Allergic asthma: Environmental factors challenging the immune system
van de Pol, M.A.

Citation for published version (APA):
van de Pol, M. A. (2013). Allergic asthma: Environmental factors challenging the immune system
Summary and General discussion
Introduction

Allergic asthma is a chronic inflammatory airway disorder with airway hyperresponsiveness in association with variable airflow obstruction. Symptom-free periods are interrupted by episodes of wheezing, coughing and chest tightness\(^1\). Airway inflammation in mild to moderate asthma patients is characterized by local accumulation of eosinophils, mast cells and T helper (Th)2-lymphocytes, producing cytokines like interleukin (IL)-4, IL-5 and IL-13, although the degree to which these are present varies between patients. As sensitized patients are often year-round exposed to airborne allergens allergic inflammation persists, which leads to damage of the airway mucosa and promotes airway hyperresponsiveness.

Wheezing-associated viral infections and allergic sensitization in early childhood are considered two major risk factors for the development of asthma\(^2\). Over the past 50 years, urbanization and associated changes to diet and lifestyle have led to a marked increase in asthma and related allergic diseases in countries with a Western lifestyle\(^3\). Therefore, the role of environmental factors such as allergens, endotoxin (lipopolysaccharide (LPS)) and microbes in the development and maintenance of allergic asthma has been an area of great interest ever since the ‘hygiene hypothesis’ was proposed by Strachan\(^4\). In this thesis, we mainly focus on the development and maintenance of allergic inflammation in asthma. Although it is well known that exposure to allergens is strongly associated with the development of allergic asthma, the specific mechanisms underlying the development of allergic inflammation are still not fully understood. In this chapter, we first summarize the main findings regarding these mechanisms as reported in this thesis and then discuss some of the results that are described in previous chapters in more detail.

Summary of the main findings

With regard to mechanisms underlying the development of allergy in a prospective cohort study on starting laboratory animal workers, we found that sensitization to rats (i.e. cases) was related to the development of a rat-specific IL-4 response (chapter 2). This IL-4 response was associated with work-related allergic symptoms and the development of rat-specific immunoglobulin (Ig)E antibodies. In contrast to suggestions in literature that allergen-specific IL-10 or IFN\(\gamma\) responses could protect against the development of allergen sensitization\(^5,6\), we did not observe differences in rat-specific IL-10- and IFN\(\gamma\)-producing cells between cases and controls at any time-point. However, mean IL-10 levels in whole blood cultures (WBC) after non-specific stimulation with Staphylococcus aureus Cowan I strain (SAC) during follow-up were negatively related to rat-specific IgE levels in serum after 2 years.
When we compared the cytokine response of peripheral blood mononuclear cells (PBMC) from house dust mite (HDM)-allergic asthmatics and non-allergic healthy controls to a very low dose of LPS (10 pg/ml) that is comparable with indoor airborne LPS exposure, we found that PBMC from allergic asthmatics produced significantly less IL-10, IL-12 and IFN-γ compared to the non-allergic subjects (chapter 3). This differential cytokine production was overcome by stimulation with LPS 100 pg/ml, leading to equally high levels of these cytokines in both groups. Moreover, addition of 100 pg/ml LPS to HDM-stimulated PBMC of allergic asthmatics reduced the production of Th2 cytokines. These results suggest that PBMC from allergic asthmatics need a higher dose of LPS to shift from a Th2 profile to a Th1 profile and that the indoor airborne LPS levels in homes of Westernized countries are currently too low to overcome this threshold. We therefore postulate that this threshold may contribute to sensitization by allergens.

In chapter 4, we showed that five weeks after allergen challenge by relatively high-dose inhalation of HDM, serum levels of HDM-specific IgE in HDM-sensitized asthmatics were still increased, in contrast to the local allergen-induced effects on lung function and bronchial inflammation that were resolved within a few weeks. The increase in HDM-specific IgE was paralleled by an enhanced systemic Th2 response of PBMC to HDM. Although this single challenge did not cause a detectable increase in baseline airway responsiveness at 5 weeks after challenge, accumulation of these systemic effects by chronic natural exposure may lead to aggravation or induction of allergic diseases. The increased systemic sensitivity to HDM after a single bronchial allergen challenge emphasizes the role of B and T cells in the development and maintenance of allergic asthma.

