Susceptibility for cigarette smoke-induced DAMP release and DAMP-induced inflammation in COPD


ABSTRACT
Cigarette smoke (CS) exposure is a major risk factor for COPD. We investigated whether CS-induced DAMP release or DAMP-mediated inflammation contributes to susceptibility for COPD.

Samples, including bronchial brushings were collected from young and old individuals, susceptible and non-susceptible for the development of COPD, before and after smoking, and used for gene profiling and airway epithelial cell (AEC) culture. AECs were exposed to CS extract (CSE) or specific DAMPs. BALB/cByJ and DBA/2J mice were intranasally exposed to LL-37 and mitochondrial (mt)DAMPs.

Functional gene-set enrichment analysis showed that CS significantly increases the airway epithelial gene-expression of DAMPs and DAMP receptors in COPD patients. In cultured AECs, we observed that CSE induces necrosis and DAMP release, with specifically higher galectin-3 release from COPD-derived compared to control-derived cells. Galectin-3, LL-37 and mtDAMPs increased CXCL8 secretion in AECs. LL-37 and mtDAMPs induced neutrophilic airway inflammation, exclusively in mice susceptible for CS-induced airway inflammation.

Collectively, we show that in airway epithelium from COPD patients, the CS-induced expression of DAMPs and DAMP receptors in vivo and the release of galectin-3 in vitro is exaggerated. Further, our studies indicate that a predisposition to release DAMPs and subsequent induction of inflammation may contribute to the development of COPD.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic lung disease that is characterized by neutrophilic airway inflammation, leading to the development of chronic bronchitis, fibrosis in the small airways and/or emphysema. The major risk factor for the development of COPD is chronic exposure to noxious gases and particles, including cigarette smoke (CS). The mechanisms underlying CS-induced airway inflammation in COPD patients are still largely unknown. Airway epithelial cells (AECs) are the first line of defense against inhaled toxicants and it has been shown that these cells show cellular damage and cell death upon CS exposure. Necrotic cell death induces the release of Damage Associated Molecular Patterns (DAMPs) into the extracellular space. DAMPs are molecules that alert and activate the innate immune system by binding to pattern recognition receptors (PRRs) upon passive or active release from damaged or necrotic cells. The most well-known PRRs are members of the Toll-Like Receptor (TLR) family. In addition, the DAMPs HMGB1, S100A8/A9 and LL-37 all bind to the Receptor for Advanced Glycation End-products (RAGE). Activation of both TLRs and RAGE leads to nuclear factor (NF)-κB-dependent pro-inflammatory cytokine release. Recently, it has been proposed that DAMPs may play a pivotal role in the pathophysiology of COPD, as several DAMPs, including HMGB1, S100A8/9, galectin-3 and LL-37, have been found increased in lung fluid or serum of COPD patients compared to smoking and non-smoking controls. Of interest, we observed that the RAGE ligands HMGB1, S100A8 and LL-37 were also increased during COPD exacerbations. Furthermore, Ager, the gene encoding RAGE, has been identified as a susceptibility gene for decreased lung function and COPD. Previously, we have shown that a specific pattern of DAMPs is released into the airways of mice susceptible for CS-exposure-induced airway inflammation, with higher levels of galectin-3, S100A9 and dsDNA upon CS exposure compared to non-susceptible mice. However, it is still unknown whether airway epithelium of COPD patients is predisposed to release DAMPs upon CS exposure and whether this contributes to the development of COPD.

We hypothesized that airway epithelium from COPD patients displays exaggerated CS-induced DAMP release and/or DAMP-induced pro-inflammatory responses. Therefore, we studied the effects of CS exposure on the expression of a set of 30 genes encoding DAMPs and DAMP receptors in RNA isolated from bronchial brushings and on DAMP release in vitro in airway epithelium from COPD susceptible and non-susceptible individuals. Furthermore, the effects of galectin-3, S100A9, HMGB1, LL-37 and mitochondrial DAMPs (mtDAMPs) on the release of neutrophil attractant CXCL8 were studied in AECs from healthy controls and COPD patients. Lastly, we investigated the effect of intranasal treatment of mtDAMPs and LL-37 on neutrophilic airway inflammation in mice either genetically susceptible or non-susceptible for CS-induced airway inflammation.

MATERIALS AND METHODS

Subjects

Serum, epithelial lining fluid (ELF) and bronchial brushings were obtained from two age groups: 1) eight old (≥ 40 year) COPD patients with GOLD stage II and ten age- and smoking history-matched current smoking individuals with normal lung function (old non-susceptible); 2) 18 young (<40 year) individuals with normal lung function and either a high (young susceptible) or low (young non-susceptible) prevalence of COPD in smoking family members. All young individuals were irregular smokers with the ability to quit smoking for at least two days. COPD patients were classified by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2007 guidelines and were recruited from outpatient clinics. Patients were included after doctors diagnose of COPD and a FEV$_1$ of <80% and a FEV$_1$/FVC of ≤0.7. Exclusion criteria for all groups include alpha-1 antitrypsin deficiency, acute pulmonary infections, treatment with antibiotics or corticosteroids within eight weeks, recent diagnosis of cancer and a doctors’ diagnosis of asthma. See table 1 for subject characteristics and figure 1 for experimental set-up and collection of blood, epithelial lining fluid (ELF) and bronchial brushings. Samples were collected at baseline and upon smoking three cigarettes within one hour. Subjects were asked not to smoke for at least two days prior to baseline visits, and to refrain from smoking between the acute smoking procedure and the 24-hrs bronchial brushings. Refraining from smoking was verified by exhaled carbon monoxide (CO) measurements.
being <5 parts per million (ppm) at baseline and sufficient inhalation of the three cigarettes was verified by a rise in CO (Micro+ Smokerlyzer®, Bedfont Scientific Ltd, Kent, England). Subjects were excluded from the study when their CO measurement was >5 ppm at baseline. The study was approved by the Medical Ethics Committee of the University Medical Center Groningen and all subjects gave their written informed consent. AECs were isolated from bronchial brushings of the patients in table 1 and from trachea-bronchial tissue of six severe ex-smoking patients with COPD GOLD stage IV (56±7 years old, 36±6 packyears and FEV\textsubscript{1} % of 25±6) who underwent lung transplantation as well as from non-COPD lung donors of whom no further information was available. The study protocol was consistent with the Research Code of the UMCG (http://www.rug.nl/umcg/onderzoek/researchcode) and national ethical and professional guidelines (http://www.federa.org). For mechanistic studies, normal human bronchial epithelial (NHBE) cells were obtained from Lonza (Walkersville, MD).

