DAMPs, endogenous danger signals fueling airway inflammation in COPD

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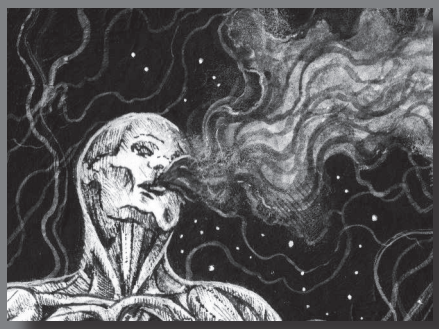

Chapter I

General introduction, background & scope of this thesis

BACKGROUND

The clinical features, causes and treatment of COPD

Chronic Obstructive Pulmonary Disease (COPD) is a severe and debilitating lung disease characterized by progressive and largely irreversible airway obstruction and accelerated lung function decline.[6] COPD is characterized by chronic inflammation of the bronchi and bronchioles, leading to mucus hypersecretion (chronic bronchitis), thickening of the (small) airway walls (remodeling), and airspace enlargement and alveolar destruction (emphysema).[9] COPD is clinically manifested by breathlessness, cough, wheezing, fatigue and chest tightness. Periodically, COPD patients can experience a sudden worsening of symptoms, also called COPD exacerbations, characterized by a further increase in inflammation and accelerated decline in lung function, resulting in a decrease in quality of life and increased mortality and healthcare costs.[2] COPD is a complex disease, with variation between patients in the type of inflammation, the experienced symptoms and severity of the disease. COPD patients can be divided into different endotypes based on their symptoms, such as disease characterized by a predominance of emphysema, or alternatively of bronchitis, or by a mixed disease presenting with a combination of the two. Endotypes can also be based on the nature of the observed inflammatory infiltrate, leading to the distinction between neutrophilic or eosinophilic/ T helper (Th)2 endotypes, distinction on basis of the severity of symptoms and on basis of responsiveness to corticosteroids.[36] This disease heterogeneity significantly complicates studies using clinical samples of COPD patients, as the different endotypes may respond differently towards various stimuli and treatments and most studies are unable to differentiate between the various endotypes.

COPD is caused by chronic inhalation of noxious gases or particles, of which cigarette smoke (CS) is the main risk factor. Recently, it has been shown that up to 30% of the COPD patients are never-smokers, underlining the importance of other risk factors in the inception of COPD, including exhaust fumes, indoor burning of biomass fuels, air pollution or secondary smoking.[12,21] In addition, exposure-independent COPD can occur in patients with alpha-1 antitrypsin deficiency, caused by a mutation in the serpin peptidase inhibitor, clade A, member 1 (*SERPINA1*) gene, which leads to the early onset of COPD.[27] This mutation is only present in 1-2% of all COPD patients.[4] In the remainder of COPD patients, the disease is also not caused by environmental exposure alone, but is thought to result from an interaction between environmental and genetic factors, supported by the fact that only 20% of the smoking population develops COPD.[38] Over the years, candidate gene-association studies, linkage studies and genome wide-association studies have identified many different candidate genes for COPD, including *IL6R*, *MMP12*, *HHIP*, *AGER*, *FAM13A* and *CHRNA3*.[27,4] These different genes are not linked to a common pathway, indicating the complexity of COPD. The multitude of genetic factors involved in COPD may in part be explained by the heterogeneity of the disease, as for instance emphysema is likely associated with different genes than bronchitis. Differentiation between different endotypes of COPD is necessary to further unravel the genetic factors involved in the pathophysiology of COPD. However, to date this is not routinely performed in genetic studies identifying candidate genes for COPD. In this thesis, we focus on cigarette smoke-induced airway inflammation as the first step in the development of exposure-dependent COPD, as the molecular mechanisms of this process are to date largely unknown.