As inflammation and coagulation have been proposed to act in parallel and eosinophils have been proposed to play a role in coagulation, we assessed the effect of intra-bronchial allergen challenge (HDM or grass pollen) on coagulation activation in the lower airways of allergic asthmatic patients and healthy controls (chapter 5). We found significantly higher levels of markers of coagulation activation in bronchoalveolar lavage fluid (BALF) from allergic asthmatics compared to that of healthy controls at baseline and at 4 hours after allergen challenge. The allergen-induced increase in thrombin-anti-thrombin complexes (TATc) and soluble tissue factor (sTF) was accompanied by reduced levels of the anticoagulant activated protein C (APC). Although the allergen challenge-induced increase of coagulation activation was significantly higher in allergic asthmatics compared to healthy controls, this was not related to the presence of eosinophils in the airways. Interestingly, while the allergic asthmatic patients received a more than 60-fold lower allergen dose than the healthy controls, they still produced similar levels of pro-inflammatory cytokines in response to the allergen challenge. These results suggest that
allergic asthmatics not only have an aberrant response to allergens, but that they are also more susceptible to increased activation of coagulation and increased activation of a-specific inflammatory responses in the airways.

In chapter 6 we described the potential anti-allergic effect of a synbiotic supplement to target allergic asthma. Treatment with this specific mixture of pre- and probiotics for 4 weeks was associated with a significant improvement in peak expiratory flow (PEF) and a reduced allergen-induced production of IL-5 both in vivo and in vitro. This implies that synbiotics may suppress the enhanced allergen-induced sensitivity of PBMC after allergen exposure, as shown in chapter 4, and thereby gradually decrease the allergen-induced immunological response in allergic patients with established asthma. Finally, we propose a new method to correct whole sputum data for saliva contamination (chapter 7). The analysis of sputum has become the major substrate for assessing local inflammation and other physiological processes in asthma and COPD, but analyses are subject to large variation due to contamination with saliva. The proposed method improves the validity of cellular and molecular biomarkers in induced whole sputum.

Cytokine responses during the development of allergy
In a 2-year follow-up study of apprentice laboratory animal workers about 20% developed a positive skin prick test (SPT) to one or more of the laboratory animals and/or animal-specific immunoglobulin (Ig)E antibodies in serum. During this study, participants were seen four times: at the start of the study (visit A), after 4 months (visit B), 1 year (visit C) and 2 years (visit D). In a nested case-control setting, we compared at each time-point the cellular responses of 18 of these laboratory animal workers that developed rat-specific sensitization (cases) with those of matched controls to investigate whether sensitization was preceded or accompanied by changes in cytokine production in vitro. Stimulation of peripheral blood mononuclear cells (PBMC) from cases as compared to controls revealed a significant increase in rat-specific IL-4-producing cells between the last time point before and the first time point after sensitization (chapter 2). By contrast, no differences in rat-specific IL-10- and IFNγ-producing cells between cases and controls were observed.

The rat-specific cytokine responses may have been biased by lipopolysaccharide (LPS) contamination of the rat-allergen extract that was used in the in vitro experiments as the extract containing rat urinary proteins (42 μg/ml) appeared to be contaminated with 22.4 endotoxin units (EU)/ml. This amount of endotoxin is equal to the biological activity of 224 pg/ml of the LPS substrate that we used in the stimulation assays described in chapter 3. As shown in chapter 3, addition of exogenous LPS (100 pg/ml) to house dust mite (HDM)-
Table 1. Exposure response relationships

<table>
<thead>
<tr>
<th>LPS exposure (EU/m³*hours/month)</th>
<th>&lt;160</th>
<th>160-360</th>
<th>360-725</th>
<th>725-1600</th>
<th>&gt;1600</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of workers</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Sensitized against lab.animals</td>
<td>2 (9%)</td>
<td>3 (14%)</td>
<td>5 (23%)</td>
<td>6 (27%)</td>
<td>6 (27%)</td>
<td>0.065</td>
</tr>
<tr>
<td>Sensitized against rats</td>
<td>2 (9%)</td>
<td>3 (14%)</td>
<td>4 (18%)</td>
<td>5 (23%)</td>
<td>6 (27%)</td>
<td><strong>0.018</strong></td>
</tr>
<tr>
<td>Sensitized against mice</td>
<td>1 (5%)</td>
<td>2 (9%)</td>
<td>4 (18%)</td>
<td>3 (14%)</td>
<td>2 (9%)</td>
<td>0.518</td>
</tr>
</tbody>
</table>