**Figure 1: Flow-chart of experimental set-up and sample collection.** Samples were collected from subjects from two age groups: 1) eight current smoking ≥40 years old patients with COPD GOLD stage II and 10 current smoking individuals with normal lung function matched for age- and smoking history (old non-susceptible); 2) 18 individuals of <40 years of age with normal lung function and either a high (young susceptible) or low (young non-susceptible) prevalence of COPD in smoking family members. Blood was taken at baseline, after 48 hours of smoking cessation and at three hours after smoking three cigarettes within one hour. Serum samples were obtained three hours after smoke exposure. Epithelial lining fluid (ELF) samples were obtained 24 hours after smoke exposure. Bronchial brushings were obtained 24 hours after smoke exposure, and either directly stored for RNA isolation or used for isolation and culture of culturing airway epithelial cells (AECs).

**Table 1: Patient characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Young susceptible (n=10)</th>
<th>Young non-susceptible (n=8)</th>
<th>COPD patients (n=8)</th>
<th>Age-matched controls (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>4/6</td>
<td>7/1</td>
<td>8/0</td>
<td>9/1</td>
</tr>
<tr>
<td>Age</td>
<td>30.4 (±2.8)</td>
<td>23.0 (±2.3)</td>
<td>64.8 (±3.0)</td>
<td>61.2 (±2.7)</td>
</tr>
<tr>
<td>Pack years</td>
<td>3.0 (±1.2)</td>
<td>1.9 (±1.1)</td>
<td>34.6 (±4.2)</td>
<td>33.1 (±4.1)</td>
</tr>
<tr>
<td>No. cigarettes per day</td>
<td>3.60 (±2.07)</td>
<td>6.88 (±2.36)</td>
<td>8.25 (±2.45)</td>
<td>12.20 (±2.90)</td>
</tr>
<tr>
<td>FEV\textsubscript{1}, %pred</td>
<td>100.5 (±2.7)</td>
<td>104.6 (±2.7)</td>
<td>55.2 (±4.5)</td>
<td>103.3 (±3.5)</td>
</tr>
<tr>
<td>FEV\textsubscript{1}/FVC, %</td>
<td>90.9 (±2.4)</td>
<td>96.6 (±2.6)</td>
<td>58.2 (±5.6)</td>
<td>93.6 (±2.3)</td>
</tr>
<tr>
<td>RV/TLC, %</td>
<td>23.6 (±0.9)</td>
<td>20.8 (±1.1)</td>
<td>42.8 (±2.4)</td>
<td>34.2 (±1.5)</td>
</tr>
<tr>
<td>BMI</td>
<td>23.4 (±1.0)</td>
<td>22.7 (±0.7)</td>
<td>26.1 (±1.1)</td>
<td>26.9 (±0.6)</td>
</tr>
</tbody>
</table>

Patient characteristics of young susceptible individuals, young non-susceptible individuals, COPD patients and age-matched controls. All young individuals were irregular smokers with the ability to quit smoking for at least two days. BMI is Body Mass Index in kg/m\textsuperscript{2}. FEV\textsubscript{1} is forced expiratory volume in one second, FVC is forced expiratory volume, RV is residual volume and TLC is total lung capacity. All data is shown as mean (±SEM).
**Genealogical study**

Young subjects with normal lung function were classified as susceptible for the development of COPD when the prevalence of COPD in smoking first or second degree relatives older than 45 years meets the following criteria: 2 out of 2, 2 out of 3, 3 out of 3, 3 out of 4 or 4 out of 4 smoking family members have developed COPD. Young healthy subjects were classified as non-susceptible to COPD only when none of the smoking first or second degree relatives who are at least 45 years of age (at least two should be identified) have been diagnosed with COPD. Families with alpha-1 antitrypsin deficiency were excluded in this study.