COPD is diagnosed by spirometry according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) standards, which states that a patient has COPD when the ratio between the post-bronchodilator forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) is below 70%.[26] The disease is categorized into four severity stages based on the FEV_1, which is ≤80% in GOLD stage I, between 50-79% in GOLD stage II, between 30-49% in GOLD stage III and <30% in GOLD stage IV. The World Health Organization showed in 2012 that COPD was the fourth leading cause of death worldwide, and the incidence is expected to rise even further for the coming 10 years.[32] The prevalence and mortality rates are still increasing and it is expected that by 2020 approximately 7.8% off all deaths worldwide will be directly caused by COPD.[23,21] Furthermore, COPD is a major economic burden for society as the healthcare costs for COPD are estimated to be €38.7 billion in the European union alone, which is approximately 4.8% of the total health care budget.[21] The economic and societal costs are

largely caused by COPD exacerbations, which is the main cause for hospitalization of COPD patients.[3] Currently, treatment is aimed at reducing the severity of symptoms, improve the quality of life and reduce complications related to COPD, but no curative treatment options are available for COPD patients.[11,1] Pharmacotherapy mostly consists of the use of bronchodilators, antibiotics and inhaled corticosteroids which can be used in several combinations.[28] The effectiveness of these medications is largely patient dependent and none of the treatments is able to fully stop the progression of COPD. A large subgroup of COPD patients is partially or completely unresponsive to inhaled corticosteroids, and this treatment is mainly effective in patients with the eosinophilic endotype and in frequent exacerbators with chronic bronchitis.[22] Furthermore, no treatment is currently available reducing or reversing the alveolar tissue damage seen in patients with emphysema, rendering the need for novel treatments aimed at tissue regeneration.

The innate immune response in COPD, a role for neutrophils and airway epithelial cells

In COPD patients, lung mucosal, submucosal and glandular tissues are infiltrated by immune cells.[10] This infiltrate exists of both innate and adaptive immune cells. With respect to innate immune cells, an increase has been observed for alveolar macrophages, neutrophils and dendritic cells in the airways of COPD patients compared to smoking controls.[6] Neutrophilic granulocytes are the most abundant cell type in the inflammatory infiltrate of the airways of COPD patients.[16] Neutrophils are responsible for the production of anti-microbial peptides, pro-inflammatory cytokines, lipid mediators, chemokines and damaging enzymes, e.g. neutrophil elastase and metalloproteases, and contribute to the mucus hypersecretion and tissue destruction in COPD patients.[30]

An important cell type of the innate immune system in the development of COPD are the respiratory epithelial cells. The bronchial epithelium is the first line of defense against inhaled toxicants. The bronchial epithelial layer forms a continuous physical barrier lining the airway lumen, and is responsible for mucus production and clearance of pathogens and foreign particles by ciliary movement.[13] Additionally, the bronchial epithelium acts as a chemical barrier, producing both anti-oxidants and anti-proteases.[15] Moreover, the bronchial epithelium contributes to the defense against invading pathogens by the production of anti-microbial peptides, including defensins, mucins, pro-inflammatory cytokines and chemokines, the latter especially when damaged.[13] The muco-ciliated pseudostratified bronchial epithelial layer consists of several cell types, including ciliated cells, goblet cells, basal cells and club cells. Approximately 50% of the bronchial epithelium consists of ciliated cells, which are important for the transport of particles trapped in the mucus. Goblet cells, which account for 5-15% of all bronchial epithelial cells in the large airways, are responsible for the production of mucus, while club cells produce anti-microbial peptides and immune regulatory cytokines and are progenitor cells for ciliated and goblet cells.[19] Additionally, basal cells serve as progenitor cells that are responsible for the regeneration of the epithelial layer. The alveolar epithelium consists of type I and type II pneumocytes. The type II cells are essential for the production of surfactant and can differentiate into type I pneumocytes, which are structural cells important for gas exchange.[5] It has been shown that bronchial epithelial cells become activated upon exposure to CS and subsequently release high levels of pro-inflammatory cytokines, including the neutrophil chemoattractant CXCL8.[42,44] Furthermore, CS induces oxidative stress in the epithelial layer, causing damage, disruption of cell-cell contacts and cell death.[46,42] In this thesis we investigate the damage induced to airway epithelial cells by CS exposure, and how this can contribute to airway inflammation and COPD.

