The pathogenesis of lung emphysema:

lessons learned from murine models

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The pathogenesis of lung emphysema:
lessons learned from murine models

De pathogenese van longemfyseem:

bevindingen verkregen uit muismodellen

(met een samenvatting in het Nederlands)

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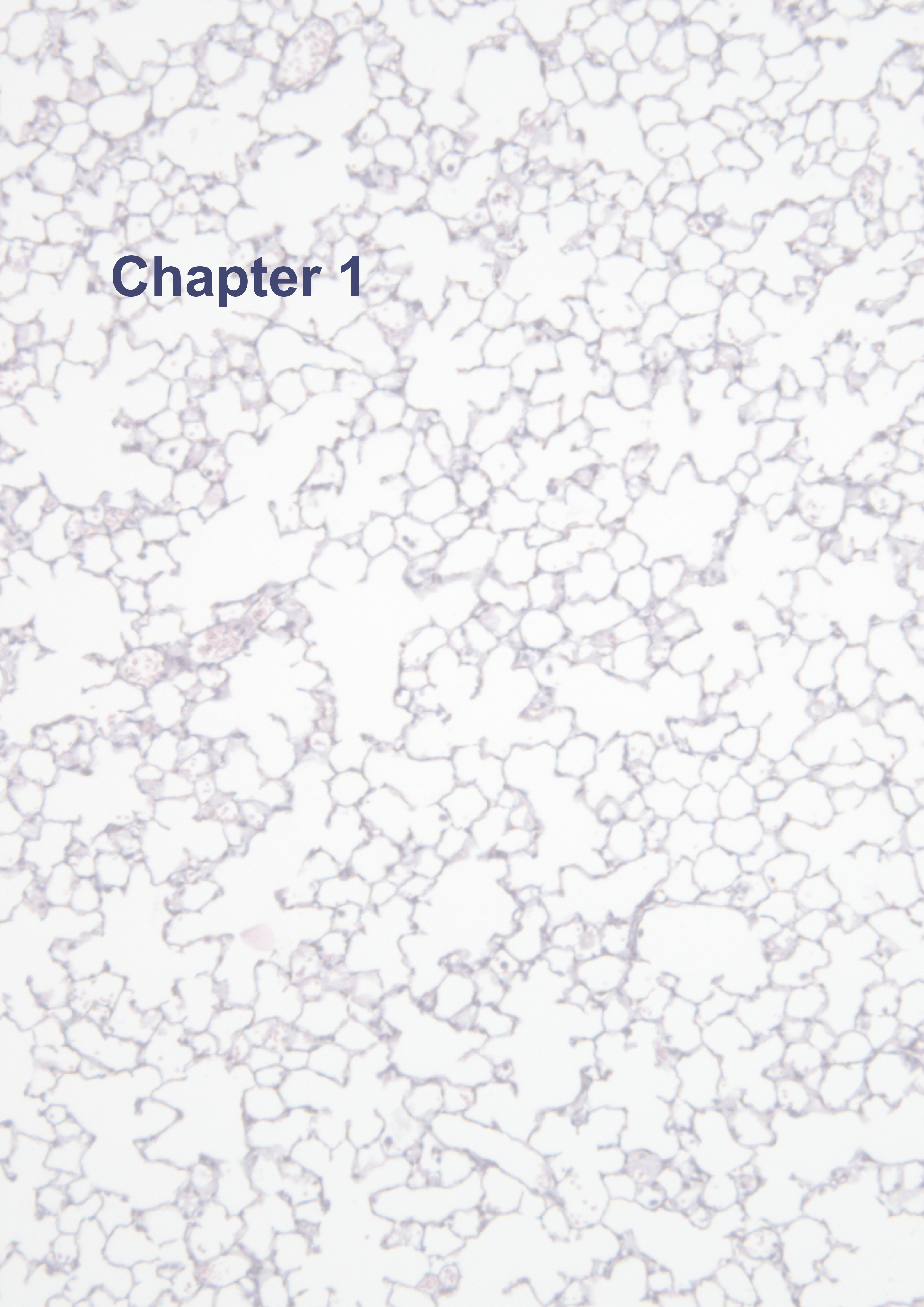

Chapter 1

General introduction and outline of the thesis

Introduction

Definition of COPD

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality throughout the world. COPD is currently listed as the fifth leading cause of death in the world and the WHO predicts that it will become the fourth leading cause of death by 2030 [1-3]. COPD is defined by the Global initiative in Obstructive Lung Disease (GOLD) as: "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" [4, 5]. COPD involves two spectra of clinical or pathological conditions, chronic bronchitis and lung emphysema.

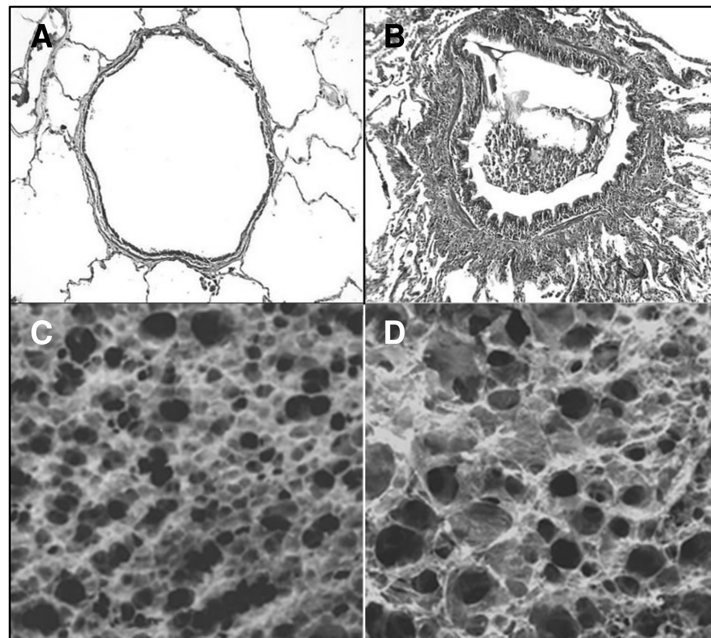

Fig.1. *Photograph of a human airway with an active inflammatory process, where the mucus extends into the lumen and the airway wall is thickened (B) compared to a normal airway with an empty lumen (A). Photograph of a surface of normal human lung tissue with thin airway walls and intact alveoli (C) compared to emphysematous human lung tissue with alveolar enlargement induced by destroyed parenchyma (D). Adapted from references [6, 7].*

Chronic bronchitis is defined as the presence of a mucus-producing chronic cough most days for a minimum of 3 months a year, for at least 2 successive years. The main characteristics of chronic bronchitis are mucus hypersecretion and chronic inflammatory cell infiltration of the bronchial wall, which could lead to airway obstruction [8, 9] (Fig.1A and B). Pulmonary emphysema is defined as abnormal permanent enlargement of airspaces distal to the terminal bronchioles accompanied by destruction of alveolar walls without obvious fibrosis [8, 9] (Fig.1C and D).

The diagnosis of COPD should be considered in patients with cough, sputum or dyspnoea and/or a history of exposure to risk factors for this disease, such as tobacco smoking, occupational dusts or chemicals, indoor or outdoor pollution and respiratory infections [10]. COPD is characterized by an accelerated decline in lung function. Airflow limitation is measured by spirometry, as this is the most widely available and reproducible test of lung function, which is used for diagnosis of COPD. The classification of severity of COPD, as issued by GOLD, is based upon Forced Expiratory Volume in one second (FEV_1) and its ratio to the Forced Vital Capacity (FVC), namely FEV_1/FVC (Table 1) [4, 5, 11].

COPD and cardiovascular diseases

Cardiovascular diseases, such as ischemic heart disease, pulmonary hypertension and heart hypertrophy, are important complications of COPD [12, 13], since heart failures are one of the leading but underrecognized causes of death in COPD patients. The epidemiologic evidence linking COPD and cardiovascular morbidity and mortality is strong and COPD patients are two to three times more at risk for cardiovascular mortality [12, 14-16]. Even modest reduction in FEV_1 elevates the risk of ischemic heart disease, atrial fibrillation and sudden cardiac deaths. The high prevalence of smoking, increased systemic inflammation, oxidative stress, increased hemodynamic abnormalities and chronic hypoxia might increase the risk of developing cardiac disease in COPD patients [17]. However, the exact relationship between COPD and cardiovascular events remains unclear.

***Table 1**. GOLD classification of COPD severity [4, 5].*

STAGE OF COPD	FEV_1 FEV_1/FVC	SYMPTOMS
Stage I: Mild COPD	$FEV_1 \geq 80\%$ predicted $FEV_1/FVC < 0.70$	± Variable
Stage II: Moderate COPD	$50\% \leq FEV_1 < 80\%$ predicted $FEV_1/FVC < 0.70$	+ Mild to moderate
Stage III: Severe COPD	$30\% \leq FEV_1 < 50\%$ predicted $FEV_1/FVC < 0.70$	++ Limit exertion
Stage IV: Very severe COPD	$FEV_1 < 30\%$ predicted or $FEV_1 < 50\%$ predicted plus chronic respiratory failure $FEV_1/FVC < 0.70$	+++ Limit daily activities

FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; Respiratory failure: arterial partial pressure of oxygen (PaO_2) less than 8.0 kPa (60 mmHg) with or without arterial partial pressure of CO_2 ($PaCO_2$) greater than 6.7 kPa (50 mmHg) while breathing air at sea level.

Risk factor for COPD

Cigarette smoking is by far the most important risk factor for COPD in more than 90% of the COPD patients. However, only 15-20% of all smokers develop COPD, this implies that there should be other co-factors and/or an individual susceptibility in order to develop COPD [18]. Smoking-induced inflammation in the lung is thought to play a key role in the pathogenesis of COPD. Since, cigarette smoking is the most common form of tobacco use, most research into the effects of tobacco smoke on humans and animals has been performed using cigarettes. Cigarette smoke is a very complex mixture and contains more than 4700 compounds. These compounds comprise human carcinogens and many toxic agents, including carbon monoxide, ammonia, acrolein, acetone, nicotine, benzopyrenes, hydroquinone and nitrogen oxides [19]. The following types of cigarette smoke are distinguished: mainstream, sidestream and environmental smoke. Mainstream smoke is tobacco smoke, which is directly inhaled into the smoker's lung before it is released into the surrounding air. Sidestream smoke includes the smoke components that are emitted into the air during the burning of a tobacco product during and between puffs. Environmental smoke is a mixture of sidestream smoke and exhaled mainstream smoke diluted in ambient air of which the physical and chemical properties considerably change with time [20-22].

Treatment of COPD

Bronchodilator medications (β_2-agonists and anticholinergics) and inhaled corticosteroids are the most prescribed drugs for COPD and are central to the symptomatic management of COPD. Regular therapy with these drugs leads to small clinical benefits in terms of symptoms, exacerbation rates and lung function levels [10, 23]. Nevertheless, smoking cessation is the only most effective therapeutic intervention shown to reduce the risk of developing COPD, to stop the disease progression and to improve survival. Moreover, patients who quit smoking experience fewer respiratory symptoms and less hyperresponsiveness as compared to those who continue smoking [24-26]. Since treatment options for COPD remain limited to the amelioration of symptoms, and the pathogenesis of COPD is not yet fully elucidated, more research into this disease is warranted.

Pathogenesis of COPD

The pathogenesis of COPD is strongly linked to the effects of cigarette smoke on the lungs. Three processes are believed to be most important in COPD: pulmonary chronic inflammation, a protease-antiprotease imbalance and oxidative stress. COPD is a complex inflammatory disease that involves several types of inflammatory and structural cells, all of which have the capacity to release multiple inflammatory mediators [6, 27, 28]. The general hypothesis on COPD states that stimuli, such as cigarette smoke, trigger airway resident macrophages and epithelium to produce chemoattractants, such as interleukin-8 (IL-8 or CXCL8). These chemoattractants facilitate the migration of inflammatory cells, like neutrophils. The activated neutrophils and macrophages release proteases which lead to alveolar wall destruction and mucus hypersecretion [28, 29] (Fig.2). The role of epithelial cells and various inflammatory cells in COPD will be discussed more extensively below.

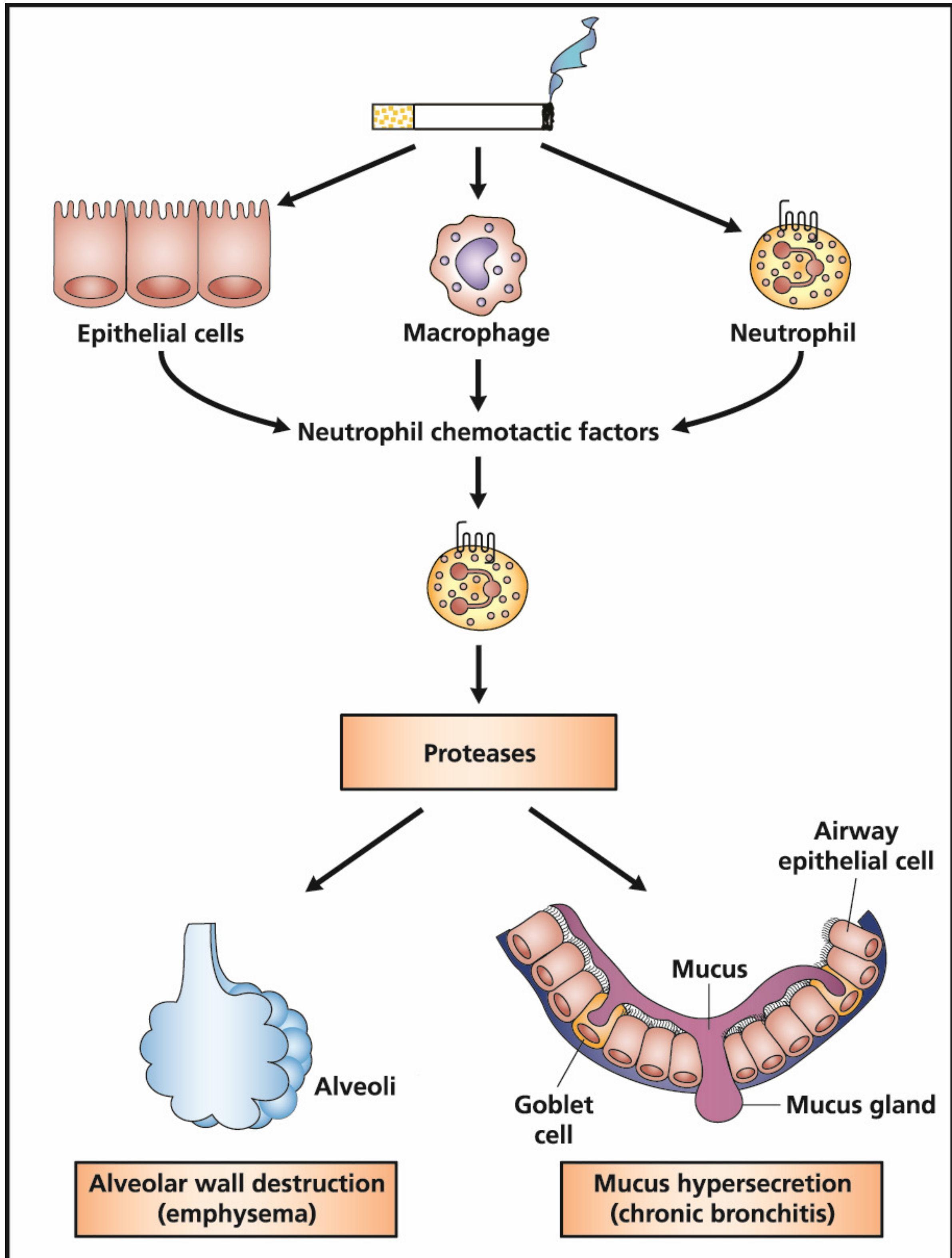

Fig.2. *The general hypothesis on COPD. Cigarette smoke stimulates epithelial cells and airway resident macrophages to release neutrophil chemotactic factors, such as CXCL8, which is also produced by activated neutrophils. The attracted neutrophils release different proteases, which lead to alveolar wall destruction (emphysema) and mucus hypersecretion (chronic bronchitis).*

Cell types involved in COPD

Epithelial cells

The airway epithelium is an important barrier between the host and the environment and contributes to an adequate maintenance of lung homeostasis by mucus production, ciliary beating, secretion of antimicrobial products and an adequate immunological drive in response to noxious stimuli. Upon cigarette smoke activation epithelial cells release inflammatory mediators and proteases, such as CXCL8, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and interleukin-1β (IL-1β) [30-32], which are important for initiating and maintaining the pulmonary inflammation and airway remodeling.

Macrophages

Macrophages appear to play a central role in the inflammatory process in COPD and can account for most of the features of the disease. Increased numbers of macrophages have been found in airways, lung parenchyma, sputum and bronchoalveolar lavage fluid (BALF) of smokers and COPD patients, which are correlated with the severity of COPD. Cigarette smoke activates macrophages to release inflammatory mediators, such as TNF-α, CXCL8 and matrix metalloproteinases (MMPs), which can contribute to the pulmonary inflammation and alveolar wall destruction [33-36].

Neutrophils

There is abundant evidence confirming the neutrophil as a crucial effector cell in COPD. Increased numbers of neutrophils are found in sputum and BALF of patients suffering from COPD. The neutrophil numbers in bronchial biopsies and induced sputum are correlated with COPD disease severity and with the rate of decline in lung function. Neutrophils can recruit to the airways and parenchyma under control of different chemotactic factors, like CXCL8 and leukotriene B_4 (LTB_4), which are increased in COPD airways. Activated neutrophils release several proteins, such as neutrophil elastase, MMPs, oxygen radicals and myeloperoxidases (MPO), which may all contribute to alveolar destruction. [33, 34, 37-42].

Dendritic cells

Dendritic cells are specialized antigen presenting cells and initiate the innate and adaptive immune responses [43]. The airways and lung parenchyma contain a network of dendritic cells localized at the interface between the inhaled air and the lung. Therefore, dendritic cells seem to play an important role in signaling the entry of foreign substances, such as cigarette smoke and they can activate a variety of other cell types including macrophages, neutrophils and T and B lymphocytes [44, 45]. Increased numbers of dendritic cells are present in the

airways and sputum of COPD patients [46], but the exact role of dendritic cells in the pathophysiology of COPD remains to be elucidated.

Lymphocytes

Inflammatory cells of the innate immune system as well as the adaptive immune system infiltrate the lungs in COPD. Increased numbers of T lymphocytes, particularly CD8+ lymphocytes, have been observed throughout the lungs of COPD patients. The amount of alveolar destruction and the severity of airflow obstruction are correlated with the number of T lymphocytes. There is also an association between CD8+ lymphocytes and apoptosis of alveolar cells in emphysema. These CD8+ lymphocytes have the capacity to contribute to parenchymal destruction in COPD via release of perforins, granzymes, and TNF-α [34, 35, 47-50].

Furthermore, B lymphocytes are increased in the large and small airways of patients with COPD, which is associated with disease severity. This B cell increase can result from a local inflammatory process, an altered T-helper (Th)1-Th2 balance and/or can reflect an antigen-specific reaction to cigarette smoke components, autoantigens, or pathogens [51-53]. B cells respond to pathogens by producing and secreting immunoglobulins. It is also documented that besides whole immunoglobulins, B cells produce and secrete immunoglobulin free light chains (IgLC) in excess over immunoglobulin heavy chains [54, 55].

Inflammatory mediators involved in COPD

Inflammatory mediators have a very critical role in the pathophysiology of COPD. Different mediators, such as chemokines, cytokines, growth factors and proteases are involved in the inflammatory process that results in alveolar destruction and airway fibrosis.

Cytokines

Several cytokines have been implicated in COPD. Cytokines are small cell-signaling proteins produced by different cell types, such as epithelial cells, macrophages, T and B lymphocytes. Cytokines associated with COPD include TNF-α, interferon-y (IFN-y), IL-1β, IL-6, IL-12 and granulocyte macrophage colony stimulating factor (GM-CSF). These cytokines are associated with the airway inflammation observed in COPD patients and these cytokine levels are elevated in sputum and/or BALF of COPD patients.

Additionally, growth factors like transforming growth factors (TGF), epidermal growth factor (EGF), vascular-endothelial growth factor (VEGF) and fibroblast growth factors (FGF) are also possible mediators in COPD. Growth factors can induce fibrosis and cell proliferation

and increased expression has been observed in the airways of patients with COPD [34, 56-59].

Chemokines

Chemokines, a family of chemotactic cytokines, play a crucial role in orchestrating inflammatory and immune responses by regulating the trafficking of inflammatory and immune cells to target organs, such as the lung. Several chemokines are involved in the recruitment of inflammatory cells in COPD. Especially, CXCL8 and its receptors CXCR1 and CXCR2 have an important role in the pathogenesis of COPD, since CXCL8 is a potent chemoattractant of neutrophils. CXCL8 levels are markedly increased in sputum and BALF of patients with COPD and correlate with the increased number of neutrophils. CXCL8 may derive from alveolar macrophages, epithelial cells and neutrophils itself [34, 60-62]. Several *in vivo* studies confirmed the role of CXCL8 and its receptor CXCR2 in the development of COPD, since a CXCR2 antagonist reduces neutrophilic inflammation in the lungs of mice exposed to cigarette smoke [63, 64]. Furthermore, neutrophil migration is inhibited by the selective CXCR2 antagonist SB225002 [65].
Other important chemokines associated with the recruitment of inflammatory cells in COPD are growth-related oncogene (GRO)-α (CXCL1), epithelial cell-derived neutrophil-activating peptide (ENA)-78 (CXCL5), monocyte chemoattractant protein (MCP)-1 (CCL2) and macrophage inflammatory protein (MIP)-1α (CCL3) [34, 62, 66, 67]. The role of chemokines and chemokine receptors in the pathogenesis of COPD will be discussed more extensively in Chapter 2.
There is now evidence that the collagen fragment N-acetyl proline-glycine-proline (N-Ac-PGP) is also a chemotactic mediator [68]. N-Ac-PGP can induce neutrophil recruitment into the airways and plays a role in the regulation of neutrophils and inflammation in COPD [29, 69].

Proteases

Proteases are enzymes involved in the breakdown of connective tissue components in lung parenchyma causing the occurrence of lung emphysema. An imbalance between proteases and antiproteases is believed to play an essential role in the development of COPD [34, 70]. The imbalance may occur either by an excessive release of proteases by inflammatory cells and lung resident cells, or by a reduced synthesis or increased breakdown of antiproteases. Initial COPD studies focused on neutrophil elastase (NE), a serine protease produced by neutrophils, which has the capacity to hydrolyze elastin and also potently stimulates mucus secretion. Many other proteases have been implicated in the development of COPD based

on their capacity to degrade elastin. These include other serine proteases and cysteine proteases. Moreover, proteases involved in the degradation of collagen and/or gelatin play a major role in COPD. These include certain members of the matrix metalloproteinases (MMPs) family, a group of zinc-based proteases that regulate the destruction of the extracellular matrix. MMPs are released by both inflammatory and structural cells. The concentration and activity of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-12 is increased in sputum of COPD patients [71-75]. Interestingly, MMPs are involved in the regulation of cytokines, chemokines and growth factors, thereby further contributing to the inflammatory response in COPD [76-79].

Purines

Numerous inflammatory mediators as described above have been implicated in the inflammatory cascade found in COPD airways. A similar role has been proposed for purines, especially adenosine-5'-triphosphate (ATP) and the product of its enzymatic breakdown, adenosine [80-83]. ATP is an important signaling molecule and can be released via lytic or non-lytic mechanisms by many cell types, such as structural and inflammatory lung cells under conditions of cellular stress [84, 85]. ATP induces the recruitment and activation of inflammatory cells including neutrophils, macrophages and dendritic cells [86]. However, little is known about the exact contribution of ATP in airway inflammation in COPD.

Oxidative stress

Oxidative stress is defined as an imbalance between oxidants and/or antioxidant capacities. Various studies have shown increased markers of oxidative stress in the airspaces, breath, blood and urine in smokers and in patients with COPD [87]. The increased oxidative stress observed in COPD patients is due to an increased burden of inhaled oxidants present in cigarette smoke, as well as increased amount of reactive oxygen species (ROS) generated by activated inflammatory and structural cells, including macrophages, neutrophils and epithelial cells [34, 87, 88]. The presence of oxidative stress has important consequences for the pathogenesis of COPD, which include damage to lipids, proteins, DNA and carbohydrates, oxidative inactivation of antiproteases, airspace epithelial injury, increased sequestration of neutrophils in the pulmonary microvasculature and gene expression of proinflammatory mediators [87].

Models for COPD

In vitro model

In vitro models usually investigate the acute response of different cell types to the exposure of cigarette smoke extract (CSE). Cigarette smoke extracts can be prepared by capturing the smoke emitted from a cigarette and bubbling it through an aqueous solution. However, these preparations have disadvantages of losing volatile and reactive components, a variety of chemical changes can take place and CSE is only a part of cigarette smoke. On the other hand, very specific questions such as the effect of CSE on the release of different mediators by inflammatory cells can be investigated [89-91].

In vivo models

Several experimental models of COPD and emphysema exist in mice, based upon different approaches.

Firstly, tracheal instillation of various proteins, chemicals and irritants has been used to study the development of lung emphysema. The administration of lipopolysaccharide (LPS), a strong pro-inflammatory compound present in the cell wall of Gram-negative bacteria, results in neutrophil recruitment and airspace enlargement. This mouse model mimics several important pathological changes that are observed in COPD patients, including irreversible alveolar enlargement and pulmonary inflammation [92, 93]. LPS was demonstrated to be present in high concentration in tobacco and bioactive LPS could be detected in both mainstream and sidestream cigarette smoke [94]. Furthermore, irritants such as pollutants, oxidants (nitrogen dioxide) and ozone have been applied to experimental animal models [95]. Tissue-degrading enzymes have been used to generate COPD in experimental animals. A variety of proteases (porcine pancreas elastase, neutrophil elastase, proteinase-3) have been instilled into the lungs of rodents and caused significant airspace enlargement and neutrophil accumulation in the lung [95].

Secondly, several mouse strains with naturally occurring genetic mutations develop emphysema spontaneously without external stimuli, such as mice with a mutation in fibrillin-1, which is involved in elastic fiber assembly or pallid mice that are α_1-antitrypsin deficient and develop mild emphysema late in life. Moreover, transgenic and gene targeted mice provide powerful techniques to determine the importance of the gene in question *in vivo.* Gain of function models may be achieved by overexpression of proteins in transgenic mice (e.g. overexpression of IL-13, IFN-y or MMP-1) and loss of function models achieved by targeted mutagenesis (e.g. elastin or MMP-12 knockout mice) [92, 95-97].

Thirdly, murine models of cigarette smoke-related COPD are mainly used to mimic the human situation, because of the overwhelming role of cigarette smoke as a causative agent (Fig. 3).

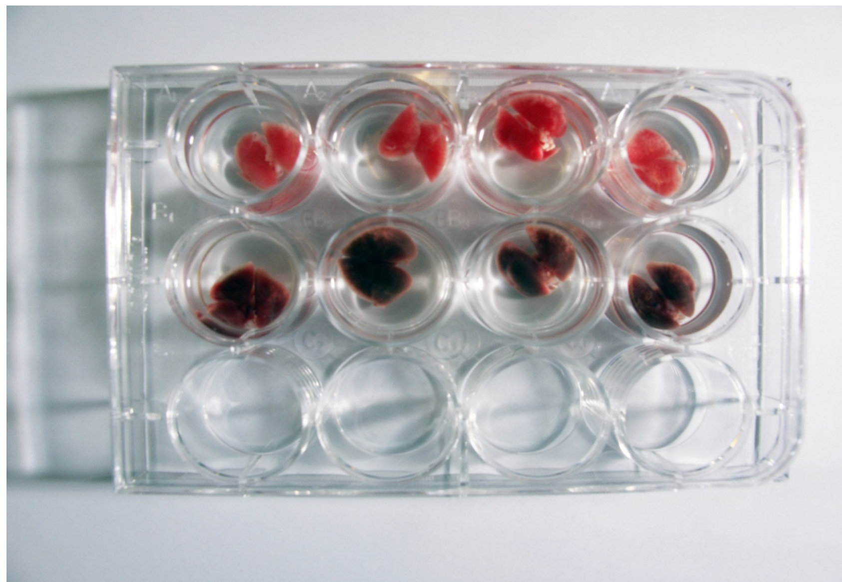

Fig.3. *Lungs of mice after 5 months air (first row) or cigarette smoke (second row) exposure via a whole body exposure system.*

Despite considerable variation in the specific pulmonary responses to acute or chronic cigarette exposure, there is no doubt that cigarette smoke adversely affects the lungs of laboratory animals in various ways, including the induction of airway wall inflammation, epithelial cell alterations and formation of emphysema as conclusively demonstrated by different studies. [22, 92, 95, 97, 98]. Mice can be exposed to cigarette smoke delivered by a smoking machine either by a nose only or by a whole body methodology. Neither the type/number of cigarettes, nor the exposure time and the time between exposure and measurement, nor species, strain, sex and age of the animals has been standardized and is a personal methodological choice [22, 89, 99]. Emphysema starts to develop in different mice strains after 3-4 months of smoke exposure. The severity of emphysema progresses with time with continuing exposure [89, 100, 101].

Enlargement of the alveoli is considered to be the most important parameter to assess the degree of emphysema in animal models. Alveolar enlargement is primarily defined on morphological criteria and the mean linear intercept (Lm) analysis is the most widely accepted method for detecting and quantifying lung emphysema. The Lm is defined as an estimate of the mean distance between alveolar walls and has been shown to increase with disease severity [102, 103].

In this thesis several lung emphysema models were used to gain insight into the underlying mechanisms of COPD and to search new compounds with a potential therapeutic activity.

Outline and research questions of the thesis

1. Which chemokines and chemokine receptors play an important role in COPD?

COPD is characterized by a chronic inflammation in the airways accompanied with the recruitment of inflammatory cells. This inflammatory cell influx is orchestrated by multiple chemokines and their receptors, which are reviewed in Chapter 2.

2. Which lung fixation method can be recommended for the use in murine lung studies?

In Chapter 3 different methods of lung fixation have been evaluated in a murine model of LPS-induced lung emphysema. The use of the most optimal lung fixation method with an appropriate fixative is a prerequisite to study the pathogenesis of lung emphysema, since alveolar enlargement is primarily defined on morphological criteria.

3. Is ATP involved in the pathogenesis of lung emphysema?

Extracellular ATP is a signaling molecule that often serves as a danger signal to alert the immune system of tissue damage. In Chapter 4 the presence of ATP was determined in the BALF of mice after chronic cigarette smoke exposure and we investigated whether CSE-stimulated neutrophils release ATP. Furthermore, we examined the effect of CSE and ATP on the release of CXCL8 and elastase by human neutrophils and the effects of apyrase (catalyses the hydrolysis of ATP to yield AMP) and suramin (P2-receptor antagonist) on CSE- and ATP-induced activation of human neutrophils were investigated.

4. Are immunoglobulin free light chains (IgLC) involved in the development of COPD and is there a role for an IgLC-mediated immune response via a neutrophil-dependent mechanism in COPD?

IgLC are able to prolong the life of neutrophils, suggesting that IgLC contribute to the chronic state of inflammation. In Chapter 5 the IgLC levels were determined in 3 different murine models of lung emphysema as well as in serum and lung tissue from patients with COPD. Furthermore, we investigated whether IgLC can bind to human neutrophils and directly contribute to neutrophil activation *in vitro*. The effect of the IgLC antagonist F991 was examined on the smoke-induced neutrophil influx in murine lungs.

5. Is there a role for the collagen breakdown product PGP and the enzyme prolyl endopeptidase (PE) in the development of COPD?

The formation of the collagen breakdown product, PGP, is a multistep process involving neutrophils, MMPs and PE. This cascade of events was investigated in a murine model of cigarette smoke-induced lung emphysema and we focused on the role of PE and PGP in the pathophysiology of COPD. Furthermore, we examined the effect of the peptide, L-arginine-threonine-arginine (RTR), that binds to PGP sequences thereby blocking its biological activity and the effect of valproic acid (VPA), a PE inhibitor, on the smoke-induced neutrophil influx in the murine lung. Finally, the presence of PE in lung tissue of COPD patients was studied, which is all described in Chapter 6.

6. Is it possible to block the PGP-induced neutrophil influx in the airways of mice with CXCR2 antagonists?

In Chapter 7 we studied the effects of two different CXCR2 antagonists in a murine model, where a neutrophil influx was induced via oropharyngeal administration of N-Ac-PGP. We investigated whether CXCR2 activation is involved in N-Ac-PGP-induced neutrophil migration and activation.

7. Are the inflammatory changes in the airways caused by cigarette smoke exposure reversible after smoking cessation?

In Chapter 8 we investigated the effect of smoking cessation on airway remodeling and pulmonary inflammation in a murine model of cigarette smoke-induced lung emphysema. The severity of airway remodeling and inflammation was studied by analyzing alveolar enlargement, inflammatory cells in the BALF and lung tissue and by determining the cytokine and chemokine profiles in the BALF. In addition, we also examined the effect of smoking cessation on the development of heart hypertrophy associated with cigarette smoke-induced pulmonary COPD features in mice.

8. Chapter 9 provides a summarizing discussion of the most relevant findings of this thesis.

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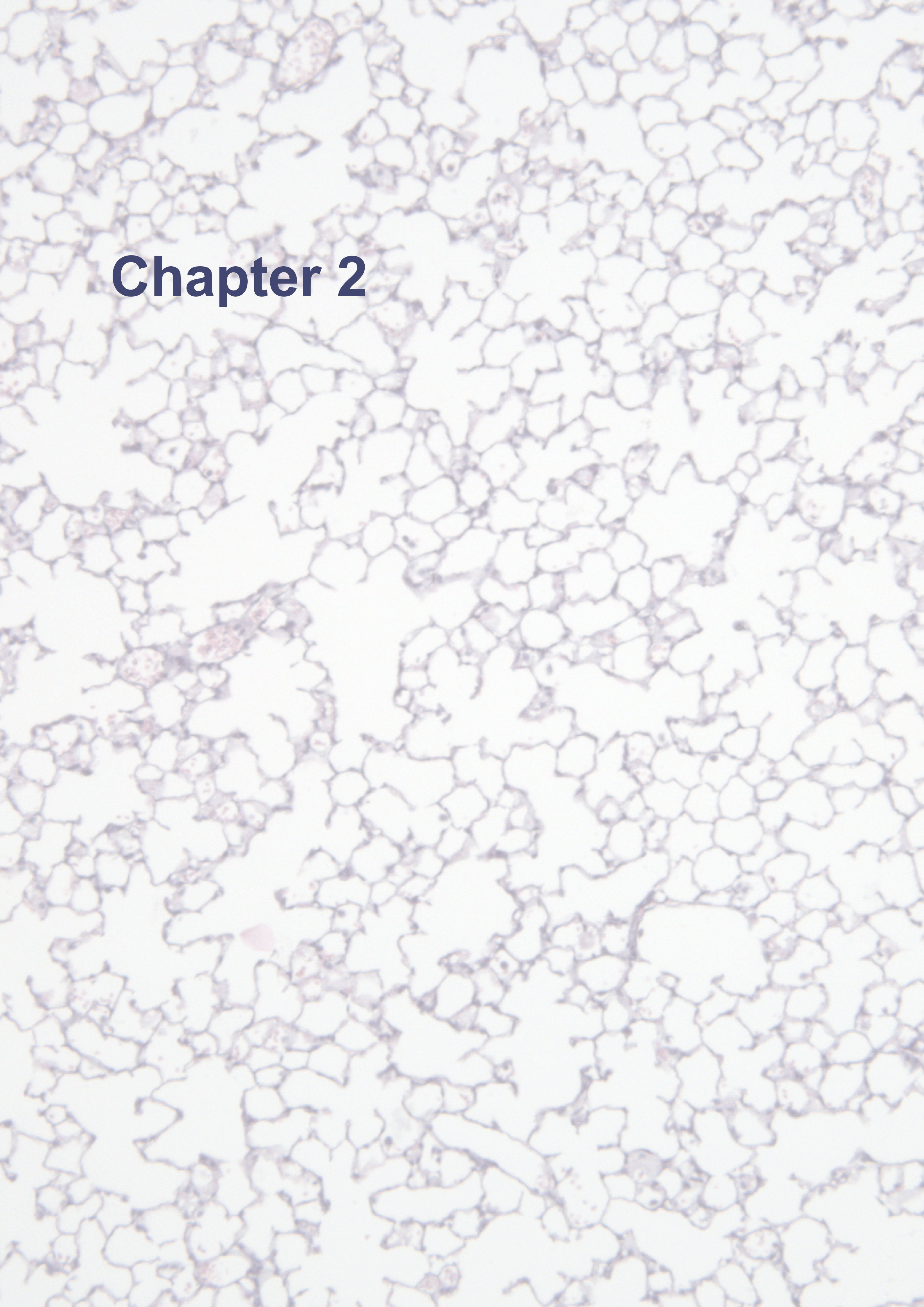

Chapter 2

Chemokine receptors in inflammatory diseases

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Chemokine receptors in COPD

In COPD the inflammatory cascade starts with exposure to cigarette smoke or other irritants, which activate the epithelial cells and the macrophages in the respiratory tract. When these cells are activated they have the capacity to release several chemotactic factors. Chemotactic factors use specific chemokine receptors to induce inflammatory cell migration to the airways (Fig. 1) [1].

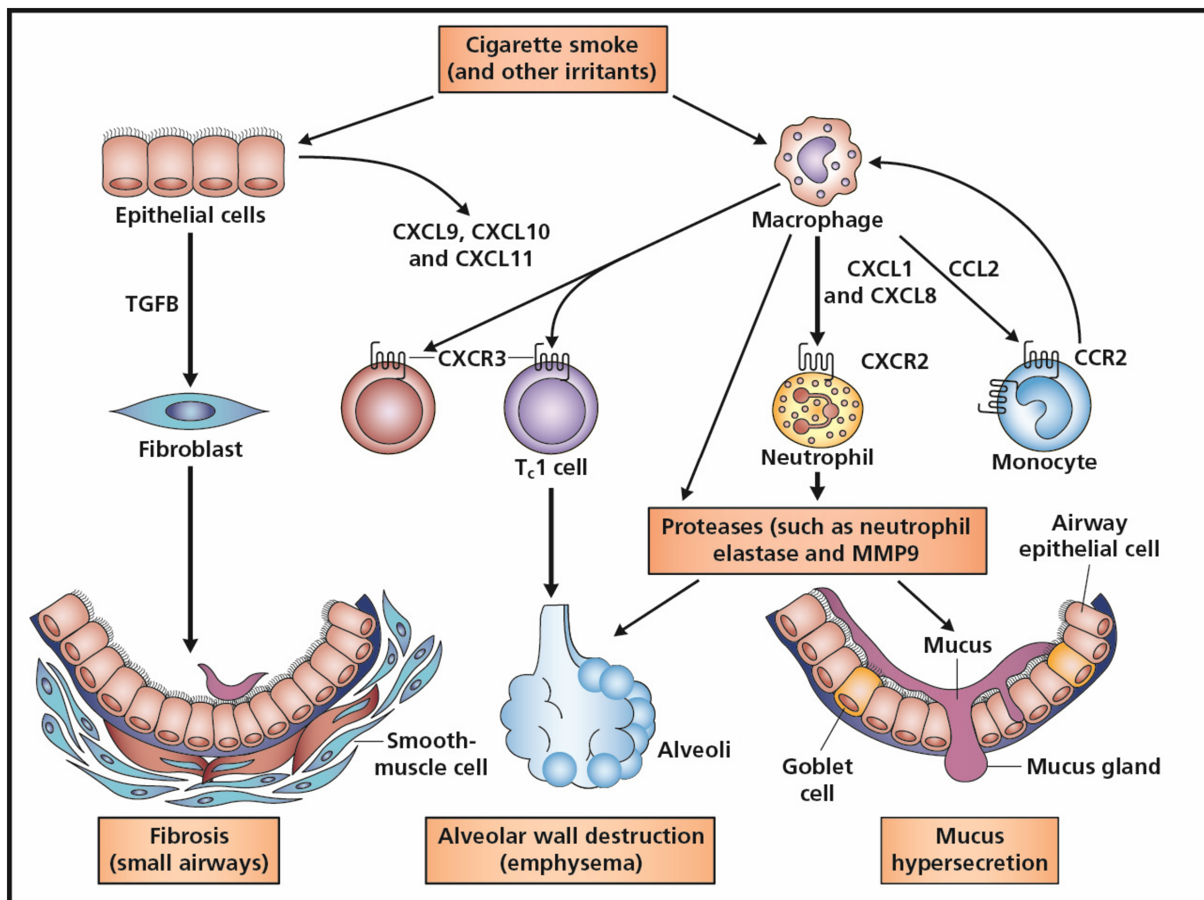

Fig.1. *Involvement of chemokines and chemokine receptors and different cell types in the inflammation of COPD. Chemokines released from epithelial cells and macrophages in the lung recruit inflammatory cells from the circulation leading to the development of COPD [1].*

First, the chemotactic factor CCL2 (MCP-1), a CC-chemokine that mediates its effects via the CCR2 chemokine receptor. This specific receptor for CCL2 is expressed by monocytes, macrophages, T lymphocytes and epithelial cells. It is known from literature that the chemoattractant CCL2 and the receptor CCR2 are involved in the recruitment of monocytes into the airway epithelium in COPD. Under migration into tissues, monocytes differentiate into macrophages. Macrophages are important in the pathogenesis of COPD, reflecting in an increased number of macrophages in the lungs of COPD patients [2-4].

Nevertheless, the CCR2 receptor might play a crucial role in COPD, since CCL2 levels are increased in the sputum, BALF and lungs of patients suffering from COPD [3].
One of the most important chemokines associated in the recruitment of inflammatory cells in COPD is the CXC-chemokine CXCL8 [5]. CXCL8 binds to both CXCR1 and CXCR2 chemokine receptors, which are expressed on a broad range of leukocytes, predominantly neutrophils. Recent studies demonstrated a significantly increased expression of CXCR1 on circulating neutrophils in COPD patients compared to healthy controls [6]. Furthermore, it is established that the expression of CXCR2 in bronchial biopsies of COPD patients is increased [7]. These two receptors have the capacity to regulate the migration of neutrophils into the pulmonary tissue during the neutrophilic inflammation in COPD. Including the fact, that activated neutrophils are undoubtedly crucial players in the pathogenesis of COPD.
CXCL8, is secreted by several cell types, like macrophages, neutrophils and airway epithelial cells and is a powerful chemotactic mediator for neutrophils. Among CXCL8, the CXCR2 receptor is also selectively activated by the CXC chemokine CXCL1 (GRO-α). CXCL1 is secreted by alveolar macrophages and airway epithelial cells and is a potent chemoattractant of neutrophils and monocytes. The concentration neutrophils is increased in the sputum and bronchoalveolar lavage fluid (BALF) of COPD patients and this is related to the increased production of CXCL8 and CXCL1 [1, 4, 5, 8, 9]. It has been considered in neutrophil chemotaxis that the CXCR2 receptor responds not only to CXCL8 and CXCL1, but also to other chemokines including, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7. CXCL5 is predominantly derived from epithelial cells and CXCL5 is more released by BALF cells from smokers than by BALF cells from non-smokers [10]. CXCL7 is chemotactic for neutrophils as well as for monocytes and shows an enhanced chemotactic activity for monocytes from COPD patients which is similar to the chemotactic activity of CXCL1 [11].
In addition to macrophages and neutrophils, the T cell is also a potentially important factor in the initial inflammatory process leading to COPD. This is supported by the finding of an increased number of T cells in the airways and in lung parenchyma COPD patients, to a greater extent in CD8+ T cells compared to CD4+ cells [12, 13]. Lymphocytes, particularly type-1 T lymphocytes (Th1/Tc1 cells), express the chemokine receptor CXCR3. In the airways of COPD patients an increase in the number of CXCR3+ T cells and an increased expression of CXCR3 was observed. T cells may be attracted to the lungs by IFNγ and IFNγ-induced CXCR3 receptor ligands: CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC). All three chemokines activate CXCR3 and are present at high levels in COPD airways [14, 15]. Kelsen et al., 2004 [16] demonstrated that human airway epithelial cells also express the CXCR3 chemokine receptor and activation of CXCR3 by CXCL9, CXCL10 and CXCL11 may contribute to airway inflammation/remodeling in the development of COPD. Furthermore,

CXCR3 knockout mice showed less lung inflammation induced by cigarette smoke exposure compared with the wildtype mice [17].
Even as the CXCR3 receptor, the CCR5 receptor is also expressed on Th1 and Tc1 cells and might have a cooperative role with CXCR3 in the recruitment of these cells into the lungs [18]. The CCR5 ligand CCL5 is elevated in sputum from COPD patients, this increase is also observed in the airways and sputum of COPD patients during exacerbations [15, 19].
Due to the activity of these inflammatory cells as well as the epithelial cells by receptor-ligand interactions, the inflammatory response in COPD is further augmented leading to the induction and release of different proteases. Including, matrix metalloproteinases (MMPs, e.g. MMP-9) and neutrophil elastase. This proteolytic cascade leads to the remodeling of the lung tissue by collagen and elastin degradation (emphysema) and mucus hypersecretion (chronic bronchitis). The protease-antiprotease imbalance hypothesis is thought to play a key role in the development of COPD [1, 20, 21].
Finally, epithelial cells and macrophages in the small airways also regulate the proliferation of fibroblasts by releasing transforming growth factor-ß (TGF-ß), resulting in fibrosis a clinical feature of COPD [22] (Fig. 1 [1]). In Table 1 the different chemokine receptors and their ligands demonstrated to be involved in COPD are summarized.

Table 1. *Chemokine receptors and their ligands demonstrated to be involved in COPD.*

RECEPTOR	LIGAND	TARGET CELLS COPD	REFERENCES
CXCR1	CXCL6, CXCL7,CXCL8	Neutrophil, Monocyte/macrophage	[1, 4, 23-27]
CXCR2	CXCL1, CXCL2, CXCL3 CXCL5, CXCL6, CXCL7, CXCL8	Neutrophil, Monocyte/macrophage	[1, 4, 23-27]
CXCR3	CXCL9, CXCL10, CXCL11	T cell	[1, 4, 17, 24, 25, 28]
CCR2	CCL2	Monocytes/macrophage T cell	[4, 23, 25]
CCR5	CCL5	T cell	[4, 15, 19, 29]

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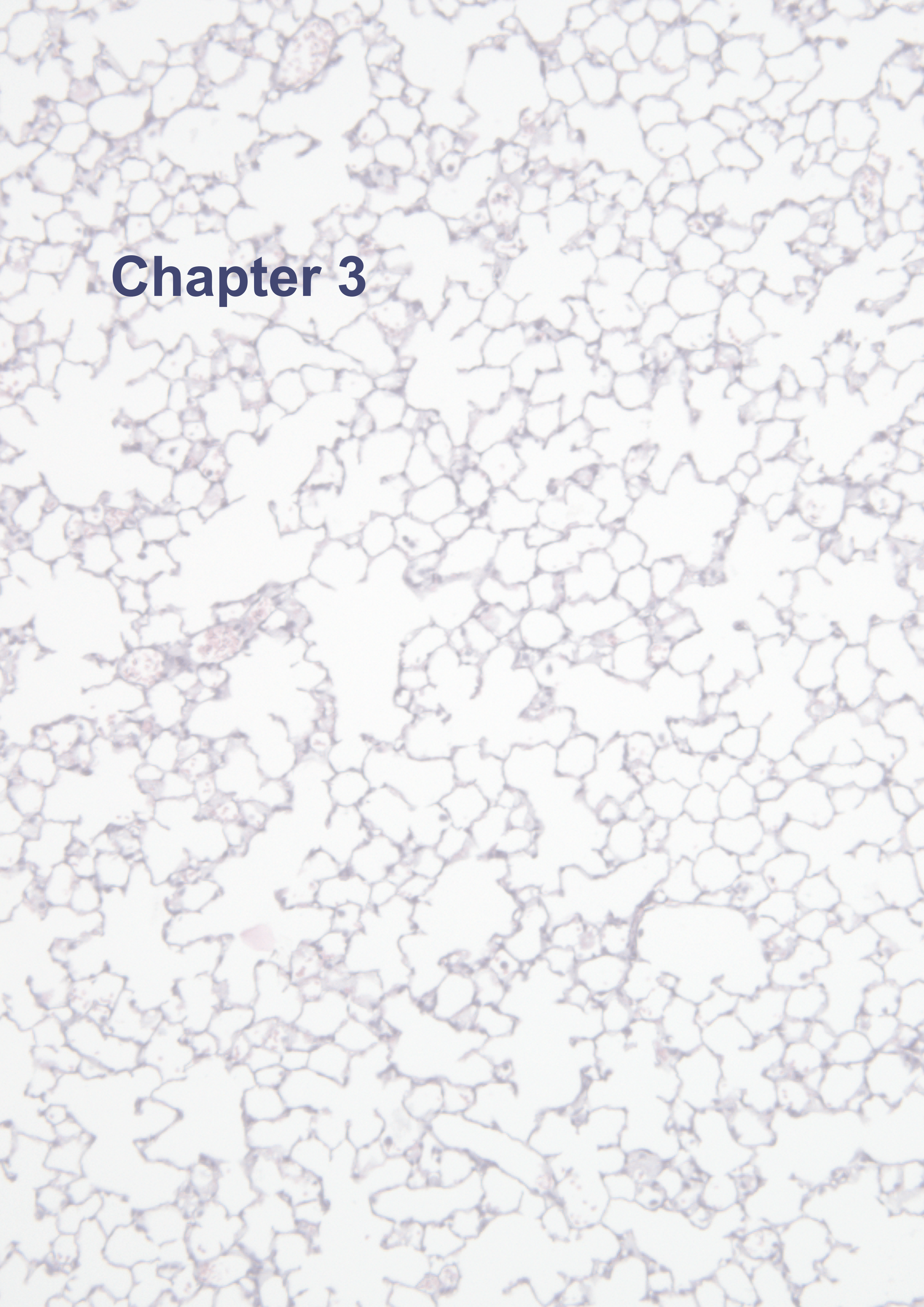

Chapter 3

A comparison of fixation methods on lung morphology in a murine model of emphysema

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Abstract

Emphysema is characterized by enlargement of the alveoli, which is the most important parameter to assess the presence and severity of this disease. Alveolar enlargement is primarily defined on morphological criteria; therefore, characterization of this disease with morphological parameters is a prerequisite to study the pathogenesis. For this purpose, different methods of lung fixation were evaluated in a murine model of LPS-induced lung emphysema. Five different methods of lung fixation were evaluated: intratracheal instillation of fixatives, *in situ* fixation, fixed volume fixation, vascular whole body perfusion and vacuum inflation. In addition, the effects of three different fixatives (10% formalin, Carnoy's and agarose/10% formalin solution), and two embedding methods (paraffin and plastic) were investigated on the murine lung morphology. Mice received intranasal administration of LPS to induce alveolar wall destruction. Quantification of airspace enlargement was determined by mean linear intercept (Lm) analysis and the histological sections were analyzed for the most optimal fixation method. Additionally, a routine immunohistological staining was performed on lung tissue of PBS-treated mice. Intratracheal instillation of formalin or agarose/formalin solution, *in situ* fixation and fixed volume fixation provided a normal lung architecture in contrast to the lungs fixed via whole body perfusion and vacuum inflation. Formalin-fixed lungs resulted in the most optimal lung morphology for lung emphysema analysis when embedded in paraffin, while for Carnoy's-fixed lungs plastic embedding was preferred. The histological findings, the Lm measurement and the immunohistochemistry data demonstrated that fixation by intratracheal instillation of 10% formalin or *in situ* fixation with 10% formalin are the two most optimal methods to fix lungs for alveolar enlargement analysis to study lung emphysema.

