Allergic asthma: Environmental factors challenging the immune system
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Uitnodiging

voor het bijwonen van de openbare verdediging
van het proefschrift:

Allergic asthma: environmental factors challenging the immune system

van

Marianne van de Pol

Dinsdag 2 juli 2013
om 10.00 uur

Agnietenkapel
Oudezijds Voorburgwal 231
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Allergic asthma: environmental factors challenging the immune system

Marianne van de Pol
Allergic asthma: environmental factors challenging the immune system

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ter verkrijging van de graad van doctor
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op gezag van de Rector Magnificus
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ten overstaan van een door het college voor promoties ingestelde
commissie, in het openbaar te verdedigen in de Agnietenkapel
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door

Marie Anna van de Pol

glezen te Barsingerhorn
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General introduction
INTRODUCTION

Asthma

Asthma is a chronic inflammatory disorder of the airways that is associated with airway hyperresponsiveness and variable airflow obstruction leading to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning\(^1\). Symptom-free periods are interrupted by periods of acute worsening of asthma (exacerbations), often caused by viral infections and/or increased exposure to allergens. In research, the diagnosis of allergic asthma is based on objective measures, including (1) lung function (reversibility in forced expiratory volume in 1 second (FEV\(_1\)), variability in peak expiratory flow), airway hyperresponsiveness (narrowing of the airways in response to triggers that have little or no effect in healthy individuals)\(^2\), (2) indirect measures of airway inflammation (nitric oxide in exhaled air, eosinophils and neutrophils and their soluble activation products (eosinophil cationic protein (ECP) and myeloperoxidase (MPO)) in induced sputum)\(^3\) and (3) responsiveness to allergens (the presence of positive skin-prick tests, levels of specific-immunoglobulin (Ig)E in serum, or the clinical or cellular response (in vivo and ex vivo) to common environmental allergens).

Although allergic sensitization in early childhood, through the induction of allergen-specific T helper (Th)2-type inflammation, is the major risk factor for children to become asthmatic, only 50% of adults who have asthma are atopic\(^4,5\). Wheezing-associated infections in the first 3 years of life with respiratory syncytial virus or with rhinovirus, which is also the main cause of asthma exacerbations in adults, are associated with the development of asthma\(^6\). Recent data have shown that also structural cells, such as airway epithelial cells and dendritic cells play an important role in the initiation and exacerbations of asthma, probably due to epigenetic changes induced by environmental factors\(^7,8\).

Allergy and asthma

Allergy is defined as a hypersensitivity reaction initiated by immunological mechanisms that can be antibody- or cell-mediated\(^9\). In the majority of cases the antibody responsible for an allergic reaction is IgE. Atopic individuals are genetically pre-disposed to become IgE-sensitized to common environmental allergens, to which everyone is exposed, but to which the majority does not produce a prolonged IgE-antibody response\(^9\). Atopy, or more specifically, sensitization to indoor allergens is strongly associated with the development of asthma\(^10,11\). For house dust mite (HDM), for example, the prevalence of sensitization and the development of asthma appears to be directly correlated with exposure\(^12,13\). Although the association between allergy and asthma is well established, the mechanisms responsible for the allergen-induced Th2-type inflammation and the initiation of asthma remain poorly understood.
Once sensitized, the antigens interact, upon inhalation of allergen, with allergen-specific IgE receptors, which are bound to the cell membrane of mast cells that are present in increased numbers in the lungs of sensitized subjects. After cross-linking the high-affinity IgE receptors, mast cells immediately start to release mediators (histamine, cysteinyl leukotrienes and prostaglandin D2) that induce typical allergic responses such as vasodilatation and smooth muscle contraction\textsuperscript{14}. This results in an acute bronchoconstriction (early asthmatic response, EAR), which is maximal 15-30 minutes after exposure and resolves in about 2 hours\textsuperscript{15}. At the same time many chemokines and cytokines, including the T helper(Th)-2 cytokines interleukin (IL)-4, IL-5 and IL-13, are released both locally and systemically\textsuperscript{16,17}. Whereas IL-4 and IL-13 are important for IgE secretion, IL-5 plays a pivotal role in the recruitment, activation and survival of eosinophils in the airways\textsuperscript{18}. These activated eosinophils subsequently release inflammatory mediators in the airways, such as eosinophil cationic protein (ECP), leukotrienes and major basic protein (MBP) that damage airway epithelial cells and thus promote the development of a second episode of bronchoconstriction (LAR), which occurs 3-8 hours after inhalation of allergen, and an increased airway hyperresponsiveness (AHR), which may last for up to several days or weeks\textsuperscript{19}.

