Mechanisms of glucocorticoid insensitivity in asthma

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Chapter 1

General introduction

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are chronic inflammatory and obstructive lung diseases (characterized by airway obstruction and inflammation) affecting millions of people worldwide. The most common type of asthma is allergic asthma, characterized by reversible airway obstruction, airway hyperresponsiveness, airway remodeling and a type of inflammation predominated by eosinophils and T helper (Th)2 cells upon inhalation of airborne allergens. For COPD, the main risk factor is the inhalation of noxious gases such as cigarette smoke, which induce abnormal inflammatory and repair responses. This leads to the classical phenotypes chronic bronchitis (mucus hypersecretion) and emphysema (alveolar destruction), which both contribute to irreversible airway obstruction. The inflammation in COPD is predominantly characterized by the presence of neutrophils, macrophages, cytotoxic Tcells, Th1 and Th17 cells, which are involved in the production of oxidants and proteases that cause lung tissue damage [1]. The treatment of obstructive airway diseases comprises predominantly of bronchodilation to treat airway obstruction, and immunosuppression to treat the inflammation. The main anti-inflammatory drugs used to treat both asthma and COPD are inhaled glucocorticoids (GCs) as maintenance and oral GCs during an exacerbation. While bronchodilation has been shown to be effective in the short term treatment of dyspnea, control of inflammation has been shown to lead to long term control of disease[2]. Long term clinical control of disease by inhaled GCs can be obtained in a majority of asthma patients and a minority of COPD patients[3]. However, a subset of asthma patients and the majority of COPD patients do not respond well to GCs. In these patients, oral GCs only temporarily relieve symptoms, but inhaled GCs do not halt the progression of their disease to the same extent as in the classical eosinophilic asthmatic patients. Indeed, GCs, provide relatively little therapeutic benefit in COPD. They reduce exacerbations, but do not effectively change the course of the disease in the majority of COPD patients.

Burden of disease in GC insensitive patients

Among asthma patients, the number of patients with difficult to treat, GC-insensitive disease is rather low, however, this subset of patients is a major contributor to hospitalization due to exacerbations and therefore to the costs of care that this entails [4]. COPD is currently a disease that, due to a lack of definitive treatment, comprises a huge burden to both patients and healthcare. Due to the ageing of the population, it is increasing in prevalence and expected to be the 3rd leading cause of death worldwide. COPD patients experience an accelerated decline in lung function, which cannot be halted by GCs and leads in a number of patients to disability through the final years of their life[5], with the amount of daily activities declining due to an ever more difficult ventilation. Eventually, this leads inevitably to their demise.

Therefore, this thesis aims to study mechanisms of GC-insensitivity. In both asthma and COPD, GC insensitivity is thought to develop gradually upon prolonged exposure to oxidative stress, although the molecular mechanisms are still largely unclear

Common pathways in the pathogenesis of asthma and COPD

In 1961 Prof Orie posed the so called Dutch hypothesis that asthma, chronic bronchitis and emphysema in essence have the same basis, as these diseases are all characterized by airway obstruction and airway inflammation. In 1965 chronic bronchitis and emphysema were on the basis of their common etiology in smoking united under the acronym COPD. Through the years both asthma and COPD have been characterized to increasing detail, which led to their subdivision into separate diseases and to the identification of various phenotypes of each disease[6,7]. Some phenotypes of asthma share properties with phenotypes of COPD, whereas other phenotypes of asthma have different properties. Additionally, in COPD some patients have some degree of airway hyperresponsiveness, classically attributed to asthma. These findings have led in recent years to the formulation of an asthma-COPD overlap syndrome (ACOS)[8] and subsequently led to the idea of not treating every patient with asthma or COPD the same, but according to their phenotype and the treatable traits observed in these patients.

In addition to the clinical syndrome ACOS, there have been shown overlapping inflammatory phenotypes as well. Especially interesting is that neutrophilia in asthma has been shown to be an independent disease phenotype that shares multiple characteristics with COPD[9]. Neutrophilic asthma has been associated with smoking as well as more severe, difficult to control disease. Furthermore, a subset of COPD patients have shown some degree of eosinophilic inflammation. One could postulate that they are all different diseases arbitrarily united under two monikers, but based on similar mechanisms and resulting in similar symptoms[10]. For the scope of this thesis, several mechanisms are addressed which can play a role in both asthma and COPD.

Inflammatory phenotypes of asthma and COPD

The inflammatory response in the respiratory system is a major contributor to disease severity in both asthma and COPD. As described above, while asthma is a mostly eosinophilic disease, COPD is a more neutrophilic disease. However, some asthmatics have a neutrophilic type of disease and some COPD subjects have an eosinophilic type of disease[9,11,12]. To complicate matters even further, with both diseases it is possible to have a mixture of both phenotypes. Past studies have shown more eosinophilic disease to be more responsive to GCs. Cigarette smoke is a major causative agent for COPD that also modulates inflammation in asthma[13]. In asthma,

cigarette smoking has been associated with a more neutrophilic phenotype as well as a diminished response to GCs. Additionally, a subgroup of patients can have persistent eosinophilic inflammation despite treatment with GCs, either through some form of inflammatory overdrive or an acquired mechanism of GC insensitivity of the eosinophil. The phenotype of inflammation can be dependent on the guidance it receives from T lymphocytes. Th cells can play a major role in orchestrating the type of inflammation. Th cells attract different types of inflammatory cells through release of specific inflammatory cytokines, leading to either a more eosinophilic or neutrophilic inflammation. The classic eosinophilic inflammation of asthma is strongly correlated to Th2 activity, while the neutrophilic subtypes can be Th1 or Th17 mediated, but can also be guided by direct stimulation by structural cells. The type of inflammation can be considered a treatable trait and different types of inflammation can lead to different treatments.

Th17 cells

The classical division in merely Th1 cells, producing IFN-γ, and Th2 cells, producing e.g. IL-4, IL-5 and IL-13, has been discarded. The Th family has been expanded to include regulatory T cells (Tregs), Th9 cells, and Th17 cells, leaving still room for more family members to be discovered. Th17 cells have been primarily associated with autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and multiple sclerosis[14]. Th17 cells mainly function by the production of IL-17 family of cytokines with IL-17A as the classical and most abundantly produced cytokine among them. Innate lymphoid cells type 3 (ILC3) have in recent years also been described to produce members of the IL-17 family of cytokines as well. This type of cell has been described to be present in the airways of asthmatic and COPD subjects. However, at present there a no studies linking the presence of ILC3 cells to neutrophilic inflammation or GC insensitivity [15].

Especially the Th17 cell has gained interest since McKinley et al showed induction of GC-insensitivity by the transfer of Th17 cells in a mouse model of asthma[16]. In a paper by the same group it was shown that cigarette smoke extract augments Th17 differentiation in an *in vitro* differentiation assay as well as in a mouse model of asthma[17]. Overexpression of the transcription factor that drives IL-17 genes, RORγ T, resulted in a neutrophilic airway inflammation with GC-insensitive airway hyperresponsiveness in a mouse model of asthma[18]. IL-17A works mostly by inducing the production of pro-inflammatory mediators in the surrounding tissues[19,20], acting on the airway epithelium to induce the secretion of pro-inflammatory cytokines (e.g. CXCL8, GM-CSF and CCL20) that recruit neutrophils to the site of inflammation. In pediatric asthma, Kerzel et al were able to show that Th17 cells are increased in children

with asthma and a higher percentage of Th17 cells amongst the Th cells is associated with worse asthma control[21]. Despite novel insights that Th17 cells are involved in the indication of GC-insensitive, neutrophilic inflammation, it is currently still unclear how Th17-mediated inflammation develops. Th17 cells are characterized as the lymphocyte expressing the chemokine receptor CCR6, which is predominantly expressed by this subtype[22]. CCR6 has been discovered as a receptor for the chemokine CCL20[23]. No other receptors have been discovered for CCL20, while CCR6 can be stimulated by CCL20 and beta-defensins. In the lung, CCL20 is produced predominantly by epithelial cells and dendritic cells[24]. Through CCL20 production they can induce Th17 chemotaxis[25,26]. Hastie et al found increased levels of CCL20 in asthmatics with sputum neutrophilia[27]. CCL20 is increased in the airways of COPD patients as well and in a murine model of COPD CCR6-deficient mice neutrophil influx was attenuated[28,29]. Therefore, there may be a role for the CCL20-CCR6-IL17A-axis in neutrophilic and GC insensitive inflammation in both asthma and COPD, although it is still unclear why this type of inflammation is insensitive to GCs.

Molecular mechanisms of GC action

In order to understand how GC insensitivity develops, insight into the molecular mechanisms of GC action is needed. The cellular response to GCs is dominated by two mechanisms: transrepression of transcription of pro-inflammatory proteins and transactivation of transcription of anti-inflammatory proteins[30]. First, the glucocorticoid diffuses over the cellular membrane to the cytosol, where it binds the GC receptor and forms a complex of two activated receptors. This complex migrates to the nucleus. Here, the transrepression and transactivation pathways diverge[30]. For the transrepression pathway, GCs act through closing the DNA for transcription. Normally, when transcription of pro-inflammatory proteins is activated, DNA is opened for transcription factors by histone acetyl transferase placing acetyl groups on the histones. GCs inhibit this transcription process by recruiting histone deacetylase (HDAC) complexes to acetylated histones and reversing the process of the histone acetylation by removing the acetyl groups and thereby making the DNA inaccessible for transcription factors[3]. In addition, GCs can induce gene transactivation by GR activation, which exerts an anti-inflammatory effect by binding to GC response element (GRE) in anti-inflammatory genes (e.g. IL-10, SLPI, MKP-1, GILZ)[31].

Molecular mechanisms of GC insensitivity

In addition to Th17-mediated inflammation, the oxidative stress that is associated with cigarette smoking has been implicated in the development of GC insensitivity. Our group has previously shown that exposure of bronchial epithelial cells to oxidative stress in vitro causes GC insensitivity[32]. Oxidative stress may induce GC insensitivity

through a variety of molecular mechanisms, including decreased GR translocation to the nucleus of the, competitive binding of an inactive splicing variant of the GR, GRβ, and a decrease in HDAC activity[3]. Irusen et al have shown that downstream signaling of the redox sensitive kinase p38 MAPK can induce GC insensitivity by phosphorylation of the GR and subsequent decreased translocation to the nucleus in peripheral blood mononuclear cells. Matthews et al showed that this might explain the failure to decrease histone acetylation and subsequent pro-inflammatory transcription[33,34]. Goleva et al showed that increased levels of GRβ were associated with a reduced GC response in GC-insensitive asthma, while in a later study the same group showed that GRβ overexpression in vitro inhibited HDAC2 expression[35,36].

HDAC2 activity has also been implicated in GC-insensitive inflammation induced by cigarette smoking[37]. The reduced expression and activation of HDAC is thought to be caused by oxidative stress leading to activation of phosphoinositol-3-kinase (PI3K) activation[38].

Cellular damage: leading to GC insensitive neutrophilic inflammation?

In addition, cigarette smoke-induced oxidative stress can cause cellular damage and cell death. This can lead to the release of danger signals (dangers) that act in a similar way as foreign invaders (strangers) to activate innate and adaptive immune responses. We aimed to investigate whether additional inflammation caused by cigarette smoke induced damage can contribute to difficult to control inflammation. One of the newer paradigms of immunologic disease is the role of danger signals[39]. Classically, the immune system had been subdivided in the innate and adaptive immune system. Where the adaptive immune system recognizes specific antigens, the innate immune system recognizes molecular patterns present in pathogens. Previously, this recognition was shown to be mediated through pattern recognition receptors (PRRs) expressed on innate immune cells, recognizing specific patterns in micro-organisms, the so-called pathogen associated molecular patterns (PAMPs). The danger hypothesis is primarily based on the (innate) immune system not just recognizing danger through PAMPs, but also by detecting cellular damage and cell death through the recognition of damage associated molecular patterns (DAMPs). These DAMPs are not necessarily dangerous or toxic substances, their presence merely implies the presence of cell damage or cell death. Importantly, these signals provide the recognition of patterns associated with danger by PRRs expressed by innate immune cells[39,40]. These DAMPs are molecules which have a different function within the cell under physiological circumstances, but once released upon cell damage or necrosis, these molecules can activate the immune system through binding to PRRs[41], such as toll like receptors (TLRs), purinergic receptors and Receptor for

Advanced Glycation Endproducts (RAGE). Examples of DAMPs are double stranded DNA (dsDNA), mitochondrial DNA (mtDNA), High Mobility Group Box 1 (HMGB1), Adenosine-Triphosphate (ATP), heat shock protein 70 and S100 proteins (S100A8/9). Here, dsDNA and mtDNA signal primarily through Toll-like receptor (TLR)9, HMGB1 signals through TLR2, TLR4 as well as Receptor for Advanced Glycation Endproducts (RAGE) and ATP . All TLRs except TLR3 can signal through MyD88 leading to activation of the downstream transcription factors Nuclear Factor (NF-)κB, which induces pro-inflammatory gene transcription. RAGE activation also results in pro-inflammatory NF-κB activation. ATP signals through purinergic receptors leading to downstream inflammasome signaling, which acts pro-inflammatory through splicing of pro-IL-1β into IL-1β[42]. In this way, PAMPs and DAMPs induce similar responses, acting through the same PPRs. For example, similar to various DAMPs, bacterial derived lipopolysaccharide (LPS) also acts on TLR4[41]. DAMPs have been shown to induce pro-inflammatory signals in numerous inflammatory diseases[43-45]. Importantly, DAMPs can also act directly on immune cells, including neutrophils, to indicate their activation and chemo-attraction.

Cigarette smoke has been described to cause cell death in a variety of ways[46]. Our group has shown that cigarette smoke causes a switch from apoptosis to necrosis and cells subsequently die in an uncontrolled manner[47]. We speculate that this may initiate lung inflammatory responses by the release of DAMPs in a GC-insensitive way, and thereby contribute to the development of GC-insensitive inflammation in COPD. In addition to directly inducing neutrophilic inflammation, Zhang et al have shown a role for HMGB1 in an mouse model of Th17 mediated neutrophilic asthma[44,48].

Scope of this thesis

This thesis aims to determine the mechanisms underlying difficult-to-control, GC insensitive airway inflammation in asthma and COPD. In chapter 2, we aimed to characterize the airway inflammation in relation to the response to oral GCs in asthma patients and define the characteristics which are associated with less responsiveness to GCs in asthma. Asthma patients were treated for 2 weeks with 30 mg prednisolone, and we investigated the relation between baseline characteristics and response to prednisolone as measured by FEV_1. In chapter 3, we aimed to determine whether cigarette smoking and GC differently affect HDAC2 expression in asthmatics. We hypothesized that HDAC2 levels were reduced upon smoking. We investigated HDAC2 expression in bronchial biopsies from an observational study in smoking and non-smoking asthma patients on different inhaled GC regimens subjects. In chapter 4, we

hypothesized that the cytokine IL-17A is able to induce GC-insensitivity in bronchial epithelial cells. We used a human bronchial epithelial cell line to determine whether pre-incubation with IL-17A led to a decreased suppression of TNF-a induced pro-inflammatory responses by GC, and used specific inhibitors of intracellular signaling pathways to unravel the involved pathways. In chapter 5, to gain more insight in the mechanisms of Th17 associated GC insensitivity, we aimed to determine whether the production of neutrophil and Th17 chemotactic cytokine CCL20 was affected by GCs. We studied the levels of CCL20 in sputum in a group of asthmatic subjects who did not use or did use u inhaled GCs and assessed the effect of GC on CCL20 production in bronchial epithelial cells in vitro, both in a cell line and primary cells derived from healthy controls and asthma patients. In chapter 6, we aimed to determine whether cigarette smoke can induce neutrophilic inflammation through a separate pathway depending on the induction of immunogenic cell death by cigarette smoke. We investigated whether cigarette smoke-induced necrosis was accompanied by the release of various DAMPs in bronchial epithelial cells and whether these necrotic products can subsequently induce pro-inflammatory gene transcription in neighboring epithelial cells. Furthermore we determined whether cigarette smoke can induce the release of DAMPs in mice through measuring this in bronchoalveolar lavage fluid (BALF), and we measured whether an inhibitor of regulated necrosis decreased the amount of inflammatory cells in the BALF of cigarette smoke treated mice.

Together, with these studies, we aimed to obtain insight in the mechanisms underlying GC insensitive inflammation in asthma and COPD.

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Chapter 2

Characterization of glucocorticosteroid response in mild-to-moderate asthma

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Manuscript in preparation

Abstract

Background

Unresponsiveness to glucocorticosteroids (GC) in asthma has been associated with several clinical and inflammatory features, including smoking. It is largely unknown whether these features contribute independently to GC-unresponsiveness. We aimed to determine the independent role of airway inflammation markers predicting GC-unresponsiveness in relation to smoking.

Methods

Inflammation was assessed in peripheral blood, induced sputum, bronchial biopsies, and exhaled Nitric Oxide (eNO) from 60 mild-to-moderate asthmatics: 20 current-smokers, 41 females, 21 inhaled GC-users, median age 50 years, median FEV_1 96% predicted. GC-unresponsiveness was defined as no improvement in pre-bronchodilator FEV_1 after a 2-week course of 40 mg methylprednisolone/day.

Results

At baseline, GC-unresponsive asthmatics (n=33) were more often current-smokers, demonstrated significantly lower levels of bronchodilator reversibility and eNO than GC-responsive asthmatics (n=27), and had higher blood lymphocyte counts. GC-response correlated positively with sputum eosinophils and ECP ($r_{spearman}$=0.36 (p=0.019) and 0.54 (p<0.001) respectively) and both alveolar and bronchial eNO ($r_{spearman}$=0.46 (p=0.001) and 0.42 (p=0.003) respectively). Neutrophil-to-eosinophil ratios correlated negatively in blood sputum and bronchial biopsies . Multivariate regression showed that higher age (p=0.038), blood lymphocyte counts (p=0.008) and neutrophil/eosinophil ratios (p=0.050), lower alveolar eNO levels (p=0.003) and sputum eosinophil percentages (p=0.048) independently predicted lower GC-response (R^2=0.71, p<0.001). Cigarette smoking did not contribute independently to the model (p=0.197)

Conclusion

As current smoking did not affect GC-response independently, we suggest that smoking-induced inflammatory changes appear to be more important than direct effects of smoking itself.

Background

Asthma is characterized by inflammation of the central and peripheral airways involving many cell types and mediators. Glucocorticosteroids (GC) inhibit virtually all components of asthmatic airway inflammation and provide beneficial clinical effects in most patients. Unfortunately, some asthmatic patients are unresponsive to GC [1,2]. This GC-unresponsiveness has been associated with higher numbers of peripheral blood lymphocytes [3], higher neutrophil [4] and lower eosinophil sputum counts [5], and lower Nitric Oxide (NO) levels in exhaled air [5,6]. Several clinical studies in asthma have demonstrated that cigarette smoking increases GC-unresponsiveness [7-9]. Interestingly, smokers with asthma have increased neutrophil counts and decreased eosinophil counts in sputum [10] and bronchial biopsies [11], as well as decreased NO levels in exhaled air [11,12] compared to non–smoking asthmatics, leading to the hypothesis of a causal role of smoking. So far, the only study comparing bronchial biopsies between GC-sensitive and GC-insensitive subjects did not show any differences in inflammatory parameters [13]. However, it is largely unknown to what extent the inflammatory characteristics contribute to GC-unresponsiveness and whether smoking affects this relation independently.

We assessed inflammatory cells in peripheral blood, induced sputum, exhaled air (NO), and bronchial biopsies in smoking and non-smoking subjects with mild-to-moderate asthma. In addition, we measured Adenosine-5'-MonoPhosphate (AMP) responsiveness, which can also be considered as an inflammatory marker as it is associated with airway eosinophilia [14]. We aimed to determine the independent role of these markers of airway inflammation in predicting GC-unresponsiveness in relation to smoking.

Methods

Subjects

Participants were recruited from previous research cohorts and were well characterized asthma patients visiting our University Medical Center. Inclusion criteria were a doctor's diagnosis of asthma, a positive histamine provocation test in the past, and age >18 years. Main exclusion criteria were an FEV_1 <1.2 L, bronchiectases, upper respiratory tract infection (e.g. colds) and/or use of antibiotics or oral corticosteroids within 2 months, serious acute infections in the previous 3 months. Non-smokers did not currently smoke and had a maximum of 2 packyears. The medical ethics committee of the University Medical Center Groningen approved the study protocol

(Approval reference number METC2004.271). All subjects provided their written informed consent.

Design

Subjects visited our research facilities 4 times. If they used inhaled GC they first were asked to stop inhaled GC for four weeks prior to visit 1. At visit 1, they underwent lung function testing, blood collection and sputum induction. At least 1 week later we performed a challenge test with Adenosine-5'-MonoPhosphate (AMP) at visit 2. Exhaled NO was measured at least 1 week later prior to a bronchoscopy at visit 3. After a two-week course of oral methylprednisolone (40 mg/day), lung function was assessed at visit 4.

Lung Function

All measurements were performed by pulmonary technicians in the University Medical Center in Groningen using standardized protocols. FEV_1 was measured with a calibrated water-sealed spirometer according to guidelines [15]. Reversibility of FEV_1 (%predicted) was measured by inhaling 400 µg albuterol at visit 1 and 4. AMP provocation was performed using a two-minute inhalation procedure with a calibrated De Vilbiss nebulizer. FEV_1 was measured 30 and 90 seconds after each inhalation. Doubling concentrations of AMP, ranging from 0.04 to 320 mg/ml, were inhaled until a fall of 20% in FEV_1 (PC_{20} AMP) occurred or the highest dose of 320 mg/ml was reached. PC_{20} AMP was determined as described previously [14].

Sputum Induction and Sputum Processing

Sputum was induced by inhalation of nebulized hypertonic saline (5%) for 3 consecutive periods of 5 min. Whole sputum samples were processed as described previously [16]. May Grünwald Giemsa (MGG) staining was used to obtain cell differentials from in total 600 viable, non-squamous cells. Sputum was not scored if the percentage squamous cells was >80 percent or the total number of non-squamous cells was <600. Induced sputum was not obtained in 8 GC-unresponsive and 10 GC-responsive subjects, because they were unable to expectorate sufficient sputum meeting the standards of our protocol.

Blood

Blood differential counts were analysed by flow cytometry (Coulter-STKS; Beckman Coulter, Miami, FL, USA) in the routine hospital laboratory. Total serum IgE (IU/L) was measured by a solid-phase immunoassay (VIDAS total IgE kit, BioMérieux, Marcy l'Etoile, France). The Phadiatop screening test was used to determine atopic status and was performed on the ImmunoCap system according to the instructions of the

manufacturer (Phadia AB, Sweden). Results were presented as quotients (fluorescence of the serum of interest divided by the fluorescence of a control serum). Atopy was defined as patient serum/control serum >1.

Exhaled Nitric Oxide

Exhaled NO was measured on the Aerocrine NO system (Niox, Aerocrine AB, Stockholm, Sweden) in accordance with international guidelines [17]. Alveolar NO concentrations and bronchial NO fluxes were assessed according to Tsoukias & George [18] with some modifications [19]. Exhaled NO values of 3 GC-unresponsive and 4 GC-responsive subjects were not used because the calibration gas was not reliable during a short period of the study.

Collection, processing and immunohistochemical staining of bronchial biopsies

Bronchial biopsies were obtained under local anaesthesia using a flexible bronchoscope from segmental divisions of the main bronchi. The biopsies were fixed in 4% formalin, processed and embedded in paraffin. Bronchial biopsies were cut in 3µm thick sections.

The inflammatory profile was assessed with specific antibodies against eosinophilic peroxidase (EPX, laboratories of NA Lee and JJ Lee, Mayo Clinic, Scottsdale, USA), mast cell tryptase (AA1, DAKO, Glostrup, Denmark), macrophages (CD68, DAKO), neutrophil elastase (NP57, DAKO), T-lymphocytes (CD3 (DAKO), CD4 (Novocastra, Newcastle upon Tyne, UK) and CD8 (DAKO) and B-lymphocytes (CD20, DAKO). In short, sections were deparaffinised and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision" Detection Kit (DAKO) followed by the chromogen NovaRED (Vector Labs, Burlingame, USA). EPX and CD8 were detected using biotinylated anti-mouse IgG1 (Southern Biotechnology, Birmingham AL, USA) and alkaline phosphatase- (DAKO) or peroxidase-labelled streptavidin conjugates (DAKO) followed by permanent Red (DAKO) and NovaRED chromogens, respectively. All stainings for inflammatory cell markers were performed in an automated system using the DAKO autostainer and were manually counterstained with methylgreen. All stainings were quantified by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). Quantification was performed on the largest of three biopsy sections. Inflammatory cell numbers were quantified by counting the number of positively stained cells in the submucosal area 100µm under the basement membrane (BM), in a total area of 0.1mm^2 per biopsy sample. Epithelial layer integrity was assessed on HE-stained biopsy sections and expressed as the percentage of BM covered

with 1) normal, intact epithelium (basal and ciliated columnar epithelial cells) and 2) metaplastic epithelium (multi-layered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells). BM thickness was calculated based on computer-assisted measurements of BM surface area and BM length.