It has been proposed by Cosio *et al*. that innate immune responses play an important role in the early phases of COPD, while the adaptive immune system is more important in the advanced stages of the disease.[9] The innate immune system is activated by pattern recognition receptors (PRRs), unlike the adaptive immune cells that carry unique antigen-binding receptors. PRRs are involved in the immune response against invading pathogens and damaged endogenous cells, triggering innate immune pathways and inducing inflammation.[37] They are expressed at the cell surface and in the endosomal compartment of a wide variety of cells in the airways, including epithelial cells, endothelial cells, macrophages and dendritic cells.[45] PRRs recognize a wide range of conserved molecular patterns present in pathogens, the pathogen associated molecular patterns

(PAMPS), e.g. LPS, lipopeptides and flaggelin, as well as molecules released from damaged and necrotic cells, the damage associated molecular patterns (DAMPs). Well known families of PRRs are Toll-like receptors (TLRs), NOD-like receptors, C-type lectin receptors, Retinoic acid-inducible gene-I-like receptors and the receptor for advanced glycation end-products (RAGE).[41] Upon activation of PRRs, pro-inflammatory signaling pathways will be activated, leading to activation of transcription factors, most notably the NF-κB pathway, and subsequently the production and release of pro-inflammatory cytokines and chemokines, e.g. CXCL8, TNF, IL-1β and IL-6.[41] These cytokines activate the immune system and attract cells of the innate immune system to the airways. The downstream signaling pathways of PRRs will be extensively discussed in chapter 2. The activation of PRRs on immature dendritic cells induces maturation, enabling them to migrate to the lymph nodes and activate T-cells.[9] This alerts and subsequently activates the adaptive immune system.

Concerning the adaptive immune system both $CD4^+$ (helper) T-cells, $CD8^+$ (cytotoxic) T-cells and B-cells are increased in the airways of COPD patients.[30] However, both in the small and the large airways, the adaptive immune response in COPD patients is thought to be mainly driven by $CD8^+$ cytotoxic T-cells, as these are the predominant cells in both the large and small airway walls.[30] The increased activity of the $CD8^+$ T-cells that has been observed in COPD,[30] may be in part due to lack of immune regulation by regulatory T-cells, as these cells are present in lower numbers in the airways of COPD patients compared to smoking and non-smoking controls.[10] Also, the pro-inflammatory, IL-17-producing Th-17 cells have shown to be increased in the airways of COPD patients compared to smoking and non-smoking controls.[40] IL-17 is an important cytokine which upon stimulation leads to release of CXCL8, an important chemo-attractant for neutrophils, from bronchial epithelial cells, further aggravating the neutrophilic infiltration in the airways of COPD patients in a self-augmenting loop.[40]

Damage associated molecular patterns, the danger from within

During recent years our understanding of the processes involved in cell death has increased. The differentiation between regulated, apoptotic cell death and unregulated, necrotic cell death is outdated and over-simplified. Recently, many cell death modalities have been described, including pyroptosis, paraptosis, NETosis, autophagy, secondary necrosis, accidental necrosis, intrinsic- and extrinsic-apoptosis and necroptosis. For the sake of simplicity we will use the term immunogenic cell death when we refer to a cell death modality which induces a pro-inflammatory response mediated by the release of DAMPs. Furthermore, we will use the term necrosis to describe all cell death modalities which lead to uncontrolled rupture of the cell membrane followed by the release of the intracellular content, including accidental necrosis, secondary necrosis which occurs as a consequence of failed apoptosis and the regulated form of necrosis, necroptosis.