\textbf{Environmental risk factors for the development of allergy and asthma}

Over the last century, the prevalence of allergic diseases has increased markedly\textsuperscript{20}, especially in industrialized countries. Although genetic factors mostly govern susceptibility to allergic diseases, the increase occurred within too short a time frame to be explained by genetic changes alone. In 1989, Strachan proposed the hygiene hypothesis\textsuperscript{21}, which stated that improved hygiene and better infection control reduced microbial exposure in early life and led to an insufficient microbial-induced Th1-immune response and thus an imbalance in the Th1/Th2 balance in favor of the pro-allergic Th2 response\textsuperscript{22}. Since, the hygiene hypothesis has been revised many times and comprises nowadays a complex interplay between innate and adaptive immune responses of the host, characteristics of symbiotic bacteria and infectious agents during early childhood, and the level and variety of environmental pollution and allergens\textsuperscript{23}.

Interestingly, the prevalence of asthma is reduced in children raised in a rural setting, which may be linked to the presence of higher levels of endotoxin in these environments\textsuperscript{24}. Endotoxin or lipopolysaccharide (LPS) is a cell wall component of Gram-negative bacteria, which is ubiquitous in our living environment. Several murine and human studies have demonstrated that the dose of endotoxin, present during allergen sensitization or allergen challenge was critical for the outcome of the immune response\textsuperscript{25}. Exposure to high doses of endotoxin during sensitization was associated with the
prevention of asthma and allergy, whereas low doses of endotoxin acted like an adjuvant and stimulated a Th2-mediated immune response\textsuperscript{26,27}. In established asthma, however, high doses of endotoxin are able to induce severe asthma exacerbations\textsuperscript{28}.

**Therapy**

Because many asthma patients respond to multiple factors in the environment, complete avoidance of these factors is usually impractical and very limiting to the patient. Thus medications to maintain asthma control have an important role because patients are often less responsive to environmental allergens and viruses when their asthma is well controlled\textsuperscript{1}. Current standard treatment is based mainly on suppression of airway inflammation with (inhaled) corticosteroids and relief of symptoms with bronchodilators. Nevertheless, these therapies only reduce symptoms and do not consistently alter the inflammatory processes in the airways of patients with asthma\textsuperscript{29}. The ‘hygiene hypothesis’ of asthma, has led to the suggestion that strategies to prevent allergic sensitization should focus on redirecting the immune response in early life toward a Th1, non-allergic response or on modulating T regulatory cells\textsuperscript{30}, but research in this area has just started and still requires further investigation.

**SCOPE OF THIS THESIS**

The role of environmental factors such as allergens, endotoxin and microbes in the development and maintenance of allergic asthma has been an area of great interest ever since the ‘hygiene hypothesis’ was proposed. Although it is well known that allergy is strongly associated with asthma, the specific mechanisms underlying this process are still not fully understood. With the studies presented in this thesis I aimed to elucidate some aspects of these mechanisms.

Allergic sensitization to common environmental allergens often occurs at early childhood, which makes it difficult to study the allergen-specific and nonspecific cellular responses during the development of allergy. Laboratory animal allergy, however, can be found in up to 30% of exposed workers and appears to be very similar to sensitization to common environmental allergens with regard to symptoms and immunologic mechanisms\textsuperscript{31,32}. Because these allergies usually develop within 2-3 years of exposure\textsuperscript{33,34} and the moment of first exposure is known, laboratory animal allergy provides an attractive model to investigate changes in cellular responses during the development of an allergy longitudinally. In chapter 2 we describe the dynamics in cytokine responses during the development of allergic sensitization to rats in a nested case-control setting. In a cohort of starting laboratory animal workers, allergen-specific and non-specific cytokine responses,
measured ex-vivo in blood samples from incident animal workers, who developed rat-specific sensitization during 2 years of follow-up, were compared to the cytokine responses in blood samples from control animal workers.

Besides exposure to allergens, also other environmental factors have been shown to be positively or negatively related to the development of allergy and asthma\textsuperscript{23,35}. Considering the observation that the dose of endotoxin present during allergen sensitization or allergen challenge is critical for the outcome of the immune response, we investigated in chapter 3 the cytokine response of peripheral blood mononuclear cells (PBMC) from allergic and non-allergic subjects to HDM to a very low dose of LPS (10 pg/ml) that is comparable with the daily indoor exposure to endotoxin and the endogenous endotoxin contamination in our HDM extract.

Experimental bronchial allergen challenge followed by sputum induction of bronchoalveolar lavage is often performed to provide insight into the cellular mechanisms of airway inflammatory processes caused by inhaled allergens\textsuperscript{36}. Although several studies concerning the safety and reproducibility of bronchial allergen challenge show that the airway-related symptoms of the early and late asthmatic response are transient and return to baseline within 2 weeks, little is known yet about any longer-lasting systemic effects of the challenge. In chapter 4 the allergen-specific B- and T-cell responses were investigated in blood samples from house dust mite (HDM)-allergic asthmatics that were collected 5 weeks after exposure to (high dose) HDM during a single bronchial challenge and compared with those in blood samples that were collected before the challenge.

