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DAMPs activating innate and adaptive immune responses in COPD

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ABSTRACT
Chronic Obstructive Pulmonary Disease (COPD), a progressive lung disease characterized by sustained neutrophilic airway inflammation, is caused by chronic exposure to noxious stimuli, e.g. cigarette smoke. This chronic exposure can induce immunogenic cell death of structural airway cells, inducing the release of Damage Associated Molecular Patterns (DAMPs). Levels of several DAMPs, including S100 proteins, defensins and High Mobility Group Box-1 (HMGB1), are increased in extracellular lung fluids of COPD patients. Since DAMPs can attract and activate immune cells upon binding to pattern recognition receptors, we propose that their release may contribute to neutrophilic airway inflammation. In this review, we discuss the novel role of DAMPs in COPD pathogenesis. Relevant DAMPs are categorized based on their subcellular origin, i.e. cytoplasm, ER, nucleus and mitochondria. Furthermore, their potential role in the pathophysiology of COPD will be discussed.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality, with a worldwide prevalence of 9-10%. Currently, COPD is the fourth leading cause of death worldwide and it is estimated to become the third leading cause of death by 2030. The disease is characterized by progressive airway obstruction that is not fully reversible and accelerated lung function decline. Furthermore, COPD is associated with an abnormal inflammatory reaction in the lungs, causing destruction of lung parenchyma (emphysema) and/or chronic bronchitis. A major risk factor for development of COPD is chronic exposure to noxious particles and gasses, e.g. cigarette smoke, coalmining dust, diesel exhaust particles and fumes from burning biomass fuels for cooking or heating. The chronic airway inflammation in COPD is characterized by activation of the innate immune system, as defined by increased numbers of innate immune cells like neutrophils, macrophages, natural killer cells and mature dendritic cells in lung tissue and airway lumen. In addition, the adaptive immune system is activated in COPD, as defined by lung infiltration of CD8+ T-cells, B-cells and both the Th17 and Th1 types of CD4+ T-cells, along with a decrease in regulatory T-cells (Tregs) in the airways.

At present, little is known about the initial steps in the activation of innate- and adaptive immune responses observed in COPD. Curtis and colleagues postulated that during early stages of COPD, innate immune inflammation increases with the progression of COPD, whereas in more advanced COPD (GOLD stages III and IV), adaptive T- and B-cell responses become increasingly important for pathologic abnormalities. Although the exact nature of the triggers for these innate- and adaptive immune responses is at present largely unknown, we hypothesize that cell damage upon environmental insults is involved. According to the danger hypothesis of Polly Matzinger, ‘danger signals’ or Damage Associated Molecular Patterns (DAMPs) from injured cells can alarm the immune system by activation of pattern recognition receptors (PRRs). Cigarette smoke and other noxious gasses and particles can cause damage to resident cells in the lungs, which can induce multiple types of both regulated and non-regulated cell death. Different forms of cell-death, e.g. apoptosis, necrosis (accidental, non-programmed) and necroptosis (programmed), may cause distinct signatures of DAMPs released into the extracellular space (ECS). Regulated forms of cell death encompass both apoptosis, a form of programmed and caspase-dependent cell death, and necroptosis, a form of receptor-interacting protein kinase-1 (RIPK1) and RIPK3 dependent regulated necrosis. Non-regulated cell death encompasses accidental necrosis, where cells when subjected to harsh physico-chemical injuries disrupt through uncontrolled physical events, releasing cellular constituents into the microenvironment. Necrotic and necroptotic cell death are the main, but not the only forms of cell death that lead to DAMP release. During early apoptosis, most DAMPs are retained in apoptotic bodies and phagocytized before they can ligate PRRs, yet during secondary necrosis DAMPs can be released. Secondary necrosis occurs when apoptotic cells are not cleared sufficiently by phagocytosis, such as has been observed in COPD patients.

The airway epithelium forms the first barrier towards inhaled insults, separating lung tissue from the environment. Consequently, epithelial cells are one of the first cells to be exposed to inhaled noxious gasses and particles present in cigarette smoke and diesel exhaust fumes. An increase in apoptotic epithelial cells has been shown in lungs of emphysema patients. Our group has shown that exposure of bronchial epithelial cells to cigarette smoke extract (CSE) causes a switch from apoptotic to necrotic cell death. Unpublished observations indicate that this switch is in fact a switch from apoptosis to necroptosis. In addition to these direct effects, decreased phagocytosis of apoptotic cells by airway macrophages has also been observed in COPD. Taken together, these two effects might result in inducing an increased DAMP release in COPD. Some studies show that danger signals released from secondary necrotic cells are often inactivated by caspases, which are expressed during apoptosis and secondary necrosis, resulting in poor immunogenicity of such DAMPs.