Introduction

Emphysema is a pulmonary disease characterized by permanent enlargement of the air spaces accompanied by destruction of the alveolar walls. This parenchymal destruction leads to the loss of elastic recoil and hyperexpansion, which contributes to airflow limitation [1, 2]. Lung emphysema is a feature of a group of diseases termed chronic obstructive pulmonary disease (COPD), which is associated with poorly reversible airflow limitation that is usually both progressive and related to an abnormal inflammatory response of the lung [3]. Cigarette smoking is the primary risk factor for the development of lung emphysema [4]. Enlargement of the alveoli is considered to be the most important parameter to assess the degree of emphysema. Alveolar enlargement is primarily defined on morphological criteria, therefore characterization of this disease with morphological parameters is a prerequisite to study the pathogenesis [5]. Many methods have been developed to assess the presence and severity of lung emphysema, of which the mean linear intercept (Lm) analysis, has been most widely accepted. The Lm is defined as a measure of the mean distance between the alveolar walls and has been shown to increase with disease severity [6, 7]. The method of lung fixation is the basis for the subsequent stages in the preparation of the lung sections necessary for the diagnosis of emphysema. Therefore, it is essential that the fixation method be effective and that the appropriate fixative be used [8]. However, problems arise when lung tissue must be prepared for morphometric analysis, since the volume of an inflated lung consists of 90% air and 10% tissue [9]. During lung fixation it is important to prevent the collapse, deflatation and disruption of lung structures and to avoid fixation artefacts, which could lead to the suggestion of alveolar wall thickening, hypercellularity, change in shape or volume and blood in the lungs [10]. Otherwise a correct morphological analysis of the lung tissue is not possible. Besides the lung fixation method, an appropriate embedding procedure is essential for tissue preservation. Meaningful interpretation of the lung histology can only be obtained if alterations in structures within the tissue are at least kept to an absolute minimum [11]. For this purpose, different methods of lung fixation have been evaluated in a murine model of LPS-induced lung emphysema: 1) intratracheal instillation of fixatives, 2) *in situ* fixation, 3) fixed volume fixation, 4) vascular whole body perfusion and 5) vacuum inflation. Moreover, the effects of three different fixatives (10% formalin, Carnoy’s and agarose/10% formalin solution) have been investigated on the murine lung morphology. Additionally, paraffin-embedded lung tissue was compared with the mice lungs embedded in plastic. The usefulness of the different lung fixation methods was validated by morphometric analysis, evaluating the Lm and by immunohistochemistry. In this study, the advantages and disadvantages of each method are described and the most optimal fixation method for the use in murine lung studies is recommended.

Material and methods

Animals

Male Balb/c mice, 5-6 weeks old were obtained from Charles River Laboratories and housed under controlled conditions in standard laboratory cages in the animal facility. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the local Ethics Committee and were performed under strict governmental and international guidelines on animal experimentation.

Induction of lung emphysema

To induce lung emphysema mice were repeatedly challenged with LPS (5µg/50µl/mouse) (Sigma-Aldrich, Zwijndrecht, the Netherlands) twice a week for a period of 8 weeks. LPS, or control fluid (PBS, 50 µl/mouse) was administered intranasally to the animals, which were anesthetized by isoflurane inhalation. After 8 weeks of treatment, there was a recovery period of 2 weeks to eliminate the direct effects of LPS administration.

Fixatives

The three fixatives used in this study were as follows: a formaldehyde, 10% formalin (Baker BV, Deventer, the Netherlands), an alcohol-based fixative, Carnoy's and an agarose/10% formalin solution. The fixative Carnoy's consisted of 60% absolute ethanol (Merck, Darmstadt, Germany), 30% chloroform (Biosolve, Valkenswaard, The Netherlands) and 10% acetic acid (Merck, Darmstadt, Germany). The agarose/10% formalin solution consisted of low gelling temperature agarose (Bio-rad Laboratories, Hercules, USA) in a concentration of 0.75 gm % (grams per 100 ml fluid), which was dissolved in 10% formalin by heating in a microwave until clear and was kept in a water bath of 45°C until use.

Lung fixation methods: intratracheal instillation of fixatives

Mice were sacrificed by an i.p. injection with an overdose of pentobarbital (Nembutal™, Ceva Santé Animale, Naaldwijk, The Netherlands). The animals were exsanguinated by cutting the caudal vena cava to prevent the flow of blood into the bases of the lungs. A cannula was inserted into the trachea and fixed with a ligature. Lungs and heart were removed en bloc and lungs were inflated via the cannula by gentle infusion of the fixative 10% formalin, Carnoy's, or agarose/10% formalin solution at a constant fluid pressure of 25 cm for 5 min. After the trachea was tied off with a ligature, the lungs were placed in a glass vial containing the corresponding fixative, while the agarose/10% formalin fixed lungs were placed in a glass vial containing 10% formalin and were kept on ice for 1 hour [11-17].

Lung fixation methods: in situ fixation

Mice were sacrificed as described above. The animals were exsanguinated by cutting the caudal vena cava to prevent the flow of blood into the bases of the lungs. The trachea was cannulated and the lungs were fixed *in situ* before opening the thorax via the cannula with 10% formalin at a constant fluid pressure of 25 cm for 5 min [18, 19].

Lung fixation methods: fixed volume fixation

Mice were sacrificed as described above. The animals were exsanguinated by cutting the caudal vena cava to prevent the flow of blood into the bases of the lungs. A cannula was inserted into the trachea and fixed with a ligature. The lungs were fixed by gentle infusion of the fixative (10% formalin or Carnoy's) through the cannula by a continuous release pump under pressure and volume-controlled conditions (12 ml/h; 5 min). This lung fixation method was performed after opening the thorax [20].

Lung fixation methods: vascular whole body perfusion

The mice were anesthetized with three i.p. injections of 0.2 ml of a 10% solution of urethane (Sigma-Aldrich, Zwijndrecht, The Netherlands). When adequately anesthetized, the right jugular vein was cannulated and the caudal vena cava was cut for draining the blood and perfusate. The mouse was flushed with a mixture of saline and heparin (50 U/ml) (Leo Pharmaceuticals, Weesp, the Netherlands) by a continuous release pump under pressure and volume-controlled conditions (120 ml/h; 5 min). After 2–3 min the drained fluid ran clear and the fixative 10% formalin was pumped through at a flow rate of 120 ml/h for 5 min to fix the whole mouse [21-23].

Lung fixation methods: vacuum inflation

Mice were sacrificed as described above and were exsanguinated by cutting the caudal vena cava to prevent the flow of blood into the bases of the lungs. The lungs and heart were removed and were placed in glass vials containing 10% formalin. The vials were placed in a vacuum dessicator and degassed under vacuum (250 mbar) for 10 min. At that time the lungs, which initially floated on the fluid surface, sank to the bottom of the vials. Sinking is a good indication of successful degassing [10, 24].

Embedding

Carnoy's fixation was continued for at least 4 hours, while lungs in 10% formalin were immersed for at least 24 hours. After rinsing the lungs in 3 changes of 100% ethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands) followed by 70% ethanol, the left lung was embedded

in acrylic copolymer consisting of 75 parts of butyl methacrylate, 25 parts of methyl methacrylate (Fluka, Buchs, Switzerland) and 0.8% organic stabilised dibenzoylperoxide (Lucidol CH 50-L; Akzo Nobel Chemicals, Emmerich, Germany). The polymerisation was pre-activated by the addition of 0.5% N,N-dimethyl-p-toluidine (Merck, Hohenbrunn, Germany) to the monomer solution. After polymerisation, sections of 3 µm were cut at 300, 600, 900 and 1200 µm depth in dorsal ventral plane. The sections were placed on poly-L-lysine (Sigma Aldrich, Zwijndrecht, The Netherlands) coated glass slides and deplasticized using acetone (Merck, Darmstadt, Germany).
The right lung was embedded in paraffin (Stemcowax, Adamas Instruments, Rhenen, The Netherlands) after dehydration in graded ethanol series followed by xylene (Sigma Aldrich, Zwijndrecht, The Netherlands). Subsequently, sections of 5 µm were cut at 200, 400, 600 and 800 µm in dorsal ventral plane. The sections were placed on poly-L-lysine coated glass slides and deparaffinized using xylene and ascending ethanol series. All the sections were stained with hematoxylin & eosin (H&E) according to standard methods and were dehydrated in graded ethanol concentrations and xylene before mounted with DePeX (Serva, Heidelberg, Germany) and coverslipped.

Mean linear intercept (Lm) analysis

Morphometric assessment of emphysema, included determination of the average inter-alveolar distance, was estimated by the mean linear intercept (Lm) analysis. The Lm was determined by light microscopy at a total magnification of 100x, whereby 24 random photomicroscopic images per lung tissue section (6 images per depth) were evaluated by microscopic projection onto a reference grid. By dividing total grid length by the number of alveolar wall-grid line intersections, the Lm (in µm) was calculated [6].

Immunohistochemistry

Paraffin sections were deparaffinized in xylene and plastic sections were deplasticized in acetone before the endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck, Darmstadt, Germany) in methanol for 30 min at room temperature. The slides were rehydrated through decreasing concentrations of ethanol ending in PBS. For antigen retrieval, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave. The slides were cooled down to room temperature, rinsed with PBS (3x) and blocked with 5% goat serum (Dakocytomation, Glostrup, Denmark) in 1% bovine serum albumin in PBS for 30 min at room temperature. Sections were incubated with the primary antibody (rabbit polyclonal antimouse Clara cell secretory protein, CC16 (diluted 1:3200, Uteroglobin antibody, Abcam, Cambridge, UK)), in 1% bovine serum albumin/PBS overnight at 4℃. The

slides were rinsed with PBS (3x) and incubated with the biotinylated secondary antibody (goat-anti-rabbit, 1:200, Dakocytomation) in 1% bovine serum albumin/PBS for 45 min at room temperature. The slides were rinsed with PBS (3x) and the biotinylated proteins were visualized by incubation with streptavidin–biotin complex/horseradish peroxidase (Vectastain Elite ABC, Vector Laboratories) for 45 min at room temperature, followed by 0.015% H_2O_2/0.05% diaminobenzidene (Sigma, Schneldorf, Germany)/0.05 M Tris–HCl (pH 7.6) for 10 min at room temperature. Sections were counterstained with Mayers' hematoxylin (Merck), dehydrated and mounted in Permount (Fisher Scientific) [25]. Negative controls without the primary antibody and normal rabbit IgG (AB-105-C, R&D systems) were included as controls. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera.

Right ventricular heart hypertrophy measurement

The right ventricle was removed from lower heart after removal of the atria. The right ventricle and the left ventricle plus septum were weighed and the ratio of the weights was calculated as follows: (right ventricle)/(left ventricle + septum) [15, 16].

Statistical analysis

Experimental results are expressed as mean ± S.E.M. Differences between groups were statistically determined by an unpaired two-tailed Student's *t*-test using GraphPad Prism (Version 4.0). Results were considered statistically significant when $P < 0.05$.

Results

Differences between the morphology of 10% formalin-fixed lungs and Carnoy's-fixed lungs

Before investigating the effect of the different fixation techniques, the two fixatives 10% formalin and Carnoy's were compared in the murine PBS-treated lungs embedded in paraffin fixed via the intratracheal instillation and via the fixed volume technique (Fig.1). 10% formalin fixation via intratracheal instillation (Fig.1A) demonstrated a normal lung architecture with normal, curled alveoli and without apparent fixation artefacts. The lungs of the 10% formalin fixation via the fixed volume method (Fig.1B) were comparable with the lungs fixed via intratracheal instillation, but the alveolar walls were slightly pressed together and thickened. In comparison with the 10% formalin-fixed lungs, the Carnoy's-fixed lungs fixed via intratracheal instillation (Fig.1C) and via the fixed volume technique (Fig.1D) showed more fixation artefacts, such as enlarged alveoli and disrupted alveolar walls. Especially the Carnoy's-fixed lungs fixed via the fixed volume technique demonstrated enlarged alveoli compared to the other PBS-treated lungs fixed. All of these fixed lungs were embedded in paraffin.

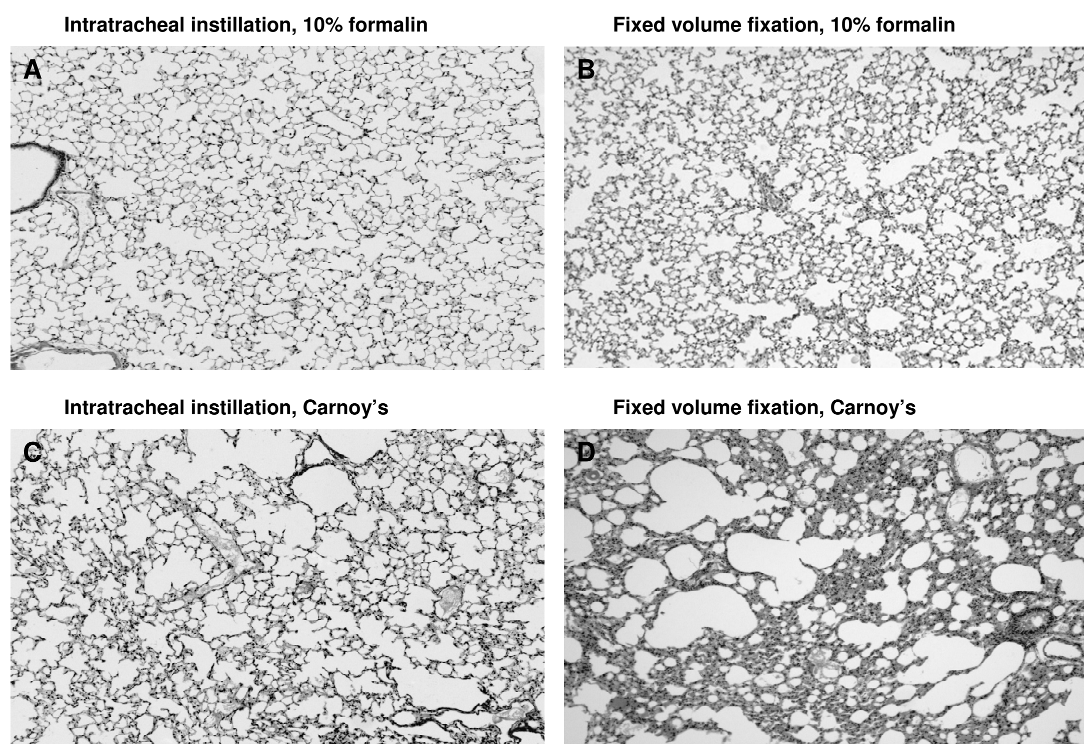

Fig.1. *Differences between the morphology of 10% formalin-fixed lungs and Carnoy's-fixed lungs embedded in paraffin. Representative photomicrographs of hematoxylin and eosin stained lung tissue of PBS-treated mice fixed via intratracheal instillation of 10% formalin (A), fixed volume fixation with 10% formalin (B) , intratracheal instillation of Carnoy's (C) and fixed volume fixation with Carnoy's (D). n = 5 animals per group. Magnification 100x.*

Increased Lm after LPS exposure in lungs fixed via intratracheal instillation of 10% formalin and embedded in paraffin

The mean linear intercept was used to quantify the presence of emphysema and to compare the size of the alveoli after the use of 10% formalin or Carnoy's fixation (Fig. 2). A significantly increased Lm was observed in the lungs of LPS-treated mice fixed via the intratracheal instillation of 10% formalin compared to the PBS-treated mice. The Lm of the lungs fixed via intratracheal instillation of Carnoy's was also increased, but not significantly different compared to the control mice. The LPS-treated lungs fixed with 10% formalin or Carnoy's via the fixed volume technique were both not significantly enhanced compared to the control lungs. It was also observed that the mean distance between the alveolar walls in the PBS-treated lungs fixed with Carnoy's via the fixed volume technique was significantly higher compared to the control lungs fixed via the other lung fixation methods. All of these fixed lungs were embedded in paraffin.

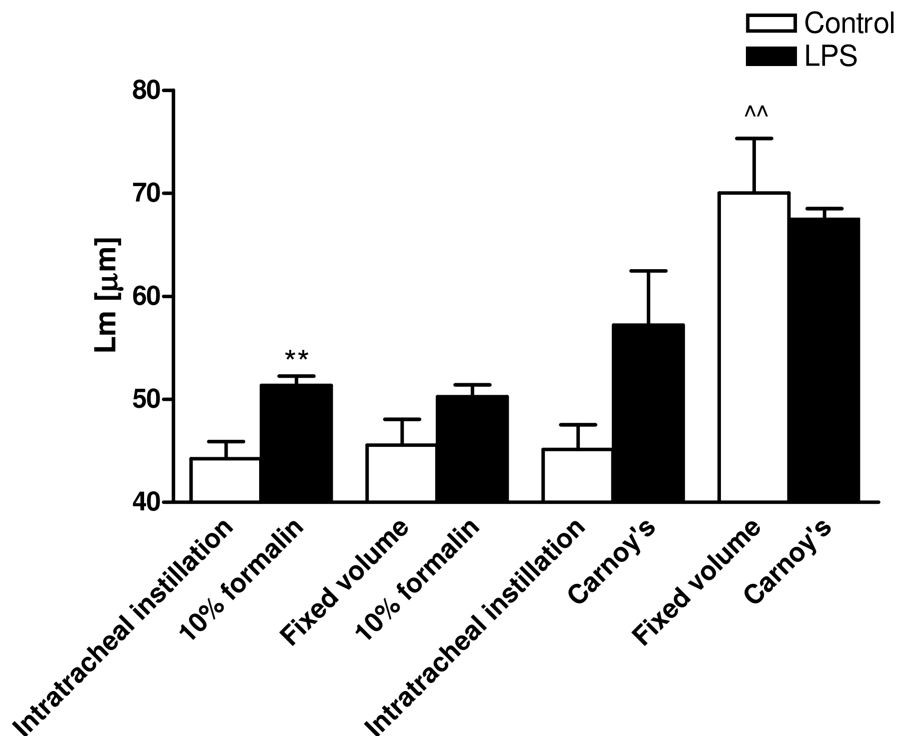

Fig.2. *Increased Lm after LPS exposure in lungs fixed via intratracheal instillation of 10% formalin and embedded in paraffin. Mean linear intercept values measured in lung tissue of PBS-treated (white bars) and LPS-treated mice (black bars), fixed via intratracheal instillation of 10% formalin, fixed volume fixation with 10% formalin, intratracheal instillation of Carnoy's and fixed volume fixation with Carnoy's. n = 5 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01; significantly different from the control group intratracheal instillation with 10% formalin. ^^P ≤ 0.01; significantly different from the control groups intratracheal instillation with 10% formalin, intratracheal instillation with Carnoy's and fixed volume fixation with 10% formalin.*

10% formalin-fixed lungs and Carnoy's-fixed lungs embedded in plastic or paraffin

Two embedding procedures: paraffin and plastic were compared in PBS-treated lungs fixed via the intratracheal instillation of 10% formalin or Carnoy's (Fig.3). As already described before, the 10% formalin fixation via intratracheal instillation embedded in paraffin (Fig. 3A) demonstrated a normal lung architecture without apparent fixation artefacts, but when these PBS-treated lungs were embedded in plastic (Fig. 3B) the alveolar walls were slightly enlarged and destructed compared to paraffin embedding. In comparison with the 10% formalin-fixed lungs embedded in paraffin, the Carnoy's-fixed lungs embedded in paraffin (Fig. 3C) show more fixation artefacts, such as enlarged alveoli and disrupted alveolar walls. In contrast, when PBS-treated lungs fixed with Carnoy's were embedded in plastic, these artefactual phenomena were not present and a normal lung architecture was displayed without fixation artefacts (Fig. 3D).

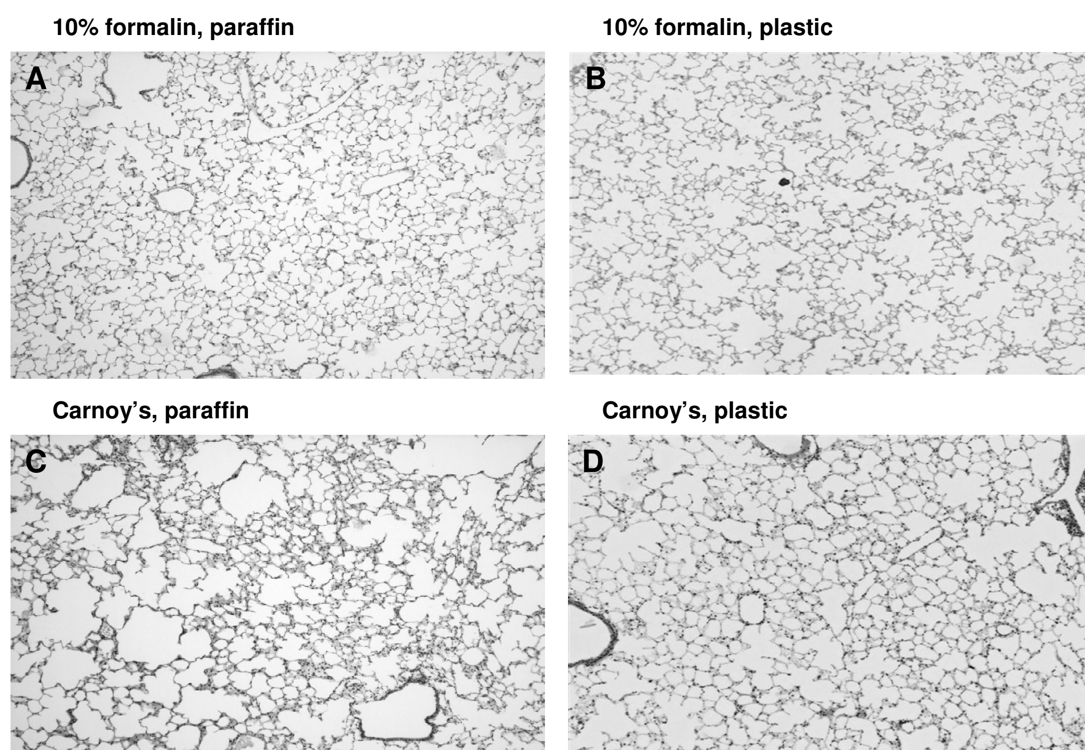

Fig.3. *10% formalin-fixed lungs and Carnoy's-fixed lungs embedded in plastic or paraffin. Representative photomicrographs of hematoxylin and eosin stained lung tissue of PBS-treated mice fixed via intratracheal instillation of 10% formalin embedded in paraffin (A), via intratracheal instillation of 10% formalin embedded in plastic (B), via intratracheal instillation of Carnoy's embedded in paraffin (C) and via intratracheal instillation of Carnoy's embedded in plastic (D). n = 5 animals per group. Magnification 100x.*

Increased Lm after LPS exposure in 10% formalin-fixed lungs embedded in paraffin and Carnoy's-fixed lungs embedded in plastic

The mean linear intercept was used to quantify the presence of emphysema and to compare the size of the alveoli after the use of the two different embedding procedures (Fig. 4). The increased Lm observed in the lung tissue of LPS-treated mice fixed via the intratracheal instillation of 10% formalin embedded in paraffin was significantly different compared to the PBS-treated mice, while the Lm of the LPS-treated lungs fixed with 10% formalin embedded in plastic was not significantly increased. The LPS-treated lungs fixed with Carnoy's embedded in paraffin showed an increase in Lm, but this was not significantly different compared to the control animals. When the LPS-treated lungs fixed with Carnoy's were embedded in plastic the Lm was significantly elevated compared to the PBS-treated animals.

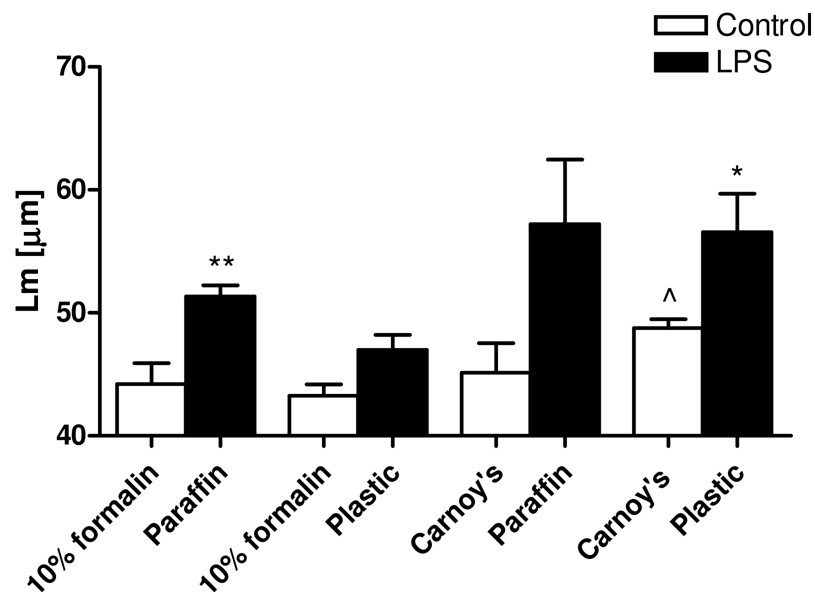

Fig.4. *Increased Lm after LPS exposure in 10% formalin-fixed lungs embedded in paraffin and Carnoy's-fixed lungs embedded in plastic. Mean linear intercept values measured in lung tissue of PBS-treated (white bars) and LPS-treated mice (black bars), fixed via intratracheal instillation of 10% formalin embedded in paraffin, via intratracheal instillation of 10% formalin embedded in plastic, via intratracheal instillation of Carnoy's embedded in paraffin and via intratracheal instillation of Carnoy's embedded in plastic. n = 5 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01; significantly different from the control group 10% formalin, paraffin. *P ≤ 0.05; significantly different from the control group Carnoy's, plastic. ^P ≤ 0.05; significantly different from the control group 10% formalin, plastic.*

Differences in lung morphology after various lung fixation techniques

Comparison of the PBS-treated lungs fixed with 10% formalin or agarose/10% formalin solution via the different lung fixation methods embedded in paraffin reveals different images (Fig. 5). First, control mice lungs fixed via the intratracheal instillation with 10% formalin (Fig. 5A) demonstrated a normal lung morphology with normal, curled alveoli, without fixation artefacts such as collapsed parenchymal tissue, hypercellularity and alveolar wall thickening. This was also observed for the PBS-treated lungs fixed via *in situ* fixation (Fig. 5C), which displayed a normal lung architecture, but the alveoli show a more angular structure. Murine lungs fixed via intratracheal instillation with agarose/10% formalin solution (Fig. 5B) demonstrated also open air spaces, but the alveoli appeared to be slightly enlarged and the agarose was still visible in the air spaces. The lungs fixed via the fixed volume technique with 10% formalin (Fig. 5D) were comparable with the lungs fixed via intratracheal instillation with 10% formalin, but the alveolar walls were slightly thickened. The lungs fixed via the vacuum inflation (Fig. 5E) showed disturbed lung architecture and thickening of alveolar walls became evident. Vascular whole body perfusion (Fig. 5F) was also not an optimal method for lung fixation, since the alveoli were enlarged, the parenchymal architecture was collapsed, apparent hypercellularity and thickening of the alveolar walls were visible.

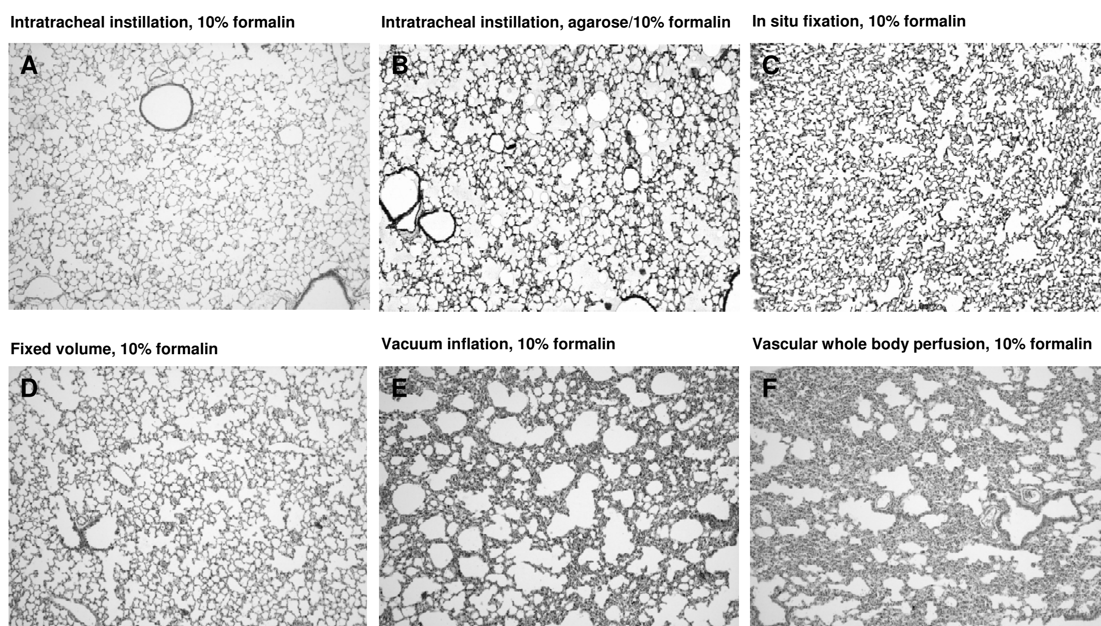

Fig.5. *Differences in lung morphology after various lung fixation techniques. Representative photomicrographs of hematoxylin and eosin stained lung tissue of PBS-treated mice fixed via intratracheal instillation of 10% formalin (A), intratracheal instillation of agarose/10% formalin solution (B), in situ fixation (C), fixed volume fixation with 10% formalin (D), vacuum inflation (E) and vascular whole body perfusion (F). All these fixed lungs were embedded in paraffin. n = 5 animals per group. Magnification 100x.*

Increased Lm after LPS exposure in lungs fixed via intratracheal instillation of 10% formalin and in situ fixation

All of the histological lung sections of the LPS-treated mice fixed via the most optimal lung fixation methods: intratracheal instillation with 10% formalin (Fig. 6A & 6E), intratracheal instillation with agarose/10% formalin solution (Fig. 6B & 6F), *in situ* fixation (Fig. 6C & 6G) and fixed volume fixation (Fig. 6D & 6H) showed a visible alveolar enlargement (Fig. 6E, F, G, H) compared with the PBS-treated mice (Fig. 6A, B, C, D). The mean linear intercept was used to quantify the presence of emphysema and to compare the size of the alveoli after the use of various lung fixation methods. The mean linear intercept values determined in the lung tissue fixed via the most optimal lung fixation methods were depicted in Fig. 7. The increased Lm values observed in the lung tissue of LPS-treated mice fixed via the intratracheal instillation of 10% formalin and via the *in situ* fixation method with 10% formalin were significantly different compared to the PBS-treated animals, while the Lm of the other two fixation techniques were not significantly increased. It was also observed that the mean distance between the alveolar walls in the PBS-treated lungs fixed via the *in situ* fixation method with 10% formalin was lower compared to the lungs fixed via intratracheal instillation of 10% formalin or agarose/10% formalin solution and via the fixed volume fixation method. All of these fixed lungs were embedded in paraffin.

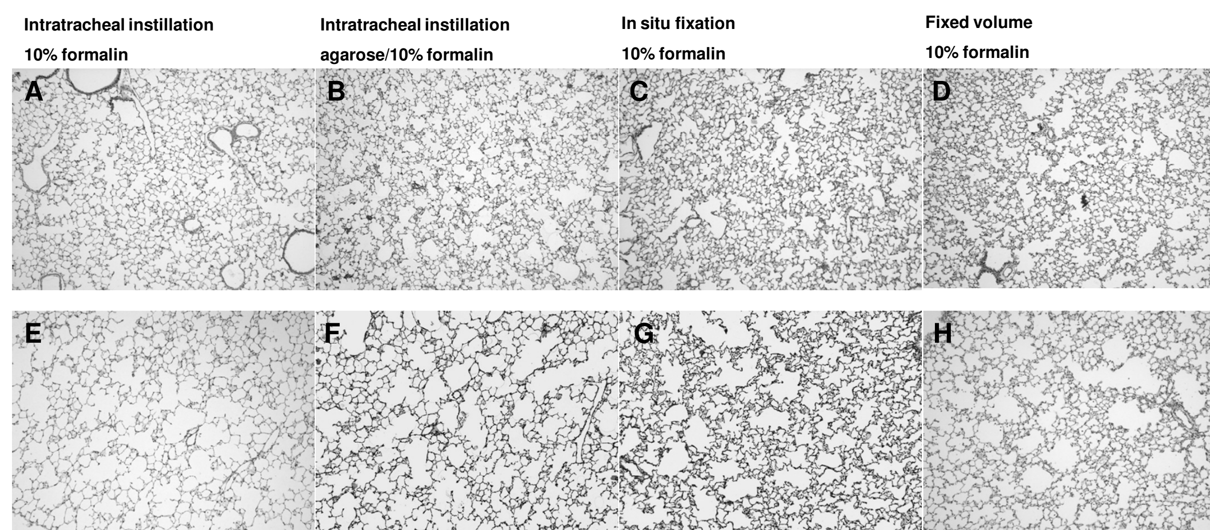

Fig.6. *Alveolar enlargement after LPS administration observed in lung tissue fixed via the various lung fixation methods. Representative photomicrographs of hematoxylin and eosin stained lung tissue of PBS-treated (A, B, C, D) and LPS-treated (E, F, G, H) mice fixed via intratracheal instillation of 10% formalin (A, E), intratracheal instillation of agarose/10% formalin solution (B, F), in situ fixation (C, G), fixed volume fixation with 10% formalin (D, H). All these fixed lungs were embedded in paraffin. n = 5 animals per group. Magnification 100x.*

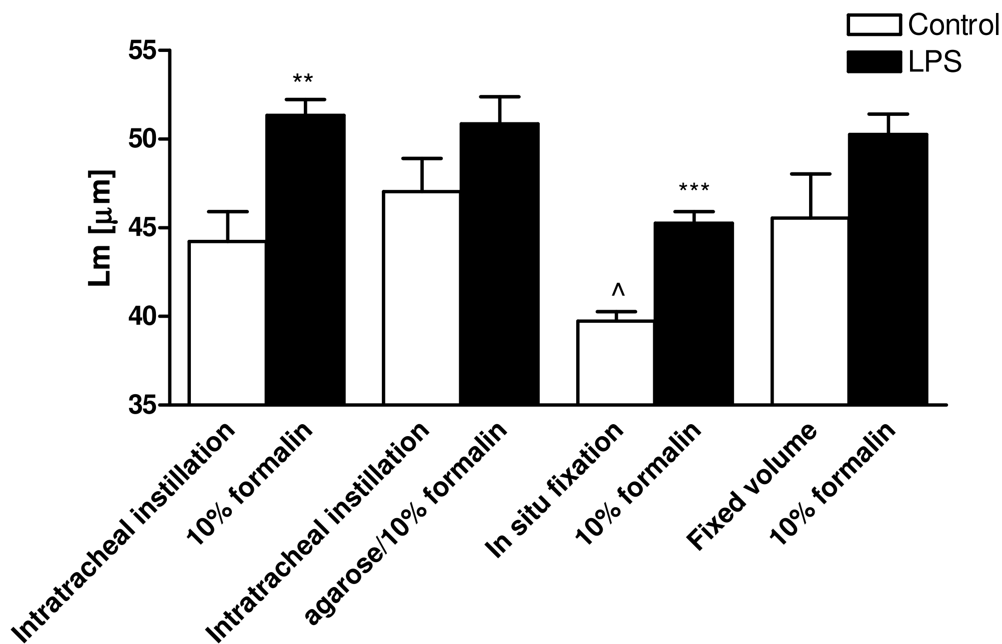

Fig.7. *Increased Lm after LPS exposure in lungs fixed via intratracheal instillation of 10% formalin and via in situ fixation. Mean linear intercept values measured in lung tissue of PBS-treated (white bars) and LPS-treated mice (black bars), fixed via intratracheal instillation of 10% formalin, in situ fixation, intratracheal instillation of agarose/10% formalin solution and fixed volume fixation with 10% formalin. All these fixed lungs were embedded in paraffin. n = 5 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01; significantly different from the control group intratracheal instillation with 10% formalin. *** P ≤ 0.001; significantly different from the control group in situ fixation with 10% formalin. ^P ≤ 0.05; significantly different from the control groups intratracheal instillation with 10% formalin and agarose/10% formalin solution.*

Differences in CC16 immunohistological staining in the lung after various fixation and embedding procedures

The type of fixation and the method of embedding affect the ability to perform successful immunohistochemistry. Therefore a routine immunohistological staining was performed on lung tissue of PBS-treated mice fixed with Carnoy's or 10% formalin embedded in plastic or paraffin. Since CC16 is one of the main secretory proteins in the lung secreted by Clara cells, a multifunctional epithelial cell type of the mammalian lung [26], cells containing CC16 were localized in lung sections with an antibody against murine CC16. The Carnoy's-fixed lungs embedded in plastic (Fig. 8A) demonstrated a very light, brown DAB reaction product in the Clara cells. Moreover, not all of the nonciliated cells lining the terminal bronchioles throughout the lung were stained and the staining intensity showed large differences between the animals. In some lung slices we observed no staining at all. When these Carnoy's-fixed lungs were embedded in paraffin the DAB staining intensity was slightly

increased and the variation in staining intensity between animals was decreased compared to the plastic-embedded lungs (Fig. 8B). In comparison with the Carnoy's-fixed lungs embedded in plastic, the 10% formalin-fixed lungs embedded in plastic (Fig. 8C) showed an increase of the DAB intensity, however there was still a difference in staining intensity between the animals. In the 10% formalin-fixed lungs embedded in paraffin the intensity of the DAB reaction product in the Clara cells was markedly increased compared to the other fixation and embedding procedures (Fig. 8D). Additionally, all the nonciliated cells lining the bronchioles throughout the lung were stained and the lungs of all PBS-treated animals gave a similar result.

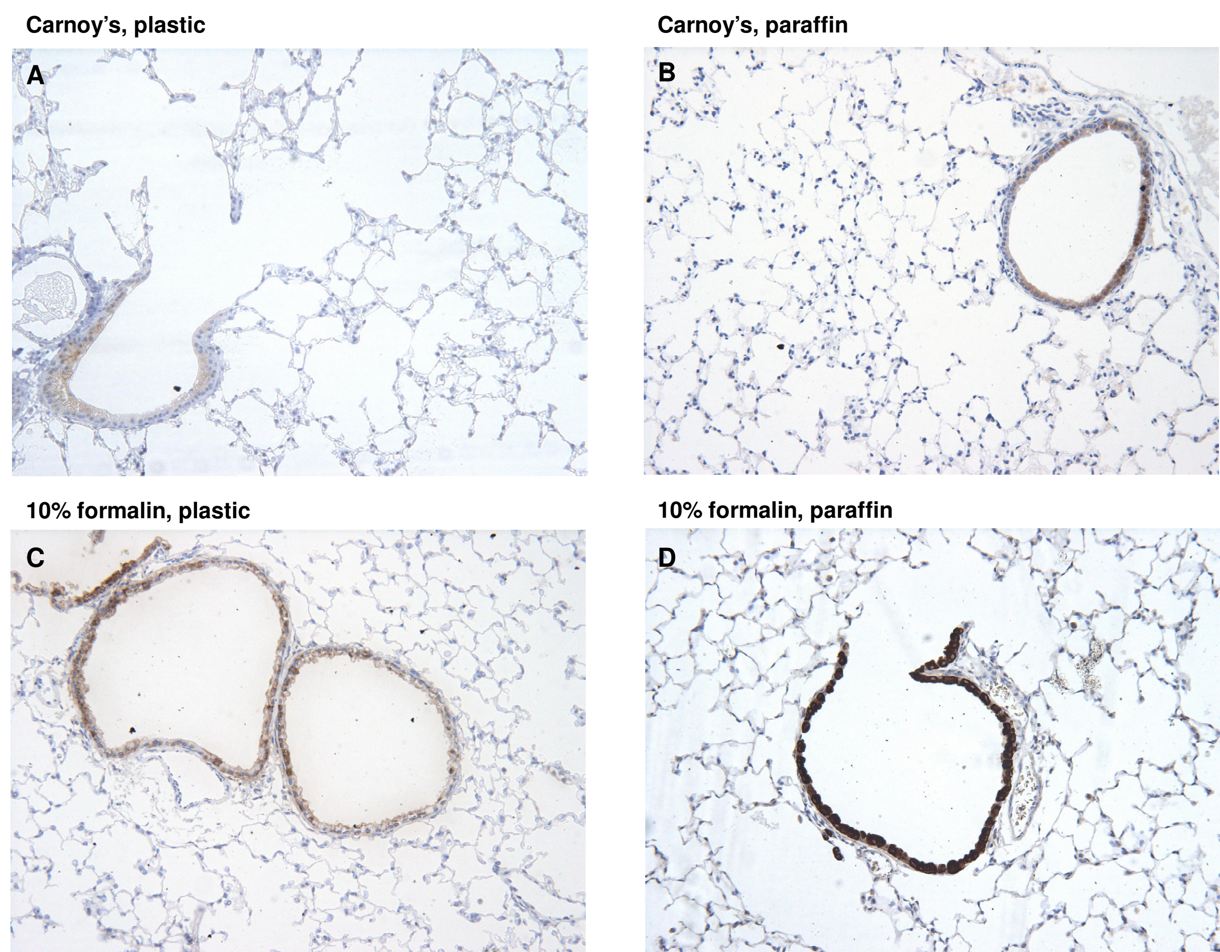

Fig.8. *Immunohistological localization of CC16 in Clara cells in the bronchioles. Representative photomicrographs of immunohistological localization of CC16 in lung tissue of PBS-treated mice fixed via intratracheal instillation of Carnoy's embedded in plastic (A) or paraffin (B) and lung tissue fixed via intratracheal instillation of 10% formalin embedded in plastic (C) or paraffin (D). n = 5 animals per group. Magnification 200x.*

Right ventricle heart hypertrophy observed in all LPS-treated animals

To determine whether the LPS model was effective and caused emphysema-like changes in each group, the right ventricle heart hypertrophy was measured as an indication of the development of lung emphysema. LPS administration twice a week for a period of 8 weeks caused right ventricular heart hypertrophy (Fig. 9). The right ventricular mass was proportionally greater than the rest of the lower heart (left ventricle and septum) in all LPS-treated mice compared to the PBS-treated animals.

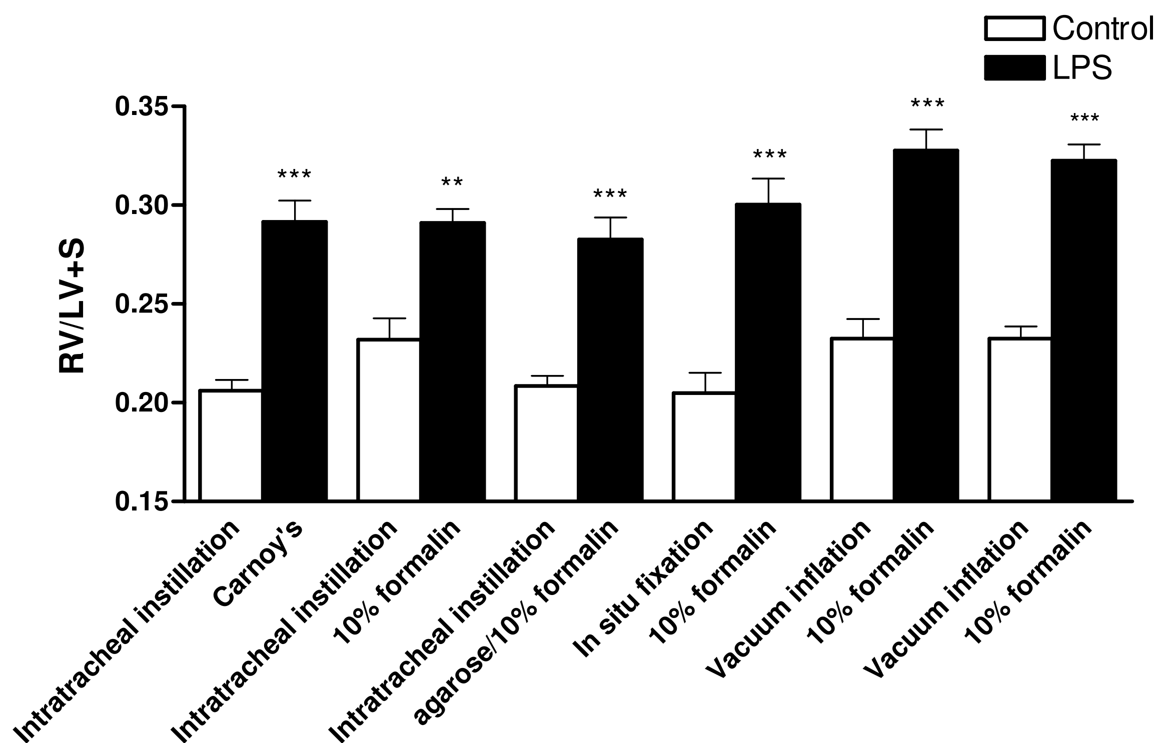

Fig.9. *Right ventricle heart hypertrophy observed in all LPS-treated animals. Right ventricle (RV) and left ventricle (LV) + septum (S) were dissected after intranasally PBS (white bar) or LPS (black bar) administration twice a week for a period of 8 weeks followed by a recovery period of 2 weeks. The weight ratio of 6 different experiment groups (intratracheal instillation of 10% formalin, intratracheal instillation of Carnoy's, in situ fixation, fixed volume fixation with 10% formalin, vacuum inflation and intratracheal instillation of agarose/10% formalin solution) was determined by the formula: (RV/(LV+S)). n = 5 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01, ***P ≤ 0.001; significantly different from the control group.*

Discussion

In rodents, alveolar enlargement is the most important parameter to assess the degree of emphysema and is primarily defined on morphological criteria. Therefore, it is essential to conserve the lung architecture, size and connectivity of all air spaces. For this purpose, different methods of lung fixation were compared in a murine LPS-induced lung emphysema model and the most optimal lung fixation technique was determined. It has to be emphasized that no lung fixation procedure revealed a perfect image of the condition in the living subject, but it is important to retain as close as possible to the *in vivo* appearance of the lung immediately preceding death to keep the structural alterations at least to a minimum.

The most widely used fixation method is the intratracheal instillation of suitable fixatives into the airways of collapsed lungs under 25 cm fluid pressure. This usually results in excellent preservation of lung tissue, which is also observed in our study, since our histological photomicrographs of murine lungs fixed via intratracheal instillation of 10% formalin and Carnoy's show a normal lung architecture with well-expanded alveoli without fixation artefacts. Reported advantages of this method are its easy performance and the homogeneity of the lung structures, because this method provides a large volume of fixative in intimate contact with the various surfaces fixing all portions of the lung uniformely and adequately compared to immersion fixation [11]. However, structural alterations and changes in the alveolar size and the removal of the alveolar surface lining layer have also been observed in lung tissue fixed via this procedure, which have to be taken into account when analyzing these lung structures [11]. Members of the Society of Toxicologic Pathology express the opinion that the advantages of intratracheal instillation of formalin for fixation of mice lungs outweigh the disadvantages. Therefore they recommends this method for use in quantitative studies involving morphometry of the alveoli [27].

Besides the fixatives 10% formalin and Carnoy's, agarose/10% formalin solution was also used as fixative in the intratracheal instillation technique. We observed almost similar photomicrographs of the lung tissue fixed via the intratracheal instillation of agarose/10% formalin solution compared to Carnoy's and 10% formalin fixation, except the agarose was still visible in the air spaces and the alveoli appeared to be slightly enlarged in the control animals compared to 10% formalin instillation. This was also detected in the mean linear intercept values of these methods, since the Lm of the control lung tissue fixed via agarose/10% formalin instillation was higher (but not significant) compared to 10% formalin instillation. The Lm observed by Bowler et al. [12] of pieces of adult rat lungs inflated with agarose was also not significantly different from lungs inflated with formalin. Halbower et al. [13] concluded that agarose infiltration is the preferred method for improving the morphology of cryostat sections of the lungs. In our study the Lm values of LPS-treated lungs fixed via

intratracheal instillation of agarose/10% formalin solution were not significantly different compared to the control mice.