The activated eosinophils that are recruited to the airways of sensitized asthmatics after allergen challenge have been proposed to play a role in local activation of coagulation in the airways\textsuperscript{37,38}. As coagulation and inflammation often act in parallel, the activation of coagulation within the airways of asthmatic patients is considered to aggravate local inflammation. To investigate the role of local coagulation activation on inflammatory processes and vice versa in the airways of allergic asthmatics, we compared, in chapter 5, the activation of coagulation in the bronchoalveolar space and the acute effect of a segmental allergen challenge hereon in asthmatic patients with the activation in healthy controls.

In search for new therapies to prevent and/or treat allergic diseases, modulation of the intestinal microbiota by the intake of probiotic bacteria has been proposed to alter, directly or indirectly, the immune response to allergens toward a non-allergic Th1 response. Although several clinical trials have successfully shown the preventive potential of probiotics on the development of allergic disease in young children, the few studies with probiotics in older populations with established allergic diseases have shown conflicting results\textsuperscript{39}. In chapter 6 we describe the results of a double-blind placebo-
controlled clinical intervention study, investigating the effect of supplementing synbiotics, a specific combination of prebiotic oligosaccharides and a probiotic *Bifidobacterium breve* strain, on the allergic responses in adults with established allergic asthma. In this study a bronchial allergen challenge was performed at study entry and after a 4 week treatment period to determine whether supplementing synbiotics would have a positive effect on allergen-induced bronchial inflammation, lung function and immunological parameters, as was previously observed in a mouse model.

The analysis of induced sputum is an important tool in the diagnosis of pulmonary disease and in pulmonary research to investigate the airway inflammatory cells and related mediators. Since the sputum plugs are coughed up from the airways and collected into a tube, certain contamination with saliva cannot be avoided. In chapter 7 we propose a new method to correct whole sputum data for saliva contamination, which improves the validity of cellular and molecular biomarkers, determined in whole induced sputum of asthma and COPD patients.

Finally, in chapter 8, the results of previously described studies are summarized and the implications for further research are discussed.

REFERENCES


Dynamics in cytokine responses during the development of occupational sensitization to rats

Esmeralda J.M. Krop, Marianne A. van de Pol, René Lutter, Dick J.J. Heederik, Rob C. Aalberse & Jaring S. van der Zee

Allergy 65 (2010): 1227-1233
ABSTRACT

Background: Occupational allergy forms an attractive model to study the development of allergic responses, as in some occupations it has a high incidence and develops quickly. In a cohort of starting laboratory animal workers, we previously found 20% sensitization to animal allergens within 2 years.

Methods: We compared cellular responses of incident laboratory animal workers who developed rat-specific sensitization (cases, n = 18) during 2 years of follow-up to control animal workers matched for atopic status but without sensitization after follow-up (controls, n = 18). Practically, this is a case–control study, nested within the cohort. Rat-specific IgE antibodies were measured in sera, and allergen-specific and nonspecific cytokine responses were measured in whole blood and in isolated peripheral blood mononuclear cells.

Results: Self-reported allergic symptoms were related to the presence of rat-specific IgE (p ≤ 0.01). Cases developed a rat allergen-specific interleukin (IL)-4 response during sensitization, while controls did not show an increased IL-4 response (at visit D: 33 vs 5 IL-4 producing cells/10⁶ cells, p < 0.001). The IL-4 response was related to the levels of rat-specific IgE in cases (visit D: rho = 0.706, p < 0.001). By contrast, allergen-specific IL-10 and interferon γ (IFNγ) responses as well as nonspecific cytokine responses were comparable between cases and controls.

Conclusion: This study is the first to show the development of an allergen-specific IL-4 response in adult human subjects during allergen-specific sensitization. This IL-4 response was quantitatively associated with the development of the specific IgE antibodies. Allergen-specific or nonspecific IL-10 and IFNγ responses showed no protective effect on the development of allergic sensitization.

INTRODUCTION

It is generally accepted that a shift in the balance of T-helper (Th) cell populations toward a polarized Th2 subset is occurring in atopic disease. The Th2-derived cytokine interleukin (IL)-4 is increased in cell cultures of patients with established atopic disease¹-⁴ and triggers B cells to produce allergen-specific Immunoglobulin (Ig)E⁵. IL-12 is suggested to be a crucial factor for Th1 cell polarization⁶. The Th1 cell-derived cytokine interferon γ (IFNγ) was shown to reverse Th2 skewing in vitro⁷, and it is decreased in atopics compared to controls⁴,⁸. IL-10, produced by monocytes as well as regulatory T cells, was also shown to be decreased in atopics¹,⁸.

