total protein concentration (A), 6 and 18 h after intratracheal instillation of Jo2 (FAS) or isotype (ISO) with (PMN-) or without previous neutrophil depletion (PMN+). Representative hematoxylin and eosin-stained lung tissue sections (B-E) from animals 48 h after i.v. neutrophil depletion (PMN-) or isotype treatment (PMN+), and consecutive additional Fas-induction (FAS) or isotype instillation for 6 h (ISO). * $p < 0.05$ vs. corresponding isotype, # vs. corresponding PMN+, @ vs. corresponding group at 18 h, Two Way ANOVA, SNK, $n = 4-6$.

4. DISCUSSION

4.1 Inflammation

The pulmonary and systemic increase of inflammatory mediators is one of the hallmarks of the acute respiratory distress syndrome. The combination of early-response cytokines, adhesion molecules, and chemokines orchestrates the recruitment of neutrophils into the lung and therefore presents an important initial and maintenance factor in its pathogenesis [159]. Once recruited into the lungs, PMN are known to have the ability to exert structural damage via the production and release of reactive oxygen species (ROS), nitric oxide species (NOS), elastases, proteases, and other lytic enzymes [18,35,153,193]. Furthermore oxidative stress activates redox-sensitive transcription factors (NF- κ B, AP-1), resulting in the rapid generation of pro-inflammatory cytokines and chemokines [70]. The Fas/FasL system is potentially involved in mediating both hallmarks of ALI/ARDS associated pulmonary damage, addressing the neutrophil as well as the epithelial cell hypothesis. Instillation of a Fas-agonistic antibody into murine lungs led to lung epithelial cell apoptosis, pulmonary cytokine release, consecutive PMN recruitment, and disruption of the alveolo-capillary barrier [118]. Among the discussed signal transduction pathways nuclear factor-kappa B (NF- κ B) [145], extracellular signal regulated kinase 1/2 (ERK 1/2), and mitogen-activated protein kinase p38 (p38 MAPK) [141] have been suggested to be involved. The observation of a marked inflammatory response in the absence of alveolar macrophages [21,152] is important because it suggests that other cell types in the lung can promote inflammation in response to Fas. An important role of neutrophils in Fas-induced inflammation has been suggested and was part of this study.

In the presence of PMN, we observed a significant increase in BALF (6 h: MCP-1; 18 h: MCP-1, IL-6, IL-10, G-CSF), lung tissue (6 h: KC; 18 h: MCP-1, KC, IL-6, G-CSF), and plasma (6 h: MCP-1, KC; 18 h: MCP-1, KC, IL-6) mediators in response to pulmonary Fas activation. With respect to kinetics, a significant increase was