**Functional gene-set enrichment analysis**

Gene expression in bronchial brushings of the 4 subjects groups in table 1 was analyzed by Affymetrix hugen ST1.0 microarrays as described before. In short, nasal brushes were immediately snap-frozen and stored at -80 °C. RNA was extracted from bronchial brushes and fractioned into low molecular weight (< 200 nt) and high molecular weight (> 200 nt) fractions, by using the miRNeasy mini kit (QIAGEN) according to manufacturer's protocol. The purity of RNA fractions was checked on NanoDrop 1000 UV-Vis spectrophotometer and the integrity of large RNA fraction was assessed by running RNA Pico assay in the Agilent 2100 BioAnalyzer. All procedures were performed at Boston University Microarray Resource Facility as described previously in GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Santa Clara, CA, current version available at www.affymetrix.com). Normalization was performed with R statistical software V3.0.2. using Robust Multichip Analysis (RMA) sketch algorithm workflow. Microarray data quality was assessed using relative log expression (RLE) plots, normalized unscaled standard error (NUSE) plots, and principle component analysis (PCA) of all genes across all samples. Based on the variability of gene expression data according to the RLE and NUSE plots. Next, we investigated the change in gene expression 24 hours after smoking of three cigarettes in susceptible and non-susceptible patients for COPD. This analysis was performed using a linear mixed effects model with time defined as a categorical variable with two levels (1 = baseline, 2 = 24 hours after smoking of three cigarettes), susceptibility for developing COPD was defined as a categorical variable with two levels (1 = non-susceptible, 2 = susceptible) or COPD and healthy which was defined as a categorical variable with two levels (1 = healthy, 2 = COPD). An interaction analysis was conducted between time: susceptibility, adjusted for age and sex. $G_{ij}$ represents the log2 gene expression value for a gene in sample i from patient j, $\varepsilon_{ij}$ represents the error that is assumed to be normally distributed and $\alpha_j$ represents the patient random effect:

$$G_{ij} = \beta_0 + \beta_1 X_{Age-i} + \beta_2 X_{Sex-i} + \beta_3 X_{Susceptibility-i} + \beta_4 X_{Time-i} + \beta_5 X_{Time-i: Susceptibility/COPD-i} + \varepsilon_{ij} + \alpha_j$$

Second, we investigated baseline gene expression in susceptible and non-susceptible patients for COPD, COPD patients and healthy individuals. This analysis was performed using a linear model with susceptibility for developing COPD defined as a categorical variable with two levels (1 = non-susceptible, 2 = susceptible) or COPD and healthy which was defined as a categorical variable with two levels (1 = healthy, 2 = COPD), adjusted for age and sex. $G_{ij}$ represents the log2 gene expression value for a gene in sample i from patient j, $\varepsilon_{ij}$ represents the error that is assumed to be normally distributed and $\alpha_j$ represents the patient random effect:

$$G_{ij} = \beta_0 + \beta_1 X_{Age-i} + \beta_2 X_{Sex-i} + \beta_3 X_{Susceptibility/COPD-i} + \varepsilon_{ij} + \alpha_j$$

Functional gene-set enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA) version 2.2.0.14, on a set of 30 genes encoding DAMPs or their receptors. Genes from the above analysis were ranked based on t-statistic values comparing patients before and after smoking, between healthy and COPD or between young susceptible and non-susceptible individuals. The list of 30 genes was then used to investigate the ranks, in order to determine if they were enriched and differentially expressed between these groups after smoking. Enrichment p-values were calculated by gene set permutation (n = 1000) and significant enrichment was determined by a false discovery rate (FDR)-corrected p-value < 0.05.
List of genes used in functional enrichment analysis

All genes of the DAMP-related gene-expression signature were selected based on the review by Pouwels et al. The following genes were used in the functional enrichment analysis: HAS2 (hyaluronan synthase 2), TLR7 (toll-like receptor-7), LGALS9 (galectin-9), LGALS3 (galectin-3), FPR2 (formyl peptide receptor 2), TLR4 (toll-like receptor-4), VCAN (versican), NLRP3 (NLR family, pyrin domain containing 3), FPR1 (formyl peptide receptor 1), TLR2 (toll-like receptor-2), S100A9 (S100 calcium binding protein A9), P2RX7 (purinergic receptor P2X, ligand gated ion channel, 7), FPR3 (formyl peptide receptor 3), S100A8 (S100 calcium binding protein A8), S100A12 (S100 calcium binding protein A12), FN1 (fibronectin 1), LGALS1 (galectin-1), CALR (calreticulin), CAMP (LL-37; cathelicidin antimicrobial peptide), HSPD1 (heat shock 60kDa protein 1), MYD88 (myeloid differentiation primary response 88), TLR3 (toll-like receptor-3), TLR9 (toll-like receptor-9), HMGB1 (high mobility group box 1), AGER (RAGE; advanced glycosylation end product-specific receptor), HSP90AB1 (heat shock protein 90kDa alpha (cytosolic), class B member 1), HSPA2 (heat shock 70kDa protein 2), CPS1 (carbamoyl-phosphate synthase 1, mitochondrial), HSPB1 (heat shock 27kDa protein 1) and DEFB1 (defensin, beta 1).

Airway epithelial cell culture

NHBE cells and AECs were cultured in bronchial epithelial growth medium (BEGM, Lonza) and used for experiments at passage three as described before. Cells were seeded in duplicates, grown to confluence, hormonally-deprived overnight and stimulated with CSE or DAMPs in the presence/absence of specific receptor antagonists, and assayed for necrosis and DAMP and CXCL8 release. Cigarette smoke extract (CSE) was prepared as described before. In short, filters were cut from two cigarettes and the smoke from these cigarettes was bubbled through 25 mL of serum-free growth medium. This solution was considered as 100 % CSE.