Allergic sensitization to environmental allergens most often occurs at young age making it difficult to study cellular immunologic events longitudinally. Cross-sectional and longitudinal studies of birth cohorts have provided insight into the significance of cytokine
responses of allergic disease\textsuperscript{1,3,4,8}. However, these results are often conflicting and differ with age and most studies had cross-sectional designs and focused on the difference between allergic individuals and controls. Therefore, changes in cellular responses and accompanying cytokine levels during the development of allergic sensitization remain largely unknown.

Laboratory animal allergy provides an attractive model to investigate changes in cytokine levels during the development of sensitization in a longitudinal design. It can be found in up to 30% of exposed workers\textsuperscript{9-12}. Occupational allergy appears to be very similar to sensitization to common environmental allergens with regard to symptoms and immunologic mechanisms and atopy is the major risk factor\textsuperscript{13}. The majority of sensitized workers develop sensitization within the first 2–3 years of exposure\textsuperscript{10,11}.

We conducted a prospective cohort study on starting laboratory animal workers in the Netherlands\textsuperscript{14}. During a two-year follow-up, 18 laboratory animal workers developed sensitization to rats. The aim of the present study was to investigate in a nested case–control setting whether sensitization to rats was preceded or accompanied by changes in the levels of cytokines in vitro.

METHODS
Study design
Our study population consisted of 36 selected apprentice laboratory animal workers from a previous described cohort of apprentice laboratory animal workers\textsuperscript{14}. In short, the cohort was followed for 2 years and development of animal specific allergic sensitization was monitored. During the 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). Blood was taken at each visit and participants completed a questionnaire based upon a previously described questionnaire on occupational allergy containing detailed questions concerning allergic symptoms, animal contact, and smoking\textsuperscript{15}.

In the present study nested within the cohort, we compared 18 workers who developed rat-specific sensitization during the 2-year follow-up (cases) to 18 workers (controls) matched by atopic status but without sensitization after the 2 years of follow-up. Development of rat-specific sensitization was determined by skin prick testing with rat allergens and development of rat-specific IgE as detected in serum. Cases developed a positive skin prick test, a positive IgE test or both. Cases and controls were matched for atopic status determined by the presence of specific IgE antibodies against common allergens.
Testing for atopy and sensitization

Rat urinary allergens, positive control (histamine 10 mg/ml) and negative control (ALK Abello, Nieuwegein, the Netherlands) were used for skin prick testing at visits A, C and D. Rat-specific tests were performed with previous described urine extracts dissolved in negative control solutions. Skin prick tests were read after 15 min and were considered positive if the wheal diameter was at least 3 mm and redness was present. Additionally, the negative control had to have no erythema and a wheal diameter of maximal 1 mm.

Atopy was defined as having at least one positive IgE test for common allergens. Specific IgE antibodies against common allergens including house dust mite, grass pollen, tree pollen, cat dander, dog dander, guinea pig dander, rabbit dander and horse dander and the occupational allergen rat urine were determined by radioallergosorbent testing as previously described. For quantifying rat urine-specific IgE, the same urine extract was used as for skin prick testing. Results were expressed in International Units IgE per milliliter (IU/ml). The detection limit was 0.15 IU/ml and levels of specific IgE above 0.35 IU/ml were considered positive.

Exposure to rat allergens

Exposure was assessed in the animal facilities and a personalized exposure to rat allergens was estimated as previously described. Cumulative exposure was obtained by multiplying the personalized exposure by the time exposed as reported in the questionnaires.

Whole blood culture (WBC)

Heparin-containing blood was diluted 10 times in Iscove’s modified Dulbecco’s medium (IMDM; BioWhittaker, Verviers, Belgium) supplemented with 0.1% FCS and 30 U/ml heparin (Leo Pharmaceutical products B.V., Weesp, the Netherlands). Blood was stimulated with Staphylococcus aureus Cowan I strain (SAC, 75 µg/ml; Calbiochem, Darmstadt, Germany) or lipopolysaccharide (LPS, 1 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) both in the absence and in the presence of recombinant IFNγ (100 U/ml; U-Cytech, Utrecht, the Netherlands) at 37°C. Supernatant was harvested after 24 h and was stored at -20°C until analysis.

