DAMPs, endogenous danger signals fueling airway inflammation in COPD
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CHAPTER XII

SUMMARY, GENERAL DISCUSSION & FUTURE PERSPECTIVES
Chapter XII

SUMMARY

Chronic obstructive pulmonary disease (COPD) is a complex, progressive and disabling pulmonary disease, characterized by chronic airway inflammation, persistent airflow limitation and accelerated lung function decline.\(^9\) COPD is caused by chronic exposure to noxious gases and particles, like cigarette smoke (CS), leading to an exaggerated inflammatory response, which can result in airway remodeling with thickening of the (small) airway wall and mucus hypersecretion (bronchitis) as well as destruction of alveolar tissue (emphysema).\(^\text{23}\) During the early phases of COPD, the innate immune system is thought to be mainly responsible for the inflammatory response, while in later stages of the disease the adaptive immune system becomes more important.\(^\text{23}\) In this thesis we postulate that DAMPs, endogenous danger signals released from damaged or dying cells, can initiate the innate response during the early phases of the disease. The group of molecules currently known as DAMPs consists of approximately 30 different molecules with a wide variety in molecular structure and physiological functions. For instance, DNA and RNA molecules can act as DAMPs, as well as proteins such as HMGB1, HSP70, S100 proteins and LL-37. Other groups of DAMPs include small molecules such as ATP, alarmins with cytokine-like functions such as IL-1α and IL-33 and extracellular matrix proteins such as fibronectin, fibrinogen and hyaluronan. All DAMPs share the ability to activate pattern recognition receptors (PRRs) on cells of the innate immune system, upon their release into the extracellular space.\(^\text{11}\) Activation of PRRs induces the activation of several pro-inflammatory signaling pathways, including an NF-κB-dependent transcriptional response, in tissue resident cells of the innate immune system, such as airway epithelial cells, alveolar macrophages and dendritic cells in case of the lungs. Release of pro-inflammatory mediators by activated innate immune cells subsequently leads to an inflammatory response, with several inflammatory cells, including neutrophils being recruited to the site of damage.\(^\text{38}\) Airway epithelial cells are the first cells to come in contact with inhaled toxicants, and are therefore likely to play an important role in the induction of an innate immune response by releasing DAMPs. In this thesis we investigated the hypothesis that toxic gases, i.e. CS, induce cellular damage in airway epithelial cells with subsequent release of DAMPs, and that these DAMPs contribute to the development of chronic inflammation which is seen in the airways of COPD patients. We tested whether sensitivity for cellular damage or for the release of DAMPs by damaged cells is increased in COPD. Furthermore, we studied whether genetic susceptibility for CS-induced DAMP release exists and which genes are responsible for this process.

In chapter 2 the available knowledge in literature at the time of the start of this project on the role of DAMPs, classified based on their subcellular origin, e.g. cytoplasm, mitochondria, other subcellular organelles and the extracellular matrix, in the development of COPD has been discussed on basis of an overview of literature. This led us to postulate a novel concept for COPD, where the inhalation of noxious gases induces immunogenic cell death of airway epithelial cells and subsequent release of DAMPs. These can activate the innate immune system, triggering neutrophilic airway inflammation in individuals genetically susceptible for the development of COPD. Several DAMPs were shown to be increased in serum, BAL fluid, epithelial lining fluid or sputum of COPD patients compared to both smoking and non-smoking controls, including HMGB1, S100A8/A9 and HSP70. In the remaining chapters of this thesis we provide further support for this DAMP theory for COPD.

In chapter 3 we tested the hypothesis that exposure of bronchial epithelial cells to cigarette smoke induces immunogenic cell death and subsequent DAMP release, leading to activation of pro-inflammatory responses in neighboring epithelial cells. Here, we exposed human bronchial epithelial cells (BEAS-2B cells) to cigarette smoke extract (CSE) \textit{in vitro} and showed that this induces necrotic cell death and DAMP release. Furthermore, we showed that these DAMPs are able to induce a CXCL8 response in bronchial epithelial cells that have not been exposed to CSE. We also found that the predominant mechanism of cell death that is induced by CSE exposure is necroptosis, a regulated form of necrosis which is known to cause DAMP release. Using a mouse model, we showed that acute CS exposure induces DAMP release \textit{in vivo} and that pharmacological inhibition of necroptosis reduces the severity of CS-induced airway inflammation. Together, this study shows that CS exposure induces airway epithelial cell death and subsequent DAMP release, which is accompanied by neutrophilic airway inflammation \textit{in vivo} and mediated, at least in part, by necroptosis. This strengthens our hypothesis that CS-
induced DAMP release is important in the initiation of neutrophilic airway inflammation.

Next, in chapter 4, we investigated whether CS-induced DAMP release and/or DAMP-induced airway inflammation contributes to the susceptibility for COPD. To this end, we first compared the CS-induced expression of a group of genes encoding DAMPs and their receptors, the DAMP gene-set, in COPD patients and matched controls and in healthy individuals genealogically determined to be either susceptible or non-susceptible for the onset of COPD. We employed a functional gene-set enrichment analysis to determine whether expression of the DAMP gene-set was altered between these groups. Here, we identified that out of the gene-set, 15 genes were core-enriched, including the genes encoding galectin-3, TLR2, TLR4, and S100A9, with Hyaluron Synthase 2, TLR7, galectin-9, galectin-3 and the Formyl Peptide Receptor 2 as the top five. Moreover, we showed that the increase in expression of the DAMP gene-set induced by cigarette smoking is significantly greater in airway epithelial cells isolated from COPD patients compared to those isolated from controls. A similar trend with a stronger CS-induced increase of the expression levels of the DAMP gene-set was found in susceptible compared to non-susceptible individuals. This indicates that the transcriptional response of specific DAMPs and DAMP receptors to CS exposure is dysregulated in COPD as well as in susceptible individuals prior to disease onset, arguing for a contribution of this response to disease etiology. In further in vitro studies, we showed that the exposure of primary airway epithelial cells to CSE leads to the release of various DAMPs, with a stronger increase in the release of galectin-3 in cells from COPD patients compared to controls. Stimulation of primary epithelial cells with galectin-3, LL-37 and mitochondrial DAMPs (MTDs) strongly induced a partially TLR-dependent CXCL8 response. In addition, using a mouse model we showed that a single intranasal injection of LL-37 or MTDs induces neutrophilic airway inflammation. Interestingly, this only occurred in mouse strains susceptible for CS-induced neutrophilic airway inflammation and not in non-susceptible mouse strains. Together, these data indicate that genetic susceptibility to enhanced expression of DAMPs and PRRs in bronchial epithelial cells upon CS exposure may contribute to the development of airway inflammation in COPD patients. In an in vivo model one single treatment with LL-37 or MTDs induced an innate inflammatory response in CS susceptible mouse strains, indicating that a predisposition to release DAMPs and subsequent induction of inflammation may contribute to the development of COPD.