Participants were divided in quintiles based on their exposure. A significant association was found for rat-specific sensitization and mean exposure per month to LPS (p = 0.018), but not for mouse-specific sensitization.

stimulated PBMC, but not HDM with 10 pg of endogenous LPS, dramatically increased the levels of IL-10 and IFNγ to levels comparable with non-allergic subjects and at the same time reduced the levels of allergen-induced Th2 cytokines. The finding that 100 pg/ml LPS reduced the HDM-induced production of Th2 cytokines implies that the LPS contamination of the rat-urine extract could have masked small differences in IL-4-producing cells during the development of rat allergy. Depletion of LPS in the rat-urine extract might have revealed an earlier and more pronounced allergen-specific IL-4 response (e.g. at visit B) and possibly even a protective role of allergen-induced IL-10 and/or IFNγ during the development of allergy, as suggested in literature. Measurements of airborne LPS levels in the working zones correlated with the levels of airborne rat allergens. Although exposure to LPS tended to be higher in sensitized cases, it was probably still too low to overcome the threshold in these subjects. Interestingly, in contrast to laboratory animal workers that developed sensitization to rats, the number of those that developed sensitization to mice seemed to decline with increasing levels of airborne LPS (table 1, unpublished data from E.J.M. Krop).

Furthermore, we did observe a negative correlation between SAC-induced IL-10 production in whole blood cultures (WBC) during follow-up and the development of rat-specific IgE (Rs = −0.449, p = 0.006, fig. 1). This suggests that an intrinsic threshold to produce IL-10, as proposed in chapter 3, may facilitate sensitization to allergens. The fact that gram-positive SAC signals via TLR-2 pathways suggests that the intrinsic threshold for the production of IL-10 in allergic asthmatics might be caused by an aberrant regulation of adapter molecules that are shared by TLR-4 and TLR-2.
Figure 1. Mean IL-10 levels in SAC-stimulated whole blood cultures during follow-up were negatively related to rat allergen-specific IgE levels in serum at the last visit.

Allergen-induced coagulation activation in asthmatic airways

Although coagulation and fibrinolysis usually take place in the vascular compartment, it has been shown that coagulation and anticoagulant mechanisms can also be initiated locally in the airways, for example during infection. Essential mediators of coagulation, such as soluble tissue factor (sTF), the main initiator of coagulation, and thrombin that transforms fibrinogen to fibrin, have been detected in induced sputum samples of asthmatic patients. Since eosinophils have been shown to express substantial amounts of tissue factor, we assessed the effect of allergen exposure on coagulation activation in the lower airways of allergic asthmatic patients and healthy controls (chapter 5). Several coagulation mediators were determined in bronchoalveolar lavage (BAL) fluid collected before and 4 hours after segmental allergen challenge by Nocker et al. At baseline, the levels of thrombin-anti-thrombin complexes (TATc) and sTF were elevated in the BAL fluid of asthmatic patients compared to that of healthy controls. These markers of coagulation activation increased after allergen challenge in both asthmatic patients and healthy controls, although the allergen-induced increase in TATc was more pronounced in asthmatics than in controls. Furthermore, intra-bronchial exposure to allergen resulted in markedly reduced levels of the anticoagulant APC in the BAL fluid of allergic asthmatic patients compared to controls. At the same time, we found a significant increase in soluble thrombomodulin (sTM) in asthmatics only, which probably contributed to the impaired APC generation. In contrast to earlier findings by Terada et al. in BAL fluid collected 48 hours after allergen challenge, the increase in coagulation activation that we found at 4 hours after challenge was not related to an allergen-induced influx of eosinophils. Since the dose of allergen administered intra-
Figure 2. Activation of coagulation and inflammatory parameters in induced sputum samples. Levels of thrombin-antithrombin complexes (TATc), soluble tissue factor (sTF), soluble thrombomodulin (sTM), interleukin (IL)-8, eosinophil cationic protein (ECP) and eosinophil counts per gram induced sputum obtained before, 6 and 24 hours after bronchial allergen challenge in HDM-allergic asthmatic patients. Mean values are shown. Significant changes from baseline (t = 0) are marked with *, p < 0.05.
Figure 3. Associations between coagulation mediators and inflammatory parameters in induced sputum samples from allergic asthmatic patients after allergen challenge. (A) Correlation between percentage of eosinophils at 6 h after challenge and the levels of TATc at 6 and 24 hours after challenge. Correlation between (B) eosinophil counts and (C) levels of IL-8 at 6 h after challenge and levels of sTF at 24 h after challenge.

bronchially by Nocker et al. was based upon skin reactivity for the allergen, the allergic asthmatic patients received less allergen than the patients in the challenge study from Terada et al. and at least 60 times less allergen than the healthy non-allergic controls (0.1-100 ng vs. >6000 ng). Although the dose received by the allergic asthmatics may have been too low to induce an allergic response with recruitment of eosinophils at 4 hours after challenge, the a-specific inflammatory response with increased levels of the granulocyte-attractant IL-8 and increased neutrophil counts may also have contributed to the increase in coagulation activation.