Levels of IL-4, IL-6, IL-10, IL-12 and IFNγ in supernatant were determined with a Bioplex assay (Bio-Rad Laboratories, Hercules, CA, USA). The assay was performed according to manufacturer’s protocol. IL-12 was measured in WBC with IFNγ, other cytokines were measured in the cultures without IFNγ.
Enzyme linked immunospot assay (ELISpot)

Cytokine production after allergen stimulation of peripheral blood mononuclear cells (PBMCs) with rat allergen was studied with ELISpot technique. PBMCs were isolated from heparin-containing blood samples using standard density gradient centrifugation techniques and subsequently cryopreserved. For the assay, PBMCs were thawed, washed twice and diluted to a concentration of 4x10^5 cells/ml in tissue culture medium (RPMI 1640; Gibco BRL, Life Technologies Ltd, Paisley, UK) in the absence or presence of rat urinary proteins (same material as used in skin prick testing and IgE assay, 42 µg/ml) or phytohaemagglutinin (PHA, 0.1 µg/ml, positive control) in round bottom tubes (Micronic, McMurray, PA, USA). Cells were preincubated at 37°C for 4 h. Multiscreen filter plates (Millipore, Billerica, MA, USA) were coated with IL-4-, IL-10- or IFNγ-specific antibodies (Mabtech, Nacka Strand, Sweden) as described in the manufacturer’s protocol. Preincubated cells were subsequently incubated on the coated microplates (IL-4: 2x10^5 cells/well; IL-10: 5x10^4 cells/well; IFNγ: 2x10^4 cells/well) for 72 h. Plates were washed and cytokine production by PBMC was quantified by incubating the plates with biotinylated secondary antibodies according to the manufacturers protocol. Spots were counted using ELISpot analysis software (A.EL.VIS GmbH, Hannover, Germany). All results were expressed as spots per 10^6 cells.

Statistical analysis

SPSS version 11.5 Statistics UK (Chicago, IL, USA) was used for statistical analysis. Antibody levels and cytokine levels from WBC were evaluated in terms of their log values. Values below the detection limit were allotted the value of half the detection limit. Differences between the cases and controls were tested with Student’s t-test, ANOVA, chi-square or Mann–Whitney test depending on distribution. Parametric correlations between variables were expressed as Spearman correlation coefficients (rho). Relations between symptoms and other variables were determined by linear and multiple logistic regression. All reported P-values were two-tailed, and P-values of <0.05 were considered statistically significant.
RESULTS

Study population

Characteristics of the cases and controls are in table 1. No significant differences were found between cases and controls but controls tended to have worked for a longer time with rats while the cases tended to have higher exposure to rat allergens. One case was lost to follow up after he developed a rat-specific IgE response at visit B. One control did not donate blood at visit D and one case did not donate blood at visit B. Rat-specific skin prick test results increased in cases from 0 mm at visit A to 7 mm (range: 0-23 mm) at visit D \((p < 0.001)\) and was significantly different from controls at visit C and D (both \(p < 0.001\)). Rat-specific IgE was <0.35 IU/ml for all participants at the start of the study. It increased over time in cases and was significantly higher in cases at visit B, C and D \((p > 0.01)\). Results from at-specific skin prick test and IgE measurements in serum correlated highly in the cases (visit C: \(\rho = 0.688, p = 0.002\); visit D: \(\rho = 0.872, p < 0.001\), fig. 1). Twelve cases developed rat-specific IgE levels above 0.35 IU/ml.

### Table 1. Characteristics of the nested case-control study

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Cases</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>n=18</td>
<td>n=18</td>
<td>1.000</td>
</tr>
<tr>
<td>Age (mean, years)</td>
<td>26</td>
<td>24</td>
<td>0.475</td>
</tr>
<tr>
<td>Atopy</td>
<td>15 (83%)</td>
<td>15 (83%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Total IgE (IU/ml, GM, range)</td>
<td>59 (9-398)</td>
<td>103 (3-1288)</td>
<td>0.175</td>
</tr>
<tr>
<td>Exposure to rats in previous job (months, mean, range)</td>
<td>3.9 (0-24)</td>
<td>3.3 (0-15)</td>
<td>0.767</td>
</tr>
<tr>
<td>Exposure to rats in current job at visit A (months, mean, range)</td>
<td>7.3 (0-18)</td>
<td>4.1 (0-10)</td>
<td>0.074</td>
</tr>
<tr>
<td>Mean exposure to rat allergens/month (ng eq/m^3 * hours/month, GM, range)</td>
<td>101 (1-11220)</td>
<td>526 (1-8913)</td>
<td>0.059</td>
</tr>
<tr>
<td>Cumulative rat exposure during follow up (ng eq/m^3 * hours, GM, range)</td>
<td>2914 (35-251189)</td>
<td>7530 (26-162181)</td>
<td>0.238</td>
</tr>
</tbody>
</table>

GM, Geometric mean; ng eq/m^3, nanogram of equivalent animal urinary proteins per cubic meter.
Cytokine responses in occupational sensitization

Whole blood culture

Unstimulated WBC

During the first year of follow-up (visit B and C), IL-12 remained significantly lower for cases compared to controls (34 pg/ml vs 17 pg/ml at visit B, \( p = 0.006 \)). After 2 years of follow-up, none of the cytokines measured showed significant differences between cases and controls in the unstimulated culture. We did find a decline in IL-10 between the last visit before development of sensitization and the first visit after sensitization in cases but this did not reach statistical significance (335-212 pg/ml, \( p = 0.059 \)).