Chapter 5 describes the genetics of CS-induced airway inflammation in mice. Here, we investigated the genetics underlying susceptibility for CS-induced neutrophilic airway inflammation in mice. Thirty different inbred mouse strains were exposed to CS or control-air for five consecutive days. Afterwards the level of neutrophilic airway inflammation was analyzed using several methods, including lung tissue myeloperoxidase (MPO) measurements. Using haplotype association mapping (HAM) analysis, a genome-wide association study for inbred mouse strains, four novel susceptibility genes for basal MPO levels and five susceptibility genes for CS-induced lung tissue MPO levels were identified. Of these genes, three showed CS-induced changes in lung tissue gene expression that correlated with susceptibility for CS-induced neutrophilic airway inflammation. Investigation of the gene ontology of the candidate genes did not reveal a single biological activity or signaling pathway to be critical for CS-induced neutrophilic airway inflammation, although several of the candidate genes are known to be involved in the regulation of cell death processes. Together, we showed that CS-induced neutrophilic airway inflammation has a strong genetic component and that several genes involved in cell death processes contribute to the susceptibility for this response in a mouse model.

In chapter 6, we hypothesized that CS-induced DAMP release underlies the susceptibility for CS-induced neutrophilic airway inflammation in mice. To this end, we selected four mouse strains from the 30 mouse strains described in chapter 5, covering a range from highly susceptible to fully resistant to develop neutrophilia in response to CS exposure. We measured a profile of six different DAMPs, i.e. calreticulin, galectin-3, S100A8, dsDNA, HSP70 and HMGB1 in BAL fluid of these mice, leading to the identification of a CS-induced DAMP release profile which was characteristic for susceptibility to CS-induced airway inflammation. Specific DAMPs, e.g. dsDNA and S100A8, were released upon CS exposure in susceptible but not in non-susceptible strains, while other DAMPs were released upon CS exposure in all mouse strains, or randomly in only one or two strains.
These data indicate that the difference in DAMP release between susceptible and non-susceptible mice is not only quantitative but also qualitative. Together, this chapter shows that genetic susceptibility for CS-induced neutrophilia is associated with a specific profile of DAMPs released into BAL fluid of mice, which can possibly be used as a biomarker for susceptibility to cigarette smoke-induced neutrophilic airway inflammation.

In chapter 7, we further explored the genetic susceptibility for CS-induced DAMP release. Here, we performed a similar study as in chapter 5, investigating the effect of genetic background on CS-induced DAMP release in mice, using the same cohort of CS-exposed inbred mouse strains. In BAL fluid of 28 mouse strains exposed to CS or air (control) we measured the levels of four DAMPs, i.e. dsDNA, mtDNA, HMGB1 and HSP70. Of these four DAMPs, dsDNA showed the highest correlation with CS-induced neutrophilic airway inflammation. HAM analyses identified 11 candidate genes for either CS-induced or basal dsDNA release in mice, of which two genes (Elac2 and Ppt1) showed differential expression in lung tissue upon CS exposure between susceptible and non-susceptible mice, in accordance to their haplotype. Furthermore, knockdown of ELAC2 and PPT1 in human alveolar epithelial cells markedly altered susceptibility to CSE-induced cell death and dsDNA release. Lastly, using primary bronchial epithelial cells from healthy individuals either susceptible or non-susceptible for the onset of COPD we found that the CS-induced gene expression of ELAC2 showed a trend towards a difference between susceptible and non-susceptible individuals, while ENOX1 and ARGHGEF11 were significantly different between these groups. Together, this chapter shows that susceptibility to CS-induced DAMP release is genetically determined, and may contribute to neutrophilic airway inflammation in vivo. This may thus have consequences for the susceptibility to develop COPD.

The previous chapters have shown that CS exposure induces immunogenic cell death and DAMP release from airway epithelial cells and that the regulation of DAMP release is dependent on the genetic background in mice. Similarly, there is a higher susceptibility to release specific DAMPs in epithelial cells from COPD patients compared to healthy controls upon exposure to CS. COPD patients show the highest rates of inflammation during exacerbations, a sudden worsening of symptoms. To study whether DAMP release may also contribute to the aggravation of airway inflammation during exacerbations, in chapter 8 we compared the levels of specific DAMPs in induced sputum and serum of COPD patients during an exacerbation and stable disease. We measured a panel of six DAMPs, i.e. dsDNA, mtDNA, galectin-3, LL-37, S100A9 and HMGB1. Of these DAMPs three were increased in serum during an exacerbation compared to stable disease. Notably, these three DAMPs, HMGB1, S100A9 and LL-37, are all ligands of the RAGE receptor, indicating that the RAGE-signaling pathway may be involved in the pathophysiology of COPD exacerbations. Furthermore, we found that this increase was higher in females and in individuals in whom the exacerbation was not associated with airway infection. Together, this chapter shows that RAGE-activating DAMPs are increased in serum of COPD patients during an exacerbation, which opens an avenue to novel therapeutic targets to decrease the inflammatory reaction in COPD patients during an exacerbation.

In chapter 9, we further studied the expression of DAMP receptors on peripheral blood neutrophils during COPD exacerbations and stable disease. Neutrophils are important inflammatory cells and the most abundant infiltrating cells in the airways of COPD patients during exacerbations. Here, we hypothesized that the expression of DAMP receptors on peripheral neutrophils is increased during exacerbations, leading to DAMP-induced migration of neutrophils to the airways during COPD exacerbations. We showed that the expression of the DAMP receptors TLR2, TLR4 and NLRP3 is increased on peripheral blood neutrophils of COPD patients during an acute exacerbation compared to stable disease. Furthermore, we showed that the levels of circulating sRAGE are decreased in COPD patients during an exacerbation. The combined findings of increased levels of circulating DAMPs and increased neutrophilic expression of DAMP receptors, further supports our hypothesis that DAMP signaling contributes to the increase in airway inflammation during COPD exacerbations.