An alternative approach used for the fixation of murine lungs is *in situ* fixation before opening the thorax. The collapse of the lung caused by the loss of the negative pleural pressure, which usually occurs after opening of the thoracic cavity, is reduced with this technique. In our study, the photomicrographs of the lung tissue fixed via *in situ* fixation and the associated mean linear intercept values show results associated with a normal state of alveolar expansion. It is preferable to leave the lungs in the chest to prevent distortions due to weight of the fluid-filled organ [28]. The *in situ* fixation technique before opening of the pleural cavity is also recommended for practical application by Hausmann et al. [19], who compared *in situ* fixation of the lung with routine fixation of the lung. When we evaluate the histological images of the lungs fixed via the *in situ* fixation method and the intratracheal instillation of 10% formalin *ex vivo*, no remarkable differences were observed in the lung morphology. The mean linear intercept values of the lung tissue fixed via *in situ* fixation show less variance and were significantly lower compared with the intratracheal instillation method. Most probably the alveoli are not artificially enlarged, since the lung collapse at autopsy was avoided.

To ensure that the murine lung was not overinflated by fixative instillation, the fixed volume fixation method was also assessed. Since the total lung capacity of the mouse is about 1 ml [29], this volume was used during this technique. Nevertheless, the lung morphology was not improved in comparison with intratracheal instillation with 10% formalin and the lungs of the LPS-treated animals show no significant alveolar enlargement compared to controls.

The fluid pressure of inflation used in these methods described above could affect the mean linear intercept values measured after fixation, since 25 cm fluid pressure might overinflate an emphysematous lung, while a fibrotic lung can be underinflated [30]. Soutiere et al. (2004) found no difference in airspace chord lengths when fixed at 14 or 7 cm H_2O [31]. The effect of different fluid pressures (16-34 cm) on murine lung tissue during intratracheal instillation was also tested in a pilot experiment (data not shown), but no remarkable differences were observed, therefore the standard 25 cm fluid pressure was used during this study. To our knowledge, the most widely accepted fluid pressure is 25 cm, since this pressure is used in several studies for lung emphysema measurement [10, 15-17, 27, 30, 32].

To preserve the alveoli in their natural state without any pressure, lung fixation by vascular perfusion is another option for lung fixation [28].The morphometrical results obtained in the study of DeFouw [33] described that the alveolar dimensions were obviously not adequately preserved in lung specimens fixed by vascular perfusion. This is in line with our observations related to vascular whole body perfusion, since the alveoli were enlarged, the parenchymal

architecture was collapsed, apparent hypercellularity and thickening of the alveolar walls were visible. On the contrary, Roberts et al. [21] and Mercer et al [22] both reported a vascular perfusion technique, that was thought to be an adequate procedure for morphometrical determination of the architecture in animal and human lungs. Hsia et al. (2009) described that as well airway instillation fixation as vascular perfusion fixation preserved cell and tissue structure for morphometry under light microscopy [17].

Vacuum inflation is a simple, quick and easy procedure to restore lung architecture, allowing correct histological evaluation of lung specimens as also mentioned by van Kuppevelt et al. [10]. Blumler et al. [24] also showed an easy and robust method to preserve lungs from small rodents, where the fixative was applied to lungs inflated by low vacuum. Since no fluid pressure is used to inflate the lungs, over-expanded alveoli and rapture of alveolar walls caused by possible overinflation by high positive airway pressures were prevented by this technique. Nevertheless, the photomicrographs of lungs fixed via the vacuum inflation method show no optimal lung architecture, but thickening of the alveolar walls become evident. Most probably, this is caused by parts of the lungs which are not totally inflated or the application of vacuum conditions does not allow sufficient inflation of the lung.

Besides the different methods of lung fixation, the choice of the fixative is also a major consideration in view of investigating the most optimal lung fixation procedure.

In this study, we compared a formaldehyde-containing fixative, 10% formalin, an alcohol-based fixative, Carnoy's and an agarose/10% formalin solution. 10% formalin, a non-coagulating fixative, is the gold standard of fixatives and widely used for morphometric analysis and routine immunohistochemistry [14, 17]. Formalin preserves mainly peptides and the general structure of cellular organelles. It penetrates the tissue quickly, but fixes slowly (24-48 hours), and usually results in cross-linking of reactive sites on tissue proteins [34-36]. Most commercially available antibodies have been optimized for formalin in contrast to Carnoy's. Carnoy's is not as commonly used as 10% formalin and is a coagulating fixative. It may be used to increase the speed of tissue processing, since only 1-2 hours is sufficient to fix the tissue completely [35].

After paraffin embedding, 10% formalin fixation via intratracheal instillation demonstrated a normal lung architecture, while Carnoy's-fixed lungs showed more fixation artefacts, such as enlarged alveoli and disrupted alveoli. However, when these PBS-treated lungs fixed with Carnoy's were embedded in plastic, these artefactual phenomena were not present and normal lung morphology was displayed. This was also observed in the Lm values found in these groups, since the Lm values of the lung tissue of LPS-treated animals fixed with 10% formalin and embedded in paraffin and fixed with Carnoy's and embedded in plastic were significantly increased compared to the control animals. When the embedding procedure was

changed, an increase in Lm values was determined in the lung tissue of LPS-treated animals compared to the control animals, but this was not significant. From these results, we can conclude that the embedding method is also an important factor in tissue preservation.

Furthermore, the Lm values of the 10% formalin-fixed lungs embedded in plastic show lower values compared to the Carnoy's-fixed lungs embedded in plastic. Possibly, this is caused by lung tissue shrinkage after 10% formalin fixation. However, prolonged fixation in formalin is known to cause shrinkage and hardening of the tissue, but this is also possible for Carnoy's fixation [35]. Shrinkage due to fixation might rarely interfere with the mean linear intercept analysis measured *in vivo*, since shrinkage take place in as well the controls as in the treated animals by using the same method. We also used low melting agarose dissolved in 10% formalin as fixative, since agarose has the advantage of being liquid at slightly above room temperature and solid at room temperature, a principle that has been exploited to prevent collapse of alveoli during cryosectioning [12]. Unfortunately, the histological images as well as the Lm values of the agarose/10% formalin-fixed lungs were less suitable compared to the 10% formalin-fixed lungs.

The immunohistochemistry results showed a strong and reproducible immunohistological staining against murine CC16 on the 10% formalin-fixed, paraffin-embedded lung tissue as compared to the other procedures. This fixative and embedding method also demonstrated a normal lung morphology without apparent fixation artefacts. Nevertheless, this will not conclude that this fixative and embedding method can be recommended for all immunohistological stainings, because there is no standard fixative and embedding method in immunohistochemistry.

Finally, the right ventricle heart hypertrophy was determined in each lung fixation group, to exclude differences in lung emphysema development induced by LPS exposure. In all LPS-treated groups right ventricle heart hypertrophy was observed, suggesting the LPS model was effective. Weathington et al. [16] also found right ventricle heart hypertrophy in LPS-treated animals.

Conclusion

In summary, the histological photomicrographs and the Lm measurement data presented here, demonstrate that intratracheal instillation of 10% formalin or agarose/10% formalin solution, *in situ* fixation and fixed volume fixation provided a normal lung architecture in contrast to the lungs fixed via whole body perfusion and vacuum inflation. Formalin-fixed lungs resulted in the most optimal lung morphology for lung emphysema analysis when embedded in paraffin, while for Carnoy's-fixed lungs plastic embedding was preferred, which resulted in a normal lung architecture without fixation artefacts. The immunohistochemistry results showed the most optimal CC16 staining in the 10% formalin-fixed paraffin-embedded lung tissue. Lung fixation by intratracheal instillation of 10% formalin or *in situ* fixation with 10% formalin embedded in paraffin are the most optimal lung fixation methods. These methods are sensitive to reveal the presence of alveolar enlargement to study lung emphysema and are appropriate to perform immunohistochemistry.

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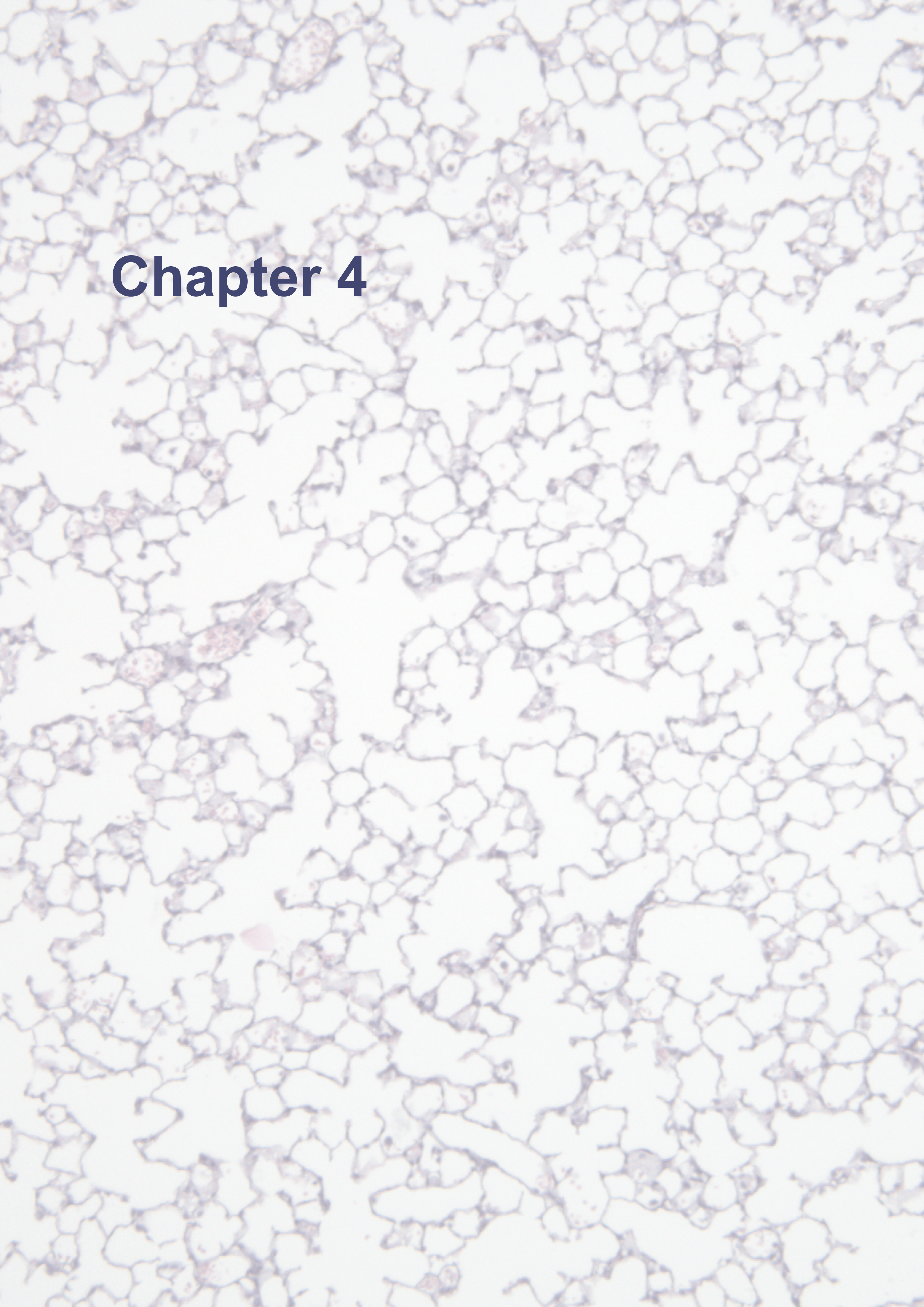

Chapter 4

ATP in the pathogenesis of lung emphysema

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Abstract

Extracellular ATP is a signaling molecule that often serves as a danger signal to alert the immune system of tissue damage. This molecule activates P2 nucleotide receptors, that include the ionotropic P2X receptors and metabotropic P2Y receptors. Recently, it has been reported that ATP accumulates in the airways of both asthmatic patients and sensitized mice after allergen challenge. The role and function of ATP in the pathogenesis of chronic obstructive pulmonary diseases (COPD) is not well understood. In this study we investigated the effect of cigarette smoke on purinergic receptors and ATP release by neutrophils. Neutrophils and their mediators are key players in the pathogenesis of lung emphysema. Here we demonstrated that in an *in vivo* model of cigarette smoke-induced lung emphysema, the amount of ATP was increased in the bronchoalveolar lavage fluid. Moreover, activation of neutrophils with cigarette smoke extract induced ATP release. Treatment of neutrophils with apyrase (catalyses the hydrolysis of ATP to yield AMP) and suramin (P2-receptor antagonist) abrogated the release of CXCL8 and elastase induced by cigarette smoke extract and exogenous ATP. These observations indicate that activation of purinergic signaling by cigarette smoke may take part in the pathogenesis of lung emphysema.

Introduction

ATP, a molecule which belongs to the purine family, is an important signaling molecule. Extracellular ATP has recently gained attention as a mediator of intercellular communication via the activation of purinergic P2X and P2Y receptors [1, 2]. Extracellular ATP serves as a danger signal to alert the immune system of tissue damage [3]. Extracellular nucleotides, primarily ATP, trigger various proinflammatory responses of neutrophils including arachidonic acid release, oxidative burst and phagocytosis [4]. Neutrophils are primary phagocytic cells with important roles in host defense and tissue repair. However, activated neutrophils damage host tissues and contribute to chronic inflammatory diseases, including rheumatoid arthritis, chronic obstructive pulmonary diseases (COPD), and asthma [5]. Recently, it has been reported that ATP accumulates in the airways of both asthmatic patients and sensitized mice after allergen challenge. In addition, sensitization to an inhaled antigen was enhanced by endogenously released or exogenously added ATP [6].

To our knowledge, the role of ATP in the disease COPD has not yet been investigated. COPD is a major and increasing global health problem and cigarette smoke has been considered a major player in the pathogenesis [7-9]. Neutrophils, neutrophil elastase, metalloproteases, and oxidants have all been shown to play a role in the pathogenesis of COPD [10]. COPD has a complex underlying pathophysiology involving inflammatory and structural cells, all of which have the capacity to release multiple inflammatory mediators, like chemokines and cytokines. Chemokines (mainly CXCL8) and proinflammatory cytokines recruit proinflammatory cells into the lungs [8, 10]. Indirect evidence support the notion that ATP might be involved in the pathogenesis of COPD [11, 12], since increased amounts of ATP induce chemotaxis of neutrophils [13]. Chemotaxis allows neutrophils to rapidly reach infected and inflamed sites. Recent findings suggest that ATP and adenosine are involved in neutrophil chemotaxis through P2Y and adenosine A_3 receptors [5, 13]. Therefore, these purinergic signaling processes may be targets for novel therapeutic approaches to ameliorate host tissue damage.

In this study, the effect of cigarette smoke on ATP release and signaling was investigated. The amount of ATP was significantly increased in the bronchoalveolar lavage fluid of cigarette smoke-exposed animals and was associated with lung emphysema. Activation of neutrophils with cigarette smoke extract induced ATP release. Furthermore, cigarette smoke extract and ATP increased the release of CXCL8 and elastase by human neutrophils, which was suppressed by suramin an apyrase. In conclusion, this study describes a new pathway for releasing proinflammatory mediators by cigarette smoke-stimulated neutrophils.

Material and methods

Animals

Five/six-week-old male Balb/c mice were obtained from Charles River (Maastricht, The Netherlands) and housed under controlled conditions in standard laboratory cages in the local animal facility. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the Animal Care Committee of Utrecht University.

Smoke exposure

Cigarette smoke was generated by the burning of commercially available Lucky Strike cigarettes without filter (British-American Tobacco, Groningen, The Netherlands) using the TE-10z smoking machine (Teague Enterprises, Davis, CA), which is programmed to smoke cigarettes according to the Federal Trade Commission protocol (35 ml puff volume drawn for 2 s, once per min). Mice were exposed nose only to the diluted main stream and side stream smoke of 3 cigarettes per session, twice every weekday for 3 months using the In-Tox-24-port nose-only exposure chamber (In-Tox Products Inc., Albuquerque, NM). Control mice were room air-exposed under similar circumstances [14].

Bronchoalveolar lavage

After 3 months of smoking, mice were sacrificed by an i.p. injection with an overdose of pentobarbital (Nembutal™, Ceva Santé Animale, Naaldwijk, The Netherlands) 24h after the last smoke exposure. The lungs were lavaged using 1 aliquot (1 ml) of saline solution (NaCl 0.9%) containing a mixture of protease inhibitors (Complete Mini, Roche Applied Science) at 37℃. The bronchoalveolar lavage fluid was centrifuged at 4℃ (400 g, 5 min) and the supernatant was immediately stored at -20℃ until the ATP measurements.

Mean linear intercept (Lm)

Lung tissue was obtained as described previously; the left lung was fixated with a 10% formalin infusion through the tracheal cannula at a constant pressure of 25 cm H_2O. After excision, the left lung was immersed in fresh fixative for at least 24h, after which it was embedded in paraffin for morphometric analyses [14]. After paraffin embedding, 5 µm sections were cut and stained with hematoxylin/eosin (H&E) according to standard methods. To evaluate the alveolar airspace enlargement we used the mean linear intercept analysis, whereby 24 random photomicroscopic images per left lung tissue section were evaluated by microscopic projection onto a reference grid. By dividing total grid length by the number of alveolar wall-grid line intersections, the Lm was calculated [15].

ATP measurements in bronchoalveolar lavage fluid of mice

ATP levels in bronchoalveolar lavage fluid of control and cigarette smoke exposed mice were measured using an ATPlite detection assay (Perkin Elmer) according to instructions, but with the cell lysis step omitted to avoid any contaminating intracellular ATP.

Isolation of neutrophils

Human neutrophils were isolated as previously described [16]. Briefly, human neutrophils were obtained from heparinized venous blood buffy coat by Ficoll-Hypaque centrifugation, followed by sedimentation in 5% dextran/0.9% saline. Neutrophils were separated from erythrocytes by lysis in a solution of 0.15 M NH_4Cl, 0.01 M $NaHCO_3$ and 0.01 M tetra EDTA. The recovered neutrophils were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplied with 10% fetal calf serum and essential amino acids and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2 and washed three times. The purity of neutrophils preparations was 93-95%, as determined by Wright's staining of cytospin preparations. Cell viability of these cells was 97%, as determined by trypan blue exclusion. Neutrophils, as isolated above, were kept on ice until used as described below.

Production of cigarette smoke extract

Cigarette smoke extract was prepared as described before [17]. Briefly, a smoking machine (Teague Enterprises) was used to direct main and side stream smoke from one cigarette through 5 ml culture medium (RPMI without phenol red). Hereafter, absorbance was measured spectrophotometrically and the media were standardized to a standard curve of cigarette smoke extract concentration against absorbance at 320 nm. This solution is considered to be 100% cigarette smoke extract. Solutions ranging from 0.75% to 3% were used in the present study following preliminary experiments, which indicated that these were nontoxic concentrations (viability ≥ 96%). Freshly prepared cigarette smoke medium was used in all experiments.

Preparation of activated neutrophil supernatants for ATP measurement

The release of ATP was measured as described before [6]. Freshly isolated neutrophils (10^8 cells/ml in Hank's balanced salt solution (HBSS)) were activated with different concentrations cigarette smoke extract (0.75-3%), and N-formyl-Met-Leu-Phe (FMLP) (1nM) at 4°C for 3 min. Neutrophils were then immediately pelleted and supernatants were filtered (0.45 m; Phenomenex). The supernatants were stored at -80°C. ATP levels were measured using an ATPlite detection assay (Perkin Elmer) according to instructions.

Pharmacological inhibitors

To analyze the action of ATP, 4 U/ml apyrase (Sigma-Aldrich) and 100 µM suramin (Sigma-Aldrich) were used as inhibitors [5]. Cells were co-incubated with apyrase and stimulated with various stimulators. Cells were pre-incubated with suramin 30 min before activation with various stimulators.

Quantification of CXCL8 and elastase

CXCL8 and elastase concentrations in cell supernatants were determined by an CXCL8 ELISA kit (BD Biosciences Pharmingen, Breda, The Netherlands) and by a human elastase ELISA kit (Hycult biotechnology B.V., The Netherlands), according to the manufacturer's instructions.

Cell viability assay

Lactate dehydrogenase (LDH) activity was measured in the supernatant (Roche Diagnostics) to evaluate lytic cell death of neutrophils.

Statistical analysis

Experimental results are expressed as mean ± S.E.M. Results were tested statistically by an unpaired two-tailed Student's *t*-test or one-way ANOVA, followed by Newman–Keuls test for comparing all pairs of groups. Analyses were performed by using GraphPad Prism (Version 4.0). Results were considered statistically significant when $P < 0.05$.

Results

Increased ATP levels in bronchoalveolar lavage fluid of mice exposed to cigarette smoke

Mice were exposed to cigarette smoke for 3 months and 24h after the last exposure, the concentration of ATP was significantly increased in the bronchoalveolar lavage fluid of the smoke-exposed mice compared to the control animals (Fig. 1A).

Airspace enlargement, as measured by the mean linear intercept (Lm), was significantly augmented in the cigarette smoke-exposed mice as compared to the control group (Fig. 1B).

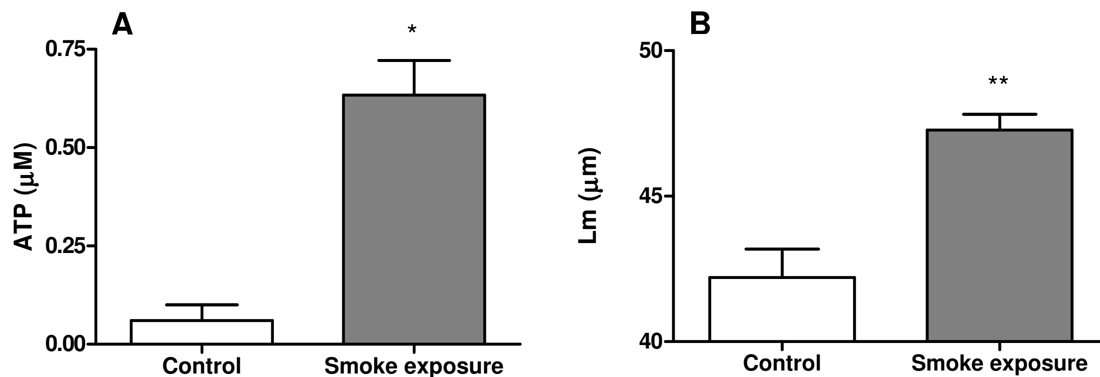

Fig.1. *Increased ATP levels in bronchoalveolar lavage fluid of mice with cigarette smoke-induced emphysema. ATP levels in bronchoalveolar lavage fluid of Balb/c mice exposed to cigarette smoke for 3 months (A). Data represent the mean ± S.E.M. (n=3 in each group). *P < 0.05; significantly different from the control group. Cigarette smoke exposure resulted in a marked increase in the mean linear intercept (Lm) compared to air exposure (B). Data represent the mean ± S.E.M. (n=6 in each group). **P < 0.01; significantly different from the control group.*

Cigarette smoke extract increased the production of ATP by human neutrophils

It has been demonstrated that neutrophils have the capacity to release adenine nucleotides in the form of ATP [5, 18]. Since, neutrophils are important in the pathogenesis of lung emphysema [19, 20], we next studied the effects of cigarette smoke extract on the release of ATP by human neutrophils. As shown in Fig. 2, exposure of cells to various concentrations of cigarette smoke extract (0.75%-3%) induced the instant release of ATP (within 1 min). FMLP was used as a positive control (Fig. 2).

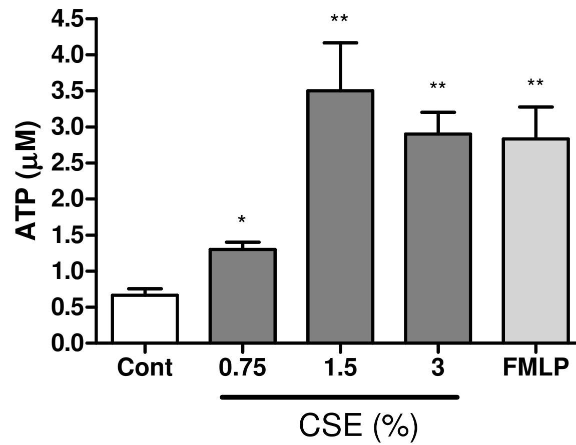

Fig.2. *Cigarette smoke extract (CSE) increased ATP release by human neutrophils. Human neutrophils (10^6/ml) were stimulated with different concentrations (0.75-3%) cigarette smoke extract for 1 min and ATP levels were measured. FMLP (1nM) was used as positive control. Data represent the mean ± S.E.M. of 3 experiments performed in duplicate. *P < 0.05, **P < 0.01; significantly different from the non-stimulated neutrophils (Cont).*

Apyrase and suramin attenuated the release of elastase and CXCL8

We have shown that cigarette smoke extract induces the release of CXCL8 of several inflammatory cells such as human neutrophils, macrophages and dendritic cells [16, 17, 21, 22]. In this experiment we investigated the possible role of ATP on the release of elastase and CXCL8 by human neutrophils. Incubation of neutrophils with cigarette smoke extract (1.5%) or exogenous ATP (10 μM) induced the release of CXCL8 and elastase (Fig. 3A). As a pharmacological approach, pre-incubation of neutrophils with the P2 receptor antagonist suramin suppressed the production of CXCL8 (Fig. 3C) and elastase (Fig. 3D) induced by cigarette smoke extract and exogenous ATP. Pre-incubation with apyrase, which catalyses the hydrolysis of ATP to yield AMP, had no effect (data not shown). However, co-incubation of neutrophils with apyrase attenuated the release of CXCL8 (Fig. 3C) and elastase (Fig. 3D) induced by cigarette smoke extract and ATP.

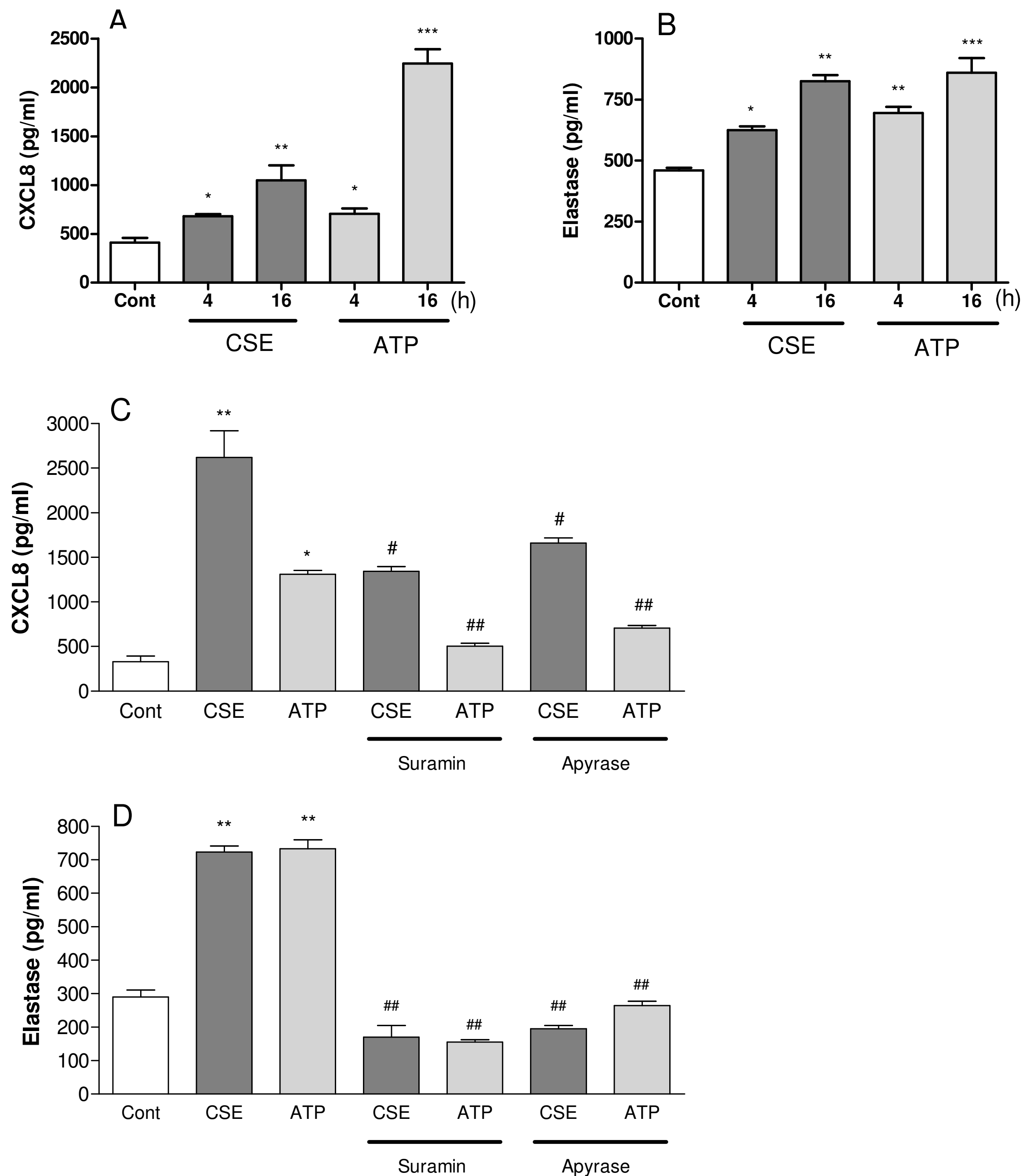

Fig.3. *Exogenous ATP increased the release of CXCL8 and elastase by human neutrophils. Human neutrophils (10^6/ml) were stimulated with 1.5% cigarette smoke extract (CSE) or exogenous ATP (10 µM) for 9h. The amount of CXCL8 (A) and elastase (B) were measured in supernatants. Data represent the mean ± S.E.M. of 3 experiments performed in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001; significantly different from the non-stimulated neutrophils (Cont). Human neutrophils (10^6/ml) were pre-treated with suramin (100 µM) or co-incubated with apyrase (4U/ml) and then stimulated with 1.5% CSE) or exogenous ATP (10 µM). The amount of CXCL8 (C) and elastase (D) were measured in supernatants. Data represent the mean ± S.E.M. of 3 experiments performed in duplicate. *P < 0.05, **P < 0.01; significantly different from the non-stimulated neutrophils (Cont). $^{\#}$P < 0.05, $^{\#\#}$P < 0.01; significantly different from the CSE-stimulated cells or ATP-stimulated cells.*

Discussion

Extracellular ATP liberated during hypoxia and inflammation can either signal directly on purinergic receptors or can activate adenosine receptors following phosphohydrolysis to adenosine. Given the association of neutrophils with adenine-nucleotide/nucleoside signaling in the inflammatory milieu and oxidative stress [5, 18, 23], we hypothesized a link between cigarette smoke and ATP release and signaling in the pathogenesis of COPD. Therefore, we aimed to investigate the role of ATP in COPD. It was demonstrated that increased amounts of ATP were present in the bronchoalveolar lavage fluid of cigarette smoke-exposed mice. These animals also showed an airspace enlargement, as measured by the mean linear intercept. It has been reported in the literature, that in healthy tissues the release of ATP is tightly controlled and its extracellular concentration is kept very low by ubiquitous ecto-ATP/ADPases (CD39). During inflammation, the inflammatory mediators (for example, bacterial endotoxin or reactive radicals) can enhance the amount ATP by down-regulation of CD39 [24]. Whether a reduced activity of CD39 could explain the increase in ATP levels in the lungs of smoke exposed-animal remains to be determined. The ATP increase could also be caused by cigarette smoke-activated neutrophils or by cell damage due to cigarette smoke exposure. Indeed, it was found that neutrophils released ATP after cigarette smoke stimulation *in vitro* (Fig. 2). This ATP release was not caused by cell death since no decreased cell viability after stimulation was observed.

Further work is being directed to elucidate the mechanisms leading to cigarette smoke-induced ATP release in the lungs.

Our study also demonstrated that activation of purinergic receptors by ATP lead to the release of CXCL8 and elastase by neutrophils (Fig. 3). CXCL8 and elastase are two important mediators in pathogenesis of lung emphysema and COPD [10, 19, 25, 26]. This release of CXCL8 and elastase was reduced by treatment with the enzyme apyrase, that catalyzes the hydrolysis of ATP to yield AMP and orthophosphate. Besides apyrase, suramin, a P2 receptor antagonist, had also a suppressive effect on the release of CXCL8 and elastase by human neutrophils. We can conclude from these results, that ATP may play a role in CXCL8 and elastase release by neutrophils. In addition, P2 receptor signaling pathways are involved in the release of inflammatory mediators by cigarette smoke-activated neutrophils, which is a potential novel pathway in understanding the pathogenesis of COPD. Further research is needed to identify the exact mechanisms responsible for the release of ATP by neutrophils after cigarette smoke exposure.

Taken together, the current study demonstrated that cigarette smoke extract induces ATP release from neutrophils, which may play a critical role in the induction of CXCL8 and elastase release. As CXCL8 is largely responsible for neutrophil recruitment into the sites of inflammation, and elastase for destruction of lung tissues, this nucleotide-dependent mechanism could be an useful target for the development of new drugs to treat inflammatory diseases like lung emphysema and COPD.

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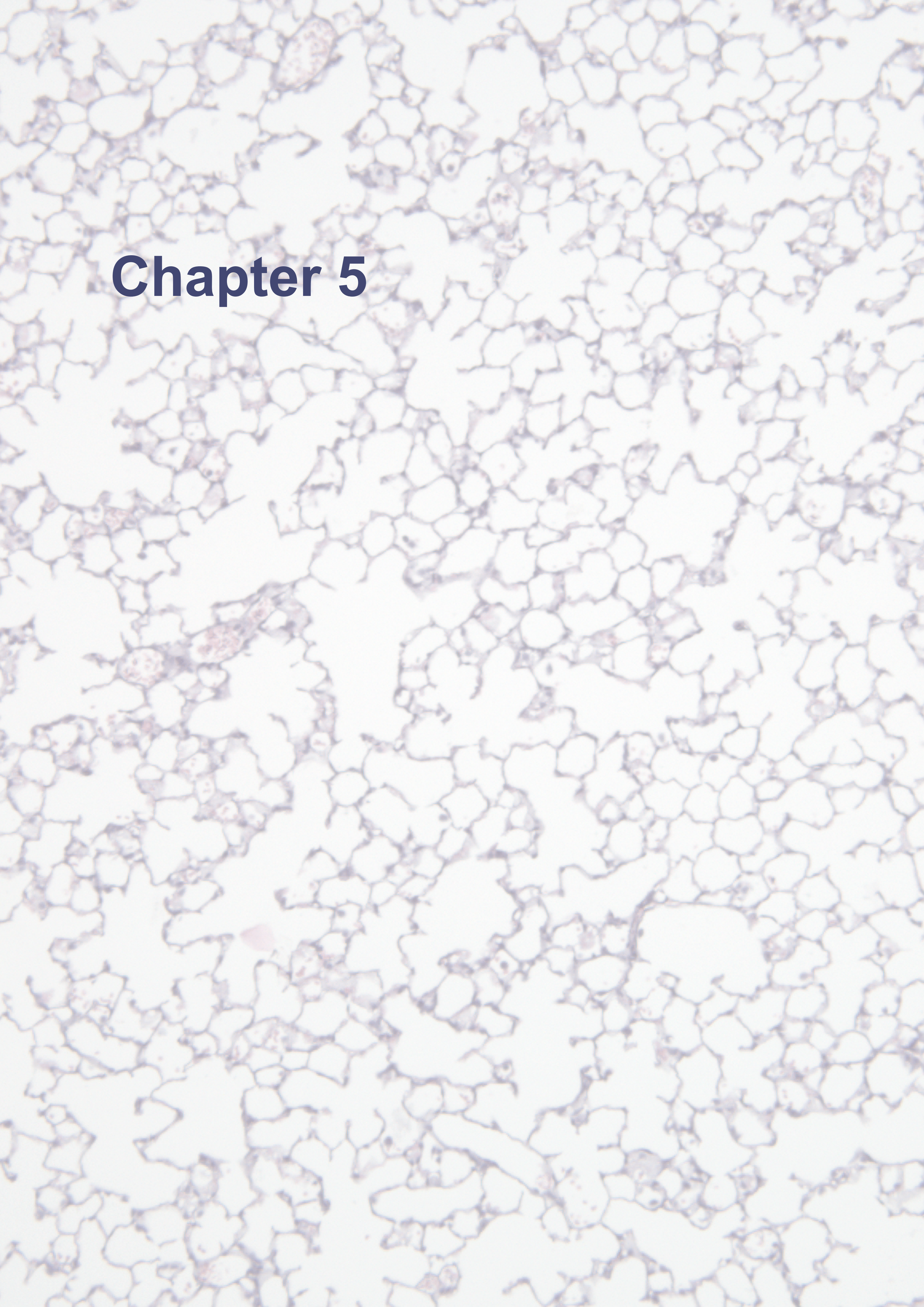

Chapter 5

An association between neutrophils and immunoglobulin free light chains in the pathogenesis of COPD

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Abstract

Neutrophils (PMNs) are key players in chronic obstructive pulmonary disease (COPD) and increased numbers of neutrophils are present in sputum and lung tissue of COPD patients. Interestingly, immunoglobulin free light chains (IgLC) are able to prolong the life of neutrophils, therefore IgLC may contribute to the chronic state of inflammation. To our knowledge, the relation between IgLC and COPD has never been investigated.

We investigated the presence of IgLC in 3 different murine models of lung emphysema: a nose-only, a whole body cigarette smoke exposure model and a short emphysema model with intraperitoneal and intratracheal injections of cigarette smoke extract (CSE). IgLC serum levels were examined by Western blot analysis. IgLC levels in serum and lung tissue from COPD patients were determined by ELISA and immunohistochemistry, respectively. FACS and immunofluorescent analysis were used to detect binding between IgLC and human PMNs. CXCL8 release by human PMNs after incubation with IgLC was measured by ELISA. Finally, we examined the effect of F991, an IgLC antagonist, on the smoke-induced neutrophil influx in murine lungs after 5 days smoke exposure.

Our studies showed increased levels of IgLC in serum of cigarette smoke-exposed and CSE-treated mice compared to control mice. COPD patients showed increased serum IgLC and expression of IgLC in lung tissue compared with healthy volunteers. Interestingly, IgLC bind to PMNs and activate PMNs to release CXCL8. F991 reduced the smoke-induced neutrophil influx in murine lungs after 5 days smoke exposure.

This study describes for the first time a neutrophil-related role for IgLC in the pathophysiology of COPD, which could open new avenues to targeted treatment of this chronic disease.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by an incompletely reversible, progressive airflow limitation and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases, most commonly cigarette smoke [1, 2]. The relation between neutrophils and COPD has been extensively investigated and several studies provide suggestive evidence that neutrophils are important key players in the development of COPD [3, 4]. Increased numbers of neutrophils are present in sputum, lung tissue and bronchoalveolar lavage fluid (BALF) of COPD patients [5, 6]. The general hypothesis on COPD states that cigarette smoke activates the epithelial cells and alveolar macrophages to release chemotactic factors, such as CXCL8, which attract neutrophils to the lung. Neutrophils then release proteases, which contribute to lung matrix breakdown resulting in emphysema [3, 4]. Neutrophils are critical effector cells during acute inflammatory processes and recruitment and activation of neutrophils can be initiated and regulated via a multitude of mechanisms. An example is the interaction of CXCL8 with the chemokine receptor CXCR2, which has been claimed to be a major mechanism of neutrophil activation in inflammatory lung diseases [7]. However, neutrophils also express receptors for different immunoglobulin isotypes. These so-called Fc receptors are important triggers of neutrophil effector functions and may modulate airway inflammatory reactions. Human neutrophils constitutively express the IgG receptors, FcγRIIIB and FcγRIIA, while expression of FcγRI is increased during inflammatory conditions [8]. Moreover, neutrophils from asthmatic subjects express the IgE high-affinity receptor (FcεRI) and the cross-linking of this receptor is involved in the release of CXCL8 [9]. It is widely accepted that FcεRI cross-linking by IgE bound to its antigen results in biological effects, however, Saffar et al. [10] demonstrated an anti-apoptotic effect exerted by monomeric IgE alone on human neutrophils *in vitro*. Interestingly, immunoglobulin free light chains (IgLC) are also able to prolong the life of neutrophils, suggesting that IgLC could contribute to the chronic state of inflammation [11]. It has been long recognized that B cells not only produce and secrete tetrameric immunoglobulins, but also secrete a substantial amount of IgLC. In the mammalian immune system, two isotypes of IgLC are produced: κ and λ [12]. IgLC can exert various biological activities: enzymatic activities, such as specific proteolysis, binding to intracellular and extracellular proteins, such as opioid peptides, antigens and chemotactic factors and cellular interactions including binding and activation of mast cells, B cells, mesangial cells and renal tubular cells [13]. Inflammatory disorders, such as asthma, rhinitis, food allergy and inflammatory bowel disease but also autoimmune diseases e.g. multiple sclerosis and rheumatoid arthritis are accompanied with elevated levels of IgLC in different body fluids [14-19]. The increased

concentrations of IgLC correlate with relapses of disease and enhanced activity of the immune system [12, 20-23].

In this study we investigated the involvement of IgLC in COPD and have focussed on the neutrophil, the major inflammatory cell involved in the pathogenesis of COPD. Our data suggest a role of IgLC in COPD, since increased levels of IgLC in sera were found in 3 different murine models of lung emphysema. Furthermore, IgLC levels were increased in serum and lung tissue from patients with COPD. Interestingly, we determined that IgLC bind to human neutrophils and cross-linking of IgLC resulted in the production CXCL8 *in vitro*. Finally, antagonizing the action of IgLC reduced the cigarette smoke-induced neutrophil influx in murine lungs.

Material and methods

Animals

Female A/J mice, 9-14 weeks old, male C57Bl/6 mice, 6-8 weeks old, male Balb/c mice, 6-8 weeks old (Charles River Laboratories) were housed under controlled conditions in standard laboratory cages. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the local Ethics Committee and were performed under strict governmental and international guidelines on animal experimentation.

Nose-only cigarette smoke exposure

C57Bl/6 mice were nose-only exposed to the diluted mainstream and sidestream smoke from commercially available Lucky Strike cigarettes without filter (British-American Tobacco, Groningen, The Netherlands), using the TE-10z smoking machine (Teague Enterprises, Davis, CA) which is programmed to smoke cigarettes according to the Federal Trade Commission protocol (35 ml puff volume drawn for 2 seconds once per minute). Before starting smoke exposure, mice were accustomed to the nose-only exposure chambers (In-Tox Products Inc., Albuquerque, NM) by gradually prolonging their stay in the tubes over the course of one week. Smoke exposures were conducted twice every weekday for 3 months after an adaptation period of 2 weeks, starting with 1×1 cigarette, increasing the dosage to 2×3 cigarettes within two weeks. Control mice underwent the same procedures, but were allowed to breathe room air throughout the whole exposure period. The average total suspended particulates (TSP) concentration of the smoke inside the exposure chamber was 85 mg/m^3 determined by gravimetric analysis of Emfab filter samples (Pall Corporation, East Hills, NY, USA). The carbon monoxide (CO) content of the smoke inside the exposure chamber was measured by sampling with the Monoxor II CO analyzer (Bacharach Inc, New Kensington, PA, USA) at 15 seconds intervals during 2 runs and was around 1200 ppm. The mice were sacrificed 16-24 hours after the last air or smoke exposure [24].

Whole body cigarette smoke exposure

A/J mice were divided into three groups. The first group was exposed to room air for 20 weeks, the second group was exposed to cigarette smoke for 20 weeks and the third group was exposed to cigarette smoke for 20 weeks followed by a period of 8 weeks without cigarette smoke exposure. The mice were exposed in whole-body chambers to air (sham) or to diluted mainstream cigarette smoke from the reference cigarettes 2R4F (University of Kentucky, Lexington, Kentucky) using a smoking apparatus. Exposures were conducted

4h/day (with a 30/60-minutes fresh air break after each hour of exposure), 5 days/week for 20 weeks to a target cigarette smoke concentration of 750 µg total particulate matter/l (TPM/l). This TPM concentration was reached after an adaptation period of 2 weeks, starting with a TPM concentration of 125 µg TPM/l. The mass concentration of cigarette smoke TPM was determined by gravimetric analysis of Cambridge filter samples. Carbon monoxide (CO) levels were monitored continuously and were around 800 ppm. The nicotine concentration in the smoke was approximately 40 µg/l. The sample sites were located in the middle of the exposure chamber at the breathing zone. The carboxyhemoglobin level measured via blood gas analysis was 50% on average in the smoke-exposed mice. The mice were sacrificed 16-24 hours after the last air or smoke exposure, or after the smoke-free period of 8 weeks.

Cigarette smoke extract administration

Cigarette smoke extract (CSE) was prepared as described before [25]. Briefly, TE-10z smoking machine was used to direct mainstream and sidestream smoke from one cigarette through 5 ml PBS. Hereafter, absorbance was measured spectrophotometrically and the OD of the CSE-PBS solution was 11 on average. Freshly prepared cigarette smoke extract was used for each injection. The control C57Bl/6 mice were intraperitoneally injected with 200 µl PBS at day 1. The CSE group was injected intraperitoneally with 100 µl CSE-PBS plus 100 µl adjuvant (Freunds Complete Adjuvant H37Ra, Difco) at day 1 and 50 µl CSE-PBS was administered intratracheally at day 11. At day 21 the mice were sacrificed.

F991, IgLC antagonist, study

Balb/c mice were exposed in whole-body chambers to air (sham) or to diluted mainstream cigarette smoke from the reference cigarettes 2R4F (University of Kentucky, Lexington, Kentucky) using a peristaltic pump. Just before the experiments, filters were cut from the cigarettes. Each cigarette was smoked in 5 minutes at a rate of 5 l/hour in a ratio with 60 l/hour air. The mice were exposed to cigarette smoke using 5 cigarettes twice daily for 5 consecutive days, except for the first day when they were exposed to 3 cigarettes. The mice were sacrificed 16 hours after the last air or smoke exposure. In this cigarette smoke exposure model, cigarette smoke- and air-exposed mice received F991 (100µg F991 in 70µl sterile PBS) by oropharyngeal administration twice daily for 5 consecutive days during isoflurane anesthesia. The IgLC antagonist F991, a 9-mer peptide (AHWSGHCCL), was synthesized by Fmoc chemistry (Ansynth, Roosendaal, The Netherlands) [26].

Bronchoalveolar lavage

Immediately after i.p. injection with an overdose of pentobarbital, the lungs of mice were lavaged 4 times through a tracheal cannula with 1 ml saline (NaCl 0.9%), pre-warmed at 37 °C. After centrifuging the bronchoalveolar lavage fluid at 4°C (400 g, 5 min), the cell pellets of the 4 lavages were used for neutrophil cell counts. The 4 cell pellets, kept on ice, were pooled per animal and resuspended in 150 µl cold saline.

Neutrophil cell count in BALF

After staining with Türk solution, total cell counts per lung lavage were made under light microscopy using a Burker-Turk chamber. Differential cell counts were performed on air-dried cytospin preparations stained by DiffQuick™ (Dade A.G., Düdingen, Switzerland). Cells were identified according to standard morphology. At least 200 cells were counted and the absolute number of neutrophils was calculated.

Histology and morphomethric analysis

Mice used for morphometric analysis, were sacrificed by an i.p. injection with an overdose of pentobarbital (Nembutal™, Ceva Santé Animale, Naaldwijk, The Netherlands). The lungs were fixed with a 10% formalin infusion through a tracheal cannula at a constant pressure of 25 cm H_2O [27]. The left lung was immersed in fresh fixative for at least 24h, after which it was embedded in paraffin. After paraffin embedding, 5 µm sections were cut and stained with hematoxylin/eosin (H&E) according to standard methods. Morphometric assessment of emphysema, including determination of the average inter-alveolar distance, was estimated by the mean linear intercept (Lm) analysis. The Lm was determined by light microscopy at a total magnification of 100x, whereby 24 random photomicroscopic images per left lung tissue section were evaluated by microscopic projection onto a reference grid. By dividing total grid length by the number of alveolar wall-grid line intersections, the Lm (in µm) was calculated [28].

Right ventricular heart hypertrophy measurement

The right ventricle was removed from lower heart after removal of the atria. The right ventricle and the left ventricle plus septum were weighed and the ratio of the weights was calculated as follows: (right ventricle)/(left ventricle + septum) [29].

Serum collection

Directly after the i.p. injection with an overdose of pentobarbital, blood was obtained by heart puncture and collected in MiniCollect Z Serum Sep tubes (Greiner Bio-one, part no.450472). After 1 hour the blood samples were centrifuged for 10 min at 20.000 g and the sera were stored at -20℃ for IgLC measurement.

Culture of thoracic lymph node (TLN) cells

Lung-draining lymph nodes were collected from the thorax and transferred to 1 ml sterile RPMI 1640 without L-glutamine and phenol red (Lonza Verviers SPRL, Belgium), supplemented with 1% Pen-Strep. TLNs from six mice were pooled and were filtered through a 70 µm nylon cell strainer (BD Falcon, Belgium) with 4 ml RPMI 1640 without L-glutamine and phenol red, supplemented with 1% Pen-Strep to obtain a single-cell suspension. The total number of cells in the TLN-cell suspension was determined using a Burker-Turk counting-chamber. TLN cells (1×10^6 cells/ml) were cultured in a culture flask at 37℃ in 5% CO_2. After 24 hours, the supernatant was harvested, and stored at -20℃ until free immunoglobulin light chains were determined.

Western Blot analysis

Before use, serum samples were precipitated to deplete high amounts of albumin by using trichloroacetic acid/acetone, as previously described [30]. Equal amounts of proteins (precipitated serum and TLN supernatants) of boiled nonreduced samples were separated electrophoretically (SDS-PAGE 12%) and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, The Netherlands). The membrane was blocked with Tween-PBS containing 2% milk proteins. HRP-labeled goat anti-mouse κIgLC (1:10.000) was applied for 1 h at room temperature as primary Ab. Blots were washed in Tween-PBS three times for 10 min, incubated in commercial ECL reagents (Amersham Biosciences, Roosendaal, The Netherlands), and exposed to photographic film. Films were scanned on a GS710 Calibrated Imaging Densitometer (Bio-Rad Laboratories) and the optical density (OD) of the immunoreactive bands was quantified.

Patient characteristics

Patient characteristics of the 6 COPD patients included in the study are presented in Table 1.