In addition to the release of DAMPs, cell damage or death also induces the release of several cytokines and chemokines that can induce or regulate immune responses. In particular, IL-1α, IL-6 and IL-33 have been described as danger signals or alarmins, released during immunogenic cell death. Although these interleukins can have important pro-inflammatory properties upon release during accidental necrosis, their function as DAMP is
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not different from their function under physiological conditions and therefore the role of interleukins in COPD will not be discussed in this review.

As mentioned above, a critical feature of DAMPs is that they specifically bind PRRs, which upon ligation lead to activation of the innate immune system. There are at least five classes of PRRs: Toll-Like Receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and the Receptor for Advanced Glycation End products (RAGE), all of which upon ligation activate downstream signaling pathways. These include, nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) and type I interferon pathways, initiating the release of pro-inflammatory cytokines and chemokines (e.g. IL-6, IL-8, Type I IFN and TNF) and ultimately resulting in activation of the immune system and attraction of immune cells to the site of damage. (See figure 2) Furthermore, some DAMPs, e.g. HSPs, HMGB1, Galectins and Cathelicidins, can stimulate the adaptive immune system by inducing maturation of dendritic cells, yet when immunogenic (auto)-antigens are presented and tolerance mechanisms (e.g. suppression by Tregs) fail. Thus, DAMPs can directly activate cells of the innate immune system and either directly or indirectly promote adaptive immune responses.

To date, no standard classification system for DAMPs is available. In this review we will divide DAMPs into several subclasses based on their physiological localization. These subclasses consist of DAMPs derived from the cytoplasm (HSPs, S100 proteins, galectins, anti-microbial peptides), subcellular organelles, i.e. the nucleus

Figure 1: Hypothetical scheme of CS-induced airway epithelial immunogenic cell death followed by DAMP release and subsequent triggering of the innate- and adaptive immune responses in COPD. Inhalation of toxic gasses and particles, e.g. cigarette smoke and diesel exhaust particles, cause damage and subsequent cell death to airway epithelial cells. The main immunogenic cell death pathway induced by chronic cigarette smoke exposure is up to date unknown. Upon immunogenic cell-death DAMPs, e.g. HMGB1, HSPs and S100 proteins, are released which can activate PRRs on adjacent epithelial cells as well as innate - and adaptive immune cells. Upon ligation of PRRs, epithelial cells become activated and release pro-inflammatory cytokines, e.g. TNF, IL-6, IL-8 and Type I IFN. Pro-inflammatory cytokines can activate and attract cells of both the innate immune system, e.g. Neutrophils, Macrophages and Dendritic cells, as well as cells from the adaptive immune system, e.g. T-lymphocytes and B-lymphocytes. Furthermore, DAMPs can also activate and mature cells of the innate as well as the adaptive immune system directly upon binding of PRRs on these cells. (see color image on page 208)
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Heat Shock Proteins

HSPs are proto-typical DAMPs derived from the cytoplasm. HSPs are chaperone proteins that are upregulated during various types of physiological and environmental stress conditions, including infections, wounding or heat. In physiological concentrations, HSPs act as intracellular molecular chaperones that assist the folding of nascent or mis-folded proteins and thereby prevent the aggregation of proteins.

The mammalian HSPs are classified into five different families based on their molecular weight, namely: HSP20, HSP60, HSP70, HSP90 and HSP100. Normally, HSPs are contained intracellularly, but during cellular stress, HSPs are also present at the cell surface or secreted into the extracellular matrix (ECM). HSPs released into ECS can function as DAMPs due to their activity as a signaling molecule. Therefore, the term chaperokine was introduced to describe the function of extracellular HSPs.

HSPs have been shown to activate both TLR2 and TLR4 signaling in a MyD88 dependent fashion, leading to the initiation of the NF-κB and IRF signaling pathways, which ultimately leads to the release of pro-inflammatory cytokines (e.g. TNF, IFNs, IL-1β, IL-6 and IL-8). Furthermore, HSPs can initiate the maturation and activation of dendritic cells.

The role of HSPs in COPD is not fully elucidated, but in the past few years several studies have been performed to investigate the role of HSPs in COPD and in vitro models. Already in 1997, in vitro studies revealed that HSP70 and HSP90 protein levels are up-regulated by cigarette smoke exposure in monocytes. More recently, it has been shown that HSPs can induce the release of pro-inflammatory cytokines in COPD patients. (see color image on page 208)
been shown that the levels of HSP27, HSP70 and HSP90 are significantly higher in serum of COPD patients compared to non-smoking individuals. Furthermore, HSP10, HSP27 and HSP40 protein levels are increased in airway epithelial cells of COPD patients in comparison to healthy controls and control smokers. Additionally, increased HSP60 expression has been observed in bronchial biopsies of patients with severe COPD (GOLD stage III/IV) compared to healthy non-smoking volunteers. This increase in HSP60 was positively correlated with neutrophil numbers in the biopsies, an important pathological hallmark of COPD. Whether this is related to increased extracellular HSP60 levels remains unknown, however, human bronchial epithelial (16HBE) cells actively released HSP60 upon H2O2 stimulation, to mimic oxidative stress in COPD. Furthermore, increased release of HSP60 was induced by CSE in human umbilical cord endothelial cell, indicating that cigarette smoke can induce active secretion of HSP60.