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found between 6 h and 18 h in lung tissue (KC, MCP-1, IL-6, G-CSF) and in plasma (MCP-1, IL-6). Regarding pulmonary Fas activation in the presence of PMN, Perl et al. presented data showing a significant release of lung tissue chemokines (MCP-1, MIP-2, KC) 4 h after i.t. administration of Jo2 [152]. Matute-Bello et al. presented data showing significantly increased mRNA concentrations in lung tissue after 6 h of intranasal Jo2, including MCP-1, MIP-2, MIP-1 α , and IL-6 [118]. Similar observations were made by Herrero et al. regarding KC, MIP-2, TNF- α , IL-1 β , and IL-6 in lung tissue after 16 h of i.t. sFasL delivery [73]. These findings are in line with our observations and suggest an early chemokine release with a consecutive increase of cytokines. Experimentally, over-expression of the CC chemokine MCP-1 (CCL2) in alveolar type 2 cells did not cause inflammation but resulted in increased monocyte and lymphocyte numbers in BALF, suggesting chemotactic properties but not necessarily activation of cells [68], for which an additional stimulus (e. g. LPS i.p.) was required. MCP-1 was also shown to act as an efficient neutrophil chemoattractant in mice [88] and was reported to induce this effect directly as well as indirectly via upregulation of the chemokines KC [57] and MIP-2 [19]. Treatment with MCP-1 antiserum lead to a reduction of plasma, lung, and liver KC concentrations as well as neutrophil numbers 4 h after laparotomy and hemorrhage [57]. Administration of intratracheal recombinant MCP-1 (rMCP-1) to MCP-1 knockout mice (MCP-1(-/-)) restored pulmonary neutrophil influx and local release of KC, MIP-2, and IL-6 in the presence or absence of *E. coli* infection at 24 h [19]. KC (CXCL1) – primarily released by mesothelial cells, endothelial cells, and PMN [6,58] – and MIP-2 (CXCL2) – mainly secreted by AM and pneumocytes [41,103,201] – present murine homologues of human IL-8, essentially responsible for PMN recruitment and activation [142]. In patients suffering from ARDS, IL-8 most consistently correlates with the neutrophil concentration in BAL and with mortality [127,128]. In conclusion, an initial MCP-1 production by pneumocytes and AM in response to pulmonary Fas activation is suggested. In our data this is mirrored by an early increase of MCP-1 at 6 h in BALF and plasma. In lung tissue however, MCP-1 levels were markedly elevated in response to Fas activation late at 18 h. The discrepancies between BALF and lung tissue levels here might be explicable by the different pulmonary compartments involved, since AM and pneumocytes present major sources for MCP-1 and mainly contribute to BALF mediator levels, whereas their influence on

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lung tissue mediator levels remains limited due to their numerical inferiority [140,144,150]. Finally, the aforementioned early increase of MCP-1 leads directly and indirectly (via MIP-2, KC) to neutrophil attraction and – in case of an additional trigger – to neutrophil activation with subsequent cytokine production.

In the absence of neutrophils, we observed a markedly increased inflammatory response to Fas (PMN+ vs. PMN-) in BALF (6 h: MCP-1, G-CSF; 18 h: KC, MIP-2, G-CSF), lung tissue (6 h: MCP-1, G-CSF; 18 h: MCP-1, MIP-2), and plasma (6 h: MCP-1, KC, G-CSF, IL-6; 18 h: KC, MIP-2, G-CSF) mediators. In this regard, similar results were shown in 2012 by Perl et al. [156] after neutrophil depletion and consecutive isolated blunt chest trauma (PMN+ vs. PMN-) in mice in BALF (3 h: KC, MIP-2, MCP-1; 30 h: MCP-1), lung tissue (3 h: MCP-1, MIP-2; 30 h: MCP-1, KC), and plasma (3 h: KC; 30 h: KC, IL-6) mediators. Moreover, in a double hit model of chest trauma-induced septic acute lung injury following PMN depletion [155], a significant increase of MCP-1 in lung tissue (PMN+ vs. PMN-) was observed at 12 h. However, in this study the lung tissue levels of IL-1 β and G-CSF were markedly decreased in the absence of PMN. In addition, no significant alterations (PMN+ vs. PMN-) regarding plasma mediators (MCP-1, KC, MIP-1 α , IL-6, IL-10, G-CSF) were observed. In contrast, our data did not show significant alterations in IL-1 β levels. In this regard, the cytokine IL-1 β is primarily produced by PMN and AM in the pulmonary environment [4,86] activated by NF- κ B [171]. Due to the application route of the Jo2 antibody (intratracheal) an earlier increase in BALF when compared to lung tissue and plasma might be expected. In this regard our data indicated a tendency towards higher concentrations in BALF IL-1 β at 6 h and 18 h subsequent to Jo2 instillation and towards lower levels in the absence of PMN. In lung tissue and plasma this was true only at the 18 h time point. Matute-Bello et al. presented data, showing a significant increase of IL-1 β 4 days after i.t. administration of Jo2. After 2 days no significant increase was observed [121]. Similar results (also with a non-significant increase in response to Fas but not to depletion) were shown by Bem et al. following depletion of AM and stimulation with Jo2 [21]. Taken together, the current data suggests a late increase of IL-1 β in response to pulmonary Fas activation. Furthermore, the divergence in results from isolated chest trauma, chest trauma with subsequent septic challenge, and i.t. instillation of Jo2 in naive lungs indicates an insult-specific modulation of