As mentioned above, CS can lead to epithelial damage, inducing the release of endogenous danger signals that activate PRRs and induce immune responses.[44,31] Already in 1994 Polly Matzinger proposed the danger hypothesis, in which she postulated that not only infectious agents originating from strangers can activate the immune system, but also endogenous danger signals originating from damaged or necrotic cells can induce an inflammatory response.[24] To date more than 20 different endogenous danger signals or DAMPs are known, all showing great homology with PAMPs and activating the same receptors. It was not until 2009 that the danger theory of Matzinger was first applied to the pathogenesis of COPD, as it was postulated that CS induces damage to the airway epithelium, leading to the release of DAMPs and triggering an innate immune response in the airways.[14,9] Later it was proposed that toxic compounds present in CS, e.g. bacterial products and genetic material of the tobacco plant, can also directly activate PRRs in addition to the DAMPs released from damaged and/or necrotic epithelial cells.[6,25] Evidence is accumulating that DAMPs indeed contribute to the pathophysiology of COPD. For instance, several DAMPs, including S100A8/A9, β-defensin, LL-37, HMGB1 and ATP have been found increased in the bronchoalveolar lavage (BAL) fluid or the epithelial lining fluid (ELF) of COPD patients compared to smoking and non-smoking controls,[33,8,47,39] indicating that the number of damaged and necrotic cells, releasing DAMPs, is increased in COPD patients. Furthermore, the expression of several PRRs e.g. TLR2, TLR4 and RAGE, was found to be increased in the lungs of COPD patients compared to both smoking

and non-smoking controls.[33] Thus, the CS-induced release of DAMPs may induce a stronger pro-inflammatory response in the lungs of COPD patients. Furthermore, the gene encoding the pattern recognition receptor RAGE, *AGER*, was identified as a susceptibility gene for the decline in lung function and the inception of COPD.[33,34,7] A single nucleotide polymorphism (SNP) within the *AGER* gene that shows a strong association with lung function decline and serum soluble RAGE levels (rs2070600), might be functionally involved in COPD, as this SNP changes the glycosylation pattern of the ligand-binding domain of RAGE.[18] This may induce increased activation of RAGE, leading both the release of pro-inflammatory mediators and increased lung tissue damage.[35]

Exposure of the airways to CS does not only induce damage to airway epithelial cells, but also to the connective tissue surrounding the epithelium.[9] Breakdown products of the extracellular matrix (ECM) have also been shown to activate PRRs and thus act as a DAMP.[20] These ECM products have found to be increased in COPD patients compared to smoking controls.[29,33] DAMPs released from damaged epithelial cells and ECM breakdown products both are able to activate neighboring epithelial cells in a PRR-mediated and NF-κB-dependent way, inducing the release of pro-inflammatory cytokines, like CXCL8. As mentioned above, CXCL8 is a strong neutrophil chemotactic factor, leading to neutrophilic infiltration in the airways. Neutrophils in turn secrete proteolytic enzymes and reactive oxygen species which together damage the lung tissue further, leading to cell death and DAMP release, inducing a positive feedback loop.[9] Thus, DAMP signaling may play an important role in the pathophysiology of COPD.

Nevertheless, much is still unknown about the role of DAMPs in the pathobiology of COPD. For instance, it is unknown which DAMPs are released upon inhalation of toxic gases like CS and whether cells in the airways of COPD patients release the same levels and profile of DAMPs in response to CS exposure as those of healthy individuals. Additionally, it is unknown whether COPD patients have an altered susceptibility for CS-induced DAMP release and DAMP-induced airway inflammation, and which genes are involved in these processes.

THE SCOPE OF THIS THESIS

The DAMP theory for COPD: a novel concept for airway inflammation induced by exposure to inhaled toxicants

We propose a novel concept for the development of the inflammatory response in the airways of COPD patients, in which we hypothesize that DAMPs play a critical role. In this theory we hypothesize that in individuals who are genetically susceptible for the development of COPD, chronic inhalation of toxicants induces exaggerated immunogenic cell death and DAMP release by lung structural cells and immune cells, which subsequently leads to the initiation and maintenance of airway inflammation, ultimately leading to the development of COPD.