In conclusion, an increased expression and release of several HSPs has been found in the circulation and lungs of COPD patients. Although some studies only examined intracellular expression of HSPs, increased expression may cause increased release upon accidental necrosis. Further studies are needed to determine if HSPs may play a causal role in the cigarette smoke-induced neutrophilic airway inflammation and pathogenesis of COPD.

**S100 proteins**

S100 proteins are a family of low-molecular weight calcium-binding proteins. To date, 25 members are known of which S100A8, S100A9 and S100A12 have been recognized as DAMP. All S100 proteins can form non-covalent homodimers and some, including S100A8/S100A9, can form heterodimers. Intracellularly, S100 dimers interact with downstream effector molecules to regulate cell differentiation and growth, cell attachment, cell cycle progression and cell motility. Furthermore, S100 proteins have anti-microbial properties. S100 proteins are expressed in a wide range of cell types, with high constitutive expression of S100A8 and S100A9 in neutrophils, their cytosolic content consists for ~45% of S100 proteins. S100 proteins can be passively released upon accidental necrosis as well as actively secreted by a regulated but unconventional pathway without using a leader sequence for secretion. Once in the ECS, S100 proteins can activate multiple receptors, including RAGE and TLR4, both leading to NF-κB activation.

Although still largely uncovered, some studies have indicated a role for S100 proteins and their receptor RAGE in the pathophysiology of COPD. Mass spectrometry has revealed that levels of S100A8 and S100A9 are increased in bronchoalveolar (BAL) fluid of COPD patients in comparison to control smokers and non-smokers. Later, a trend towards higher S100A12 levels was observed in sputum of COPD patients compared to healthy controls, although the levels of S100A8 and S100A9 were not different between the groups in this study. Furthermore, a recent meta-analysis shows a 1.6 fold increase in S100A12 in serum of COPD patients compared to healthy smokers and non-smokers. The discrepancy in results between studies can have multiple causes. For instance, studies may differ in their technique to assess S100 proteins, their collection of lung specimen, or in COPD population characteristics like smoking status and disease severity.

Additionally, studies were performed concerning the role of S100 receptor RAGE in COPD, showing an increased expression of RAGE in lung mucosal cells, bronchial epithelial cells, airway smooth muscle cells and lung macrophages of COPD patients compared to healthy controls. Additionally, RAGE expression was increased in bronchial epithelial cells and airway smooth muscle cells from COPD patients compared to control smokers. Other studies show that levels of soluble RAGE (sRAGE), which blocks binding of ligands to RAGE, are reduced in plasma and BAL fluid of COPD patients compared to healthy controls. Furthermore, an association has been shown of RAGE with lung function using a genome-wide association study and RAGE has been proposed as susceptibility gene for COPD. The combined increase in S100 proteins and RAGE and decrease in sRAGE highlights the importance of further studies on the involvement of the S100 - RAGE pathway in the pathophysiology of COPD.
Galectins

Galectins are β-Galactoside-binding lectins that have a variety of physiological functions in humans, including the control of intracellular trafficking of glycoproteins.47 Yet, upon release from damaged or dead cells, galectins exhibit a pro-inflammatory function, qualifying them as DAMPs.47 Most galectins are widely expressed in many cell types, including structural and immune cells of the lungs.48 Galectins can be secreted both passively upon accidental necrosis and actively by a leaderless secretory pathway similar to the secretion of HMGB1 and S100 proteins.48 Galectin-1 and Galectin-3 are the most studied galectins and have the strongest reported pro-inflammatory properties.63 The pro-inflammatory properties of galectin-3 include induction of oxidative bursts in neutrophils, chemoattraction of monocytes, neutrophils and macrophages and the induction of IL-8 production by naïve and primed neutrophils.49,50 For Galectin-3 an increase in intracellular protein expression has been observed in the small airways of COPD patients compared to control smokers and non-smokers, while no significant difference was observed between non-smoking and smoking controls, suggesting that the increase in Galectin-3 is specific for COPD.51 Unfortunately, to our knowledge no studies have been performed studying the levels of Galectin-3 in lung fluids.

Similarly to Galectin-3, increased Galectin-1 protein expression has been observed in epithelial cells of the small airways of control smokers in comparison to non-smokers and COPD patients, yet, the levels of COPD patients were still significantly higher than the levels of non-smokers.51 However, the BAL levels of yet another galectin, Galectin-9, were not different between COPD patients and healthy controls.52 Thus, specifically Galectin-1 and 3 could contribute to the innate immune response involved in the pathogenesis of COPD, although more research will be required to confirm this.