Epithelial cell stimulation

AECs were stimulated with CSE (0-100%) for four hours before the CSE was washed away and the cells were incubated in serum free medium overnight, or for 24 hours with recombinant human galectin-3 (2 µg/mL; Peprotech, Neuilly Sur Seine, France), High Mobility Group Box-1 (HMGB1; 20 µg/mL; Prospek, Ness-Ziona, Israel), S100A8 (250 µg/mL; Sino Biological, Beijing, China), LL-37 (40 µg/mL; Invivogen, Toulouse, France), Tumor Necrosis Factor (TNF)-α (10 ng/mL; Sigma, St Louis, MO, USA) as positive control for epithelial cell activation or Mitochondrial DAMPs (mtDAMPs; 10 µg/mL). mtDAMPs were prepared from A549 cells by isolating the mitochondria using the mitochondria isolation kit for cultured cells according to manufacturer’s protocol (Thermo Fisher Scientific, Rockford, USA) and subsequently releasing the DAMPs by sonicating the isolated mitochondria for three minutes. The stock concentration of mtDAMPs was determined measuring DNA levels using a NanoDrop ND-1000 spectrophotometer (NanoDrop Tech, Wilmington, DE). All stimulations were performed upon pre-treatment with and without antagonists for TLR2/4 (30 µg/mL OxPAPC; Invivogen, Toulouse, France), TLR9 (50 µg/mL ODN-TTACGG; Invivogen, Toulouse, France) and RAGE (60 µg/mL RAGE antagonist peptide (RAP); Calbiochem, San Diego, USA) for 60 minutes. Supernatant was harvested after 24 hours and DAMPs or CXCL8 was measured in cell free supernatant. In addition, cells were collected in TRIzol for mRNA isolation and sample buffer for western blot analysis and both the cells attached to the bottom of the plate and the free floating cells were harvested for cell viability assays.

CXCL8 ELISA

CXCL8 measurements in cell free supernatants were performed according to manufacturer’s protocol (Human CXCL8/CXCL8 DuoSet; R&D Systems, Minneapolis, USA).

DAMP and PRR measurements

DAMP profiles were determined in cell free supernatant of airway epithelial cells and serum using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Carlsbad, USA) for dsDNA and ELISA for galectin-3 (detection limit (DL) is 62.5 pg/mL; R&D Systems, Minneapolis, USA), HMGB1 (DL is 800 pg/mL; Chondrex, Redmond, USA), HSP70 (DL is 156 pg/mL; R&D Systems, Minneapolis, USA), LL-37 (DL is 140 pg/mL; Hycult Biotech, Uden, The
Netherlands) and S100A9 (DL is 31.2 pg/mL; R&D Systems, Minneapolis, USA). The mRNA expression levels of S100A9, LL-37 and galectin-3 were determined using qRT-PCR as described before.\textsuperscript{16} Commercial primer/probe sets specific for target genes were purchased from Life Technologies (Invitrogen Life Technologies, Carlsbad CA, USA), S100A9 (Hs00610058_m1), LL-37 (CAMP; Hs00189038_m1) and galectin-3 (Lgals3; Hs00173587_m1) as target gene and B2M (Hs00984230_m1) and PPIA (Hs04194521_s1) as housekeeping genes.

**Cell viability**

The percentage of viable, apoptotic and necrotic cells was determined using Annexin-V/Pi staining for flow cytometry. Cells were trypsinized and washed two times using cell staining buffer (BioLegend, San Diego, USA) and stained in Annexin-V binding buffer (BioLegend, San Diego, USA) using 2.5 µL of Annexin-V-FITC (Immunotools, Friesoythe, Germany) and 2.5 µg/mL Propidium Iodide (Sigma-Aldrich, Saint Louis, USA). The percentage of necrotic, apoptotic and viable cells was measured afterwards using the BD FACSCalibur (BD Biosciences) flow-cytometer and data was analyzed using Winlist software (Verity Software House, Topsham, ME, USA). Cell viability was tested using Trypan blue staining.

**Animal experiments**

Mice experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen. Specified pathogen-free female 8-week old BALB/cByJ and DBA/2J mice (n=8 per group, Jackson Laboratories, Bar Harbor) received a single instillation of 67.4 µg LL-37, 30 µg mtDAMPs or 30 µg BSA in saline by intranasal administration under oxygen/isoflurane anesthesia and were sacrificed, two or six hours after the instillation. bronchoalveolar lavage (BAL) fluid and BAL cells were collected and stored at -80 °C until further use. BAL cell differentials were counted as described previously.\textsuperscript{22}

**Statistics**

The Mann Whitney-U test was used to test for significant differences between subject groups, the Wilcoxon Signed Rank test for differences within groups, two-way ANOVA for differences over a range of CSE concentrations and the one-way ANOVA for differences between treatment groups in mice.

**RESULTS**

*The effect of cigarette smoking on DAMP-related gene-expression in airway epithelium of COPD patients, age-matched smoking controls and young susceptible and non-susceptible individuals*