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the inflammatory response by PMN. In this regard, Ayala et al. proposed an inflammatory priming of neutrophils by an initial insult (e.g. hemorrhage), as shown by increased respiratory burst capacity and decreased apoptosis. Exposure to the second hit (here sepsis) then induced the influx of primed PMN into the lung with the development of ALI [14]. In a double-hit model (chest trauma and sepsis) of ALI Fas was shown to be significantly blunted in lung tissue [155]. A reduction of Fas concentration in lung tissue after chest-trauma induced apoptosis may be the result of receptor internalization after early activation [100]. Fas activation can lead to transcription of NF- κ B by utilizing c-FLIP_L as an adapter-like molecule [190], and to activation of the ERK 1/2 and the p38 MAPK pathway [141]. Thus, a consecutive rise of a plethora of mediators, including TNF- α , IL-1 β , IL-6, MIP-1 α , MIP-2, MCP-1, and KC [117,196], as well as G-CSF in response to stimulation by IL-1 β and TNF- α , may be induced [20,27,38]. In summary, an insult-specific modulation of the inflammatory response by neutrophils appears reasonable to explain the subtle differences observed among the different animal models.

PMN have been proposed to mediate inflammation. MCP-1, a key chemokine for lung monocyte recruitment and an early mediator in ALI, appears to be elevated independently of the type of insult (Fas, single hit, double hit) in the presence and further in the absence of neutrophils in lung tissue. Pulmonary activation of Fas induced an initial chemokine production, followed by cytokine upregulation, which was further increased in the absence of PMN. This finding suggests that PMN possess anti-inflammatory properties containing the local and systemic inflammatory response to Fas activation. Also, it highlights the importance of cell-cell interactions and imposes as an interrupted feedback mechanism. The increase in mediator concentrations appears compensatory in order to recruit neutrophils and indicates an inflammatory priming of the pulmonary environment by previous neutrophil depletion. The sequestration of PMN into the lung may inhibit MCP-1, MIP-2, KC, and MIP-1 α production by AM and lung epithelial cells [103], thus explaining the additional chemokine increase in the absence of PMN.

Interestingly and in contrast to the results mentioned above, depletion of PMN blunted the Fas-induced inflammatory response in the case of KC and IL-6 in lung tissue at 18 h. In this regard, Lomas-Neira et al. [103] also observed a suppression of KC and IL-6 in lung tissue in response to neutrophil depletion and indirect ALI

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from hemorrhagic shock and sepsis at 48 h. However, in a murine model of isolated blunt chest trauma, no decrease in lung tissue KC or IL-6 was observed subsequent to neutrophil depletion [156] but a marked increase of KC concentrations in the lungs were observed 30 h after blunt chest trauma. The divergence of these results might be explained by mediator kinetics since samples were collected at 18 h post i.t. administration of Jo2 in our study, as opposed to a 30 h or 48 h time point in the studies by Perl et al. [156] and Lomas-Neira et al. [103]. Since KC is then primarily released by PMN and endothelial cells [6,58], the latter of which were shown to be susceptible to Fas induced apoptosis [73], the decrease in lung tissue levels of KC after neutrophil depletion and Fas activation may be explicable. IL-6 is produced by a wide array of different cells including monocytes/macrophages, endothelial cells, and fibroblasts in response to endotoxin, IL-1 β , and TNF- α [23-25,37]. In addition, alveolar type 2 cells have been reported as a source for IL-6 after stimulation with IL-1 β , or TNF- α [40]. Since PMN present a major source of IL-1 β in the lung [4], a subsequent reduction of IL-1 β levels is expected in response to PMN depletion. In this regard our results showed a trend towards a reduction of IL-1 β concentrations in lung tissue at 18h after i.t. administration of Jo2 (PMN+ vs. PMN-), which in turn might have lead to a local reduction of lung tissue levels of IL-6. Interestingly, after local AM depletion following i.t. instillation of clodronate and consecutive pulmonary Fas challenge, no significant changes were observed regarding KC and IL-6 in lung tissue [21], suggesting the requirement of PMN but not necessarily AM in order to potentiate the local inflammatory response to Fas regarding KC and IL-6 in lung tissue.