Anti-microbial peptides

Antimicrobial peptides derived from the airway epithelium protect the lungs against infections. Some of these antimicrobial peptides can also function as DAMPs. The most well-known antimicrobial peptides with DAMP properties are defensins and cathelicidins. α- and β-defensins are expressed in the human lungs and are categorized by their molecular weight and the arrangement of their cysteine disulfide bonds.53 Defensins belong to a family of small (3-6 kDa) proteins that share a characteristic β-sheet and six cysteine residues forming three intra-chain disulfide bonds.54 Defensins may function as a DAMP by activating TLR4 downstream signaling.55,56 In addition, α-defensins 1-3 have chemotactic activity towards monocytes, naïve T-cells and immature dendritic cells.54 Furthermore, α-defensins have been shown to activate the production of pro-inflammatory cytokines, including IL-1α and TNF-α by monocytes, leading to the upregulation of adhesion molecules, including ICAM-1, CD11b and CD11c by neutrophils.57,58 Human β-defensin 1-2 attract memory T-cells, especially of the Th17 subtype, neutrophils and immature DCs by binding to the chemokine receptor CCR6.54,58 Furthermore, β-defensins increase the expression of several pro-inflammatory cytokines and chemokines (e.g. CXCL5, IL-6, IL-8, MCP-1 and GM-CSF) and induce necroptotic cell death.59

The concentrations of α-defensins 1-3 are higher in the sputum of COPD patients than in non-symptomatic smokers and β-defensin-1 mRNA expression is significantly higher in bronchial epithelial cells of COPD patients compared to healthy volunteers.56,61 Contradictory findings have been reported on β-defensin-2 levels in COPD patients. Pace and co-workers showed an increased concentration of human β-defensin-2 in mini-BAL samples of COPD patients compared to non-symptomatic smokers and healthy volunteers,62 whereas Tsoumakidou et al. found no detectable levels of human β-defensin in BAL samples of COPD patients, while levels were detectable in control smokers and non-smokers.63 Limited data is available from (pre)-clinical studies, where it has been shown that the level of β-defensin-2 was increased after cigarette smoke exposure in rats, in a NF-κB dependent fashion.64 Of note, COPD patients have a higher bacterial and viral load in their lungs compared to healthy controls especially during exacerbations.56,66 Although it is possible that increased levels of defensins in COPD patients are a consequence of this increased bacterial load, visa versa, the increased bacterial load could also be consequence of an impaired antimicrobial response in COPD patients. Nevertheless, the increased β-defensin levels in COPD do not support the latter and increased β-defensin release could exert a pro-inflammatory DAMP.
function, contributing to the disease pathogenesis.

In addition to defensins, cathelicidins are also antimicrobial peptides with DAMP properties. Cathelicidins are characterized by a highly conserved pre- and pro-region, where the pre-region is located at the N-terminus and the pro-region has a structure similar to the cathepsin-L-inhibitor cathalin. At the C-terminus, cathelicidins are very variable. The C-terminus forms the mature peptide with the antimicrobial properties.\textsuperscript{67} LL-37/hCAP-18 is the only cathelicidin that is known to be expressed in humans to date.\textsuperscript{68} LL-37 is expressed by various cell types, including airway epithelial cells, macrophages, lymphocytes, neutrophils, natural killer cells, monocytes, B-cells and mast cells.\textsuperscript{53} Airway epithelial cells are thought to secrete cathelicidins in the airway surface fluid, since LL-37 is found in human BAL fluid and in supernatant of primary bronchial epithelial cell cultures.\textsuperscript{69,70} Cathelicidins have a clear DAMP function, as they can provoke a pro-inflammatory response through TLR7, TLR9 and RAGE activation, either alone or in complex with extracellular DNA.\textsuperscript{71,72,73} Furthermore, LL-37 has chemotactic activity towards eosinophils and neutrophils, which is mediated via the formyl peptide receptor.\textsuperscript{74} LL-37 can also induce necrosis upon stimulation in human airway epithelial cells, although this only occurs when concentrations exceed a certain threshold, rendering the clinical relevance of this finding debatable.\textsuperscript{75} A dose-dependent increase in IL-8 release and apoptosis has been observed in airway epithelial cells stimulated with recombinant LL-37.\textsuperscript{76} Furthermore, CSE dose-dependently increased the protein expression of LL-37 in airway epithelial cells.\textsuperscript{76} Multiple studies show that the levels of LL-37 in sputum of COPD patients during exacerbations as well as in stable disease are significantly higher compared to smoking and non-smoking controls.\textsuperscript{77,78,76} Importantly, this increase in LL-37 levels is inversely correlated with lung function, although further research needs to elucidate whether this is cause or consequence of COPD.\textsuperscript{77,78,76} No increase was found in serum of COPD patients compared to smoking and non-smoking controls,\textsuperscript{76} indicating a local increase of LL-37 instead of a systemic increase. Recently it was shown that LL-37 levels in BAL fluid and epithelial lining fluid (ELF) of early stage COPD (GOLD stage I-II) patients were significantly increased compared to healthy controls. The same study showed that in late stage COPD (GOLD stage III-IV) patients, BAL and ELF levels of LL-37 are significantly decreased compared to healthy controls.\textsuperscript{79} Interestingly, it has been shown that LL-37 can bind extracellular DNA and facilitate binding of DNA to TLR9 inducing a pro-inflammatory response.\textsuperscript{72} Thus, LL-37 has properties that may be relevant in COPD pathogenesis and higher levels have been observed in extracellular fluids of COPD patients, although it needs to be established whether increased LL-37 levels actually lead to increased airway inflammation in COPD.