Statistics

Differences in continuous variables between groups were tested with Mann-Whitney U-test. Chi-square test was used to test differences in dichotomous variables between groups. To investigate which factors were associated with GC-unresponsiveness, we assigned subjects into two groups: GC-responsive and GC-unresponsive. Subjects with any increase in pre-bronchodilator FEV_1 (%predicted), i.e. >0% in response to a 2-week course of GC were assigned to the responder group and subjects without increase to the non-responder group. Correlations were described using Spearman's rank correlation coefficient. We performed linear regression analysis with the pre-bronchodilator FEV_1 response to GC as dependent variable and smoking status, age, gender, PC_{20} AMP, blood lymphocytes, neutrophil/eosinophil ratio in blood, sputum eosinophils and alveolar NO as independent variables entered simultaneously into the model. The model was corrected for FEV_1 at baseline. Since there existed a close relation between reversibility to albuterol and FEV_1 at baseline, reversibility to albuterol was excluded from the model. PC_{20} AMP, blood lymphocytes, sputum eosinophils, and NO values were transformed logarithmically to obtain normal distributions. Model assumptions were checked visually by inspection of the distribution of the residuals. P-values <0.05 were considered to be statistically significant (tested 2-sided). We used SPSS 14.0 for all statistical analyses.

Results

Demographic characteristics

The proportion of currently smoking subjects was significantly higher in the GC-unresponsive than in the GC-responsive group. Because of the distribution of smokers, the numbers of packyears and cigarettes/day were also significantly different between groups. Subjects who used inhaled GC prior to the study and had to stop this medication were equally distributed over both groups (Table 1).

Lung function

Overall, the median (range) pre-bronchodilator improvement in FEV_1% predicted after a 2-week course of methylprednisolone was –0.6 (-22.1-90.1) %. The median (range) value was: –3.5 (-22.1-0) % in the GC-unresponsive and +7.1 (0.5-90.1) % in the GC-responsive group. Table 1 demonstrates the differences between the GC-unresponsive

and GC-responsive subjects at baseline. Reversibility to albuterol (FEV_1 %predicted) was significantly lower in GC-unresponsive than in GC-responsive subjects.

Blood

GC-unresponsive subjects had more leukocytes in blood than GC-responsive subjects, which may be primarily due to higher numbers of both neutrophils and lymphocytes in this group (Table 2). Furthermore, a higher neutrophil/eosinophil ratio was significantly correlated with a lower response to GC ($r_{spearman}$=0.433, p<0.001) (Fig.2A).

Table 1. Subject characteristics at baseline

	GC-responsive (n=27)	**GC-unresponsive (n=33)**
Female, number (%)	19 (59)	22 (58)
Age, years	52 (22-67)	50 (19-70)
BMI, kg/m^2	26.8 (19.3-40.3)	27.0 (21.8-42.4)
Atopy, number (%)	21(78)	16 (53) p=0.054
Current-smoking, number (%)	**4 (15)**	**16 (49)***
Pack years of current-smokers	**15.7 (8.0-30.0)**	**23.5 (5.8-47.3)***
Cigarettes/day of current-smokers	**16 (5-20)**	**15 (7-25)***
Inhaled GC use prior to study	11 (41%)	10 (30%)
Dosis equivalent beclomethason	800 (28-2000)	900 (200-2000)
FEV_1 pre-bd, %pred	94.6 (53.5-110.1)	96.6 (67.4-118.0)
FEV_1/VC pre-bd, %	71.4 (40.5-94.4)	74.0 (50.0-89.6)
PEF pre-bd, L/s	7.02 (3.97-11.9)	7.96 (4.68-14.86)
MEF_{50} pre-bd, L/s	2.55 (0.96-5.03)	2.69 (1.00-5.55)
Reversibility, %pred	11.86 (-1.39-38.43)	7.56 (-2.23-23.27)*
PC_{20} AMP, mg/mL	32.27 (0.02-640)	155.79 (0.25-640)
Total IgE, IU/L	42.0 (0-1302.0)	43.0 (0-698.0)

Values are medians (ranges) or numbers (percentages), *: p≤0.05 vs. GC-responsive, bd: bronchodilator, AMP: Adenosine Mono-Phosphate, GC: glucocorticosteroid.

Table 2. Inflammation in blood, sputum and exhaled air

	GC-responsive	GC-unresponsive
Blood		
Leukocytes, 10^9/L	5.8 (3.5-9.7)	7.4 (4.1-14.2)*
Neutrophils, 10^9/L	3.14 (1.80-6.77)	4.28 (2.17-10.08)*
Eosinophils, 10^9/L	0.21 (0.08-1.16)	0.20 (0.01-0.51)
Basophils, 10^9/L	0.05 (0-0.22)	0.04 (0-0.90)
Lymphocytes, 10^9/L	1.82 (0.80-4.05)	2.48 (1.01-4.35)*
Monocytes, 10^9/L	0.28 (0-0.98)	0.24 (0-0.87)
Sputum		
Total cells, 10^5/mL	365.8 (188.6-1481.0)	450.8 (90.9-1800.0)
Neutrophils, %	44.3 (16.5-87.7)	58.2 (15.3-83.8)
Neutrophils, 10^3/mL	189.9 (35.2-734.7)	199.0 (18.3-1443.0)
Eosinophils, %	3.3 (0.2-65.8)	1.5 (0-16.7)
Eosinophils, 10^3/mL	12.4 (0.4-177.8)	4.7 (0-81.7)
Lymphocytes, %	0.7 (0-5.5)	0.3 (0-3.7)
Lymphocytes, 10^3/mL	2.8 (0-52.0)	1.0 (0-19.1)
Macrophages, %	30.5 (11.0-63.6)	35.2 (11.2-80.7)
Macrophages, 10^3/mL	106.6 (24.8-578.4)	114.1 (24.7-826.4)
ECP, ng/mL	50.9 (5.8-601.0)	21.0 (5.6-2467.0)*
Elastase, µg/mL	1.13 (0.23-5.0)	0.81 (0.19-5.0)
Exhaled air		
Alveolar NO, ppb	6.28 (0.88-51.7)	4.75 (2.33-18.34)*
Bronchial NO, nL/s	1.44 (0.19-10.38)	0.45 (0.06-3.17)*

Values are medians (ranges), *: p≤0.05 vs. GC-responsive, GC: glucocorticosteroid

Exhaled NO

Bronchial NO flux and alveolar NO concentration in exhaled air were both significantly lower in GC-unresponsive than GC-responsive subjects (Table 2). Lower GC-responses correlated significantly with lower bronchial NO fluxes and lower alveolar NO concentrations ($r_{spearman}$=0.42 (p=0.003) and 0.46 (p=0.001) respectively; Figure 1A & 1B, including stratification for smoking). Bronchial NO flux and alveolar NO concentration were both significantly lower in smoking than in non-smoking subjects: (median (range)) 0.26 (0.06-0.59) nL/s vs 1.33 (0.32-10.38) nL/s (p<0.001), and 3.44 (2.33-6.24) ppb vs 6.73 (3.57-51.72) ppb (p<0.001), respectively.

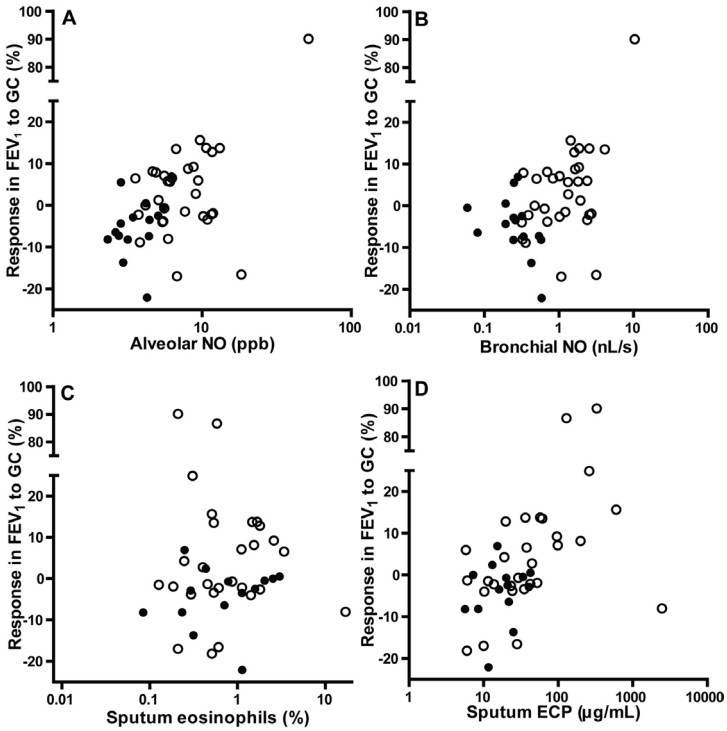

Figure 1. Scatter plots of GC-response (Y-axis) in relation to (A) alveolar NO concentration (ppb), (B) bronchial NO flux (nL/s), (C) sputum eosinophils (%), (D) sputum ECP concentration (ng/mL). Closed circles are smokers, open circles are non-smokers. GC response (%) correlated significantly with alveolar NO ($r_{spearman}$ = 0.46, p=0.001), bronchial NO ($r_{spearman}$ = 0.42, p=0.003), sputum eosinophils ($r_{spearman}$ = 0.36, p=0.019), sputum ECP ($r_{spearman}$ = 0.54, p<0.001). GC= Glucocorticosteroid.

Sputum

The total numbers and the proportions of the various inflammatory cells in sputum were not different between the two groups. However, the concentration of ECP was significantly lower in GC-unresponsive subjects than in GC-responsive subjects (Table 2). Lower GC-responses correlated significantly with lower percentages of sputum eosinophils and lower concentrations of ECP: $r_{spearman}$=0.36 (p=0.019) and $r_{spearman}$ = 0.54 (p<0.001) respectively (Figure 1C & 1D, including identification of smokers). A higher neutrophil/eosinophil ratio was significantly correlated with a lower response to GC ($r_{spearman}$=-0.33, p=0.041) (Fig.2B), as was the elastase-to-ECP ratio ($r_{spearman}$ =-0.42, p=0.005). Sputum eosinophil numbers were comparable in smokers and non-smokers.

Biopsies

Biopsies were obtained from 23 GC-responsive subjects and 20 GC-unresponsive subjects. No significant differences could be observed between GC-unresponsive and GC-responsive subjects. However, a trend was observed for a higher neutrophil/eosinophil ratio in GC-unresponsive subjects (p=0.071) (Table 3). Furthermore, a higher neutrophil/eosinophil ratio was significantly correlated with a lower response to GC: $r_{spearman}$ = -0.37 (p=0.01) (Fig.2D).

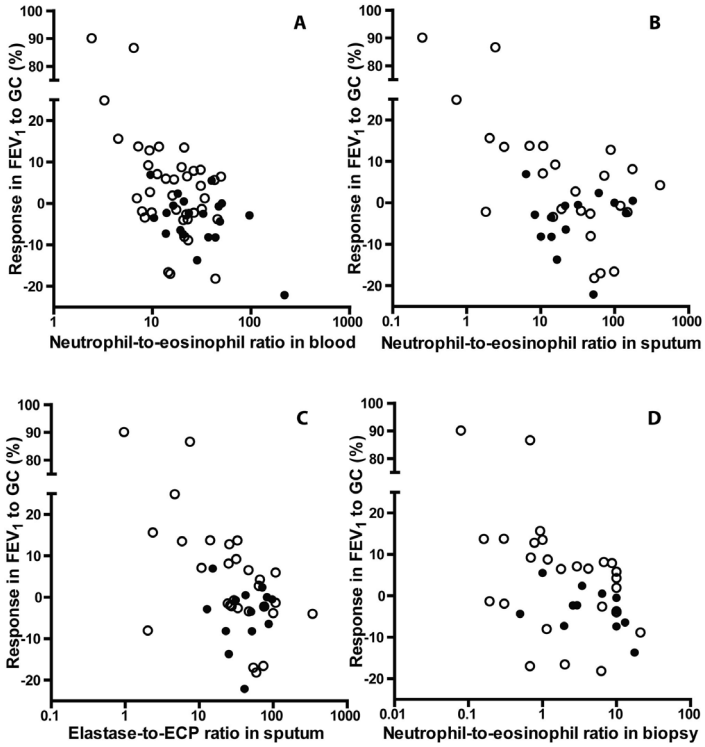

Figure 2. Scatter plots of GC-response (Y-axis) in relation to (A) neutrophil/eosinophil ratio in blood, (B) neutrophil/eosinophil ratio in sputum, (C) elastase-to-ECP ratio in sputum, (D) neutrophil/eosinophil ratio in biopsies. Closed circles are smokers, open circles are non-smokers. GC response (%) correlated significantly with neutrophil/eosinophil ratio in blood ($r_{spearman}$=-0.43, p=0.001), neutrophil/eosinophil ratio in sputum ($r_{spearman}$=-0.33, p=0.041), elastase-to-ECP ratio in sputum ($r_{spearman}$=-0.42, p=0.005), neutrophil/eosinophil ratio in biopsies ($r_{spearman}$=-0.37, p=0.014). GC= Glucocorticosteroid.

Multivariate regression analyses

Multiple linear regression analysis on GC-response provided a model with an R^2 of 0.71 ($p<0.001$). Lower alveolar NO, lower percentage of sputum eosinophils, older age, higher neutrophil/eosinophil ratio and numbers of lymphocytes in blood were all independently and significantly associated with a lower GC-response (Table 4). Current smoking and PC_{20} AMP did not affect the GC-response significantly in our model.

Table 3. biopsy data

	GC-responsive	GC-unresponsive
Inflammatory cells		
NP57$^+$ Neutrophils, cells/0.1mm^2	4.45 (0-19.81)	7.46 (0-46.02)
EPX$^+$ Eosinophils, cells/0.1mm^2	2.5 (0-25.6)	2.0 (0-25.8)
AAI$^+$ Mast cells, cells/0.1mm^2	7.46 (0-18.71)	11.14 (0-19.08)
CD3$^+$ T-Lymphocytes, cells/0.1mm^2	75.26 (21.31-216.19)	67.45 (18.02-294.20)
CD8$^+$ T-Lymphocytes, cells/0.1mm^2	18.33 (1.02-91.95)	24.09 (1.94-103.17)
CD68$^+$ Macrophages, cells/0.1mm^2	13.05 (0.31-57.01)	12.05 (0-37.15)
Neutrophil/eosinophil ratio	0.85 (0-8.67)	2.01 (0-21.12)*
Remodelling		
Goblet, n	35.16 (3.68-97.37)	36.36 (7.08-92.23)
Mucus, %	8.60 (1.49-26.44)	8.07 (0.94-32.67)
Epithelial Thickness, µm	18.85 (8.13-48.13)	20.15 (7.92-36.76)
Ki67$^+$, %intact	3.20 (0.97-13.61)	5.42 (0-15.50)
Ki67$^+$, %basal	3.43 (0.57-19.34)	5.45 (0.66-25.47)
Normal epithelium, %	3.87 (0-29.09)	10.14 (0-65.27)
Basal membrane thickness, µm	5.67 (3.06-12.55)	6.24 (3.80-9.04)

Values are medians (ranges), GC: glucocorticosteroid, *: p=0.071 vs GC-responsive asthma.

Table 4. Multivariate linear regression analysis on GC-response

	B	Standard Error	p-value
Log alveolar NO	17.987	5.513	**0.003**
Log blood lymphocytes	-13.808	4.498	**0.008**
Age	-0.431	0.196	**0.038**
Log % sputum eosinophils	4.855	2.327	**0.048**
Neutrophil/eosinophil ratio in blood	-0.143	0.069	**0.050**
Log PC_{20} AMP	2.089	1.184	0.091
Current-smoking	-9.414	7.079	0.197

R-squared: 0.71, $p<0.001$. Corrected for gender and FEV_1 %predicted at baseline.

Discussion

This study demonstrates that lower exhaled NO levels, lower sputum eosinophil numbers, higher neutrophil/eosinophil ratios in blood, higher blood lymphocyte numbers and older age were independent predictors of a poorer FEV_1 response to a 2-week course of oral glucocorticosteroids in subjects with mild-to-moderate asthma. Current smoking did not contribute independently to GC-unresponsiveness, in contrast with findings in the literature [8,20].

That we found lower exhaled NO values to be associated with GC-unresponsiveness can be due to a variety of mechanisms. First, lower exhaled NO values may reflect a lower degree of lung inflammation [21], thereby giving less room for improvement with anti-inflammatory treatment. Second, lower exhaled NO values may be present in a subset of patients with a different *type* of lung inflammation, i.e. in this case less response to the anti-inflammatory effects of methylprednisolone.

Generally, the neutrophil is seen as the least GC-sensitive and the eosinophil is seen as the most GC-sensitive inflammatory cell type. In our study, a higher percentage of eosinophils in sputum was significantly correlated with a better GC-response. Little et al [5] did not find a correlation between sputum eosinophils and GC response but demonstrated that sputum eosinophilia (≥4%) significantly predicts an increase in FEV_1 ≥15%. In contrast to our study, Little *et al* only included *non*-smoking asthma patients with a lower mean FEV_1 %predicted of 76%, whereas sputum was processed using the plug method [16,22]. Our results are more in line with the study of Meijer et al [23], who included both smoking and non-smoking subjects and demonstrated higher numbers of sputum eosinophils to be associated with GC response. A drawback of their study was that effects of inhaled GC and oral prednisolone were analysed together. Overall, we conclude that our data is in line with the literature showing that a low percentage of eosinophils in sputum associates with a low response to GC.

We observed a significant correlation between a higher neutrophil/eosinophil ratio in biopsies and decreased GC-response. In another biopsy study Chakir *et a*l [13] did not observe baseline differences between oral GC-sensitive and insensitive subjects, similar to our study. However, they did not determine the ratio of neutrophils to eosinophils. Furthermore, the studies are not easy to compare because Chakir investigated a lower number of subjects, who had moderate-to-severe asthma while our subjects had mild-to-moderate asthma. A strong point of our study is that association of a higher neutrophil/eosinophil ratio with a lower GC-response was also present in both sputum and blood. We regard this as evidence of a shift away from the GC-responsive eosinophilic inflammation to the more GC-unresponsive neutrophilic inflammation.

Our results show that a higher number of blood lymphocytes is associated with a lower response to GC. Unfortunately, we do not know which lymphocyte subtype is responsible for this higher number of blood lymphocytes in our study population. Based on findings in the literature we can speculate about this. First, CD8+ T-lymphocytes tend to respond less to GC than CD4+ T-lymphocytes [24,25]. Second, within CD4+ T-lymphocyte subsets Th1 and Th17 are associated with a lower response to GC [26,27], whereas Th2 lymphocytes respond well to GC [28]. Therefore, we postulate that the association between higher blood lymphocyte counts and lower response to GC can be due to an increase in CD8+, Th1, or Th17-lymphocytes, but not an increase in Th2-lymphocytes.

Interestingly, older age was also associated with GC-unresponsiveness. Older asthmatics generally have a higher prevalence of irreversible airway obstruction, which may be due to airway remodelling and parenchymal changes [29]. Such processes may reflect accumulation of on-going airway inflammation and repetitive exposures to e.g. cigarette smoke, occupational irritants and microbial agents. In our study epithelial airway remodelling parameters did not differ between GC-sensitive and GC-insensitive asthmatics. However, other parts of the airway wall and surrounding lung tissue may contribute more extensively to airway wall stiffness. Another explanation for the observation that older age is associated with GC-unresponsiveness may be that aging has profound effects on the immune system, e.g. elderly individuals have increased neutrophil counts in broncho-alveolar lavage fluid, cells known to be less responsive to GC [30]. In our study, older age was not associated with changes in inflammatory cell types in blood, sputum or biopsies. Finally, older subjects are more likely to have co-morbidities, e.g. subclinical heart failure, which can limit expiratory flow and does not likely respond to GC [31].

To our surprise, current smoking was not independently associated with GC-unresponsiveness when tested in the multivariate regression analysis. Nevertheless, this does not rule out that smoking affects GC-responsiveness. First, the number of current-smokers was significantly higher in the GC-unresponsive group in the univariate analysis, in line with earlier reports [7,8]. Second, cigarette smoking has been associated with lower numbers of sputum eosinophils and exhaled NO levels [10,12,21,32,33]. In the present study lower sputum eosinophil counts and exhaled NO levels were significantly associated with less GC-responsiveness. Consequently, smoking subjects have a low GC-response, low exhaled NO and low sputum eosinophils. Together these data suggest that smoking changes the inflammatory profile into one that is not responsive to GCs, and is not directly responsible for the observed GC-unresponsiveness itself.

A potential drawback of our study is the inclusion of relatively mild-to-moderate asthmatic subjects. GC-responsiveness may not be easy to measure in this subgroup of asthmatics due to a low degree of airway inflammation and a ceiling effect for GC-responsiveness in airway patency. However, we believe it is important to study GC-unresponsiveness, particularly in mild-to-moderate asthmatics because of the high prevalence of this disease stage and its relatively high GC use [34]. In the past a frequently used cut-off value for GC responsiveness in (severe) asthma was a 15% improvement in $FEV_{1;}$ but as only 4 subjects in our study showed a response larger than 15%, we decided to present our results on basis of a more realistic cut-off value (0%). Furthermore, we decided to perform linear regression instead of logistic regression analysis, in order to take the presence of some "high"-responders on GC into account. Indeed, GC-unresponsiveness is generally assumed to be a characteristic of severe asthma, but our study shows that this may also occur in milder disease states and with mild airway inflammation. A second potential drawback of our study is the lack of a placebo control in our study. We did not deem the placebo control essential in our study, since we aimed to describe the predictive value of inflammatory parameters on GC-responsiveness. We feel safe to investigate this in a non- placebo controlled study, because we only investigated objective variables as both dependent and independent variables.

Do our results imply that our group of GC-unresponsive asthmatics should not receive inhaled or oral GC at all? We believe this is not necessarily the case. Even if FEV_1 does not improve, GC may have beneficial effects on other parameters relevant to asthma like airway wall inflammation, remodelling and bronchial hyperresponsiveness [35-38]. Symptoms may change when treated with GC even without a change in FEV_1 and exacerbations can be prevented through treatment with GC. Treatment of asthmatics should not solely be based on FEV_1 but on other important clinical outcome variables as well [39].

Conclusion

We conclude that GC-unresponsiveness in mild-to-moderate asthmatics is associated with lower exhaled NO values, lower sputum eosinophil numbers, higher neutrophil/eosinophil ratios in blood, higher blood lymphocytes, and higher age. To our surprise, smoking did not affect GC-responsiveness independently, but our data suggests that smoking appears to inhibit GC-responsiveness by changing the type of inflammation.

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Chapter 3

Short Communication: Smoking and inhaled corticosteroid use are independently associated with higher histone deacetylase-2 expression in bronchial epithelial cells in asthma

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Manuscript in preparation

Corticosteroids, the most effective therapy available for asthma, suppress inflammatory genes by inhibiting histone acetyltransferase (HAT) and in particular by recruiting histone deacetylase-2 (HDAC-2) to the nuclear factor-κB-activated inflammatory gene complex[1]. The epithelium is a major site of inflammatory gene expression and a main localization site for HDAC-2 expression within the airway wall, and therefore target for (inhaled) corticosteroid action[2]. Examination of complete bronchial biopsies from non-smoking mild/moderate persistent asthmatics treated with budesonide revealed higher HDAC-2 levels compared to non-smoking mild intermittent asthmatics untreated with budesonide[2].

Cigarette smoke has been shown to down-regulate expression and activity of HDAC-2 in bronchial biopsies and alveolar macrophages from young healthy smokers.[3] Reduced HDAC-2 expression was suggested as one of the underlying mechanisms for reduced corticosteroid responsiveness in COPD, severe asthma and smoking asthma.[1] However, HDAC-2 expression data in bronchial epithelial cells in severe asthma did not support this hypothesis[4;5]. Moreover, a recent study unexpectedly demonstrated that bronchial epithelial cells from COPD patients and healthy smokers have *higher* HDAC-2 expression than healthy non-smokers[6]. Until now, HDAC-2 expression of bronchial epithelial cells has not been investigated in smoking asthmatics. Because epithelial cells constitute the first barrier affected by smoking, we compared epithelial HDAC-2 expression in smoking and non-smoking asthmatics, taking into account inhaled corticosteroids (ICS) use.

ICS= inhaled corticosteroid, Atopy is based on a positive phadiatop, FEV_1= forced expiratory volume in 1 s (FEV_1 was measured after inhalation of 800 µg Albuterol), Reversibility FEV_1= change in FEV_1, expressed as increase in percentage predicted normal value after 400 µg of Albuterol, PC_{20} AMP (mg/ml)= provocative concentration of adenosine 5'-monophosphate causing a 20% fall in $_{FEV1.}$ *= non-smoking ICS use vs. smoking ICS use $p<0.05$, #= smoking non-ICS use vs. non-smoking non-ICS use $p<0.05$, $= non-smoking ICS use vs. non-smoking non-ICS use $p<0.05$, †= smoking non-ICS use vs. non-smoking ICS use $p<0.05$, ‡= smoking non-ICS use vs. smoking ICS use $p<0.05$, ∞= smoking ICS use vs. non-smoking non-ICS use $p<0.05$.

Endobronchial biopsies from 96 non-smoking asthmatics and 27 current smoking asthmatics (Table 1) were immunostained for HDAC-2. The percentage of HDAC-2 nucleus positive cells was determined in the intact epithelium (Figure 1) and compared between asthmatics with and without ICS treatment.