Table 1. *Patient characteristics*

COPD PATIENT	SEX, M/F	AGE, YEARS	CURRENT SMOKER	SMOKING HISTORY, PACKYEARS	FEV_1, %predicted	FEV_1/FVC, %
1	m	67	-	37	40	45
2	m	67	-	35	57	58
3	m	52	+	31	33	23
4	m	59	+	42	35	42
5	m	63	+	50	42	41
6	m	62	-	45	55	58

FEV_1: Forced Expiratory Volume in one second. FVC: Forced Vital Capacity.

ELISA for kappa IgLC proteins

The concentration of κIgLC in serum samples obtained from patients with COPD and healthy volunteers was measured by ELISA with mAbs specific for human IgLC (purchased from A. Solomon, University of Tennessee, Knoxville, TN) as described previously [31, 32].

Immunohistochemical staining for IgLC

Paraffin sections of lung tissue were routinely deparaffinized and blocked for endogenous peroxidase for 30 min at room temperature. Sections were incubated with the primary mouse antibodies directed against human κ and λ IgLC [33] for 60 min at room temperature. Post-incubation with the primary antibody, the sections were incubated with anti-mouse HRP-labeled polymer (Dakocytomation, Heverlee, Belgium) for 30 min at room temperature. Color was developed with AEC substrate chromogen. Between incubation steps, the sections were intensively rinsed with 0.05 M TBS containing 0.05% Tween. Within each test, negative controls were included, and they were all found not to contain any specific staining.

Isolation of human PMNs

PMNs were isolated from buffy coats, which were purchased from Sanquin Blood Bank (Amsterdam, The Netherlands). The PMNs were obtained by gradient centrifugation on Ficoll-Paque^{TM} PLUS (density: 1.077 g/ml, GE Healthcare, Diegem, Belgium), followed by hypotonic lysis of erythrocytes with sterile lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$ and 0.1 mM EDTA, pH 7.4) on ice. Contamination by monocytes was prevented by incubating the isolated PMN fraction at 37℃ in RPMI/10%FBS medium for 1h. After incubation, the PMNs were washed with PBS and finally resuspended in RPMI 1640 medium (without L-glutamine and phenol red) supplemented with 1% heat-inactivated FBS. Resulting PMN preparations consisted of approximately 95-97% PMNs.

Flowcytometry

Freshly isolated PMNs ($2x10^5$ cells/200 µl) were incubated with 7 µg FITC labeled κIgLC (κIgLC isolated and purified from the urine of multiple myeloma patients were kindly donated by dr B. Bast, UMC Utrecht, the Netherlands) for 30 min on ice. After incubation, the cells were washed to remove the unbound FITC-IgLC and fixed in PBS/1%BSA containing 1% PFA (Sigma Aldrich, Zwijndrecht, the Netherlands). Binding of FITC labeled κIgLC to PMNs was analyzed using a FACS Calibur (BD Biosciences, Breda, the Netherlands).

Immunofluorescence staining for IgLC on PMNs

Freshly isolated PMNs ($1x10^6$ cells/ml) were incubated with 7 µg FITC labeled κIgLC for 30 min on ice. After incubation, the cells were washed to remove the unbound FITC-IgLC. The labeled cells were spun onto slides by using a cytospin centrifuge. The FITC signal was amplified using a FITC signal amplification kit (Invitrogen, Breda, the Netherlands). Fluorescent imaging was performed on a Zeiss microscope.

PMN stimulation assay

96-well plates (Greiner, Alphen a/d Rijn, the Netherlands) were coated with κIgLC (10, 30 and 100 µg/ml) in carbonate buffer (pH 9.6) by overnight incubation. Prior to adding the cells, the coated surface was blocked with RPMI containing 10% immunoglobulin free BSA (Sigma Aldrich, Zwijndrecht, the Netherlands) for 1h at room temperature. Buffy coat isolated PMNs (200 µl per well, cell density adjusted to $1x10^6$ cells/ml using RPMI/10%FBS) were added to the 96-well plate coated with κIgLC and incubated for 4 hours. As a positive control 1 µg/ml LPS (Sigma Aldrich, Zwijndrecht, the Netherlands) was added to the isolated PMNs and

incubated for 4 hours. After incubation the cells were centrifuged at 450 g for 5 min and the supernatant was collected for CXCL8 measurement.

CXCL8 ELISA

CXCL8 levels were measured using a human CXCL8 ELISA kit (BD Biosciences, Breda, the Netherlands) according to manufacturer's instructions.

Statistical analysis

Experimental results are expressed as mean ± S.E.M. Differences between groups were statistically determined by an unpaired two-tailed Student's *t*-test and differences between (*in vitro*) data were analyzed using two-way ANOVA, with post hoc Bonferroni's *t*- test for multiple comparisons using GraphPad Prism (Version 4.0). Results were considered statistically significant when $P < 0.05$.

Results

Increased production of IgLC after nose-only cigarette smoke exposure

IgLC levels were measured in serum of C57Bl/6 mice which were nose-only exposed to cigarette smoke for 1, 3, 6, 9 and 12 weeks. The concentrations of IgLC in serum of cigarette smoke-exposed mice were significantly enhanced compared to the air-exposed mice, which was dependent on the duration of cigarette smoke exposure (Fig. 1A). The increase of IgLC in serum was most pronounced after 9 and 12 weeks of cigarette smoke exposure. This nose-only exposure model leads to characteristics of lung emphysema, since airspace enlargement was observed in mice after 12 weeks of cigarette smoke exposure compared to the air-exposed mice (Table 2). Furthermore, 12 weeks nose-only cigarette smoke exposure caused right ventricle heart hypertrophy, since the right ventricular mass was proportionally greater than the rest of the lower heart (left ventricle and septum) in smoke-exposed mice compared to air-exposed mice (Table 2). In order to investigate if production of IgLC occurred in local lymph nodes of cigarette smoke-exposed C57Bl/6 mice, cells from the thoracic lymph nodes (n=6 pooled) were cultured for 24 hours, and supernatants were analyzed for presence of IgLC. Samples fractionated by SDS-PAGE under non-reducing conditions showed complete immunoglobulins (about 180-200 kDa), IgLC dimers (about 45 kDa) and IgLC monomers (20 kDa). The TLN cells of cigarette smoke-exposed mice produced higher levels of IgLC compared to TLN cells of control animals (Fig. 1B and 1C). Increases in IgLC were most pronounced after 12 weeks smoke exposure.

Table 2. *Lung emphysema characteristics in the different emphysema models*

Animal model	Groups	Mean linear intercept (μm)	Heart hypertrophy
Nose-only exposure	Control	38.9 ± 0.5	0.18 ± 0.005
	Smoke	40.1 ± 0.4	0.21 ± 0.004 ***
Whole body exposure	Control	42.5 ± 0.8	0.21± 0.010
	Smoke	52.6 ± 1.7 **	0.27 ± 0.010 ***
	Smoke cessation	49.6 ± 1.4 **	0.26 ± 0.008***
CSE administration	Control	38.7 ± 0.1	0.17 ± 0.010
	CSE	41.9 ± 0.5 **	0.21 ± 0.007 **

*Mean linear intercept values and heart hypertrophy measurement ((weight of the right ventricle)/(weight of the left ventricle + septum)) of mice nose-only or whole body exposed to air or cigarette smoke or after smoking cessation and of PBS- and CSE-treated mice. n = 4-6 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01, ***P ≤ 0.001; significantly different from the control group.*

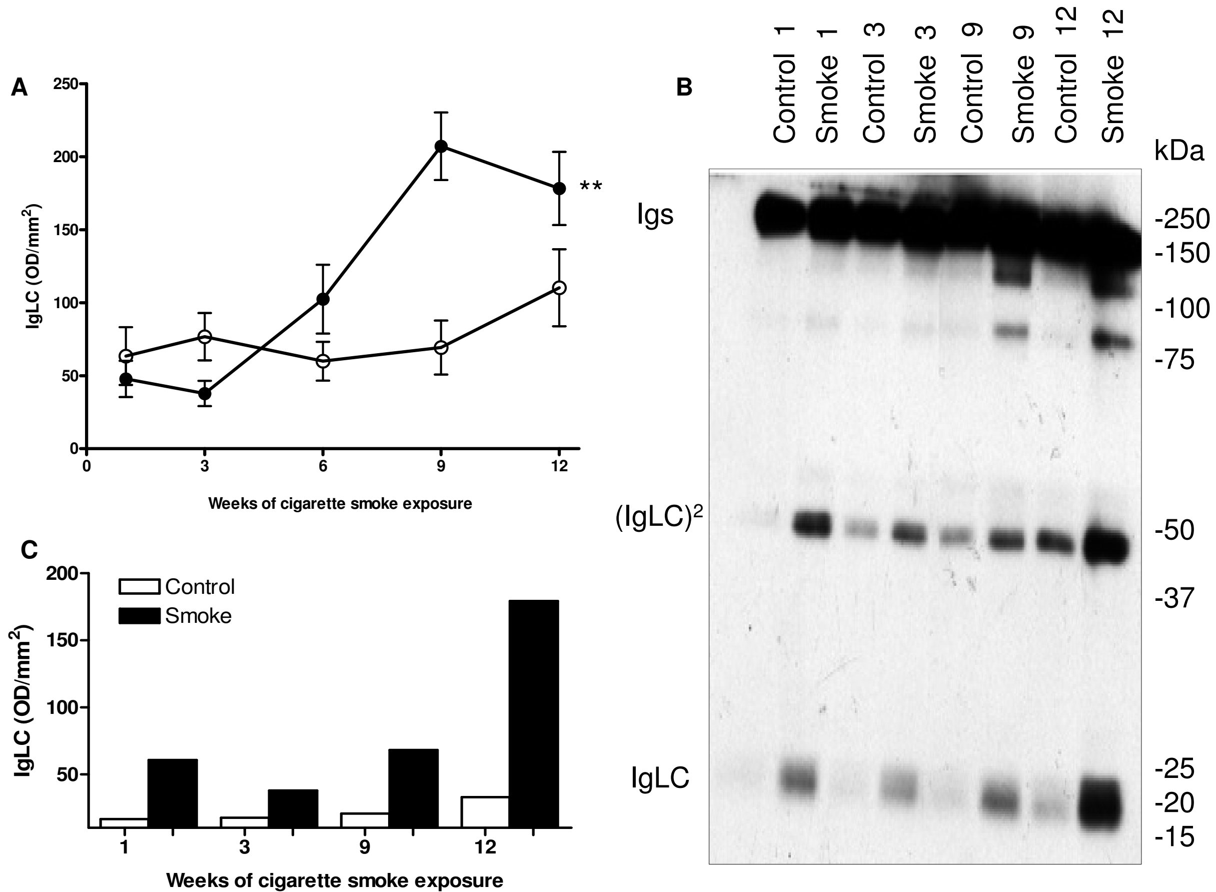

Fig.1. *Increased IgLC levels in serum and supernatant of thoracic lymph node cells after nose-only cigarette smoke exposure. Densitometric analysis after Western Blotting of IgLC in serum of C57Bl/6 mice after 1, 3, 6, 9 and 12 weeks nose-only cigarette smoke exposure (closed symbols) compared to air-exposed mice (open symbols) (A). n = 4-6 animals per group. Values are expressed as mean (OD/mm^2) ± S.E.M. **P ≤ 0.01; significantly different from the control group. Representative Western Blot of IgLC levels in supernatants of thoracic lymph node (TLN) cells of C57Bl/6 mice after 1, 3, 9 and 12 weeks nose-only cigarette smoke exposure compared to air-exposed mice (B). Igs, total immunoglobulins (IgA, IgD, IgE, IgG, and IgM); IgLC^2, IgLC dimers; IgLC, monomers. Quantification of the TLN supernatant samples of the air-exposed mice (n=6 pooled, white bars) and the smoke-exposed mice (n=6 pooled, black bars) used for Western blotting via densitometric analysis (C). Values are expressed as OD/mm^2.*

Increased IgLC levels in serum after whole body cigarette smoke exposure

In the next experiments, IgLC production was monitored in a different model for experimental emphysema. A/J mice were whole body exposed to cigarette smoke for 1, 4 and 20 weeks. The IgLC concentrations in serum of cigarette smoke-exposed mice were increased compared to the air-exposed mice at all time points (1, 4 and 20 weeks) (Fig. 2A, B, C). After

smoking cessation for 8 weeks, the IgLC serum levels were significantly decreased compared to the smoke-exposed mice. The mean linear intercept was measured to quantify the presence and severity of lung emphysema. Air space enlargement was observed in the mice after 20 weeks cigarette smoke exposure compared to the air-exposed mice (Table 2). The increased Lm was not reduced after a period of 8 weeks without cigarette smoke exposure demonstrating irreversible lung damage. Furthermore, lung emphysema was accompanied by right ventricle heart hypertrophy, since twenty weeks of whole body cigarette smoke exposure caused right ventricle heart hypertrophy. The right ventricular (RV) mass was proportionally greater than the rest of the lower heart (left ventricle and septum, LV+S) in smoke-exposed mice compared to air-exposed mice (Table 2). The heart hypertrophy ratio (RV/(LV +S)) was not significantly decreased in the smoking cessation group compared to smoke-exposed group.

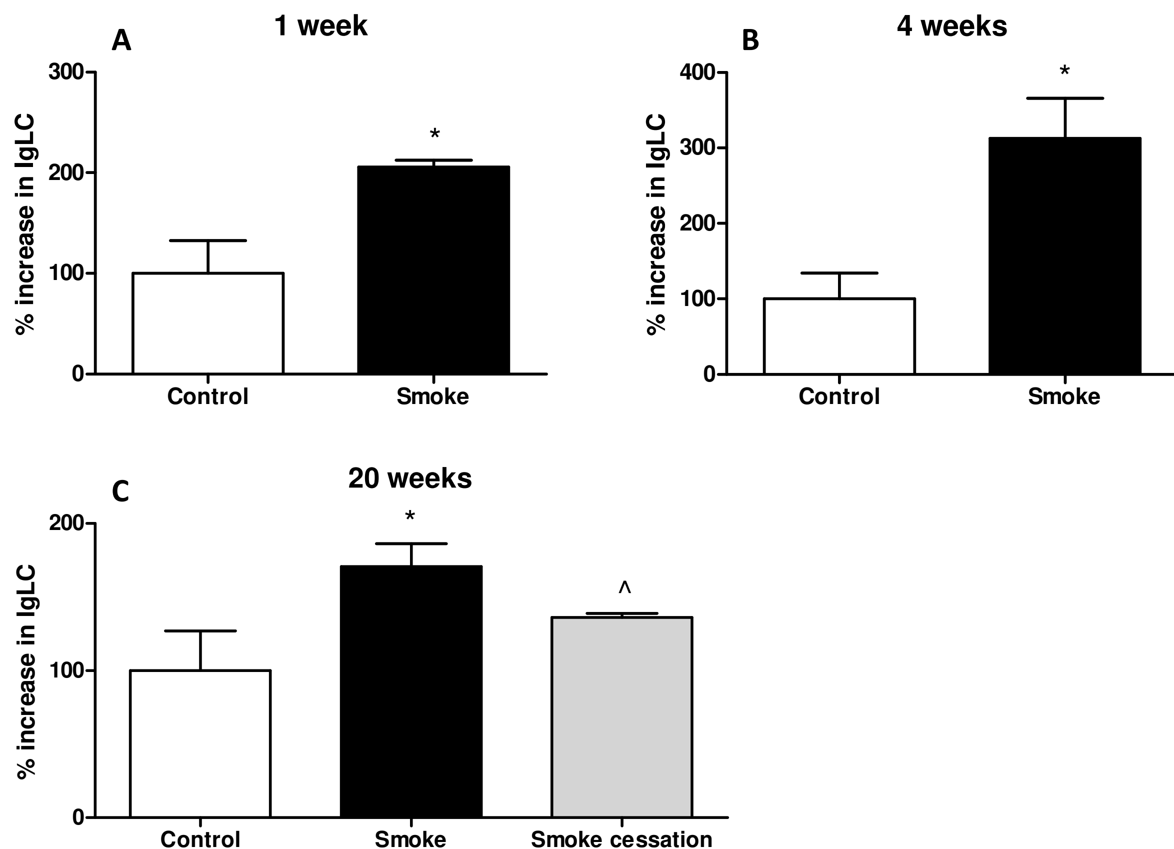

Fig.2. *Increased IgLC levels in serum after whole body cigarette smoke exposure. Densitometric analysis after Western Blotting of IgLC in serum of A/J mice after whole body cigarette smoke exposure (black bars) for 1 week (A), 4 weeks (B), 20 weeks and 20 weeks plus 8 weeks smoking cessation (gray bar) (C), all compared to air-exposed mice (white bars). n = 4-6 animals per group. Values are expressed as percentage increase in IgLC (OD/mm^2) ± S.E.M. * P ≤ 0.05; significantly different from the control group. ^ P ≤ 0.05; significantly different from the smoke group.*

Increased IgLC levels in serum of mice intraperitoneally and intratracheally injected with cigarette smoke extract

Previously, it has been shown that injection of cigarette smoke extract (CSE), containing all water soluble components of cigarette smoke, caused lung emphysema in rats [34] and we explored if IgLC production was also increased under these circumstances in mice. At day 1 C57Bl/6 mice were i.p. injected with CSE plus an adjuvant followed by an i.t. administration at day 11 and at day 21, serum IgLC concentrations in CSE-treated mice were significantly enhanced compared to control mice (Fig. 3). Characteristics of lung emphysema, alveolar enlargement and right ventricle heart hypertrophy were measured to quantify the presence and severity of emphysema. An increased Lm was measured in the CSE-treated mice compared to the control mice (Table 2). Furthermore, the heart hypertrophy ratio was significantly increased in the CSE-treated mice compared to control mice (Table 2).

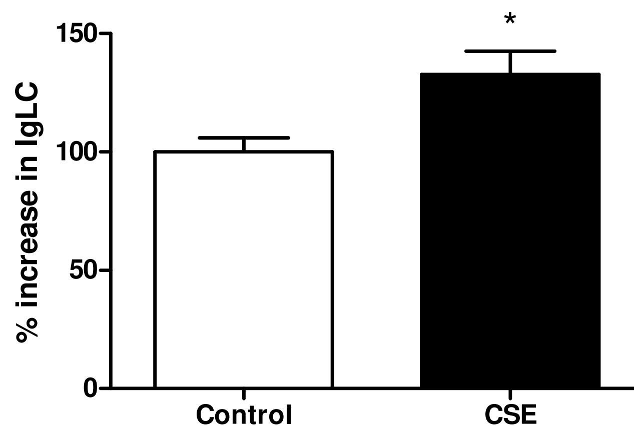

Fig.3. *Increased IgLC levels in serum after CSE administration. Densitometric analysis after Western Blotting of IgLC in serum of C57Bl/6 mice after an i.p. injection of CSE + adjuvant at day 1 followed by an i.t. administration of CSE at day 11 (black bar). Mice were sacrificed at day 21 and compared to PBS-treated mice (white bar). n = 5 animals per group. Values are expressed as percentage increase in IgLC (OD/mm^2) ± S.E.M. *$P \leq 0.05$; significantly different from the control group.*

IgLC in serum and lung tissue specimens of COPD patients

To investigate if IgLC are also involved in the pathogenesis of human emphysema, IgLC levels were examined in serum samples obtained from COPD patients and healthy controls. As depicted in Fig 4A, the IgLC serum levels of COPD patients were significantly increased compared to the healthy controls. Immunohistochemical staining with antibodies specific for κ and λ free light chain was performed to analyze the localization of IgLC in lung tissue specimens of patients with COPD. Fig. 4 demonstrates representative photomicrographs of κIgLC (B) and λIgLC (C and D) staining in lung tissue of COPD patients. Positive IgLC staining was observed around the larger airways and within follicles.

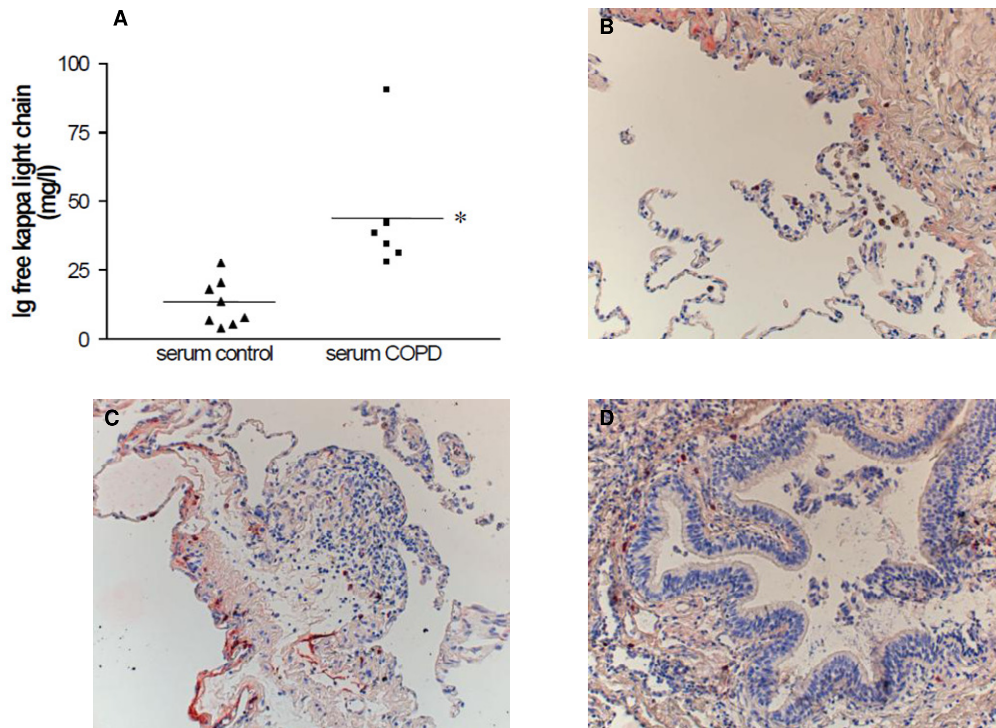

Fig.4. *IgLC in serum and lung tissue of COPD patients. IgLC levels in serum of healthy control subjects (▲, n = 8) and patients with COPD (■, n = 6) were analyzed using a specific ELISA (A). *P < 0.05; significantly different from the healthy controls. Representative photomicrographs of an immunohistological staining for IgLC (red color, AEC staining) in human lung tissue of patients suffering from COPD. Magnification, 200x. The presence of both kappa (B) and lambda (C and D) IgLC was demonstrated around the larger airways and within follicles.*

IgLC bind to PMNs and cross-linking bound IgLC induces CXCL8 production
Since neutrophils are key players in the development of COPD and previous studies showed that IgLC could influence neutrophil activity [11], we first analyzed whether IgLC could bind to human PMNs. FITC-labeled IgLC showed extensive binding to PMNs determined via FACS analysis (Fig. 5A). In addition, the immunofluorescent analysis showed focal binding of IgLC on the cell membrane of PMNs (Fig. 5B). To determine whether cross-linking bound IgLC induced activation of PMNs, PMNs were incubated in IgLC-coated wells to mimic crosslinking. PMN activation was monitored by CXCL8 release. CXCL8 release by PMNs was significantly increased in the wells coated with κIgLCs compared to the non-coated wells (Fig. 5C). A dose dependent increase of CXCL8 production by PMNs was observed after incubation with 10, 30 and 100 μg/ml κIgLC. The highest levels for CXCL8 were detected after incubation with 30-100 μg/ml κIgLC. As a positive control, PMNs were incubated with 1 μg/ml LPS. In addition, incubating PMNs with free floating κIgLC (100 μg/ml) did not result in an increased release of CXCL8 (data not shown).

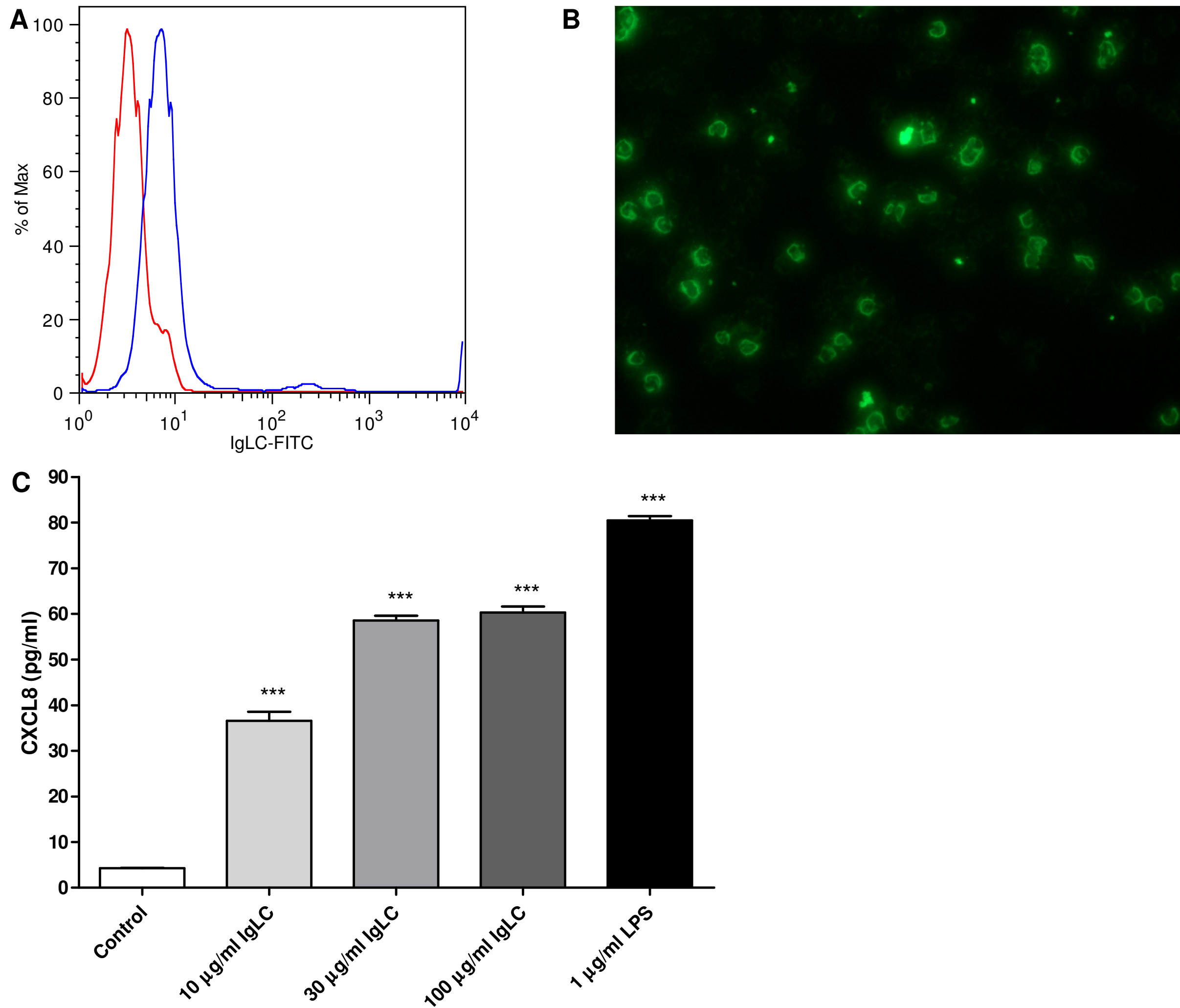

Fig.5. *Binding of IgLC to human PMNs and cross-linking IgLC induce CXCL8 production. Freshly isolated human PMNs were incubated with FITC labeled κIgLC. Subsequent flow cytometric analysis of the live cell population showed significant binding of IgLC to PMNs (blue line) compared to unstained cells (red line) (A). Representative photomicrograph of an immunofluorescent staining on cytospin preparations of PMNs incubated with FITC labelled κIgLC. IgLC binding was detected along the cell membrane of PMNs. Magnification, 400x (B). This experiment was repeated in two different donors with similar results. CXCL8 production of freshly isolated human PMNs incubated in 96 well-plates coated with medium (control, white bars), κIgLC (10 µg/ml, 30 µg/ml, 100 µg/ml, gray bars) for 4h (C). As a positive control LPS (1 µg/ml, black bars) was added to the PMNs and also incubated for 4h. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group. This experiment was repeated in two different donors with similar results.*

Cigarette smoke-induced neutrophil influx in the BALF is significantly decreased after F991 administration

To investigate whether IgLC in the airways were of functional importance in COPD, we explored whether antagonizing its biological action with a specific IgLC antagonist, F991, affected cigarette smoke-induced neutrophil migration and activation in the airways. When mice were exposed for 5 days to cigarette smoke, a significant increase of BALF neutrophils was observed compared to the air-exposed mice. The cigarette smoke-induced neutrophil influx in the BALF was significantly decreased after F991 administration (Fig. 6). The TLNs of cigarette smoke-exposed mice produced higher levels of IgLC compared to control animals (IgLC levels; control: 166.51 OD/mm^2 versus smoke: 245.43 OD/mm^2). After F991 administration *in vivo*, the IgLC levels produced by TLNs *ex vivo* were reduced to normal level (IgLC levels; 171.96 OD/mm^2). Furthermore, we observed significantly increased KC, the murine CXCL8 homologue, levels in the BALF of smoke-exposed mice compared to air-exposed (KC levels; control: 44.5 ± 7.3 pg/ml versus smoke: 191.1 ± 18.2 pg/ml, P ≤ 0.001), while after F991 administration the cigarette smoke-induced KC levels were decreased (169.5 ± 17.5 pg/ml KC).

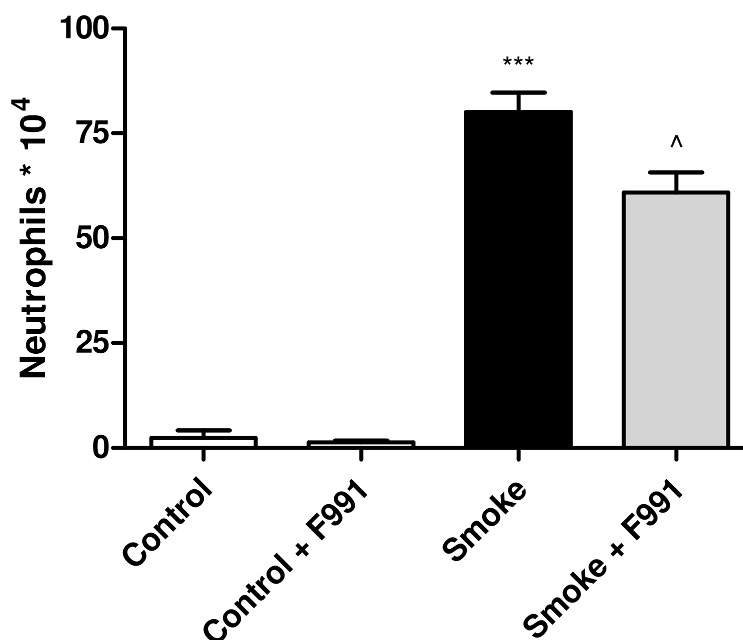

Fig.6. *Cigarette smoke-induced neutrophil influx in the BALF was significantly decreased after F991 administration. Absolute neutrophil numbers in the BALF of mice exposed to air (white bar), F991-treated mice exposed to air (light gray bar), mice exposed to cigarette smoke (black bar) and F991-treated mice exposed to cigarette smoke (gray bar) for 5 days. The mice received vehicle (PBS) or F991 by oropharyngeal aspiration (100µg F991 / 70µl sterile PBS) twice daily. n = 4-6 animals per group. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group. ^P ≤ 0.05; significantly different from the smoke group.*

Discussion

In this study, we describe a possible role of increased IgLC production in the development of COPD. First, IgLC serum levels were enhanced in cigarette smoke-exposed mice and CSE-treated mice compared to control animals. Additionally, local lymph nodes of smoke-exposed mice showed enhanced production of IgLC *ex vivo* compared to air-exposed mice. Furthermore, the concentration of IgLC is significantly higher in sera of COPD patients than healthy controls and positive IgLC staining was found in lung tissue of COPD patients. To date, little is known about the role of immunoglobulins in COPD. The presence of B cells has been demonstrated in human and in murine lung tissue in association with COPD [35-37]. Increased IgG and IgA levels were observed in serum from COPD patients [38] as well as higher levels of IgA positive cells in blood [39]. A recent study by Singh et al. [40] suggests that IgE serum levels correlate with clinical aspects of disease severity in COPD and that production of IgE in the airways of COPD patients may be related to smoking. Additionally, COPD is associated with a higher prevalence of antinuclear autoantibodies [41]. Nevertheless, the biological role of these immunoglobulins in the development of COPD remains unclear. To our knowledge, IgLC has never been investigated in the pathogenesis of COPD. Increased IgLC serum or plasma levels have been assessed in other (auto)immune/inflammatory diseases [16, 17, 42-46]. Rises in IgLC levels appear to be associated with relapses of disease, enhanced activity of the immune system, disease progression and severity and this may indicate increased polyclonal B cell activity, hypersecretion of (auto)antibodies or increased local synthesis [18]. Many of these inflammatory diseases are also associated with increased numbers of activated PMNs. Since PMNs are one of the major inflammatory cell types implicated in COPD, and it has been shown that whole immunoglobulins can induce CXCL8 production by PMNs, we investigated the relation between IgLC and neutrophils in COPD.

We observed that IgLC can bind to the cell membrane of human PMNs. The fluorescent signal suggests a patchy distribution of IgLC-binding proteins on the neutrophil membrane. Such clustered distribution has also been demonstrated previously for mast cells [26]. In earlier studies we described that crosslinking of receptor-bound IgLC by an antigen leads to mast cell degranulation, release of pro-inflammatory mediators and the induction of a local inflammatory response [26]. The current study provides evidence that IgLC may also act on neutrophils and *in vitro* studies demonstrate that cross-linking of IgLC on neutrophils resulted in an increase of the inflammatory mediator CXCL8. In COPD, CXCL8 is a prominent and selective attractant of neutrophils. CXCL8 levels in sputum from COPD patients correlate with the extent of neutrophilic inflammation and disease severity [4, 6, 47]. Interestingly,

Gounni et al. [9], showed that cross-linking of the high-affinity IgE receptor on human neutrophils [10] leads to the release of CXCL8. Furthermore, *in vitro* IgE alone (without cross-linking) can delay programmed cell death of neutrophils [10]. Our study suggests that the presence of IgLC could be responsible for a sustained neutrophilic inflammation as observed in COPD [37, 48]. Moreover, it is described that IgLC may contribute to a pre-stimulation of PMNs, since IgLC increase the basal levels of neutrophil oxidative metabolism [11, 49] and interfere with essential PMN functions, such as the activation of glucose uptake and chemotaxis [11, 49, 50]. Cohen et al. [11] demonstrated that IgLC increase the percentage of viable PMNs, by reducing the PMN apoptosis, thereby inducing increased PMN survival. It was further investigated that tyrosine phosphorylation was essential for this IgLC-induced increase of PMNs survival [11, 51-53]. These data are in line with our results and suggest the presence of a possible IgLC receptor on the cell membrane of the neutrophil. To date, no receptor for IgLC proteins has been characterized and the identity of this protein is currently under investigation.

IgE levels are increased in COPD patients [9-11, 40] and we now also demonstrate increased IgLC levels. This is of interest, since both IgE and IgLC can exert similar pro-inflammatory effects via PMNs. This remarkable common functionality leads to the speculation whether these immunoglobulins and IgLC play a crucial role in the onset and development of neutrophil-related diseases, such as COPD. It is yet unknown which antigen(s) in cigarette smoke or in the airways may be responsible for PMN activation through either IgE or IgLC.

An important finding of our study is that intra-airway treatment with the antagonist of IgLC, F991, reduces the cigarette smoke-induced neutrophil influx and activation in the BALF of mice. This means that IgLC in the smoke-exposed mice plays a role in the smoke-induced neutrophil influx and activation in the BALF. A possible explanation for this could be that F991 inhibits the IgLC-induced decrease in PMN apoptosis [11] and inhibits the possible IgLC-mediated binding and cross linking-induced activation of neutrophils. Earlier studies showed that F991 effectively inhibits the development of non-atopic asthma in mice [17] by inhibition of IgLC-induced mast cell activation, but it cannot be excluded that F991 may also have additional inhibitory effects on the associated neutrophil influx in this model.

In conclusion, our studies demonstrate production of IgLC after cigarette smoke exposure in various experimental models of COPD. This IgLC phenomenon is also observed in sera from COPD patients and in lung tissue of COPD patients. Antagonizing the action of IgLC reduces the cigarette smoke-induced neutrophil influx and activation in murine lungs. IgLC were shown to bind to and activate PMNs. This can serve as an attractive therapeutic target and may open new avenues towards effective treatment of COPD.

Acknowledgements

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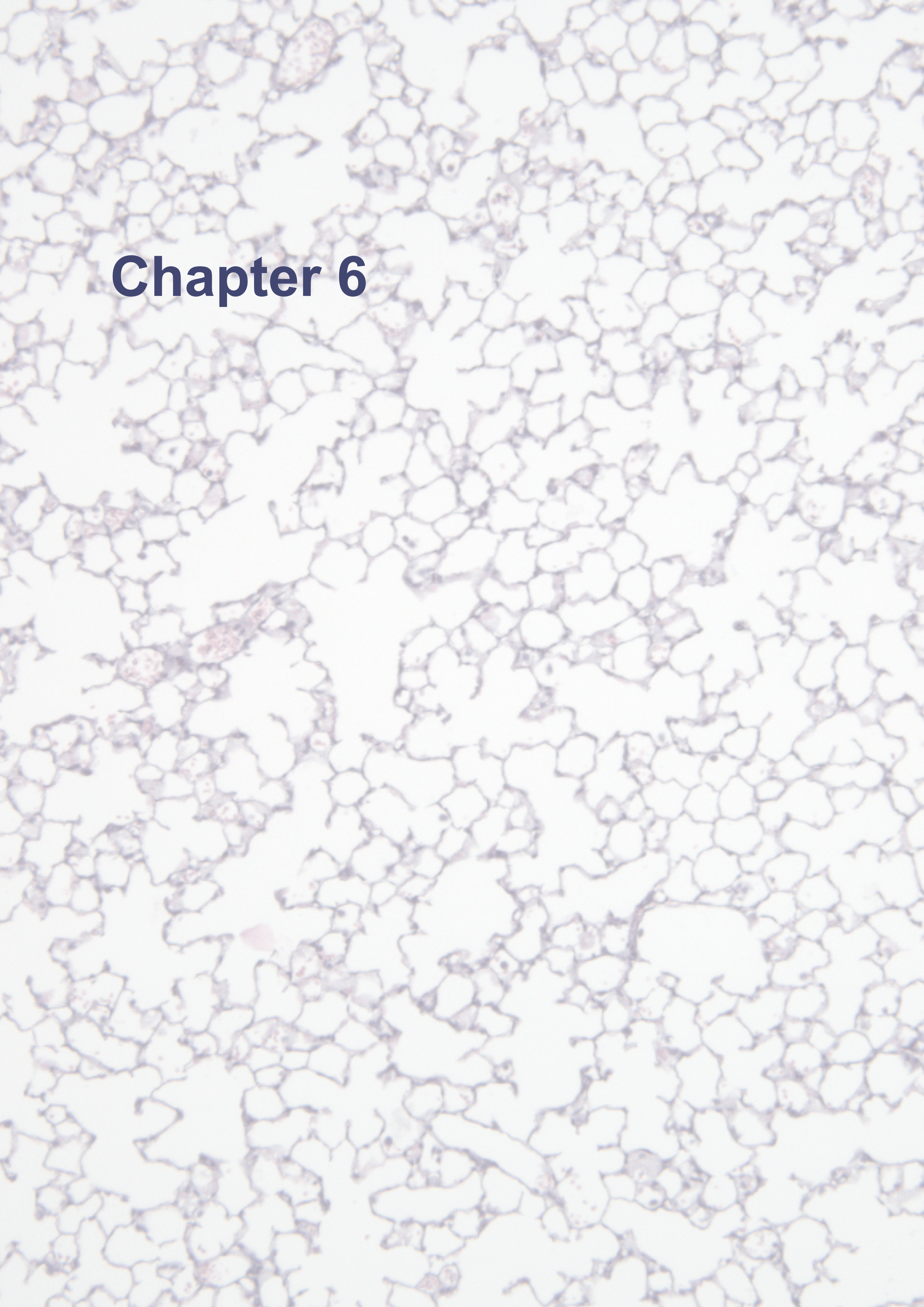

Chapter 6

Cigarette smoke-induced lung emphysema in mice is associated with prolyl endopeptidase, an enzyme involved in collagen breakdown

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(additional data included)

Abstract

There is increasing evidence that the neutrophil chemoattractant proline-glycine-proline (PGP), derived from the breakdown of the extracellular matrix, plays an important role in neutrophil recruitment to the lung. PGP formation is a multistep process involving neutrophils, metalloproteinases (MMPs) and prolyl endopeptidase (PE). This cascade of events is now investigated in the development of lung emphysema.

A/J mice were whole body exposed to cigarette smoke for 20 weeks. After 20 weeks or 8 weeks after smoking cessation animals were killed and bronchoalveolar lavage fluid (BALF) and lung tissue were collected to analyze the neutrophilic airway inflammation, the MMP-8 and MMP-9 levels, the PE activity and the PGP levels. Lung tissue degradation was assessed by measuring the mean linear intercept. Moreover, we investigated the effect of the peptide, L-arginine-threonine-arginine (RTR), that binds to PGP sequences and the effect of the PE inhibitor, valproic acid (VPA), on the smoke-induced neutrophil influx in the lung after 5 days smoke exposure. Additionally, an immunohistological staining for PE was performed on lung tissue specimens of current smokers, ex-smokers and COPD patients.

Neutrophilic airway inflammation was induced by cigarette smoke exposure. MMP-8 and MMP-9 levels, PE activity and PGP levels were elevated in the lungs of cigarette smoke-exposed mice. PE was highly expressed in epithelial and inflammatory cells (macrophages and neutrophils) in lung tissue of cigarette smoke-exposed mice and in lung tissue specimens of current smokers and COPD patients. After smoking cessation, the neutrophil influx, the MMP-8 and MMP-9 levels, the PE activity and the PGP levels were decreased or reduced to normal levels. Moreover, RTR and VPA inhibited the smoke-induced neutrophil influx in the lung after 5 days smoke exposure.

In the present murine model of cigarette smoke-induced lung emphysema, it is demonstrated for the first time that all relevant components (neutrophils, MMP-8, MMP-9, PE) involved in PGP formation from collagen, are upregulated in the airways. Together with MMPs, PE may play an important role in the formation of PGP and thus in the pathophysiology of lung emphysema.

Introduction

Chronic obstructive pulmonary disease (COPD) is a term referring to two separate chronic lung disorders: emphysema and chronic bronchitis [1]. The pathogenesis of COPD is complex and multifactorial, where neutrophilic airway inflammation and protease–antiprotease imbalance play a pivotal role [2, 3]. The inflammatory response of the lungs to noxious particles and gases, predominantly characterized by increased neutrophil numbers, contributes to the progressive airflow limitation [4]. Besides neutrophils, macrophages and CD8+ T lymphocytes have also been implicated in the development and progression of COPD [5-9]. Since tobacco smoking is the major risk factor in the development of COPD [10], the pathogenesis of COPD is strongly linked to the effects of cigarette smoke on the lungs. The general hypothesis on COPD states that cigarette smoke initiates an inflammatory immune response, characterized by a cascade of events that culminate in alveolar wall destruction, a characteristic of lung emphysema. First, cigarette smoke can act on airway epithelial cells and alveolar macrophages to release several inflammatory mediators and chemoattractants, such as tumor necrosis factor (TNF)-α and interleukin-8 (IL-8, CXCL-8) [11]. Subsequently, the chemoattractants facilitate the migration of neutrophils and other cell types, like CD8+ T cells, to the site of inflammation [12]. As a consequence, the activated neutrophils and macrophages release a variety of proteolytic enzymes, like matrix metalloproteinases (MMPs), finally resulting in degradation of the lung tissue [13, 14]. It is proposed that the increased protease activity leads not only to the lung matrix breakdown, but also to the generation of the tripeptide proline-glycine-proline (PGP) from collagen [15, 16]. PGP is chemotactic for neutrophils *in vivo* as well as *in vitro* [17, 18] and can also stimulate neutrophils to release CXCL8 [19]. At this point, both CXCL8 and PGP might be involved in the continuous recruitment and activation of neutrophils in the airways, which will lead to an excessive release of proteases and an ongoing PGP formation. This process finally results in a chronic airway inflammation with tissue destruction and remodeling [16]. Furthermore, it has been shown that airway exposure to PGP can induce lung emphysema in mice as indicated by alveolar enlargement and right ventricle heart hypertrophy [17, 20]. Clinical data demonstrated that PGP was detected in the BALF and sputum of COPD patients, but not in asthmatics or controls [17, 21]. PGP generation is a multistep process involving members of the MMP family, MMP-9, and/or MMP-8, and the serine protease family, prolyl endopeptidase (PE) [22]. MMP-8 and MMP-9, proteolytically cleave collagen to smaller fragments and creates an optimal substrate for PE. These collagen fragments are then further cleaved to PGP by PE. To our knowledge, PE is the only enzyme directly capable of cleaving PGP from shorter portions of collagen [23, 24].

The aims of this study were to investigate whether the different components involved in the proteolytic cascade generating the chemoattractant PGP were indeed present in the lungs of mice chronically exposed to cigarette smoke with emphasis on the PE and PGP levels in the lung and we were interested in the effect of smoking cessation on the different components. First, we determined whether neutrophils were present in the airways of mice after cigarette smoke exposure. Secondly, MMP-8 and MMP-9 levels, necessary to cleave collagen into smaller fragments for PE, were measured in the lung. PE activity in murine airways was examined to confirm that the necessary enzyme for PGP generation was present. Additionally, PE expression in lung tissue specimens of current smokers, ex-smokers and COPD patients was determined. Furthermore, the presence of PGP was measured in the BALF of mice exposed to cigarette smoke. Finally, we investigated the effect of the peptide, L-arginine-threonine-arginine (RTR), that binds to PGP sequences and the effect of the PE inhibitor, valproic acid (VPA), on the smoke-induced neutrophil influx in the murine lung.

Material and methods

Patient characteristics

Patient characteristics of the COPD patients included in the study are presented in Table 1.

Table 1. *Patient characteristics*

	CURRENT SMOKER	**EX-SMOKER**	**COPD PATIENT GOLD STAGE II**	**COPD PATIENT GOLD STAGE IV**
Sex (m/f, n)	0/6	0/6	5/3	0/6
Age (yrs)	58 ± 9	59 ± 8	67 ± 9	54 ± 5
Current smoker / not current smoker (n/n)	6/0	0/6	4/4	0/6
Packyears (yrs)	30 ± 15	31 ± 13	38 ± 17	34 ± 13
FEV_1, % predicted	94.2 ± 9.5	88.2 ± 5.0	65.6 ± 10.0	19.5 ± 5.8
FEV_1/FVC, %	78.4 ± 7.4	71.9 ± 9.0	58.7 ± 8.5	35.6 ± 14.7

Data are presented as mean ± standard deviation. FEV_1: Forced Expiratory Volume in one second. FVC: Forced Vital Capacity. The FEV_1 of the current smoker and ex-smoker is based on pre-bronchodilator values and the FEV_1 of the COPD patients is based on post-bronchodilator values.

Lung tissue specimens

Tissue from the current smokers and ex-smokers was derived from noninvolved lung tissue from patients undergoing resective surgery for pulmonary carcinoma. Patients had no airway obstruction and no chronic airway symptoms, such as cough and sputum production. Material was always taken from as far away as possible from the tumor, or from a noninvolved lobe. No histopathological lesions were present.

Tissue of GOLD stage II COPD patients was derived from noninvolved lung tissue from patients undergoing resective surgery for pulmonary carcinoma. Tissue was always taken as far away as possible from the tumor, or from a noninvolved lobe. Histopathologically emphysematous lesions were present, however, of limited but varying severity. The moderate forms can be histopathologically demonstrated by the finding of isolated or free-lying segments of viable alveolar septal tissue or isolated cross sections of pulmonary vessels.

Tissue of GOLD stage IV COPD patients was obtained from patients with COPD undergoing surgery for lung transplantation or lung volume reduction. All individuals had to quit smoking for at least 1 yr before surgery. The resected tissue showed both macroscopically and microscopically severe emphysematous lesions, often accompanied by bullae. Subpleural, fibrous areas were avoided. The study protocol was consistent with national ethical and professional guidelines ("Code of Conduct; Dutch Federation of Biomedical Scientific Societies"; http://www.federa.org/?s=1&m=68).
Immunohistochemistry was performed on 3-4 µm formalin-fixed, paraffin-embedded lung tissue.

Animals

Female A/J mice, 9-14 weeks old and male Balb/c mice, 5-6 weeks old (Charles River Laboratories) were housed under controlled conditions in standard laboratory cages. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the local Ethics Committee and were performed under strict governmental and international guidelines on animal experimentation.

Chronic cigarette smoke exposure

Female A/J mice were divided into three groups. The first group was exposed to room air for 20 weeks, the second group was exposed to cigarette smoke for 20 weeks and the third group was exposed to cigarette smoke for 20 weeks followed by a period of 8 weeks without cigarette smoke exposure. The mice were exposed in whole-body chambers to air (sham) or to diluted mainstream cigarette smoke from the reference cigarettes 2R4F (University of Kentucky, Lexington, Kentucky) using a smoking apparatus [25]. Exposures were conducted 4h/day (with a 30/60-minute fresh air break after each hour of exposure), 5 days/week for 20 weeks to a target cigarette smoke concentration of 750 µg total particulate matter/l (TPM/l). This TPM concentration was reached after an adaptation period of 2 weeks, starting with a TPM concentration of 125 µg TPM/l. The mass concentration of cigarette smoke TPM was determined by gravimetric analysis of Cambridge filter samples. The carbon monoxide (CO) was monitored continuously and was around 800 ppm. The nicotine concentration in the smoke was approximately 40 µg/l. The sample sites were located in the middle of the exposure chamber at the breathing zone. The carboxyhemoglobin level measured via blood gas analysis was determined as 50% on average in the smoke-exposed mice. The mice were killed 16-24 hours after the last air or smoke exposure, or after the smoke-free period of 8 weeks. A/J mice were used in the present chronic COPD model, since this strain is

characterized as moderately susceptible to the development of lung emphysema and to the lung inflammatory response after acute cigarette smoke exposure [26, 27].