Our results also showed that depletion of neutrophils induces a strong inflammatory response in BALF (6 h: MCP-1, MIP-2, KC, IL-6, G-CSF; 18 h: MCP-1, MIP-2, KC, MIP-1 α , IL-6, IL-10, G-CSF), lung tissue (6 h: MCP-1, MIP-2, KC, MIP-1 α , G-CSF; 18 h: MCP-1, MIP-2, KC, MIP-1 α , G-CSF), and plasma (6 h: MCP-1, MIP-2, KC, IL-6, IL-10, G-CSF; 18 h: MCP-1, MIP-2, KC, IL-6, G-CSF). In this regard, Stirling et al. observed a similar inflammatory response pattern regarding MCP-1, KC, IL-6, and G-CSF after depletion of Gr-1 positive cells [179]. Similar results were also shown in lung tissue for MCP-1 and in plasma for KC [156] and MIP-1 α [113]. However, this observation was not made for other mediators in BALF (KC, MIP-2, MCP-1, IL-6, IL-10), lung tissue (KC, MIP-2, IL-6),

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or plasma (IL-6) after depletion using the Gr-1 antibody [156]. As elaborated above, the finding of increased local and systemic chemotactic mediators after neutrophil depletion highlights the importance of cell-cell interactions and suggests an interrupted feedback mechanism. Another explanation for the increase of inflammatory mediators in response to neutrophil depletion resides within the nature of the Gr-1 antibody. The lack of neutrophil knockout animals has mandated the use of pharmacological agents or antibody-mediated depletion strategies. We depleted mice of neutrophils using the anti-Gr-1 antibody RB6-8C5. This approach has been used frequently by different groups [103,106,139,179]. We refrained from use of the Ly-6G specific antibody 1A8 because of inconsistent effect and duration of neutrophil depletion as described elsewhere [42]. Other agents like cyclophosphamide were excluded due to their considerable immunoregulatory effects [206]. Our depletion analysis showed a significant reduction of the number of PMN within blood and BALF. Moreover MPO activity, a combined marker for presence and activation of neutrophils, was significantly reduced by depletion at both time points. However, depletion did not completely eradicate Gr-1 positive cells, but rather lead to a reduction of neutrophils of about 90%, which has been also reported in other studies [103,179]. This leaves opportunity for residual neutrophil activity, which might have influenced the results. Nevertheless, with respect to clinical implications a reduction of PMN causes a much more desirable therapeutic effect as opposed to a complete eradication rendering the subject unable to fight infecting pathogens. The Gr-1 antibody features binding to the Ly-6C and Ly-6G antigen. Ly-6G is specific for neutrophils, however, the Ly-6C receptor has been reported on other cell populations, including lymphocytes and monocytoid cells [42,54]. Among those are the so called myeloid-derived suppressor cells (MDSC) [161]. Down regulation of these cells has been shown to significantly increase inflammation [197]. However, the effect of the Gr-1 antibody on Ly-6C positive cells was not observed by other authors [133,179]. In conclusion, systemic PMN depletion leads to increased local and systemic chemokine and cytokine levels, suggesting an interrupted feedback mechanism and an anti-inflammatory capacity of PMN.