In chapter 10 we investigated whether CS exposure induces necrotic cell death and DAMP release in neutrophils and whether these DAMPs are able to activate bronchial epithelial cells. In previous chapters we describe a prominent role of airway epithelial cells in the initiation of CS-induced and DAMP-mediated airway
inflammation. Here, we tested the hypothesis that neutrophils contribute to the chronicity and severity of this process by acting as an additional source of DAMPs upon re-exposure to CS once the airway inflammation has been induced. Because airway epithelial cells are the first cells to be encountered by inhaled toxicants, the DAMP-mediated inflammatory response is likely to be initiated by airway epithelial cells, which subsequently promotes the influx of neutrophils into the lungs. Consequently, these neutrophils are exposed to inhaled toxicants, possibly leading to neutrophilic cell death and DAMP release. In chapter 10, we found that neutrophils can undergo necrotic cell death and release DAMPs upon exposure to CS, and that these DAMPs are able to activate primary bronchial epithelial cells. The immunostimulatory effects of these released DAMPs were partially inhibited by blocking TLR2, TLR4 and RAGE, indicating the involvement of these receptors. Together, this chapter describes that not only airway epithelial cells but also neutrophils are sensitive to CS-induced cell death and DAMP release, indicating that neutrophilic cell death can aggravate the DAMP-mediated airway inflammatory response seen in the lungs of COPD patients.

Finally, in chapter 11 we further explored the importance of RAGE signaling in COPD patients. Here, we assessed whether there is an association of RAGE ligands and sRAGE with the severity of COPD and the decrease in lung function. We measured the levels of sRAGE in serum and induced sputum and the accumulation of advanced glycation end-products (AGEs) in the skin. COPD patients showed increased accumulation of AGEs in the skin and lower sRAGE levels in serum compared to controls, and both of these values correlated with lung function and the severity of COPD. Furthermore, lower serum sRAGE levels significantly and independently predicted the accumulation of AGEs in the skin. Moreover, we identified two SNPs flanking the AGER gene, which showed significant association with lower serum sRAGE levels and higher accumulation of AGEs in the skin respectively. This chapter adds additional evidence to the theory that RAGE signaling is important in the pathogenesis of COPD.

In summary, our studies support the concept of CS-induced DAMP release and subsequent neutrophilic airway inflammation in the development of airway inflammation in COPD. Together, this thesis provides extensive evidence for the DAMP theory for COPD, using both in vitro and in vivo models.

**GENERAL DISCUSSION**

With this thesis we aimed to test the DAMP theory for COPD, a novel concept that the inhalation of toxic gases induces immunogenic cell death and subsequent DAMP release which will lead to chronic neutrophilic airway inflammation in genetically susceptible individuals, which ultimately contributes to the development of COPD. We provide several lines of support for this theory. First of all, we have shown using in vitro and in vivo models that CS exposure induces immunogenic cell death and DAMP release from airway epithelial cells and that these DAMPs are able to induce neutrophilic airway inflammation (Figure 1). This process of CS-induced neutrophilic airway inflammation can be reduced by pharmacologically blocking the execution of necroptosis. Additionally, inhibition of specific DAMPs like galectin-3 or DAMP receptors such as RAGE may also reduce CS-induced DAMP release and subsequently airway inflammation. Secondly, we have shown that susceptibility for CS-induced neutrophilic airway inflammation is associated with susceptibility for CS-induced DAMP release in a murine in vivo model (Figure 2). Correspondingly, the intranasal application of LL-37 and mtDAMPs only induced airway inflammation in mouse strains susceptible for CS-induced neutrophilic airway inflammation and DAMP release. This indicates that release of DAMPs is a major factor in the susceptibility for CS-induced airway inflammation. Furthermore, we found that several susceptibility genes for CS-induced DAMP release, including Ppt1, Elac2 and Cflar, are involved in the regulation of cell death processes. These genes are likely to be associated with the early inflammatory responses in COPD, although their relevance for human disease should be validated in future studies. Lastly, we showed that RAGE signaling plays an important role in COPD pathophysiology. Most notably during COPD exacerbations, for which we have shown that specific RAGE-activating DAMPs are increased in BAL fluid and during which the expression of specific DAMP receptors is increased on peripheral blood neutrophils (Figure 3).
In conclusion, our studies support an important role for CS-induced DAMP release in the induction of airway inflammation during the early stages of COPD and during exacerbations of this disease. Signaling of DAMPs through their receptors is likely to be a critical step in the initiation of neutrophilic airway inflammation that will eventually culminate in the chronic obstructive disease, for which no effective interventions exist to date. We propose to further characterize DAMP profiles as biomarkers for disease susceptibility and PRRs and their downstream signaling pathways as targets for intervention.

**Figure 1: The DAMP theory for COPD.** Schematic representation of the DAMP theory for COPD. In genetically susceptible individuals and COPD patients inhalation of cigarette smoke induces exaggerated cellular damage and immunogenic cell death, e.g. necrosis or necroptosis, of structural airway cells and immune cells. This causes release of Damage Associated Molecular Patterns (DAMPs) which can activate Pattern Recognition Receptors (PRRs) on neighboring epithelial cells and immune cells. The activated airway epithelial cells secrete pro-inflammatory cytokines which attract cells of both the innate and the adaptive immune system. During COPD exacerbations the amount of DAMPs that are released and the expression of PRRs is even further increased. This process can be reduced using several inhibitors. Necroptosis can be pharmacologically inhibited by Necrostatin-1, RAGE can be inhibited by sRAGE and RAGE Antagonistic Peptide (RAP) and DAMPs can be inhibited by specific antagonists including a galectin-3 inhibitor. (see color image on page 217)

In conclusion, our studies support an important role for CS-induced DAMP release in the induction of airway inflammation during the early stages of COPD and during exacerbations of this disease. Signaling of DAMPs through their receptors is likely to be a critical step in the initiation of neutrophilic airway inflammation that will eventually culminate in the chronic obstructive disease, for which no effective interventions exist to date. We propose to further characterize DAMP profiles as biomarkers for disease susceptibility and PRRs and their downstream signaling pathways as targets for intervention.