In **chapter 2**, we provide an overview of the available literature on DAMPs in COPD. Here, we divided most known DAMPs into different categories based on the sub-cellular origin of the DAMPs, e.g. cytoplasm, mitochondria, other subcellular organelles and the extracellular matrix. For all these DAMPs we provide an overview of the available data on their role in COPD at the start of this thesis.

In **chapter 3** we investigated the basic molecular mechanism underlying the DAMP theory for COPD. Here, we tested the hypothesis that exposure of bronchial epithelial cells to CSE induces immunogenic cell death and subsequent DAMP release and that these DAMPs are able to activate pro-inflammatory responses in neighboring epithelial cells. Furthermore, we investigated whether this process of CS-induced DAMP release and airway inflammation could be pharmacologically inhibited *in vitro* and *in vivo*.

Next in **chapter 4**, we aimed to study whether these processes are dysregulated in COPD patients. To this end we utilized samples from the TIP study, which was designed to investigate genetic susceptibility for COPD.[43,17] In this cohort, subjects were divided in four groups of old (40-75 year old) and young (18-40 year old) individuals, where the old individuals were either COPD patients (GOLD stage I-IV) or age- and smoking history-matched controls, while the young individuals were party smokers with normal lung function and either a high or low familial risk to develop COPD. Party smoking was defined as irregular smoking with the ability to quit smoking for at least two days. The young subjects were classified as susceptible for the development of COPD when the

prevalence of COPD in smoking first or second degree relatives older than 45 years meets the following criteria: 2 out of 2, 2 out of 3, 3 out of 3, 3 out of 4 or 4 out of 4 smoking family members have developed COPD and were classified as non-susceptible to COPD only when none of the smoking first or second degree relatives who are at least 45 years of age (at least two should be identified) have been diagnosed with COPD. Families with alpha-1 antitrypsin deficiency were excluded in this study. Bronchial brushings were collected from all subjects as a source for bronchial epithelial cells. Furthermore, serum, induced sputum and ELF samples were taken at two time-points: 1) after two days of smoking cessation and 2) upon smoking three cigarettes within one hour.

Next, we investigated whether the processes of CS-induced immunogenic cell death and levels of released DAMPs are different in primary epithelial cells from these subjects. Here, we tested the hypothesis that the airway epithelium from COPD patients displays exaggerated CS-induced DAMP release and/or DAMP-induced pro-inflammatory responses. To this end we studied the effect of CS on DAMP release from epithelial cells *in vitro* and *in vivo,* on DAMP release in serum and on the expression of 30 genes encoding DAMPs and their receptors, the DAMP gene-set, in airway epithelial cells from COPD patients, matched controls and individuals either susceptible or non-susceptible for COPD. Furthermore, to test whether differences in CS-induced inflammation are caused by a different sensitivity to DAMPs, we investigated the effect of specific DAMPs *in vitro* as well as *in vivo* by investigating the effect of intranasal treatment with one single DAMP, i.e. mtDAMPs or LL-37, on neutrophilic airway inflammation in mice either genetically susceptible or non-susceptible for CS-induced airway inflammation.