We observed that i.t. instillation of the Fas-agonistic antibody Jo2 lead to a significant raise in mediator plasma levels (6 h: MCP-1, KC; 18 h: MCP-1, KC, IL6)

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similar to their local (BALF, lung homogenate) counterparts. This finding may be interpreted as a consequence of alveolo-capillary barrier disruption with a reversed flow direction, leading to an overspill of pulmonary mediators into the blood. In this regard, i.t. instillation of human IL-6 lead to a dose-dependent correlation between BALF and plasma levels after blunt chest trauma, constituting a direct relationship between the local pulmonary and systemic concentration of inflammatory mediators [150]. As opposed to the plethora of consequences following blunt chest trauma, isolated pulmonary Fas-activation leads to the apoptosis of pneumocytes, which has been described as a major contributor regarding a compromise of alveolar wall permeability [73]. While this may work as an explanation regarding MCP-1 and IL-6, which we observed to be markedly increased at 6 h or 18 h respectively in plasma and BALF, our data does not support this hypothesis with respect to KC. KC was increased significantly in plasma at 6 h after Jo2, whereas no significant increase in BALF could be observed. The fact that endothelial cells represent a main source of KC with respect to lung tissue but not BALF may play a role here. In line with this hypothesis we observed a significantly increased concentration of KC in lung tissue at 6 h. Moreover, mediators might be even more increased locally than systemically. In response to blunt chest trauma, Kupffer cells (KU) and splenocytes (SPL) have been suggested as a mediator source at 30 h after the impact [156]. Regarding our data, pulmonary instillation of Jo2 may also have lead to chemokine/cytokine release by distant immune cells. In this regard, Fas activation has been reported to lead to significant inflammatory mediator releases from peritoneal macrophages [76], human blood monocytes and monocyte-derived macrophages [145], human endothelial cells (MCP-1, IL-8) [200], and dendritic cells from mice (MCP-1, MIP-2, MIP-1 α) [71]. After incubation with the Jo2 mAB, Fas activation induced pro-inflammatory gene expression via nuclear translocation of NF- κ B in human monocytes and monocyte-derived macrophages (1 h: MCP-1, TNF- α , IL-1 β , IL-6; 4/8 h: MCP-1, IL-1 β , IL-6; but not IL-10) [145]. Activated circulating PMN express KC, MIP-2, TNF- α , and present the predominant source for IL-1 β in the pulmonary environment [4]. Activated PMN also show the capacity to express and release MCP-1 [202]. Taken together, the combination of pulmonary mediator overspill and systemic upregulation via distant cells, including endothelial cells, monocytes, macrophages, neutrophils, Kupffer

cells, splenocytes, and dendritic cells, may both lead to an increase in mediator plasma levels following Fas activation in the lungs.

4.2 Apoptosis

Apoptosis is mediated via a family of death receptors, including the TNF receptors and the Fas receptor. TNF- α is not regularly detectable in BALF of patients suffering from ALI/ARDS. In addition, the levels of soluble TNF receptors far exceed the concentrations of free TNF- α , suggesting that the existing TNF activity is localized to lung tissue [12,112,147]. The Fas/FasL pathway has been suggested to play an important role in inducing apoptosis during ALI [34] in addition to direct epithelial necrosis caused by mechanical factors, ischemia or pathogens. It presents an important pathomechanism of alveolar epithelial cell injury in the lungs of patients with ALI [115]. Human pneumocytes, i.e. lung epithelial cells type I and II, express Fas and are responsive to FasL-induced apoptosis, especially in the distal airways [134]. Fas activation has also been shown to cause apoptosis of alveolar endothelial cells, but this effect was less pronounced than the effect on the alveolar epithelium [73], rendering the second particularly susceptible to Fas induced apoptosis. In addition, under physiological conditions, the epithelial barrier in the lungs is less permeable compared to the endothelium [195]. Apoptosis of pneumocytes has been described as a major pathomechanism promoting increased alveolar wall permeability. In contrast, Fas-mediated inflammation did not exert a deleterious effect on protein permeability. In vitro data of Herrero et al. showed that low level stimulation of the Fas pathway in isolated lung epithelial cells is sufficient to increase permeability (11.5% cell death), whereas cytokine expression remained unchanged. Blockage of cell death by a pan-caspase inhibitor prevented changes in permeability, while leading to increased Fas dependent cytokine expression [73]. An increased amount of biologically active soluble Fas ligand (sFasL) was detectable in the airspaces of patients with ALI [7,115]. Moreover, FasL is expressed on lymphocytes [11,132,163], monocytes [143], neutrophils [102,167], eosinophils [132], and platelets [5]. Soluble FasL has the potential to induce apoptosis in leukocytes, pneumocytes and other parenchymal cells [110]. Immunohistochemical analysis in