SAC- and LPS-stimulated WBC

Results from the stimulated WBC for all cytokines are in table 2. In stimulations with SAC, IL-4 increased slightly but significantly in controls during follow-up (from 132 to 161 pg/ml, \( p = 0.011 \)) and was higher in controls compared to cases at visit B, C and D (\( p = 0.02, p = 0.03, p = 0.07 \), respectively). None of the other cytokines showed ongoing significant differences in production between cases and controls during follow-up.

Mean IL-10 levels after SAC stimulation during follow-up were negatively related to rat allergen-specific IgE production at the last visit (\( r = -0.449, p = 0.006 \)) and to mean skin prick test (SPT) results during follow-up (\( r = -0.357, p = 0.032 \)).

For LPS-stimulated WBC, we found no significant differences in cytokine production between cases and controls at any time point nor did we find changes in cytokine production over time.

Rat-specific stimulation of isolated mononuclear cells

To determine rat allergen-specific cytokine production by PBMC, ELISpots were performed. For IL-4, no significant differences were found in the number of IL-4 producing cells (spots) in the unstimulated cultures between cases and controls. For PHA stimulation (positive control), we found that cases had significantly more spots than controls only at visit C (1155 vs 835 spots/10^6 cells, \( p = 0.02 \)). At the start of the study, there was no difference in rat-specific IL-4 spots between cases and controls, but during follow-up, the number of rat-specific IL-4 spots increased in cases and was significantly different from controls (B: \( p = 0.03; C: p = 0.02; D: p < 0.001 \), fig. 2A). There was a significant increase in rat-specific IL-4 spots between the last time point before and the first time point after sensitization became apparent in cases (10 vs 35 spots/10^6 cells, \( p = 0.03 \)). The number of rat-specific IL-4 spots correlated with the amount of rat-specific IgE of cases at visit B (\( \text{rho: 0.480, } p = 0.004 \)), C (\( \text{rho: 0.429, } p = 0.01 \), fig. 3) and D (\( \text{rho: 0.706, } p < 0.001 \), Fig. 3), but not at the start of the study or in controls.
Table 2. Cytokine responses in the whole blood culture (WBC) stimulated with *Staphylococcus aureus* Cowan I strain (SAC) or lipopolysaccharide (LPS)

<table>
<thead>
<tr>
<th>Visit</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAC-stimulated WBC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>Cases</td>
<td>131</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>135</td>
<td><strong>165</strong></td>
<td><strong>167</strong></td>
</tr>
<tr>
<td>IL-6</td>
<td>Cases</td>
<td>23099</td>
<td>13095</td>
<td>22408</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>25055</td>
<td><strong>49454</strong></td>
<td>22331</td>
</tr>
<tr>
<td>IL-10</td>
<td>Cases</td>
<td>246</td>
<td>256</td>
<td>248</td>
</tr>
<tr>
<td></td>
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Results are expressed in pg/ml as geometric means. Bold results are significantly higher (p < 0.05). IFNγ, interferon γ; IL, interleukin.

*Cultured in the presence of IFNγ.

Figure 2. IL-4-, IL-10- and IFNγ-specific spots in ELISpot of cases and controls after stimulation with rat allergen. Visits are on the x-axes. (A) IL-4 spots in cases significantly increase during follow-up. At the first visit, there is no significant difference between cases and controls but after follow-up the difference in number of spots increases (*p < 0.05, **p < 0.001). (B) IL-10-specific spots after stimulation with rat allergen show no significant differences between cases and controls. IL-10 spots declined in controls between visits C and D (p = 0.023). (C) IFNγ-specific spots after stimulation with rat allergen are not significantly different between cases and controls. At the beginning of follow-up, there is a significant increase in IFNγ spots followed by a reduction in both cases and controls.
No significant differences between cases and controls were found in IL-10 spots, neither in the unstimulated culture, nor after PHA stimulation or after rat-specific stimulation (Fig. 2B). No significant changes in IL-10 were found during follow-up in cases, but controls showed a small but significant decline in rat-specific IL-10 spots from visit C to D ($p = 0.02$, fig. 2B). If results from the last visit before sensitization and the first with a positive allergy test were considered, we found a significant decrease in IL-10 spots in the unstimulated culture only ($p = 0.03$). IL-10 spots in unstimulated cultures, in PHA cultures or in rat-specific cultures showed no relationships with rat-specific IgE.