In **chapter 5** we asked whether there is a genetic factor involved in CS-induced DAMP release and the subsequent airway inflammatory response. Therefore, we utilized a mouse model for CS-induced airway inflammation. In this thesis we aimed to investigate CS-induced DAMP release and subsequent neutrophilic airway inflammation, in which the early responses to CS exposure are important, before chronic manifestations of COPD have developed. Therefore, we used a short-term smoke exposure model with five subsequent days of CS exposure. In this model, mice were exposed to CS via the full body smoke exposure system by placing mice in a 16 liter Perspex box and pumping CS mixed with ambient air in the box.[50] Upon exposure of mice to CS for five days with 20 cigarettes per day, divided over two exposure sessions, neutrophilic airway inflammation and airway hyper-responsiveness is induced without the formation of emphysema.[24] In this chapter we utilized 30 inbred mouse strains with known genetic background to identify which genes are associated with CS-induced neutrophilic airway inflammation. Furthermore, with this study we showed which mouse strains are susceptible for CS-induced neutrophilic airway inflammation and which strains are non-susceptible. In **chapter 6** we aimed to test whether susceptibility for CS-induced airway inflammation is associated with an altered DAMP release profile upon CS exposure. Therefore, we selected two susceptible and two non-susceptible mouse strains and studied whether the levels of a panel of six DAMPs in BAL fluid correlate with susceptibility for CS-induced airway inflammation. Next, in **chapter 7** we investigated which genes are associated with the susceptibility for CS-induced DAMP release. To this end, we utilized the genetic screen of 28 different inbred mouse strains which were all exposed to CS and control air and measured the BAL levels of several DAMPs. Furthermore, the identified candidate genes were validated by measuring the expression of the candidate genes in lung tissue of susceptible and non-susceptible mouse strains. In addition, we evaluated whether knockdown of these genes altered the susceptibility of human alveolar epithelial cells to CS-induced cell death and DAMP release, and tested whether these genes were differentially regulated by CS exposure in human primary bronchial epithelial cells from individuals with a family history indicative of either presence or absence of susceptibility for COPD.

Next, in **chapter 8** we investigated the role of DAMP signaling during COPD exacerbations, for the reason that the inflammatory reaction in the airways of COPD patients is most severe during exacerbations. Therefore, we hypothesized that the levels of released DAMPs are increased during an exacerbation compared to stable disease. To this end, we used samples from the exacerbation study cohort. This cohort was designed to be able to directly compare COPD patients when they are in stable disease and when they are experiencing an exacerbation. Serum, induced sputum and ELF samples were collected from COPD patients in stable disease,

after which the treatment with inhaled corticosteroids and long acting β2-agonists was discontinued. After the collection of samples the patients had to be in stable disease for at least 60 days. The moment they report an exacerbation, a second set of samples was taken, allowing the direct comparison between stable disease and exacerbation within the same patients. Here, we measured a profile of DAMPs in serum and induced sputum of COPD patients during an exacerbation and in the same patients when they are in stable disease. Furthermore, in **chapter 9** we hypothesized that the expression of DAMP receptors is increased on circulating neutrophils during exacerbations. To assess this, we measured the expression of several PRRs on peripheral blood neutrophils of COPD patients during exacerbation and stable disease. Additionally, we measured the levels of soluble RAGE, the decoy receptor for RAGE, in serum of these patients, as the levels of soluble RAGE may influence the activity of RAGE.

After the initial response of CS-induced damage and DAMP release from airway epithelial cells, neutrophils are attracted to the site of damage. The neutrophil is the predominate cell type in the inflammatory infiltrate in the airways of COPD patients. Therefore, in **chapter 10** we asked whether neutrophils are also an important source of DAMPs upon inhalation of CS and whether DAMPs released from epithelial cells can directly activate PRRs on neutrophils to aggravate the inflammatory response in COPD. To this end, we investigated the CS-induced DAMP release and airway inflammation in human and murine neutrophils using *in vitro* and *in vivo* models. Hitherto, mice were exposed to CS for five days and the level of inflammation and DAMP release in the airways was determined. Furthermore, neutrophils were isolated from healthy subjects and were subsequently exposed to smoke to investigate the effects on DAMP release and cell death.

RAGE is one of the most important DAMP receptors and has shown to be strongly associated with COPD. Therefore, we hypothesized that the expression of RAGE and RAGE-ligands is increased with the severity of COPD and that the expression of the decoy receptor sRAGE, is decreased with the severity of COPD. In **chapter 11** we studied the association between the expression of RAGE, sRAGE and RAGE-ligands in different body compartments and the severity of COPD. Finally, in **chapter 12** the findings of this thesis are summarized and discussed and the future perspectives are described.

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