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a murine model of LPS-induced ALI showed that the expression of Fas was seen in the alveolar wall cells, namely lung epithelial cells type I and II, and endothelial cells, as well as in the infiltrating PMN and AM at 24 h, whereas expression of FasL was limited to the macrophage-like cells in the air space [91]. Fas expression as well as TUNEL-positive staining was observed in 30 % of alveolar wall cells, as well as in 70% of inflammatory cells (AM, PMN), indicating that Fas-induced apoptosis plays a role in both cell types. The amount of TUNEL-positive cells was significantly reduced in inflammatory and alveolar wall cells by instillation of LPS in combination with an antagonistic anti-Fas antibody [91].

Here we observed a significant increase in activated caspase-3 and TUNEL-positive cells at 18 h in response to pulmonary Fas activation in the presence of neutrophils. In line with our results, Matute-Bello et al. presented an increased pulmonary apoptotic rate 24 h after i.n. application of Jo2 [118]. In addition, Bem et al. showed a significant increase in caspase-3 activity 18 h after i.t. delivery of Jo2 [21]. After 16 h of i.t. administration of recombinant human sFasL, a significant increase in TUNEL-positive cells as well as caspase-3 activity was observed by Herrero et al. [73]. Wild-type mice and mutant mice lacking Fas in myeloid cells (e.g. AM, PMN), but not mutant mice expressing Fas in myeloid cells only, displayed significantly increased pulmonary apoptosis 24 h after i.t. Jo2 [120]. Taken together, pulmonary activation of Fas induced an elevation of apoptosis only in the presence of Fas in lung epithelial and endothelial cells. This suggests the alveolar epithelium to be the primary target of Fas-induced apoptosis and consecutive lung injury.

Our results show a significant increase of cleaved active caspase-3 at 6 and 18 h after i.t. instillation of Fas in the absence of PMN (ISO vs. FAS). However, we did not observe a significant alteration in apoptosis comparing depleted and not depleted mice in response to Fas (PMN+ vs. PMN-) at 6 or 8 h. Bem et al. observed a significant increase in caspase-3 activity 18 h after i.t. administration of Jo2 in the absence of alveolar macrophages (AM+ vs. AM-) [21]. Increased Fas induced apoptosis seen after depletion of resident AM as opposed to depletion of PMN might be explained by deficient phagocytosis and degradation of apoptotic PMN by AM [92]. A potential pathway of lung injury by neutrophils, next to the

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release of cytokines, proteases, elastases, NOS and ROS, is the induction of AT II cell apoptosis by release of sFasL from neutrophils undergoing spontaneous apoptosis [173]. Apoptotic cells, including neutrophils, are cleared by resident macrophages [51,173]. Apoptotic cells that have not been removed in time can progress to secondary necrosis with loss of cell membrane integrity and subsequent cleavage and release of membrane bound sFasL as well as release of MPO and esterase resulting in further tissue necrosis, apoptosis, and inflammation [168]. Therefore, in the absence of AM, an increased apoptotic rate of lung epithelial cells may be observed. However, after PMN depletion a reduced amount of apoptotic neutrophils may be present in the lung, thus reducing pulmonary apoptosis and secondary damage. In conclusion, pulmonary Fas activation leads to apoptosis of a variety of cell types, including pneumocytes which are of great importance to the integrity of the alveolo-capillary barrier function. Pulmonary apoptosis in response to Fas was increased in the absence of AM [21], but independent of the presence of PMN as observed in our study. This observation consolidates the role of Fas as an initiator of lung epithelial cell apoptosis with the potential consequence of alveolo-capillary barrier disruption and suggests a direct impact of Fas on epithelial cells independent of the presence of PMN. In line with this observation, 16 h after i.t. instillation of recombinant human sFasL and s.c. application of the caspase inhibitor zVAD-fmk decreased apoptosis and lung damage were observed [73]. Furthermore, silencing of caspase-3 via small interfering ribonucleic acid (siRNA) improved alveolo-capillary barrier function and histological appearance in a murine model of trauma-induced septic acute lung injury [123]. Thus, Fas-induced lung epithelial cell apoptosis is suggested as a necessary premise for Fas-induced lung injury and is not mediated by AM [21] or PMN as indicated by our results.