**The cellular source of DAMPs in COPD; a role for macrophages in the cellular interplay responsible for DAMP release in COPD**

Although we have gathered substantial evidence to support the DAMP theory for COPD, many questions remain to be answered. These questions include unraveling which immunogenic cell death modalities and types of damage are induced upon cigarette smoke exposure and identification of the cellular source of the released DAMPs in COPD patients. We provided evidence that the CS-induced and DAMP-mediated inflammatory response is initiated by damage to airway epithelial cells and aggravated by CS-induced necrosis of neutrophils.
Additionally, it was recently suggested that also airway macrophages are involved in the complex cellular interplay during DAMP-mediated airway inflammation in COPD patients. In healthy individuals approximately 95% of the cells found in BAL fluid are alveolar macrophages. These macrophages are responsible for the efficient phagocytosis, also called efferocytosis, of invading pathogens and apoptotic cells. The efferocytic function of airway macrophages has been shown to be significantly impaired in COPD, as macrophages from the airways of COPD patients were unable to efficiently clear apoptotic epithelial cells and neutrophils. Failure to clear damaged cells can cause apoptotic cells to go into secondary necrosis, leading to the release of DAMPs. Efferocytosis by airway macrophages can be inhibited by HMGB1, which we and others have observed to

**Figure 2: Genetic susceptibility determines the severity of DAMP or CS-induced neutrophilic airway inflammation.** Inbred mouse strains can be highly susceptible, totally non-susceptible or anything in between, for the onset of either DAMP- or CS-induced neutrophilic airway inflammation, based on their genetic background. One intranasal treatment with the DAMPs, LL-37 or mitochondrial DAMPs (mtDAMPs) is sufficient to induce neutrophilic airway inflammation in susceptible mouse strains. Similarly, five consecutive days of exposing mice to two times 10 cigarettes per day is sufficient to induce neutrophilic airway inflammation in susceptible mice. Whether a mouse develops neutrophilic airway inflammation or not is dependent on susceptibility genes. Several susceptibility genes for CS-induced neutrophilic airway inflammation and DAMP release have been identified. Multiple genes, including Elac2, Ppt1 and Cflar are associated with cell death processes. Therefore, genetic susceptibility for CS-induced immunogenic cell death may be involved in the onset of CS-induced neutrophilic airway inflammation in mice. (see color image on page 218)
be increased in BAL fluid of COPD patients. Therefore, the dysfunctional efferocytosis of neutrophils and airway epithelial cells by airway macrophages may also contribute to DAMP-induced airway inflammation in COPD, in addition to the observed direct induction of epithelial cell and neutrophil necrosis by cigarette smoke exposure. In our in vitro models macrophages were not present, which could account for the discrepancy with our in vivo model, where genetic susceptibility for CS-induced DAMP release was associated with genes involved in the regulation of cell death, while we did not observe differences in cell death between epithelial cells from individuals susceptible and non-susceptible for COPD in vitro. The susceptibility for CS-induced necrosis and subsequent DAMP release might in part be related to the ability of macrophages to phagocytose apoptotic epithelial cells and neutrophils. In addition, the discrepancy between the in vitro and in vivo results with respect to the finding that genetic susceptibility determines DAMP-induced inflammation in vivo, might be explained by the notion that DAMP-induced inflammation may require gene-environment interaction in humans, which was not taken into account in our in vitro assays. Furthermore, DAMP-induced inflammation may include genetic factors, while the criteria for susceptibility in the human study relied merely on a genealogical study, and not all susceptibility genes may be equally spread amongst first and second degree family members.

**Susceptibility for COPD; your fate resides in your genes**

One of the most important research questions of this thesis is whether DAMPs are involved in disease susceptibility. To date, many susceptibility genes for COPD, including *IL6R, MMP12, HHIP, AGER, FAM13A* and
CHRNA3 have been identified by candidate gene-association studies, linkage studies and genome-wide-association studies. However, these studies did not identify one or several distinct signaling pathways involved in the development of COPD, and therefore the biological mechanisms that underlie the inception of COPD are still not understood. The fact that so many different genes have been identified as susceptibility genes for COPD is likely a consequence of COPD being a complex, multi-factorial disease that can be divided into different endotypes. It is plausible that susceptibility for the emphysema endotype is controlled by different genes compared to the bronchitis endotype. In this thesis we investigated the genetics of the early processes of CS-induced DAMP release and neutrophilic airway inflammation. These processes may contribute to the initiation of chronic airway inflammation in COPD. The HAM analyses for both dsDNA release and neutrophilic airway inflammation identified several genes involved in the regulation of cell death, indicating that susceptibility for dysregulated cell death increases CS-induced DAMP release and subsequent neutrophilic airway inflammation. Exposing multiple mouse strains to a damaging stimulus or environmental insult before performing HAM analysis to identify candidate genes has proven to be successful before, as similar studies identified several susceptibility genes for ventilator-, chlorine-, acrolein-, and phosgene-induced acute lung injury.

Three examples of genes we showed to be involved in CS-induced dsDNA release are Elac2, Ppt1 and Cflar. Elac2 encodes the protein elaC ribonuclease Z 2, which catalyzes the removal of the 3’ tail from precursor tRNAs. Interestingly, Elac2 is also involved in the regulation of cell death, as Elac2 is important for the DNA damage response, which upon failure leads to the induction of cell death.75 We did not find dysregulated ELAC2 expression upon CS exposure in young individuals susceptible or non-susceptible for COPD and no studies have been performed investigating the role of Elac2 in COPD to date, opening avenues for future research.

Ppt1 encodes the protein palmitoyl-protein thioesterase 1, a small glycoprotein which induces catabolism of lipid-modified proteins upon lysosomal degradation. In similarity with Elac2 also Ppt1 is involved in the initiation of apoptosis, as Ppt1 knock-out mice and PPT1 down-regulation in human cells showed decreased levels of apoptosis.64,66 The function of PPT1 in COPD has yet to be uncovered, although recently it was shown in two independent genome-wide association studies that SNPs within PPT2 were associated with emphysema and lung function decline.64,59 However, it cannot be excluded that these signals derive from the strong association of AGER with emphysema and lung function decline, as the AGER gene is in close proximity to PPT2.

CASP8 And FADD Like Apoptosis Regulator (Cflar), also called Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (C-FLIP), is another gene we identified to be a susceptibility gene for CS-induced dsDNA release. This gene plays an important multifunctional role in the regulation of both the extrinsic apoptotic pathway and the necroptotic pathway.77 In short, C-FLIP can form a heterodimer with Caspase-8 which prevents the cleavage and thereby activation of Caspase-3, leading to failure to execute the extrinsic apoptotic pathway. In addition, the formation of this Caspase-8:C-FLIP heterodimer also abolishes the inhibitory function of Caspase-8 on Receptor Interacting Serine/Threonine Kinase 3 (RIPK3), a key regulator of necroptosis, thereby stimulating RIPK3-dependent and mixed lineage kinase domain like (MLKL)-mediated necroptosis.28 This places C-FLIP at a crucial position on the balance between extrinsic apoptosis and necroptosis, subsequently contributing to the decision whether a cell dies in a non-immunogenic (apoptotic) or immunogenic (necroptotic) fashion. This provides a possible explanation for the association with CS-induced dsDNA release as necroptosis is associated with DAMP release, while apoptosis is not. To date, the role of C-FLIP in COPD is completely unknown. Nevertheless, our preliminary studies suggest an important role of C-FLIP in CS-induced apoptosis, necroptosis and subsequent DAMP release studied in an in vitro model (data not shown).