**DAMPS DERIVED FROM SUBCELLULAR ORGANELLES**

**HMGB1**

One of the most extensively studied DAMPs is HMGB1, a 215 amino acid non-histone molecule that is normally resident in the cell nucleus, where it binds DNA to facilitate the assembly of nucleoprotein complexes.\textsuperscript{80} HMGB1 can exert pro-inflammatory functions when it resides in the ECS.\textsuperscript{81} This occurs either when HMGB1 is released actively by a non-conventional secretory mechanism upon stimulation with pro-inflammatory mediators (e.g. LPS, pro-inflammatory cytokines, nitric oxide) or passively upon necrosis.\textsuperscript{82} During apoptosis and secondary necrosis, HMGB1 cannot be released due to its irreversible binding to chromatin which undergoes structural modifications upon apoptosis.\textsuperscript{80} This can result from post-translational modifications, including histone acetylation and DNA methylation.\textsuperscript{81} When HMGB1 is secreted, either passively or actively, it has been demonstrated to bind to different PRRs, including TLR2, TLR4 and RAGE.\textsuperscript{83,84} TLR9 is also mentioned as a receptor for HMGB1, although this is controversial,\textsuperscript{85} as HMGB1 readily forms complexes with various molecules including DNA, a known TLR9 ligand, indirect binding of HMGB1 to TLR9 cannot be ruled out.\textsuperscript{87,88} Moreover, HMGB1 exerts direct chemotactic activity towards monocytes, macrophages, neutrophils and dendritic cells.\textsuperscript{89} Furthermore HMGB1 can promote the activation, migration and maturation of dendritic cells.\textsuperscript{90,91} However, there is debate about the direct immunostimulatory properties of HMGB1, as some studies show that highly purified HMGB1 does not exert pro-inflammatory properties itself,\textsuperscript{92} but that the pro-inflammatory properties are caused by formation of complexes of HMGB1 with DNA, lipids, LPS or cytokines.\textsuperscript{93,91} These complexes are possibly highly
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inflammatory and may stimulate cytokine production via binding of TLRs or IL-1R. The fact that other studies have shown an immunostimulatory effect of purified HMGB1 may be explained by LPS contamination.\(^93\) Another reason for discrepancies in the results could be that the redox state of HMGB1 was not taken into account, which is important for the regulation of its functions. When the three cysteine residues (C23, C45 and C106) of HMGB1 are all in a reduced form, HMGB1 has chemotactic but no immunostimulatory properties, while HMGB1 has no chemotactic or immunostimulatory activities at all when all three cysteine residues are present in their oxidized form.\(^94\) Finally, when C106 is reduced, while C23 and C45 form an intermolecular disulfide-bond, HMGB1 has immunostimulatory properties.\(^91\) In this intermediate state it will bind RAGE and the TLR2/4 receptors, leading to activation of the NF-κB pathway and subsequent secretion of pro-inflammatory cytokines.\(^94\)

Some interesting studies have been performed in the past few years to investigate the role of HMGB1 in cigarette smoke-induced inflammation and COPD. Ferhani et al. were the first to show elevated levels of HMGB1 in BAL fluid of current smoking COPD patients compared to smokers without COPD and non-smokers.\(^41\) This up-regulation was positively correlated with IL-1β levels and negatively correlated with the Forced Expiratory Volume (FEV\(_1\)), the most important parameter for the severity of COPD. Later, it was shown that levels of HMGB1 are also higher in sputum and serum of GOLD stage II, III and IV COPD patients compared to healthy controls and in ELF derived from the peripheral airways of COPD patients compared to control smokers and non-smokers.\(^95,96\)

Altogether, these data suggest that HMGB1 levels in the ECS are increased in COPD patients compared to control individuals, however, it is at present unclear if this is the result of necrotic cell death and subsequent release or active production and/or secretion. Moreover, it is currently unknown if the release of HMGB1 is a direct consequence of smoking or whether it is related to the underlying inflammatory process in COPD, since HMGB1 levels are also increased in other unrelated chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, scleroderma and pulmonary fibrosis.\(^80\)

In C57BL/6 mice chronic cigarette smoke exposure (12 cigarettes per day, 60 days) was shown to induce an up-regulation of HMGB1 protein expression in the lungs.\(^97\) Additionally, the amount of HMGB1 was shown to increase in the serum of rats after sub-chronic cigarette smoke exposure (8 cigarettes per day, 4 weeks, 5 days per week),\(^98\) together indicating that cigarette smoke-exposure alone is sufficient to induce increased extracellular HMGB1 levels. For future studies it is important to assess the role of cigarette smoke-exposure in increased HMGB1 levels in extracellular fluids of COPD patients. Furthermore, the redox state of extracellular HMGB1 is important to determine as different redox forms have different functions.