Interestingly, PMN depleted mice presented a significant increase in TUNEL-positive cells at 18 h independent of i.t. Jo2. Whether a reduced resolution of dead cells due to a reduced amount of reparative Ly-6C positive monocytes may have influenced this finding, needs to be further investigated. Furthermore, due to the non-specific nature of the detection technique the observed increase in TUNEL-positive cells might be caused by other means of cell death, including necrosis [79]. Thus, a reduced resolution of debris may have influenced our results.

4.3 Lung Damage

Our data shows an early significant increase in BAL protein concentration after pulmonary activation of the Fas system at 6h in the presence of PMN. Supporting this finding, microscopic evaluation of lung tissue sections (H&E) from control mice displayed few inflammatory cells, intact alveolar architecture, and regular alveolar wall constitution. In contrast, Fas-treated animals showed inflammatory cell influx, alveolar architecture disruption, and alveolar wall thickening, illustrating lung damage. In line with our observation, Matute-Bello et al. indicated an increased BAL protein concentration 6 h after intranasal application of Jo2 in wild-type mice, whereas no changes were seen at 24 h. These results were supported by corresponding lung histology sections and findings were blunted in *lpr* mutant mice, devoid of Fas [118]. Pulmonary instillation of recombinant FasL (rFasL) lead to increased BAL protein concentration at 3, 6, and 24 h as shown by Wortinger et al. in 2003 [196]. Divergences in significant BAL protein concentration peaks may be explicable due to the different antibodies and dosages (62.5 µg Jo2 vs. 500 ng rFasL/mouse) used.

The Fas-induced increase in BAL protein concentration was significantly reduced in the absence of neutrophils (PMN+ vs. PMN-), suggesting a protective effect of PMN depletion on the alveolo-capillary barrier dysfunction. Lung histology sections confirmed this finding at 6 and at 18 h, since PMN depleted animals exhibited these pathological findings mentioned above to a much lesser degree. The protective capacity of neutrophil depletion was shown in various settings and lead to the distinction between neutrophil dependent- and neutrophil independent forms of ALI [4,67]. Reperfusion-, immune complex-, sepsis-, or even LPS-induced forms of acute lung injury responded with a reduction of lung damage parameters after previous neutrophil depletion. However, for example oleic acid- or hyperoxia-induced ALI did not respond favourable to previous PMN depletion [89,122,176]. Neutrophil induced lung damage also seems to depend on the presence of an infectious agent. Over-expression of the anti-apoptotic protein Bcl-2 in a myeloid restricted fashion, thus prolonging the lifespan of PMN, worsened lung injury and

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reduced survival after SIRS (LPS), but displayed a protective capacity with a survival benefit after sepsis (CLP) in a murine model of hemorrhage-induced septic lung injury [151]. Fas-induced lung injury requires Fas-expression on non-myeloid cells of the lung [120], emphasizing the role of lung epithelial cell dependent mediator release, and apoptosis. In the absence of resident AM, Fas-induced lung damage was worsened [21], displayed by significantly increased BAL protein concentration and lung histology. Furthermore, depletion of AM increased lung damage and inflammation following non-lethal unilateral lung contusion in mice [109]. The protective effects of neutrophil depletion fall into place particularly in synopsis with the exacerbation of lung injury in the absence of AM as a result of deficient phagocytosis and reduced degradation of apoptotic PMN [92]. Pulmonary Fas activation leads to production of MCP-1, which in turn induces macrophage differentiation towards the protective M2 phenotype [107] with increased migration in response to Fas [204] and elevated capacity for phagocytosis of necrotic cells and debris, representing neutrophil chemotactic signals [183]. In summary, PMN depletion blunted lung damage but not lung apoptosis in response to pulmonary Fas activation. Therefore, the absence of Fas-induced PMN influx, activation, as well as the consecutive release of cytokines, proteases, elastases, NOS, and ROS blunted lung damage even though apoptosis of lung epithelial cells remained unaffected. Apoptosis then may present an essential premise for Fas-induced lung damage, but appears not to be sufficient by itself.