Table 1. Characteristics of asthma patients

Characteristics	Non-smoking non-ICS use (50 cases)	Smoking non-ICS use (19 cases)	Non-smoking ICS use (46 cases)	Smoking ICS use (8 cases)
Female sex (%)	26 (52%)	5 (35.7%)	24 (52.2%)	4 (50%)
Age (yr)	47 (25-70)	52 (19-64)	50.5 (19-71)*	36.5 (24-64)
Packyears (yr)	0 (0-44.6)	25.6 (1.4-44)#,†,‡	0.2 (0-63.8)*,§	5.6 (0.4-33.6)¤
Cigarettes/day (n)	0 (0-18)	15 (3-23)	0 (0-0)	10 (3-14)
ICS dose (µg/day) beclomethasone equivalent	--	--	800 (28-2000)	650 (200-1000)
Atopy (%)	33 (66%)	13 (68.4%)	35 (76.1%)	6 (75%)
FEV_1 (% pred)	104 (77.9-127.8)	94.6 (59.8-134.5)	96.3 (42.5-135.5)§	101.4 (81.3-108.6)
FEV_1/VC (%)	78.2 (56.4-97.7)	71.9 (47.6-93.6)	72.5 (39.4-96.7)§	75.9 (54.9-84.4)
Reversibility (% pred)	6.2 (-1.4-28.7)	9.2 (-2.2-17.8)	7.5 (-0.8-38.4)	10.5 (4.6-25.6)
Log PC_{20} AMP	2.8 (-1.7-2.8)	2.3 (-1.7-2.8)	2.3 (-2.6-2.8)	0.9 (0.6-1.5)*,‡,¤

Values are medians (ranges) or numbers (proportions).

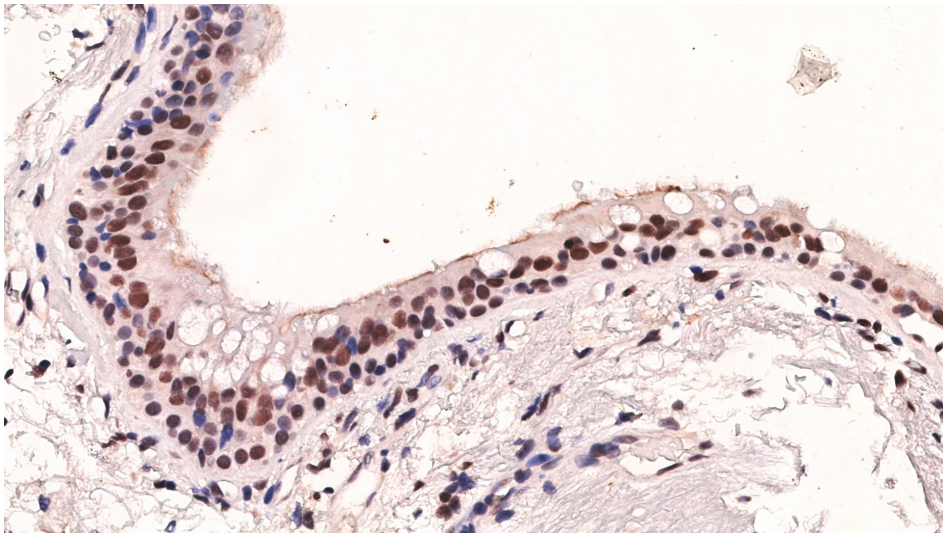

Figure 1. Expression of HDAC-2 in a representative bronchial biopsy from a non-smoking asthmatic on ICS use.

Our results show that current smoking is associated with higher HDAC-2 expression in the epithelium in asthmatics not treated with ICS (Figure 2). ICS-use is associated with higher HDAC-2 expression in non-smoking asthmatics, but we did not observe this in currently smoking asthmatics. Linear regression analysis confirmed that smoking and ICS-use contribute independently to HDAC-2 expression (B: 14.47, 95% CI: 8.21–20.73 and B: 10.86, 95% CI: 6.12–15.61 respectively). Smoking interacted *negatively* with ICS-use (B: -19.62, 95% CI: -30.50– -8.74).

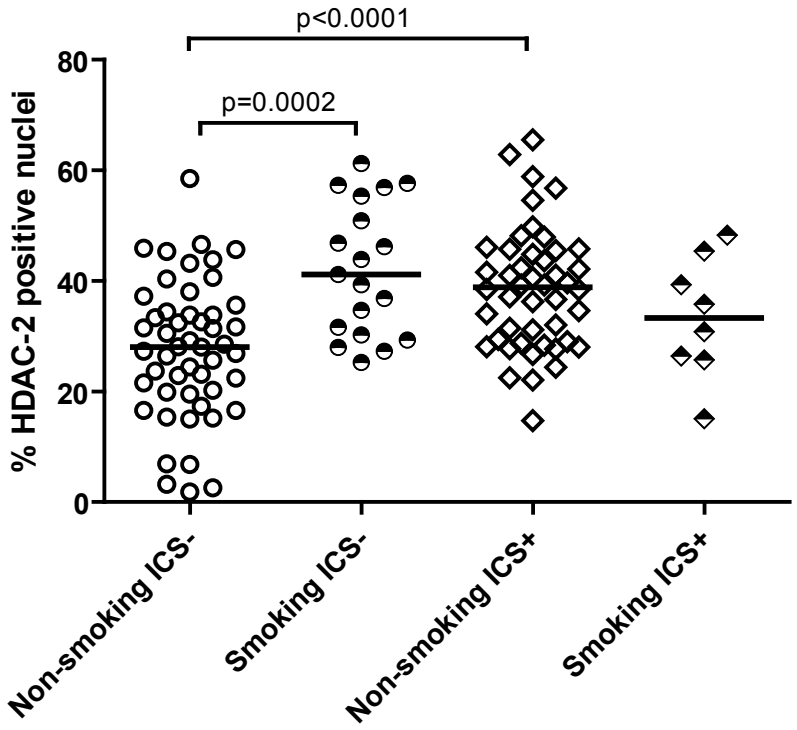

Figure 2. Percentage of HDAC-2 nucleus positive cells in intact epithelium.

Our study for the first time demonstrated that HDAC-2 expression is higher in epithelial cells from smoking asthmatics. This is compatible with recent findings in epithelial cells from healthy smokers and COPD patients[6], but incompatible with findings in alveolar macrophages from healthy smokers[3] and COPD patients[7]. Together, these observations suggest that effects of smoking on HDAC-2 expression may vary between compartments. It is intriguing that smoking might lead to higher HDAC-2 expression in epithelial cells, suggesting an anti-inflammatory effect in that compartment.

Additionally, HDAC-2 expression was higher in bronchial epithelial cells from non-smoking asthmatics using ICS, in line with previous findings.[2] We did not observe this beneficial effect of ICS on HDAC-2 expression in smoking asthmatics, reflecting a possible mechanism for smoking-induced corticosteroid unresponsiveness. However, we cannot draw a definitive conclusion because we did not investigate the balance between HAT and HDAC-2, nor their activity. Additionally, we performed an observational study, not randomizing for corticosteroids or smoking. Therefore, further studies on smoking-induced corticosteroid unresponsiveness in asthma need to confirm our preliminary results.

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Chapter 4

Interleukin-17A induces glucocorticoid insensitivity in human bronchial epithelial cells

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ABSTRACT

A subset of asthma patients suffer from glucocorticoid (GC) insensitivity. T-helper cell type 17 cells have an emerging role in GC insensitivity, although the mechanisms are still poorly understood.

We investigated whether interleukin (IL)-17A induces GC insensitivity in airway epithelium by studying its effects on responsiveness of tumour necrosis factor (TNF)-a-induced IL-8 production to budesonide in human bronchial epithelial 16HBE cells. We unravelled the underlying mechanism by the use of specific pathway inhibitors, reporter and overexpression constructs and a histone deacetylase (HDAC) activity assay.

We demonstrated that IL-17A-induced IL-8 production is normally sensitive to GCs, while IL-17A pre-treatment significantly reduced the sensitivity of TNF-a-induced IL-8 production to budesonide. IL-17A activated the p38, extracellular signal-related kinase (ERK) and phosphoinositide-3-kinase (PI3K) pathways, and the latter appeared to be involved in IL-17A-induced GC insensitivity. Furthermore, IL-17A reduced HDAC activity, and overexpression of HDAC2 reversed IL-17A-induced GC insensitivity. In contrast, IL-17A did not affect budesonide-induced transcriptional activity of the GC receptor, suggesting that IL-17A does not impair the actions of the ligated GC receptor.

In conclusion, we have shown for the first time that IL-17A induces GC insensitivity in airway epithelium, which is probably mediated by PI3K activation and subsequent reduction of HDAC2 activity. Thus, blockade of IL-17A or downstream signalling molecule PI3K may offer new strategies for therapeutic intervention in GC-insensitive asthma.

Key words

Airway epithelial cell, airway inflammation, glucocorticosteroids, interleukin-17

Introduction

Asthma is an obstructive lung disorder, characterised by airway hyperreactivity, airway remodelling and invasion of inflammatory cells, *e.g.* eosinophils, mast cells and T-helper (Th) cells. Inhaled glucocorticoids (GCs) are currently the most effective anti-inflammatory treatment for asthma. However, a subset of asthmatic subjects is relatively insensitive to this treatment [1, 2].

GCs exert a broad spectrum of anti-inflammatory effects upon binding to their receptor (GR). The ligated receptor translocates to the nucleus and suppresses pro-inflammatory gene transcription by recruitment of histone deacetylases (HDACs). HDAC2, in particular, is able to induce deacetylation of histones containing inflammatory genes, thereby restricting access of the transcriptional machinery to these genes and inhibiting transcription [3]. In addition to the recruitment of HDACs, GCs are able to exert anti-inflammatory effects through activation of GC response elements (GREs), which are present in the promoter of several anti-inflammatory genes, inducing their transcription.

Reduced sensitivity to GCs has been clinically associated with neutrophilic airway inflammation [4, 5], but it is still largely unclear which cellular and molecular mechanisms contribute to this GC insensitivity. Th17 lymphocytes have an emerging role in the induction of neutrophilic airway inflammation [6]. Moreover, Th17-induced neutrophilic airway inflammation in mice was reported to be GC insensitive [7]. Th17 cells act by producing various inflammatory cytokines, including interleukin (IL)-17A, IL-17F, IL-21 and IL-22, which act locally to induce pro-inflammatory transcription in tissue cells, example IL-8 [6]. Interestingly, IL-17A is known to enhance chemotaxis of neutrophils by inducing IL-8 release in bronchial epithelial cells [8] and airway smooth muscle cells [9]. Despite these novel insights, it is still unknown why GCs are unable to efficiently suppress Th17-mediated inflammation. Although neutrophils are considered relatively unresponsive to GCs, the production of chemokines involved in neutrophilic infiltration, *e.g.* IL-8 by airway epithelium, can be suppressed by GCs [10].

Therefore, we hypothesise that Th17-mediated GC-insensitive inflammation is not mediated by direct induction of GC-insensitive neutrophil chemoattractant production, but by IL-17A inducing epigenetic changes diminishing the possibilities for GCs to inhibit cytokine production. In the present study, we demonstrate that IL-17A significantly reduces GC responsiveness in the human bronchial epithelial cell line 16HBE, which is probably mediated by activation of phosphoinositide-3-kinase (PI3K) signalling and subsequent reduction in HDAC2 activity.

METHODS

Cell culture

16HBE14o-human bronchial epithelial cells (16HBE; kindly provided by D.C. Gruenert, University of California, San Francisco, CA, USA) were cultured in flasks coated with 30 μg-mL^{-1} collagen and 10 μg-mL^{-1} bovine serum albumin in Eagle's minimal essential medium (EMEM; Lonza, Walkersville, MD, USA) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA) and supplemented with 100 μg-mL^{-1} penicillin and 100 μg-mL^{-1} streptomycin (Biowhittaker, Verviers, Belgium), as previously described [11].

Stimulation of the cells

Cells were seeded in duplicates at a concentration of 10^5 cells-mL^{-1} in 24-well plates, grown to ,90% confluence and serum-deprived overnight. For measurement of IL-8 levels, cells were pre-treated for 2 h with budesonide (BUD; AstraZeneca, Lund, Sweden) in concentrations ranging from 10^{-11} to 10^{-9} M, washed and subsequently stimulated with/without 10 ng-mL^{-1} IL-17A (R&D Systems, Abingdon, UK) or 10 ng-mL^{-1} tumour necrosis factor (TNF)-a (Sigma, St. Louis, MO, USA) for 24 h. Alternatively, IL-17A was added 2 h prior to BUD treatment, in the presence and absence of specific inhibitors of extracellular signal-related kinase (ERK) (U0126, 10 μM), p38 (SB203580, 1 μM) and PI3K (LY294002, 10 μM) pathways, which were added 30 min prior to IL-17A incubation. All inhibitors were purchased from Tocris Bioscience (Bristol, UK). For measurements of GRE-mediated transcription, cells were incubated for 2 h with 10 ng-mL^{-1} IL-17A and subsequently incubated with BUD in a concentration ranging from 10^{-9} to 10^{-7} M.

Measurement of IL-8 production

Cell-free supernatants were harvested 24 h after stimulation with IL-17A or TNF-a. IL-8 production was measured by ELISA (R&D Systems) according to the manufacturer's instructions.

Immunodetection

Upon stimulation with/without IL-17A (10 ng/mL^{-1}) for 5– 120 min, total cell lysates were obtained by resuspension of cells in sample buffer (2% sodium dodecyl sulphate (SDS), 10% glycerol, 2% 2-mercapto-ethanol, 60 mM Tris-HCl (pH 6.8) and bromophenol blue) and boiling for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. Immunodetection was performed with anti-phospho-p38, anti-phospho-ERK, anti-phospho-Akt (Cell Signaling Technology, Hitchin, UK) and pan-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), as previously described [11].

HDAC activity

Cells were stimulated for 2 h with 10 ng/mL^{-1} IL-17A, resuspended in radio-immunoprecipitation assay buffer containing 150 mM NaCl, 1% nonyl phenoxylpolyethoxylethanol-40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris pH 8.0, and sonicated to obtain total cell lysates. Protein levels were determined using a BCA kit (Thermo Scientific, Rockford, IL, USA). HDAC activity was assessed by a fluorometric HDAC activity assay (BioVision, Mountainview, CA, USA) performed according to the manufacturer's instructions.

Transfection with GRE-luciferase construct

The GRE-luciferase construct was kindly provided by S.A. Asgeirsdottir (University Medical Center Groningen, Groningen, the Netherlands). The thymidine kinase-driven renilla luciferase vector (pRL-TK; Clonetech, Paris, France) was used as an internal control. 16HBE cells were grown to 80–90% confluence in EMEM containing 10% FCS. For GRE promoter activity measurements, cells were transfected with OptiMEM (Invitrogen, Carlsbad, CA, USA), 1 µL-mL^{-1} Lipofectamine™ 2000 (Invitrogen), 1,000 ng-mL^{-1} of GRE-luciferase and 100 ng/mL^{-1} of renilla luciferase construct. After 20 h, cells were washed and stimulated with the indicated amounts of BUD for 24 h. Next, cells were lysed in 50 mL passive lysis buffer (Promega, Madison, WI, USA) and subjected to freezing/thawing. Firefly and renilla luciferase activities were determined using the dual-luciferase reporter assay kit (Promega) on a Luminoskan Ascent microplate luminometer (Thermo Scientific) according to manufacturer's instructions. Results were normalised by dividing the firefly luciferase activity with the renilla luciferase activity of the same sample.

Transfection with HDAC2 construct

Cells were grown to 80–90% confluence in EMEM containing 10% FCS, transfected overnight in OptiMEM with 1 mL-mL^{-1} lipofectamine and 1,000 ng-mL^{-1} of a pcDNA3.1-HDAC2 construct (kindly provided by K. Ito, Imperial College London, London, UK) or an empty vector. After transfection, stimulations were performed as described before.

Statistical analysis

We tested for normal distribution with the Shapiro–Wilks normality test and observed that TNF-a-induced IL-8 production by 16HBE cells was normally distributed. We used the t-test for paired observations to test for statistical significance and the ANOVA with Dunnett's post-test when we compared different concentrations.

RESULTS

IL-17A-induced IL-8 production in 16HBE cells is normally sensitive to BUD

First, we tested whether IL-17A induced IL-8 production in 16HBE cells. We observed that treatment with increasing doses of IL-17A (24 h) induced an approximate two-fold increase in IL-8 production (fig. 1a). To explore the possibility that IL-17A induced IL-8 production through a GC-insensitive pathway, we tested whether IL-17A-induced IL-8 production was sensitive to BUD. We observed that BUD dose-dependently decreased IL-17A-induced IL-8 production by 16HBE cells, resulting in a reduction of 49.6±7.2% at a concentration of 10^{-9} M (fig. 1b). 10^{-9} M BUD reduced TNF-a-induced IL-8 production to a similar extent, *e.g.* by 46.7±7.1% (fig. 2), indicating that IL-17A-induced IL-8 production in bronchial epithelium is normally sensitive to GCs.

IL-17A induces GC insensitivity of TNF-a-induced IL-8 production

Next, we aimed to determine whether IL-17A was able to induce GC insensitivity of TNF-a-induced IL-8 production in 16HBE cells and we assessed the effect of pre-treatment with 10 ng-mL^{-1} IL-17A prior to the addition of BUD. 2 h pre-treatment with IL-17A did not affect baseline or TNF-a-induced IL-8 levels (fig. 2), but importantly, it significantly reduced the inhibitory effect of 3×10^{-10} M BUD from 34.1±6.7% to 12.5±5.8% (p50.006, n54) (fig. 2). Furthermore, we observed that pre-treatment with IL-17 increased the mean inhibitory concentration of BUD on TNF-a-induced IL-8 production from 5.20×10^{-10} to 1.15×10^{-9} M (n54, p,0.05; data not shown). This indicates that IL-17A pre-treatment reduces sensitivity of TNF-a-induced IL-8 production to BUD. Increasing the period of incubation (up to 6 h) or altering the concentration of IL-17A (1–100 ng-mL^{-1}) did not further affect GC sensitivity (data not shown).

IL-17A induces phosphorylation of ERK, p38 and Akt

To unravel the molecular mechanisms involved in the reduced responsiveness of IL-8 production to BUD upon IL-17A treatment, we first studied whether IL-17A was able to activate PI3K/Akt, mitogen activated protein kinase (MAPK)/ERK kinase (MEK)/ERK-1/2 and p38 MAPK signalling pathways in 16HBE cells. Immunodetection revealed that IL-17A induced phosphorylation of Akt, ERK-1/2 and p38 in 16HBE cells, with a peak between 5 and 20 min (fig. 3a). Analysis by densitometry revealed that levels of phosphorylated p-Akt, p-ERK and p-p38 were significantly increased upon 5 min of incubation with IL-17 (fig. 3b–d).

Inhibition of PI3K abrogates IL-17A-induced GC insensitivity

Next, we aimed to determine whether activation of these pathways was involved in IL-17A-induced GC insensitivity. We used the specific inhibitors LY294002, U0126 and

SB203580 to inhibit the PI3K, MEK/ERK and p38 pathways, respectively. Notably, IL-17A-induced GC insensitivity was, at least partially, reversed upon addition of the PI3K inhibitor LY294002, resulting in stronger inhibition of IL-8 production by BUD in the presence of IL-17A (from 1.8±9.0% to 24.9±6.6%, p50.04). In contrast, the effect of IL-17A on GC sensitivity was not significantly affected by the presence of U1026 and SB203580 (fig. 3e). These data indicate that PI3K, but not ERK and p38, activity contributes to the effect of IL-17A on GC sensitivity.

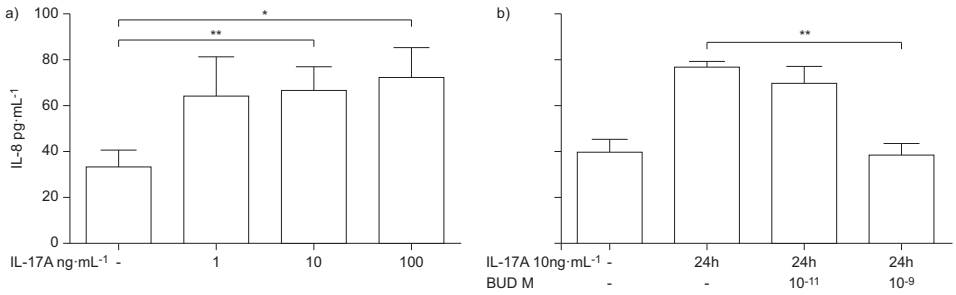

Figure 1. a) Interleukin (IL)-17A induced IL-8 production in human bronchial epithelium cells (16HBE) cells. Cells were serum-deprived overnight, washed and treated for 24 h with the indicated amounts of IL-17A. b) IL-17A-induced IL-8 production is dose-dependently inhibited by budesonide (BUD) in 16HBE cells. Cells were pre-treated with control medium, 10^{-11} M BUD or 10^{-9} M BUD for 2 h, washed and treated for 24 h with 10 ng·mL^{-1} IL-17A. Absolute±SEM values of IL-8 are shown in pg·mL^{-1} (n53).
*: p,0.05; **: p,0.01 between the indicated values.

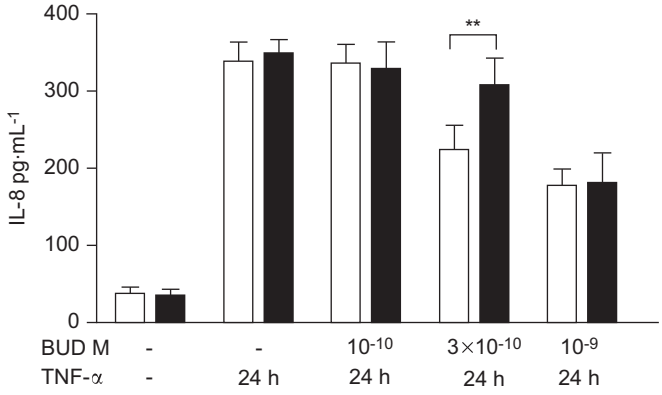

Figure 2. Inhibition of tumour necrosis factor (TNF)-a-induced interleukin (IL)-8 production by budesonide (BUD) is impaired by pre-incubation with IL-17A. Cells were serum-deprived overnight, pre-treated with 10 ng·mL^{-1} IL-17A (&) or control medium (h) for 2 h, washed, treated for 2 h with control medium or BUD, washed, and finally incubated for 24 h with 10 ng·mL^{-1} TNF-a. Absolute±SEM values of IL-8 are shown in pg·mL^{-1} (n54). **: p,0.01 between the indicated values.

Reduced HDAC activity may be involved in IL-17A-induced GC insensitivity

Since PI3K has been implicated in the phosphorylation of HDAC2, leading to its inactivation and a subsequent reduction in GC sensitivity [12], we were interested to see whether IL-17A can reduce HDAC activity in bronchial epithelium. We analysed HDAC activity upon 2 h of incubation with or without IL-17A. Importantly, IL-17A significantly reduced HDAC activity compared with untreated cells (fig. 4a). In contrast to HDAC activity, IL-17 did not significantly affect total protein expression of HDAC2 (data not shown).

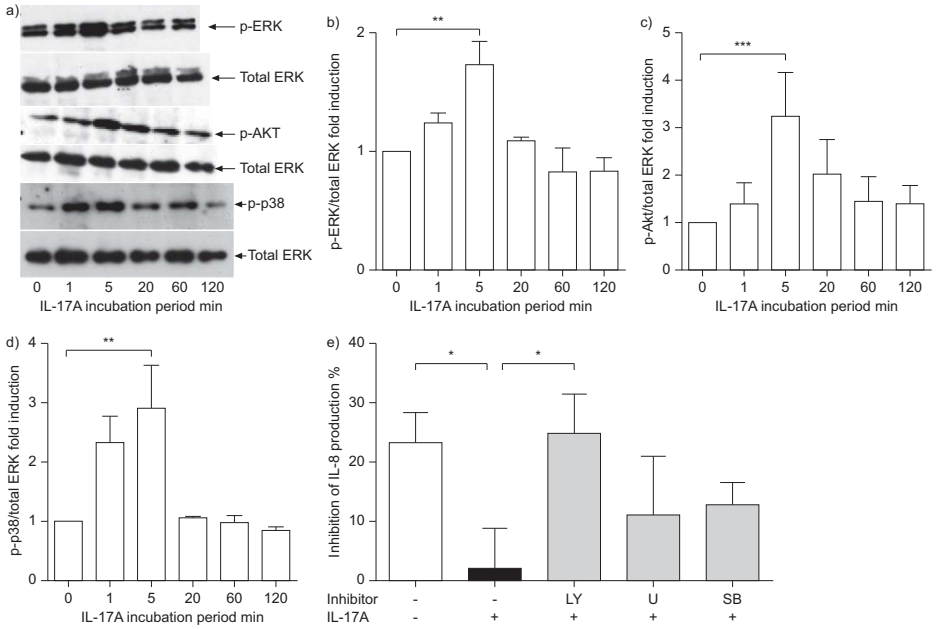

Figure 3. a) Interleukin (IL)-17A induced phosphorylation of extracellular signal related kinase (ERK), p38 and Akt. Cells were serum-deprived overnight. IL-17A was added for the indicated period. Phosphorylation (p) of ERK, p38 and Akt was assessed by immunodetection. Representative blots of four independent experiments are shown. Analysis by densitometry of immunodetection of b) phosphorylated ERK (p-ERK), c) phosphorylated Akt (p-Akt) and d) phosphorylated p38 (p-p38) (n54). e) An inhibitor of phosphoinositide-3-kinase (PI3K) negates the effects of IL-17A on inhibition of IL-8 production by budesonide (BUD). 30 min prior to IL-17A treatment, the PI3K, ERK and p38 inhibitors were added: LY294002 (LY; 10 mM), U0126 (U; 10 mM) and SB203580 (SB; 1 mM), respectively. 10 ng?mL^{-1} of IL-17A was added and cells were incubated for 2 h, washed and incubated for 2 h with 3610^{-10} M BUD, washed and then incubated for 24 h with 10 ng?mL^{-1} tumour necrosis factor (TNF)-a. Cell-free supernatants were harvested and IL-8 was determined with ELISA. Data are shown as %± SEM of inhibition by BUD of corresponding TNF-a stimulated control (n54).

