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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