4.4 Conclusions

We found Fas/FasL to be an effective trigger of pulmonary inflammation, apoptosis, and lung damage. Our data suggests that following activation of the Fas-apoptotic pathway the presence of PMN contains inflammation but also facilitates structural organ damage. Fas induced apoptosis was independent of the presence of PMN, while pulmonary inflammation and lung damage both depended on the presence of neutrophils. With the exception of KC and IL-6 in lung tissue, inflammation was increased, whereas lung damage was blunted by PMN depletion. Moreover, pulmonary activation of the Fas signalling pathway was sufficient to induce alveolo-capillary barrier disruption only in the presence of neutrophils. Thus, secondary lung damage via recruitment of activated PMN into the lungs in response to Fas mediated inflammation is an important mechanism in the development of structural lung damage, and needs to be differentiated from the direct effects of Fas induced lung epithelial cell death.

5. SUMMARY

Pulmonary Fas activation is a hallmark of the acute respiratory distress syndrome (ARDS). Fas-signalling induces chemokine release by lung epithelial cells (LEC) with subsequent immigration of activated neutrophils (PMN), LEC apoptosis, and lung damage. So far it remained unclear whether lung damage after Fas activation is the consequence of LEC apoptosis, PMN influx, or both.

Therefore, mice (C57Bl/6) were injected with PMN-depleting antibody (Gr-1) or isotype 48 h prior to intratracheal (i.t.) instillation of Fas-activating antibody (Jo2) or isotype. 6 or 18 h later lung tissue, plasma, and bronchoalveolar lavage fluid (BALF) were harvested. Lung cytokines were quantified by multiplex or enzyme-linked immunosorbant assay (ELISA), lung active caspase-3 (C-3) by western blotting. Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) and hematoxylin-eosin (H&E) staining were performed in lung sections. Lung myeloperoxidase activity (MPO) was evaluated. Total protein concentration in BALF was assessed. PMN depletion markedly reduced the increase of lung MPO activity and PMN influx in BALF observed after Fas activation in non-depleted mice. In the presence of PMN, keratinocyte-derived chemokine (KC) (lung, plasma), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) (lung, plasma, BALF) were markedly increased 18 h after Jo2; in the absence of PMN no such increase was observed for lung KC or IL-6, whereas MCP-1 was even further enhanced in lungs and BALF but not in plasma. KC was also further enhanced in BALF and plasma, whereas IL-6 remained similar to mice treated with Fas in the presence of PMN. 6 h after Jo2 BALF protein was markedly increased in the presence but not in the absence of PMN. Following Fas activation apoptosis was increased (TUNEL, C-3) irrespectively whether PMNs were present or absent.

Taken together, in the absence of PMN, Fas induced lung inflammation was divergently affected, whereas pulmonary apoptosis remained unaffected. The degree of lung injury, however, was blunted after PMN depletion. This indicates, that secondary lung damage via recruitment of activated PMN into the lungs in response to Fas mediated inflammation is an important mechanism in the development of structural lung damage and needs to be differentiated from the direct effects of Fas induced lung epithelial cell death.

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7. PUBLICATIONS

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9. CURRICULUM VITAE

Der Lebenslauf wurde aus Gründen des Datenschutzes entfernt.

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Inhalte der Dissertation wurden bereits in folgendem Artikel veröffentlicht:

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