*: p,0.05; **: p,0.01; ***: p,0.001.

Since HDAC2 has previously been described as the predominant HDAC involved in GC action, we were interested to further underscore the role of HDAC2 in IL-17A-induced GC insensitivity and we studied the effect of IL-17A on GC insensitivity upon overexpression of HDAC2. In cells expressing the control vector, BUD (3×10^{-10} M) inhibited TNF-a-induced IL-8 production by 14.2±4.3%. Pre-treatment with IL-17A significantly reduced this effect to 0.6±5.4%. In contrast, IL-17A was not able to reduce the effect of BUD in cells overexpressing HDAC2, as BUD still reduced TNF-a-induced IL-8 production by 11.1±3.2% (fig. 4b). Overexpression of HDAC1 and HDAC3 did not result in a significant abrogation of the IL-17-mediated effect (see online supplementary fig. 1). These data further support the role for reduced HDAC2 activity in IL-17A-induced GC insensitivity.

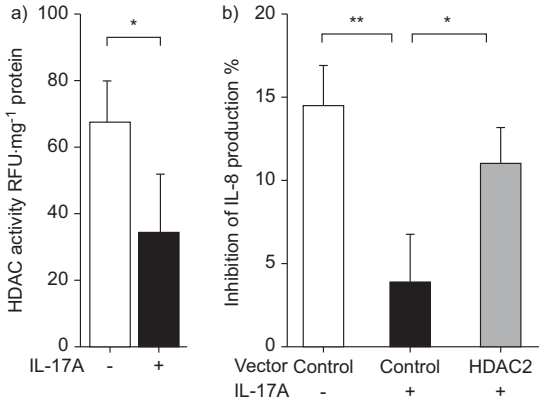

Figure 4. a) Interleukin (IL)-17A decreased histone deacetylase (HDAC) activity. Cells were serum-deprived overnight, treated for 2 h with and without 10 ng·mL^{-1} IL-17A, resuspended in radio-immunoprecipitation assay buffer and sonicated. HDAC activity was measured using a fluorometric activity assay. HDAC activity is shown as a ratio of the fluorescent units (RFU) per mg of protein (n54). b) Effect of HDAC2 overexpression on IL-17A-induced glucocorticoid insensitivity. Cells were grown to 90–95% confluence before transfection with control vector or HDAC2 overexpression. Cells were then pre-treated with 10 ng·mL^{-1} IL-17A or control medium for 2 h, washed, treated for 2 h with control medium or budesonide (BUD), washed, and finally incubated for 24 h with 10 ng·mL^{-1} tumour necrosis factor (TNF)-a. Cell-free supernatants were harvested and IL-8 was determined with ELISA. Data are shown as %±SEM of inhibition by BUD of corresponding TNF-a stimulated control (n58). *: p,0.05; **: p,0.01.

IL-17A has no effect on GRE-mediated transcription

Another mechanism that has been implicated in cytokineinduced GC insensitivity is the reduced translocation of the ligated GR [13]. Nuclear translocation of the ligated GR is required for GCs to exert their suppressive effect on transcription pro-inflammatory genes as well as on transcription of antiinflammatory genes that contain a GRE in their promoter [3, 13]. To test whether IL-17A affects transcriptional activity of the GR, we determined the effect of IL-17A treatment on a GRE reporter construct in 16HBE

cells. Our data demonstrated that BUD induced a dose-dependent increase in GRE transcriptional activity, which reached an approximate seven-fold increase at 3×10^{-8} M BUD over basal GRE transactivation (p,0.001). Pretreatment with IL-17A did not affect BUD-induced activity of the GRE promoter at any of the used concentrations (fig. 5). This indicates that IL-17A does not decrease GRE transcriptional activity or translocation of the ligated receptor within 2 h of incubation, the time frame in which IL-17A induced GC sensitivity. Therefore, IL-17A-induced GC insensitivity is not likely to be caused by a decrease in translocation of the ligated receptor, but the reduced HDAC activity appears to be the main contributor to this effect.

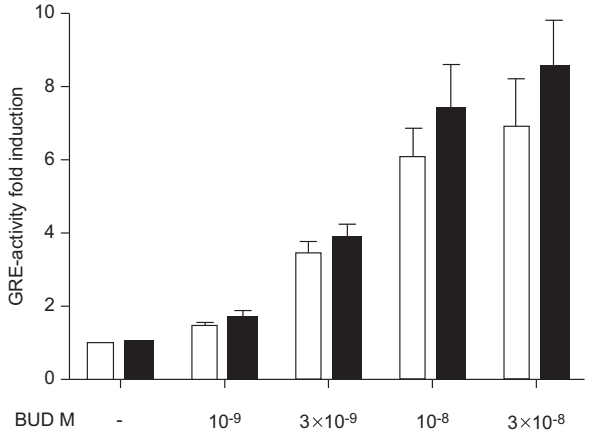

Figure 5. Interleukin (IL)-17A has no effect on glucocorticoid response element (GRE)-mediated transcription. Cells were grown to 90–95% confluence before transfection with GRE-driven firefly luciferase with renilla luciferase as a control for transfection efficiency. Cells were pre-treated for 2 h with (&) or without (h) 10 ng·mL^{-1} IL-17A, treated for 24 h with budesonide (BUD) (concentrations 10^{-9}–3×10^{-8} M) and resuspended in lysis buffer. Activity was assessed for both luciferases. Data is shown as fold induction±SEM over control (n54).

DISCUSSION

There is increasing evidence that Th17 cells play a role in the development of GC-insensitive airway inflammation in asthma, although it is still poorly understood why Th17-dependent inflammation is less responsive to GCs. Here, we have shown that IL-17A induced GC insensitivity of TNF-a-induced IL-8 production in human bronchial epithelial cells. Furthermore, our data demonstrate that IL-17A-induced IL-8 production is normally sensitive to GCs and that IL-17A does not induce GC-insensitive transcription, as previously suggested [8, 14]. We are the first to show that IL-17A-induced GC insensitivity is mediated by PI3K downstream signalling and a reduction

in HDAC activity. As a result, this leads to impaired ability of GCs to efficiently suppress IL-8 production upon stimulation with pro-inflammatory cytokines (*e.g.* TNF-α). In contrast to PI3K downstream signalling, the MEK/ERK, p38 MAPK pathways and/or decreased GR translocation to the nucleus do not seem to play a role in IL-17A-induced GC insensitivity in bronchial epithelial cells.

Although inhaled GCs are currently the most effective anti-inflammatory treatment of asthma, a subset of patients suffer from a difficult-to-treat and relatively GC-insensitive asthma, which is a burdensome problem in the management of asthma.

The mechanisms underlying the poor response in this subset of patients are still largely unknown. Previous studies have shown a link between neutrophilic inflammation and GC insensitivity in asthma [4, 5] as well as between neutrophilic inflammation and the presence of Th17 cells in the airways [6]. This led to the hypothesis that Th17-mediated inflammation related to GC-insensitivity. In line with this hypothesis, MCKINLEY et al. [7] showed that the transfer of Th17 cells in mice results in GC-insensitive airway inflammation and airway hyperresponsiveness when compared with the inflammation induced upon the transfer of Th2 cells. Thus, Th17 cells have an emerging role in GC-insensitive neutrophilic inflammation, although the underlying mechanism of GC insensitivity has remained unknown so far.

Our results demonstrate that Th17-induced GC insensitivity is not mediated by activation of GC-insensitive pathways by IL-17A. Similar effects were observed by PRAUSE *et al.* [15], showing that IL-17A-induced production of IL-8, as well as granulocyte chemotactic protein-2 and growth-related oncogene-α, in 16HBE cells is sensitive to hydrocortisone. In contrast to our findings and those of PRAUSE *et al.* [15], JONES *et al.* [8] showed that IL-17A-induced IL-8 production is insensitive to dexamethasone in primary bronchial epithelial cells. One of the dissimilarities is that JONES *et al.* [8] seem to have conducted experiments under subconfluent conditions. However, a relationship between cell-cell contacts and the actions of GCs has, to our knowledge, not been described. Another important difference is that JONES *et al.* [8] performed their experiments in primary epithelial cells, while the study by PRAUSE *et al.* [15] and our study used cell lines. In future studies we plan to compare the effects in cell lines to primary cells, although it may be of concern that the GC hydrocortisone is customarily present in the culture medium for primary cells.

We do not consider it probable that IL-17A plays a major role in the release of IL-8 in the airways and subsequent development of neutrophilia, since IL-17A was relatively inefficient in inducing IL-8 production when compared with the pro-inflammatory cytokine, TNF-α. Thus, we hypothesised that IL-17A exerts a different role in GC sensitivity, reducing the

responsiveness of IL-8 transcription to GCs.

In line with this hypothesis, we showed that the pre-incubation with IL-17A reduced the capacity of GCs to inhibit TNF-α-induced IL-8 production. Thus, the presence of IL-17A secreting cells in the airways may render airway epithelial production of IL-8 less responsive to GCs. It is of importance to note that we did not observe an effect on the inhibitory effect of BUD at high concentrations. One of the explanations for the lack of effect on the maximum inhibition of BUD is that IL-17A did not affect GR binding to the GRE region. Possibly, the inhibitory effect of GR binding to the IL-8 promoter predominates over the effect of HDAC2 on IL-8 production at higher concentrations of GCs. In addition to our novel findings on the role of IL-17A in GC insensitivity, earlier studies concerning cytokine-induced GC insensitivity have shown possible roles for different cytokines in GC insensitivity, *i.e.* IL-2 in combination with IL-4 in T-cells [16], IL-13 in monocytes [17], TNF-α in combination with interferon (IFN)-γ in airway smooth muscle cells [18] and IL-27 in combination with IFN-γ in macrophages [19]. However, none of these cytokines can explain the association between Th17 cells and GC insensitivity, and clinical data on the association between these cytokines and GC insensitivity in asthma is rather scarce. Since IL-17A induces GC insensitivity of IL-8 production in epithelial cells, we postulate that IL-17A itself plays an important role in the development of GC-insensitive neutrophilic inflammation.

We showed that IL-17A-induced PI3K/Akt signalling is involved in the observed effects on GC sensitivity. IL-17A has previously been shown to activate PI3K signalling [20]. Furthermore, PI3K activation has been implicated in H_2O_2-and cigarette smoke-induced GC insensitivity in A549 cells, *i.e.* by a mechanism involving HDAC2 phosphorylation and subsequent inactivation [12]. In line with this, we found that IL-17A activated the PI3K downstream signalling and decreased overall HDAC activity. Specific overexpression of HDAC2 abrogated IL-17A-induced GC insensitivity, strongly suggesting that PI3K activation and subsequent HDAC2 inactivation play a role in IL-17A-induced GC insensitivity.

In addition to the PI3K-signaling pathway, IL-17A has been described to activate the MEK/ERK signalling in A549 cells and primary tracheal cells [21, 22], and both the p38 MAPK and the MEK/ERK signalling in 16HBE cells [23]. We confirmed this in our cells. Activation of the MEK/ERK and p38 pathways has been shown to induce phosphorylation of the GR and decrease its translocation to the nucleus causing GC insensitivity in various cell types [13, 24, 25]. However, our data do not support a role for the MEK/ERK and p38 MAPK pathways in IL-17A-induced GC insensitivity in airway epithelial cells, since specific inhibition of these pathways could not block the IL-17A-

induced effects. Moreover, GRE-mediated transcription was not impaired upon IL-17A exposure in airway epithelium, suggesting that IL-17A-induced MAPK activity does not result in altered translocation of the ligated receptor.

Previous studies in peripheral blood mononuclear cells and broncholaveolar lavage (BAL) fluid of asthmatic patients have suggested that GC insensitivity in asthma patients is associated with increased GR-b [26, 27], which acts as a negative competitor to the active variant GR-a. In line with these findings, VAZQUEZ-TELLO *et al.* [14] recently demonstrated that IL-17A induced GR-b. However, this probably does not contribute to the effect observed in our study, since we observed the strongest effect of IL-17A on GC sensitivity within 2 h of pre-incubation, whereas VAZQUEZ-TELLO *et al.* [14] showed that IL-17A does not induce GR-b mRNA until after 6 h in primary bronchial epithelial cells. Moreover, if GR-b does play a role in our model, we would expect an effect on the transactivation of the GRE reporter construct due to the competitive nature of this mechanism. Since we did not observe such an effect, we do not render it probable that GR-b plays a role in our experiments.

Together with VAZQUEZ-TELLO *et al*. [14], we are the first to describe mechanisms of GC insensitivity in bronchial epithelial cells. Previous studies on GC insensitivity have mainly focused on leukocytes. However, bronchial epithelial cells have an emerging role in the innate immune response and we speculate that changes in sensitivity to GCs in airway epithelial cells strongly contribute to GC insensitivity of inflammatory responses in the lung.

Cigarette smoking has been associated with a poor response to GCs in asthma [28] and smoking asthmatics displayed increased levels of sputum neutrophils [29]. Interestingly, IL-17A is increased in the BAL of a mouse model of cigarette smoke-induced inflammation [30, 31], and therefore it is tempting to speculate that Th17 cells also play a role in smoking-induced GC insensitivity. Strikingly, similar mechanisms have been proposed in smoking-induced GC insensitivity, *i.e.* PI3K-dependent HDAC2 inactivation [32]. Therefore, it will be of interest to study whether cigarette smoke-induced GC insensitivity is (partly) mediated by IL-17A in future studies.

Given our novel results on the role of PI3K in GC insensitivity, we propose that inhibition of the PI3K pathway may be a promising tool to improve GC function and revert GC insensitivity. In macrophages it has been demonstrated that theophylline treatment restores response to GCs by its inhibitory effect on PI3K activity [33]. Therefore, we anticipate that treatment with theophylline might serve as a strategy to revert GC insensitivity associated with IL-17A, especially given the fact that inhibition of PI3K was

also able to restore GC sensitivity in our study. In asthma as well as chronic obstructive pulmonary disease, clinical pilot studies have been performed on the effect of theophylline combined with inhaled GCs [34, 35]. Although these first data show some improvement in lung function, the authors of both studies also stress the need for more extensive investigation into this possible treatment, because both studies were relatively small and single centred. No *in vivo* and clinical data are available on the action of theophylline on Th17-mediated inflammation and the associated steroid insensitivity in severe asthma, thus further studies are warranted.

In conclusion, we have demonstrated that IL-17A is able to induce GC insensitivity in bronchial epithelial cells by activating the PI3K pathway, which may lead to a decrease in HDAC2 activity. Therefore, we propose that therapeutic strategies to inhibit PI3K, as well as therapies focused on downregulating Th17 activity and secretion of IL-17A, may lead to novel ways to improve the efficacy of GCs.

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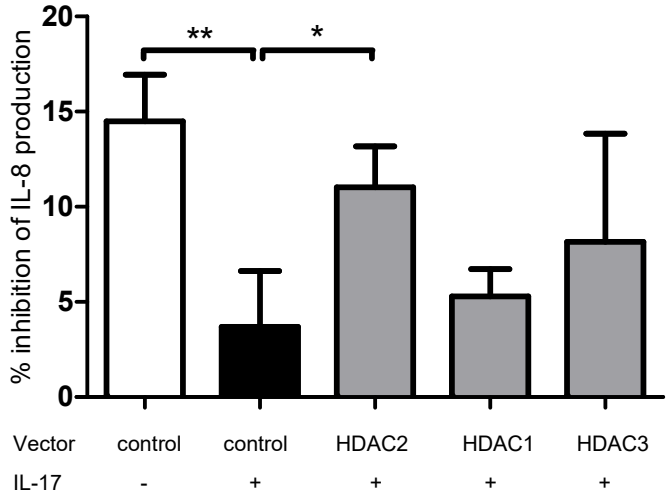
Supplementary information



Effect of HDAC overexpression on IL-17Ainduced GC-insensitivity. Cells were grown to 90-95% confluency before transfection with control vector or HDAC1, HDAC2 or HDAC3 overexpression (grey bar). Cells were then pretreated with 10 ng/ml IL-17A (black bar) or control medium (white bar) for 2h, washed, treated for 2h with control medium or budesonide, washed, and finally incubated for 24h with 10 ng/ml TNF-α. Cell-free supernatants were harvested and IL-8 was determined with ELISA. Data are shown as percentage± SEM of inhibition by budesonide of corresponding TNF-α stimulated control (n=48) *=p<0.05; **=p<0.01.

Chapter 5

Glucocorticoids induce the production of the chemo-attractant CCL20 in airway epithelium

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European Respiratory Journal (2014); 44; 361-370

Abstract

Th17-mediated neutrophilic airway inflammation has been implicated in decreased response to glucocorticoids (GC) in asthma. We aimed to investigate the effect of GCs on the airway epithelial release of the neutrophilic and Th17-cell chemo-attractant CCL20.

We studied CCL20 and CXCL8 sputum levels in asthmatic subjects using inhaled GCs or not, and the effect of budesonide (BUD) on CCL20 and CXCL8 production in primary bronchial epithelial cells. The mechanism behind the effect of BUD-induced CCL20 production was studied in 16HBE cells using inhibitors for the GC receptor (GR), intracellular pathways, and metalloproteases.

We observed higher levels of CCL20, but not CXCL8, in sputum of asthmatics who used inhaled GCs. CCL20 levels correlated with inhaled GC dose and sputum neutrophils. BUD increased TNF-α-induced CCL20 by primary bronchial epithelium, while CXCL8 was suppressed. In 16HBE cells, similar effects were observed at the CCL20 protein and mRNA level, indicating transcriptional regulation. Although TNF-α-induced CCL20 release was dependent on the ERK, p38 and STAT3 pathways, the increase by BUD was not. Inhibition of GR or ADAM17 abrogated the BUD-induced increase in CCL20 levels.

We show that GCs enhance CCL20 production by bronchial epithelium, which may constitute a novel mechanism in Th17-mediated GC-insensitive inflammation in asthma.

Introduction

Asthma is a chronic obstructive airway disease affecting millions of people worldwide, characterized by airway hyperresponsiveness, remodelling and inflammation, the latter predominantly characterised by eosinophils and Th2 cells. Inhaled Glucocorticoids (GCs) are currently the cornerstone of asthma treatment due to their broad anti-inflammatory effects, including the suppressive effect on chemokine production by structural airway cells. Despite this, a subset of asthmatic subjects is relatively insensitive to GC treatment. This insensitivity has been associated with a neutrophilic type of airway inflammation (1), which is thought to play a prominent role in acute exacerbations and chronic severe asthma (2).

Recently, neutrophilic airway infiltration has been associated with T lymphocytes of the Th17 subset (3). Th17 cells specifically secrete cytokines from the IL-17 family, although they are not the only source of these cytokines, which act on the airway epithelium to induce the secretion of pro-inflammatory cytokines (e.g. CXCL8, GM-CSF and CCL20) that recruit neutrophils to the site of inflammation (4-6). Interestingly, it has recently been demonstrated in mice that passive transfer of Th17 cells and subsequent airway challenge induces GC-insensitive neutrophilic airway inflammation and hyperresponsiveness (7). Despite these novel insights, it is still unknown how Th17-mediated inflammation develops and why GCs are unable to efficiently suppress Th17-mediated neutrophilic airway inflammation. Neutrophils are relatively insensitive to GCs, however, the production of their chemo-attractants, including CXCL8, by airway epithelium is GC-sensitive (8).

In addition to CXCL8, chemoattraction of neutrophils as well as Th17 cells can be induced by CCL20 (9). CCL20 acts on CCR6, which is expressed on memory T cells, predominantly of the Th17 subtype, on a subset of neutrophils and on dendritic cells (DCs) (10). Airway epithelium is a major producer of CCL20 (11). Interestingly, increased CCL20 levels have been observed in asthma patients, with a further increase upon allergen challenge (12). In addition, severe asthma patients displayed higher CCL20 levels in sputum than non-severe asthma patients, which was associated with higher neutrophil counts (13). Moreover, increased levels of CCL20 mRNA have been observed in bronchoalveolar lavage fluid of GC-insensitive asthmatic subjects (14). However, it is still unknown if and how airway epithelial CCL20 production is regulated by GC.

In this study, we were interested to assess whether the epithelial release of CCL20 is sensitive to GCs. We investigated CCL20 levels in sputum from asthmatics using inhaled GCs or not, as well as the release of CCL20 by primary bronchial epithelial

cells from asthma patients upon treatment with GCs *in vitro*. Interestingly, we found that CCL20 levels were higher in the sputum of inhaled GCs using subjects and that GCs increased the release of CCL20 by primary bronchial epithelial cells, instead of inhibited. Therefore, we further unravelled the mechanism of CCL20 upregulation by GCs in the bronchial epithelial cell line 16HBE.

Material & Methods

Subjects

Samples from 89 asthmatic individuals were included in a cross-sectional, observational study and classified by the use of inhaled GCs, rendering a group of 50 subjects using inhaled GCs and a group of 39 subjects who did not use GCs. See Table 1 for clinical characteristics.

Primary bronchial epithelial cells were obtained from bronchial brushings from 4 asthmatic patients and 4 healthy subjects. All subjects were non-smokers (< 10 packyears, no smoking in the last year) and between 18-65 years. Asthma patients were free of other lung diseases and included on basis of the presence of allergy (either by skin test or Phadiatop), FEV_1 > 80% predicted, documented bronchial hyperresponsiveness defined as either a PC_{20} AMP <320 mg/ml or a PC_{20} methacholine < 8 mg/ml or a PC_{20} histamine < 8 mg/ml.

The Medical Ethics Committee of the University Medical Center Groningen approved the study and signed informed consent was given to participate.

Sputum induction and processing

Sputum was induced by inhalation of nebulized hypertonic saline (5%) for 3 consecutive periods of 5 min. Whole sputum samples were processed as described previously (15).

Cell culture and stimulation

Primary bronchial epithelial cells and human bronchial epithelial 16HBE14o- cells (16HBE; kindly provided by dr. D.C. Gruenert, University of California, San Francisco) were cultured in hormonally-supplemented bronchial epithelium growth medium (BEGM, Lonza, Walkersville, MD) containing bovine pituitary extract, EGF, epinephrine, hydrocortisone, retinoic acid and triiodothyronine or in EMEM/10%FCS and collagen/fibronectin- or collagen-coated flasks respectively, as previously described (16). Primary cells were used for experimentation in passage 3. Cells were seeded in duplicates at a concentration of 10^5 cells/mL in 24-well plates, grown to ~90%

confluence and hormonally- or serum-deprived (16HBE) overnight, pre-treated for 2 hours with BUD (AstraZeneca, Lund, Sweden) in concentrations ranging from 10^{-10} to 10^{-7} M and subsequently stimulated with/without 10 ng/mL TNF-α (Sigma, St. Louis, MO), upon 60 min of pre-incubation with/without specific inhibitors for the ERK (U0126, 10 μM), p38 (SB203580, 1 μM), STAT3 (S3I-201, 100 μM) and PI3K (LY294002, 10 μM) pathways, GC receptor inhibitor (RU486, 1 μM), general metalloprotease inhibitor (TAPI-2 (Calbiochem, Omnilabo International BV, Breda, The Netherlands, 20 μM), ADAM10/17-inhibitor (GW280264X, 10 μM) or ADAM10-inhibitor (GI254023X, 1 μM) prior to BUD treatment. GW280264X and GI254023X were kindly provided by GlaxoSmithKline. Unless stated otherwise, inhibitors were purchased from Tocris Bioscience (Bristol, UK).

Air-liquid interface (ALI) culture

Normal human bronchial epithelial cells (NHBE, Lonza) were grown on semi-permeable collagen/fibronectin-coated membranes in a 1:1 mixture of DMEM (Lonza) and BEGM supplemented with retinoic acid (RA, 15 ng/ml; Sigma) and exposed to an air–liquid interface (ALI) for 4 weeks as described previously (17). At day 14 of air-exposure, cells were placed submerged in growth-factor deprived medium overnight and subsequently treated with 10^{-8} M BUD for 24 hrs. Cell-free supernatants were harvested from the apical side.

Methods for cytokine measurements and real-time RT-PCR are described in the online data supplement.

Statistics

We used the Student's t-test for paired observations for differences between condition within the cell experiments, the Mann-Whitney U test for differences in continuous data between subject groups, and Chi-square test for differences in ordinal data between groups. Spearman's rho test was used for analysis of correlations in patient groups. When analyzing the correlation with GC dose, only subjects using GC were tested.

Results

Higher sputum levels of CCL20 in asthma patients using inhaled GCs than in patients who do not.