Using both mouse and human studies we have added novel information useful for understanding the complex genetics of CS-induced DAMP release and COPD. Novel genes and pathways were uncovered ultimately pointing at a dysregulation of regulated cell death pathways in individuals susceptible for COPD. Future studies should be aimed at addressing these regulated cell death pathways guiding lung structural and resident cells towards less immunogenic cell death and mapping which genes in these pathways can be used as biomarker for the early detection of susceptibility for COPD.
**DAMPs, a melting pot of molecules activating the immune system**

The group of DAMP molecules consists of approximately 30 different molecules and this list is still growing. Intracellularly these DAMPs possess a wide variety of functions, while upon release they all share the ability to activate pattern recognition receptors on cells of the innate immune system. In this thesis we showed that although all DAMPs possess similar functions upon release, there are specific DAMPs which are released in higher levels upon CS exposure or are more potent in inducing airway inflammation. For most DAMPs the levels of CS-induced release were not different between COPD patients and controls. This indicates that in general the process of CS-induced necrotic cell death and passive DAMP release in bronchial epithelial cells is not dysregulated in airway epithelial cells from COPD patients. Of all the different DAMPs, our data indicate that galectin-3 is of specific interest for COPD. Galectin-3 was more released upon CSE exposure by bronchial epithelial cells of COPD patients compared to controls. This is in agreement with our GSEA analysis where the gene encoding galectin-3 is one of the most significantly changed genes from our DAMP gene-set upon CS exposure in COPD patients compared to controls and our murine study in which the BAL galectin-3 levels upon CS exposure are related to susceptibility for neutrophilic airway inflammation. Furthermore, stimulation with galectin-3 induced CXCL8 release in epithelial cells *in vitro*, and this response could be partly inhibited by blocking TLR2/4 in cells from controls but not in cells isolated from COPD patients, indicating that the response towards galectin-3 is differentially regulated in COPD patients compared to controls. These combined results identify galectin-3 as a DAMP that may play an important role in COPD pathophysiology. Galectin-3 is a carbohydrate-binding lectin which contains a carbohydrate-recognition-binding domain (CRD) binding β-galactose sugars. Galectin-3 has been implicated in a wide variety of biological functions, including anti-microbial and anti-fungal functions, cell adhesion, cell activation, chemo-attraction, cellular differentiation and apoptosis. In COPD patients the expression of galectin-3 is increased in the small airway epithelium compared to smoking controls. Interestingly, it has been shown that increased levels of extracellular galectin-3 can increase the efferocytic potential of alveolar macrophages. Thus, the increased expression of galectin-3 upon CS exposure as observed in our study may contribute to the impaired efferocytosis that has been observed in COPD patients. Additionally, it has been shown that galectin-3 can be actively secreted via a non-conventional transport pathway dependent on caspase-1 activation. Caspase-1 can be activated by extracellular ATP-activated NLRP3, as described in chapter 2, causing a potential positive feedback loop of DAMPs activating caspase-1, initiating the release of galectin-3, which can then also act as a DAMP by activating β-galactose-containing receptors like the pro-inflammatory receptor β1-integrin. The conditions which induce active galectin-3 secretion are largely uncovered, although it has been described that galectin-3 secretion is cell-type specific and dependent on cellular differentiation and stress. It is plausible that these conditions are different in bronchial epithelial cells isolated from COPD patients compared to controls. However, the increased mRNA expression of galectin-3 upon CS exposure identified in our studies is the most likely explanation of why galectin-3 responses were found to be different between COPD patients and controls but other DAMPs are not.

Galectin-3 is not the only DAMPs that is released in high levels upon CS exposure. Upon investigating CS-induced DAMP release in BAL fluid of mice, we found that the release profile of DAMPs differed strongly among the different susceptible and non-susceptible mouse strains (chapter 6). In both mouse studies, we observed that CS exposure differently affected the release of specific DAMPs. Different cell death pathways, including necrosis, necroptosis, pyroptosis and autophagy in combination with the active secretion of specific DAMPs, all contribute to the release of specific DAMPs. Until recently it was thought that apoptosis is a form of non-immunogenic cell death, which does not induce the release of DAMPs. However, recently it was shown that under specific conditions apoptosis can also be pro-inflammatory and cause DAMP release. Especially, death receptor Fas-mediated and Cflar-dependent apoptotic cell death showed to induce the release of DAMPs, including calreticulin. Interestingly, we identified Cflar as a susceptibility gene for dsDNA release in mice and knockdown of CFLAR in human alveolar epithelial cells strongly associated with susceptibility for CS-induced apoptotic cell death and DAMP release (See chapter 7). Together, we have shown that, although DAMPs can
be considered as one group of molecules, they comprise a wide variety of molecules which are not all released in a similar fashion. Specific conditions can induce the release of specific DAMPs, indicating the necessity to investigate which DAMPs are key in COPD pathophysiology.