Calreticulin

Another well-known DAMP is Calreticulin\(^99\), a 46-kDa Ca\(^{2+}\)-binding chaperone molecule that usually resides in the lumen of the ER. CRT is translocated from the lumen of the ER to the cell membrane during immunogenic apoptotic cell death.\(^100,21,101\) When CRT is expressed at the outer surface of the cell membrane it serves as an 'eat me' signal, being an essential recognition signal for phagocytosis.\(^102\) Exposure of CRT at the cell surface is a result of activation of ER stress pathways.\(^100\) ER stress is an imbalance between ER protein folding load and capacity, and severe ER stress can result in the activation of pro-apoptotic signaling pathways.\(^21\) ER stress is induced in bronchial epithelial cells upon cigarette smoke-exposure,\(^103,104\) and an exaggerated ER stress response was found in the lungs of COPD patients, potentially leading to the release of CRT.\(^105,106\)

Very limited data are available on the role of CRT as a DAMP in the pathophysiology of COPD. It has been shown that CRT protein expression is significantly up-regulated in CSE-treated human bronchial epithelial 16-HBE cells.\(^107\) Furthermore, the expression of CRT is up-regulated in lung tissue lysates of smokers in comparison with non-smokers and ex-smokers, which may serve as a regulatory mechanism to cope with misfolded proteins.\(^107\) Although CRT release has to our knowledge not been determined in COPD patients, increased protein expression may lead to increased levels upon immunogenic cell death and trigger inflammatory responses contributing to the development of COPD.\(^102\)
DAMPS DERIVED FROM THE MITOCHONDRION

mtDNA
Besides DAMPs derived from the cytoplasm, nucleus or ER recent data show that mitochondria are also an important source for DAMPs, the so-called mitochondrial DAMPs (mtDAMPs).\textsuperscript{108} This is a group of DAMPs that is currently known to exist of mitochondrial DNA (mtDNA), N-formylated peptides (NFPs), ATP and Carbamoyl phosphate synthetase-1 (CPS-1).\textsuperscript{108} The concept that mitochondria are a rich source of DAMPs derives from the endosymbiont hypothesis, which states that mitochondria originate from protobacteria that have committed an endosymbiotic relationship with ancestral, phagocytic, unicellular anaerobes more than a billion years ago.\textsuperscript{109} Mitochondria still possess morphological and biochemical features of their prokaryotic ancestors, which may explain why many of their molecular patterns are recognized by PRRs.\textsuperscript{108} MtDNA contains more unmethylated CpG motifs than genomic DNA.\textsuperscript{110} These unmethylated CpG motifs are responsible for the immunogenic properties of mtDNA, acting on TLR9.\textsuperscript{111} As for genomic DNA, mtDNA readily forms complexes with other DAMPs, including HMGB1 and LL37, which increases its immunostimulatory effects by facilitating uptake via RAGE after which it can bind to intracellular DNA sensors, including TLR9.\textsuperscript{72} The role of mtDNA in COPD remains to be elucidated, although it has been shown that mice develop rapid inflammation in the lungs when mitochondrial lysates are intravenously injected.\textsuperscript{112} This suggests a potential role of mtDAMPs in the pathophysiology of neutrophilic lung inflammation and COPD, although levels in extracellular lung fluids have not been studied.

N-formylated peptides
The protein synthesis process of mitochondria resembles that of prokaryotes. Unlike eukaryotes, where protein synthesis is initiated by a non-formylated methionine residue, mitochondria and prokaryotes initiate protein synthesis by N-formylmethionine, creating N-formylated peptides or NFPs. Already in 1972 it has been shown that NFPs derived from bacteria are very potent chemo-attractants for neutrophils,\textsuperscript{113,114} and in 1982 it was shown that NFPs derived from mitochondria also have this activity.\textsuperscript{115} NFPs are recognized by high-affinity formyl-peptide receptors (FPRs), which are G-coupled receptors expressed by numerous cells, including neutrophils, monocytes, dendritic cells, hepatocytes and endothelial cells.\textsuperscript{116} There are three human FPR receptors, FPR1, FPR2 and FPR3.\textsuperscript{117} NFPs can activate neutrophils by binding FPR1, leading to the release of pro-inflammatory signals like MMP-8 and IL-8.\textsuperscript{118} NFPs released from necrotic cells have been shown to attract neutrophils to the site of injury causing sterile inflammation.\textsuperscript{119}