First, we tested CCL20 levels in sputum from asthmatic individuals using inhaled GCs and those who do not use inhaled GCs. Both groups had similar disease severity as ascertained by clinical parameters (Table 1). Importantly, we observed significantly

higher levels of CCL20 in the sputum of asthma patients using inhaled GCs compared to the subjects who do not (Fig. 1A), while CXCL8 levels were not different (Fig.1B). Within the group of subjects using inhaled GCs, we observed a significant correlation between the dose of inhaled GCs and the level of CCL20 in the sputum samples ($r_{spearman}$=0.28, p=0.04, supplementary fig. 1A). Moreover, CCL20 levels in sputum correlated to the number of neutrophils in sputum ($r_{spearman}$=0.34, p=0.01, supplementary fig. 1B), although the numbers of sputum neutrophils did not differ between asthma patients using inhaled GC and those who did not (Table I). As expected, sputum CXCL8 levels correlated significantly with sputum neutrophils ($r_{spearman}$=0.24, p=0.03, supplementary fig. 1D).

Table 1. Subject characteristics

	Not using inhaled GC (n=39)	**Using inhaled GC (n=50)**
Females (%)	19 (48)	25 (50)
Age, years	50 (24-70)	51 (22-71)
Atopy, number (%)	26(68)	36 (75)
Current-smoking, number (%)	17 (43)	9 (18)*
Pack years	8.4 (0-47.3)	0.2 (0-63.8)
FEV_1 pre-bd, L	2.9 (1.6-5.9)	2.7 (1.4-4.5)
FEV_1 pre-bd, %pred	90.3 (53.9-113.7)	86.3 (51.6-128.7)
FEV_1/VC pre-bd, %	69.6 (45.8-89.5)	66.5 (40.3-94.4)
PEF pre-bd, L/s	7.2 (4.7-14.9)	7.9 (4.0-14.2)
MEF_{50} pre-bd, L/s	2.4 (1.0-5.6)	2.3 (0.6-5.3)
Reversibility, %pred	10.0 (-2.2-33.2)	9.5 (1.5-38.4)
PC_{20} AMP, mg/mL	78.7 (0.02->640)	51.5 (0.01->640)
Total IgE, IU/L	45 (0-604)	2 (0-1668)
Blood Eosinophils, 10^9/L	0.20 (0.01-0.51)	0.20 (0.06-1.16)
Sputum Eosinophils, %	1.2 (0-7.3)	0.8 (0-65.8)
Sputum Neutrophils, %	53.0 (11.8-87.7)	54.0 (15.3-90.0)
Alveolar NO, ppb	5.57 (2.13-18.34)	5.63 (1.49-51.72)
Bronchial NO, nL/s	0.64 (0.06-3.17)	0.89 (0.20-10.38)
Control according to GOAL criteria	24 (72)	24 (51)

Values are medians (ranges) or numbers (percentages), *: p ≤0.05 vs. not using inhaled GC, bd: bronchodilator, AMP: Adenosine Mono-Phosphate, GC= glucocorticoids.

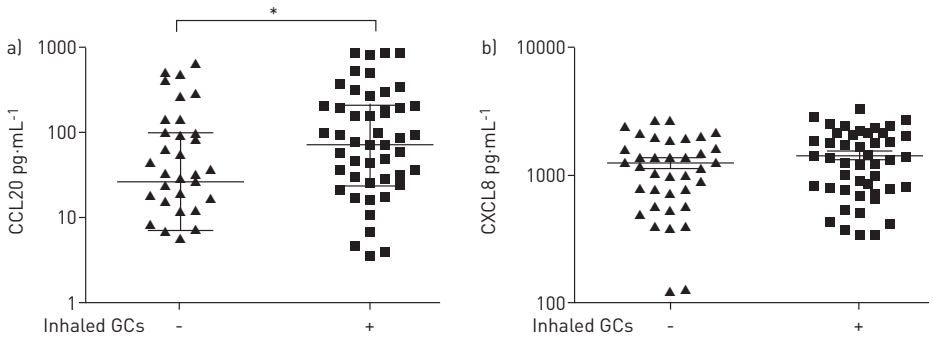

Figure 1. CCL20 levels are significantly higher in sputum of asthmatics using inhaled glucocorticoids (GCs, n=39) than in those who do not (n=50) (A), while IL-8 levels are similar between groups (B). Levels of CCL20 (ng/ml) and IL-8 (ng/ml) were measured in induced sputum by ELISA. Median ± IQR is indicated, *=p<0.05

Glucocorticoids increase CCL20 release in primary bronchial epithelial cells

Next, we examined whether the GC budesonide (BUD) regulates CCL20 secretion by primary bronchial epithelial cells from asthma patients. We used TNF-α as a relevant cytokine to induce a pro-inflammatory response. TNF-α significantly increased CCL20 and CXCL8 secretion (Fig. 2A & B). BUD significantly inhibited TNF-α-induced CXCL8 secretion (Fig. 2B). In striking contrast, the TNF-α-induced secretion of CCL20 was significantly increased upon treatment with BUD (Fig. 2A). In addition, we observed that BUD induced a significant increase of baseline CCL20 levels and BUD significantly enhanced the house dust mite (HDM)-induced CCL20 secretion (Fig. 2C). BUD did not significantly decrease levels of CXCL8, probably due to a lack of power (Fig. 2D). Similar effects were observed in bronchial epithelial cells from healthy controls (Fig 2E and 2F) with no significant differences between asthma patients and healthy controls.

To increase the relevance of our findings, we also studied the effect of BUD on CCL20 secretion in primary human bronchial epithelial cells cultured at ALI to induce mucociliary differentiation, reflecting the *in vivo* situation better. Again, treatment with BUD (10^{-8}M, 24 hours, significantly increased CCL20 levels (Fig. 2G), while CXCL8 levels were not affected (Fig. 2H).

Mechanisms of GC-induced CCL20 secretion in 16HBE cells

To further elucidate the underlying mechanisms of GC-induced CCL20 upregulation in airway epithelium, we used the human bronchial epithelial cell line 16HBE due to the limited numbers of primary cells. In these cells, TNF-α also induced a significant increase in CCL20 secretion, which was again further upregulated by BUD (Fig.3A),

while CXCL8 secretion was strongly reduced (Fig. 3B). Furthermore, BUD induced a significant increase in baseline levels of CCL20 (data not shown). To determine whether the increased CCL20 secretion by BUD was mediated by GR activation, we used the competitive GR antagonist, mifepristone (RU486) and found that the presence of RU486 completely prevented the BUD-induced increase in CCL20 secretion (Fig.3C). Next, we studied whether CCL20 was regulated at the transcriptional level, and we observed that BUD was able to increase CCL20 mRNA levels (Fig. 3D).

TNF-α induced CCL20 production is dependent on ERK, p38 and STAT3

Since CCL20 was regulated by BUD at the transcriptional level, we aimed to further unravel the signal transduction pathways involved in these effects. Since the STAT3, ERK and p38 pathways have been implicated in CCL20 transcription as well as in

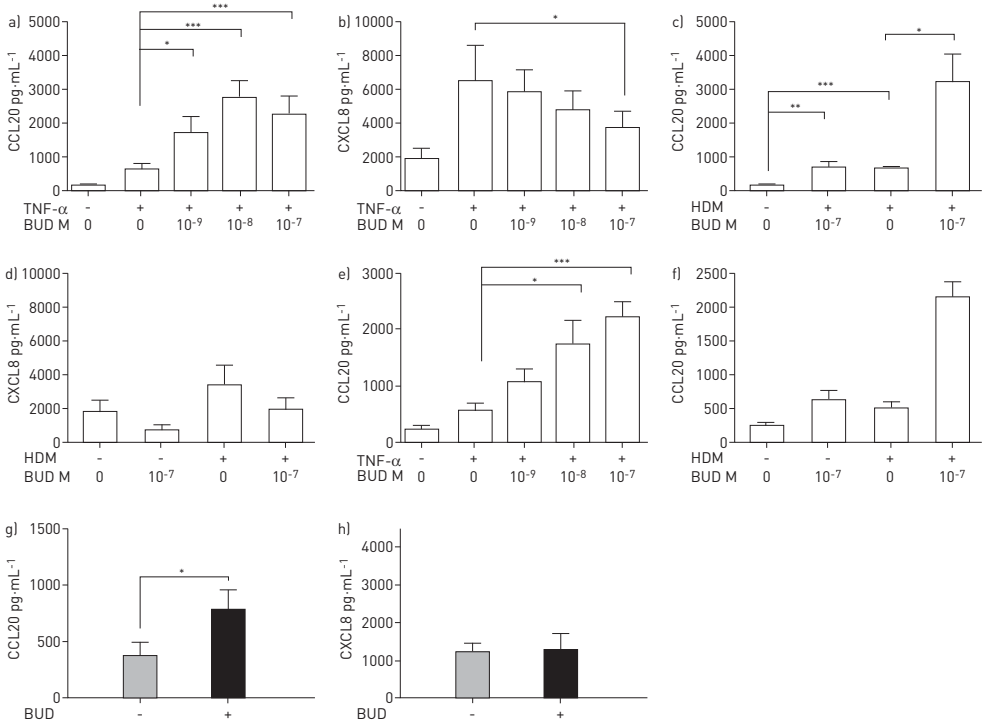

Figure 2. BUD enhances the TNF-α and house dust mite (HDM)-induced CCL20 release, but suppress the TNF-α induced IL-8 release in primary bronchial epithelial cells from asthma patients (A-D) and healthy donors (E, F). Cells were obtained from 4 donors per group. Cells were pre-treated for 2 hours with or without BUD (10^{-7}-10^{-10}M) and left unstimulated or stimulated for 24 hours with 10 ng/ml TNF-α (A,B,E) or 50 µg/ml HDM (C,D,F). In panel G and H, cells were first grown at air-liquid interface (ALI) culture for 2 weeks. CCL20 (A,C,E,F,G) and CXCL8 (B,D,H) levels were measured in cell-free supernatants (pg/ml) from the apical side after treatment with (grey bars) our without (black bars) BUD (10^{-8}) for 24 hours. Mean ± SEM (n=4) levels are shown, *=p<0.05, **=p<0.01 and ***=p<0.001.

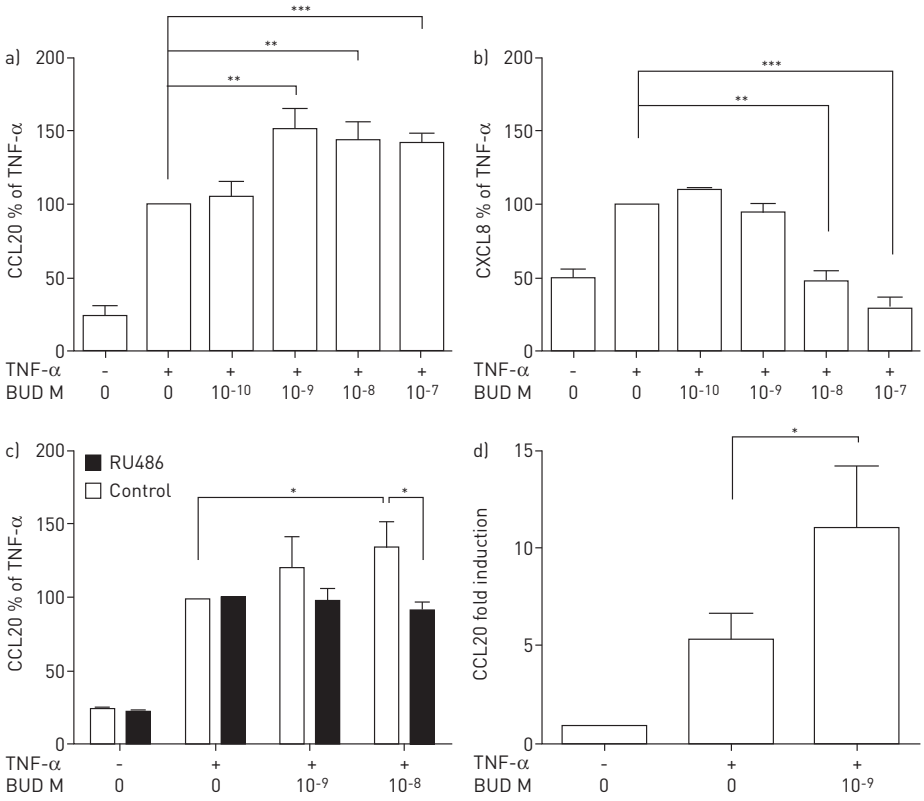

Figure 3. BUD enhances the TNF-α-induced CCL20 release and mRNA expression, which is dependent on GR activity, but suppressed TNF-α-induced IL-8 release in 16HBE cells. Cells were pre-treated with BUD (10^{-7}-10^{-10}M) for 2 hours, stimulated with 10 ng/ml TNF-α, and mRNA and cell-free supernatants were collected after 6 hours and 24 hours respectively. CCL20 (A) and IL-8 (B) were measured in cell-free supernatants and expressed as percentage of the TNF-α levels without BUD (mean ±SEM, n=4 independent experiments). (C) Prior to BUD, cells were treated for 1 hour with 10 μM RU486 (black bars) and CCL20 levels are expressed as percentage of the TNF-α levels without BUD. (D) CCL20 mRNA levels were related to the housekeeping gene and expressed as fold change compared to the unstimulated control ($2^{-\Delta\Delta Ct}$). Mean ± SEM levels are depicted (n= 4 independent experiments). *=p<0.05 **=p<0.01, and ***=p<0.001

GC-insensitive airway inflammation(18-20), we tested the effect of their specific inhibitors on the TNF-α- and BUD-induced CCL20 production in 16HBE cells. Pre-incubation with the inhibitors of the ERK (U0126), p38 (SB203580) and STAT3 (S3I-201) pathways, significantly reduced the TNF-α-induced CCL20 production, indicating a role for these signalling molecules in CCL20 production (Fig. 4A). Inhibition of the PI3K pathway did not affect CCL20 production, although it significantly inhibited IL-8 secretion under the same conditions (supplementary fig. 2A). Next, we determined

whether the BUD-induced increase was dependent on the above described pathways observed. However, no decrease in the upregulatory effect of BUD was found upon the use of LY294002, U0126, SB203580 and S3I-201 (Fig. 4B), nor did BUD increase the phosphorylation of p38, ERK or STAT3 (data not shown). Thus, our data indicate that while TNF-α-induced CCL20 production is dependent on the ERK, p38 and STAT3 pathways, the additional upregulatory effect of GCs is not mediated by these pathways in human bronchial epithelium, suggesting involvement of additional pathways.

GC-induced CCL20 secretion is ADAM17 dependent

Previously, Kim et al have described that ADAM17-dependent EGFR stimulation can increase CCL20 production (21), while GCs have been reported to increase EGFR activity (22). Indeed, ADAM17 has been described as a key sheddase of ligands of EGFR (23). Therefore, we used the broad-spectrum metalloprotease inhibitor TAPI-2, the selective ADAM10/17 inhibitor GW280264X and the selective ADAM10 inhibitor GI254023X as well as the EGFR inhibitor AG1478 to determine if an ADAM/EGFR-dependent mechanism could be involved in GC-induced CCL20 secretion. TAPI-2 did not significantly inhibit TNF-α-induced CCL20 secretion, but completely abrogated the upregulatory effect of BUD. A similar effect was observed for the selective ADAM10 and ADAM17- inhibitor GW280264X, while the more ADAM10 specific inhibitor GI254023X did not show a significant effect on the secretion of CCL20 (Fig. 5A). Since both GW280264X and TAPI-2 have a higher affinity for ADAM17 than ADAM10, these data suggest that the BUD-induced increase in CCL20 is dependent on ADAM17 activity. In contrast to the data of Kim *et al*, we did not observe an effect of the EGFR inhibitor AG1478 on the BUD-induced increase in CCL20 release (Fig. 5A), although it significantly inhibited CXCL8 secretion under the same conditions (supplementary fig. 2B). This excludes the involvement of EGFR ligand shedding and subsequent EGFR activation in BUD-induced CCL20 secretion.

Subsequently, we aimed to determine whether the GC-induced CCL20 secretion is due ADAM17-mediated shedding of CCL20 itself, or is a consequence of downstream signalling induced by the shedding of another ADAM17 substrate than EGFR ligands. We assessed this by studying the effect of ADAM17 inhibition at the CCL20 mRNA level. Of note, BUD was no longer able to upregulate CCL20 mRNA when cells were pre-treated with GW280264X (Fig.5B). Thus, our results indicate that the upregulatory effect of GC on CCL20 is dependent on ADAM17 activity and downstream signalling of an as yet unknown substrate of ADAM17.

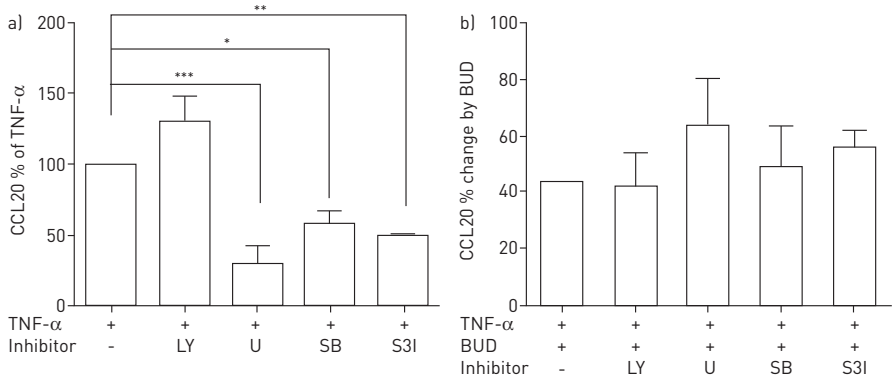

Figure 4. Inhibition of the ERK, p38 and STAT3 pathways reduces TNF-α-induced CCL20 release, but did not block the upregulatory effect of BUD in 16HBE cells. Cells were treated with LY294002 (LY, 10 μM), U0126 (U, 10 μM), SB203580 (SB, 1 μM) and S3I-201 (S3I, 100 μM) for 30 min prior to pre-treatment with BUD (10^{-8}M) for 2 hours and subsequently stimulated with TNF-α (10 ng/ml) for 24 hrs. (A) Effect of the inhibitors on TNF-α-induced CCL20 release. CCL20 levels are expressed as percentage of the TNF-α levels without inhibitors (Mean ± SEM, n=4 independent experiments). (B) Effect of the inhibitors on the BUD-induced CCL20 release.CCL20 levels are expressed as percentage increase over the levels with TNF-α alone. (Mean ± SEM n=4 independent experiments). *=p<0.05, **=p<0.01 and ***=p<0.001

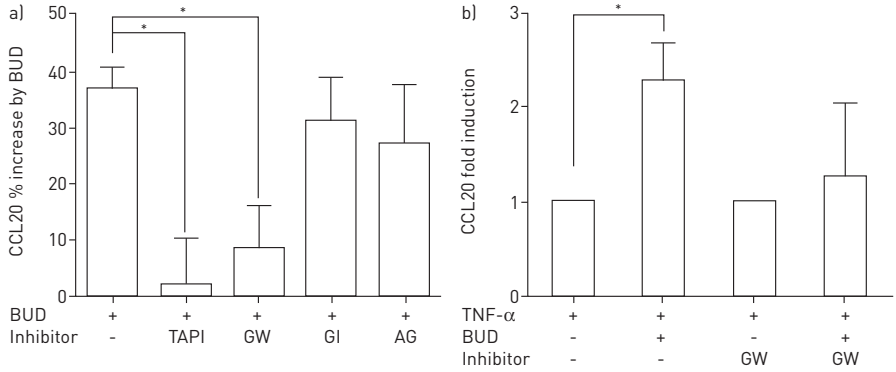

Figure 5. The effect of BUD is dependent on activity of ADAM17. Cells were pre-treated for 1 hour with broad-spectrum metalloprotease inhibitor TAPI-2 (TAPI, 20 μM), ADAM17 and ADAM10 inhibitor GW280264X (GW, 10 μM), ADAM10 inhibitor GI254023X (GI, 1 μM) and EGFR inhibitor AG1478 (AG, 1 μM), and subsequently incubated with BUD (10^{-9}M) for 24 hours (cell-free supernatants) or stimulated with TNF-α for 6 hours and harvested for mRNA isolation. (A) CCL20 was measured in cell-free supernatants and levels are expressed as % increase over the levels with BUD alone (Mean ± SEM n=4 independent experiments). (B) CCL20 mRNA levels were related to the housekeeping gene and expressed as fold change compared to the levels in the absence of BUD ($2^{-\Delta\Delta Ct}$). Mean ± SEM levels are depicted of 3 independent experiments. *p=<0.05

Discussion

The mechanism of GC-insensitive Th17-mediated neutrophilic airway inflammation in asthma has remained unclear, and the effect of GC on the airway epithelial secretion of the Th17 cell and neutrophil chemoattractant CCL20 has not been studied before. We show for the first time that asthma patients using inhaled GCs display higher sputum levels of CCL20 than asthmatics who do not use inhaled GCs, while CXCL8 levels did not differ between the groups. Furthermore, we demonstrate that GCs upregulate CCL20 secretion in cultured bronchial epithelial cells from asthma patients, whereas CXCL8 is inhibited by GCs. Our experiments in 16HBE cells further reveal that this effect of GCs is regulated at the transcriptional level by an ADAM17- and GR-dependent mechanism.

Our findings may have important implications for our understanding of the initiation GC-insensitive Th17 and neutrophilic airway inflammation in asthma, since CCL20 has been known to attract both Th17 cells and neutrophils. In addition to allergen-induced airway inflammation, a crucial role for CCL20 has been demonstrated in cigarette smoke-induced airway infiltration of neutrophils, T cells and dendritic cells (DCs) in a mouse model of COPD (24). Importantly, both Th17-mediated neutrophilic airway inflammation and cigarette smoking have been related to GC-insensitivity in asthma (25) and smoking has been shown to induce airway infiltration of both neutrophils and Th17-type cells (26). Thus, we propose a novel paradigm for the development of GC-insensitive airway inflammation in both asthma and COPD, where GCs enhance CCL20 release, inducing airway infiltration of $CCR6^+$ neutrophils and Th17 cells. In line with this hypothesis, the increased sputum levels of CCL20 in asthma patients using inhaled GC were associated with neutrophil counts. Furthermore, COPD patients were found to display higher sputum levels of CCL20 than never-smokers and smokers without COPD. Here, the majority of COPD patients, but none of the control subjects, used inhaled GCs. It is tempting to speculate that GC use contributes to the increased levels of COPD observed in this study, although the comparison of CCL20 levels in COPD patients using and not using ICS would be required to support this.

We observed that the upregulatory effect of GCs on CCL20 was mediated at the transcriptional level and involved GR activation. GCs have been shown to induce gene transcription through binding to glucocorticoid response element (GRE) in the promoter region. A GRE has been described in an intron downstream of the transcription start site of the CCL20 gene (27). The regulatory properties of this GRE have not been extensively studied to our knowledge, but our results suggest that ADAM17 activity is indispensable for the effect of GCs on CCL20. To our knowledge,

GRE binding has not been described to be metalloprotease-dependent. Since ADAM17 inhibition also abrogated the upregulatory effect of BUD at the transcriptional level, we anticipate that the upregulatory effect of BUD is not mediated by ADAM17-dependent shedding of CCL20 itself, but rather involves downstream signalling of an as yet unknown ADAM17 substrate. ADAM17 plays a role in the shedding of many signalling molecules (23), e.g. Notch (28) and EGFR ligands. The latter has been implicated in many autocrine loops involving pro-inflammatory transcription, including CCL20 (21). However, our results do not support a role for EGFR activation in the GC-induced CCL20 production. Indeed, the EGFR-induced CCL20 release described in H292 cells by Kim et al could not be confirmed in NHBE cells (21). Further studies will be of interest to elucidate which specific pathways downstream of ADAM17 substrates are affected by BUD and are involved in the upregulatory effect on CCL20.

Our findings exclude a role for the STAT3, p38 ERK and PI3K pathways in the GC-induced CCL20 upregulation. We observed that TNF-α-induced CCL20 production was dependent on STAT3, p38 and ERK. In line with this, IL-17-induced CCL20 was shown to be dependent on ERK activity in primary human tracheal cells (18) on both ERK and p38 activity in human gingival fibroblasts (19) and on phosphorylation of STAT3 in naïve T-cells (29). ERK and p38 phosphorylation has previously been shown to be inhibited by GC (30), while GCs induce IL-10 in a STAT3-dependent way in B cells (31). In our setting, BUD did not affect phosphorylation of STAT3 nor the p38/ERK pathway, and the use of their inhibitors revealed that these pathways were not involved in the GC-mediated enhancement of CCL20 release in human bronchial epithelium.

Lannan and colleagues have shown a possible mechanism of co-regulation between TNF-α and GCs (32). In their study, the upregulation of *serpinA3* required both GR activation and the soluble TNF receptor (TNFSR1). TNFSR1 can be shed by ADAM17 (33). However, we render it unlikely that this mechanism plays a major role in the GC-induced increase of CCL20, since the effect occurred regardless of the presence of TNF-α.

Though epithelial cells play an emerging role in the regulation of airway inflammation in asthma (34), we must acknowledge the possibility of other cell types to play a role in the CCL20-induced chemotaxis of Th17 cells and neutrophils to the inflamed lungs in asthma as well. Especially macrophages have been shown to produce CCL20 (35). We cannot exclude that macrophages also contribute to the increased levels of CCL20 in sputum of asthma patients using inhaled GCs. The same mechanisms could also apply in macrophages as higher levels of CCL20 mRNA have been described in macrophages from GC-insensitive subjects compared to GC-sensitive subjects (14).