Furthermore, in sputum samples collected at 6 and 24 hours after inhalation of a relatively high dose of HDM by allergic asthmatic patients at the start of an intervention study (described in chapter 4 and 6), we found similar increases in TATc, sTF and sTM and a decrease in APC compared to the levels before challenge (fig. 2). In contrast to the levels in BAL fluid, in sputum the values of most coagulation mediators were just above (or below for APC) detection limit. This could possibly be due to sputum processing with dithiotreitol (DTT), but also because sputum is coughed up from the bronchial
compartment whereas BAL fluid is obtained from the alveolar compartment, where there is maybe more leakage from the vascular compartment. Despite the low coagulation values, we found a correlation between several coagulation mediators and allergen-induced inflammatory parameters. Interestingly, the percentage of sputum eosinophils at 6 hours after challenge was not only related to the levels of TATc and sTF at 6 hours after challenge ($r^2 = 0.722$, $p = 0.016$ for TATc and $r^2 = 0.607$, $p = 0.039$ for sTF; for sTM, $r^2 = 0.522$, $p = 0.067$) but also to the level of TATc at 24 hours after challenge ($r^2 = 0.824$, $p = 0.012$, fig. 3A). Moreover, the number of sputum eosinophils and the level of IL-8 in sputum samples at 6 hours after allergen challenge appeared to be strongly related to the levels of sTF ($r^2 = 0.761$, $p = 0.023$ for eosinophils, fig. 3B; $r^2 = 0.830$, $p = 0.012$ for IL-8, fig. 3C) and to some extent to sTM ($r^2 = 0.564$, $p = 0.086$ for IL-8) at 24 hours after challenge, but not at the 6-hours time-point. These results suggest that allergen-induced activation of coagulation in the airways of asthmatics runs in parallel with the increased inflammatory activity in the airways and coagulation may serve to inhibit local inflammation and prevent bleeding of the inflamed lung tissue\textsuperscript{14,15}. It remains to be established whether coagulation drives inflammation or vice versa, or that there are mutual interactions in the airways.

**Probiotic bacteria for prevention and treatment of allergic diseases**

Several epidemiologic studies have reported that changes in the composition of intestinal commensal bacteria often precede the onset of allergic disease in infants\textsuperscript{16-18}. Recently, asthmatic patients were also found to have an altered microbial composition in the airways compared with non-asthmatics\textsuperscript{19}. Commensal bacteria have been shown to play an important role in the maturation of mucosal-associated lymphoid tissue (MALT), which largely regulates the tolerogenic nature of the immune response to innocuous antigens in the surface lining of the gastrointestinal, respiratory and genitourinary tracts\textsuperscript{20,21}. This process starts immediately after birth when the intestines become colonized by commensal bacteria, which increase in diversity and stability over the first 3 years of life\textsuperscript{22-24}. The composition of the human gut microbiota is different for each individual and can be affected by factors such as environmental exposures, levels of hygiene, diet and antibiotic use\textsuperscript{25}. In the past decade many studies have been performed to investigate the therapeutic and/or preventive potential of probiotic bacteria to reduce the pathological features of allergic diseases. Although the use of probiotics for prevention of allergic disease has been successful in several clinical trials, the evidence for a beneficial effect of probiotic bacteria in the treatment of atopic eczema and asthma is inconclusive\textsuperscript{26}. The lack of a probiotic treatment effect can be explained by differences in study populations and
the variety of probiotic strains used in different trials, which makes it hard to determine the overall effectiveness of treatment with probiotic bacteria in meta-analyses.