We first investigated whether CS-induced DAMP expression is a component of the susceptibility to develop COPD. To this end, we compared expression of DAMPs and their receptors in bronchial brushings, which predominantly comprise epithelial cells, between GOLD stage II COPD patients and age- and smoking history-matched controls with normal lung function, and between younger individuals susceptible or non-susceptible for COPD. GSEA was conducted comparing the expression of a set of 30 genes encoding DAMPs and their receptors at baseline and 24 hours post-smoking. We observed a significantly stronger increase in the expression of the DAMP gene-set in COPD patients compared to smoking controls following cigarette smoking (Figure 2A). 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. Interestingly, also a trend towards an increased expression of the DAMP and PRR gene-set was observed in the young COPD-susceptible compared to the non-susceptible group (p=0.08, Figure 2B). We next investigated whether the differences in the DAMP gene-set were caused by differences at baseline. Here, no overall significant differences were observed, although we observed a trend towards a lower basal gene expression of the DAMP gene-set in COPD patients compared to controls (p=0.062) (Figure 2C-D). Correction for body mass index did not change the outcome of any of the GSEA analysis (data not shown). Together, these results show that cigarette smoking induces higher induction of epithelial expression of specific DAMPs and PRRs in COPD patients compared to controls, and that this difference may already exist in susceptible individuals prior to the development of the disease.
Figure 2: Cigarette smoke exposure induces a stronger increase in expression of a DAMP gene-set in COPD patients compared to smoking controls as shown by functional gene enrichment analysis. A) Gene set enrichment analyses (GSEA) in bronchial brushings of COPD patients (n=8) and age- and smoking history matched controls (n=10) 24 hours post smoking of three cigarettes (p=0.003). The color bar indicates the genes ranked according to their difference between COPD patients and controls (blue representing genes decreased in COPD, red indicating increased genes). The vertical bars in all plots indicate the location of DAMP related genes within the ranked gene list and the height of the bars indicate the running GSEA enrichment score (black bars = core enriched genes, grey bars = core non-enriched genes). B) GSEA in bronchial brushings of young susceptible individuals (n=10) and non-susceptible individuals (n=8) 24 hours post smoking of three cigarettes (p=0.08). The vertical bars in all plots indicate the location of DAMP related genes within the ranked gene list and the height of the bars indicate the running GSEA enrichment score (black bars = core enriched genes, grey bars = core non-enriched genes). C) GSEA were conducted comparing the gene-expression at baseline. A trend towards an increase of the DAMP-related gene-expression signature was found in COPD patients compared to healthy controls in bronchial brushings (p=0.062). D) No increase of the DAMP-related gene-expression signature was found in young susceptible individuals (n=10) compared to non-susceptible individuals (n=8) in bronchial brushings (p=0.48). Lgals3 = Lectin Galactoside-binding Soluble 3 (encodes galectin-3), Tlr4 = Toll-like Receptor 4, Tlr2 = Toll-like Receptor 2, S100A9 = S100 Calcium Binding Protein A9, Camp = Cathelicidin Antimicrobial Peptide (encodes LL-37), Hmgb1 = High Mobility Group Box 1, Ager = Advanced Glycosylation End Product-Specific Receptor (encodes RAGE), Hsp70 = Heat Shock Protein 70. (see color image on page 210)
The effect of cigarette smoking on serum and ELF levels of DAMPs in COPD susceptible and non-susceptible individuals

To assess whether the CS-induced changes in DAMP gene-expression also result in differences in secreted DAMPs, we analyzed the levels of RAGE-activating DAMPs (LL-37, S100A9 and HMGB1) and TLR-activating DAMPs (galectin-3, dsDNA and HSP70) in the four study groups, based on previous data and the results above. At baseline, no differences in serum levels were found between the groups. Smoking resulted in significantly higher serum levels of LL-37, but none of the other DAMPs in all groups, except the young susceptible group (Figure 3A-F). In ELF, baseline LL-37 levels were significantly higher in the young susceptible compared to the young non-susceptible group, although no changes were observed in LL-37 (Figure 3G) nor any of the other DAMPs (data not shown) upon cigarette smoking.

The effect of CSE exposure on cell death and DAMP release in COPD- and control-derived epithelium

In addition, we investigated whether cultured AECs from COPD patients are more susceptible for CS-induced necrosis and subsequent DAMP release. In response to a dose-range of CSE, AECs from severe ex-smoking COPD patients and controls showed a strong increase in necrotic cells and a slight increase in apoptotic cells, without significant differences between the subject groups (Figure 4A-C). Furthermore, release of dsDNA, HSP70, galectin-3 and HMGB1 increased with CSE concentration in both subject groups (Figure 4D-H), while the levels of LL-37 were below the detection limit of the ELISA (data not shown). The levels of S100A9 did not show a dose-dependent increase, although the release upon CSE exposure was significantly higher in control-derived compared to COPD-derived AECs. Importantly and in line with the GSEA data, CSE caused a significantly stronger increase in the levels of galectin-3 in AECs from COPD patients compared to controls. To assess whether the observed differences in galectin-3 and S100A9 were a consequence of differences in gene-expression at baseline, we analyzed mRNA expression of galectin-3 (p=0.0830) and S100A9 (p=0.8148) in these cells, showing no significant differences between COPD and controls (Figure 4I-J).

In AECs from young susceptible and non-susceptible individuals, CSE induced a similar effect on necrosis and apoptosis as observed for the older subjects, without significant differences between the groups (Figure 5A-C). Similarly, CSE induced a dose-dependent and significant increase in the release of HMGB1, galectin-3 and dsDNA with increasing concentrations of CSE (Figure 5D-G), again without significant differences between the groups.