1-week cigarette smoke exposure

To confirm a smoke-related effect in another strain, Balb/c mice were exposed in whole-body chambers to air (sham) or to diluted mainstream cigarette smoke from the reference cigarettes 2R4F using a peristaltic pump. Just before the experiments, filters were cut from the cigarettes. Each cigarette was smoked in 5 minutes at a rate of 5 l/h in a ratio with 60 l/h air. The mice were exposed to cigarette smoke using 5 cigarettes twice daily for five consecutive days, except for the first day when they were exposed to 3 cigarettes. The mice were killed 16 hours after the last air or smoke exposure.

L-arginine-threonine-arginine (RTR) and valproic acid (VPA) administration

During 1-week cigarette smoke exposure, the mice received vehicle (50 µl or 70 µl sterile PBS), RTR (50 µg RTR (Anaspec) / 50 µl sterile PBS) or VPA (100 µg VPA (Sigma-Aldrich) / 70µl sterile PBS) by oropharyngeal aspiration under light isoflurane anesthesia twice daily before and after smoke exposure [28].

Bronchoalveolar lavage

Immediately after i.p. injection with an overdose of pentobarbital, the lungs of a separate groups of mice (n=4-5) were lavaged 4 times through a tracheal cannula with 1 ml saline (NaCl 0.9%), pre-warmed at 37 °C. After centrifuging the bronchoalveolar lavage fluid at 4°C (400 g, 5 min), the supernatant of the first ml was used for MMP-8, MMP-9 and PGP analysis and the cell pellets of the 4 lavages were used for cell counts. The 4 cell pellets, kept on ice, were pooled per animal and resuspended in 150 µl cold saline.

Neutrophil accumulation in BALF

After staining with Türk solution, total cell counts per lung were made under light microscopy using a Burker-Turk chamber. Differential cell counts were performed on air-dried cytospin preparations stained by DiffQuick™ (Dade A.G., Düdingen, Switzerland). Cells were identified as macrophages, neutrophils and lymphocytes according to standard morphology. At least 200 cells were counted and the absolute number of neutrophils was calculated.

Preparation of lung homogenates

In brief, lung samples were homogenized in a potter glass tube with a Teflon pestle in 1 ml ice cold PBS. Homogenates were centrifuged at 14,000 g for 5 min and supernatants were collected. The protein concentration of each sample was assayed using the Pierce BCA protein assay kit standardized to BSA, according to the manufacturer's protocol (Thermo Fisher Scientific, Rockford, IL, USA). The homogenates were diluted to a final concentration of 2 µg protein/µl.

Myeloperoxidase (MPO) activity in lung homogenates

Lung homogenates were assessed biochemically for the neutrophil marker enzyme MPO according to a previously reported method [29]. 50 µl lung homogenate (2 µg protein/µl)was incubated with 50 µl 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 5.5) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) for 30 min at room temperature. The enzymatic reaction was started by mixing the sample (100 µl) with 150 µl of 50 mM phosphate buffer (pH 5.5) containing 0.26 mg/ml o-dianisidine dihydrochloride and 0.52 mM 30% hydrogen peroxide (37°C). The enzyme activities were determined spectrophotometrically. The changes in absorbance were measured at 450 nm over 20 minutes with an iMark Microplate absorbance reader (Bio-Rad). The reaction was standardized by a series of pooled human neutrophils and MPO activity was expressed in arbitrary units.

MMP-8 and MMP-9 ELISA

Total MMP-9 (pro- and active MMP-9) and pro-MMP-9 levels were measured in BALF and lung homogenates by sandwich ELISA using the Quantikine mouse total and pro–MMP-9 ELISA kit (R&D systems) according to manufacturer's instructions. Active MMP-9 levels were calculated by subtracting the pro-MMP-9 levels from the total MMP-9 levels. Total MMP-8 levels were measured in BALF by ELISA using the mouse MMP-8 ELISA kit (Cusabio Biotech Co., Ltd) according to manufacturer's instructions.

Gelatin zymography

Presence of active and latent forms of MMP-9 was analysed by zymography on 11% polyacrylamide gels containing 1% gelatin (Sigma Aldrich) as previously described [30]. Samples were diluted in nonreducing sample buffer (0.5 M Tris-HCl, pH 6.8, 8% sucrose, 20% sodium dodecylsulphate (SDS) and 0.05% bromophenol blue). Samples volumes were adjusted to obtain a uniform protein content of 15 µg per sample and 10 µl sample was added to each lane. Gels were electrophoresed at 20 mA at 4°C until the bromophenol blue

stained front reached the bottom of the gel. After running the gels were washed twice in 2.5% Triton X-100 for 15 min at room temperature to remove the SDS, followed by two washes of 5 min in 50 mM Tris/HCl, pH 8.0, containing 5 mM $CaCl_2$, 10 µM $ZnCl_2$ and incubated overnight in the same buffer at 37℃. The gels were stained for 1 h with 0.5% Coomassie Brilliant Blue R-250 dissolved in 50% methanol and 5% acetic acid and subsequently destained 2 h. Proteolytic activities were visualised by clear zones against a dark blue background indicating lysis of gelatin.

MMP-8 Western Blot analysis

Equal amounts (20µl) of boiled BALF samples were subjected to 11% SDS–PAGE under reducing conditions and blotted to nitrocellulose membranes (Whatman, Dassel, Germany). Blots were blocked with PBS/0.1% Tween 20 (PBST) containing 5% milk proteins (Laboratories, Hercules, CA, USA) for 1 h at room temperature and afterwards incubated overnight at 4 ℃ with primary antibody (rabbit anti-MMP-8 polyclonal antibody, 1:500, Lifespan Biosciences) in PBST containing 2% milk proteins. After subsequent incubation with horse–radish peroxidase (HRP)-conjugated secondary antibody (Dakocytomation, Glostrup, Denmark, 1:1000) for 1 h in PBST containing 2% milk proteins, the antibodies were visualized with commercial ECL reagents (Amersham Biosciences, Roosendaal, The Netherlands), and exposed to photographic film. Films were scanned on a GS710 Calibrated Imaging Densitometer (Bio-Rad Laboratories) and the optical density (OD) of the immunoreactive bands was quantified.

PE activity assay

PE activity was measured in the lung homogenates using the fluorogenic substrate Z-Gly-Pro-AMC (Bachem, Inc.) as previously described [31]. 20 µl of the lung homogenate (1 µg/µl) was added to each well in a black 96-well-flat-bottom plate, followed by addition of 80 µl assay buffer (25mM Tris, 0.25M NaCl, pH 7.5, 2mM DTT) containing 100 µM substrate Z-Gly-Pro-AMC. The fluorescence from liberated AMC was monitored every 1 min over 60 min at 37 ℃ using a Fluostar reader at excitation wavelength of 355 nm and an emission wavelength of 460 nm. Fluorometric intensities observed were converted to pmol AMC released per minute using appropriate AMC standard curves. The enzyme activity is defined as pmol AMC released per minute at 37 ℃ per 20 µg protein.

Electrospray ionization liquid chromatography–MS/MS (ESI-LC/MS/MS) for PGP detection

PGP and N-Ac-PGP were measured using a MDS Sciex (Applied Biosystems, Foster City, CA) API-4000 spectrometer equipped with a Shimadzu HPLC (Columbia, MD). HPLC was done using a 2.0 x 150 mm Jupiter 4u Proteo column (Phenomenex, Torrance, CA) with buffer A: 0.1% HCOOH and buffer B: MeCN + 0.1% HCOOH: 0 min-0.5 min 5% buffer B/95% buffer A, then increased over 0.5-2.5 min to 100% buffer B/0% buffer A. Background was removed by flushing with 100% isopropanol / 0.1% formic acid. Positive electrospray mass transitions were at 270-70, 270-116 and 270-173 for PGP and 312-140 and 312-112 of N-Ac-PGP.

Histology and morphomethric analysis

Mice (n=4-5), used for morphometric analysis, were killed by an i.p. injection with an overdose of pentobarbital (Nembutal™, Ceva Santé Animale, Naaldwijk, The Netherlands). The lungs were fixed with a 10% formalin infusion through a tracheal cannula at a constant pressure of 25 cm H_2O [32]. The left lung was immersed in fresh fixative for at least 24h, after which it was embedded in paraffin. After paraffin embedding, 5 µm sections were cut and stained with hematoxylin/eosin (H&E) according to standard methods. Morphometric assessment of emphysema, included determination of the average inter-alveolar distance, was estimated by the mean linear intercept (Lm) analysis. The Lm was determined by light microscopy at a total magnification of 100x, whereby 24 random photomicroscopic images per left lung tissue section were evaluated by microscopic projection onto a reference grid. By dividing total grid length by the number of alveolar wall-grid line intersections, the Lm (in µm) was calculated [33].

Immunohistochemistry

Paraffin sections were deparaffinized, endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck, Darmstadt, Germany) in methanol for 30 min at room temperature and rehydrated in a graded ethanol series to PBS. For antigen retrieval, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave. The slides were cooled down to room temperature, rinsed with PBS (3x) and blocked with 5% goat serum (Dakocytomation, Glostrup, Denmark) in 1% bovine serum albumin in PBS for 30 min at room temperature. Sections were incubated with the primary antibody (rabbit-anti-PE, 6 ug/ml (murine lung tissue), 0.6 ug/ml (human lung tissue) PREP antibody, ProteinTech Group, USA) in 1% bovine serum albumin/PBS overnight at 4℃. The slides were rinsed with PBS (3x) and incubated with the biotinylated secondary antibody (goat-anti-rabbit, 1:200, Dakocytomation)

in 1% bovine serum albumin/PBS for 45 min at room temperature. The slides were rinsed with PBS (3x) and the biotinylated proteins were visualized by incubation with streptavidin–biotin complex/horseradish peroxidase (Vectastain Elite ABC, Vector Laboratories) for 45 min at room temperature, followed by 0.015% H_2O_2/0.05% diaminobenzidene (Sigma, Schneldorf, Germany)/0.05 M Tris–HCl (pH 7.6) for 10 min at room temperature. Sections were counterstained with Mayers' hematoxylin (Merck), dehydrated and mounted in Permount (Fisher Scientific). Negative controls without the primary antibody and normal rabbit IgG (AB-105-C, R&D systems) were included as controls. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera.

Statistical analysis

Experimental results are expressed as mean ± S.E.M. Differences between groups were statistically determined by an unpaired two-tailed Student's *t*-test using GraphPad Prism (Version 4.0). Pearson's correlation was used to compare the relationship between the different parameters. Calculations were made using SPSS version 16. Results were considered statistically significant when $P < 0.05$.

Results

Cigarette smoke exposure induces neutrophilic airway inflammation

In order to investigate whether chronic smoke exposure induces neutrophilic airway inflammation, the alveolar lumen (by BALF) and the lung tissue of air and smoke-exposed mice were analyzed. In cytospins, neutrophils were absent in the BALF of air-exposed mice (Fig. 1A), while the smoke-exposed mice developed a BALF neutrophilia (Fig. 1B). After the smoking cessation period of 8 weeks almost no neutrophils in the BALF were detected (Fig. 1C). Quantification of neutrophil cell counts on these cytospin preparations confirmed the observed increase of BALF neutrophils after smoke exposure and a decrease in neutrophil numbers after smoking cessation (Fig. 1D). Neutrophil accumulation in lung tissue was quantitated by measuring MPO activity in lung homogenates. The MPO activity in the lung homogenates of mice exposed to cigarette smoke was significantly higher compared to control animals (Fig. 2). After a smoking cessation period of 8 weeks, the smoke-induced increase in MPO activity was restored towards normal levels.

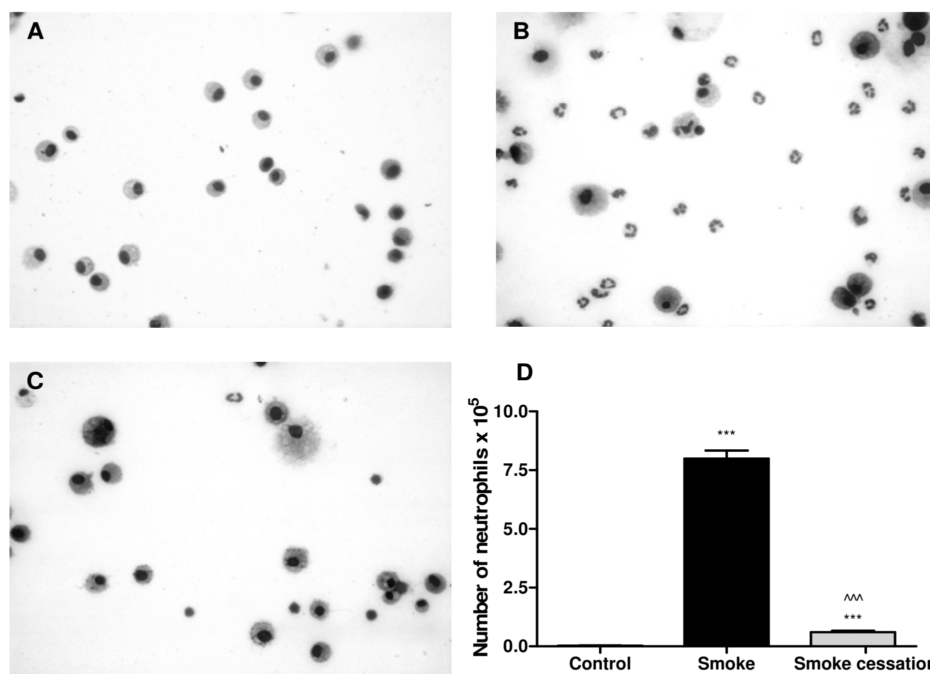

Fig.1. *Neutrophil influx in the BALF is related to smoke exposure. Representative photomicrographs of DiffQuick stained cytospins on glass slides of BALF from air-exposed mice (A), smoke-exposed mice (B), smoke-exposed mice 8 weeks after smoke cessation (C). Magnification, 1000x. Absolute neutrophil numbers in the BALF of mice exposed to air, mice exposed to cigarette smoke for 20 weeks (black bar) and mice exposed to cigarette smoke for 20 weeks plus a smoking cessation period of 8 weeks (grey bar) (D). n = 4-5 animals per group. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group. ^^^P ≤ 0.001; significantly different from the smoke group.*

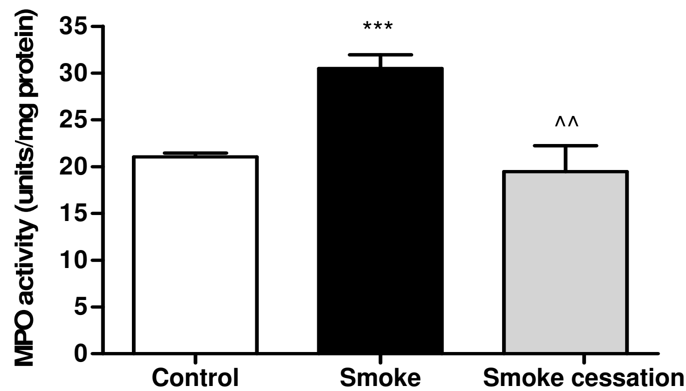

Fig.2. *Elevated MPO activity in lung homogenates after chronic smoke exposure. MPO activity in lung homogenates of mice exposed to air (white bar), mice exposed to cigarette smoke for 20 weeks (black bar) and mice exposed to cigarette smoke for 20 weeks plus a smoking cessation period of 8 weeks (grey bar). n = 4-6 animals per group. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group. ^^P ≤ 0.01; significantly different from the smoke group.*

Cigarette smoke-induced increase in MMP-8 and MMP-9 levels in BALF and lung homogenates

MMP-8 and MMP-9 are normally present in the granules of neutrophils and can be released following stimulation [34-36]. Since an increased neutrophil cell count and MPO activity was observed in the mice exposed to cigarette smoke, the next step was measuring the MMP-8 and MMP-9 levels in the BALF and lung tissue homogenates of air- and smoke-exposed mice. An ELISA was performed to detect the total MMP-8 and total and pro-MMP-9 levels. The mice exposed to cigarette smoke for 20 weeks showed significantly increased total MMP-8 levels in the BALF measured via ELISA (Fig. 3A). After smoking cessation, the concentrations of MMP-8 in the BALF were significantly decreased compared to smoke-exposed mice (Fig. 3A). To further support these data, BALF samples were also analyzed for MMP-8 levels via Western blotting. Via Western blot analysis elevated MMP-8 levels were observed after 20 weeks smoke exposure and after a smoking cessation period of 8 weeks the MMP-8 levels were reduced (Fig. 3C). The molecular weight bands at ~65kDa likely represent active MMP-8. The optical density of the molecular weight bands was measured and depicted in Fig. 3B. Unfortunately, no clear differences in MMP-8 levels were observed in the lung homogenates. The mice exposed to cigarette smoke for 20 weeks showed also increased total MMP-9 levels in the BALF (Fig. 4A) as well as in the lung homogenates (Fig. 4B). The active form of MMP-9 was also significantly elevated after smoke exposure (Fig. 4A and 4B). After smoke cessation, the BALF and lung homogenates concentrations of both total and active MMP-9 were decreased compared to the smoke-exposed mice (Fig. 4A and 4B). To further support these data, lung homogenates were analyzed for the presence and

activity of MMP-9 by gelatin zymography. In lung homogenates of cigarette smoke-exposed mice the MMP-9 gelatinase activity was higher than in lung homogenates of the control mice. After a smoking cessation period of 8 weeks reduced MMP-9 activity in gelatin zymography was observed as compared to the smokers (Fig. 4C). Since the BALF (and lung homogenates) showed elevated activity of MMP-8 and MMP-9, the first necessary proteases to generate PGP from collagen are present during chronic smoke exposure.

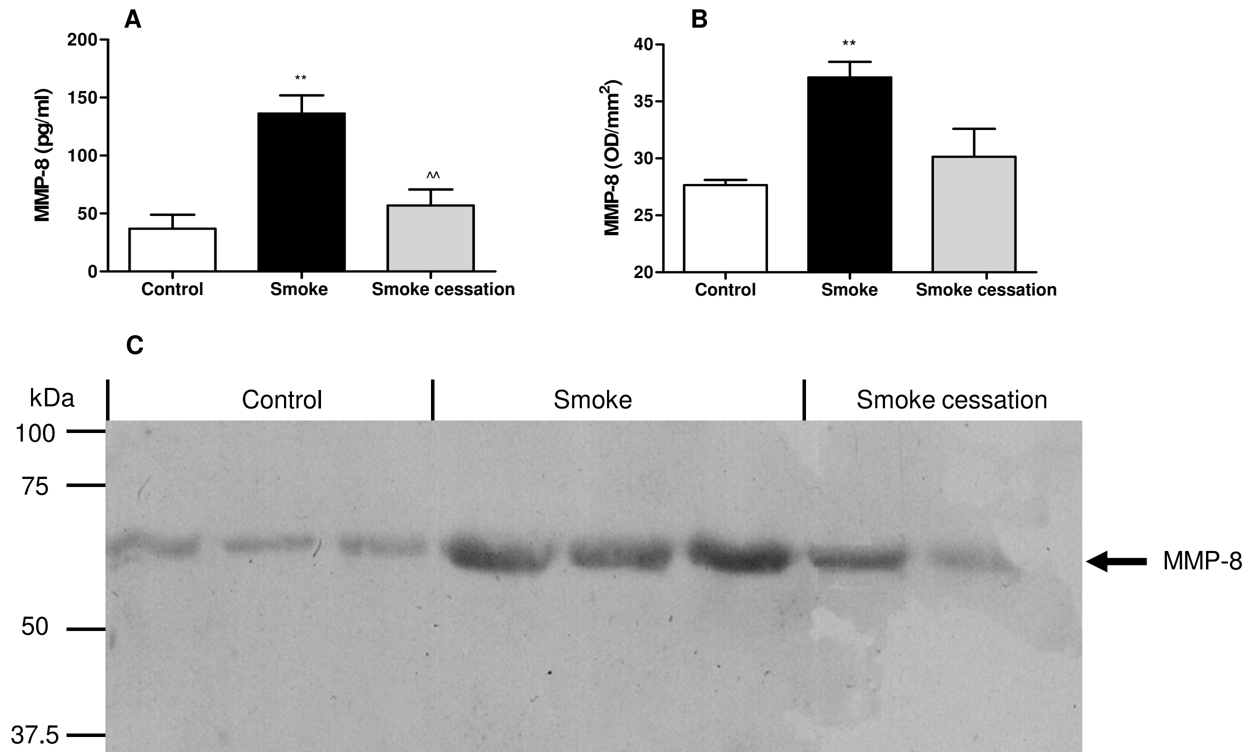

*__Fig.3.__ Smoke-related MMP-8 increase in BALF. The total MMP-8 levels were determined in the BALF (A) after 20 weeks air exposure (white bar), after 20 weeks smoke exposure (black bar) and after 20 weeks smoke exposure plus a smoking cessation period of 8 weeks (grey bar) via ELISA. n = 4-5 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01; significantly different from the control group . ^^P ≤ 0.01; significantly different from the smoke group. BALF samples from 3 control mice, 3 smoke-exposed mice and 2 smoke-exposed mice after a smoking cessation period were randomly chosen and analysed via Western blotting (C). The molecular weight bands at ~65kDa likely represent active MMP-8 and the optical density of these molecular weight bands was measured (B). The position of the bands for MMP-8 is indicated by an arrow.*

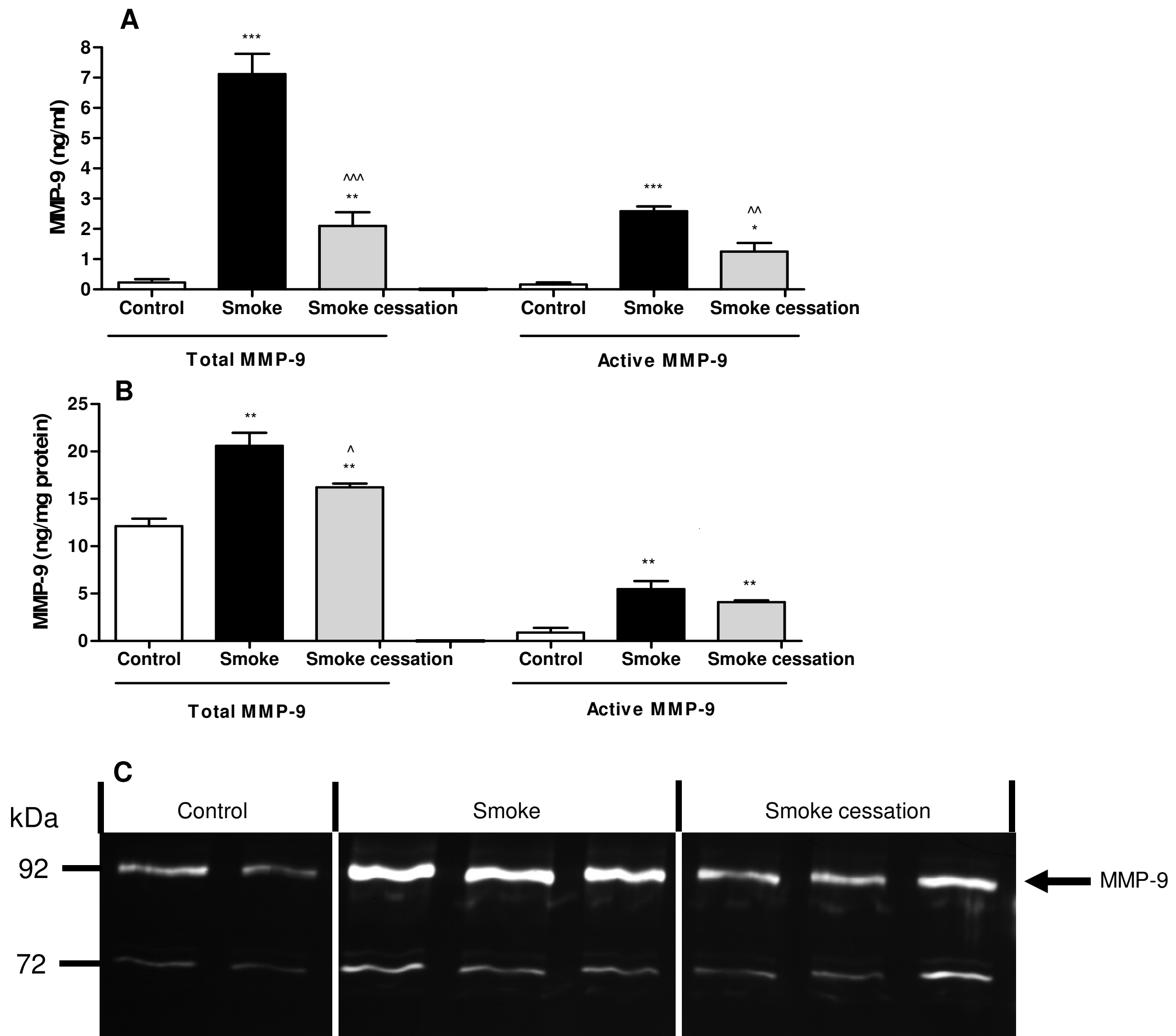

Fig.4. *Smoke-related MMP-9 increase in BALF and lung homogenates. The total and active MMP-9 levels were determined in the BALF (A) and lung homogenates (B) after 20 weeks air exposure (white bars), after 20 weeks smoke exposure (black bars) and after 20 weeks smoke exposure plus a smoking cessation period of 8 weeks (grey bars). n = 4-5 animals per group. Values are expressed as mean ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; significantly different from the control group. ^P ≤ 0.05, ^^P ≤ 0.01, ^^^P ≤ 0.001; significantly different from the smoke group. Lung homogenates from 2 control mice, 3 smoke-exposed mice and 3 smoke-exposed mice after a smoking cessation period were randomly chosen and analysed by gelatin zymography (C). Gelatinolytic activity of MMP-9 was increased in the lung homogenates after 20 weeks of smoke exposure compared to the air-exposed mice, whereas after smoking cessation the activity of MMP-9 was decreased. The position of the bands for MMP-9 is indicated by an arrow.*

PE activity is elevated in lung homogenates after chronic smoke exposure

The smaller collagen fragments generated by proteases, such as MMP-9, are hypothesized to be further cleaved by PE to the formation of PGP [23, 24]. To determine whether PE may play a role in PGP generation related to lung emphysema, lung homogenates of the mice were examined for PE activity using a colorimetric assay. We found a 2-fold increase in PE activity in lung homogenates of smoke-exposed mice compared to air-exposed mice (Fig. 5). This increase returned to normal levels after a smoking cessation period of 8 weeks. It can be concluded that PE activity is strongly related to cigarette smoke exposure.

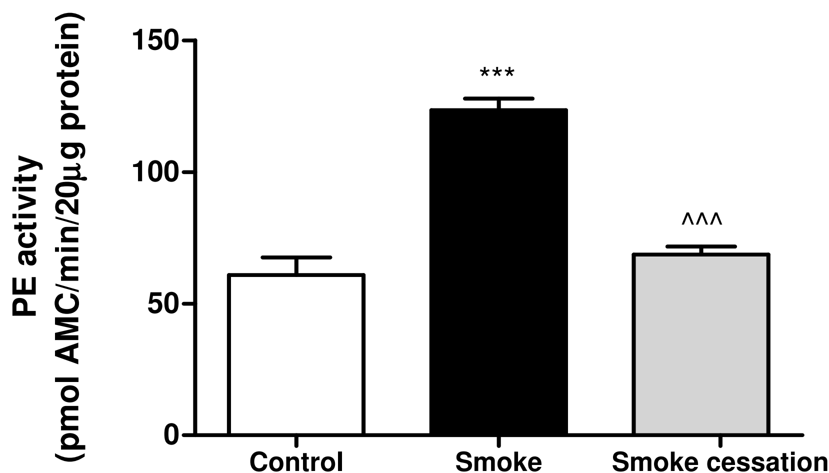

Fig.5. *PE activity was elevated in lung homogenates after chronic smoke exposure. PE activity in lung homogenates of mice exposed to air (white bar), mice exposed to cigarette smoke for 20 weeks (black bar) and mice exposed to cigarette smoke for 20 weeks plus a smoking cessation period of 8 weeks (grey bar). n = 4-5 animals per group. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group. ^^^P ≤ 0.001; significantly different from the smoke group.*

Various cell types in the murine lung express PE after chronic cigarette smoke exposure

Immunohistochemical analysis was performed to identify which cell types in the lung tissue express PE and to compare the PE expression in the lung tissue of PBS-exposed mice, cigarette smoke-exposed mice and cigarette smoke-exposed mice after a smoking cessation period of 8 weeks. In the lung tissue of the air-exposed mice the epithelial cells express PE (Fig. 6A), while in the lung tissue of the smoke-exposed mice the epithelial cells as well as the inflammatory cells (macrophages and neutrophils) highly express PE (Fig. 6B). The inflammatory cells were still present in the lung tissue of the smoke-exposed mice after a smoking cessation period of 8 weeks and PE was expressed in the epithelial cells and in the

inflammatory cells in the lung tissue of these animals (Fig. 6C). In figure 6D it is clearly demonstrated that murine lung epithelial cells contain PE and figure 6E shows a profound staining of PE in inflammatory cells in the lung tissue.

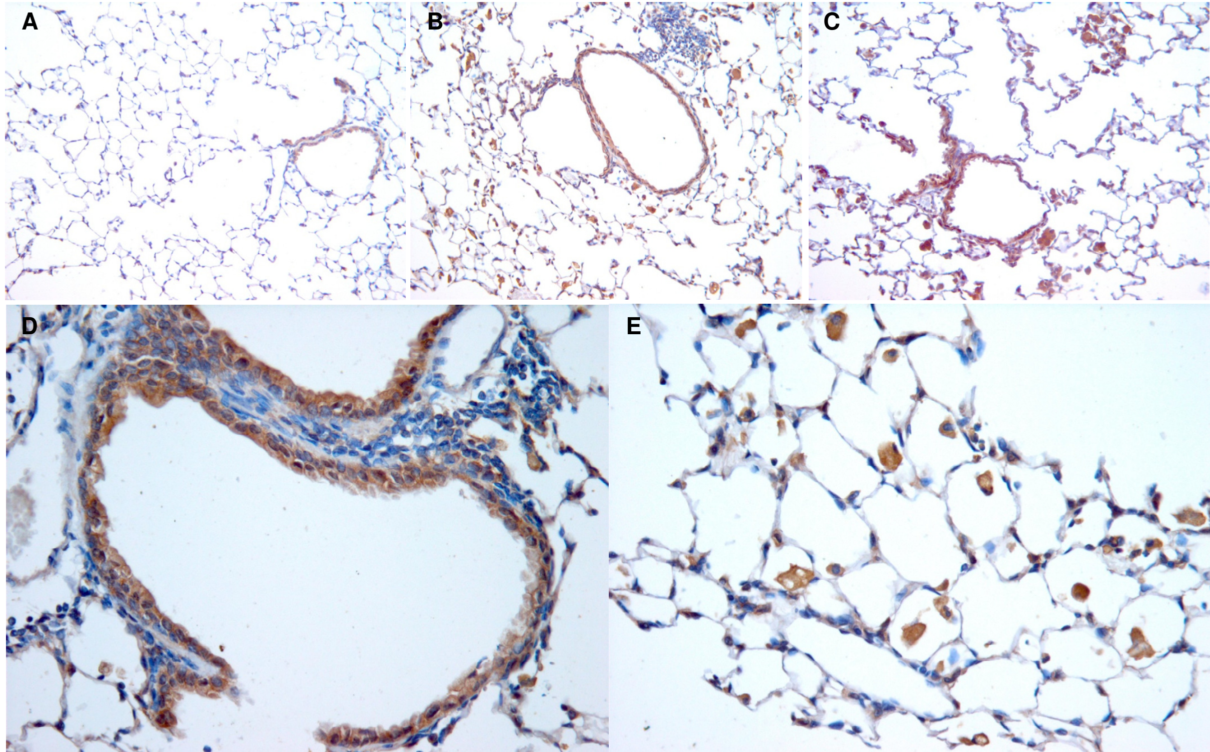

Fig.6. *Localization of PE in the murine lung. Representative photomicrographs of an immunohistological staining for PE (brown color, DAB staining) in lung tissue of air-exposed mice (A), smoke-exposed mice (B) and smoke-exposed mice after a smoking cessation period of 8 weeks (C). Representative photomicrographs of lung tissue of a smoke-exposed mouse with a pronounced expression of PE in the epithelial cells (D) and the inflammatory cells (E). Magnification, 200x (A, B, C), 400x (D, E). n = 3 animals per group.*

Inflammatory cells in lung tissue of smokers and COPD patients express PE

Immunohistochemical analysis was performed to compare the PE expression in lung tissue of current smokers, ex-smokers, COPD patients with GOLD stage II and COPD patients with GOLD stage IV. In the lung tissue of current smokers the enormous amount inflammatory cells highly express PE (Fig. 7A). The number of inflammatory cells and consequently the PE expression was decreased in the lung tissue of ex-smokers (Fig. 7B). Furthermore, the inflammatory cells observed in the lung tissue of COPD patients with GOLD stage II and IV express PE (Fig. 7C and 7D). Additionally, the bronchial epithelial cells observed in human lung tissue also express PE.

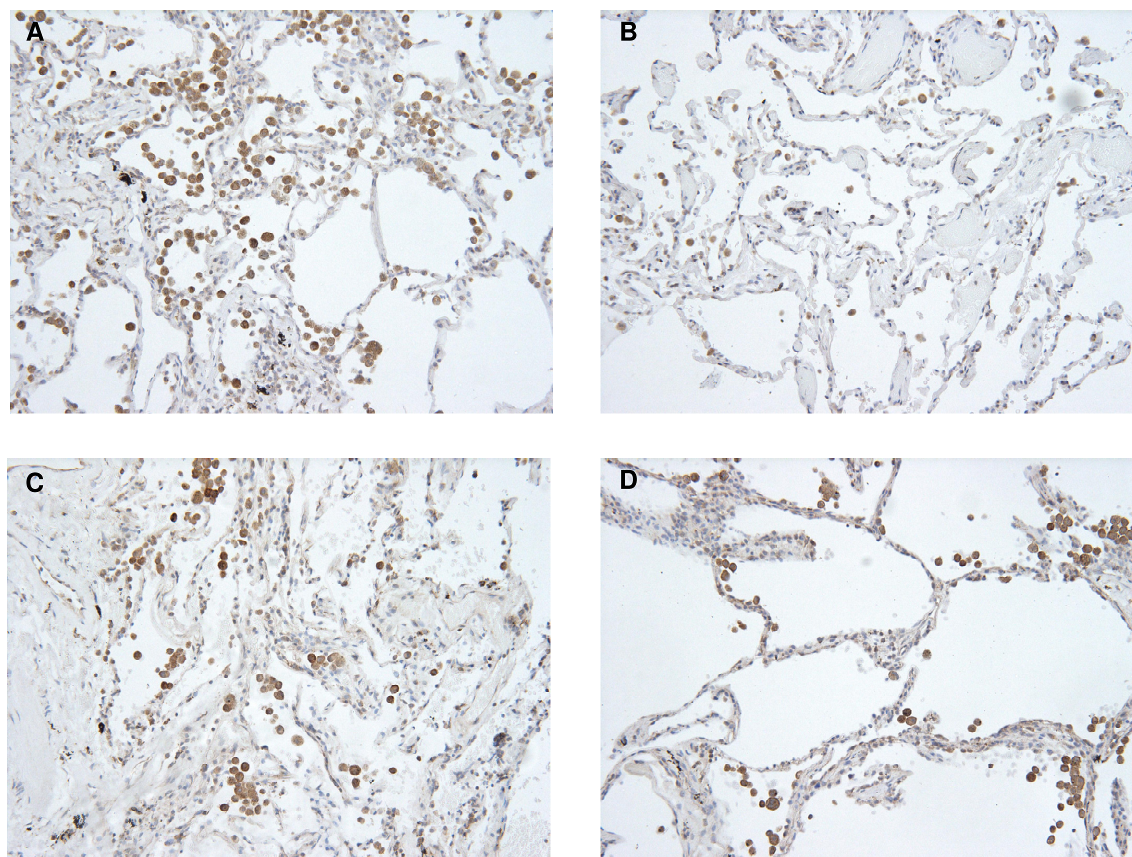

__Fig.7.__ Localization of PE in the human lung. Representative photomicrographs of an immunohistological staining for PE (brown color, DAB staining) in lung tissue of a current smoker (A), ex-smoker (B), COPD patient with GOLD stage II (C) and a COPD patient with GOLD stage IV (D). Magnification, 200x. n = 6-8 patients per group.

PGP and N-Ac-PGP levels are increased in BALF after chronic smoke exposure

The components necessary for the PGP formation, namely MMP-8, MMP-9 and PE, were present in the airways of the mice after chronic smoke exposure. At this point, the MS technique of ESI-LC/MS/MS was used for examining PGP and N-Ac-PGP levels in BALF from mice before and after smoking cessation and healthy controls. PGP levels were significantly increased in the BALF of mice exposed to cigarette smoke compared to the control animals. After smoking cessation, the PGP levels were returned to basal levels (Fig. 8A). Figure 8B shows that the BALF of all cigarette smoke-exposed mice were positive for N-Ac-PGP above our detection limit of 10 pg/ml, but all control BALF samples and the samples of the mice after smoking cessation were negative. These data pointed out that the PGP generation is related to chronic cigarette smoke exposure.

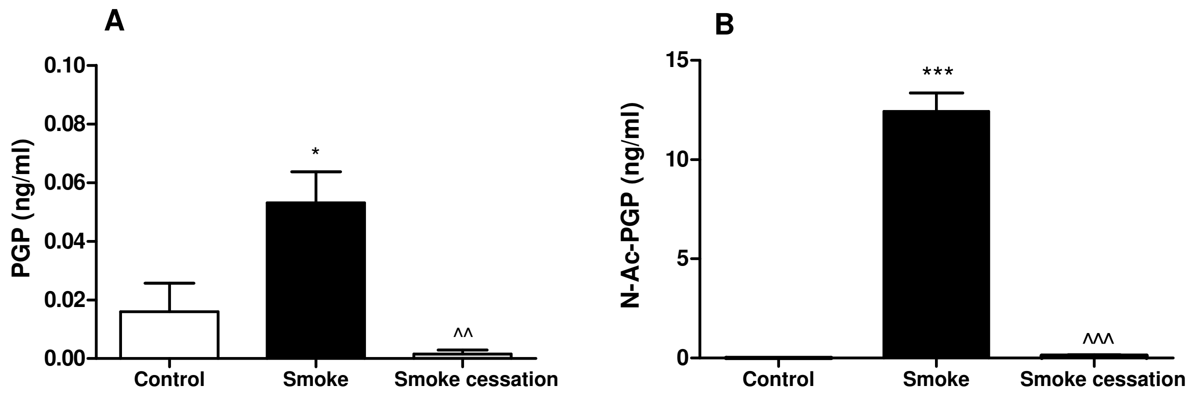

Fig.8. *PGP and N-Ac-PGP were detectable in BALF after chronic smoke exposure. The PGP (A) and N-Ac-PGP (B) levels were determined in the BALF after 20 weeks air exposure (white bars), after 20 weeks smoke exposure (black bars) and after 20 weeks smoke exposure plus a smoking cessation period of 8 weeks (grey bars). n = 4-5 animals per group. Values are expressed as mean ± S.E.M. *P ≤ 0.05, ***P ≤ 0.001; significantly different from the control group. ^^P ≤ 0.01, ^^^P ≤ 0.001; significantly different from the smoke group.*

Chronic cigarette smoke exposure induces irreversible alveolar enlargement

PGP and N-Ac-PGP levels were observed in the BALF of smoke-exposed mice, which indicates that collagen breakdown occurs in these airways. To ensure that tissue degradation was present after chronic smoke exposure, the mean linear intercept, a quantification method for alveolar size, was used to quantify the presence and severity of emphysema. Significant airspace enlargement was observed in the mice after 20 weeks cigarette smoke exposure (Lm of the air-exposed mice: 42.5 ± 0.8 µm versus the Lm of the smoke-exposed mice: 52.6 ± 1.7 µm, P ≤ 0.01). Furthermore, the airspace enlargement induced by cigarette smoke exposure was not reversible, since the increased Lm was not reduced after a period of 8 weeks without cigarette smoke exposure (Lm after smoking cessation: 49.6 ± 1.4 µm).

Correlations between different parameters

The air-exposed mice and the smoke-exposed mice were grouped together and the relationship between the various parameters was calculated. The most relevant correlations are described in Table 2. First, when we correlated the amount of neutrophils in the BALF and the MMP8 and MMP-9 levels in the BALF, the correlation coefficient (R^2) was high (Table 2). The MPO activity and the MMP-9 levels in the lung homogenates were also strongly correlated. A highly significant correlation was found between the neutrophils in the BALF and the PE activity. Furthermore, a tight correlation between the neutrophil influx in the BALF and the N-Ac-PGP levels in the BALF was observed (Table 2). The neutrophil influx was also significantly correlated with the PGP levels in the BALF. Moreover, the MMP-8 and

MMP-9 levels as well as the PE activity revealed strong correlation with the N-Ac-PGP levels in the BALF, which indicates that both proteases are related to N-Ac-PGP formation. The MMP-9 levels, the PE activity and the N-Ac-PGP levels showed a significant association with the lung destruction measured by the mean linear intercept (Table 2), indicating that MMP-9, PE and N-Ac-PGP are related to the development of lung emphysema.

Table 2. *Correlations between the different parameters measured in the airways of air- and smoke-exposed mice*

Parameter	versus	R^2
Neutrophils	MMP-8 in BALF	0.863***
Neutrophils	MMP-9 in BALF	0.977***
Neutrophils	PE activity	0.957***
Neutrophils	N-Ac-PGP	0.987***
MMP-8 in BALF	N-Ac-PGP	0.823***
MMP-9 in BALF	N-Ac-PGP	0.961***
PE activity	N-Ac-PGP	0.925***
MMP-9 in BALF	Lm	0.890**
PE activity	Lm	0.821**
N-Ac-PGP	Lm	0.916**

***P < 0.01; ***P < 0.001*

Cigarette smoke-induced neutrophil influx in the BALF is significantly decreased after RTR and VPA administration

To investigate whether inhibition of PGP or PE in the airways can decrease or prevent the neutrophil migration to the lung, the complementary peptide L-arginine-threonine-arginine (RTR), which can bind to PGP sequences [20] and the PE inhibitor, valproic acid (VPA) were tested in a murine smoking model of 5 days cigarette smoke exposure. We observed a significant increase of BALF neutrophils after 5 days smoke exposure compared to the air-exposed mice (Fig. 9A and B). The cigarette smoke-induced neutrophil influx in the BALF was significantly decreased after RTR administration (Fig. 9A) as well as after VPA administration (Fig. 9B).

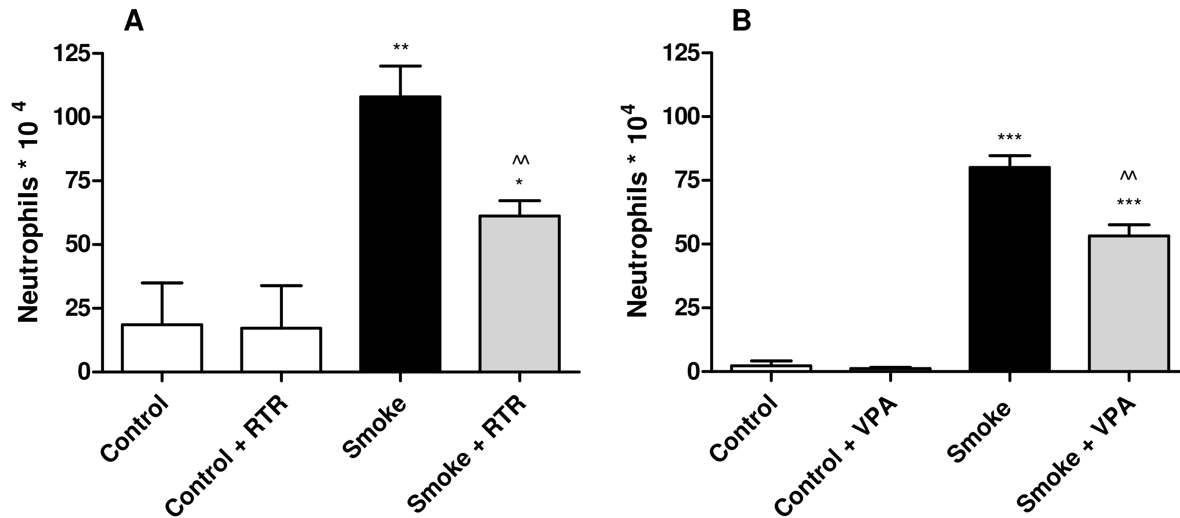

Fig.9. *Cigarette smoke-induced neutrophil influx in the BALF was significantly decreased after RTR and VPA administration. Absolute neutrophil numbers in the BALF of mice exposed to air, mice exposed to cigarette smoke, RTR-treated mice exposed to air and cigarette smoke (A), and VPA-treated mice exposed to air and cigarette smoke (B) for 5 days. The mice received vehicle (PBS), RTR (50µg / 50µl PBS) or VPA (100µg / 70µl PBS) by oropharyngeal aspiration twice daily. n = 4-6 animals per group. Values are expressed as mean ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; significantly different from the control group. ^^P ≤ 0.01; significantly different from the smoke group.*

Discussion

In this report, we describe the involvement of PE in the development of lung emphysema. Since lung emphysema is characterized by the destruction of alveolar walls, the role of proteases is extensively studied in this disease [37]. Neutrophil elastase and MMPs have attracted the most attention, while little is known about PE in inflammation biology or lung pathology. PE activity has been detected in a variety of tissues, body fluids and various physiological roles are suggested [38], such as neuropeptide processing and secretion related to psychiatric diseases [39, 40] and affecting the renin-angiotensin system [41]. Gaggar et al. [22] were the first, who demonstrated that PE could be involved in airway inflammation, showing an elevated PE activity in sputum from cystic fibrosis patients. They identified a novel pathway for generating the tripeptide PGP from collagen, in which PE is involved. This pathway is based on *in vitro* experiments, where sputum from cystic fibrosis and COPD patients has the ability to generate PGP *de novo* from collagen and they demonstrated a role for MMP-1, MMP-8, MMP-9 and PE in this process. These data were further expanded with *in vivo* murine data, where PGP production in the BALF was examined after intratracheal administration of MMPs or human neutrophil elastase (HNE) with or without PE [21]. At first, this pathway for generating chemotactic PGP from collagen can be activated via an inflammatory stimulus, such as CXCL8, which will lead to the recruitment and activation of neutrophils. In lung emphysema, this cascade will start with cigarette smoke exposure to the lungs, which was mimicked in the present murine lung emphysema model. After chronic cigarette smoke exposure an excessive neutrophil influx in the BALF and an increased MPO activity in the lung tissue was observed, which is in agreement with other *in vivo* lung emphysema studies [42-44]. Secondly, when neutrophils are activated, they will release different proteases, like MMP-8 and MMP-9, since neutrophils are a rich source of these MMPs [34, 45]. There is an abundance of literature regarding MMP-9 related to lung pathology, reviewed in Atkinson et al. [46]. In our study, the total and active MMP-8 and MMP-9 levels were increased in the BALF of the smoke-exposed mice compared to the control animals. Other *in vivo* studies also demonstrated increased MMP-9 levels in the BALF of mice after cigarette smoke exposure [47, 48]. Additionally, elevated MMP-8 and MMP-9 levels were observed in the airways of COPD patients compared to healthy controls [30, 36, 49]. In this study, the MMP-8 and MMP-9 levels in the airways are most probably related to the amount of neutrophils as reflected in the high correlation coefficient. Besides neutrophils, alveolar macrophages and lymphocytes can also synthesize and release MMP-9 [46], which could explain the difference between the normalized MPO activity in the lung tissue after 8 weeks smoking cessation and the MMP-9 levels in the lung homogenates, which did not return completely to basal level. MMP-8 and MMP-9 have been proposed to

induce airway remodeling, because of their capacity to cleave structural proteins, such as collagens and elastin [50]. At this point, collagen in the lung can be cleaved in smaller fragments caused by the release of MMP-8 and MMP-9 in the airways after smoke exposure. MMPs alone are not sufficient to generate PGP from collagen. Therefore, the next step was measuring PE activity in the lung homogenates. To our knowledge, PE is the only enzyme directly capable of cleaving PGP from shorter portions of collagen, like peptides and oligopeptides, generated by the prior digestion of MMPs [23, 24]. In this lung emphysema model, a significant increase in PE activity was observed in the lung homogenates of smoke-exposed mice compared to air-exposed mice. Furthermore, we can emphasize that both MMP-8/MMP-9 and PE are important for the formation of N-Ac-PGP from collagen, since the MMP-8/MMP-9 levels and the PE activity were strongly correlated to the N-Ac-PGP levels in the BALF. From our immunohistological staining for PE, it can be concluded that the PE expression is increased in lung tissue of cigarette smoke-exposed mice compared to air-exposed mice. This increase in PE expression is caused by the inflammatory cell influx in the lungs of the smoke-exposed mice, since the inflammatory cells (macrophages and neutrophils) highly express PE. This increase in PE expression in mice related to the inflammatory cell influx in the lungs is confirmed with our human data. The inflammatory cells present in lung tissue of current smokers and COPD patients with GOLD stage II and IV highly express PE, while the number of inflammatory cells and consequently the PE expression was decreased in the lung tissue of ex-smokers. The epithelial cells in the lung tissue of air- and cigarette smoke-exposed mice as well as the epithelial cells in human lung tissue contain PE, indicating that epithelial cells are an important source for PE in the lung. Exposure of human bronchial epithelial cells to cigarette smoke extract for 16 hours *in vitro* leads to an elevated PE activity in the supernatant (E. Mortaz, unpublished data), suggesting that cigarette smoke exposure might lead to a release of PE. However, PE could also be released as a result of airway epithelial necrosis or necrosis of other inflammatory cell types (macrophages and neutrophils) after cigarette smoke exposure. After smoking cessation, the inflammatory cells were still present in the lung tissue and express PE, but the PE activity in the lung homogenates returned to normal levels. One explanation could be that the activity of PE is decreased in the inflammatory cells despite they contain PE. However, Polgar et al. [51] described that PE does not have a zymogen (or proenzyme) form and is synthesized as an active peptidase. Secondly, the PE activity could also be related to the amount of neutrophils in the lung tissue, which was also reduced after smoking cessation, since the MPO activity in these lung homogenates was returned to normal levels. Recently, O'Reilly et al. [52] described that neutrophils contain PE and are capable themselves to generate PGP from collagen after LPS exposure. In our study, we found a strong correlation between the

neutrophils in the BALF and the PE activity, indicating that the PE activity is related to the neutrophil influx. The importance of PE in neutrophil migration to the lung is also enforced by the finding that the cigarette smoke-induced neutrophil influx in the BALF was significantly decreased after treatment with the PE inhibitor valproic acid. As with MMPs, PE can be released by neutrophils through degranulation in response to pro-inflammatory stimuli and could both play a role in a wide variety of chronic inflammatory diseases. Whether cigarette smoke exposure is able to activate this pathway for generating PGP from collagen is the subject of ongoing studies. Furthermore, it has also been demonstrated that PE is present in both macrophages and lymphocytes [53, 54], while its presence in other cell types is as yet unknown.