**DAMP release during COPD exacerbations; cause, consequence or coincidence?**

Exacerbations are episodes in which a sudden increase in symptoms takes place. This coincides with increased inflammation and accelerated decline in lung function, resulting in a decreased quality of life and increased hospitalization rates. The definition for COPD exacerbations is according to the WHO and GOLD “an event in the natural course of the disease characterized by a change in baseline dyspnoea, cough, and/or sputum that is beyond normal day-to-day variations, is acute in onset, and may warrant a change in medication strategy.” COPD exacerbations are heterogeneous events which can be induced by several causes. The number of studies investigating the cause of exacerbations are limited and the results are greatly dependent on different parameters, including the inclusion criteria, the used definition for exacerbation, medication used by the patients and the method of detection of airway infections. The most common causes for COPD exacerbations are either viral or bacterial airway infections and air pollution. The causal relationship between COPD exacerbations and air pollution is hard to establish, however upon investigating the temporal relationship it was shown that there is a strong correlation between the level of air pollution and the amount of exacerbations. Nevertheless, results about the portion of COPD exacerbations induced by air pollution are greatly dependent on the geographical location of the study subjects. It has been estimated that approximately two third of the exacerbations is caused by either a viral or bacterial airway infection, leaving a large portion of the patients with unknown cause of the exacerbation. In our studies investigating COPD exacerbations, we found in chapter 8 that only 28% of the exacerbations was associated with either a bacterial or a viral airway infection, while in chapter 9 we found that 86% of the exacerbations was associated with either bacterial or viral airway infection. This difference is probably caused by variation in the study set-up. In chapter 8 patients experienced mild to moderate self-reported exacerbations, while in chapter 9 the patients experienced severe acute exacerbations needing hospitalization, indicating that severe exacerbations are more associated with airway infections compared to mild exacerbations. Very little is known to date about the role of DAMPs during COPD exacerbations. Previously, it was shown that the levels of HMGB1 were higher in plasma during acute exacerbation compared to stable disease. This was confirmed in our study also showing an increase in serum levels of HMGB1 during COPD exacerbations. Furthermore, we showed that out of a panel of six DAMPs, HMGB1, S100A9 and LL-37 were significantly higher in serum during an exacerbation compared to stable disease, while the serum levels of dsDNA, galectin-3 and mtDNA were not different. The increased release of DAMPs may be either a cause or a consequence of the increased inflammation during COPD exacerbations. It is possible that the increased level of neutrophilic inflammation during COPD exacerbations coincides with higher levels of pro-inflammatory cytokines, chemokines, elastase and other damaging proteins, leading to more cellular damage and thus higher release of DAMPs. However, the fact that only specific DAMPs are increased during COPD exacerbations suggests that the increased DAMP levels are not merely a result of dying (epithelial) cells. Furthermore, in chapter 9 we showed that the expression of TLR2, TLR4 and NLRP3 is increased on peripheral blood neutrophils of COPD patients during exacerbation compared to stable disease. In literature it was shown that there is no association between the frequency of exacerbations and the expression of TLR2 or TLR4 in induced sputum. This suggests that it is not the susceptibility for exacerbations but the type (cause) of exacerbation which is associated with high DAMP levels. In summary, specific DAMPs and DAMP receptors are increased in COPD patients during exacerbations, irrespective of infection, indicating that next to airway infections also levels of DAMP signaling may be causally involved in part of the COPD exacerbations.

**RAGE; more than just another DAMP receptor**

Several chapters point out that RAGE-signaling is important in the pathophysiology of COPD. In chapter 8 we showed that specifically RAGE-activating DAMPs are increased in serum of COPD patients during an exacerbation.
Furthermore, in chapter 9 we showed that the decoy receptor for RAGE, sRAGE is decreased in serum during exacerbations. Finally, in chapter 11 we showed that the levels of sRAGE and RAGE ligands are correlated with the severity of COPD, further indicating the importance of this specific receptor in the pathophysiology of COPD. RAGE is a multi-ligand pattern recognition receptor that belongs to the immunoglobulin super-family and to the class of type I cell-surface receptors. In humans, RAGE is expressed at low levels in all tissues, and in lung tissue it is even highly expressed, with the highest expression in type I alveolar epithelial cells. Ligands activating RAGE comprise a wide variety of molecules, including Advanced Glycation End-products (AGEs), β-sheet-fibrils, Amyloid-β-protein, Phosphatidylserine and DAMPs like HMGB1, S100A9 and LL-37. The fact that the group of RAGE ligands comprises so many structurally different molecules is induced by the unusually large positive surface change, which acts as an electrostatic trap for negatively charged molecules, a unique property of the RAGE receptor. Upon activation of the RAGE receptor, NF-κB-dependent pro-inflammatory responses are induced. Furthermore, under specific conditions RAGE-signaling is involved in other biological processes, including cellular differentiation, proliferation, wound healing and apoptosis. Interestingly, it has recently been shown that RAGE signaling is involved in alveolar tissue loss, as over-expression of RAGE has been shown to induce spontaneous and progressive loss of alveolar structure, to induce leukocyte infiltration and culminate in airspace enlargement in mice, while RAGE knock-out mice are significantly protected against CS- and elastase-induced emphysema. Next to the membrane-bound form of RAGE, a free circulating soluble form of RAGE exists (sRAGE), which can be produced by proteolytic cleavage of the membrane-bound form of RAGE or by transcription of alternative splice variants lacking the transmembrane domain. Soluble RAGE functions as a decoy receptor, binding and inactivating free circulating RAGE ligands and preventing dimerization of membrane-bound RAGE, which is necessary for down-stream signaling. In COPD patients it has been shown that the levels of sRAGE are significantly lower compared to smoking and non-smoking controls, and are associated with neutrophilic airway inflammation, recovery from an exacerbation, decline in FEV₁, and especially emphysema. Furthermore, during COPD exacerbations the plasma levels of sRAGE have been shown to be decreased even further, a finding we were able to confirm in chapter 9 but not in chapter 8. This difference between our two analyses might stem from differences in the COPD patient population studied: in chapter 8 the exacerbations were relatively mild self-reported, while in chapter 9 the exacerbations were severe acute exacerbations necessitating hospitalization. In chapter 11 we showed that the levels of sRAGE and the accumulation of AGEs in the skin are associated with the severity of COPD. During the progression of COPD the levels of AGEs increase with time as a consequence of the increased levels of reactive oxygen species, and these RAGE ligands possibly bind to and inactivate free circulating sRAGE, ultimately inducing increased RAGE activation.

Next to the decrease of sRAGE in COPD patients, the expression of the RAGE receptor was found to be increased in mucosal cells, airway smooth muscle cells, macrophages and bronchial epithelial cells of COPD patients compared to smoking and non-smoking controls. A role for RAGE in the pathophysiology of COPD is further supported by the finding that the gene encoding RAGE, AGER, has been identified in several genome-wide association studies as a susceptibility gene for lung function decline and COPD. The variance is caused by a non-synonymous single nucleotide polymorphism (SNP), rs2070600, located on exon 3 in which a glycine is changed into a serine in the minor allele. This SNP is exactly located in one of the two N-linked glycosylation sides found in the ligand-binding domain of RAGE. In the minor allele the serine promotes glycosylation leading to increased ligand binding and activation of RAGE. Increased activation of RAGE possibly contributes to the inflammation and lung tissue damage seen in COPD patients. Interestingly, in our murine genome-wide association studies investigating susceptibility for CS-induced DAMP release and neutrophilic airway inflammation (Chapters 6 and 7), we did not identify AGER as a susceptibility gene. This may indicate that RAGE is predominantly involved in the late responses upon chronic CS exposure, including alveolar tissue damage leading to emphysema.