The effect of DAMPs on CXCL8 secretion in AECs

In addition to differences in susceptibility for CS-induced DAMP release, enhanced responsiveness to DAMPs may also contribute to the aberrant inflammatory response to CS in COPD patients, as supported by the observed differences in TLR2 and 4 expression in the GSEA. Therefore, we compared the pro-inflammatory response of AECs from COPD patients and controls to DAMPs. Both recombinant LL-37 and isolated mtDAMPs induced a strong and significant increase in the secretion of the neutrophil attractant CXCL8, while this was not observed for recombinant S100A8 and HMGB1 (Figure 6A). To investigate the involved receptors in the mtDAMP- or LL-37-induced CXCL8 secretion, we used specific PRR antagonists and observed that the mtDAMP-induced CXCL8 secretion was only reduced by the TLR2/4 antagonist (Figure 6B), while LL-37-induced CXCL8 secretion was significantly reduced by blocking the TLR2/4, TLR9 and RAGE receptors (Figure 6C). None of the antagonists affected basal CXCL8 secretion and cell viability (Figure 6D-F). In AECs from COPD patients and controls, HMGB1, LL-37 and mtDAMPs also induced a significant increase in CXCL8 secretion. Of note, galectin-3 only significantly increased CXCL8 secretion in COPD-derived cells. Although, no significant differences were found in the CXCL8 induction between subject groups (Figure 6G), the LL-37-induced CXCL8 release was inhibited to a greater extent by all three PRR antagonists in AECs from controls compared to COPD patients, with the RAGE blocker failing to suppress LL-37-induced CXCL8 release in COPD AECs. Furthermore, the galectin-3-induced CXCL8 secretion was significantly reduced by blocking TLR2/4 in control, but not COPD-derived AECs, with a significant difference between the groups (Figure 6H-J).

Together, AECs from COPD patients and controls secrete equal levels of CXCL8 in response to DAMP exposure, but the CXCL8 release is less sensitive to TLR antagonists in COPD patients.
Figure 3: Smoking induces an acute increase of LL-37 in serum of both COPD patients and controls. Serum and epithelial lining fluid (ELF) samples were used from COPD patients (n=8), age- and smoke-status-matched controls (Old NS; n=10) and young healthy controls that were either susceptible (Young S; n=10) or non-susceptible (Young NS; n=8) for the development of COPD. Serum and ELF samples were obtained after a smoking cessation period of two days (before smoking) and either directly (serum) or 24 hours (ELF) after smoking of three cigarettes within one hour (after smoking). The levels of the DAMPs A) LL-37, B) S100A9, C) HMGB1, D) galectin-3, D) dsDNA and F) HSP70 were measured in serum and the levels of G) LL-37 were measured in ELF. For HMGB1, the lowest data points were below the detection limit of the ELISA, which is set at 0.8 ng/mL. Significance between groups was tested using a Mann Whitney-U test and between pre- and post-smoking was tested using a Wilcoxon Signed-Rank test, * = P<0.05 and ** = P≤0.01.
Figure 4: Cigarette smoke extract exposure induces increased release of galectin-3 and decreased release of S100A9 in airway epithelial cells from COPD patients. Tracheal-bronchial epithelial cells isolated from COPD patients (n=6) and controls (n=6) were grown to ~90% confluence, hormonally deprived overnight and exposed to various concentrations of cigarette smoke extract (CSE, 0-100%) for four hours and incubated with serum free medium for 24 hours thereafter. The percentage of A) viable B) apoptotic and C) necrotic cells was determined using Annexin-V/Pi staining for flow cytometry. In cell free supernatant the levels of D) S100A9, E) HMGB1, F) galectin-3, G) dsDNA and H) HSP70 were measured. The mRNA expression levels of I) galectin-3 and J) S100A9 were measured in airway epithelial cells from COPD patients (n=8) and controls (n=8). All data is shown as mean ± SEM. To test for differences between COPD patients and controls over a range of CSE concentrations the interaction term of a 2-way ANOVA was used, * = P<0.05 at the right side of the figure. To test for differences between the groups at every CSE concentration separately, a Mann-Whitney-U test was used, * = P<0.05 and ** = P ≤0.01 between the indicated values. To test for the difference in overall effect of CSE between groups, a 1-way ANOVA was used, # = P<0.05 at the right side of the figure.
Figure 5: Genetic predisposition for the development of COPD does not influence cigarette smoke extract-induced cell death or DAMP release. Bronchial epithelial cells isolated from young healthy individuals that were either susceptible (n=10) or non-susceptible (n=8) for the development of COPD were grown to ~90% confluence, hormonally deprived overnight and exposed to a range of cigarette smoke extract (CSE) concentrations (0-100%) for 4 hours and incubated with serum free medium for 24 hours thereafter. The percentage of A) viable, B) apoptotic and C) necrotic cells was determined using Annexin-V/Pi staining for flow cytometry. In cell free supernatant the levels of D) S100A9, E) HMGB1, F) galectin-3 and G) dsDNA were measured. To test for differences between susceptible and non-susceptible individuals over a range of CSE concentrations the interaction term of a 2-way ANOVA was used, *=P<0.05 at the right side of the figure. To test for differences between the groups at every CSE concentration separately, a Mann-Whitney-U test was used, *=P<0.05 and **=P ≤0.01 between the indicated values. To test for the difference in overall effect of CSE between groups, a 1-way ANOVA was used, # = P<0.05 at the right side of the figure. All data is shown as mean ± SEM.
Figure 6: LL-37 and mtDAMPs induce a partially TLR dependent CXCL8 response in airway epithelial cells. Normal human bronchial epithelial (NHBE) (n=5) cells were grown to ~90% confluence, hormonally deprived overnight and stimulated with the recombinant DAMPs A) S100A8 [250 pg/mL], HMGB1 [10 µg/mL] and LL-37 [20 µg/mL], isolated mitochondrial DAMPs (mtDAMPs) [10 µg/mL] and TNF-α [40 ng/mL] as a positive control for 24 hours. Afterwards, the concentration of CXCL8 was measured in the cell free supernatant. Control conditions are presented as light grey bars and DAMP stimulations are presented as dark grey bars. The receptor by which B) mtDAMPs and C) LL-37 induce an CXCL8 response was investigated by pre-incubating the cells for one hour with specific antagonists for TLR2/4 (OxpapC; 30 µg/mL), TLR9 (ODN-TTAGGG; 50 µg/mL) and RAGE (RAP; 60 µg/mL) before being stimulated with mtDAMPs or LL-37 for 24 hours and the CXCL8 concentration is determined. D) The effect of 24 hour stimulation with the receptor antagonists ODN-TTAGGG, OxpapC, RAP and the solvent DMSO on cell viability was studied using Tryphan blue staining in NHBE cells (n=5). E) The effect of OxpapC, ODN-TTAGGG and RAP on basal CXCL8 production in NHBE cells (n=5) and F) tracheal-bronchial epithelial cells isolated from COPD patients (n=6) and healthy controls (n=6). Tracheal-bronchial epithelial cells isolated from COPD patients (n=6) and controls (n=6) were grown to ~90% confluence, hormonally deprived overnight and stimulated with the recombinant DAMPs G) S100A8 [250 pg/mL], HMGB1 [10 µg/mL], LL-37 [20 µg/mL] and galectin-3 [2 µg/mL], isolated mitochondrial DAMPs (mtDAMPs) [10 µg/mL] and TNF-α [40 ng/mL] as a positive control for 24 hours. Afterwards, the concentration of CXCL8 was measured in the cell free supernatant. The receptor by which H) mtDAMPs, I) LL-37 and J) galectin-3 induce an CXCL8 response was investigated by pre-incubating the cells for one hour with specific antagonists for TLR2/4 (OxpapC; 30 µg/mL), TLR9 (ODN-TTAGGG; 50 µg/mL) and RAGE (RAP; 60 µg/mL) before being stimulated with mtDAMPs, LL-37 or galectin-3 for 24 hours and the CXCL8 concentration was determined. To test for significant differences between two groups a Mann Whitney-U test was used and to test for differences within a group a Wilcoxon Signed Rank test was used, * = P<0.05, ** = P≤0.01 and *** = P≤0.001. All data is shown as mean ± SEM.
Finally, we investigated whether exposure of mice to the most potent pro-inflammatory DAMPs identified in our in vitro experiments, LL-37 and mtDAMPs, induced neutrophilic airway inflammation in vivo and whether this response correlates to susceptibility for CS-induced airway inflammation. We selected two mouse strains that were previously identified as either genetically susceptible (BALB/cByJ mice) or non-susceptible (DBA/2J mice) to develop neutrophilic airway inflammation in response to CS exposure\(^5\), and exposed these to a single installation of LL-37 and mtDAMPs. First, in a subset of mice it was shown that intranasal treatment with LL-37 increased the release of several mouse CXCL8 homologues, i.e. KC, MIP-2 and LIX respectively (data not shown) with the most significant induction for KC. Upon measurement of KC in all mice, we showed that both LL-37 and mtDAMPs significantly increased the BAL levels of KC two hours after DAMP exposure in both mouse strains. The percentage of neutrophils was determined in BALF using cytospin cell differentiation. All data is shown as mean ± SEM with n=8 per group. Significance within one mouse strains was tested using a one-way ANOVA, * = P<0.05, ** = P≤0.01 and *** = P≤0.001 and significance between strains was tested using a Mann-Whitney U test, * = P<0.05, ** = P≤0.01, *** = P≤0.001.