In our clinical trial, described in chapter 6, treatment with synbiotics, a mixture of pre- and probiotics, for 4 weeks did not affect the primary outcomes, i.e. allergen-induced bronchial inflammation and changes in lung function during the late asthmatic response after allergen challenge. Therefore, this probiotic treatment study will be considered negative in prospective meta-analyses, although we did see an improvement in daily peak flow values during treatment in the synbiotics group and a reduced systemic Th2 response after allergen challenge. The study population in our study consisted of adults with established allergic asthma for whom the treatment period of 4 weeks was probably too short to affect the primary outcome. Since the composition of commensal bacteria in adults seems to be quite stable\textsuperscript{24}, it might take a longer period of diet change or probiotic intake to shift from an ‘atopic’ gut microbiota to a healthy one\textsuperscript{27,28}. Furthermore, prolonged administration of synbiotics would also be necessary to investigate the long-term effect on Th2 suppression and allergen-induced inflammatory responses.

Probiotics can exert their beneficial effect by competition with pathogens for mucosal colonization, by modulation of the permeability of epithelial barriers, by altering the inflammatory response of epithelial and innate cells or directly by modifying the activity of immune cells\textsuperscript{29}. However, the molecular mechanisms behind these immunomodulatory properties are still not fully understood. In a recent study, LPS was identified as the key molecule of \textit{Acinetobacter Iwoffii} F78, a bacterium from farming environment with possible allergy-protective properties, for activation of a Th1-polarizing program in human dendritic cells\textsuperscript{30}. In another study, oral application of a bacterial lysate, containing a heat-killed nonpathogenic gram-positive and gram-negative strain with high levels of LPS, decreased the risk of atopic dermatitis in children with single heredity for atopy\textsuperscript{31}. The same bacterial lysate reduced allergen-specific IgE levels in serum and intestinal permeability in a rat model of food allergy, which was accompanied by an increased production of IL-10\textsuperscript{32}. These studies are consistent with our findings, described in chapter 3, that PBMC from allergic asthmatics require exposure to high doses of LPS to adjust Th2 responses. In our experiments the reduced Th2-production was also accompanied by increased production of IL-10, which suggests a regulatory role for this cytokine.

LPS is one of the microbial-associated molecular patterns (MAMPs) derived from the intestinal microbiota, that bind to pattern recognition receptors (PRRs), like Toll-like receptors (TLRs), C-type lectin receptors and nucleotide oligomerisation domain-like (NOD) receptors, which are expressed by intestinal epithelial cells and innate immune cells\textsuperscript{33}. Together with other MAMPs, LPS can signal via these PRRs and determine the nature of the immune response to bacteria and food in the gut\textsuperscript{34}. 
As suggested in chapter 3, intrinsic differences in the regulatory cascade of the TLR pathways might explain the higher threshold to LPS in allergic asthmatics. Genetic defects in MyD88 or IRAK-4, two pivotal components of the TLR signaling pathways, have been shown to lead to high serum levels of IgE and an increased Th2-cytokine production, probably due to an impaired generation of Th1 responses\(^ {35,36} \). Similar exaggerated Th2 responses were also found in mice that were treated with antibiotics to alter the composition of commensal microbiota\(^ {37} \). In these mice, MyD88-expression in B-cells was required to limit serum IgE concentrations and circulating basophil populations. These results suggest that altering the MAMP-derived signals by changing the composition of commensal microbiota by diet change and/or intake of probiotics may alter the signaling cascade via PRRs and thereby change the immune response to environmental antigens.

**Perspective**

Despite an enormous research effort, the processes that drive the development and maintenance of allergic inflammation in asthma are still far from clear. The studies reported in this thesis have indicated that in allergic asthmatics there is an enhanced threshold for LPS and probably other TLR-driven pathways to induce IL-10 and reverse a dominant Th2 response. It is of interest that the capacity to modulate Th2 responses by LPS is still manifest in established allergic individuals. Exogenous LPS in an ‘in vitro’ setting and probiotics in allergic asthmatic patients attenuated established allergen-driven responses. These results suggest that proper activation of innate responses may attenuate Th2 responses, warranting further studies with probiotics to limit sensitization and possibly reverse allergic inflammation.

Another issue that is quite apparent from the studies presented here is that the systemic compartment reflects many aspects of the allergic inflammation and also over a prolonged period, whereas this is less apparent locally in the lung. On the other hand, a-specific inflammation and coagulation run in parallel with allergic inflammation in the lungs. In fact, it appears that the aspecific inflammation and coagulation are far more pronounced in asthmatics in response to even lower doses of allergen than in controls. In recent challenge studies performed in our department the link between the circulation and the local responses has been substantiated further (van der Sluijs et al.). In view of this, further studies into the regulation of allergic inflammation by the circulation are warranted.
REFERENCES