All the components (neutrophils, MMPs and PE) necessary for the generation of PGP from lung collagen were present in the airways of the mice after chronic smoke exposure. Consequently, PGP and N-Ac-PGP levels were detected in the BALF of cigarette smoke-exposed mice. Low levels of PGP were observed in the BALF of air-exposed mice, which could be a result of normal collagen turnover. Unlike PGP, no N-Ac-PGP was detected in the BALF of air-exposed mice, suggesting that the acetylation of PGP might be an important step in the pathogenesis of lung emphysema. *In vitro*, it was shown that N-Ac-PGP is four to seven times more potent as a neutrophil chemoattractant than PGP [55]. Moreover, in sputum of COPD patients the PGP levels were also significantly increased compared with healthy controls (58 ± 12 ng/ml vs. 22 ± 12 ng/ml, $P < 0.05$) [21]. The N-Ac-PGP levels were much higher in the BALF of smoke-exposed mice compared to the levels in sputum of COPD patients [21]. A possible explanation for this could be that the subjects were stable outpatients with advanced COPD and most PGP generation might occur early when matrix destruction is most active. Snelgrove et al. [56] observed that acetylation of PGP occurred when PGP was incubated with different concentrations cigarette smoke condensate. The extent of smoking will be related to the amount of N-Ac-PGP, which could be another possible explanation for the differences between the human and mice observations. However, it is still very difficult to compare the results from sputum of COPD patients with the results of the BALF of the smoke-exposed mice.

The correlation between N-Ac-PGP/PGP and neutrophils in the BALF could imply that N-Ac-PGP and PGP are neutrophil chemoattractants or are both generated due to neutrophilic airway inflammation. It is difficult to conclude that the observed increase in PGP levels is only neutrophil-related, however different results pointed out that the neutrophils play an important role in this process. First, the amount of neutrophils is extremely increased after cigarette smoke exposure. Moreover, the PE staining pointed out that neutrophils are an important source of PE. Furthermore we found strong correlations between the neutrophil influx, the PE

activity and the N-Ac-PGP levels. It would be very interesting for future research to repeat this study in neutrophil-depleted animals to investigate whether this cascade of events leading to PGP formation is really neutrophil dependent.

The importance of PGP in chronic inflammatory diseases, characterized by neutrophils as potential target cells, is reinforced by several other studies. First, in COPD patients PGP was observed in the BALF, but also in sputum and serum [17, 21], suggesting a local as well as a systemic effect for PGP in COPD. *In vivo* research pointed out that PGP generation correlates well with a neutrophil influx in the BALF [22]. Furthermore, neutrophil infiltration and lung emphysema were observed in mice after chronic airway exposure to PGP [17, 20]. PGP was also measured in the BALF of mice after exposure to aerosolized LPS [17]. Finally, PGP derived from collagen was identified as being chemotactic for neutrophils *in vitro* as well as *in vivo* [17, 18].

The importance of PGP in neutrophil migration to the lung in a cigarette smoke exposure model is now enforced, since the cigarette smoke-induced neutrophil influx was significantly decreased in the BALF after RTR administration. In addition, van Houwelingen et al. [20] found that RTR reduces the emphysema-like changes in the airways after LPS and PGP exposure. We propose that after PGP formation from the extracellular matrix, the neutrophils in the airways of the smoke-exposed mice are attracted and activated by both PGP and the mouse equivalents of CXCL8. Subsequently, the neutrophils will release more proteases, like MMP-8, MMP-9 and PE, ultimately leading to lung tissue degradation and ongoing PGP formation. This vicious circle (Fig. 10) of events initiated by cigarette smoking is a continuous process and may potentially end in the development of lung emphysema, which was also observed in the present murine model via Lm analysis. All the different components (MMP-8, MMP-9, PE and N-Ac-PGP) were significantly correlated to the alveolar enlargement, suggesting a role for these components in the pathophysiology of lung emphysema.

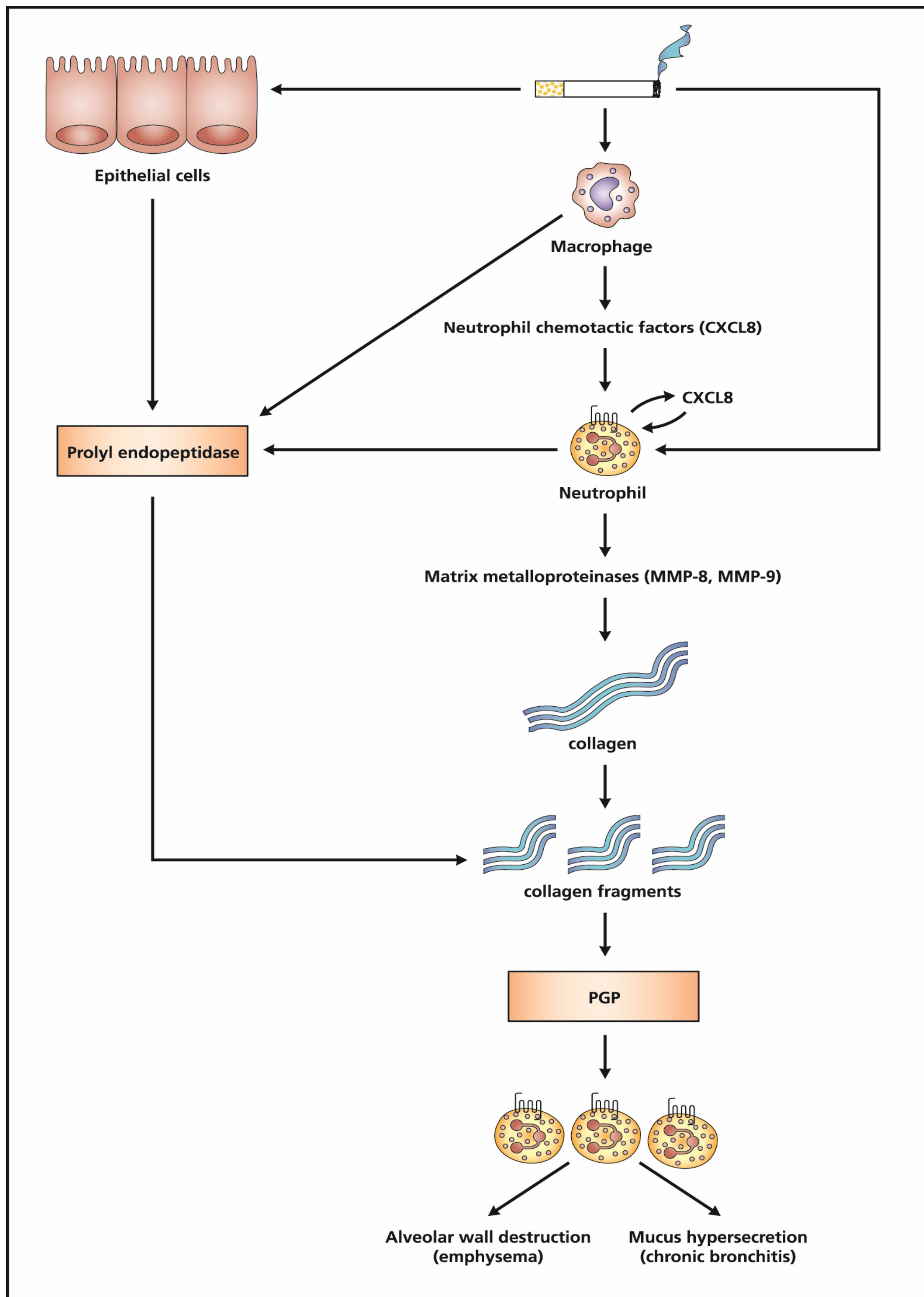

Fig.10. *PGP generation is a multistep process. In lung emphysema, the cascade of events leading to PGP formation will start with cigarette smoke exposure. Cigarette smoke can stimulate alveolar macrophages to release several chemoattractants, such as CXCL8. Subsequently, CXCL8 facilitates the migration of neutrophils to the site of inflammation. The activated neutrophils are also capable themselves to produce CXCL8 and the cigarette smoke exposure will also affect these neutrophils. PGP formation is a multistep process initially involving release of proteases from the MMP family, like MMP-8 and MMP-9. MMP-8 and MMP-9 are released by activated neutrophils and can proteolytically*

cleave collagen to smaller fragments resulting in an optimal substrate for PE activity. These collagen fragments are then further cleaved to PGP by PE, a member of the serine protease family. Various cell types, like neutrophils, macrophages and epithelial cells express PE. The generated PGP is chemotactic for neutrophils and results in an environment of chronic inflammation with proteolytic damage and PGP formation. Finally, this will lead to alveolar wall destruction (emphysema) and mucus hypersecretion (chronic bronchitis).

After a smoking cessation period of 8 weeks, the neutrophilic airway inflammation, the MMP-8 and MMP-9 levels, the PE activity and the PGP formation in the lungs are all decreased or reduced to normal levels. Other studies also pointed out that after smoking cessation the neutrophil levels and MMP-9 levels in the BALF were diminished [43, 47]. Unlike all other parameters in our study, lung emphysema was still present after smoking cessation, suggesting that the alveolar wall destruction is not reversible. Consequently, smoking cessation can interrupt the vicious circle of an ongoing neutrophil influx into the lung. This will result in a decrease in neutrophils and proteases, which will stop the lung matrix breakdown, and thus the PGP formation, while the lung emphysema stays present. This suggests that PE activity and PGP formation are associated with cigarette smoke exposure and not directly with emphysema. Additionally, after 1 week smoke exposure the PE activity and PGP levels were also increased in the lung (data not shown), but not as obvious as after 20 weeks smoke exposure. This indicates that PE and N-Ac-PGP could be biomarkers for the state of inflammation during chronic inflammatory diseases.
In conclusion, in the present murine model of cigarette smoke-induced lung emphysema, it is demonstrated for the first time that all relevant components (neutrophils, MMP-8/MMP-9 and PE), which may contribute to the neutrophilic airway inflammation by generating PGP from lung collagen, were upregulated in the airways and were related to alveolar destruction.

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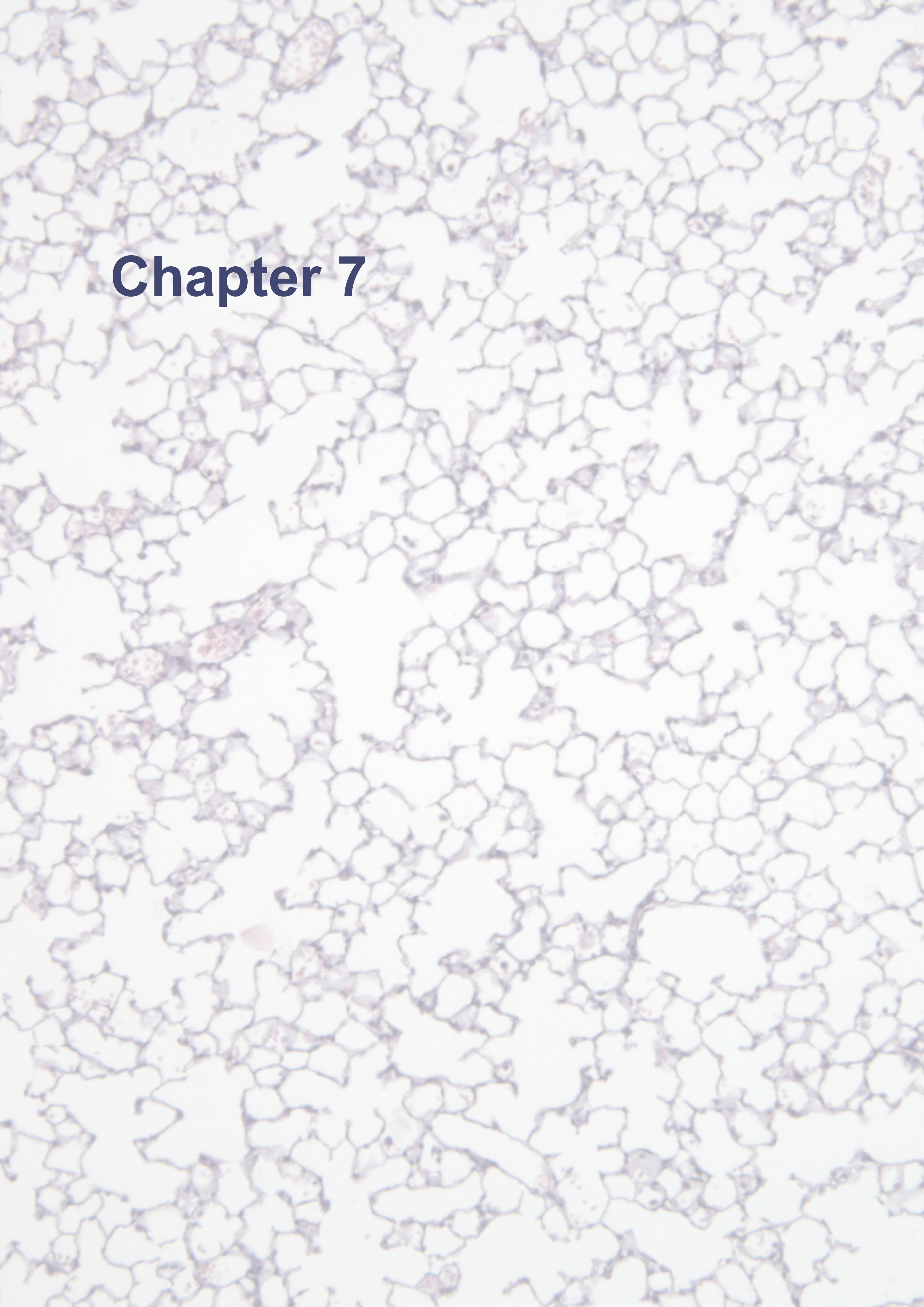

Chapter 7

CXCR2 antagonists block the N-Ac-PGP-induced neutrophil influx in the airways of mice, but not the production of the chemokine CXCL1

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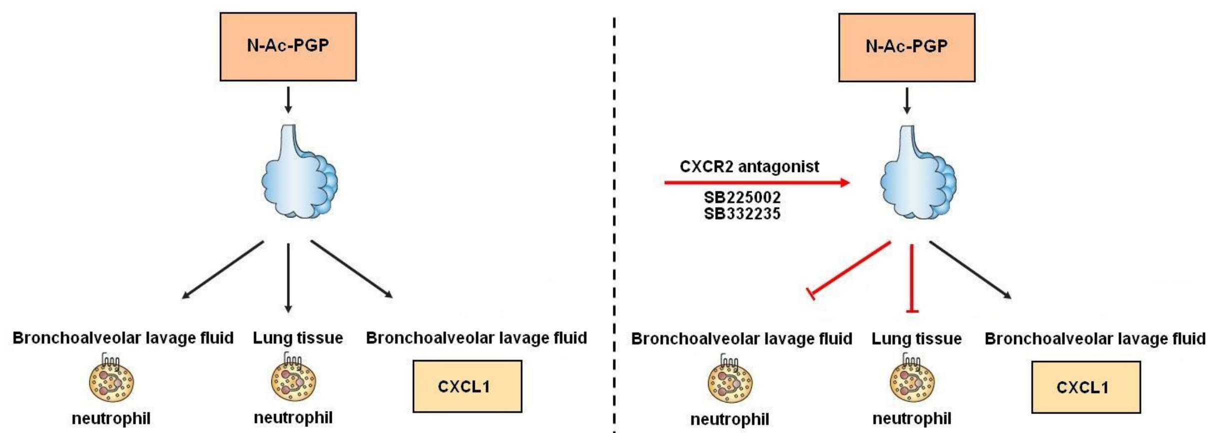
Abstract

Neutrophils are innate immune cells in chronic inflammatory diseases including chronic obstructive pulmonary disease (COPD) and can be attracted to the site of inflammation via the collagen breakdown product N-acetyl proline-glycine-proline (N-Ac-PGP). To elucidate whether CXCR2 is involved in N-Ac-PGP-induced neutrophil migration and activation, studies using specific antagonists were performed *in vivo*. N-Ac-PGP and keratinocyte cell-derived chemokine (KC, CXCL1) were administered in C57Bl/6 mice via oropharyngeal aspiration. Intraperitoneal applications of CXCR2 antagonist SB225002 or SB332235 were administered 1 h prior and 1 h after oropharyngeal aspiration. Six hours after oropharyngeal aspiration mice were sacrificed. Neutrophil counts and CXCL1 levels were determined in bronchoalveolar lavage fluid, myeloperoxidase (MPO) levels were measured in lung tissue homogenates and an immunohistological staining for neutrophils was performed on lung tissue. N-Ac-PGP and CXCL1 induced a neutrophil influx in the bronchoalveolar lavage fluid and lung tissue, which was also reflected by increased MPO levels in lung tissue. The N-Ac-PGP- and CXCL1-induced neutrophil influx and the increased pulmonary tissue MPO levels were inhibited by the CXCR2 antagonists SB225002 and SB332235. Moreover, N-Ac-PGP administration enhanced the CXCL1 levels in bronchoalveolar lavage fluid, which could not be attenuated by both CXCR2 antagonists.

In conclusion, neutrophil migration induced by N-Ac-PGP is mediated via direct CXCR2 interaction. The N-Ac-PGP-induced release of CXCL1 is independent of CXCR2. Related to the maximal effect of CXCL1, N-Ac-PGP is more potent at inducing neutrophil migration in the pulmonary tissue than into the bronchoalveolar lavage fluid, or N-ac-PGP may be more potent at inducing MPO levels in the lung tissue.



N-Ac-PGP-induced neutrophil migration into the lungs of mice is inhibited by CXCR2 antagonists, while the N-Ac-PGP-induced release of CXCL1 is independent of CXCR2.

Introduction

Neutrophils are innate inflammatory cells in chronic inflammatory diseases including chronic obstructive pulmonary disease (COPD) and are attracted to the site of inflammation via chemoattractants, such as interleukin-8 (CXCL8) in humans and keratinocyte cell-derived chemokine (KC; CXCL1) or macrophage inflammatory protein-2 (MIP-2, CXCL2) in mice [1]. CXCL8, CXCL1 and CXCL2 bind to the CXCR2 that is predominantly expressed by neutrophils. Activated neutrophils release proteases, which contribute to collagen breakdown leading to lung emphysema. In our hypothesis, the increased protease activity leads not only to alveolar wall destruction but also to the formation of N-acetyl proline-glycine-proline (N-Ac-PGP) from collagen [2]. N-Ac-PGP is chemotactic for neutrophils *in vitro* as well as *in vivo* [3] and activates human neutrophils to release CXCL8 [4]. The importance of N-Ac-PGP in inflammatory diseases, such as COPD, is reinforced by several studies. Clinical data demonstrated that N-Ac-PGP can be detected in the bronchoalveolar lavage fluid, sputum and serum of COPD patients [3, 5]. Chronic airway exposure to N-Ac-PGP causes neutrophil infiltration and lung emphysema in mice [3, 6].

It has been suggested that the basis for N-Ac-PGP effects lies in its structural homology with the GP motif present in all ELR^+ CXC chemokines, such as CXCL8. This GP motif is essential for cell activation and ligand binding to CXCR1/2 receptors on neutrophils [3, 7]. Overbeek et al. [4] and Weathington et al. [3] reported that N-Ac-PGP activity is mediated via the G-protein coupled receptor CXCR2, since CXCR2 antibodies suppressed the N-Ac-PGP-induced neutrophil chemotaxis *in vitro*. Furthermore, the accumulation of neutrophils seen in mice upon intratracheal administration of N-Ac-PGP, was not detected in $CXCR2^{-/-}$ mice [3].

The aim of this study was to investigate whether the N-Ac-PGP-induced neutrophil influx was mediated via CXCR2 *in vivo.* We investigated the effects of two different selective nonpeptide CXCR2 antagonists, SB225002 and SB332235, in a murine model where a neutrophil influx was induced via oropharyngeal administration of N-Ac-PGP or CXCL1. In this report, we demonstrate that N-Ac-PGP and CXCL1 can induce a neutrophil influx in lung tissue and bronchoalveolar lavage fluid of mice, which can be inhibited by CXCR2 antagonists. The neutrophil migration induced by N-Ac-PGP is mediated via direct interaction with CXCR2 and by a small, if any, release of CXCL1. The N-Ac-PGP-induced release of CXCL1 is independent of the CXCR2.

Material and methods

Animals

Male C57Bl/6 mice, 6-7 weeks old were obtained from Charles River Laboratories and housed under controlled conditions in standard laboratory cages in the animal facility. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the local Ethics Committee and were performed under strict governmental and international guidelines on animal experimentation.

Oropharyngeal aspiration

Oropharyngeal aspiration of CXCL1 (0.5 µg in 70 µl PBS) (R&D systems, Minneapolis, USA), N-Ac-PGP (500 µg in 70 µl PBS) (Anaspec, San Jose, USA) or vehicle (PBS) was performed after induction of light isoflurane anesthesia. The dosages used for CXCL1 and N-Ac-PGP are previously described by Frevert et al. [8] and Van Houwelingen et al. [6]. The anesthetized mice were held vertically and the tongue was gently pulled out of the mouth using forceps in order to visualize the base of the tongue and the pharynx. The CXCL1, N-Ac-PGP or PBS solution was pipetted onto the back of the tongue and to force breathing through the mouth, the nose was pinched. Respiration was monitored to ensure the solution was fully aspirated before the tongue was released [9, 10].

CXCR2 antagonist administration

The selective nonpeptide CXCR2 antagonist SB225002 (*N*-(2-hydroxy-4-nitrophenyl)-*N'*-(2-bromophenyl)urea) was purchased from Alexis Biochemicals (Nottingham, UK). The CXCR2 antagonist SB332235 (*N*-(2-hydroxy-3-sulfamyl-4-chlorophenyl)-*N'*-(2,3-dichlorophenyl)urea) was synthesized at Merck Research Laboratories as described in the International Application published under the Patent Cooperation Treaty (2000, WO 00/35442).

SB225002 was dissolved according to manufacturer's instructions at a stock concentration of 10 mg/ml ($28x10^{-3}$ M). For *in vivo* application, aliquots of SB225002 were diluted in vehicle (0.9% NaCl solution containing 0.33% Tween-80) just before use [11].

SB332235 was dissolved according to manufacturer's instructions at a stock concentration of 150 mg/ml ($380\ x10^{-3}$ M). For *in vivo* application, aliquots of SB332235 were diluted in vehicle (0.9% NaCl solution containing 5% caster oil) just before use [12].

The mice received intraperitoneal applications of vehicle, SB225002 (50 µg ($1.4x10^{-7}$ moles) in 200 µl per animal) as previously described by Herbold et al. (2010) or SB332235 (20 µg ($0.5x10^{-7}$ moles), 100 µg ($2.5x10^{-7}$ moles) and 300 µg ($7.6x10^{-7}$ moles) in 200 µl per animal) as previously described by Mihara et al. (2005) 1 h prior and 1 h after oropharyngeal aspiration of CXCL1, N-Ac-PGP or PBS.

Bronchoalveolar lavage

Six hours after the oropharyngeal aspiration of CXCL1, N-Ac-PGP or PBS, the mice were i.p. injected with an overdose of pentobarbital. The lungs of these mice were lavaged 4 times through a tracheal cannula with 1 ml saline (NaCl 0.9%), pre-warmed at 37 °C. The first lavage was performed with 1 ml saline containing a mixture of protease inhibitors (Complete Mini, Roche Applied Science, Penzberg, Germany). After centrifugation of the bronchoalveolar lavage fluid at 4°C (400 g, 5 min), the supernatant of the first ml was used for CXCL1 analysis and the cell pellets of the 4 lavages were used for neutrophil cell counts. The 4 cell pellets, kept on ice, were pooled per animal and resuspended in 150 µl cold saline. After staining with Türk solution, total cell counts per lung were made under light microscopy using a Burker-Turk chamber. Differential cell counts were performed on cytospin preparations stained by DiffQuick™ (Dade A.G., Düdingen, Switzerland). Cells were identified as neutrophils according to standard morphology. At least 200 cells were counted and the absolute number of neutrophils was calculated [13].

Measurement of CXCL1

A standard mouse cytokine/chemokine kit was used to determine CXCL1 concentrations in the bronchoalveolar lavage fluid according to the manufacturer's instructions (Millipore, Billerica, USA). The concentrations of CXCL1 were expressed as pg/ml bronchoalveolar lavage fluid.

Preparation of lung homogenates

Right lung samples were homogenized in a potter glass tube with a Teflon pestle in 1 ml ice cold PBS. Homogenates were centrifuged at 14,000 g for 5 min and supernatants were collected. The protein concentration of each sample was assayed using the Pierce BCA protein assay kit standardized to BSA, according to the manufacturer's protocol (Thermo Fisher Scientific, Rockford, IL, USA). The homogenates were diluted to a final concentration of 2 µg protein/µl [14].

Myleoperoxidase (MPO) ELISA

MPO levels were measured in lung homogenate supernatants by ELISA using the Mouse MPO ELISA kit (Hycult Biotechnology, Uden, the Netherlands) according to manufacturer's instructions.

Immunohistochemistry

Left lung samples were fixed with 10% formalin for at least 24h, after which the left lung was embedded in paraffin. Paraffin sections were deparaffinized, endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck, Darmstadt, Germany) in methanol for 30 min at room temperature and rehydrated in a graded ethanol series to PBS. For antigen retrieval, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave. The slides were cooled down to room temperature, rinsed with PBS (3x) and blocked with 5% rabbit serum (Dakocytomation, Glostrup, Denmark) in PBS containing 1% bovine serum albumin for 30 min at room temperature. Sections were incubated with the primary antibody (rat-anti-mouse neutrophils (MCA771GA), 1:2000, AbD serotec, UK) in PBS containing 1% bovine serum albumin overnight at 4℃. The slides were rinsed with PBS (3x) and incubated with the biotinylated secondary antibody (rabbit-anti-rat, 1:200, Dakocytomation) in 1% bovine serum albumin/PBS for 45 min at room temperature. The slides were rinsed with PBS (3x) and the biotinylated proteins were visualized by incubation with streptavidin–biotin complex/horseradish peroxidase (Vectastain Elite ABC, Vector Laboratories) for 45 min at room temperature, followed by 0.015% H_2O_2/0.05% diaminobenzidene (Sigma, Schneldorf, Germany)/0.05 M Tris–HCl (pH 7.6) for 10 min at room temperature. Sections were counterstained with Mayers' hematoxylin (Merck), dehydrated and mounted in Permount (Fisher Scientific). Negative controls without the primary antibody were included as controls. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera [14].

Statistical analysis

Experimental results were expressed as mean ± S.E.M. Differences between groups were statistically determined by an unpaired two-tailed Student's *t*-test using GraphPad Prism (Version 4.0). Results were considered statistically significant when $P<0.05$.

Results

N-Ac-PGP-induced neutrophil infiltration in lung tissue is more pronounced than the influx into the bronchoalveolar lavage fluid

Oropharyngeal aspiration of N-Ac-PGP and CXCL1 resulted in a neutrophilic airway inflammation, since an increased number of neutrophils was observed in the bronchoalveolar lavage fluid of N-Ac-PGP- and CXCL1-treated mice compared to control mice (Fig. 1 and 2). However, N-Ac-PGP at a dose of 500 µg/mouse only induced 5% of the amount of neutrophils in bronchoalveolar lavage fluid compared to that observed after treatment with 0.5 µg CXCL1 per mouse. Next, we examined whether the appearance of neutrophils in bronchoalveolar lavage fluid represents the infiltration of neutrophils in the pulmonary tissue. Neutrophil accumulation in lung tissue was assessed by immunohistological analysis and quantitated by measuring MPO levels in lung tissue homogenate supernatants. Besides an increased neutrophil influx in the bronchoalveolar lavage fluid, oropharyngeal aspiration of N-Ac-PGP induced a neutrophil accumulation in the lung tissue (Fig. 3B) and increased MPO levels were observed in the lung homogenate supernatants of N-Ac-PGP-treated mice compared to control mice (Fig. 4). In addition, CXCL1 also induced a neutrophil accumulation in lung tissue (Fig. 3E) and elevated MPO levels were observed in supernatants of lung tissue homogenates of CXCL1-treated mice when compared to control mice (Fig. 5). When compared to CXCL1-induced infiltration of neutrophils in lung tissue, N-Ac-PGP was able to induce 31% compared to the amount of pulmonary tissue MPO levels observed after CXCL1 treatment.

N-Ac-PGP-induced neutrophil influx in the bronchoalveolar lavage fluid is significantly decreased after CXCR2 antagonist administration

Since CXCL1 is one of the main CXCR2 ligands in mice [15], CXCL1 was used as positive control for testing two CXCR2 antagonists: SB225002 and SB332235. SB225002 and SB332235 on their own had no effect on the number of neutrophils in bronchoalveolar lavage fluid of control mice (data not shown). Intraperitoneal administration of mice with either SB225002 or SB332235, given 1 h before and 1 h after oropharyngeal aspiration of CXCL1, inhibited the CXCL1-induced neutrophil influx in the bronchoalveolar lavage fluid with 42% and 72%, respectively (Fig. 2). To investigate whether N-Ac-PGP-induced neutrophil infiltration in bronchoalveolar lavage fluid is mediated via CXCR2, the two different CXCR2 antagonists (SB225002 and SB332235) were administered to N-Ac-PGP-treated mice. Intraperitoneal treatment of mice with SB225002 (50 µg/animal) inhibited the N-Ac-PGP-induced neutrophil influx in the bronchoalveolar lavage fluid with 77% (Fig. 1). Moreover, the N-Ac-PGP-induced neutrophil influx in the bronchoalveolar lavage fluid of mice was also

significantly decreased after different doses of SB332235 (20 µg, 100 µg and 300 µg/animal) (Fig. 1). The SB332235-induced inhibition was for all doses tested more than 90%.

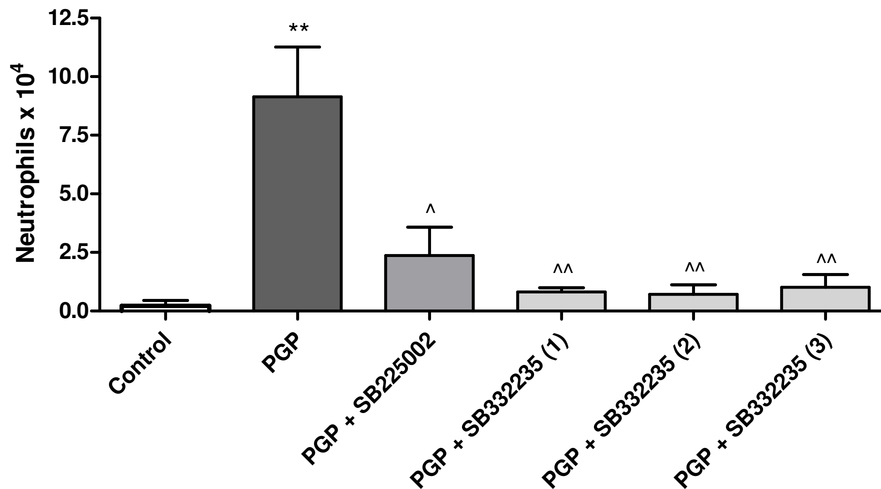


Fig.1. *N-Ac-PGP-induced neutrophil influx in the bronchoalveolar lavage fluid is significantly decreased after CXCR2 antagonist administration. Neutrophil numbers in the bronchoalveolar lavage fluid of C57Bl/6 mice after oropharyngeal aspiration of PBS or N-Ac-PGP (500 µg/70 µl PBS). The PBS and N-Ac-PGP-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (20 µg (1), 100 µg (2) and 300 µg (3)/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01; significantly different from the control group. ^P ≤ 0.05; ^^P ≤ 0.01 significantly different from the N-Ac-PGP-treated group.*

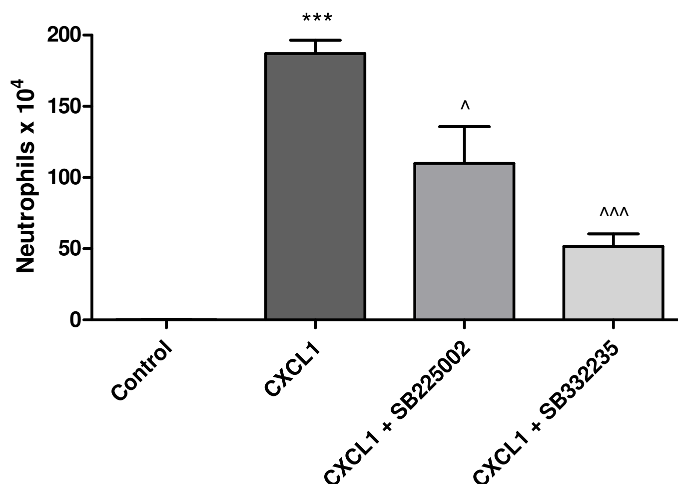


Fig.2. *CXCL1-induced neutrophil influx in the bronchoalveolar lavage fluid is significantly decreased after CXCR2 antagonist administration. Neutrophil numbers in the bronchoalveolar lavage fluid of C57Bl/6 mice after oropharyngeal aspiration of PBS or CXCL1 (0.5 µg/70 µl PBS). The PBS and CXCL1-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (300 µg/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group. ^P ≤ 0.05; ^^^P ≤ 0.001 significantly different from the CXCL1-treated group.*

N-Ac-PGP-induced neutrophil infiltration in pulmonary tissue is decreased after CXCR2 antagonist administration

Immunohistochemical analysis was performed to observe neutrophils in the lung tissue of PBS-, CXCL1- and N-Ac-PGP-treated animals with or without CXCR2 antagonist administration. As stated before, this immunological staining confirmed the results obtained from the MPO measurement in the lung homogenates, since an enormous neutrophil influx was observed in the lung tissue after oropharyngeal aspiration of CXCL1 (Fig. 3E) compared to the control mice (Fig. 3A). Furthermore, N-Ac-PGP also induced a neutrophil accumulation in the lung tissue (Fig. 3B).

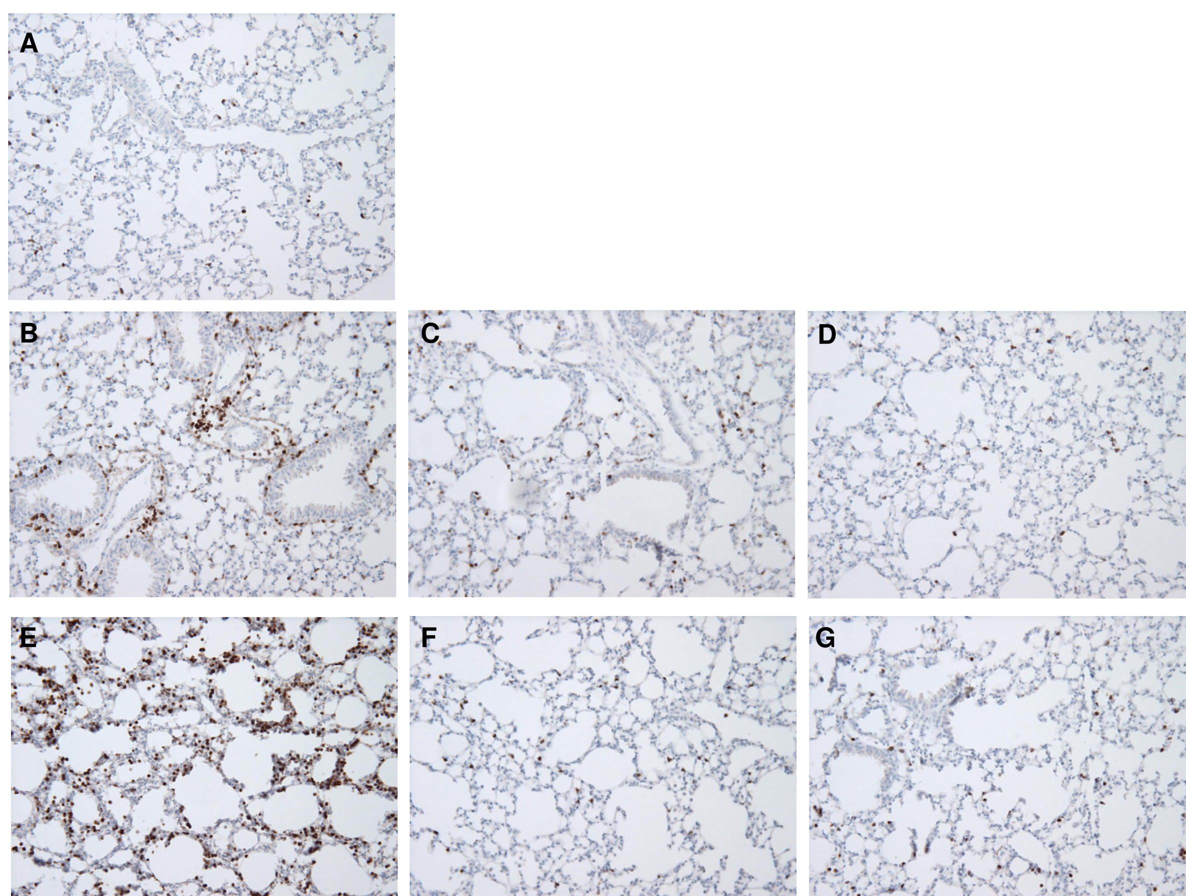


Fig.3. *N-Ac-PGP- and CXCL1-induced neutrophil influx in the lung is decreased after CXCR2 antagonist administration. Representative photomicrographs of an immunohistological staining for neutrophils (brown color, DAB staining) in lung tissue of PBS-treated mice (A), N-Ac-PGP-treated mice (B) and CXCL1-treated mice (E). The neutrophils in lung tissue of N-Ac-PGP-treated mice are decreased after i.p. administration with SB225002 (50 µg/animal) (C) or SB332235 (300 µg/animal) (D). The neutrophil accumulation in lung tissue of CXCL1-treated mice are inhibited by i.p. administration with SB225002 (50 µg/animal) (F) or SB332235 (100 µg/animal) (G). Magnification, 200x.*

The N-Ac-PGP-induced neutrophil infiltration was primarily located in peribronchial and perivascular areas in the airways, whereas CXCL1 induced a more scattered pattern throughout the pulmonary tissue (Fig. 3B and E). Intraperitoneal treatment of mice with SB225002 (50 µg/animal) reduced the amount of neutrophils in the lung tissue induced by CXCL1 (Fig. 3F). The increased neutrophil numbers induced by CXCL1 were also decreased after application of SB332235 (300 µg/animal) (Fig. 3G). Moreover, the N-Ac-PGP-induced neutrophil influx in the lung tissue was inhibited after treatment with as well SB225002 (50 µg/animal) (Fig. 3C) as SB332235 (100 µg/animal) (Fig. 3D). SB225002 and SB332235 on their own had no effect on the neutrophil influx in pulmonary tissue of control mice (data not shown).

N-Ac-PGP-induced increased MPO levels in the lung tissue homogenates are significantly decreased after CXCR2 antagonist administration

In addition to the examination of the neutrophil influx in bronchoalveolar lavage fluid, the effect of the two CXCR2 antagonists was investigated on the N-Ac-PGP- and CXCL1-induced neutrophil infiltration into the lung tissue by determining MPO levels in lung tissue homogenate supernatants. SB225002 and SB332235 had no effect on basal MPO levels in lung tissue of PBS-treated mice (data not shown).

Again, CXCL1 was used as positive control for testing two CXCR2 antagonists. Treatment of mice with SB225002 (50 µg/animal) inhibited the increased MPO levels in lung homogenates with 62% induced by CXCL1 (Fig. 5). Moreover, after application of SB332235 (300 µg/animal), the increased MPO levels induced by CXCL1 were also significantly decreased by 54% (Fig. 5).

Treatment of mice with SB225002 (50 µg/animal) completely abolished the increased MPO levels in lung tissue homogenate supernatants induced by N-Ac-PGP (Fig. 4). Furthermore, the increased MPO levels induced by N-Ac-PGP were also decreased after different doses of SB332235 (20 µg, 100 µg and 300 µg/animal) by 66%, 88% and 42%, respectively. The most pronounced effect in reducing the MPO levels by the CXCR2 antagonist SB332235 appeared after administration of the 100 µg dose (Fig. 4).

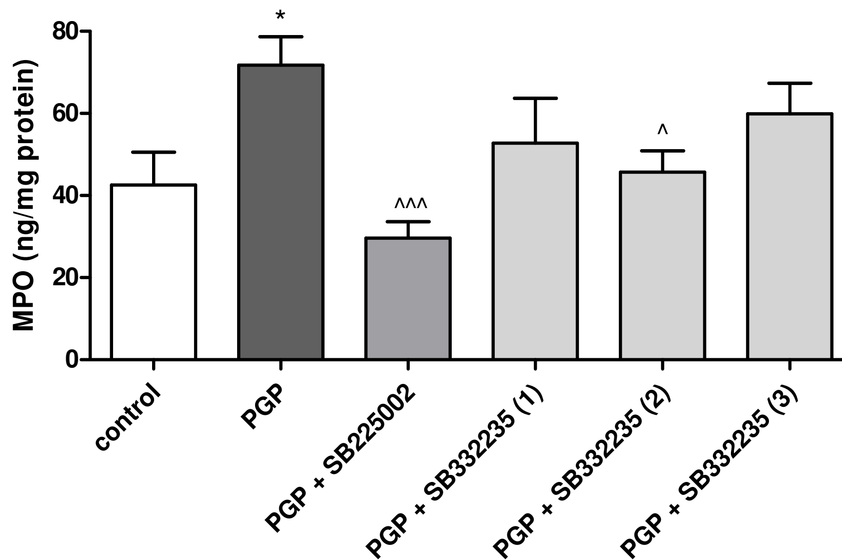


Fig.4. *N-Ac-PGP-induced MPO levels in the lung homogenates are decreased after CXCR2 antagonist administration. MPO levels in lung homogenates of C57Bl/6 mice after oropharyngeal aspiration of PBS or N-Ac-PGP (500 µg/70 µl PBS). The PBS and N-Ac-PGP-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (20 µg (1), 100 µg (2) and 300 µg (3)/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± S.E.M. *P ≤ 0.05; significantly different from the control group. ^P ≤ 0.05; ^^^P ≤ 0.001 significantly different from the N-Ac-PGP-treated group.*

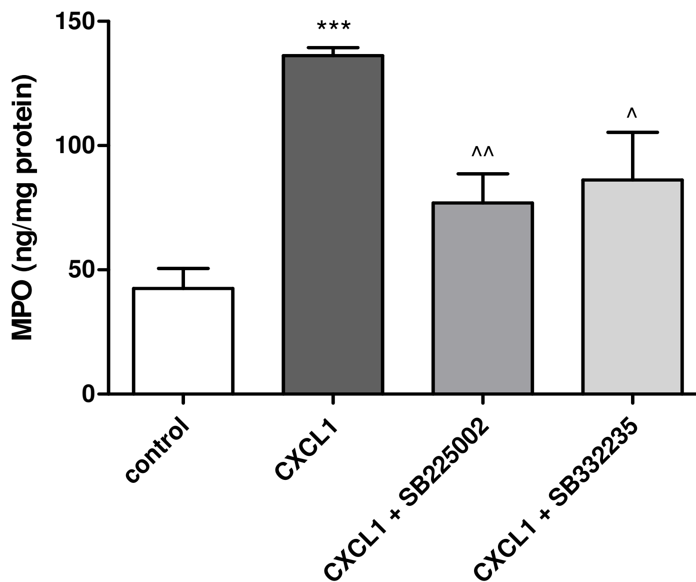


Fig.5. *CXCL1-induced MPO levels in the lung homogenates are significantly decreased after CXCR2 antagonist administration. MPO levels in lung homogenates of C57Bl/6 mice after oropharyngeal aspiration of PBS or CXCL1 (0.5 µg/70 µl PBS). The PBS and CXCL1-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (300 µg/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group. ^P ≤ 0.05; ^^P ≤ 0.01 significantly different from the CXCL1-treated group.*

CXCR2 antagonists did not significantly attenuate the N-Ac-PGP-induced CXCL1 release in the bronchoalveolar lavage fluid

The levels of the chemokine CXCL1 were measured in the bronchoalveolar lavage fluid of PBS and N-Ac-PGP-treated mice with or without administration of the CXCR2 antagonists SB225002 or SB332235. The concentrations of CXCL1 were significantly elevated in the bronchoalveolar lavage fluid of N-Ac-PGP-treated mice compared to the PBS-treated mice (Fig. 6). The N-Ac-PGP-induced CXCL1 levels in the bronchoalveolar lavage fluid were not significantly decreased after treatment with the CXCR2 antagonist SB225002 (50 µg/animal). Moreover, the N-Ac-PGP-induced CXCL1 levels were not reduced after applications of different doses of SB332235 (20 µg, 100 µg and 300 µg/animal) (Fig. 6). Although at a dose of 100 µg, SB332235 demonstrated a small but not significant reduction of N-Ac-PGP-induced CXCL1 release in bronchoalveolar lavage fluid.

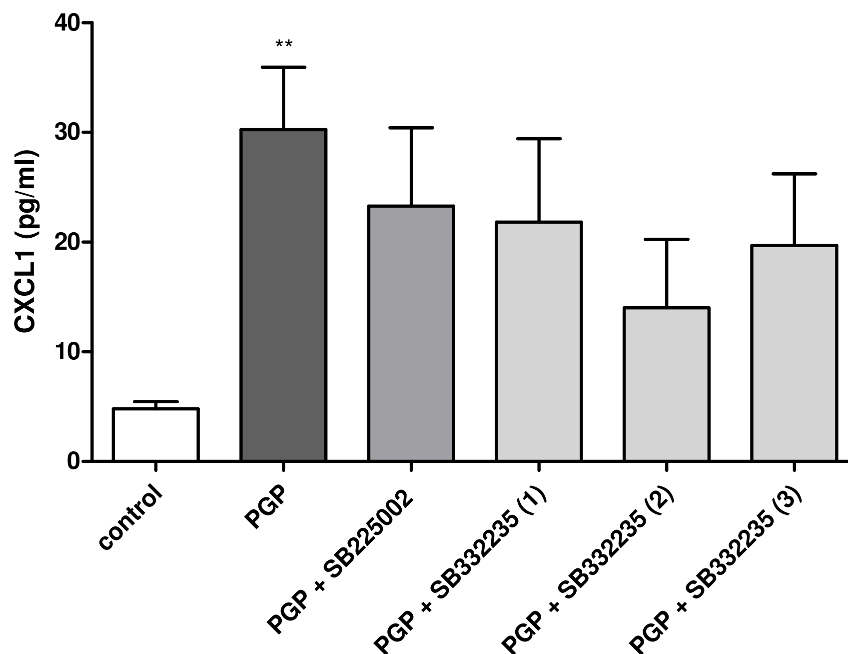


Fig.6. *CXCR2 antagonists do not significantly affect N-Ac-PGP-induced CXCL1 release in the bronchoalveolar lavage fluid. Levels of the chemokine CXCL1 in the bronchoalveolar lavage fluid of C57Bl/6 mice after oropharyngeal aspiration of PBS or N-Ac-PGP (500 µg/70 µl PBS). The PBS and N-Ac-PGP-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (20 µg (1), 100 µg (2) and 300 µg (3)/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01; significantly different from the control group.*

Discussion

The aim of this study was to investigate the role of CXCR2 in neutrophilic airway inflammation induced by N-Ac-PGP. Neutrophils are the first cells to be recruited to the site of inflammation in response to chemoattractants, such as CXCL8 in humans and CXCL1 and CXCL2 in mice [1]. In chronically inflamed tissues, neutrophilic mediator release leads to extracellular matrix breakdown and subsequently to the formation of collagen fragments with chemotactic properties, such as N-Ac-PGP [16-18]. It has been demonstrated that N-Ac-PGP is chemotactic for neutrophils *in vitro* as well as *in vivo* [3, 4, 19, 20]. The importance of N-Ac-PGP in chronic lung diseases is pointed out in different murine and human studies [3, 5, 6]. It has been proposed that N-Ac-PGP acts as a neutrophilic chemoattractant via CXCR1 and CXCR2 *in vitro* and *in vivo* [3, 4].

The most important murine ligands for CXCR2 in the early state of inflammation are CXCL1 and CXCL2 and these ligands bind with high affinity to mouse CXCR2, whereas the affinity for CXCR1 is low [3, 21-23]. Upon stimulation with cigarette smoke, the major risk factor in COPD, neutrophils are major producers of CXCL1 and CXCL2 [24]. Since CXCL1 levels are more prominent than CXCL2 levels in airway inflammation [3], CXCL1 was chosen in this study as a positive control. The effect of oropharyngeal aspiration of CXCL1 in mice lungs was tested and a significant increased neutrophilic airway accumulation was observed in the bronchoalveolar lavage fluid and lung tissue and the MPO levels in the supernatant of lung tissue homogenates were increased. This finding is in agreement with the results of Frevert et al. [8], who demonstrated a dose dependent increase of neutrophils in the bronchoalveolar lavage fluid of rats after intratracheal administration of recombinant CXCL1.