**Figure 7:** Intranasal application of LL-37 and mtDAMPs induced airway inflammation in the susceptible BALB/cByJ mice but not in the non-susceptible DBA/2J mice. BALB/cByJ and DBA/2J mice received 67.4 µg LL-37, 30 µg mtDAMPs or 30 µg BSA in saline by intranasal administration under oxygen/isoflurane anesthesia. Mice were sacrificed two or six hours after the intranasal application. The concentrations of A,B) KC and C,D) MPO were determined in BALF. The percentage of E,F) neutrophils was determined in BALF using cytospin cell differentiation. All data is shown as mean ± SEM with n=8 per group. Significance within one mouse strains was tested using a one-way ANOVA, * = P<0.05, ** = P≤0.01 and *** = P≤0.001 and significance between strains was tested using a Mann-Whitney U test, * = P<0.05, ** = P≤0.01, *** = P≤0.001.

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strains (Figure 7A) and only in BALB/cByJ mice six hours after exposure (Figure 7B). In addition, myeloperoxidase (MPO) levels in BAL were significantly increased by LL-37 and mtDAMPs at two and six hours in BALB/cByJ, but not in DBA/2J mice (Figure 7C/D). Similarly, the numbers of neutrophils in BAL were increased in BALB/cByJ mice upon treatment with both LL-37 and mtDAMPs at two and six hours, while in DBA/2J mice only LL-37 induced a significant increase at six hours (Figure 7E/F). Collectively, both LL-37 and mtDAMPs are able to induce neutrophilic airway inflammation in mice susceptible for CS-induced neutrophilic airway inflammation, but not in the non-susceptible mice, indicating that genetic susceptibility for CS-induced neutrophilic airway inflammation associates with susceptibility for DAMP-induced airway inflammation.

**DISCUSSION**

Here, we investigated whether CS-induced DAMP release and/or DAMP-induced pro-inflammatory responses contribute to the susceptibility to develop COPD. Our data indicate that CS-induced release of specific DAMPs and subsequent PRR-mediated activation of innate immune responses contribute to the susceptibility for COPD. In *vitro*, CSE exposure induced dose-dependent release of DAMPs with a significantly stronger release of galectin-3 in AECs from COPD patients compared to controls. Moreover, galectin-3, LL-37 and mtDAMP induced a pro-inflammatory response in AECs from COPD patients as well as controls. Finally, we demonstrate that LL-37 and mtDAMPs also induce pronounced neutrophilic airway inflammation in mice susceptible for CS-induced airway neutrophilia.