Since the data on CCL20 levels in sputum of asthma patients were obtained in a cross-sectional, observational study, we cannot be sure whether GC treatment will indeed increase CCL20 levels in the airways. Nevertheless, our sputum data in combination with our *in vitro* findings strongly suggest that GC use in asthma patients leads to increased sputum levels of CCL20 as a consequence of direct effects of GC, inducing CCL20 release by airway epithelium. To confirm this, a future randomised clinical trial on the effect of GCs on CCL20 and Th17 cells will be required.

In conclusion, we show that levels of CCL20 are higher in asthmatic subjects using inhaled GCs and that GCs increase the production of CCL20 in human bronchial epithelium, which is mediated by the GR and dependent on ADAM17 activity. Our data may provide new opportunities for therapeutic intervention of GC-insensitive asthma.

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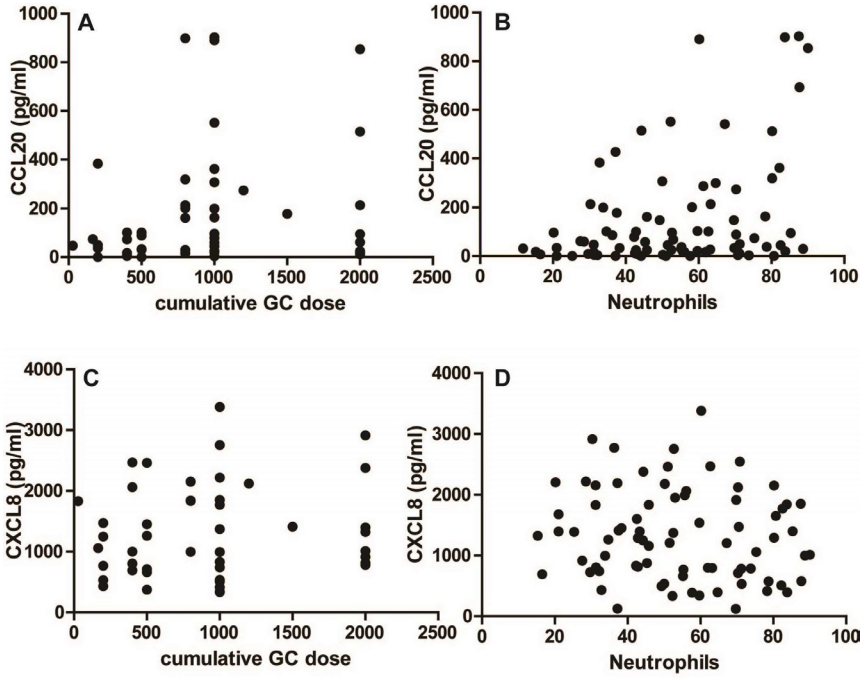
Supplementary methods:

Measurement of cytokine production

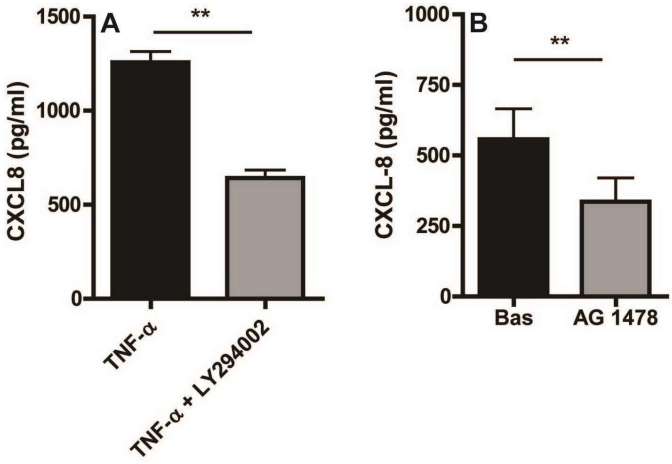
Cell-free supernatants were harvested 24 hour after stimulation. CCL20 and CXCL8 production was measured by ELISA (R&D Systems) according to manufacturer's instructions.

Realtime RT-PCR

RNA was isolated from 16HBE cells 6 hours after stimulation and cDNA was synthesized as previously described [17]. Gene expression was analysed by real-time PCR using the TaqmanÒ according to the manufacturers guidelines (Applied Biosystems, Foster City, CA). Validated probes for CXCL8, CCL20 and the housekeeping genes β2-microglobulin and Peptidylprolyl isomerase A (PPIA) and the TaqMan Master Mix were purchased from Applied Biosystems.



Supplementary figure 1. CCL20 levels correlated significantly with beclomethason equivalent dose of inhaled GC (A, $r_{spearman}$=0.28, p=0.04) and neutrophils (B, $r_{spearman}$=0.34, p=0.01), while IL-8 levels did not correlate significantly with beclomethason equivalent dose of inhaled GC (C), but did correlate significantly with sputum neutrophils (D, $r_{spearman}$=0.24, p=0.03). Levels of CCL20 (ng/ml) and IL-8 (ng/ml) were measured in induced sputum by ELISA.



Supplementary figure 2. PI3K inhibition (LY294002) and EGFR inhibition (AG1478) decrease CXCL-8 production. (A) Cells were treated with LY294002 (LY, 10 μM) for 30 min prior to stimulation with TNF-α (10 ng/ml) for 24 hrs. (B) Cells were pre-treated for 1 hour with EGFR inhibitor AG1478 (AG, 1 μM) and subsequently incubated for 24 hours. IL-8 was measured in cell-free supernatants (pg/ml, mean ±SEM, n=4 independent experiments). Black bars are control conditions, grey bars inhibitor treated. **=p<0.01

Chapter 6

Cigarette smoke-induced necroptosis and DAMP release trigger neutrophilic airway inflammation in mice

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ABSTRACT

Cigarette smoke-induced necroptosis and DAMP release trigger neutrophilic airway inflammation in mice. *Am J Physiol Lung Cell Mol Physiol* 310: L377–L386, 2016. First published December 30, 2015; doi:10.1152/ajplung.00174.2015.—Recent data indicate a role for airway epithelial necroptosis, a regulated form of necrosis, and the associated release of damage-associated molecular patterns (DAMPs) in the development of chronic obstructive pulmonary disease (COPD). DAMPs can activate pattern recognition receptors (PRRs), triggering innate immune responses. We hypothesized that cigarette smoke (CS)-induced epithelial necroptosis and DAMP release initiate airway inflammation in COPD. Human bronchial epithelial BEAS-2B cells were exposed to cigarette smoke extract (CSE), and necrotic cell death (membrane integrity by propidium iodide staining) and DAMP release (i.e., double-stranded DNA, high-mobility group box 1, heat shock protein 70, mitochondrial DNA, ATP) were analyzed. Subsequently, BEAS-2B cells were exposed to DAMP-containing supernatant of CS-induced necrotic cells, and the release of proinflammatory mediators [C-X-C motif ligand 8 (CXCL- 8), IL-6] was evaluated. Furthermore, mice were exposed to CS in the presence and absence of the necroptosis inhibitor necrostatin-1, and levels of DAMPs and inflammatory cell numbers were determined in bronchoalveolar lavage fluid. CSE induced a significant increase in the percentage of necrotic cells and DAMP release in BEAS-2B cells. Stimulation of BEAS-2B cells with supernatant of CS-induced necrotic cells induced a significant increase in the release of CXCL8 and IL-6, in a myeloid differentiation primary response gene 88-dependent fashion. In mice, exposure of CS increased the levels of DAMPs and numbers of neutrophils in bronchoalveolar lavage fluid, which was statistically reduced upon treatment with necrostatin-1. Together, we showed that CS exposure induces necrosis of bronchial epithelial cells and subsequent DAMP release in vitro, inducing the production of proinflammatory cytokines. In vivo, CS exposure induces neutrophilic airway inflammation that is sensitive to necroptosis inhibition.

INTRODUCTION

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is a major cause of morbidity and mortality worldwide and is characterized by irreversible airway obstruction, accelerated lung function decline, and a heterologous combination of bronchitis and emphysema (12). The pathogenesis of COPD involves an uncontrolled inflammatory response to noxious particles and gases, including cigarette smoke (CS) (10). COPD patients show chronic neutrophilic inflammation in the airways, which is accompanied by aberrant tissue repair and remodeling (6, 37, 40). However, little is known about the initial events in CS-induced airway inflammation that set off the cascade of events inducing chronic inflammation (5). Susceptibility to COPD has a strong genetic component, and only 10 –20% of all smoking individuals eventually develop COPD (28). Multiple susceptibility genes for COPD have recently been identified, including *AGER* encoding the receptor for advanced glycation end products (RAGE) receptor, Toll-like receptor (*TLR*) *2* and *TLR4* (2, 3, 29), providing information on the pathways involved in CS-induced airway inflammation. These data indicate that pattern recognition receptors (PRRs) have a relevant role in the pathophysiology of COPD. Upon activation of PRRs by pathogenor damage-associated molecular patterns (DAMPs) proinflammatory responses are induced in innate immune cells.

The airway epithelial layer forms the first physical barrier to inhaled noxious substances, and, therefore, epithelial cells play an important regulatory role in the induction of subsequent proinflammatory responses (16). Previously, we have shown that exposure of airway epithelial cells to CS causes cell death in vitro (34) and is associated with a switch from apoptotic to necrotic cell death, mainly due to the inhibition of caspase activity (15, 39, 42). Upon necrotic cell death DAMPs are released (14), including ATP, high-mobility group box 1 (HMGB1), heat shock protein 70 (HSP70), double-stranded DNA (dsDNA), and S100 proteins (44), which all trigger inflammation, whereas upon apoptotic cell death no DAMPs are released and no inflammatory reaction is initiated, and even tolerance can be promoted (13, 33, 35). However, recently it has been shown that certain types of cellular stresses can also induce a programmed form of necrosis, called necroptosis. Necroptosis is initiated by the activation of receptor-interacting protein kinase (RIPK)-1, RIPK3, and mixed-lineage kinase domain-like protein, leading to loss of cellular integrity and release of cytoplasmic contents and DAMPs (14, 22, 38). Interestingly, necroptosis was recently shown to potentially contribute to the pathogenesis of COPD, through activation of RIPK3 and the autophagy-dependent elimination of mitochondria induced by CS exposure of airway epithelial cells (18). DAMP release upon necrosis and/or necroptosis and the subsequent activation of PRRs on lung resident cells have been proposed

to initiate and maintain the chronic airway inflammation observed in COPD (25). In support of this, several DAMPs have been found elevated in lung fluid and serum of COPD patients compared with smoking and nonsmoking healthy controls (25), and several DAMPs were found increased in serum of COPD patients during exacerbations compared with stable disease (24). Nevertheless, it is still unknown whether exposure of airway epithelial cells to CS induces DAMP release and whether this contributes to innate immune activation in vivo. Recently, we showed that susceptibility for CS-induced neutrophilic airway inflammation was associated with the release of a specific DAMP profile in bronchoalveolar lavage (BAL) of inbred mouse strains (26). Therefore, we hypothesized that CS-induced necrotic or necroptotic cell death of airway epithelial cells results in DAMP release, activation of PRRs, and initiation of neutrophilic airway inflammation. We tested this by in vitro and in vivo approaches and demonstrate that CS exposure induces necrotic cell death and DAMP release in bronchial epithelial cells. This leads to the production of the neutrophil attractant C-X-C motif ligand 8 (CXCL8) in vitro, while the use of a RIPK1 inhibitor demonstrates the involvement of necroptosis in DAMP release and neutrophilic airway inflammation in vivo.

MATERIALS AND METHODS

Cell culture. The human bronchial epithelial cell line BEAS-2B was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI-1640 growth medium (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (BioWhittaker) and 100 U/ml penicillin, 100 µl/ml streptomycin (Penstrep; BioWhittaker). Cells were grown on 6-well, 24-well, and 75-cm^2 plastic culture flasks (Costar, Cambridge, MA) coated with 10 µg/ml BSA (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) and 30 µg/ml Purecol collagen (Purecol; Advanced BioMatrix, San Diego, CA) at 37°C in an atmosphere of 5% CO_2 until 90% confluency was reached and then passaged. Before experiments were performed, cells were incubated for 16 h in serum-free RPMI-1640 medium. Fresh cigarette smoke extract (CSE) was prepared just before experiments using Kentucky 3R4F research reference cigarettes with cut filters (Tobacco Research Institute, University of Kentucky, Lexington, KY). Smoke from two cigarettes was bubbled through 25 ml RPMI-1640 medium using a peristaltic pump, and this was considered as 100% CSE for in vitro experiments.

Ethical statement. Mouse experiments were performed after ethical review by, approval from, and in accordance to the guidelines of the Institutional Animal Care and Use Committee at the University of Groningen (Permit no.: 6018).

Animals. Specified pathogen-free female BALB\cByJ mice (8 wk old) were obtained from Charles River (Wilmington, MA). Mice were housed in individually ventilated cages with food and water ad libitum.

For in vivo smoke experiments, each Kentucky 3R4F research reference cigarette (Tobacco Research Institute, University of Kentucky) was smoked in 5 min at a rate of 5 l/h in a ratio with 60 l/h air using whole body exposure as described previously (26). Mice were placed inside a 6-liter Perspex box and exposed to smoke from 10 cigarettes per session. Six hours after the first smoking session mice received another exposure of 10 cigarettes. Two, 6, and 18 h after the final smoking session, mice were killed, and BAL fluid was collected. Control mice were exposed to air with similar conditions.

Necrostatin-1 (6.25 μg/g ip) (Bachem, Bubendorf, Switzerland) was administered 24 h before the CS exposure. A second dose of Necrostatin-1 was administered 1 h before the first CS exposure. Control animals were injected with an identical volume of dimethyl sulfoxide dissolved in saline (Sigma-Aldrich). Mice were exposed to CS or ambient air as before and killed 18 h after the final smoking session.

DAMP release by sonication or freeze-thawing. BEAS-2B cells were suspended in 1 ml of RPMI-1640 medium (0.75×10^6 cells/ml) and lysed by sonication using a Bandelin Sonoplus HD2070 Sonifier (Bandelin Electronic, Berlin, Germany) set to 70% power. The sonication was performed in three treatments of 10 s each while the cells were cooled on ice. Cell debris was pelleted by centrifugation for 5 min at 3,000 *g*, and the DAMP-containing supernatant was collected. Freeze-thawing was performed in six-well culture plates (Costar) containing 0.75×10^6 cells/well, which were placed at -80°C for 1 h, before being thawed at room temperature. Medium from thawed cells was collected and centrifuged for 5 min at 3,000 *g*, and the DAMP-containing supernatant was stored at -80°C.

DAMP release by CSE exposure. BEAS-2B cells were grown in 75-cm^2 plastic culture flasks. Cells were preincubated for 6 h with 35% CSE. Afterwards, the CSE-containing medium was removed, and cells were washed with PBS and incubated for 18 h in 5 ml of fresh RPMI-1640 medium. The supernatant was centrifuged for 5 min at 3,000 *g*, and the DAMP-containing supernatant was collected. Propidium iodide (PI) staining was used to confirm CS-induced cell death.

Flow cytometric analysis of cell death. Plasma membrane disruption was measured by PI staining according to the manufacturer's instructions (IQ Products, Groningen, The Netherlands). Cells were analyzed by flow cytometry (Calibur; Becton-Dickinson Medical Systems, Heidelberg, Germany).

Detection of DAMPs in BEAS-2B cell-free supernatant. In supernatant of untreated, freeze-thawed, sonicated, and CSE-exposed cells the several DAMPs were measured. HMGB1 was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for HMGB1 (IBL International, Hamburg, Germany). HSP70 was analyzed by Western blotting in cell-free supernatant of BEAS-2B cells and by ELISA in mouse samples. Supernatants were suspended in 5× sample buffer. The samples were loaded on SDS 10% PAGE gel and blotted to a nitrocellulose membrane (0.2 μm by Bio-Rad, Veenendaal, The Netherlands). Immunodetection was performed using an antibody against HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA) using enhanced chemiluminescence and a Bio-Rad Universal Hood II Gel Docking Station (Bio-Rad, Veenendaal, The Netherlands). PCR reactions were performed to detect mitochondrial DNA (mtDNA). Primer pairs for mtDNA were obtained from Biolegio (Malden, The Netherlands): 5 -CCCCACAAACCCCATTAC-TAAACCCA-3 sense and 5 -TTTCATCATGCGGAGATGTTG-GATGG-3 antisense. The PCR reaction occurred in a Bio-Rad iCycler (Bio-Rad), initial denaturation at 94°C for 2 min during one cycle, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s all during 30 cycles; final extension at 72°C for 7 min during one cycle. PCR products were put for 45 min on a standard 1.5% agarose gel at 100 volts. ATP levels were measured using the Enliten ATP assay from Promega (Leiden, The Netherlands) and a microplate luminometer (Berthold Microplate Luminometer). Release of dsDNA in the supernatant was analyzed using the Quant-iT Picogreen assay from Invitrogen (Invitrogen, Breda, The Netherlands) and a FL600 fluorescence plate reader (Bio-Tek Instruments) with an wavelength of 485 nm through a 590-nm bandpass filter.

Initiation of an innate immune response in human bronchial epithelial cells. BEAS-2B cells were cultured in 24-well plates. After starvation, cells were stimulated for 24 h with CSE-free DAMP-containing supernatants from CSE-exposed cells. Supernatants of healthy, freeze-thawed, and sonicated cells were used as control. At the end of the incubation period, supernatant was collected. IL-6 and IL-8 were measured in cell-free supernatant, using ELISA (Sanquin, Amsterdam, The Netherlands), according to the manufacturer's instructions.

Blocking Toll-like receptor pathways using a myeloid differentiation primary response gene 88 inhibitor. Healthy BEAS-2B cells were cultured in 24-well plates. After starvation, cells were treated with or without myeloid differentiation primary response gene 88 (MyD88) inhibitory peptide Pepinh-MYD (20 μM; InvivoGen, San Diego, CA) for 6 h before adding the DAMP-containing supernatants from sonicated and CSE-exposed cells. At the end of the 24-h incubation period, supernatant was centrifuged for 5 min at 1,000 *g*, and the cell-free supernatant was collected. IL-8 was measured in cell-free supernatant, using ELISA, according to the manufacturer's instructions.

BAL and cell differentiation. BAL was performed immediately after mice were anesthetized. In short, the airways were lavaged four times through a tracheal cannula with 1-ml aliquots of pyrogen-free saline. The first lavage was performed with 1 ml saline containing BSA (5%) and protease inhibitors (Complete mini tablet; Roche Diagnostics, Penzberg, Germany) of which the supernatant was stored at -80°C. Recovered BAL fluid of the second, third, and fourth milliliter was pooled together with the cell pellet of the first BAL aliquot. The cells in these fractions were pelleted (590 *g*, 4°C, 10 min) and resuspended in 0.2 ml cold PBS. The total number of cells in the BAL fluid was determined using a Coulter counter (Z1 Series; Beckman Coulter Nederland). For differential cell counts, cytospin preparations were made using a cytocentrifuge (Shandon Life Science, Cheshire, UK). Cells were fixed and stained with Diff-Quick (Dade A. G., Dudingen, Switzerland). All cytospin preparations were evaluated using immersion-oil microscopy (magnification: × 400). Cells were identified and differentiated into mononuclear cells, neutrophils, and epithelial cells by standard morphology and staining characteristics. Per cytospin, 300 cells were counted, and the absolute number of each cell type was calculated.

Detection of DAMPs in BAL fluid. Levels of HSP70 were measured by sandwich ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Levels of S100A8 were measured by sandwich ELISA according to the manufacturer's protocol (Uscn Life Science, Wuhan, China). Quantitative PCR reactions were performed to detect mouse mtDNA in the BAL. Primer pairs for mouse mtDNA were obtained from Invitrogen: 5 -ATGAACGGCTAAAC-GAGGG-3 sense and 5 -CCAACATCGAGGTCGTAAAC-3 anti-sense. The quantitative PCR reaction occurred in a Bio-Rad iQ5 system (Bio-Rad); initial denaturation at 95°C for 5 min during one cycle, denaturation at 95°C for 10 s, annealing and extension at 58°C for 30 s during 40 cycles. The iQ5 C_t values and amplification data were analyzed using the Bio-Rad iQ5 optical system software, version 2.1. Relative mtDNA expression was calculated as the difference between the C values, determined using the equation 2^{-LIC}[†].

Detection of inflammatory markers in BAL. Levels of myeloperoxidase (MPO) were measured by sandwich ELISA according to the manufacturer's protocol (Hycult Biotech, Uden, The Netherlands). The levels of keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2) were measured in BAL fluid using Luminex according to the manufacturer's protocol (LXSAMS-04; R&D Systems).

Statistics. In all cell line experiments significance was tested using a one-way ANOVA with Dunnett's post hoc analysis. In in vivo experiments significance was tested using a Mann-Whitney *U*-test. All in vitro data are shown as means + SE, and all in vivo data are shown as median and single measurements.

RESULTS

CS exposure induces necroptotic cell death in cultured bronchial epithelial cells. We first assessed whether CS exposure induces necrotic cell death in human bronchial epithelial cells. Exposure of BEAS-2B cells to an optimized concentration of 35% CSE for 6 h followed by 18 h of incubation in CSE-free medium resulted in loss of cell membrane integrity, as shown by positivity for PI staining in 34.3 + 5.8% of CSE-exposed cells compared with 3.7 + 0.3% in untreated cells, whereas 99.9 + 0.1% of BEAS-2B cells treated by freezing and thawing (as a positive control) showed loss of membrane integrity (Fig. 1*A*). To distinguish between necrotic and apoptotic cell death, we determined activity of caspases-3, -7, and -8 (39) and observed that CSE exposure decreased activity of caspase-3 and caspase-7, which was even more pronounced upon the induction of apoptosis using stausporine (Fig. 1*B*). This indicates that the observed loss of membrane integrity is not associated with execution of apoptosis. In addition, we observed that CSE inhibits caspase-8 activity, suggestive for the induction of necroptosis (Fig. 1*C*) (14). The necroptosis inhibitor Necrostatin-1 inhibits the CSE-induced decrease in cell viability (Fig. 1*D*), and the CSE-induced increase in extracellular dsDNA release (Fig. 1*E*), indicating that CSE induces cell death in a necroptotic fashion. Together, these data confirm previous studies by us and others (11) that CSE induces necrotic and/or necroptotic cell death in human bronchial epithelial cells in vitro.

CS-induced necrosis results in the release of DAMPs. To test whether CS-induced necroptotic cell death is associated with DAMP release, we assessed the levels of several DAMPs in supernatant of BEAS-2B cells exposed to CSE, using BEAS-2B cells forced into necrosis by sonication or freeze-thawing as positive controls. Upon exposure of BEAS-2B cells to CSE a significant increase in several DAMPs, i.e., dsDNA, HMGB1, HSP70, and mtDNA release, was observed (Fig. 2, *A–D*). Strikingly, HSP70 levels were significantly higher in the supernatant of CSE-exposed cells compared with those of the positive controls, suggesting that CSE induces de novo synthesis of HSP70 before or during CSE-induced necrotic cell death (Fig. 2*C*). No increase in the levels of ATP release were observed in supernatants of CSE-exposed cells (Fig. 2*E*), which is in line with our previous data showing that CSE exposure induced intracellular ATP depletion in cultured bronchial epithelial cells (39). Taken together, these data indicate that CSE exposure induces necrotic cell death and DAMP release in bronchial epithelial cells.

CS-induced DAMP release induces release of proinflammatory cytokines. Next, we assessed whether DAMPs released by CSE exposure induce proinflammatory cytokine production in bronchial epithelial cells that have not been exposed to CSE.

