Mechanisms of glucocorticoid insensitivity in asthma

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DOI:
10.33612/diss.136678943

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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