In our current study we show for the first time that the expression of a DAMP gene-set is more strongly increased in bronchial brushings from COPD patients than from age- and smoking history-matched controls upon cigarette smoking, with galectin-3 being one of the core-enriched genes. Importantly, a similar trend was observed for bronchial brushings obtained from young individuals susceptible for COPD compared to young non-susceptible individuals, suggesting that differences are present prior to disease onset.

We observed a stronger release of galectin-3, but none of the other DAMPs, in COPD-derived compared to control-derived AECs *in vitro*, indicating that COPD-derived epithelium is more prone to the active release of galectin-3 rather than the passive release of DAMPs. Indeed, it has been shown that galectin-3 can be secreted actively.14,15 In line with our GSEA data in bronchial biopsies, galectin-3 mRNA expression was not different at baseline. Thus, CSE may either induce a stronger increase in galectin-3 mRNA expression in COPD-derived cells or promote its active release. Nevertheless, the strongest increase in release of galectin-3 was observed at higher concentrations of CSE that also induced cytotoxicity. This indicates the involvement of necrosis/necroptosis in CSE-induced galectin-3 release. The difference in CSE-induced galectin-3 release between COPD and control-derived epithelial cells was found at high concentrations of CSE, while we did not find a difference in cell death here. Together, these data suggest that the difference in galectin-3 release between COPD and control-derived epithelium may be due to a CS-induced increase in its mRNA expression. Our findings are in line with a previous study showing increased galectin-3 protein expression in small airway epithelium of ex-smoking COPD patients compared to smoking controls.13 In contrast, it was shown that the levels of galectin-3 were decreased in BAL of COPD patients and smokers compared to non-smoking and ex-smoking controls11, an observation we did not reproduce in our ELF samples. However, ELF did not reflect the observed changes in gene expression nor the observed increase of serum levels of LL-37 upon cigarette smoking. As ELF sampling relies on highly local probing at one specific time point, further research is needed to address whether local DAMP release is increased in COPD patients briefly after CS exposure, as our GSEA analysis would predict. Furthermore, we observed that the galectin-3-induced CXCL-8 response was only inhibited by TLR2/4 blockage in AECs from controls and not in AECs from COPD patients. Since our GSEA analysis did not show differences in the basal expression of TLR2, TLR4 and RAGE between COPD patients and controls, our data indicates that the sensitivity of these specific PRRs in COPD-derived AECs is reduced or that alternative receptors for the galectin-3-induced responses are involved in COPD-derived AECs. Future studies should be focused on further elaborating the role of galectin-3 in COPD, and identifying whether intranasal application of galectin-3 induces airway inflammation.
In our experimental set-up, cultured AECs did not secrete detectable levels of LL-37, indicating that LL-37 may also have other cellular sources than the airway epithelium. Nevertheless, LL-37 may still contribute to pro-inflammatory processes induced by CS exposure, as supported by our in vivo findings. Furthermore, accumulating data from literature highlight LL-37 as one of the key mediators in CS-induced pathology in COPD, as LL-37 was implicated in airway remodeling, mucus hyper-secretion and airway inflammation.\textsuperscript{18,19,20} Indeed, the levels of LL-37 have been shown to be increased in BAL, ELF and induced sputum of COPD patients compared to smoking and non-smoking controls\textsuperscript{21,22,20}, as well in serum during COPD exacerbations.\textsuperscript{19} Here, we show that LL-37 as well as mtDAMPs are highly potent inducers of pro-inflammatory responses, both in vitro and in vivo. mtDAMPs are a mixture of mitochondrial molecules, including mtDNA, cardiolipin and N-formylated peptides which act as DAMP and have been shown to be involved in several diseases, including acute lung injury.\textsuperscript{4,23,24} No assays are commercially available to measure cardiolipin and N-formylated peptides, while the lack of effect of TLR9 inhibition excludes a role for mtDNA in the observed epithelial pro-inflammatory response. Furthermore, the observed levels of released HMGB1 and S100A9 were higher in supernatant from old subjects compared to young subjects, an observation supported by recent literature.\textsuperscript{18}

Our in vivo data further indicate that enhanced responsiveness to DAMPs can contribute to the susceptibility for CS-induced neutrophilic airway inflammation. In contrast, we did not observe significant differences in DAMP-induced CXCL8 responses between AECs of COPD patients and controls. Thus, other cell types than airway epithelial cells may be required or responsible for the observed differences in susceptibility to DAMP-induced pro-inflammatory responses. On the other hand, the discrepancy between the in vitro and in vivo data may be explained by the notion that DAMP-induced inflammation may require gene-environment interaction in humans, which were not taken into account in our in vitro assays. In this respect, our GSEA data indicate that differences in epithelial TLR expression between COPD patients and controls were only observed upon cigarette smoking. Furthermore, DAMP-induced inflammation may include genetic factors, while the criteria for susceptibility in the human study rely merely on a genealogical study, and not all susceptibility genes may be equally spread amongst first and second degree family members.

In summary, we show for the first time that the gene expression of DAMPs and PRRs are dysregulated upon cigarette smoking in COPD patients. Our mouse model shows that genetic susceptibility for CS-induced airway inflammation is related to genetic susceptibility for DAMP-induced airway inflammation. Furthermore, we show that the CS-induced release of specific DAMPs and the pro-inflammatory response to specific DAMPs is abnormal in airway epithelial cells from COPD patients. Thus, susceptibility for CS-induced DAMP release may contribute to the development of COPD.

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