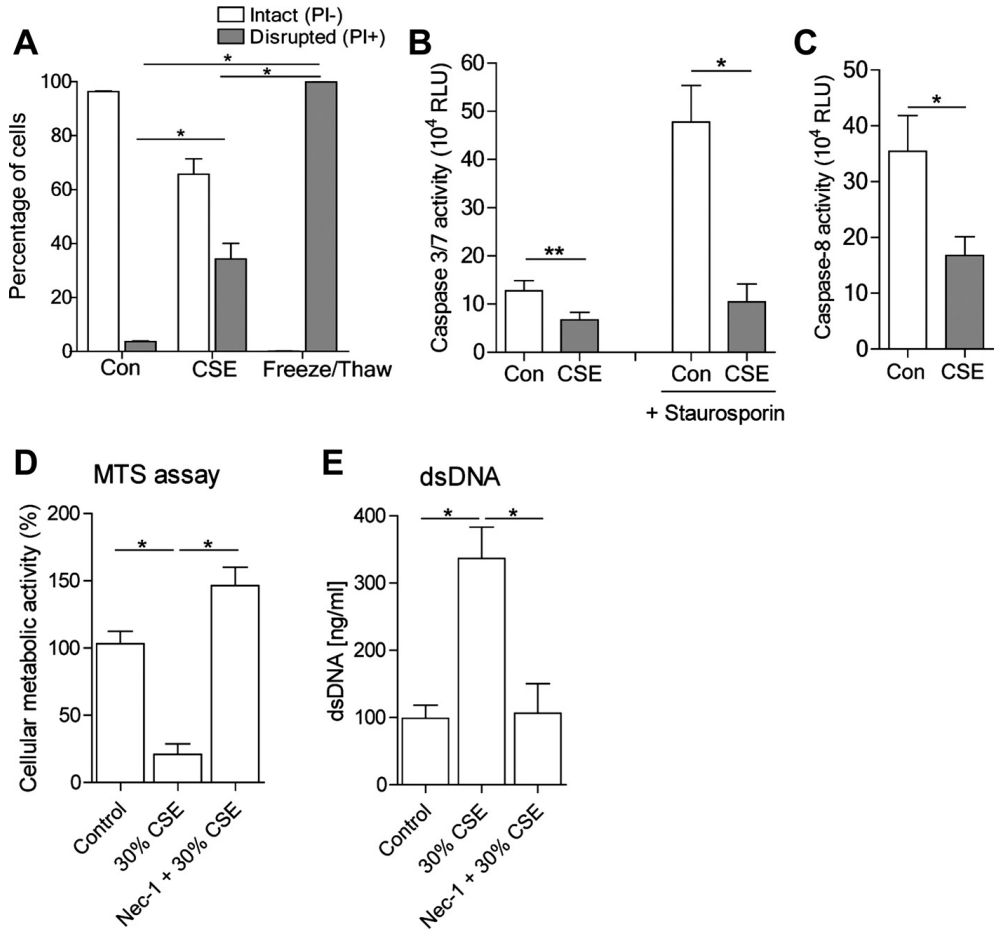

Fig. 1. Cigarette smoke extract (CSE) exposure induces epithelial necrosis and prevents apoptosis through inhibition of caspases. *A*: BEAS-2B cells were exposed to 35% of CSE for 6 h or freeze- thawing, and necrotic cell death was measured by analysis of membrane integrity by propidium iodide (PI) using flow cytometry. PI-positive cells are viable cells that are shown as intact, and PI-negative cells are necrotic cells that are shown as disrupted. *B*: BEAS-2B cells were stimulated with 35% CSE for 6 h with and without 1 h of preincubation with stausporine (2 μM). Caspase-3 and -7 activation by staurosporine is prevented by CSE. *C*: caspase-8 activation is prevented in BEAS-2B cells by stim- ulation with 35% CSE. *D*: viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy- methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The cellular metabolic activity is shown as a percentage of the control group. Necro- statin-1 was added 1 h before 6 h of 30% CSE incubation. The MTS assay was performed after 18 h incubation in CSE-free medium. *E*: double- stranded DNA (dsDNA) was measured in CSE-free supernatant of BEAS2B cells exposed to CSE for 6 h, with or without preincubation of 1 h with Necro-statin-1. All data are shown as means + SE (n = 3– 6 experiments). Significance was tested using a 1-way ANOVA and Dunnett's post hoc analysis,
*P < 0.05 and **P < 0.01.

Incubation of BEAS-2B cells with CSE-free supernatants of CSE-exposed BEAS-2B cells induced a significant increase in the levels of IL-8 and IL-6. Similar levels of IL-8 and IL-6 were secreted by BEAS-2B cells treated with supernatant from freeze-thawing or sonication-treated cells (Fig. 3, *A* and *C*). Of note, IL-6 and IL-8 were not detectable in the supernatants from CSE, freeze-thaw, or sonication-treated cells (data not shown). Because the supernatant of CSE-exposed cells contains both soluble fractions and insoluble cellular debris, we next determined which fraction of the supernatants induced the proinflammatory response in BEAS-2B cells. In line with a role for soluble DAMPs in the production of IL-6 and IL-8, we observed that the soluble fraction induced IL-6 and IL-8 to a similar extent as the total fraction, whereas the insoluble fraction was not able to do so (Fig. 3, *B* and *D*).

Induction of proinflammatory cytokines by CSE-induced DAMPs is largely dependent on MyD88 signaling. To confirm the involvement of DAMPs and subsequent PRR signaling in this response, BEAS-2B cells were pretreated with MyD88 inhibitory peptide before the addition of CSE-exposed cell supernatants. Inhibition of MyD88 significantly reduced the IL-8 production induced by supernatant of sonification-treated and CSE-exposed cells (Fig. 4, *A* and *B*). The specificity of the MyD88 inhibitor was confirmed by the finding that the TNF-a-induced IL-8 production was not suppressed but even increased by inhibition of MyD88 (Fig. 4*A*). Taken together, these data show that exposure of bronchial epithelial cells to CSE induces necrosis and DAMP release, which activates unexposed bronchial epithelial cells to produce proinflammatory cytokines in an, at least partially, MyD88-dependent fashion.

CS exposure induces neutrophilic inflammation and DAMP release in mice. To test whether our in vitro observations of CS-induced DAMP release in bronchial epithelial cells are also present in an in vivo model, we next determined the effects of acute CS exposure on release of DAMPs and subsequent airway recruitment of inflammatory cells in mice. Mice were exposed two times during 1 day to the smoke of 10 cigarettes/ exposure, and DAMPs and inflammatory cells were measured in BAL fluid 2, 6, and 18 h after the final CS exposure. The short-term CS exposure did not increase the total cell count in BAL fluid, which was even significantly reduced 18 h after the final CS exposure compared with air-exposed mice (Fig. 5*A*). This was mainly caused by a reduction in mononuclear cells upon CS exposure (Fig. 5*B*). Interestingly, absolute numbers of neutrophils were significantly higher at all measured time points in CS-exposed compared with air-exposed mice (Fig. 5*C*). Furthermore, the levels of the neutrophil-attracting cytokines KC and MIP-2 were also increased in BAL fluid by exposure to CS (Fig. 5, *D* and *E*). The levels of KC were only increased 18 h after CS exposure and not at 2 h after CS exposure, whereas the levels of MIP-2 were increased both at 2 and at 18 h

after CS exposure. Of note, 6 h after the final CS exposure, detached ciliated epithelial cells were observed in BAL fluid of CS-exposed mice, which were absent in BAL fluid of air-exposed mice (Fig. 6, *A–C*) and may reflect cell death in the airway epithelium.

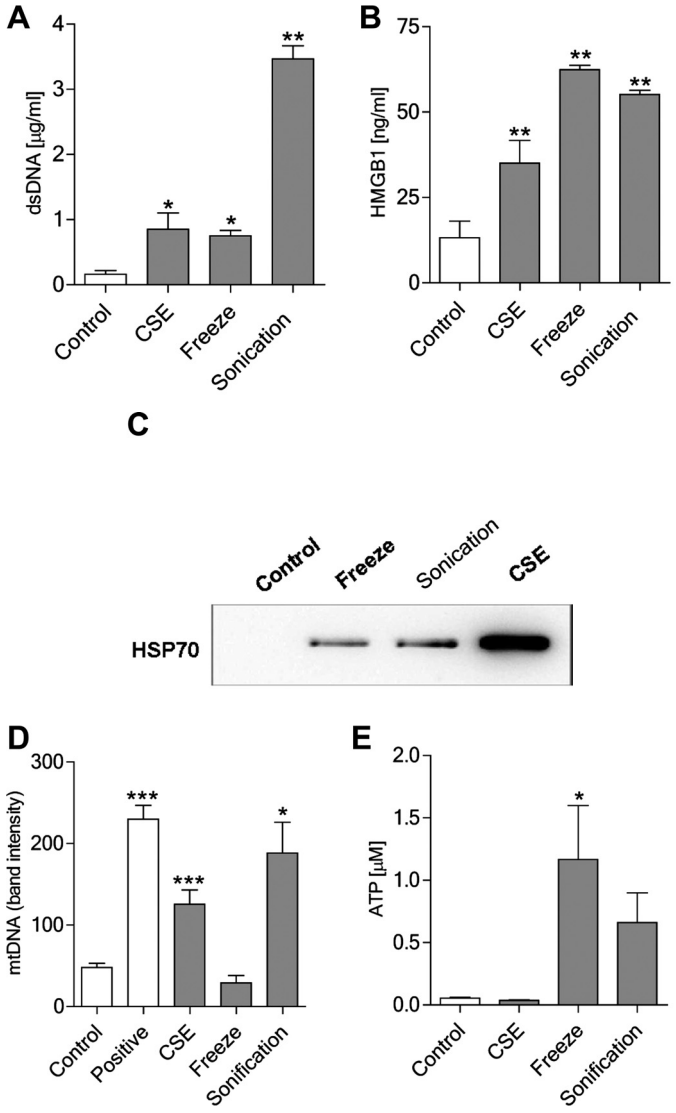

Fig. 2. CSE exposure induces the release of damage-associated molecular patterns (DAMPs) in bronchial epithelial cells. BEAS-2B cells were exposed to 35% of CSE for 6 h, freeze-thawing (freeze), or sonication, and in cell-free supernatant the levels of the DAMPs dsDNA (*A*), high-mobility group box 1 (HMGB1, *B*), heat shock protein-70 (HSP70, *C*), mitochondrial DNA (mtDNA, *D*), and ATP (*E*) were measured. All data are shown as means + SE (n = 3– 6). Significance was tested using a 1-way ANOVA and Dunnett's post hoc analysis, *P < 0.05, **P < 0.01, and **P < 0.001.

To determine whether CS exposure induces necrotic cell death and subsequent DAMP release in vivo, several DAMPs were measured in BAL fluid of mice that were exposed to CS or air. In line with our in vitro findings, significantly higher levels of HSP70, HMGB1, mtDNA, dsDNA, and S100A8 were observed in BAL fluid at multiple time points after CS exposure compared with air-exposed mice, of which only S100A8 showed a significant time-dependent increase (Fig. 6, *D–I*), while ATP levels were not significantly increased at any time point after CS exposure (Fig. 6*D*).

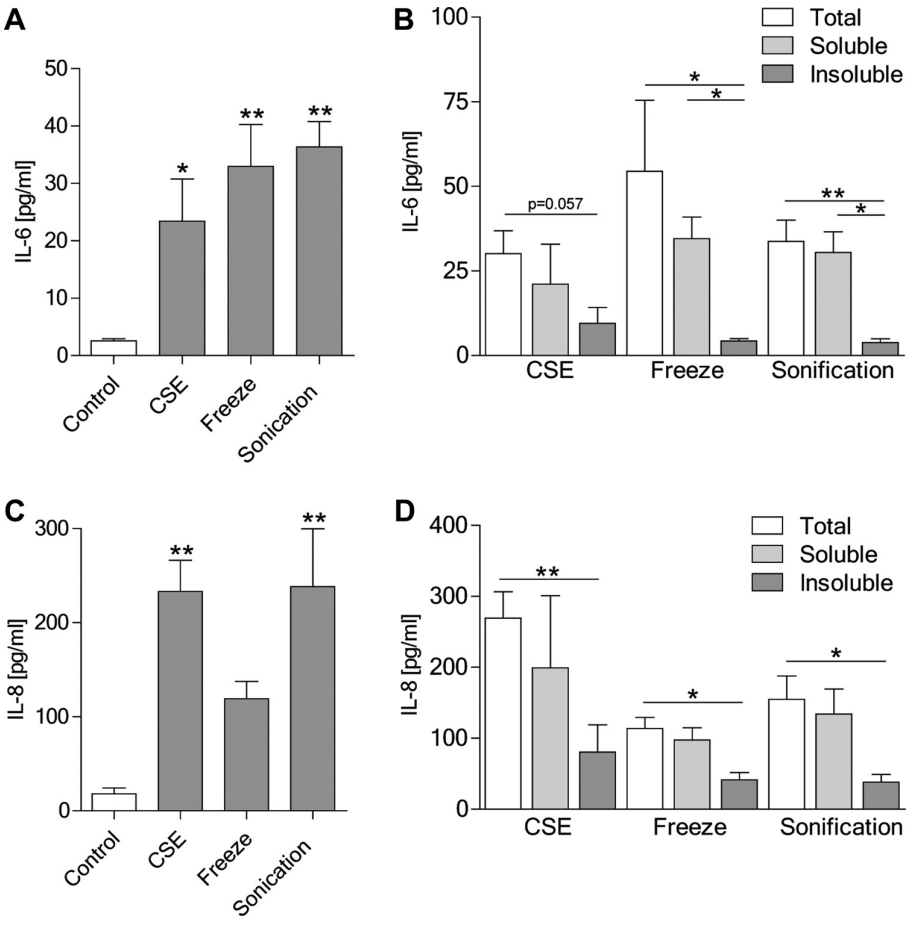

Fig. 3. DAMPs released from necrotic cells induce the release of proinflammatory cytokines in BEAS-2B cells. BEAS-2B cells were stimulated with the cell-free supernatant of BEAS-2B cells that were exposed to 35% CSE for 6 h, freeze-thawed (freeze), or sonicated. The cytokine levels of IL-6 (A) and IL-8 (C) were measured in the cell-free supernatant. To study which content of necrotic cells causes the inflammatory response, BEAS-2B cells were treated with the total, soluble, or insoluble content of necrotic cells. The cytokine levels of IL-6 (B) and IL-8 (D) were measured in the cell-free supernatant. All data are shown as means + SE (n = 3– 6). Significance was tested using a 1-way ANOVA and Dunnett's post hoc analysis, *P < 0.05 and **P < 0.01.

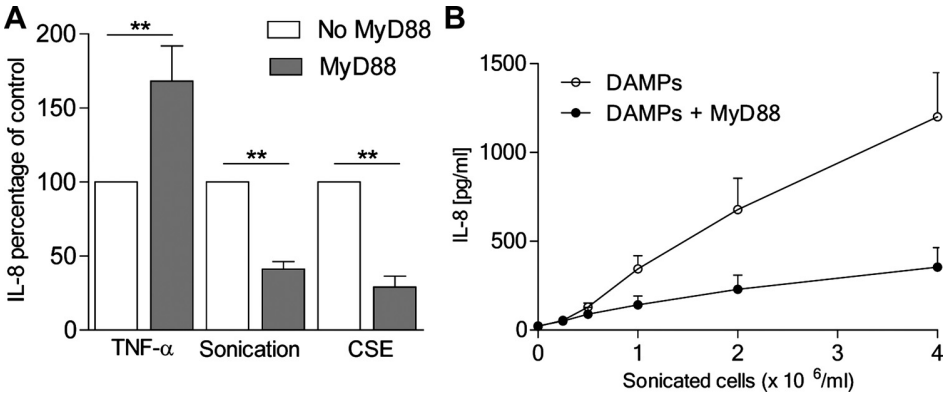

Fig. 4. Induction of proinflammatory cytokine production by DAMPs is regulated by the adaptor protein myeloid differentiation primary response gene 88 (MyD88). BEAS-2B cells were stimulated with TNF-a as positive control for non-DAMP-mediated IL-8 release, or with the cell-free supernatant of BEAS-2B cells that were exposed to 35% CSE for 6 h or sonicated, all with and without preincubation with the MyD88 inhibitory peptide. Afterwards, the cytokine levels of IL-8 were measured in the cell-free supernatant (*A*). The effect of MyD88 inhibition on IL-8 production by BEAS-2B cells was tested with a dose response of DAMPs from sonicated cells (*B*). All data are shown as means + SE (n = 3– 6). Significance was tested using a 1-way ANOVA and Dunnett's post hoc analysis, $^{**}P < 0.01$.

Treatment with the necroptosis inhibitor necrostatin-1 reduces neutrophilic inflammation induced by CS. To study the involvement of necroptotic cell death in the CS-induced inflammatory response, we used the RIPK1 inhibitor Necrostatin-1 (7, 18). After intraperitoneal injection of Necrostatin-1, mice were exposed to CS as described above. Interestingly, pretreatment with Necrostatin-1 strongly and significantly reduced the CS-induced infiltration of neutrophils (Fig. 7*A*), which was no longer significant when compared with air-exposed mice. Accordingly, the levels of the neutrophil effector molecule MPO were significantly reduced in BAL of Necrostatin-1-treated mice (Fig. 7*B*). CS-exposed mice still displayed higher MPO levels in BAL compared with air-exposed mice, suggesting that remaining neutrophils present upon Necrostatin-1 treatment were still activated by CS at this dose of Necrostatin-1. Together, these data indicate that necroptosis is indeed involved in CS-induced neutrophil influx in the airways.

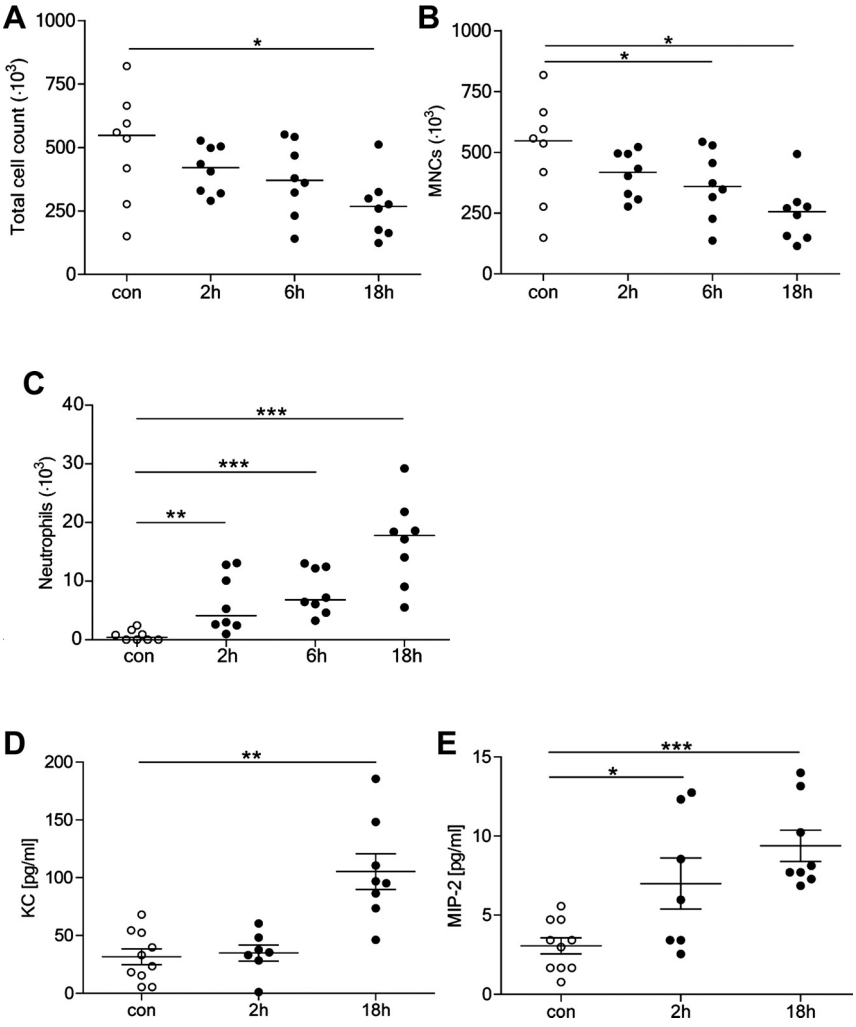

Fig. 5. Cigarette smoke (CS) exposure induces neutrophilic airway inflammation in BALB/cByJ mice. BALB/cByJ mice were exposed to CS or air as a control (con) two times during 1 day with 10 cigarettes/exposure. Mice were killed either 2, 6, or 18 h after the final exposure, and total cell count (*A*), mononuclear cell (MNCs) count (*B*), and neutrophil counts (*C*) were determined in bronchoalveolar lavage (BAL) fluid. The levels of the cytokines keratinocyte chemoattractant (KC, *D*) and macrophage inflammatory protein-2 (MIP-2, *E*) were measured in BAL fluid in air-exposed and CS-exposed mice 2 and 18 h after the final exposure. Significance was tested using a Mann Whitney *U*-test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

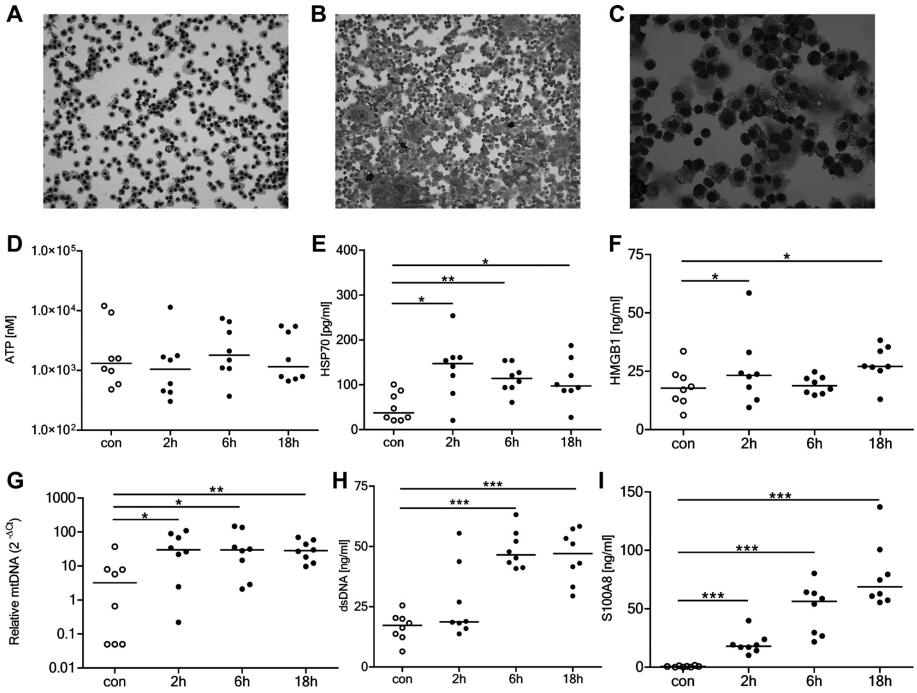

Fig. 6. CS exposure induces epithelial detachment and DAMP release in BAL fluid of mice. Microscopic imaging of BAL fluid cytospins from air-exposed control mice (*A*), CS-exposed mice (*B*), and CS-exposed mice at X60 magnification (*C*). BALB/cByJ mice were exposed to CS or air as a control two times during 1 day with 10 cigarettes/exposure. Two, 6, and 18 h after the final exposure the levels of ATP (*D*), HSP70 (*E*), HMGB1 (*F*), mtDNA (*G*), dsDNA (*H*), and S100A8 (*I*) were measured in BAL fluid. Data are shown as median and single measurements. Significance was tested using a Mann Whitney *U*-test, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

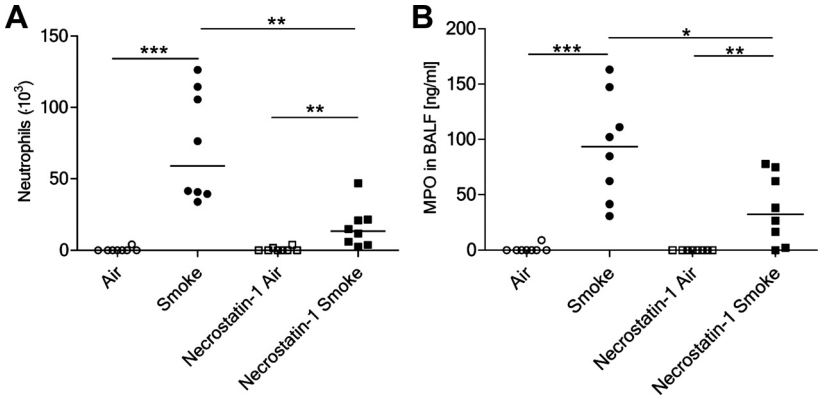

Fig. 7. Necrostatin-1 treatment suppresses the CS-induced neutrophilic airway inflammation in mice. BALB/cByJ mice were exposed to CS or air as a control two times during 1 day with 10 cigarettes/exposure. Mice were treated either with the necroptosis inhibitor Necrostatin-1 or with dimethyl sulfoxide (DMSO) as a control. Eighteen hours after the final CS exposure the neutrophil counts were determined in BAL fluid (*A*). Neutrophil activation was assessed by myeloperoxidase (MPO) measurements in BAL fluid (*B*). Data are shown as medians and single measurements. Significance was tested using a Mann Whitney *U*-test, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

DISCUSSION

In the current study we show that exposure of bronchial epithelial cells to CS induces cell death that is characterized by the release of DAMPs in the absence of caspase-3, -7, and -8 activity, consistent with necroptotic cell death (14). Moreover, DAMPs released by CS-exposed bronchial epithelial cells activate the epithelium to produce proinflammatory cytokines in a partially MyD88-dependent fashion. Finally, we showed that CS exposure induces DAMP release and neutrophilic airway inflammation in vivo and that this neutrophilic airway inflammation is sensitive to the inhibition of necroptosis.

Previously, it has been shown that CS exposure induces neutrophilic airway inflammation in both humans (40) and mice (26) and that this acute response to CS has been implicated in the early phases of the development of chronic airway inflammation in COPD. Furthermore, in line with our studies, multiple studies have shown that individual mouse strains respond differently toward CS exposure, with BALB/cJ mice among the highest responders, and that the amount of neutrophilic inflammation is highest between 3 and 24 h after the final exposure (19, 23). We show that short-term CS exposure increases DAMP levels in BAL fluid of mice, accompanied by neutrophilic airway inflammation within several hours after the last exposure. Unfortunately, to date we were unable to detect the degree or the exact type of cell death in cells isolated from murine BAL fluid to support our in vitro data showing that CS exposure induces necroptotic cell death. Previously, it was shown that human neutrophils release DAMPs upon exposure to CS (9). Moreover, we observed a decrease in mononuclear cells upon CS exposure, suggesting that cell death is also induced in this subpopulation (Fig. 5*B*). This indicates that at least part of the DAMPs released in BAL fluid of mice upon CS exposure comes from nonepithelial cells, including alveolar macrophages and neutrophils. Nevertheless, because airway epithelial cells have an important barrier function in the airways and come in direct contact with the inhaled CS, and given our current in vitro data showing that epithelial cells release DAMPs upon exposure to CSE, it is plausible that at least part of the DAMPs released in vivo upon CS exposure is coming from epithelial cells.