For IFN$\gamma$ spots, no differences were found between cases and controls in the unstimulated culture or after stimulation with PHA or rat allergen. There was a significant increase in rat-specific IFN$\gamma$ spots in cases (A-C: $p = 0.001$) and in controls (A-C: $p = 0.03$) in the first year of follow-up (fig. 2C). After the first year of follow-up, the rat-specific IFN$\gamma$ spots declined (cases: C-D: $p = 0.006$; controls: C-D: $p = 0.08$, fig. 2C). No relationships were found between IFN$\gamma$ spots and rat-specific IgE. No relations between ELISpot results and cytokine responses in WBC were found.

**Self-reported allergic symptoms**

Allergic symptoms were mainly reported by cases (table 3). Reporting work-related allergic symptoms was related to SPT results at visit C ($p = 0.01$) and visit D ($p < 0.001$). Symptoms were also related to the level of IgE at visits B ($p = 0.002$), C ($p = 0.002$) and D ($p < 0.001$) but not to WBC results. At visit C and D, the number of IL-4 spots in the rat-specific ELISpot were related to symptoms ($p = 0.02$ and $p < 0.001$, respectively). In a multiple logistic regression model, specific IgE related to symptoms at both time points, but rat-specific IL-4 spots or SPT results did not contribute independently to the model.

**DISCUSSION**

This cohort study on the development of occupational allergy provided a unique opportunity to study cellular responses during the development of allergic sensitization in a nested case-control setting. We showed the development of a rat allergen-specific IL-4 response that was quantitatively related to the development of rat-specific IgE antibodies. By contrast, allergen-specific IL-10 and IFN$\gamma$ responses and nonspecific cytokine responses were similar for sensitized animal workers and nonsensitized workers.

In this nested case-control study, we matched controls by atopic status, the best known risk factor for the development of laboratory animal allergy$^{9,12,14}$. Because of the matching of atopic status and the low percentage of nonatopics (17%) in this study,
Table 3. Self-reported work-related allergic symptoms

<table>
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<tr>
<th>Visit</th>
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<th>Cases (n=18)</th>
<th>Controls (n=18)</th>
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<td>ns</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Nose</td>
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Figure 3. Relation between rat-specific IgE and rat-specific IL-4 spots in ELISpot in cases (A) and controls (B). There is a correlation between rat-specific IgE and rat-specific IL-4 spots at visit C (●) and at visit D (○) in cases but not in controls. *p ≤ 0.001.

Differences between atopics and nonatopics during sensitization could not be studied. The study focuses, therefore, on differences between atopics that became sensitized and nonsensitized atopics.

Because of the design of the study with only four sampling time points during 2 years of follow-up, we may have missed short-term changes in cytokine responses or antibody levels. We matched cases with negative controls at the end of follow-up. This maximized the contrast between cases and controls. However, it cannot be ruled out that prolonged follow-up would have resulted in the development of sensitization in some controls. This may have resulted in underestimation of differences between lifelong negative controls and sensitized individuals.

We found a negative correlation between SAC-induced IL-10 production and development of IgE. This cytokine produced by monocyte, regulatory T cells, and possibly
by a subset of memory B cells was suggested to interfere in production of IgE. However, we did not find a protective effect of rat allergen-induced IL-10. Possibly, the protective effect of IL-10 occurs in an allergen-independent manner. IL-10 also enhances the IgG4 production by B cells. IgG4 antibodies may have a protective effect on IgE sensitization or development of allergic symptoms. However, we found no relation between IgG4 antibodies to rat allergens and IL-10 responses (data not shown). Likewise, there was no relation between symptoms and IL-10 levels in WBC or ELISpot.

Van der Pouw Kraan showed that IL-12 levels in WBC were significantly lower in allergic patients compared to controls. We found no consistent differences in IL-4, IL-6, IL-10, IL-12 or IFNγ levels in WBC between cases and controls. However, matching of controls on atopic status may have reduced the differences reported for atopics and nonatopics.

It was suggested in literature that allergen-specific IL-10 or IFNγ responses could protect against the development of allergic sensitization. Our ELISpot results showed no significant difference in allergen-specific or nonspecific IFNγ responses between cases and controls. This suggests that there is no significant role for allergen-specific T-helper 1 cells in the protection against development of sensitization in the population studied. The same was found for IL-10 producing cells.

Our study is the first in which development of an allergen-specific Th2 response in human adults during sensitization was followed. Differences in allergen-specific IL-4 production between allergic patients and nonallergic controls were shown before. However, from those studies, it remained unclear when these differences in IL-4 response developed in time. We showed a quantitative relation between the allergen-specific IL-4 response and development of IgE antibodies. This is in line with the assumption that an increased antigen-specific IL-4 response is the main factor responsible for the production of antigen-specific IgE antibodies. It was suggested in literature that IL-4 responses in nonallergic subjects could lead to IgG4 responses instead of IgE responses. However, we found no evidence for such a protective mechanism in our study.