Next to RAGE, three other receptors for advanced glycation end-products are known, AGE-R1, AGE-R2 and
AGE-R3 respectively. Interestingly, AGE-R3 is another name for galectin-3, which upon exposure at the cell surface acts as receptor for AGEs. Upon binding of AGEs to cell surface galectin-3, the AGEs are internalized, processed and subsequently detoxified by the lysosomal system, preventing the accumulation of AGEs. Possibly, the increased release of galectin-3 in COPD patients and smoke exposed mice seen in chapters 4 and 6 is the result of shedding and proteolytic cleavage of membrane-bound galectin-3. This could contribute to the accumulation of AGEs in COPD patients seen in chapter 11. The accumulation of AGEs may contribute to the excessive activation of RAGE in COPD patients, contributing to both airway inflammation and lung tissue damage. Inhibition of this pathway may be a fruitful strategy which can be ultimately utilized as therapeutic treatment in COPD patients. Specific blockage of RAGE using sRAGE is subject of future studies, whereas also inhibition of galectin-3 might be a novel target to reduce airway inflammation and tissue damage. Inhibition of the pro-inflammatory extracellular galectin-3 can be achieved using naturally occurring ligands, such as pectins, or inhibitory peptides, which have been shown beneficial before in preventing heart failure, as described below.

In summary, several of our studies indicate that RAGE signaling contributes to the pathophysiology of COPD, providing a promising target for future studies investigating airway inflammation and lung tissue damage in COPD.

In vitro and in vivo experimental cigarette smoke exposure models

The hypothesis that CS-induced DAMP release from airway epithelial cells initiates the inflammatory reaction in COPD patients was tested by various experimental in vitro models using bronchial epithelial cells (See chapters 3 and 4). Here, we cultured submerged bronchial epithelial cells, meaning in a monolayer without air exposure. Although this is a widely applied method for studying cellular mechanisms in vitro that allows CSE stimulations, there are some limitations to this experimental set-up. For instance, epithelial cell polarization and differentiation into mucociliated cells only occurs upon exposure to air at the apical side. In our experimental in vitro models, bronchial epithelial cells were exposed to CSE, where all hydrophilic particles from cigarette smoke are dissolved in culture medium, which can be used to stimulate cells in various concentrations. In agreement with our method of using freshly prepared CSE, it was shown that it is necessary to stimulate cells with CSE immediately upon preparation, because the amount of fast reacting toxic compounds, like reactive oxygen species (ROS), decreases over time upon preparation of CSE. Importantly, the hydrophobic, along with the volatile compounds of cigarette smoke do not dissolve in CSE. This is physiologically relevant, as inhaled smoke needs to dissociate through the mucus layer before reaching the epithelial cell layer, also diminishing the volatile and hydrophobic compounds. The CSE preparation procedure is critical for interpreting data, as different research groups use different protocols for CSE preparation. These protocols can differ in the number of cigarettes, the volume of medium through which gas-phase CS has been led while preparing the CSE, the brand of cigarettes, the method and duration of CSE storage prior to usage and whether the filters were removed or not while smoking the cigarettes. All these differences in CSE can affect the outcome of the results, making comparison between research groups challenging. Nevertheless, in vitro CSE exposure has proven to be a relevant experimental model for smoking as it was shown that the genome-wide genetic changes in bronchial epithelial cells from heavy smokers in vivo are strongly correlated with genetic changes induced by CSE exposure in vitro.

Additionally, the in vivo smoke exposure murine models utilized as experimental model for COPD and its sub-phenotypes have been also subject to discussion. Firstly, the intranasal instillation of tissue-degrading enzymes or chemicals can be used to quickly and potently induce emphysema in mice. Furthermore, a combination of emphysema and airway inflammation can be induced by exposure of mice to cigarette smoke. This can be done by nose-only or whole-body exposure. Long term exposure to cigarette smoke induces emphysema, airway remodeling and airway inflammation, while short-term exposure of mice to smoke mainly induces airway inflammation. In this thesis, we were interested in the early effects of cigarette smoke on neutrophilic airway inflammation. Therefore, we used a short-term whole-body cigarette smoke exposure model, exposing mice for five consecutive days with two exposures per day and ten cigarettes per exposure,
leading to neutrophilic airway inflammation and airway hyper-responsiveness.\textsuperscript{50} Here, we showed that the level of induced airway inflammation was strongly dependent on the used mouse strain (See chapter 5). Furthermore, the level of airway inflammation in these models has also been shown to be dependent on the experimental set-up, including the brand of cigarettes used, the number of cigarettes used, the instruments used for exposing the mice and the exposure scheme.\textsuperscript{50} Another important consideration to take into account when interpreting the data is the fact that susceptibility for neutrophilic airway inflammation upon short-term CS exposure and susceptibility for emphysema upon long-term CS exposure do not necessarily overlap, as CS7/Bl6J and A/J mice are non-susceptible for CS-induced neutrophilic airway inflammation while these strains are amongst the most susceptible strains for CS-induced emphysema.\textsuperscript{37,83} This indicates that additional genetic factors determine whether CS exposure will ultimately lead to emphysema, at least in the mouse model. In summary, both for \textit{in vitro} and \textit{in vivo} models the exact experimental protocol is important for the study outcomes, which illustrates the need for international guidelines for the experimental usage of cigarette smoke, facilitating increased reproducibility of data, ultimately increasing the scientific value of cigarette smoke research.

\textbf{Future perspectives}

COPD has a world-wide prevalence of more than 65 million people and is the cause of more than 3 million deaths per year.\textsuperscript{64} Furthermore, COPD is a major cause of morbidity and mortality and patients often need hospitalization.\textsuperscript{57} To date no curative treatments are available for COPD patients. Current treatment is aimed at reducing the severity of symptoms, improve quality of life and reduce complications related to COPD.\textsuperscript{29,1} Thus, there is an urgent unmet medical need to develop novel therapeutic strategies for COPD and gain more insight into the pathophysiology of COPD. Therefore, basic research investigating the various pathways and biological processes involved in airway inflammation induced by chronic inhalation of toxic gases is paramount. In particular investigating possibilities to pharmacologically induce lung tissue regeneration will be one of the major challenges in the coming years. In this thesis we provided evidence that especially inhibitors for necroptosis, RAGE signaling and galectin-3 might be used as therapeutic targets for COPD.