Recent data indicate that CS-induced necroptosis of airway epithelial cells potentially plays a role in COPD pathogenesis (18). Here, we show that inhibition of RIPK1-mediated necroptosis completely prevents neutrophilic influx in the airways of mice in response to CS, stressing the critical role for this programmed form of necrotic cell death in the activation of CS-induced innate immune responses. Furthermore, we observed that CS exposure induces the release of DAMPs both in vitro and in vivo. These

DAMPs subsequently induce proinflammatory responses, including the production of neutrophil attractant CXCL8, in airway epithelium by activating MyD88-coupled PRRs in vitro. In line, CS exposure resulted in neutrophil infiltration and the release of the mouse homolog for IL-8, KC and MIP-2 in BAL fluid in vivo. Furthermore, our data identify necroptosis-induced DAMP release as a mechanism for CS-induced neutrophilic airway inflammation.

Recently, a role for DAMPs has been suggested in the pathophysiology of COPD (25). Several DAMPs, including HMGB1 and ATP, have been found increased in BAL fluid of COPD patients compared with smoking and nonsmoking controls (9, 17). Although we did observe increased HMGB1 levels upon CS exposure in vitro and in vivo, in the current study CS did not induce the release of ATP. This is presumably a consequence of the direct effect of CSE on mitochondrial activity, which induces acute ATP depletion in CS-exposed cells (39). Interestingly, we additionally observed a significant increase in HSP70, dsDNA, and mtDNA upon CS exposure in bronchial epithelial cells and in mice.

Furthermore, our data show that the CS-induced proinflammatory response in bronchial epithelial cells is at least partially regulated by MyD88-dependent signaling, e.g., TLR and RAGE signaling. Therefore, it is tempting to speculate that differences in PRR expression contribute to the increased epithelial release of IL-8 and subsequent attraction of neutrophils in that has been observed in COPD (31). Indeed, higher numbers of TLR4-and TLR9-positive cells were found in BAL fluid of COPD patients (20, 21). Moreover, increased expression of RAGE was shown in airway epithelium and smooth muscle cells of COPD patients compared with smoking and nonsmoking controls (9). In addition, polymorphisms in the genes encoding RAGE, TLR2, and TLR4 have been shown to be associated with a decline in forced expiratory volume in 1 s and higher inflammatory cell numbers in COPD (3). Experimental models of CS-induced airway inflammation indicate that both TLR4 and RAGE signaling contributes to CS-induced inflammatory responses (8, 30). Furthermore, RAGE signaling has been shown to promote CS-induced emphysema, as evidenced by data showing that RAGE knockout mice are protected against both CS-and elastase-induced emphysema, whereas RAGE-overexpressing mice develop spontaneous emphysema (27, 32, 36, 41). On the other hand, TLR4 signaling appears to have an opposing function, since downregulation of TLR4 promotes the progression of emphysema (1, 4, 43). Future studies are needed to determine whether epithelial cells from COPD patients are more susceptible to CS-induced DAMP release and/or to subsequent proinflammatory responses due to increased expression of PPRs, including RAGE, TLR2, and TLR4 compared with cells derived from smoking controls. Additional studies are required to address the role of CS-induced necroptosis and DAMP signaling pathways in COPD

pathogenesis.

In conclusion, our data show that CS exposure induces necroptotic epithelial cell death with subsequent release of DAMPs. These DAMPs signal through PPRs and induce the production of proinflammatory cytokines, leading to neutrophilic airway inflammation.

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Chapter 7

Summary, general discussion and future perspectives

In this thesis we set out to investigate inflammatory and molecular mechanisms of difficult to control, GC-insensitive obstructive pulmonary diseases.

In **chapter 2**, we aimed to characterize the airway inflammation in relation to the response to oral GCs in asthma patients and define the characteristics associated with less responsiveness to GCs in asthma. We expected GC insensitivity to be associated with smoking, less eosinophilic inflammation and more neutrophilic inflammation, because smoking is associated with neutrophilic airway inflammation and neutrophils are described to be less responsive to GC. We show that GC-insensitivity to a 2-week course of oral GC in mild-to-moderate asthmatics is associated with lower exhaled NO values, lower sputum eosinophil numbers, higher neutrophil/eosinophil ratios in blood, higher blood lymphocytes, and higher age. To our surprise, smoking was not related to GC-insensitivity independently, but our data suggests that smoking induces GC-insensitivity by changing the type of inflammation to a more neutrophilic phenotype.

In **chapter 3**, we assessed the expression of HDAC2 in bronchial biopsies of asthma patients to study whether smoking and ICS use affect HDAC2 expression, a mediator of anti-inflammatory effects in asthma. Unexpectedly, we observed that smoking was associated with higher epithelial HDAC2 expression in asthmatics. ICS use in non-smoking (but not smoking) asthmatics is associated with higher HDAC2 expression in bronchial epithelial cells compared to subjects not using ICS. The latter is in line with previous findings, whereas this beneficial effect of ICS with respect to HDAC2 expression was not found in smoking asthmatics, indicating that smoking may reduce the responsiveness to ICS with respect to induction of HDAC expression.

In **chapter 4**, we studied whether Th17 cytokine IL-17A can reduce the ability of GC to inhibit pro-inflammatory transcription in bronchial epithelial cells. We show that IL-17A pre-treatment is able to induce GC insensitivity with respect to production of neutrophil attractant CXCL8 in bronchial epithelial cells by activating the PI3K pathway, in which a decrease in HDAC2 activity is likely involved. We concluded that the PI3K-HDAC2 pathway is a possible mechanism for Th17 cells to induce GC-insensitivity. Therefore, we propose that therapeutic strategies to inhibit PI3K, as well as therapies focused on downregulating Th17 activity and secretion of IL-17A, may lead to novel ways to improve the efficacy of GCs.

To gain further insight in the mechanisms of Th17 associated GC insensitivity, in **chapter 5** we determined whether the production of neutrophil and Th17 chemotactic cytokine CCL20 was affected by GCs. Similar to CXCL8, CCL20 acts as chemoattractant for neutrophils. Moreover, it attract T lymphocytes, especially Th17 cells, as its receptor CCR6 is predominantly expressed on Th17 cells. We show that glucocorticoids enhance CCL20 production by bronchial epithelium, which may constitute a novel mechanism in Th17-mediated glucocorticoid-insensitive inflammation in asthma. Indeed, CCL20 levels were higher in sputum from asthma patients using GCs.

Smoking and neutrophilic airway inflammation have been associated with GC insensitivity in asthma. In **chapter 6**, we aimed to determine whether cigarette smoke can induce neutrophilic inflammation through the induction of immunogenic cell death by cigarette smoke. We show that cigarette smoke extract induces necroptotic epithelial cell death and subsequent DAMP release, PRR signaling and production of pro-inflammatory cytokines. In mice, we observed that cigarette smoke exposure induces DAMP release and neutrophilic airway inflammation that is sensitive to necroptosis inhibition. Thus, CS induced epithelial cell death may lead to neutrophilic airway inflammation by the release of DAMPs.

General Discussion

We have set out to investigate the mechanisms through which difficult-to-control, GC insensitive inflammation in obstructive airway disease develops. Specifically, we studied whether this may involve common mechanisms including cigarette smoke-induced neutrophilic inflammation, CCL20 release, Th17–mediated inflammation and IL-17A downstream signalling. Nevertheless, asthma and COPD are heterogeneous diseases and in different subjects different mechanisms play a role. We described several mechanisms that may play a role in difficult-to-control inflammation in asthma and/or COPD and will discuss them below.

The relation between GC unresponsiveness and neutrophils

We hypothesized that glucocorticoid insensitivity is predominantly an inflammatory phenotype driven problem. Patients with a more eosinophilic inflammatory profile tend to respond better to glucocorticoids than patients suffering from neutrophilic inflammation. This could be the result of the type of inflammation that is induced by cigarette smoking, which has been associated with neutrophilic inflammation, and neutrophils are generally assumed to be less responsive to GCs than eosinophils [1]. In addition, neutrophilic infiltration is accompanied by oxidative stress, which has been implicated in GC insensitivity [2]. Our group previously showed lower GC responsiveness in epithelial cells from asthma and COPD patients and demonstrated that cigarette smoke-induced oxidative stress results in reduced GC responsiveness in epithelial cells [3]. With respect to neutrophilic airway inflammation, our observational study (chapter 2) is in concurrence with literature that has shown a relation between eosinophils and response to GCs as well as a relation between neutrophils and unresponsiveness [4-9]. Chapter 2 shows that asthmatic smokers have a lower response to GCs with respect to changes in FEV_1 compared to non-smoking asthmatics, but in a multivariate regression analysis this did not prove significant. Presumably this is an effect of the analysis including both cigarette smoking and inflammatory changes influenced by cigarette smoking. It appears that cigarette smoke acts for a major part through these inflammatory changes. In the literature there is an abundance of evidence linking glucocorticoid insensitivity to smoking [10-12]. In line with our findings in asthma patients, eosinophilic inflammation in COPD has also been linked to GC sensitivity and better response to GC treatment during exacerbations [2]. In addition, Th17 cells have an emerging role in the induction of neutrophilic airway inflammation and have been also linked to GC insensitivity. However, since the production of neutrophil chemo-attractants, including CXCL8, by airway epithelium is GC-sensitive, GCs are still expected to be able to reduce the perpetuation of neutrophilic inflammation in asthma and COPD. Therefore, we hypothesized that IL-17A may induce changes that lead to reduced GC responsiveness of pro-inflammatory responses in asthma and COPD.

Th17 cell-mediated GC insensitivity

Chapter 4 and 5 address the interaction between glucocorticoids, bronchial epithelial cells and Th17 cells. The bronchial epithelium has a crucial role in innate immune defence, both as the physical barrier to the exterior of the body as well as a producer of antimicrobial peptides, pro-inflammatory cytokines and chemo-attractants, especially when damaged e.g. by cigarette smoke exposure [13]. The interaction between Th17 cells and bronchial epithelial cells has been proposed to play an important part in the signalling towards neutrophilic inflammation[14]. These mechanisms are not restricted to Th17 cells, but may also involve their counterparts in the innate immune system: innate lymphoid type 3 cells (ILC3) and γδ-T cells.

Firstly, in chapter 4 we set out to investigate whether Th17-type cytokine IL-17A can directly induce glucocorticoid insensitivity in bronchial epithelial cells. In this study we observed that IL-17A reduced responsiveness to GC by activating the previously extensively described PI3K-HDAC2 pathway [2,15-18]. Indeed, activation of this pathway has been implicated in the induction of GC-insensitivity[2], but in the current study we did not study whether this pathway is essentially different between GC responsive and unresponsive asthmatic subjects. In future studies it will be of interest to assess whether reduced GC sensitivity of airway epithelial cells from asthma patients is related to increased activity of the PI3K pathway, reduced levels of HDAC2 or increased levels of IL-17A in these individuals. While we have shown IL-17A to induce GC insensitivity through decreased HDAC2 activity, others have shown that IL-17A induces GRβ upregulation in peripheral blood mononuclear cells and thereby GC insensitivity [19,20]. As a possible integrator of these pathways, Li et al showed that HDAC2 expression is inhibited by GRβ [21], which we have not studied here.

While in chapter 3 we did not observe that smoking is associated with lower expression of HDAC2 in bronchial biopsies of asthma patients, GC use in non-smoking (but not smoking) asthmatics was associated with higher HDAC2 expression in bronchial epithelial cells. Thus, reduced upregulation of HDAC expression by ICS in smoking asthma patients may indeed be involved in GC unresponsiveness in these patients.

In chapter 5, we further studied the involvement of neutrophils and Th17 in GCs unresponsiveness, focusing on their attractant CCL20, which has been found increased in the airways of both asthma and COPD patients[22-24]. We observed that GCs upregulate the expression of CCL20 in bronchial epithelial cells, constituting a novel mechanism of Th17-mediated GC-insensitive inflammation in asthma. This mechanism was studied in cell lines and epithelial cells of asthmatic patients both submerged and in air-liquid-interface cultures. However, we did not compare

insensitive and sensitive subjects to assess whether differences in CCL20 secretion exist between these groups, so to what extent this mechanism contributes to GC-insensitive obstructive pulmonary disease remains to be studied further. In both chapters 4 and 5 we focused on asthma, although the described mechanisms may not be exclusive to asthma, and similar mechanisms could be involved in GC insensitivity in other inflammatory diseases associated with GC-insensitivity, e.g. rheumatoid arthritis and inflammatory bowel disease, where increased Th17-mediated responses have been observed as well [25]. Furthermore, it Th17-mediated responses may play a role in GC insensitivity in COPD. CCL20 signals through its interaction with CCR6 and the CCL20/CCR6 axis has already been shown to play a role in COPD [23,24]. In addition, increased lung infiltration of IL-17-positive cells has been observed in COPD [26].

In support of a role for Th17-mediated inflammation in GC insensitivity, Th17 cells have been shown to be related to GC insensitivity in a mouse model of asthma [27]. Lambrecht et al have postulated neutrophilic asthma to be a Th17 disorder [28], and as described above, neutrophilia is associated with reduced responsiveness to GC. Moreover, IL-23, an essential cytokine for the differentiation into Th17 cells, is strongly and inversely correlated to FEV1 in children with asthma [29], further highlighting the role of Th17 cells in the pathology of asthma. Various of these studies used the positivity for either IL-17 or for RORγT as marker for Th17 cells [26,27]. Since ILC3 and a subset of γδ-T-cells also express the transcription factor RORγt [30-32], it cannot be excluded that these cells contribute to the observed GC insensitivity. Expression of RORγT leads to responsiveness to IL-23 and CCL20 by transcription of IL23R and CCR6 respectively as well as production of IL-17A and IL-22 [33]. Thus, these RORγT cells may also contribute to CCL20 and IL-17-mediated GC insensitivity. The inflammation mediated by RORγT+-cells has been proposed as type 3 inflammation [34] and there may be a crucial role for this type 3 inflammation in difficult-to-control obstructive airway diseases.

The role of DAMPs in neutrophilic airway inflammation in obstructive lung disease

In chapter 6 we endeavoured to show whether the damage of bronchial epithelial cells by cigarette smoke leads to inflammation through the necroptotic cell death of epithelial cells and the subsequent release of intracellular DAMPs, in turn inducing an inflammatory response in neighbouring epithelial cells. This could be a first step in the development of the inflammatory response eventually leading to COPD [35,36].

We have shown in a follow-up study that the toxic effects of cigarette smoke are not exclusive to epithelial cells, but within the airway the intraluminal inflammatory cells are also affected [37]. However, this is more likely to be involved in the perpetuation of

chronic airway inflammation rather than the initial steps leading to the inflammatory response in COPD, as the inflammatory cells first need to be attracted to the airways. In this, secretion of cytokines/chemokines by damaged epithelial cells is thought to play a crucial role. Upon their binding to PRRs on epithelial cells, DAMPs can lead to secretion of CXCL8 to induce neutrophil attraction [36]. In addition, neutrophils express various PRRs themselves [38], potentially contributing to neutrophilic airway inflammation in a GC-independent manner through direct activation by DAMPs. We speculated that GCs may even promote the release of DAMPs by increasing immunogenic cell death, as steroid treatment has been shown to induce cell death [39,40]. We have not studied this, but first aimed to test our hypothesis that cigarette smoke induced immunogenic cell death contributes to neutrophilic airway inflammation. This was indeed supported by our findings.

Among the identified DAMPs, several have been described to have a role in neutrophilic and type 3 inflammation- mediated airway inflammation, including HMGB1 [41]. In this respect, HMGB1 has been shown to potentiate CCL20 secretion in synoviocytes [42], while the purinergic receptors for ATP have been implicated in CCL20 production in bronchial epithelial cells [43].

GCs have been described as inducers of apoptosis in epithelial cells [39,40]. Our group has shown that treatment of epithelial cells undergoing apoptosis with cigarette smoke induces a switch to necrotic cell death through mitochondrial dysfunction [44]. Therefore, the interaction between GCs and cigarette smoke in epithelial cells potentially increases the immunogenic cell death and DAMP release, which in turn inducing pro-inflammatory responses, negating the anti-inflammatory effect of GCs. However, the combined effect of cigarette smoke and corticosteroids has to our knowledge not been studied and whether this may constitute a mechanism underlying GC insensitive inflammation will be subject of future studies. Our group has shown that several DAMPs are elevated in the serum of COPD patients during exacerbation [45], suggesting that DAMPs may also be involved in the sudden worsening of inflammation during exacerbations of COPD.

In addition to COPD, DAMPs may play a role in chronic allergic asthma. Increased levels of HMGB1 have been observed in asthma, and inhibition of HMGB-1 reduced airway inflammation in a mouse model [46]. Also other DAMPs have been implicated in asthma, including ATP, uric acid and S100A9 [47,48]. Whether the release of these DAMPs is sensitive to GCs is largely unknown and will be of interest for future investigation.

Future perspectives

Type 3 inflammation could be a target for therapy in both asthma and COPD and potentially increase GC effectiveness. For example, it has been shown that Vitamin D decreases Th17 differentiation and effector function in young asthmatic children, leading to lower expression of CCR6, IL23R, IL-17A and Th17-related transcription factor RORC, in peripheral blood T cells [49]. Especially in the field of psoriasis multiple monoclonal antibodies have been studied that have an effect in the Th17 pathway. However, few studies have been performed in obstructive pulmonary diseases. Busse et al have conducted a randomized controlled trial into the effect of brodalumab, an anti-IL-17 receptor A monoclonal antibody, in severe asthma [50]. Unfortunately this study showed no treatment effect in the total group of severe asthmatics as measured by asthma control questionnaire. One could argue that this is due to the heterogeneity of asthma. In the subgroup analysis, groups based on peripheral eosinophils and the amount of exhaled nitric oxide were studied, but within these groups Busse at al could not show an effect of brodalumab either. However, Busse et al did not perform a subgroup analysis on neutrophilic disease, which would be our prime target. Even better would be to target subjects with increased levels of IL-17A and/or lung infiltration of Th17 cells. Kirsten et al performed a study with secukinumab, an anti-IL-17A monoclonal antibody, in an experimental model of neutrophilic airway inflammation induced by ozone [51]. In their study secukinumab did not decrease the number of airway neutrophils measured by sputum induction. This is a highly experimental model in healthy volunteers and not necessarily similar to the disease mechanism in obstructive airway disease. However, in mouse models ozone-induced inflammation has been shown to be IL-17A mediated [52]. Collectively, inhibition of IL-17A still is a promising therapy to study, but research should be focused on finding the right phenotype to treat, presumably more neutrophilic disease and preferably subjects where the number of Th17 cells has been determined to be elevated. Unfortunately, we do not have a simple and reliable biomarker that reflects a Th17 imbalance to guide inhibition of IL-17A as a treatment. Furthermore, the possible role of ILC3 in the mechanisms we have described needs to be elucidated, as well as its role in the obstructive pulmonary diseases. Since we showed that the downstream effects of IL-17 were mediated by PI3K kinase activation and potentially involve reduced HDAC2 expression, this pathway may also be of interest to target [17,53,54].

In addition to strategies targeting Th17-mediated inflammation, DAMP-mediated inflammation may constitute a novel therapeutic strategy. Different DAMPs can cause inflammation through a variety of routes, acting on different receptors. In all likelihood it will not be possible to pinpoint a single DAMP to neutralize in order to

decrease inflammation, and it is more likely that an intricate meshwork of sometimes redundant signalling needs to be targeted. Therefore, the chances of success in the treatment of neutrophilic airway inflammation may be higher when aiming to decrease the release of DAMPs or to target common downstream pathways. It may theoretically be possible to decrease the amount of relatively uncontrolled cell death, and possibly inhibitors of necroptosis can inhibit progression of disease. Therefore, it will be of interest to test Necroptosis inhibitors currently under development for auto-immune disease, in preclinical and clinical COPD [55,56]. The novel insights into the role of type 3 inflammation and DAMPs may ultimately lead to novel therapeutic strategies to improve steroid responsiveness in severe asthma and COPD.

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Chapter 8

Nederlandse Samenvatting

In dit proefschrift zijn we de mechanisms gaan onderzoeken van moeilijk te controleren, glucocorticoïd (GC) ongevoelige obstructieve longziekten op zowel inflammatoir als moleculair niveau.

In **hoofdstuk 2** was het doe om luchtwegonsteking te karakteriseren in relatie tot de reactiviteit op een kuur orale GCs en de karakteristieken die een verminderde gevoelighed op GCs in astma voorspellen vaststellen. We verwachten dat een verminderde gevoeligheid op GCs geassocieerd zou zijn met roken, minder eosinofiele ontsteking en meer neutrofiele ontsteking, omdat roken geassocieerd is met meer neutrofiele ontsteking en neutrofielen beschreven zijn als minder gevoelig voor GCs.

We laten zien dat GC-ongevoeligheid op een 2-weekse kuur met orale GCs in mild-tot-matig astma geassocieerd is met een lager hoeveelheid stikstofoxide in uitgeademde lucht, lagere aantallen eosinofielen in sputum, een hogere neutrofiel/eosinofiel ratio in bloed, ene hoger aantal lymfocyten in bloed en een hogere leeftijd. Tot onze verrassing, was roken niet onafhankelijk geassocieerd met GC-ongevoeligheid, maar onze data suggereren dat roken GC-ongevoeligheid induceert door het type ontsteking te veranderen naar een meer neutrofiel fenotype.

In **hoofdstuk 3**, onderzochten we de expressie van het molecuul HDAC2 in bronchusbiopten van astmapatiënten, om vast te stellen of roken en het gebruik van geïnhaleerde GCs de expressie beinvloed van dit eiwit, dat een mediator van anti-inflammatoire effecten in astma is. Tegen onze verwachting in zagen we dat roken geassocieerd was met een hogere expressievan HDAC2 in bronchiaal epitheel. Gebruik van geïnhaleerde GCs was in niet-rokende astmatici geassocieerd met een hogere HDAC2 expressie in bronchiaal epitheel in vergelijking met astmatici die deze niet gebruikten. Dit laatste was in lijn met eerdere bevindingen, maar dit potentieel voordelige effect van het gebruik van geïnhaleerde GCs op HDAC2 expressie werd niet gezien in rokende astmatici. Mogelijk volgt hieruit dat roken de gevoeligheid voor GCs verminderd op het gebied van de inductie van HDAC2 expressie.

In **hoofdstuk 4** bestudeerden we of de Th17 cytokine L-17A het vermogen van GCs om pro-inflammatoire transcriptie in bronchiaal epitheel te remmen kan verminderen. We laten zien dat IL-17A voorbehandeling GC-ongevoeligheid kan induceren doordat de productie van neutrofiel chemo-attractant minder geremd wordt in bronchiale epitheelcellen via de activatie van de PI3K intacellulaire route, waarbij waarschijnlijk een vermindering van HDAC2 activiteit betrokken is. We concludeerden dat de PI3K-HDAC2 route een mogelijk mechanisme is waarlangs Th17 cellen GC-ongevoeligheid kunnen induceren.

Daarom stellen we voor dat therapeutische strategieën om PI3K te remmen, naast therapieën gericht op het verminderen van Th7 activiteit en de secretie van IL-17A kunnen leiden tot nieuwe manieren om de doelmatigheid van GCs te verbeteren.

Om veder inzicht te krijgen in de mechanismes van Th17 geassociëerde GC-ongevoeligheid hebben we in **hoofdstuk 5** vastgesteld of the productie van de neutrofiel en Th17 aantrekkende cytokine CCL20 werd beïnvloed door GCs. Vergelijkbaar met CXCL8 is CCL20 een chemoattractant voor neutrofielen. Daarnaast trekt het T-lymfocyten, met name Th17 cellen, aan, omdat receptor CCR6 als receptor voor CCL20 met name op Th17 cellen zit. We laten zien dat GCs de productie van CCL20 versterken in bronchiaal epitheel, wat een nieuw mechanisme in Th17 gemedieerde GC-ongevoelige ontsteking in astma kan zijn. Daarbij werden er inderdaad verhoogde spiegels van CCL20 gevonden in het sputum van astmatici die geïnhaleerde GCs gebruikten.

Roken en neutrofiele onsteking van de luchtwegen zijn geassocieerd met GC-ongevoeligheid in astma. In **hoofdstuk 6** streefden we ernaar om vast te stellen of sigarettenrook neutrofiele inflammatie kan induceren door de inductie van immunogene celdood.

We laten zien dat sigarettenrookextract necroptotische dood van epitheelcellen induceert en daaruit volgende vrijkomen van “Damage-associated Molecular patterns” (DAMP) en de signaaltransductie door “Pattern recognition receptor” leidt tot pro-inflammatoire cytokineproductie. In muizen zagen we dat blootstelling aan sigarettenrook leidde tot vrijkomen van DAMPs en een neutrofiele inflammatie die gevoelig is voor de inhibitie van necroptose. Zo kan sigarettenrook geinduceerde dood van eptiheelcellen leiden tot neutrofiele ontsteking van de luchtwegen door het vrijkomen van DAMPs.

.

Chapter 9

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Kijk opa, toch nog een boekje bij leven en welzijn.