N-Ac-PGP mimicked CXCL1 in attracting neutrophils into the bronchoalveolar lavage fluid 6 h after oropharyngeal aspiration. This observation is in accordance with the study of Van Houwelingen et al. [6], who described that intratracheal N-Ac-PGP administration provoked neutrophilic airway inflammation that was dependent on the N-Ac-PGP concentration and the time after application. Besides an increase in neutrophil numbers in bronchoalveolar lavage fluid caused by N-Ac-PGP administration, we also observed a neutrophil accumulation in the lung tissue of N-Ac-PGP-treated mice as measured indirectly by MPO in lung tissue homogenates and directly via an immunohistological staining for neutrophils. The histological lung sections of the N-Ac-PGP-treated animals demonstrated an increase in neutrophils in mainly peribronchial and perivascular areas in the airways while histological lung sections of CXCL1-treated mice showed a more diffused neutrophilic distribution throughout the airways.

In general, the N-Ac-PGP-induced neutrophil levels in bronchoalveolar lavage fluid and lung tissue were less pronounced than the neutrophil levels measured after oropharyngeal

aspiration of CXCL1. An explanation for this can be that N-Ac-PGP contacts a single site on the CXCR2 [3], whereas CXCL1 contacts multiple binding sites on the receptor [25]. Strikingly, N-Ac-PGP was more potent at inducing neutrophil infiltration in lung tissue than in bronchoalveolar lavage fluid, when related to the maximal effect of CXCL1. An alternative explanation is that N-Ac-PGP may be more potent at inducing MPO levels in the lung tissue. When compared to the CXCL1-induced influx of neutrophils in pulmonary tissue and bronchoalveolar lavage fluid, N-Ac-PGP treatment resulted in a 31% and only 5% of the CXCL1 response, respectively.

This study confirmed that N-Ac-PGP has chemotactic activities as measured by the neutrophils in the bronchoalveolar lavage fluid and lung tissue. Furthermore, we show that CXCL1 levels in the bronchoalveolar lavage fluid of N-Ac-PGP-treated mice were significantly enhanced compared to control mice, suggesting that N-Ac-PGP may indirectly initiate a continuous inflammatory response in the lung caused by the release of CXCL1, probably by infiltrated macrophages and neutrophils in bronchoalveolar lavage fluid. However, the CXCL1 levels in bronchoalveolar lavage fluid induced by N-Ac-PGP were more than 10,000 fold lower than the amount CXCL1 used for inducing neutrophil migration into the lung. Therefore, it is unlikely that N-Ac-PGP is acting via CXCL1.

The N-Ac-PGP- as well as the CXCL1-induced neutrophil infiltration in the pulmonary tissue and the influx in the bronchoalveolar lavage fluid were decreased by the CXCR2 antagonists SB225002 and SB332235. Both CXCR2 antagonists were equally effective in reducing the CXCL1-induced neutrophil accumulation in pulmonary tissue and seemed to be more potent at inhibiting the N-Ac-PGP response in the lung tissue as well as in the bronchoalveolar lavage fluid. SB225002 was more effective in inhibiting N-Ac-PGP-induced pulmonary tissue neutrophilia than SB332235, since lower as well as higher doses of SB332235 did not lead to the reduction in MPO levels as observed after SB225002 administration. Contrary, with regard to the N-Ac-PGP-induced neutrophil influx in bronchoalveolar lavage fluid, SB332235 appears to be more effective than SB225002, since lower and higher doses of SB332235 caused a more pronounced effect.

The effect of the selective antagonists SB225002 and SB332235 has been described in other inflammatory diseases where neutrophils play a major role. Application of SB225002 resulted in decreased alveolar neutrophil recruitment in mice suffering from a pneumococcal lung infection [11] and this CXCR2 antagonist was able to attenuate the inflammatory cell influx and tissue damage in a murine colitis model [26]. In addition, SB225002 selectively blocked CXCL8-induced neutrophil chemotaxis in ears of rabbits [27]. *In vitro*, SB225002 potently inhibited human and rabbit CXCL8-induced neutrophil chemotaxis [27, 28]. SB332235 effectively inhibited cigarette smoke-induced neutrophilia in a dose-dependent

manner in rats [29]. Moreover, it was reported that SB332235 can inhibit acute and chronic models of arthritis in rabbits [30] and an inhibition of CXCL8 neutrophil migration into the ear of hCXCR2 knock-in mice was observed after SB332235 administration [12]. To our knowledge, we are the first to show that the CXCR2 antagonists affect the neutrophilic airway inflammation induced by CXCL1 and N-Ac-PGP as indicated by the decreased neutrophil influx in the lung tissue (observed by the reduced MPO levels in the lung tissue homogenates and the immunohistological staining for neutrophils) as well as in the bronchoalveolar lavage fluid. This shows that the observed N-Ac-PGP-induced neutrophil influx is mediated via CXCR2. A role for CXCR2 in N-Ac-PGP-induced neutrophilic airway inflammation has been confirmed by other studies, since antibodies directed against CXCR2 suppressed the N-Ac-PGP-induced neutrophilic chemotaxis *in vitro* [3, 4]. Furthermore, the accumulation of neutrophils seen in mice upon intratracheal administration of N-Ac-PGP was not detected in $CXCR2^{-/-}$ mice [3].

We also show that oropharyngeal aspiration of N-Ac-PGP induces a CXCL1 release *in vivo*. This is in agreement with our *in vitro* experiments, where we demonstrated that human neutrophils release CXCL8 after N-Ac-PGP incubation [4]. In that study, it was demonstrated that the N-Ac-PGP-induced release of CXCL8 was not responsible for the N-Ac-PGP-induced chemotaxis. Although, the concentration of CXCL1 (30 pg/ml) observed in the bronchoalveolar lavage fluid after N-Ac-PGP administration is much lower than the concentration realized after oropharyngeal aspiration of CXCL1 (0.5 μg/70 μl), we can not exclude from our studies that a small part of the N-Ac-PGP induced neutrophil infiltration is via the release of CXCL1. Finally, administration of CXCR2 antagonists SB225002 and SB332235 did not result in significantly decreased N-Ac-PGP-induced CXCL1 levels in the bronchoalveolar lavage fluid.

In summary, it can be concluded that the N-Ac-PGP-induced neutrophil influx in lung tissue and bronchoalveolar lavage fluid can be inhibited by CXCR2 antagonists, showing that the neutrophil migration induced by N-Ac-PGP is mediated via direct interaction with CXCR2 and by a small, if any, release of CXCL1. The N-Ac-PGP-induced release of CXCL1 is independent of the CXCR2. Related to the maximal effect of CXCL1, N-Ac-PGP is more potent at inducing the neutrophil migration in the pulmonary tissue than into the bronchoalveolar lavage fluid. In addition, N-Ac-PGP may be more potent at inducing MPO levels in the lung tissue.

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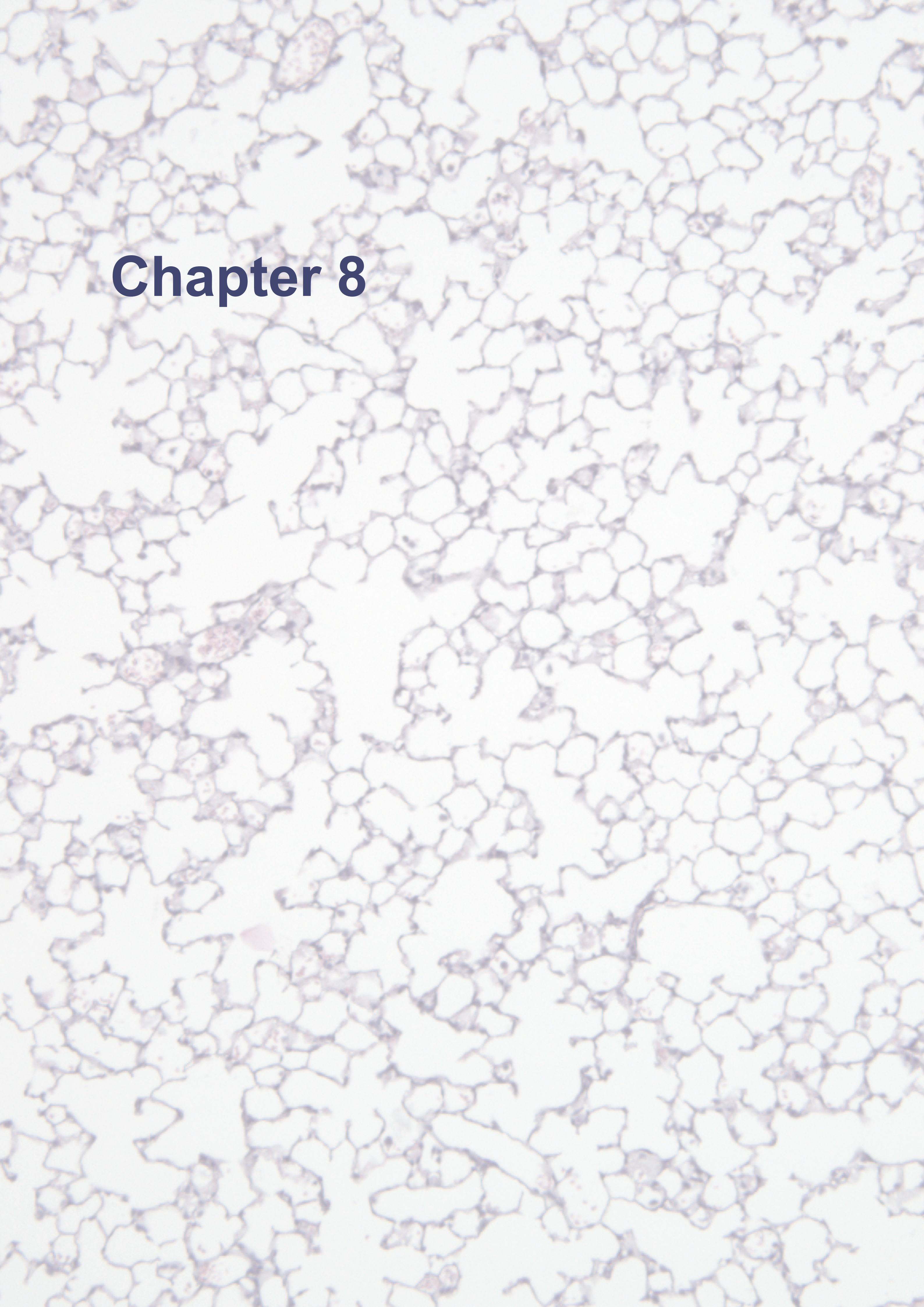

Chapter 8

Inflammatory changes in the airways of mice caused by cigarette smoke exposure are only partially reversed after smoking cessation

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(additional data included)

Abstract

Tobacco smoking irritates and damages the respiratory tract and contributes to a higher risk of developing lung emphysema. At present, smoking cessation is the only effective treatment for reducing the progression of lung emphysema, however, there is hardly anything known about the effects of smoking cessation on cytokine and chemokine levels in the airways. To the best of our knowledge, this is the first reported *in vivo* study in which cytokine profiles were determined after cessation of cigarette smoke exposure.

The severity of airway remodeling and inflammation was studied by analyzing alveolar enlargement, heart hypertrophy, inflammatory cells in the bronchoalveolar lavage fluid (BALF) and lung tissue and by determining the cytokine and chemokine profiles in the BALF of A/J mice exposed to cigarette smoke for 20 weeks and 8 weeks after smoking cessation.

The alveolar enlargement and right ventricle heart hypertrophy found in smoke-exposed mice remained unchanged after smoking cessation. Although the neutrophilic inflammation in the BALF of cigarette smoke-exposed animals was reduced after smoking cessation, a sustained inflammation in the lung tissue was observed. The elevated cytokine (IL-1α and TNF-α) and chemokine (CCL2 and CCL3) levels in the BALF of smoke-exposed mice returned to basal levels after smoking cessation, while the increased IL-12 levels did not return to its basal level. The cigarette smoke-enhanced VEGF levels did not significantly change after smoking cessation. Moreover, IL-10 levels were reduced in the BALF of smoke-exposed mice and these levels were still significantly decreased after smoking cessation compared to the control animals.

The inflammatory changes in the airways caused by cigarette smoke exposure were only partially reversed after smoking cessation. Although smoking cessation should be the first step in reducing the progression of lung emphysema, additional medication could be provided to tackle the sustained airway inflammation.

Introduction

There are currently more than 1.3 billion tobacco smokers worldwide according to the World Health Organization (WHO) [1]. Cigarette smoke contains more than 4000 hazardous chemical compounds, of which 200 are highly toxic [2]. It is generally accepted that cigarette smoking is the most important risk factor for the development and progression of chronic obstructive pulmonary disease (COPD) and accounts for about 80% of COPD cases [3, 4]. COPD, a term referring to two lung diseases: chronic bronchitis and emphysema, is characterized by an airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases [5]. Pulmonary hypertension and right ventricular failure are also often associated with COPD [6, 7]. Since a chronic airway inflammation with alveolar wall destruction and airway remodeling is central to the pathogenesis of COPD, it is not surprising that several types of inflammatory cells play a role in this condition [8]. Increased numbers of macrophages and neutrophils are observed in sputum and bronchoalveolar lavage fluid (BALF) of COPD patients [9-11]. In addition, COPD patients have elevated levels of T lymphocytes, in particular CD8+ cells, in lung parenchyma and airways [11-14]. Migration and activation of inflammatory cells to the lung is regulated by the release of different mediators, including proteases, cytokines and chemokines secreted by a variety of inflammatory and resident cells. These mediators contribute to the chronic inflammatory process with tissue damage and repair processes seen in emphysema [15, 16]. Several cytokines and chemokines have been implicated in the airway inflammation in COPD. Increased levels of interleukin-8 (IL-8), interleukin-12 (IL-12), tumour-necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1; CCL-2), and macrophage inflammatory protein-1α (MIP-1α; CCL3) have been observed in COPD patients [9, 17-21].

In general, the treatments available for COPD reduce the number and severity of exacerbations and relieve symptoms, but do not tackle the cause of the disease and have a limited effect on slowing down the progression of lung damage [22]. At present, smoking cessation is the only effective treatment for avoiding or reducing the progression of COPD [23]. However, there is contradictory evidence regarding the effect of smoking cessation on airway inflammation associated with COPD. Several studies in COPD patients reported that smoking cessation improves respiratory symptoms, reduces loss of pulmonary function and decreases lung inflammation [24-28], while other studies have shown that smoking cessation fails to reverse the chronic airway inflammation [29-32]. Unfortunately, there is insufficient evidence regarding the effects of smoking cessation on cytokine and chemokine levels, which do play an important role in airway inflammation and tissue remodeling seen in COPD. Therefore, a murine model of cigarette smoke-induced lung emphysema was used to

investigate the effect of smoking cessation on airway remodeling and pulmonary inflammation. The severity of airway remodeling and inflammation was studied by determining alveolar enlargement, heart hypertrophy, inflammatory cells in the bronchoalveolar lavage fluid (BALF) and lung tissue and by analyzing the cytokine and chemokine profiles in the BALF of mice exposed to cigarette smoke for 20 weeks and 8 weeks after smoking cessation.

Material and methods

Animals

Female A/J mice, 9-14 weeks old (Charles River Laboratories) were housed under controlled conditions in standard laboratory cages. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the local Ethics Committee and were performed under strict governmental and international guidelines on animal experimentation.

Cigarette smoke exposure

Female A/J mice were divided into three groups. The first group was exposed to room air for 20 weeks, the second group was exposed to cigarette smoke for 20 weeks and the third group was exposed to cigarette smoke for 20 weeks followed by a period of 8 weeks without cigarette smoke exposure. 20-weeks-old mice are adult mice and should have almost no alveolar growth in the additional 8 weeks [33, 34].

In the life-span of a laboratory mouse 20 weeks smoking and 8 weeks smoking cessation represents approximately 21 years smoking and 8 years smoking cessation in humans. The mice were exposed in whole-body chambers to air (sham) or to diluted mainstream cigarette smoke from the reference cigarettes 2R4F (University of Kentucky, Lexington, Kentucky) using a smoking apparatus. Exposures were conducted 4h/day (with a 30/60-minute fresh air break after each hour of exposure), 5 days/week for 20 weeks to a target cigarette smoke concentration of 750 µg total particulate matter/l (TPM/l). This TPM concentration was reached after an adaptation period of 1 week, starting with a TPM concentration of 125 µg TPM/l. The mass concentration of cigarette smoke TPM was determined by gravimetric analysis of Cambridge filter samples. The carbon monoxide (CO) was monitored continuously and was around 800 ppm. The nicotine concentration in the smoke was approximately 40 µg/l. The sample sites were located in the middle of the exposure chamber at the breathing zone. The mice were sacrificed 16-24 hours after the last air or smoke exposure, or after the smoke-free period of 8 weeks.

Histology and morphometric analysis

Mice (n=4-5), used for morphometric analysis, were sacrificed by an i.p. injection with an overdose of pentobarbital (Nembutal™, Ceva Santé Animale, Naaldwijk, The Netherlands). The lungs were fixated with a 10% formalin infusion through the tracheal cannula at a constant pressure of 25 cm H_2O. After excision, the volume of the fixed lungs was measured by fluid displacement. Then, the left lung was immersed in fresh fixative for at least 24h, after which it was embedded in paraffin. After paraffin embedding, 5 µm sections were cut and stained with hematoxylin/eosin (H&E) according to standard methods. These histological lung

sections were used to determine lung inflammation and pigmented macrophages. Lung inflammation was scored by a treatment-blind observer. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0-3, as described elsewhere [35, 36]. A value of 0 was assigned when no inflammation was detectable, a value of 1 was adjudged for occasional cuffing with inflammatory cells, a value of 2 when most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and a value of 3 was given when most bronchi or vessels were surrounded by a thick layer (more than five cells thick) of inflammatory cells. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Four lung sections per mouse were scored and inflammation scores were expressed as a mean value.
Morphometric assessment of emphysema, included determination of the average inter-alveolar distance, was estimated by the mean linear intercept (Lm) analysis. The Lm was determined by light microscopy at a total magnification of 100x, whereby 24 random photomicroscopic images per left lung tissue section were evaluated by microscopic projection onto a reference grid. By dividing total grid length by the number of alveolar wall-grid line intersections, the Lm (in µm) was calculated [37].

Bronchoalveolar lavage

Immediately after i.p. injection with an overdose of pentobarbital, the lungs of a separate group mice (n=4-5) were lavaged 4 times through a tracheal cannula with 1 ml saline (NaCl 0.9%), pre-warmed at 37℃. The first lavage was performed with 1 ml saline containing a mixture of protease inhibitors (Complete Mini, Roche Applied Science, Penzberg, Germany). After centrifuging the bronchoalveolar lavage fluid at 4℃ (400 g, 5 min), the supernatant of the first ml was used for cytokine analysis and the cell pellets of the 4 lavages were used for cell counts. The 4 cell pellets, kept on ice, were pooled per animal and resuspended in 150 µl cold saline. After staining with Türk solution, total cell counts per lung were made under light microscopy using a Burker-Turk chamber. Differential cell counts were performed on cytospin preparations stained by DiffQuick™(Dade A.G., Düdingen, Switzerland). Cells were identified as macrophages, neutrophils and lymphocytes according to standard morphology. At least 200 cells were counted and the absolute number of each cell type was calculated.

Cotinine ELISA

Directly after the i.p. injection with an overdose of pentobarbital, blood was obtained by heart puncture and collected in MiniCollect Z Serum Sep tubes (Greiner Bio-one, part no.450472). After 1 hour the blood samples were centrifuged for 10 min at 14.000 rpm and the sera were stored at -20˚C for cotinine measurement. Cotinine levels were measured in serum and BALF

by ELISA using the mouse/rat cotinine ELISA kit (Calbiotech, CA, USA) according to manufacturer's instructions.

Right ventricular hypertrophy measurement

The right ventricle was removed from lower heart after removal of the atria. The right ventricle and the left ventricle plus septum were weighed and the ratio of the weights was calculated as follows: (right ventricle)/(left ventricle + septum) [38, 39].

Measurement of cytokines and chemokines

A standard mouse cytokine 20-plex assay was used to determine cytokine and chemokine concentrations in the BALF (n=4-5) according to the manufacturer's instructions (Luminex; Biosource, Invitrogen, Breda, The Netherlands). The most relevant cytokines and chemokines (IL-1α, IL-10, IL-12, TNF-α, CCL2, CCL3, VEGF and macrophage inflammatory protein-2 (MIP-2; CXCL2)) were discussed in this study. The concentrations of these cytokines and chemokines were expressed as pg/ml BALF.

Statistical analysis

Experimental results were expressed as mean ± S.E.M. Differences between groups were statistically determined by an unpaired two-tailed Student's *t*-test using GraphPad Prism (Version 4.0). Results were considered statistically significant when $P < 0.05$.

Results

Increased cotinine levels after cigarette smoke exposure

Cotinine is the principal metabolite of nicotine and is a reliable indicator of smoke exposure. To confirm adequate cigarette smoke exposure, cotinine levels were measured in serum and BALF of air- and smoke-exposed mice and in smoke-exposed mice after a smoking cessation period of 8 weeks. Mice exposed to cigarette smoke for 20 weeks had significantly increased serum (Fig. 1A) and BALF (Fig. 1B) cotinine levels compared to the air-exposed mice. These cotinine levels returned completely towards basal levels after smoking cessation (Fig. 1A and B).

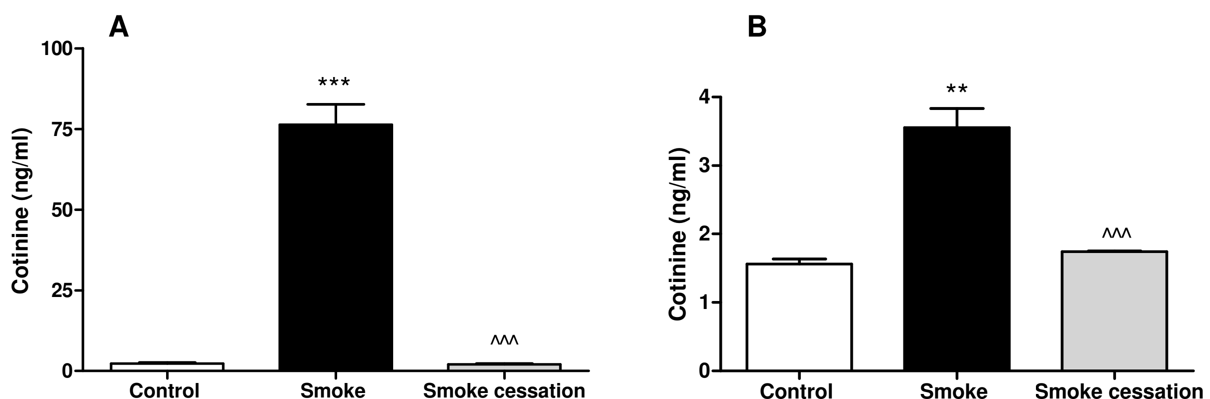

Fig.1. *Increased cotinine levels after cigarette smoke exposure. The cotinine levels were determined after 20 weeks air exposure (white bar), after 20 weeks smoke exposure (black bar) and after 20 weeks smoke exposure plus a smoking cessation period of 8 weeks (grey bar) in serum (A) and BALF (B). n = 3-5 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01, ***P ≤ 0.001; significantly different from the control group. ^^^P ≤ 0.001; significantly different from the smoke group.*

Alveolar enlargement induced by cigarette smoke exposure is irreversible

The histological lung sections of the smoke-exposed mice showed an increased air space enlargement and destruction (Fig. 2B) compared with the air-exposed mice (Fig. 2A). The alveolar enlargement is still present after a smoking cessation period of 8 weeks (Fig. 2C). The mean linear intercept, a quantification method for alveolar size, was used to quantify the presence and severity of emphysema [37]. Significant airspace enlargement was observed in mice after 20 weeks exposure to cigarette smoke (Fig. 2D). Furthermore, airspace enlargement induced by cigarette smoke exposure was not reversible, since the increase in Lm was not significantly reduced after a period of 8 weeks without exposure to cigarette smoke (Fig. 2D).

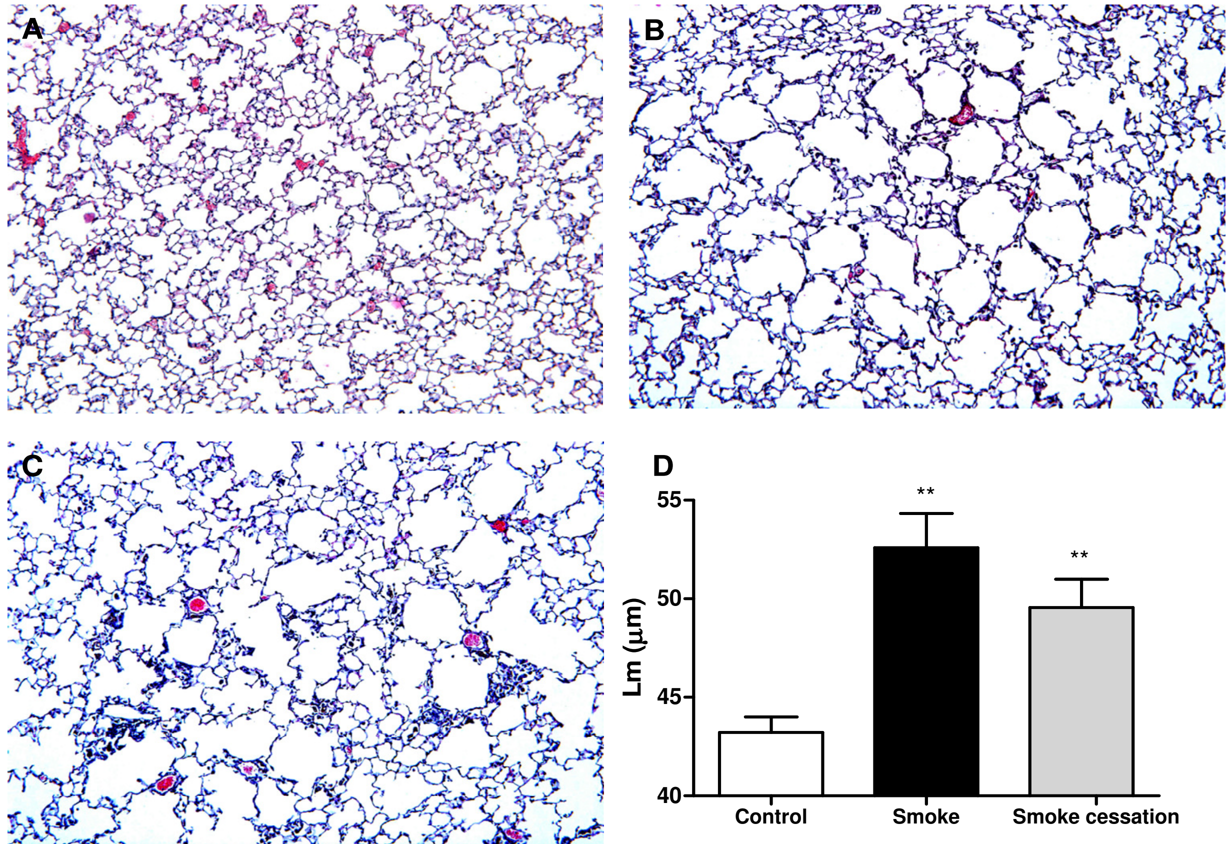

Fig.2. *Cigarette smoke-induced alveolar enlargement is irreversible. Representative photomicrographs of hematoxylin and eosin stained lung tissue of air-exposed mice (A), smoke-exposed mice (B), smoke-exposed mice 8 weeks after smoking cessation (C). Magnification, 100x. Mean linear intercept (Lm) values of mice exposed to air (white bar), mice exposed to cigarette smoke for 20 weeks (black bar) and mice exposed to cigarette smoke for 20 weeks plus a smoking cessation period of 8 weeks (grey bar) (D). n = 4-5 animals per group. Values are expressed as mean ± S.E.M. **P ≤ 0.01; significantly different from the control group.*

Right ventricle heart hypertrophy related to cigarette smoke exposure is irreversible

Twenty weeks cigarette smoke exposure caused right ventricular heart hypertrophy (Fig. 3). The right ventricular mass was proportionally greater than the rest of the lower heart (left ventricle and septum) in smoke-exposed mice compared to air-exposed mice. Moreover, right ventricle heart hypertrophy was not reversible after a period of 8 weeks without cigarette smoke exposure, because the heart hypertrophy ratio (RV/(LV +S)) was not significantly decreased in the smoking cessation group compared to smoke-exposed group.

Fig.3. *Cigarette smoke-induced right ventricle heart hypertrophy is irreversible. Right ventricle (RV) and left ventricle (LV) + septum (S) were dissected after 20 weeks air exposure (white bar), after 20 weeks smoke exposure (black bar) and after 20 weeks smoke exposure plus a smoking cessation period of 8 weeks (grey bar) to determine their weight ratio (RV(LV+S)). n = 6-7 animals per group. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group.*

Lung volume increase after cigarette smoke exposure is irreversible after smoking cessation

It has been demonstrated that chronic inflammation in the airways ultimately leads to alveolar enlargement, increased pulmonary compliance as well as enhanced lung volumes [40]. We measured the lung volumes in the murine lung emphysema model and the lung volume was significantly increased in mice exposed to cigarette smoke for 20 weeks compared to the control mice (Fig. 4). After a period of 8 weeks without cigarette smoke exposure, the lung volume was still significantly enhanced compared to the control group.

Fig.4. *Lung volume increase after cigarette smoke exposure is not reversible after smoking cessation. The relative lung volume was measured by fluid displacement. The relative lung volumes were determined after 20 weeks air exposure (white bar), after 20 weeks smoke exposure (black bar) and after 20 weeks smoke exposure plus a smoking cessation period of 8 weeks (grey bar). n = 4-5 animals per group. Values are expressed as mean ± S.E.M. *P ≤ 0.05; significantly different from the control group.*

Smoking cessation reduces the inflammatory cell influx in bronchoalveolar lavage fluid

Progression of COPD is associated with the accumulation and activation of inflammatory cells in the BALF. In the present lung emhysema model, the total number of inflammatory cells was 5-fold increased in the BALF after 20 weeks of cigarette smoke exposure (Table 1). Differential cell counts demonstrated that most of the cells in the BALF of the air-exposed mice were macrophages, with a few neutrophils and lymphocytes. The number of all these inflammatory cells in the BALF was significantly increased after cigarette smoke exposure, especially the neutrophils. Cigarette smoke exposure also affected the BALF cell composition, since there was a shift observed from mainly macrophages in the control animals towards neutrophils in the BALF of smoke-exposed mice. After smoking cessation of 8 weeks, we found a significant decline in inflammatory cells in the BALF, although the total cell number was still significant different compared to the control group (Table 1). First, the amount of neutrophils was strongly reduced after smoking cessation, but these cell numbers were still significantly increased compared to the control mice. The macrophages were also decreased compared to the smoke-exposed mice, however these numbers were not returned to basal levels. Finally, the cigarette smoke-induced increase of lymphocytes was not changed after cessation of cigarette smoke exposure. These results indicate that smoking cessation leads to a reduction in inflammatory cell types and a change in cell composition in the BALF, mainly caused by a decline in neutrophils.

Table 1. *Immune cells in BALF recovered from air-exposed mice, smoke-exposed mice and smoke-exposed mice 8 weeks after smoking cessation*

	CONTROL	**SMOKE**	**SMOKE CESSATION**
Total cell count, x 10^4	30.0 ± 3.2	140.4 ± 2.6 ***	52.8 ± 5.0 ** ^^^
Differential cell count, x 10^4			
Macrophages	29.2 ± 3.1	56.1 ± 1.1 ***	42.4 ± 4.2 * ^
Neutrophils	0.27 ± 0.1	79.9 ± 3.5 ***	6.1 ± 0.5 *** ^^^
Lymphocytes	0.51 ± 0.1	4.4 ± 1.0 *	4.4 ± 1.0 *

*n = 4-5 animals per group. Values are expressed as mean ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; significantly different from the control group. ^P ≤ 0.05, ^^^ P ≤ 0.001; significantly different from the smoke group.*

Lung inflammation is still present in lung tissue after smoking cessation

Histological lung sections demonstrated that pulmonary inflammation with peribronchial and perivascular inflammatory cell infiltrates was present in the airways of smoke-exposed mice (Fig. 5B). The air-exposed animals had no detectable lung inflammation (Fig. 5A). The smoking cessation group showed that the peribronchial and perivascular airway inflammation was still present after a smoke-free period of 8 weeks (Fig. 5C), since there was no notable difference in the leukocyte aggregates compared to those found in smoke-exposed lungs. The scores of peribronchial, perivascular and total lung inflammation were significantly increased after 20 weeks cigarette smoke exposure compared to air-exposed mice and these scores were still significantly enhanced after a smoking cessation period of 8 weeks (Fig. 5D). Moreover, there was an accumulation of brown-pigmented macrophages in lung tissue of smoke-exposed mice (Fig. 6B) compared to the lung tissue of the control mice (Fig. 6A). These pigmented macrophages were still present after a smoking cessation period of 8 weeks (Fig. 6C).

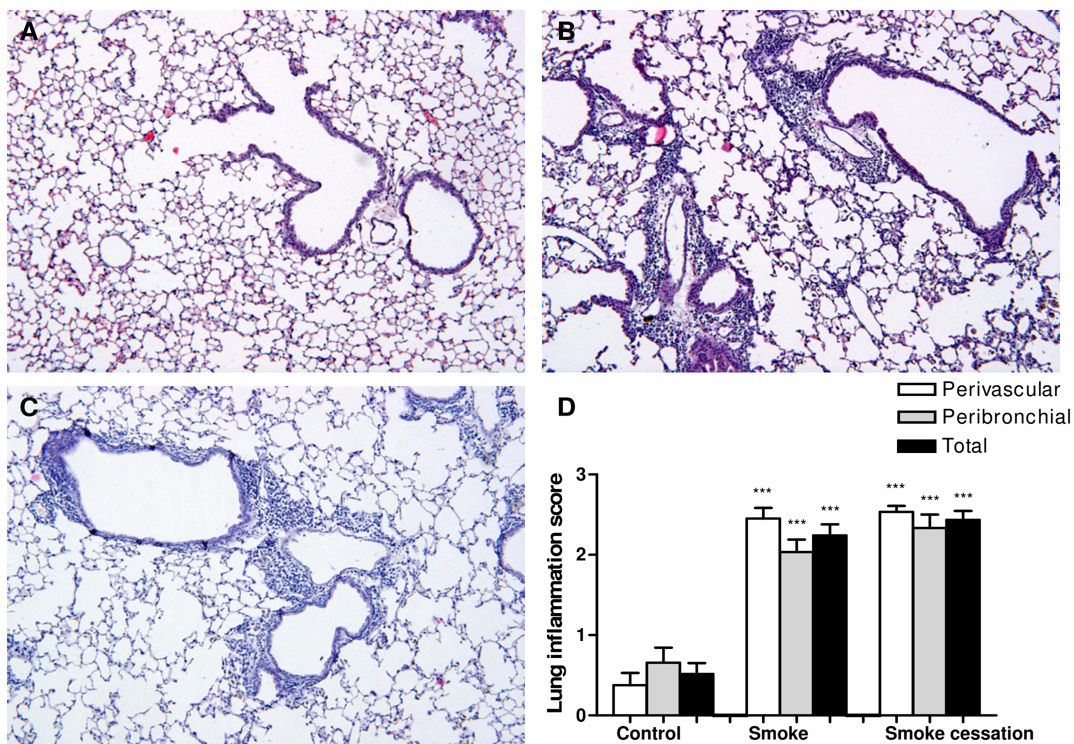

Fig.5. *Lung inflammation is still present in lung tissue after smoking cessation. Representative photomicrographs of hematoxylin and eosin stained lung tissue of air-exposed mice (A), smoke-exposed mice (B), smoke-exposed mice 8 weeks after smoking cessation (C). Magnification, 100x. The histological sections were scored for the presence of peribronchial and perivascular inflammation (D). Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. n = 4-5 animals per group. Values are expressed as mean ± S.E.M. ***P ≤ 0.001; significantly different from the control group.*

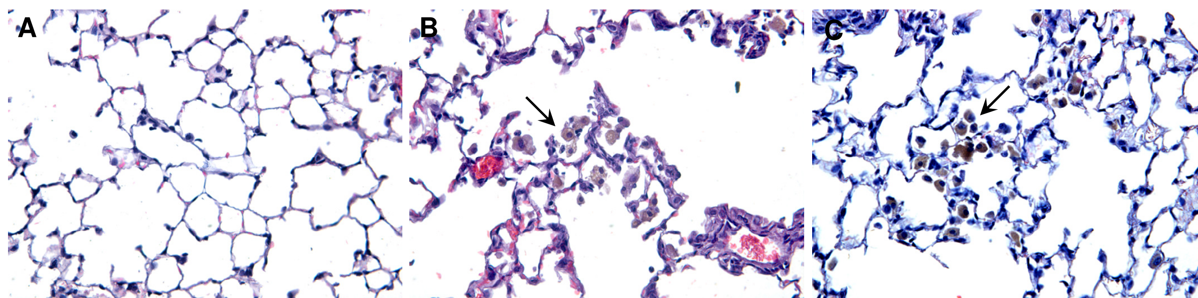

Fig.6. *Pigmented macrophage accumulation in the lung tissue before and after smoking cessation. Representative photomicrographs of hematoxylin and eosin stained lung tissue of air-exposed mice (A), smoke-exposed mice (B), smoke-exposed mice 8 weeks after smoking cessation (C). n = 4-5 animals per group. Magnification, 400x.*

The effect of smoking cessation on smoke-induced changes in cytokine and chemokine levels in BALF

The levels of different cytokines and chemokines (IL-1α, IL-10, IL-12, TNF-α, CCL2, CCL3 and VEGF) were measured in the BALF of control mice and in smoke-exposed mice before and after smoking cessation. Differences between the cytokine/chemokine profiles in the BALF before and after smoking cessation were observed. The concentrations of the pro-inflammatory cytokines IL-1α and TNF-α were significantly elevated in the BALF of the cigarette smoke-exposed mice compared to the air-exposed mice (IL-1α: control: 0 pg/ml BALF versus smoke: 73.7 ± 8.7 pg/ml BALF, $P \leq 0.001$; TNF-α: control: 17.1 ± 0.3 pg/ml BALF versus smoke: 33.1 ± 2.6 pg/ml BALF, $P \leq 0.01$). Both IL-1α and TNF-α returned completely to basal levels after smoking cessation. The cigarette smoke-enhanced IL-12 levels in the BALF did not completely return to its basal level after smoking cessation (Fig. 7A). In contrast to the pro-inflammatory cytokines, the levels of the regulatory cytokine IL-10 were significantly decreased in the BALF after cigarette smoke exposure. Although IL-10 levels were rising after smoking cessation, the smoke-induced reduction was still significantly different from the control group (Fig. 7B). Furthermore, the chemokine levels CCL2 and CCL3 were increased in the BALF of cigarette smoke-exposed mice as compared to the control mice (CCL2: control: 17.8 ± 0.2 pg/ml BALF versus smoke: 298.8 ± 47.7 pg/ml BALF, $P \leq 0.01$; CCL3: control: 12.1 ± 3.7 pg/ml BALF versus smoke: 133.6 ± 26.8 pg/ml BALF, $P \leq 0.01$), while these chemokines returned completely towards basal levels after smoking cessation. The VEGF levels were enhanced in the BALF after chronic cigarette smoke exposure and were still significantly elevated compared to the air-exposed mice after 8 weeks smoking cessation (Fig. 6C).

Since no CXCL2 levels were detected in the BALF of the smoke-exposed mice, CXCL2 levels were also examined in the lung homogenates of these animals. A significant increase of the CXCL2 concentration was observed in the lung homogenates of the smoke-exposed mice (4820.7 ± 820.1 pg/ml/mg protein, P ≤ 0.05) compared to the control animals (1108.1 ± 727.2 pg/ml/mg protein). After smoking cessation the smoke-induced increase of CXCL2 levels was still evident (4175.6 ± 1338.6 pg/ml/mg protein).

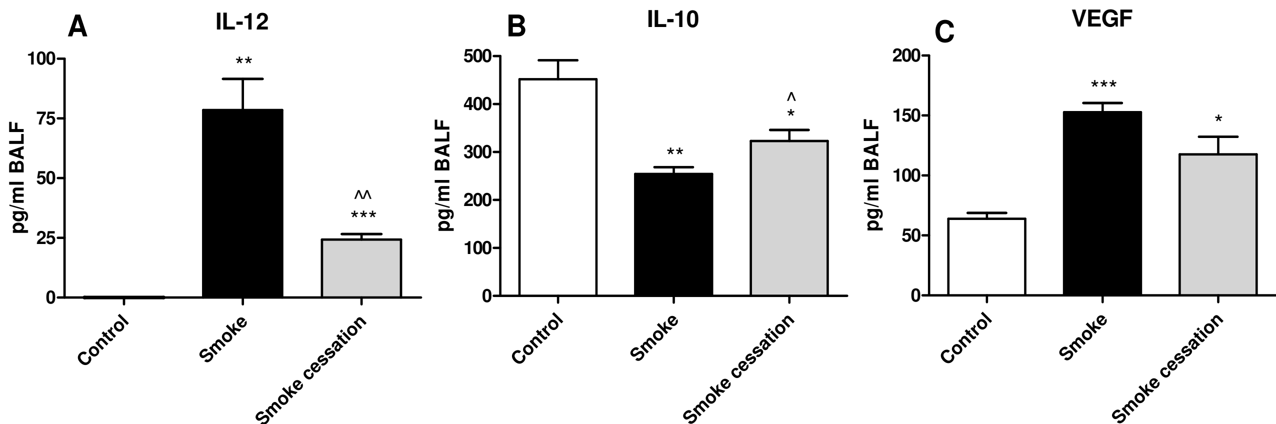

Fig.7. *The effect of smoking cessation on smoke-induced changes in cytokine and chemokine levels in BALF. Levels of the pro-inflammatory cytokine IL-12 (A), the regulatory cytokine IL-10 (B) and the growth factor VEGF (C) in the BALF of air-exposed mice (white bars), smoke-exposed mice (black bars), smoke-exposed mice 8 weeks after smoking cessation (grey bars). n = 4-5 animals per group. Values are expressed as mean ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; significantly different from the control group. ^P ≤ 0.05, ^^P ≤ 0.01; significantly different from the smoke group.*

Discussion

This study investigated the effects of smoking cessation on airway remodeling and pulmonary inflammation. First, airspace enlargement in the animal model for lung emphysema was evident after 20 weeks cigarette smoke exposure. This enlargement was not significant reduced after smoking cessation, suggesting that induction of lung emphysema by alveolar wall destruction is not reversible. These findings are in agreement with the *in vivo* data of Wright and Sun [41] and March et al. [42], who demonstrated that emphysema was still present in guinea pigs and mice after smoke exposure followed by a smoking cessation period. Vernooy et al. [43] also found that long-term LPS exposure results in irreversible alveolar enlargement in mice. The effect of cigarette smoke is believed to be strain dependent. A/J mice were used in the present COPD model, since this strain is characterized as moderately susceptible to the development of lung emphysema and to the lung inflammatory response after acute cigarette smoke exposure [44, 45].

The persistent emphysema observed in the present murine model is also similar to findings in people who have stopped smoking. The alveolar enlargement and destruction seen in lung emphysema is generally thought to be irreversible [46-48]. Besides the determination of lung emphysema, we were interested in the lung volume. In the current study, cigarette smoke-exposed mice showed a significantly increased relative lung volume compared to the air-exposed mice, which is a characteristic feature of lung emphysema [40]. This lung volume was still significantly enhanced after smoking cessation, which supported the irreversible alveolar changes after cigarette smoke exposure.

Furthermore, right ventricle heart hypertrophy was found in mice exposed to cigarette smoke, indicating changes in the structure of the heart. Other authors also demonstrated right ventricle heart hypertrophy as well in animal models for lung emphysema as in COPD patients [6, 7, 38, 39, 49]. A possible explanation for the development of right ventricle heart hypertrophy could be pulmonary hypertension, caused by hypoxic pulmonary vasoconstriction or remodeling of the pulmonary vessels, two important complications of COPD [6, 50, 51]. VEGF is identified as an endothelial cell specific growth factor that contributes to angiogenesis and vascular permeability [52]. In the current study the increased VEGF levels observed in the BALF of the smoke-exposed mice could be involved in the pulmonary vascular remodeling as a result of pulmonary hypertension, ultimately leading to right ventricle heart hypertrophy. An enhanced expression of VEGF was also observed in the pulmonary vessels and arteries of COPD patients, suggesting an important role for VEGF in the development of pulmonary hypertension [53, 54]. However, other studies suggest that VEGF may have a protective role in the development of pulmonary hypertension [55-57]. Like alveolar enlargement, the right ventricle heart hypertrophy and the increased VEGF in

the BALF were irreversible after smoking cessation. It is possible that the pulmonary hypertension continued after the recovery period due to the sustained lung damage and elevated VEGF levels, which could lead to the ongoing heart hypertrophy. It remains to be determined whether right ventricle heart hypertrophy is directly related to lung emphysema or whether other factors can play a role in the development and maintaining of heart hypertrophy in COPD patients.

Airway inflammation was present in the airways of mice exposed to cigarette smoke as shown by an increase in total cell number in the BALF and by inflammatory cell infiltration in the lung tissue. Analysis of differential cell counts in BALF revealed a significant increase in the number of macrophages, neutrophils and lymphocytes in the smoke-exposed mice compared to air-exposed mice, which is described in several *in vivo* studies [58-61]. The histological lung sections and lung inflammation scores of the smoke-exposed mice confirmed pulmonary inflammation with perivascular and peribronchial cellular infiltrates, which has also been demonstrated in other *in vivo* studies [62, 63]. After smoking cessation, the reduced numbers of inflammatory cells in the BALF did not correlate with the sustained inflammatory cell infiltration observed in lung tissue. These results support the studies by Seagrave et al. [64] and March et al. [42, 64], who also observed airway inflammation and lower levels of inflammatory cells in the BALF after smoking cessation. It should be noted that it is very difficult to compare the numerous studies, since the smoking cessation period, the duration of smoking and the experimental set-up varied between the studies, which could lead to discrepancies. Additionally, several studies in COPD patients found a normalized cell count in the BALF and sputum after smoking cessation [24, 25]. In contrast, other studies indicate that there is an ongoing airway inflammation in COPD patients who had stopped smoking [29-32]. These findings indicate that inflammatory changes in the airways of smoke-exposed mice are at least partially reversed after smoking cessation. The persistent airway inflammation (especially macrophages and lymphocytes) could be related to the irreversible tissue damage in the lungs, or to an ongoing microbial stimulus in the “sensitive” airways of smokers [65-67] as discussed by Willemse et al. [31]. Another explanation could be that COPD may have an autoimmune component that regulates the sustained airway inflammation after smoking cessation [68, 69].

Little is known about cytokine and chemokine levels in the BALF after smoking cessation. To the best of our knowledge, this is the first reported *in vivo* study in which cytokine profiles were determined after cessation of cigarette smoke exposure. Increased levels of the pro-inflammatory cytokines IL-1α, IL-12 and TNF-α were observed in the BALF of cigarette smoke-exposed mice. IL-1α and TNF-α levels returned to basal levels after smoking cessation, while IL-12 was not normalized. The cytokines IL-1α, IL-12 and TNF-α are mainly

produced by macrophages [70]. The alterations in these cytokine levels are in line with the accumulated macrophage levels before and reduced levels after smoking cessation. As IL-12 is a potent Th1 skewing cytokine, we suggest a Th1 polarization after cigarette smoke exposure. The decreased IL-10 levels after smoke exposure will amplify this polarization towards Th1, since IL-10 down-regulates the expression of Th1 cytokines [71]. Other authors also describe a possible association between COPD and a Th1-driven immune response [72, 73]. Moreover, after smoking cessation the IL-10 levels were still significantly reduced compared to the air-exposed animals. IL-10 could also play a role in function and differentiation of the regulatory T cell, which is likely to be associated with the control of immune responses in COPD [74, 75].

A significant increase of the CXCL2 concentration was observed in the lung homogenates of the smoke-exposed mice compared to the control animals. The CXCL2 increase is most probably important for the neutrophil recruitment to the lungs following cigarette smoke exposure, which is also indicated by Thatcher et al. [63].

The chemokines CCL2 and CCL3 were also elevated during COPD progression. This is in accordance with the accumulated macrophage, neutrophil and lymphocyte levels in the BALF of the smoke-exposed mice, since CCL2 is a monocyte chemoattractant and is produced by multiple cell types, including monocytes, macrophages, endothelial cells and epithelial cells [76]. CCL3 is mainly released by monocytes/macrophages and is involved in the recruitment and activation of pro-inflammatory cells, such as T cells, monocytes/macrophages and neutrophils [77, 78]. Like IL-12, the synthesis of CCL3 is typically associated with a Th1 milieu [79]. The CCL3 receptor, CCR1 is upregulated on Th1 cells by IL-12 [80, 81], while CCR5, is primarily expressed on Th1 cells and promotes Th1 skewing [82, 83]. Th1 cells secrete IL-2, IFN-y and TNF-α, which activate CD8+ T cells. Since CCL3 attracts CD8+ lymphocytes, the elevated CCL3 in the smoke-exposed mice could be related to the increase in CD8+ T cells seen in tissues of COPD patients [84]. These Th1-related cytokines and chemokines were markedly reduced after smoking cessation, suggesting that the Th1 skewing will diminish after smoking cessation.

Despite of the decrease in cell numbers and the reduction in cytokine and chemokine levels in the BALF after smoking cessation, the current study demonstrated that smoking cessation does not result in a profound reduction of airway inflammation, which is associated with the sustained emphysema. First, the neutrophils in the BALF were strongly reduced after smoking cessation to almost basal levels, but were still significantly increased compared to the control group. The macrophages in the alveolar cavity were also not completely restored toward basal levels after smoking cessation. Furthermore, the cigarette smoke-induced increase of lymphocytes was not changed after cessation of cigarette smoke exposure.