Although at present no studies have been performed investigating the role of NFPs as DAMPs in COPD patients, studies performed in mice indicate a critical role for NFP in cigarette smoke-induced lung inflammation and emphysema. Intratracheal application of fMLP, a synthetic FRP ligand, leads to increased inflammation, as shown by increased IL-13 staining, as well as emphysema and goblet cell metaplasia, characteristics of COPD.\textsuperscript{120} Importantly, it was recently shown that genetic ablation of the \textit{fpr1} gene, encoding for the mouse homologue of the FPR1 receptor, provides protection against cigarette smoke induced emphysema in mice.\textsuperscript{121} In addition, a deficiency for \textit{fpr1} in mice resulted in a strong decrease in lung infiltration of neutrophils and macrophages after exposure to cigarette smoke.\textsuperscript{121} This effect could also be achieved by using a FPR1 or FPR1/2 antagonist.\textsuperscript{121} Taken together, these data strongly suggest that NFP release upon cigarette smoke activates FRP1 leading to lung inflammation and emphysema. In line with the previous study, suggesting a role for NFPs in COPD, increased expression of FPR receptors on peripheral neutrophils has been observed in COPD patients and smoking controls, compared to non-smoking controls, reflecting a smoking-related, thus not disease-related, effect.\textsuperscript{122}

ATP
Adenosine 5'-triphosphate (ATP), a molecule that belongs to the purine family, is critical for transport of chemical energy within a cell, while it also has many signaling functions. ATP can be released into the ECS both actively and passively upon apoptotic and (secondary) necrotic cell death, where it can function as DAMP.\textsuperscript{123} ATP is produced in the mitochondrion but can be released from multiple subcellular compartments, including the cytoplasm, ER and the mitochondrion. Extracellular ATP can activate purinergic receptors, which consists of two classes:
the G-coupled protein P2Y class and the cation-permeable ligand gated ion channel P2X class of receptors.\textsuperscript{124} Activation of P2Y receptors induces recruitment of neutrophils, macrophages and dendritic cells to the side of injury.\textsuperscript{124,126} On the other hand, binding to P2X7 receptor leads to NLR-family pyrin domain containing 3 (NLRP3) inflammasome activation and subsequent release of the pro-inflammatory cytokine IL-1β from innate immune cells, such as macrophages and dendritic cells.\textsuperscript{127} A role of extracellular ATP in COPD pathogenesis has been proposed, based on experimental animal models.\textsuperscript{128,126} Balb/c mice exposed to cigarette smoke for three months showed increased ATP in BAL fluid compared to air exposed control mice.\textsuperscript{128} Furthermore, CSE induces the release of ATP in human neutrophils.\textsuperscript{128} This increase in ATP may initiate release of IL-8 and elastase by immune cells. The role of ATP in the pathogenesis of COPD was further confirmed by the observation that ATP levels were increased in BAL fluid of COPD patients.\textsuperscript{126} Moreover, higher ATP levels have been observed in current and ex-smoking COPD patients, compared to both smoking and non-smoking controls, while levels were also increased in smokers compared to non-smokers.\textsuperscript{126} Importantly, this increase in ATP was negatively correlated with the FEV\textsubscript{1}%. Furthermore, acute smoke exposure (8 cigarettes in 4 hours) has been shown to induce an immediate increase of ATP in BAL fluid of healthy controls, indicating a direct effect of smoking on BAL ATP levels. Since ATP levels in BAL of COPD patients were even higher, there may be an additional disease-related effect.\textsuperscript{126} The same study also showed that in current smoking and ex-smoking COPD patients, the expression of P2Y2 and P2X7 receptors on blood neutrophils and macrophages is higher than in smoking and non-smoking controls. The role of purinergic receptors in smoke-induced lung inflammation was supported by in vivo mice studies showing an up-regulation of P2X7 and P2Y2 receptors on neutrophils, macrophages and lung tissue in mice after short-term smoke exposure.\textsuperscript{129,130} When either one of these receptors was knocked-out, less inflammation and emphysema was observed in lungs of mice after smoke exposure.\textsuperscript{129,130} Furthermore, P2Y2R knock-out mice showed attenuated inflammation in the lungs after acute cigarette smoke exposure compared to C57Bl/6J wild type mice.\textsuperscript{130} Together, these studies suggest that there may be a role for ATP in the pathogenesis of COPD, both with respect to neutrophilic airway inflammation and emphysema.

Besides the mtDAMPs discussed above, additional mtDAMPs may exist, e.g. carbamoyl phosphate synthetase-1\textsuperscript{108} and cardiolipin\textsuperscript{131}. To date there is little evidence indicating a role in COPD, although it has previously been shown that BAL levels of cardiolipin are significantly higher in COPD patients than in non-smoking controls.\textsuperscript{132} Further research needs to clarify whether all or specific mtDAMPs are increased in lungs of COPD patients and whether they play a role in disease pathogenesis.