Until novel treatment options are available for COPD patients the most significant decrease in COPD prevalence can be obtained by a change in behavior of individuals and nations. The most important change that individuals can implement in order to decrease the chance of developing COPD and attenuate the decline in lung function is to quit smoking. However, also other behavioral changes can reduce the progression of COPD, including increased physical activity and consuming a more balanced diet.\textsuperscript{20,17} Furthermore, both national and international politics can change the prevalence of COPD. Nations should commit to reduce the emission of toxic gases by industry and individuals, thus reducing air pollution. Furthermore, the transportation sector should be stimulated to reduce the emission of fine dust and exhaust fumes and individuals should be stimulated to give up smoking. These changes are most needed in countries with the highest population densities and highest levels of airway pollution, including China and India, which are also the countries with the highest prevalence of COPD.\textsuperscript{64}

With this thesis we have shown that airway epithelial cells go into necrosis and release immunogenic DAMPs upon CS exposure and that this process is dysregulated in COPD patients. However, to fully unravel the mechanism of CS-induced DAMP release and subsequent airway inflammation and to identify where this process is different between COPD patients and controls, it is necessary to use novel state-of-the-art \textit{in vitro} and \textit{in vivo} techniques. In the coming years air-liquid-interface (ALI) cultures will be necessary to further investigate the mechanism of CS-induced DAMP release. In ALI cultures, epithelial cells polarize and differentiate into a pseudostratified mucociliary epithelium, which reflects the \textit{in vivo} situation better and may also impact the expression and release of DAMPs. Furthermore, when grown in a trans-well system, ALI-differentiated epithelial cells can be co-cultured with mesenchymal cell types, including fibroblasts and airway smooth muscle cells. In order to reflect the \textit{in vivo} situation even more closely. This also allows for the investigation of interaction between different cell types in an \textit{in vitro} setting. Lastly, ALI culture allows the epithelial cells to be exposed to gas-phase CS at the apical side. Here, the gas-phase CS can dissolve into the mucus layer on the epithelial cells,
mimicking the in vivo situation. Recently, in vitro culture models of the airways have evolved even further, as
the airway-on-a-chip system has been developed. This system consists of a co-culture of epithelial cells and
endothelial cells, with flowing medium at the basal side and flowing air at the apical side, which leads to a fully
differentiated airway epithelial layer. In this system gas-phase CS can be added to the apical side and immune
cells, e.g. neutrophils, can be added to the basal medium flow to investigate airway infiltration. Furthermore, also
the culture of primary airway material has developed recently. Increasingly, primary epithelial cells have been
cultured in 3D culture systems, first using gels consisting of extracellular-matrix proteins, e.g. Matrigel, collagen-I
gels, and later using ex vivo cultured precision-cut tissue slices. Recently, the Eickelberg research group
developed an elaborate 3D tissue culture system using thick lung slices obtained from primary lung material,
in which the 3D lung tissue cultures preserves lung structure and function for at least five days. Furthermore,
using vital and membrane-permeable dyes for live-cell imaging with 4D time-lapse confocal microscopy, it was
shown to be possible to study the kinetics of specific proteins in 3D tissue cultures over time. Moreover, 3D
lung tissue cultures can also be prepared from murine lungs, allowing several conditions to be tested in tissue
from one mouse, reducing the amount of animals needed for experiments. These techniques allow studying
the role of DAMPs on the 3D structure of the airways, e.g. investigating the effect of specific DAMPs on alveolar
tissue damage. Additionally, in the past years most basic research on COPD has been performed using CS as a
model. However, recently it has become clear that also other factors, e.g. exhaust fumes and air pollution, can
contribute greatly to the onset of COPD, as up to 30% of all COPD patients is currently a non-smoker. In vitro
and in vivo models should also implement other exposures next to CS, including diesel exhaust particles and fine
dust particles. This will increase the willingness of the general public to invest in research focused on COPD, as
this reduces the image of COPD as a self-inflicted disease. To date, no studies have been performed investigating
the effect of non-CS-related COPD-inducing stimuli on DAMP release from airway epithelial cells. Together, in
the coming years both in vitro and in vivo models for the investigation of COPD will change and will resemble
the in vivo model more accurately. This is necessary to fully understand the cellular and molecular mechanisms
involved in the pathophysiology of COPD.

With the knowledge obtained in this thesis several promising pharmacological targets have been identified
and are suitable for future studies. First, as shown in chapter 3 the inhibition of necroptosis using necrostatin-1
greatly reduces the amount of CS-induced DAMP release and airway inflammation using both in vitro and in vivo
models. Nevertheless, the therapeutic potential of necrostatin-1 has not been investigated to date. Today,
necroptosis inhibitors which are more potent and specific are available, including the selective RIP3 inhibitors
GSK'657 and GSK'840. Next to the inhibition of necroptosis also the inhibition of other immunogenic cell death
modalities, e.g. pyroptosis, paraptosis and NETosis should be investigated in relation to CS-induced DAMP release
and subsequent inflammation. Secondly, the inhibition of specific DAMPs or their receptors provides therapeutic
potential. Especially, RAGE-signaling is a potential target for future studies. RAGE is not only involved in DAMP-
mediated airway inflammation but is also involved in the development and progression of emphysema.
Future experiments are planned to investigate the role of RAGE-signaling in CS-mediated tissue damage and
tissue repair. The inhibition of RAGE using either blocking peptides (RAGE antagonistic peptides) or recombinant
human sRAGE, might reduce the amount of alveolar tissue damage and DAMP release induced by CS exposure
and might even induce tissue regeneration. Next to RAGE also the inhibition of galectin-3 may have therapeutic
potential. Inhibition of galectin-3 can be achieved by application of scavenging naturally occurring molecules,
e.g. pectins, in the circulation or by treatment with specific inhibitory peptides. As membrane-bound RAGE and
galectin-3 are both receptors for AGEs, which are accumulated in COPD patients, a combination treatment with
inhibitors for both galectin-3 and RAGE might act synergistically.

In conclusion, with this thesis we have obtained evidence supporting the DAMP hypothesis for COPD.
Additional research using state-of-the-art in vitro and in vivo techniques is necessary to fully elucidate the role
of DAMPs in COPD. This might eventually lead to novel therapeutic targets, including RAGE, galectin-3 and
necroptosis which might reduce the chronic airway inflammatory reaction and induce lung tissue regeneration,
ultimately increasing the quality of life of COPD patients.
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