Finally, the histological lung sections showed that the inflammatory cells and the brown-pigmented macrophages were still present in the lung tissue after smoking cessation of 8 weeks, confirming the results described by Seagrave et al. [64]. The pigmented macrophage has been a consistently reported inflammatory cell type in COPD and contains characteristic brown-pigmented cytoplasmic inclusions believed to be by-products of cigarette smoke [85-87]. It could be that these brown-pigmented macrophages together with the elevated lymphocytes in the BALF are responsible for the sustained airway inflammation observed in the lung tissue after smoking cessation. Future research is needed to investigate whether this ongoing inflammation is permanent after smoking cessation.

In conclusion, cigarette smoke exposure leads to irreversible lung damage and heart hypertrophy. The inflammatory changes in the airways caused by cigarette smoke exposure were only partially reversed after smoking cessation. Although smoking cessation should be the first step in reducing the progression of lung emphysema, additional medication could be provided to tackle the sustained airway inflammation.

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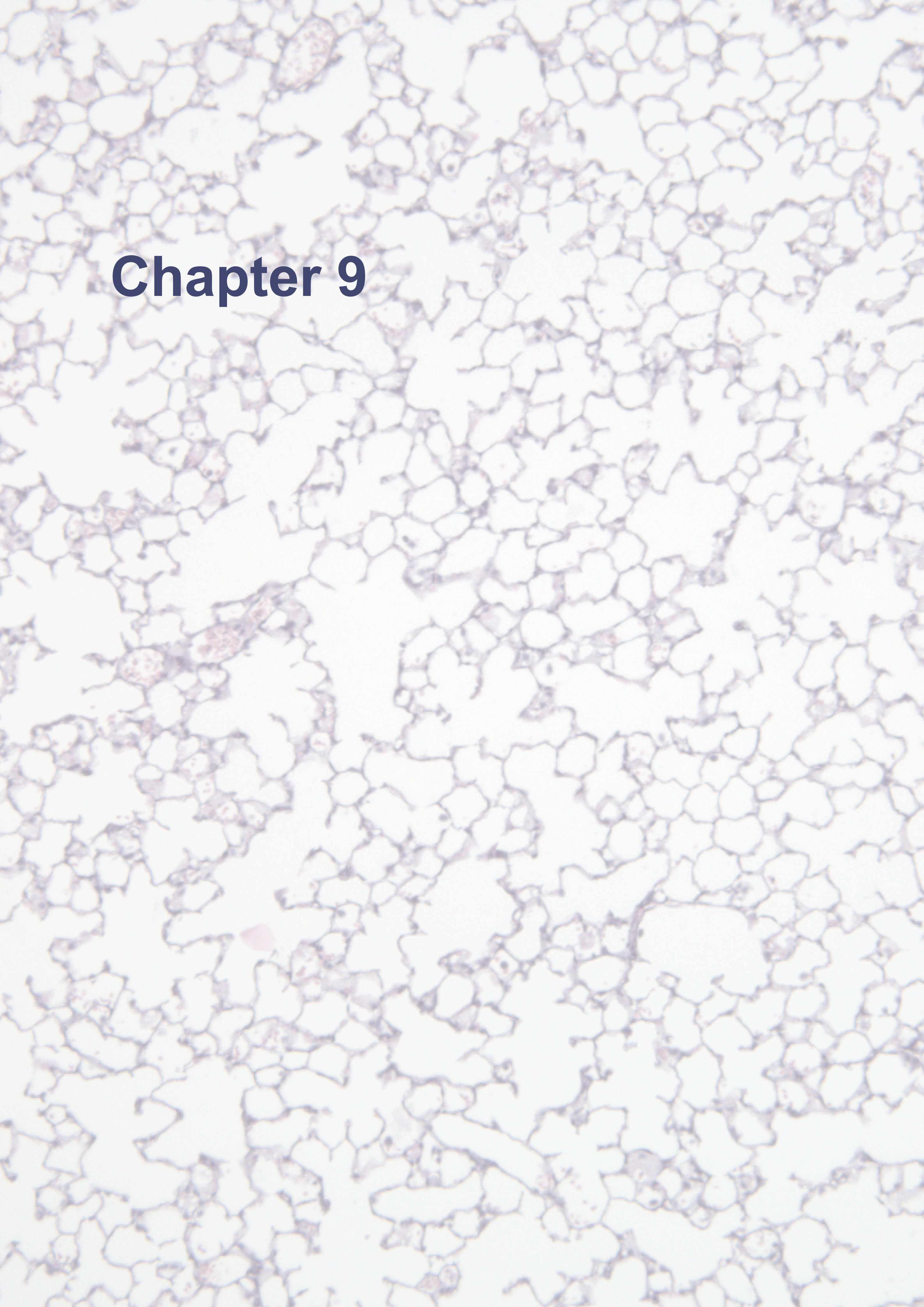

Chapter 9

Summarizing discussion

Discussion

The experiments described in this thesis were performed to gain more knowledge about the mechanisms underlying cigarette smoke-induced lung emphysema. The pathophysiology of COPD is multifactorial and is strongly linked to the effects of cigarette smoke on the lungs. COPD is associated with the accumulation and activation of several types of inflammatory cells in the lung tissue and bronchoalveolar lavage fluid (BALF) [1-3]. These activated inflammatory cells, but also structural cells in the respiratory tract release many inflammatory mediators, which are probably involved in the development of COPD. During this discussion the most important findings are summarized and discussed in the same order as depicted in Fig.1.

In Chapter 6 we demonstrated that after 5 days smoke exposure the number of neutrophils in the BALF of cigarette smoke-exposed mice were significantly increased compared to the air-exposed mice. The enormous influx of neutrophils in the BALF of cigarette smoke-exposed mice was also present after 5 months smoke exposure and the effect of cigarette smoke on the influx of neutrophils was more pronounced compared to the cigarette smoke-induced increase in macrophages and lymphocytes in the BALF (Chapter 8). This is in agreement with other *in vivo* lung emphysema studies [4-6]. These *in vivo* models largely mimic the features of human COPD, since increased numbers of neutrophils were observed in sputum and airways of COPD patients [7-10]. Neutrophils play a crucial role in the pathophysiology of COPD and have the capacity to release multiple (inflammatory) mediators in response to cigarette smoke exposure.

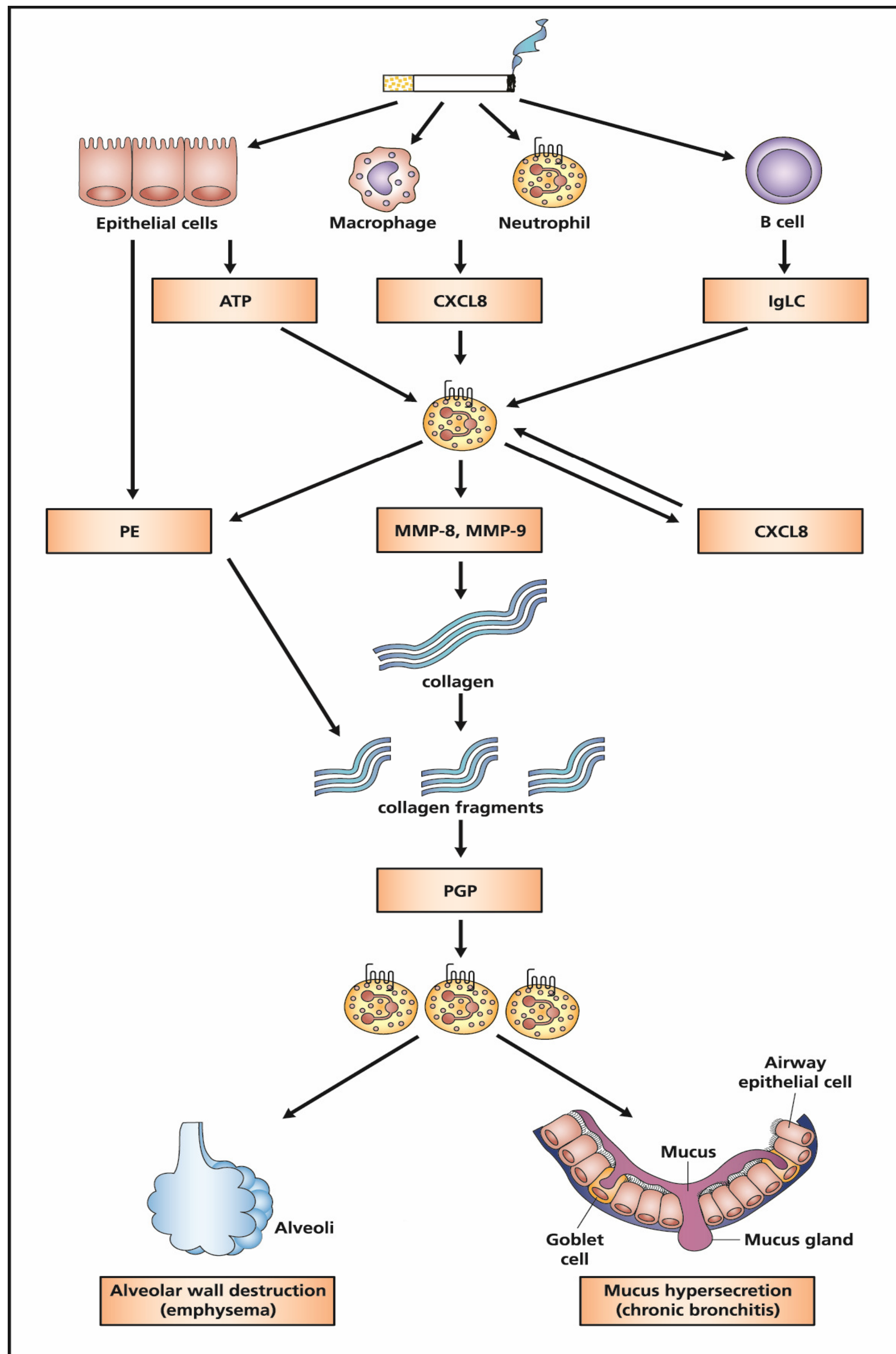

Fig.1. *Cigarette smoke stimulates various cell types, such as epithelial cells, alveolar macrophages, neutrophils and B cells to release different mediators including, ATP, CXCL8 and IgLC. These mediators have the capacity to attract and/or activate neutrophils. Subsequently, the activated neutrophils release CXCL8 and different proteases, such as MMP-8 and MMP-9, which can proteolytically cleave collagen to smaller fragments resulting in an optimal substrate for the enzyme prolyl endopeptidase. Various cell types, like neutrophils, macrophages and epithelial cells, express PE. These collagen fragments are then further cleaved to PGP by PE. Besides CXCL8, the generated PGP is also chemotactic for neutrophils and this results in an environment of chronic inflammation with proteolytic damage and ongoing PGP formation. Finally, this will lead to alveolar wall destruction (emphysema) and mucus hypersecretion (chronic bronchitis).*

First, CXCL8 is one of the most important chemokines associated with the recruitment of neutrophils in COPD [10-12]. In Chapter 4 we demonstrated that cigarette smoke extract induces the release of the neutrophil chemoattractant CXCL8 by human neutrophils *in vitro*, which is currently enforced by the data of Mortaz et al. [13]. In COPD patients, increased CXCL8 levels in sputum are correlated with the extent of neutrophilic inflammation and disease severity [14-16]. We confirmed the importance of CXCL8 in COPD by detecting a significant increase of CXCL1 (mouse CXCL8 homolog, keratinocyte-derived chemokine) levels in the BALF of mice after 1 week smoke exposure (data not shown) and the CXCL2 (mouse CXCL8 homolog, macrophage inflammatory protein-2) levels in the lung homogenates were significantly elevated after 5 months smoke exposure compared to the air-exposed mice (Chapter 8). Other *in vivo* studies also showed increased levels of CXCL1 and CXCL2 in the BALF after cigarette smoke exposure [17, 18]. Furthermore, in Chapter 7 we demonstrated that CXCL1 can induce a neutrophil influx in murine lung tissue and BALF after oropharyngeal aspiration, indicating that in mice the chemoattractant CXCL1 might be involved in the recruitment of neutrophils to the site of inflammation. CXCL8 binds to the CXCR1 and CXCR2 chemokine receptors, which are predominantly expressed on human neutrophils, while CXCL1 and CXCL2 only bind to the CXCR2, since CXCR1 is present in humans, but not in mice [19, 20]. We confirmed that the CXCL1-induced neutrophil infiltration in the murine lung is mediated via CXCR2, since the CXCL1-induced neutrophil influx in lung tissue and BALF was inhibited by two different specific CXCR2 antagonists: SB225002 and SB332235 (Chapter 7).

Besides neutrophils, macrophages play an important role in orchestrating the inflammation in COPD lungs. Most of the cells in the BALF of air-exposed mice are macrophages and these cells were significantly increased after cigarette smoke exposure (Chapter 8). Resident alveolar macrophages are considered to be a key cell in modulating the transmigration of circulating neutrophils into the lung via the release of the important neutrophil chemoattractant CXCL8 [21, 22]. Moreover, other chemokines, such as CCL2 and CCL3, were also elevated in the BALF after 5 months smoke exposure (Chapter 8). These chemokines can be related to the macrophage influx, since CCL2 is a monocyte chemoattractant produced by multiple cell types including macrophages/monocytes and epithelial cells and CCL3 is mainly released by monocytes and macrophages and is involved in the recruitment and activation of proinflammatory cell types such as monocytes/macrophages, neutrophils and T cells. Besides chemokines, increased levels of the proinflammatory cytokines IL-1α, IL-12 and TNF-α were observed in the BALF after 5 months smoke exposure and these cytokines are mainly produced by macrophages (Chapter 8).

Besides the inflammatory cells (macrophages and neutrophils), cigarette smoke can also activate structural cells, such as airway epithelial cells. Epithelial cells not only provide a barrier between the host and environment but may also contribute to airway inflammation in smokers, since exposure to cigarette smoke induce bronchial epithelial cells to release CXCL8 [10, 12, 23].

From this part we can conclude that cigarette smoke exposure leads to an excessive neutrophil influx in the lung. The increased CXCL8 (CXCL1 or CXCL2) levels in the lung, secreted by macrophages, neutrophils and epithelial cells, may play a role in the recruitment of these neutrophils. Furthermore, the increased numbers of macrophages can also contribute to the chronic inflammatory response in the lung and are another feature of COPD.

Another important signaling molecule, that can induce the recruitment and activation of different inflammatory cells, including neutrophils, macrophages, lymphocytes and dendritic cells, is ATP [24-28]. ATP belongs to the purine family and extracellular ATP serves as a danger signal to alert the immune system and triggers various inflammatory responses of neutrophils including oxidative burst, phagocytosis and arachidonic acid release [29]. In Chapter 4 we demonstrated that ATP is released by cigarette smoke extract-stimulated human neutrophils. Besides our *in vitro* studies, we also found increased amounts of ATP in the BALF of cigarette smoke-exposed mice compared to air-exposed mice, which is now confirmed by the study of Cicko et al. [30]. Recently, Lommatzsch et al. [31] showed that chronic smokers had increased ATP levels in the BALF compared to non-smokers and the amount of ATP correlated positively with neutrophil numbers and negatively with lung function. Furthermore, we demonstrated for the first time that incubation of human neutrophils with ATP induces the release of CXCL8 and elastase, two important mediators in the pathogenesis of COPD (Chapter 4). Moreover, neutrophils and macrophages obtained from COPD patients responded with an enhanced release of pro-inflammatory and tissue-degrading mediators after ATP stimulation as compared with control subjects. [31]. Extracellular ATP has recently gained attention as a mediator of intercellular communication via the activation of the purinergic P2X and P2Y receptors [32, 33]. In our study, the increased release of CXCL8 and elastase by ATP- or cigarette smoke extract (CSE)-stimulated neutrophils was abrogated by treatment with apyrase (a catalyzer of the hydrolysis of ATP to yield AMP) and suramin (P2-receptor antagonist), indicating a role for purinergic receptor signaling pathways (Chapter 4). This is a novel pathway involved in the release of inflammatory mediators by cigarette smoke-activated neutrophils [34]. Interestingly, our results are confirmed *in vivo* in a murine model for lung emphysema, where

neutralizing intrapulmonary ATP levels or blocking airway P2 receptors reduced smoke-induced lung inflammation and emphysema in mice [30]. It needs to be determined whether other cell types than neutrophils have also the capacity to release ATP after cigarette smoke exposure and whether ATP can induce the release of pro-inflammatory mediators by these cell types. Anyhow, ATP could also be released after cell damage due to cigarette smoke exposure.
In conclusion, our observations indicate that ATP release from neutrophils, plays a critical role in CXCL8 and elastase release via activation of purinergic signaling after cigarette smoke exposure and this nucleotide-dependent mechanism could be a useful target for the development of new therapeutic drugs for suppressing inflammation in lungs of COPD patients.

The third interesting mediator, that stimulates neutrophils to release proinflammatory mediators described in this thesis, is immunoglobulin free light chain. Immunoglobulin free light chains (IgLC) can exert various biological activities: enzymatic activities, binding to intracellular and extracellular proteins and cellular interactions [35]. It has been long recognized that B cells not only produce and secrete tetrameric immunoglobulins, but also secrete a substantial amount of IgLC [36]. To date, little is known about a possible role of immunoglobulins in COPD. However, higher numbers of B cells in central and small airways have been shown in COPD patients [37, 38]. Besides the CXCR2 chemokine receptor, neutrophils also express receptors for different immunoglobulin isotypes. These Fc receptors are important triggers of neutrophil effector functions and may modulate airway inflammatory reactions [39]. Neutrophils from asthmatic subjects express the IgE high affinity receptor and crosslinking of this receptor is involved in the release of CXCL8 *in vitro* [40] and IgE alone (without cross-linking) can delay programmed cell death of neutrophils [41]. Interestingly, IgLC are also able to prolong the life of neutrophils, suggesting that IgLC could contribute to the chronic state of inflammation [42]. In Chapter 5 we demonstrated that IgLC bind to human neutrophils and cross-linking of IgLC resulted in the production of the important neutrophil chemoattractant CXCL8 *in vitro.* Moreover, IgLC may contribute to a pre-stimulation of neutrophils, since IgLC increase the basal levels of neutrophil oxidative metabolism and IgLC interfere with essential neutrophil functions, such as glucose uptake and chemotaxis [42-44]. Our studies showed increased IgLC levels in serum of cigarette smoke-exposed and CSE-treated mice compared to control mice. Moreover, IgLC were elevated in serum and lung tissue of COPD patients. More importantly, antagonizing the action of IgLC by F991 was found to reduce the cigarette smoke-induced neutrophil influx in murine lungs.

In conclusion, we described a neutrophil related role for IgLC in the development of COPD, which could be an attractive therapeutic target and could open new avenues of treatment for COPD.

The three major mediators described in this thesis, CXCL8, ATP and IgLC, do all have the capacity to activate neutrophils to release pro-inflammatory mediators, such as the neutrophil chemoattractant CXCL8 (Fig.1). These mediators can be released by different cell types including, neutrophils, macrophages, epithelial cells and B cells, upon cigarette smoke exposure.

Activated neutrophils do not only release chemokines, but also tissue-degrading mediators, such as proteases, which contribute to collagen breakdown observed in COPD. Matrix metalloproteinases (MMPs) currently receive the most attention in chronic inflammatory lung disease [45]. Neutrophils are a rich source of MMP-8 and MMP-9 and release these MMPs after activation [46, 47]. In our study, total and active MMP-8 and MMP-9 levels were increased in the airways of mice after 5 months smoke exposure compared to control animals and these levels are related to the amount of neutrophils as reflected by the high correlation coefficient (Chapter 6). The importance of these MMPs is supported by studies in human, where elevated MMP-8 and MMP-9 levels were observed in the airways of COPD patients compared with healthy controls [48-50]. MMP-8 and MMP-9 have been proposed to induce airway remodeling, because of their capacity to cleave structural proteins, such as collagens and elastin [51]. At this point, collagen in the lung can be cleaved in smaller fragments by MMP-8 and MMP-9 in the airways after smoke exposure (Fig.1). Additionally, we demonstrated that CSE- and ATP-stimulated human neutrophils induced the release of elastase (Chapter 4). Neutrophil elastase (NE) is a serine protease and is the first protease implicated in the development of COPD [45]. Beyond the capacity of this protease to cleave elastin, NE has also a variety of immunologic modulatory effects seen in COPD, such as cleavage of neutrophil chemokine receptors, activation of MMPs and induction of CXCL8 expression from airway epithelia [52-55].
Interestingly, we found that the activity of another serine protease, prolyl endopeptidase (PE), was significantly increased in lung homogenates of smoke exposed-mice compared to air-exposed mice (Chapter 6). This finding opened new avenues for investigating the role of this protease in the development of COPD. Furthermore, immunohistological staining for PE showed an increased PE expression in the lung tissue of cigarette smoke-exposed mice, where inflammatory cells highly express PE. This increase in PE expression in mice lungs related to the inflammatory cell influx was confirmed by a PE staining on human lung tissue.

The inflammatory cells in lung tissue of current smokers and COPD patients with GOLD stage II and IV highly express PE, while the number of inflammatory cells and consequently the PE expression was decreased in the lung tissue of ex-smokers (Chapter 6). The epithelial cells in the lung tissue of air- and cigarette smoke-exposed mice as well as the epithelial cells in human lung tissue contain PE, indicating that epithelial cells are an important source for PE in the lung. Exposure of human bronchial epithelial cells to cigarette smoke extract for 16 hours *in vitro* leads to an elevated PE activity in the supernatant (E. Mortaz, unpublished data), suggesting that cigarette smoke exposure might lead to a release of PE. The importance of PE in the inflammatory response in the lung observed after cigarette smoke exposure is enforced by the finding that the cigarette smoke-induced neutrophil influx in the BALF was significantly decreased after the administration of the PE inhibitor valproic acid (VPA) (Chapter 6).

We hypothesize that the increased protease activity not only leads to the lung matrix breakdown, but also to the generation of the tripeptide proline-glycine-proline (PGP) from collagen. Related to this hypothesis, we found increased PGP and N-Ac-PGP levels in BALF of mice after chronic smoke exposure (Chapter 6). PGP formation is a multistep process involving members of the MMP familiy (MMP-8 and MMP-9), which proteolytically cleave collagen to smaller fragments and create an optimal substrate for the serine protease, PE [56]. These smaller collagen fragments are then further cleaved to PGP by PE (Fig.1), since PE is the only enzyme directly capable of cleaving PGP from shorter portions of collagen.

One important characteristic of PGP is its chemotactic activity for neutrophils *in vitro* [57, 58]. In Chapter 7 we demonstrated that *in vivo* PGP can induce a neutrophil influx in the lung tissue and BALF after oropharyngeal aspiration, which is in agreement with the findings of Weathington et al. [57]. Moreover, compared to CXCL1, PGP is more potent at inducing neutrophil migration in pulmonary tissue than into BALF, since the CXCL1-induced neutrophil influx in the BALF was enormous compared to PGP-induced neutrophil influx and the pulmonary tissue MPO levels induced by CXCL1 and PGP were slightly comparable. The importance of PGP in COPD is supported by studies demonstrating that chronic airway exposure to PGP can induce lung emphysema in mice and that PGP is detected in the BALF, sputum and serum of COPD patients [57, 59, 60]. Moreover, we showed that inhibition of PGP by treatment with the complementary peptide L-arginine-threonine-arginine (RTR), which can bind to PGP sequences, resulted in an inhibition of neutrophil migration to the lung of smoke-exposed mice (Chapter 6). These findings confirm earlier results generated by van Houwelingen et al. [60], who found that RTR reduces the emphysema-like changes in the airways after LPS and PGP exposure. To investigate whether the PGP-induced neutrophil influx in the BALF and lung tissue was mediated via CXCR2, we examined the effects of two

different CXCR2 antagonists: SB225002 and SB332235 on the PGP-induced neutrophil influx observed in mice lungs after oropharyngeal aspiration (Chapter 7). The PGP-induced neutrophil influx can be inhibited by the CXCR2 antagonists *in vivo*, suggesting a role for CXCR2 in PGP-induced neutrophilic airway inflammation. This has been confirmed by other studies, since antibodies directed against CXCR2 suppressed the PGP-induced neutrophilic chemotaxis *in vitro* and the accumulation of neutrophils observed in mice upon intratracheal administration of PGP was not detected in CXCR2-/- (knockout) mice [57, 58].
At this point, CXCL8 as well as PGP play a major role in neutrophil activation and migration into the lung in airway inflammation after cigarette smoke exposure. Subsequently, more proteases, such as MMP-8, MMP-9, elastase and PE, are released by the neutrophils and even more PGP is formed. This process might go on and on, resulting in a chronic airway inflammation with lung tissue remodeling, ultimately leading to mucus hypersecretion and alveolar wall destruction, the two main characteristics of COPD [61, 62] (Fig.1).

Alveolar enlargement is the most important parameter to assess the degree of emphysema and is primarily defined on morphological criteria. It is essential that an effective lung fixation method with an appropriate fixative is used to prepare the lung sections necessary for the diagnosis of lung emphysema, otherwise a correct morphological analysis of the lung tissue is not possible [63]. The mean linear intercept analysis (Lm) is the most widely accepted morphometric method to assess the presence and severity of lung emphysema and is defined as a measure of the mean distance between the alveolar walls [64, 65]. In Chapter 3 we evaluated different methods of lung fixation in a murine model of LPS-induced lung emphysema and the usefulness of the different methods was validated by morphometric analysis, evaluating the Lm and immunohistochemistry. Lung fixation by intratracheal instillation of 10% formalin and in situ fixation with 10% formalin embedded in paraffin is recommended, since these methods are sensitive to reveal the presence of alveolar enlargement to study lung emphysema and are appropriate to perform immunohistochemistry. The fixation method of intratracheal instillation of 10% formalin is standard used in this thesis and is also used in other studies describing lung emphysema [57, 60, 66].
In the different lung emphysema models described in this thesis, including the whole body cigarette smoke exposure model, the nose only cigarette smoke exposure model, LPS-induced lung emphysema model and the CSE intraperitoneally and intratracheally injected mice, the Lm was the most important parameter to confirm the development of lung emphysema. In all these models an increased Lm was observed confirming the process of alveolar wall destruction (Chapter 3, 4, 5, 6 and 8). Additionally, the right ventricle heart

hypertrophy, another characteristic of lung emphysema, was determined. In all the *in vivo* models described above right ventricle heart hypertrophy was observed in mice with alveolar enlargement (Chapter 3, 5 and 8), indicating changes in the structure of the heart. Other authors also demonstrated heart hypertrophy in animal models for lung emphysema as well as in COPD patients [57, 60, 67-69]. A possible explanation for the development of right ventricle heart hypertrophy could be pulmonary hypertension, caused by hypoxic pulmonary vasoconstriction or remodeling of the pulmonary blood vessels, two important complications of COPD [67, 70, 71].

Our treatment interventions with F991, VPA and RTR showed a significantly decreased neutrophil influx in the BALF of smoke-exposed mice (Chapter 5 and 6) and CXCR2 antagonists inhibited the CXCL1- and PGP-induced neutrophilic inflammation in the BALF and lung tissue (Chapter 7). Future research is needed to investigate the effect of these therapeutic inventions on the development of lung emphysema. Until now smoking cessation is the single most effective treatment shown to reduce the risk of developing COPD, to stop the disease progression and to improve survival. However, there is contradictory evidence regarding the effect of smoking cessation on airway inflammation associated with COPD. Several studies reported that smoking cessation improves respiratory symptoms, reduces loss of pulmonary function and decreases lung inflammation [72-76], while other studies have shown that smoking cessation fails to reverse the chronic airway inflammation [77-80]. In Chapter 8 we demonstrated that cigarette smoke exposure leads to irreversible lung damage and heart hypertrophy. Furthermore, we observed that the inflammatory changes in the airways caused by cigarette smoke exposure were only partially reversed after smoking cessation in a murine model of cigarette smoke-induced lung emphysema. Although the neutrophilic inflammation in the BALF of cigarette smoke-exposed mice was strongly reduced after smoking cessation, a sustained inflammation in the lung tissue was observed and the cigarette smoke-induced increase of lymphocytes was not changed after smoking cessation. The elevated cytokine levels, IL-1α and TNF-α, and chemokine levels, CCL2 and CCL3, in the BALF of smoke-exposed mice returned to basal levels after smoking cessation, while the increased IL-12 levels did not return to its basal level. The persistent airway inflammation could be related to the irreversible lung tissue damage, the brown pigmented macrophages with by-products of cigarette smoke, which are still present after smoking cessation or the elevated lymphocytes in the BALF. Moreover, COPD may have an autoimmune component that regulated the sustained airway inflammation. It is proposed that an acquired immune response to self- or foreign antigens may be an essential component in the pathogenesis of COPD [81-83]. We hypothesize that CSE can act directly or indirectly as

an antigen triggering an immune response that leads to lung destruction. This statement is enforced by our *in vivo* model described in this thesis (Chapter 5), where the cigarette smoke extract i.p. and i.t. injected mice develop lung emphysema and heart hypertrophy.
In Chapter 6 we demonstrated that after smoking cessation the MMP-8 and MMP-9 levels in the BALF and/or lung homogenates are reduced compared to the smoke-exposed mice, but are not always returned to basal level. However, the PE activity increase in lung homogenates and the PGP as well as the N-Ac-PGP levels in the BALF of smoke-exposed mice returned to normal levels after smoking cessation. Consequently, smoking cessation can interrupt the vicious circle of an ongoing neutrophil influx into the lung. This interference results in a decrease in neutrophils and proteases, which will stop the lung matrix breakdown and thus the PGP formation, while the lung emphysema stays present. This suggests that the PE activity and PGP formation are both associated with cigarette smoke exposure and not directly with emphysema.

Conclusion

In summary (Fig.1), the cascade of events leading to the development of lung emphysema will start with cigarette smoke exposure. Cigarette smoke exposure can stimulate epithelial cells, alveolar macrophages, neutrophils and B cells to release inflammatory mediators, including CXCL8, ATP and IgLC. These mediators have the capacity to attract and/or activate neutrophils. Subsequently, activated neutrophils release CXCL8 and different proteases, such as MMP-8 and MMP-9, which can proteolytically cleave collagen to smaller fragments resulting in an optimal substrate for the enzyme prolyl endopeptidase. Various cell types, like neutrophils, macrophages and epithelial cells, express PE. These collagen fragments are then further cleaved to PGP by PE. Besides CXCL8, the generated PGP is also chemotactic for neutrophils and this results in an environment of chronic inflammation with proteolytic damage and ongoing PGP formation. Finally, this will lead to alveolar wall destruction (emphysema) and mucus hypersecretion (chronic bronchitis). We found different possible treatment interventions to tackle the ongoing inflammation observed in COPD patients. First, a significantly decreased neutrophil influx in the BALF of smoke-exposed mice was observed after treatment with F991, VPA and RTR. Secondly, the increased release of CXCL8 and elastase by ATP- or CSE-stimulated human neutrophils was inhibited by treatment with apyrase and suramin. Thirdly, CXCR2 antagonists inhibited the CXCL1- and PGP-induced neutrophilic inflammation in the murine BALF and lung tissue. Although smoking cessation should be the first step in reducing the progression of lung emphysema, additional medication could be provided to tackle the sustained airway inflammation.

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Nederlandse samenvatting

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Wat is COPD?

COPD is een Engelse afkorting en staat voor chronic obstructive pulmonary disease, in het Nederlands chronisch obstructieve longziekten en is een ziekte waarbij er een obstructie van de luchtwegen optreedt. Deze luchtwegobstructie is permanent aanwezig en grotendeels onomkeerbaar. COPD is een verzamelnaam voor de luchtwegaandoeningen longemfyseem (verlies van longweefsel door destructie van longblaasjes, waardoor "de rek uit de long" raakt en er minder goede opname van zuurstof en afgifte van koolzuur mogelijk is) en chronische bronchitis (chronische ontsteking van het slijmvlies in de luchtwegen waarbij dagelijks slijm opgehoest wordt). COPD is onder te verdelen in vier stadia, van mild tot zeer ernstig. Elk stadium kent zijn eigen klachten. Ze variëren van kortademigheid tot voortdurende benauwdheid, hoesten en overmatige slijmproductie.

Veruit de belangrijkste oorzaak voor het ontstaan van COPD is roken: 90% van de COPD-patiënten rookt of heeft dat in het verleden gedaan. Sigarettenrook bestaat uit meer dan 4700 chemische stoffen, zoals koolmonoxide, nicotine en teer, die schade kunnen toebrengen aan het lichaam. Andere risicofactoren zijn onder meer: luchtvervuiling, overgevoeligheid voor bepaalde stoffen (bv. huismijt en stuifmeel), infecties en een beroepsmatige blootstelling aan irriterende stoffen. COPD krijgt de laatste jaren steeds meer aandacht en verdient dat ook, want op wereldschaal is COPD nu de vijfde doodsoorzaak, maar binnen twintig jaar kan het de vierde doodsoorzaak worden. De behandeling van COPD bestaat voornamelijk uit het toedienen van luchtwegverwijders, middelen die de luchtwegen verwijden zodat de benauwdheid wordt verlicht zoals β_2-agonisten en anticholinergica en ook uit het toedienen van ontstekingremmers, middelen die ontstekingsremmend werken en de luchtwegen tegen prikkels beschermen zoals corticosteroïden. Tot nu toe is gebleken dat stoppen met roken de meest effectieve interventie is in de behandeling van COPD.

Belangrijke processen die een rol spelen in de pathogenese van COPD zijn: een chronische ontsteking van de long, een disbalans tussen proteases en anti-proteases en een disbalans tussen reactieve zuurstofradicalen en anti-oxidanten.

COPD is een complexe ziekte, waarbij verschillende cel types, zoals macrofagen, neutrofielen, CD8+ cytotoxische T cellen en epitheelcellen een rol spelen. Na blootstelling aan sigarettenrook, zullen deze cel types een scala aan mediatoren vrijzetten, zoals cytokines en chemokines (bijvoorbeeld CXCL8), die betrokken zijn bij de activatie van ontstekingscellen en de migratie van ontstekingscellen naar de long.

Geactiveerde macrofagen en neutrofielen zetten vervolgens verschillende proteases vrij, zoals elastase en matrix metalloproteinases (MMP), die het bindweefsel in het longparenchym afbreken en de mucussecretie stimuleren, dat uiteindelijk leidt tot de ontwikkeling van longemfyseem en chronische bronchitis.

De onderzoeksvraagstellingen en conclusies uit dit proefschrift

Met de studies beschreven in dit proefschrift wordt bijgedragen aan de ontrafeling van de mechanismen die een rol kunnen spelen bij longemfyseem geïnduceerd door sigarettenrook. Verschillende diermodellen voor longemfyseem zijn gebruikt om de kennis wat betreft de pathogenese van longemfyseem te verbreden en om nieuwe middelen te testen met een potentiële therapeutische werking ter behandeling of preventie van deze ziekte. In dit proefschrift zijn de volgende zaken onderzocht aan de hand van onderstaande vraagstellingen.

1. Welke chemokines en chemokine receptoren spelen een belangrijke rol bij COPD?

COPD wordt gekarakteriseerd door een chronische ontsteking in de luchtwegen, die gepaard gaat met de migratie van ontstekingscellen naar de plaats van ontsteking. Deze influx van ontstekingscellen wordt geregeld door verschillende chemokines en chemokine receptoren, die zijn besproken in hoofdstuk 2.

Conclusie: Ten eerste is bekend dat de chemoattractant CCL2 en de CCR2 receptor een grote rol spelen bij de migratie van monocyten naar de long. Tijdens migratie naar de long differentiëren monocyten naar macrofagen. Een van de belangrijkste chemokines die betrokken is bij de migratie van onstekingscellen in COPD is CXCL8. CXCL8 trekt neutrofielen naar de luchtwegen en activeert deze cellen. CXCL8 bindt zowel aan de CXCR1 als aan de CXCR2 receptor, die beide aanwezig zijn op de neutrofiel. Naast macrofagen en neutrofielen spelen ook

T lymfocyten een belangrijke rol bij de ontwikkeling van COPD. T lymfocyten bezitten de CXCR3 receptor en activatie van CXCR3 door de chemokines CXCL9, CXCL10 en CXCL11 draagt bij aan het migreren van T lymfocyten naar de long tijdens de luchtwegontsteking in COPD.

2. Welke longfixatiemethode kan worden aanbevolen voor het fixeren van muizenlongen in diermodellen voor longemfyseem?

Longemfyseem wordt gekarakteriseerd door het uitrekken en kapotgaan van longblaasjes, de belangrijkste parameter om de diagnose van COPD te kunnen stellen. In diermodellen

wordt het uitrekken en kapotgaan van longblaasjes voornamelijk bepaald aan de hand van morfologische criteria en daarbij behorende technieken. De methode om de longen te fixeren is de basis voor een correcte interpretatie van de longhistologie. Daarom is het gebruik van de meest optimale longfixatiemethode met een geschikt fixatief een voorwaarde voor nauwkeurig onderzoek naar de pathogenese van longemfyseem. In hoofdstuk 3 worden verschillende longfixatiemethodes geëvalueerd in muizen met en zonder LPS-geïnduceerd longemfyseem.

Conclusie: Uit hoofdstuk 3 is gebleken dat de intratracheale toediening van het fixatief 10% formaline en *in situ* fixatie via de trachea met 10% formaline, de twee meest optimale longfixatiemethodes zijn, indien ingebed in paraffine, die gebruikt kunnen worden in muismodellen voor longemfyseem. Deze fixatiemethodes zijn nauwkeurig genoeg om de diagnose longemfyseem te stellen door de vergroting van de longblaasjes te meten en deze gefixeerde longen zijn uitermate geschikt voor immunohistochemie.

3. Is adenosine-5'-trifosfaat (ATP) betrokken bij de pathogenese van longemfyseem?

ATP is een ribonucleotide, dat in de celstofwisseling een sleutelrol vervult als drager van chemische energie. Tevens is extracellulair ATP een belangrijk signaalmolecuul dat het immuunsysteem waarschuwt bij weefselschade, maar ook kan zorgen voor de migratie en activatie van onstekingscellen, zoals neutrofielen, macrofagen en dendritische cellen. Tot nu toe is er weinig bekend over de bijdrage van ATP in de ontwikkeling van COPD. In hoofdstuk 4 is de aanwezigheid van ATP bepaald in de bronchoalveolaire lavagevloeistof van muizen die chronisch blootgesteld zijn aan sigarettenrook en is onderzocht of neutrofielen gestimuleerd met sigarettenrookextract ATP vrijzetten. Tevens is er gekeken naar het effect van sigarettenrookextract en ATP op het vrijzetten van CXCL8 en elastase door neutrofielen en of dit effect te remmen is met apyrase (katalyseert de hydrolyse van ATP naar AMP) en suramin (P2-receptor antagonist).

Conclusie: Verhoogde hoeveelheden ATP zijn gevonden in de bronchoalveolaire lavagevloeistof van muizen die chronisch zijn blootgesteld aan sigarettenrook en longemfyseem hebben ontwikkeld in vergelijking met controle muizen. Activatie van humane neutrofielen met sigarettenrookextract leidt tot het vrijzetten van ATP. Indien neutrofielen met sigarettenrookextract of ATP worden gestimuleerd, wordt er een verhoogde concentratie CXCL8 en elastase gemeten in het supernatant. Tevens kon deze vrijzetting van CXCL8 en elastase door neutrofielen geremd worden door de farmacologische remmers apyrase en suramin. Deze studie impliceert dat ATP en activatie via purinerge receptoren een rol speelt in de pathogenese van longemfyseem.

4. Zijn vrije lichte ketens van antilichamen betrokken bij de ontwikkeling van COPD en is er een rol voor de neutrofiel in dit proces?

B lymfocyten kunnen naast gehele antilichamen ook vrije lichte ketens van antilichamen produceren. Deze vrije lichte ketens zijn in staat de levensduur van neutrofielen te verlengen, dit suggereert dat vrije lichte ketens zouden kunnen bijdragen aan het in stand houden van chronische ontstekingen. Omdat COPD wordt geassocieerd met een chronische ontsteking aan de luchtwegen, is onderzocht of vrije lichte ketens aanwezig zijn in het serum van muizen in drie verschillende modellen voor longemfyseem. Tevens is in serum en longweefsel van COPD patiënten gekeken of er vrije lichte ketens aanwezig zijn. Een andere vraag was of vrije lichte ketens kunnen binden aan humane neutrofielen en of ze direct bijdragen aan neutrofiel activatie *in vitro*. Tenslotte is bepaald of de sigarettenrook geïnduceerde neutrofiel influx in de muizenlong geremd kan worden met de vrije lichte keten antagonist, F991 (hoofdstuk 5).

Conclusie: Deze studie laat zien dat er verhoogde hoeveelheden vrije lichte keten aanwezig zijn in serum van muizen die longemfyseem hebben ontwikkeld in verschillende longemfyseemmodellen. Ook COPD patiënten vertonen een verhoogde vrije lichte keten concentratie in het serum in vergelijking met gezonde vrijwilligers en er zijn vrije lichte ketens aanwezig in het longweefsel van COPD patiënten. Vrije lichte ketens kunnen binden aan neutrofielen en kunnen ze tevens activeren tot de vrijzetting van CXCL8. Tenslotte wordt de neutrofiel influx geïnduceerd door sigarettenrook in de bronchoalveolaire lavagevloeistof van muizen geremd na toediening van F991. Hiermee is aangetoond dat er een mogelijke neutrofiel gerelateerde rol is voor vrije lichte ketens in de pathofysiologie van COPD.

5. Is er een rol voor het collageen afbraakproduct proline-glycine-proline (PGP) en het enzym prolyl endopeptidase (PE) in de ontwikkeling van COPD?

De verhoogde protease activiteit in de long tijdens COPD zal niet alleen leiden tot de afbraak van de longmatrix, maar tevens tot de formatie van de tripeptide PGP uit collageen. Net als CXCL8 is PGP ook chemotactisch voor neutrofielen. De formatie van het collageen afbraakproduct, PGP, is een proces waarbij onder andere neutrofielen, MMPs en PE (een serine protease) betrokken zijn. Dit proces is stap voor stap bekeken in een muismodel door sigarettenrook geïnduceerd longemfyseem. Hoofdstuk 6 is voornamelijk gericht op de rol van PE en PGP in de pathofysiologie van COPD. Er is onderzocht of de PE remmer valproïnezuur en de PGP antagonist RTR een effect hebben op de door sigarettenrook geïnduceerde neutrofiel influx in de muizenlong. Tenslotte is de aanwezigheid van PE in longweefsel van COPD patiënten bestudeerd.

Conclusie: De hoeveelheden neutrofielen, MMP-8, MMP-9 en PGP en de PE activiteit zijn verhoogd in de muizenlong na chronische blootstelling aan sigarettenrook. Alle relevante componenten betrokken bij de formatie van PGP zijn aanwezig in muizenlongen na chronische blootstelling. Ook was het enzym PE te vinden in het longweefsel van muizen na chronische blootstelling aan sigarettenrook en in longweefsel van COPD patiënten. In dit longweefsel brengen voornamelijk de ontstekingscellen en het longepitheel PE tot expressie. Indien de muizen na chronische sigarettenrookblootstelling, acht weken geen sigarettenrook inhaleerde, daalden de hoeveelheid neutrofielen, de MMP-8 en MMP-9 concentraties, de PE activiteit en de hoeveelheid PGP aanzienlijk in de long. Tenslotte kon zowel de PE remmer valproïnezuur als de PGP antagonist RTR de door sigarettenrook geïnduceerde neutrofiel influx in de bronchoalveolaire lavagevloeistof verminderen. Samen met MMPs speelt PE mogelijk een rol in de formatie van PGP en dus in de pathofysiologie van longemfyseem.

6. Is het mogelijk om de neutrofiel influx geïnduceerd door PGP in de muizenlong te remmen met CXCR2 antagonisten?

In hoofdstuk 7 is het effect van twee verschillende CXCR2 antagonisten bestudeerd in een muismodel, waar een neutrofiel influx in de long is geïnduceerd door orofaryngeale toediening van PGP. Met dit experiment is onderzocht of CXCR2 activatie is betrokken bij een door PGP geïnduceerde neutrofiel migratie en activatie in de muizenlong.

Conclusie: De door PGP geïnduceerde neutrofiel influx in de bronchoalveolaire lavagevloeistof en longweefsel van de muis kan worden geremd door de twee CXCR2 antagonisten SB225002 en SB332235. De door PGP geïnduceerde vrijzetting van CXCL1 is onafhankelijk van CXCR2 activatie. Tevens was PGP in verhouding met CXCL1 meer in staat om neutrofielen aan te trekken naar het longweefsel dan naar de bronchoalveolaire lavagevloeistof. Dit hoofdstuk wijst uit dat neutrofielmigratie geïnduceerd door PGP veroorzaakt wordt door interactie met de CXCR2 receptor.

7. Zijn de veranderingen in de luchtwegen veroorzaakt door roken omkeerbaar na het stoppen met roken?

Stoppen met roken is de meest effectieve interventie in de behandeling van COPD. Tot nu toe is er weinig bekend over het effect van stoppen met roken op de cytokine en chemokine profielen in de luchtwegen. In hoofdstuk 8 is het effect van stoppen met roken onderzocht op veranderingen in de luchtwegen van muizen, die chronisch blootgesteld zijn aan sigarettenrook en longemfyseem hebben ontwikkeld. De mate van veranderingen in de long zijn bestudeerd aan de hand van het analyseren van de vergroting van de longblaasjes, de hoeveelheid ontstekingscellen in de bronchoalveolaire lavagevloeistof en longweefsel en de

cytokine en chemokine profielen in de bronchoalveolaire lavagevloeistof. Tevens is er gekeken naar het effect van stoppen met roken op de ontwikkeling van harthypertrofie.

Conclusie:

De mate van afbreken van de longblaasjes en de harthypertrofie na chronische blootstelling aan sigarettenrook verandert niet na het stoppen met roken. Hoewel de neutrofiel gerelateerde onsteking in de bronchoalveolaire lavagevloeistof was verminderd na het stoppen met roken, was er een blijvende onsteking aanwezig in het longweefsel. De verhoogde cytokine en chemokine concentraties (IL-1α, TNF-α, CCL2 en CCL3) in de bronchoalveolaire lavagevloeistof dalen tot basaal niveau na het stoppen met roken, maar de verhoogde IL-12 concentratie neemt niet af tot basaal niveau. De verlaagde IL-10 concentratie na chronische sigarettenrook blootstelling, is nog steeds significant verlaagd na het stoppen met roken. Uit deze studie is gebleken dat de ontsteking in de luchtwegen alleen gedeeltelijk omkeerbaar is na het stoppen met roken.

Eindconclusie

Met deze studies is weer een deel van de pathogenese van longemfyseem opgehelderd. De opeenvolging aan gebeurtenissen die leiden tot de ontwikkeling van longemfyseem starten met de blootstelling aan sigarettenrook (zie Fig.1 van hoofdstuk 9). Epitheelcellen, alveolaire macrofagen, neutrofielen en B lymfocyten blootgesteld aan sigarettenrook kunnen verschillende mediatoren vrijzetten, zoals CXCL8, ATP en vrije lichte immunoglobulineketens. Deze mediatoren hebben op hun beurt weer de capaciteit om neutrofielen aan te trekken en deze te activeren. Daaropvolgend produceren geactiveerde neutrofielen CXCL8 en zetten zij verschillende proteases vrij, zoals MMP-8 en MMP-9. MMP-8 en MMP-9 kunnen collageen afbreken tot kleinere fragmenten, zodat het een optimaal substraat wordt voor het enzym PE. Deze collageenfragmenten worden dan door PE verder afgebroken tot PGP. PGP is ook chemotactisch voor neutrofielen en dit resulteert in een chronische ontsteking met schade aan het longweefsel en continue PGP formatie. Dit zal leiden tot de ontwikkeling van chronische bronchitis en longemfyseem. Ook zijn er tijdens deze studie verschillende middelen getest met een potentiële therapeutische werking ter behandeling van COPD. Ten eerste was er een afname in de hoeveelheid neutrofielen in de longen van aan sigarettenrook blootgestelde muizen na behandeling met de vrije lichte keten antagonist F991, de PE remmer valproïnezuur en de PGP antagonist RTR. Ten tweede werden de CXCL8 en elastaseconcentraties vrijgezet door ATP en sigarettenrook gestimuleerde neutrofielen geremd na incubatie met apyrase en suramin. Ten derde kon de door PGP geïnduceerde neutrofiel influx in de bronchoalveolaire lavagevloeistof en in het longweefsel van de muis geremd worden door CXCR2 antagonisten. Hoewel het stoppen met roken een effectieve eerste stap is om de progressie van COPD te stoppen is additionele medicatie een goede optie om de blijvende ontsteking in de long te behandelen.

Dankwoord

Dankwoord

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Curriculum Vitae & Bibliography

Curriculum Vitae

The author of this thesis was born on December 13, 1981 in Brielle, the Netherlands. In 2000, she graduated from secondary school at the Maerlant College in Brielle and started the bachelor program Animal Healthcare at the Hogeschool InHolland Delft, the Netherlands. In 2004 she started the master Animal Sciences with the specialization Cell biology and Immunology at the Wageningen University, Wageningen, the Netherlands. During this master program, she performed an eight months research project about immunomodulation by probiotics in laying hens at the department of Immunology at the Wageningen University supervised by Prof. Dr. H.F.J. Savelkoul, Dr. H.K. Parmentier and Dr. A. Lammers. Subsequently, she performed a five months research project about the role of collagen breakdown products in osteoarthritis at Danone Research in Wageningen, the Netherlands, which was supervised by Prof. Dr. J. Garssen and Dr. A. Hartog. In January 2007 she joined the department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University in order to perform a PhD study supervised by Prof. Dr. G. Folkerts, Dr. A.D. Kraneveld and Dr. P.A.J. Henricks. During this PhD project she worked at the Division of Pulmonary, Allergy and Critical Care Medicine at University of Alabama in Birmingham, USA for one month. Currently, the author is employed as a post doc at the department of Veterinary Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Sciences, Utrecht University under the supervision of Prof. Dr. J. Fink-Gremmels. The subject of this postdoctoral project is immunomodulation by oligosaccharides and is coordinated by the Carbohydrate Competence Center.

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