**DAMPs derived from the ECM**

Recently, the awareness has arisen that not only intracellular molecules, but also molecules from the ECM can activate the immune system in response to danger when released from the ECM upon cleavage by metalloproteases.\textsuperscript{133,27} As this review focuses on cellular derived DAMPs, we will not extensively discuss ECM DAMPs, although there is some evidence that ECM DAMPs are involved in COPD pathogenesis. Increased protein expression of versican\textsuperscript{134,135,136} and fibronectin,\textsuperscript{137,138} has been observed in lungs of COPD patients, whereas for other ECM DAMPs, e.g. low molecular weight (lmw)-hyaluronan,\textsuperscript{139,140} higher levels of pro-inflammatory breakdown products have been found in BAL fluid of COPD patients. (See table 1) These DAMPs may play a role in the pathogenesis of COPD, since most of these DAMPs can activate TLR2 and TLR4 downstream NF-κB pathways, thereby inducing or maintaining pro-inflammatory activation of the innate immune system.\textsuperscript{8}

**CONCLUDING REMARKS**

In this review, various relevant DAMPs and their potential roles in COPD pathogenesis are discussed. Since the proposal of the danger hypothesis by Matzinger in 1994, many danger signals have been discovered, several of which may have a pathogenic role in COPD.\textsuperscript{11} Increasing numbers of publications have studied the presence of DAMPs in extracellular lung fluids, including BAL, ELF and sputum. Higher levels of HMGB1, S100A8/A9, lmw-Hyaluronan, ATP and β-defensin have been observed in BAL fluid of COPD patients compared to controls, providing possible biomarkers for the detection of COPD at early disease state. (See table 1) To date, no studies
have been performed to assess a larger set of DAMPs in order to identify the DAMP signature in COPD patients. This could be of interest considering that not all DAMPs are equally increased in lung tissue and/or released in the ECS of COPD patients. Furthermore, it is currently unknown what the contribution of individual DAMPs is in the progression of COPD, and whether specific DAMPs are more pathogenic than others or whether simultaneous release of multiple DAMPs is required to drive innate and adaptive immune responses in COPD. The latter can be examined by intervention studies both in vitro and in pre-clinical in vivo models. After identifying the DAMP signature in lungs of COPD patients, the next step will be to interfere with the immune stimulatory effect of DAMPs and to assess whether this will attenuate chronic lung inflammation. The immunologic effects of DAMPs can be inhibited by the use of neutralizing peptides or antibodies to specific DAMPs, preventing their binding to PRRs. Another possibility is to inhibit the release of DAMPs by inhibiting immunogenic cell death. This can be done by: promoting damaged cells to go into non-immunogenic apoptosis instead of necrosis or necroptosis, since apoptosis does not lead to release of immunogenic DAMPs,19,30 by inhibiting necroptosis141 or by initiating more efficient phagocytosis.15 Furthermore, DAMP mediated inflammation can be reduced by inhibition of specific PRRs, thereby disabling specific DAMPs to exert their functions. It has already been shown that TLR4, fpr1 and P2Y2R knock-out mice have attenuated inflammation in the lungs after acute cigarette smoke exposure.130,142,121 However, an even stronger reduction in inflammation was observed after acute cigarette smoke exposure when multiple PRRs were inhibited simultaneously by knocking out MyD88, a crucial signaling adaptor molecule for most TLRs.142 Nevertheless, this decrease in inflammation was only seen after acute and not after prolonged cigarette smoke exposure, when the

<table>
<thead>
<tr>
<th>DAMP group</th>
<th>Receptor(s)</th>
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<th>Effect</th>
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<tr>
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<td></td>
<td></td>
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<td>↑ HV</td>
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<td>HMGB1</td>
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<tr>
<td></td>
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<tr>
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<tr>
<td>Versican</td>
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<td></td>
<td></td>
<td>↓ HV</td>
<td>Expression alveolar walls</td>
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<td></td>
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<td>Production by isolated fibroblasts</td>
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<td>↑ SC/NSC</td>
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</tr>
</tbody>
</table>

Effect in (ex)-smoking COPD patients compared to NSC, Non-Smoking Controls; SC, Smoking Controls; HV, Healthy Volunteers with unknown smoking status. HSPs, Heat Shock proteins; TLRs, Toll-Like Receptors; RAGE, Receptor for Advanced Glycation End products; BAL, Bronchoalveolar Lavage; ELF, Epithelial Lining Fluid; HMGB1, High-Mobility Group Box-1; NLRP3, NLR-family Pyrin domain containing 3; ATP, Adenosine 5’-triphosphate.
adaptive immune reaction is thought to crucially contribute to COPD pathogenesis.\(^4\)

To the best of our knowledge, targeting DAMPs or their signaling pathways in COPD patients have not yet been performed. More research into the critical forms of cell-death and DAMP signatures in COPD patients as well as the critical down-stream PRRs may open new avenues for therapeutic intervention in this chronic disease for which current medication is lacking.

In conclusion, multiple studies have shown that the levels of specific DAMPs are higher in BAL fluid of COPD patients compared to healthy individuals and there is suggestive evidence for a role in the initiation of chronic airway inflammation in COPD. Thus, DAMPs may provide potential therapeutic targets to reduce the chronic inflammation in lungs of COPD patients as well as potential biomarkers for the detection of COPD at an early disease state.

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