In contrast to the rat allergen-induced IL-4 response, SAC-induced IL-4 responses in WBC were higher in controls than in cases. This indicates that the increased response in cases is highly allergen-specific. Self-reported allergic symptoms were related to allergen-specific IgE serum levels and to allergen-specific IL-4 spots. SPT appeared to be more sensitive compared to specific IgE tests in our study. However, IgE results showed the best relation to self-reported work-related allergic symptoms. Prolonged follow-up may have resulted in more IgE, more IL-4 spots and/or more symptoms in the cases that were only SPT after 2 years of follow-up.

A correlation between IL-4 production from WBC and ELISpot was lacking. Likewise, we found no relations between the other cytokines as detected in the ELISpot and in the
WBC. In the WBC, we used nonspecific stimuli but cells were stimulated with occupational allergens in the ELISpot. The results from the WBC are therefore a reflection of the overall immune response, while the ELISpot results show the allergen-specific immune reaction. The fact that more cell types are present in whole blood than in isolated PBMC may have contributed to the different cytokine production pattern.

In conclusion, we showed that development of rat allergen-specific IL-4 responses in PBMCs of sensitized rat workers in a nested case-control study of laboratory rat workers was the main change in the cytokine production profile. This IL-4 response was quantitatively related to the development of an allergen-specific IgE response against rat allergens and to occupational allergic symptoms.

REFERENCES


Very low doses of endotoxin reveal differential cytokine responses between peripheral blood mononuclear cells from allergic asthmatics versus healthy individuals

Marianne A. van de Pol, Tamara Dekker, Barbara S. Smids, Esmeralda J.M. Krop, Jaring S. van der Zee, René Lutter

*Manuscript in preparation*
Chapter 3

ABSTRACT

Introduction: Multiple epidemiological studies suggest an inverse association between the development of T helper (Th)2-driven allergic inflammation and (early-life) exposure to endotoxin (lipopolysaccharide (LPS)), which is ubiquitous in our living environment. Most *in vitro* studies, investigating the underlying mechanisms, are performed with relatively high doses of LPS, as compared to daily airborne exposure.

**Aim:** To investigate the Th1/Th2-cytokine response of peripheral blood mononuclear cells (PBMC) from house dust mite (HDM)-allergic and non-allergic subjects to very low doses of LPS that are comparable to that by airborne indoor exposure.

**Methods:** PBMC from 29 allergic asthmatics and 30 non-allergic controls, matched by age and gender, were stimulated with very low doses of LPS (10 and 100 pg/ml). The numbers of cells producing Th1- and Th2-cytokines were determined in ELISPOT assays and cytokine production was quantified in 24 h culture-supernatant by luminex assays.

**Results:** PBMC from allergic subjects produced significantly less IL-10, IL-12p40 and IFNγ to LPS 10 pg/ml, compared to those from non-allergic asthmatics (p < 0.001). Interestingly, allergic subjects also had less IL-10 producing cells/10⁵ cells (mean (se): 101.7 (20.2) vs. 182.9 (23.9), p < 0.001). This differential cytokine production was overcome by exposure to LPS 100 pg/ml, leading to an equally pronounced IL-10, IL-12p40 and IFNγ production by PBMC from both groups. LPS 100 pg/ml also reduced HDM-stimulated production of Th2 cytokines by PBMC from allergic subjects (-69%, p < 0.001 for IL-5, -31%, p = 0.15 for IL-13).

**Conclusion:** There is an enhanced threshold at very low doses of LPS (10 pg/ml, comparable with indoor LPS exposure) in PBMC from allergic subjects that prevents induction of mediators that can inhibit the production of Th2 cytokines in response to HDM. We postulate that this threshold may contribute to sensitization by allergens.

INTRODUCTION

Allergic asthma is characterized by T helper (Th)2-driven, often eosinophilic inflammation of the airways, with airway hyperresponsiveness (AHR) and recurrent episodes of shortness of breath with variable airflow obstruction¹. The Th2-driven inflammation is characterized by the cytokines interleukin (IL)-4, IL-5 and IL-13 that are responsible respectively for the secretion of IgE, activation and recruitment of eosinophils, and airway remodeling, leading to the pathology characteristic of asthma²,³. Allergic asthmatics have been sensitized to airborne allergens via initiation of a type 2 immune response, which mechanistically is still not fully elucidated⁴. A complex interplay between genetic and environmental factors appears to determine sensitization to allergens.
Epidemiological studies have revealed an inverse association between the development of allergic diseases and (early-life) exposure to endotoxin or lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria, which is ubiquitous in our living environment. In established asthma, however, exposure to LPS is known to aggravate the allergen-induced inflammation. In addition, several studies have demonstrated that the dose of LPS present during allergen sensitization or allergen challenge is critical for the outcome of the immune response. For example, inhalation of low levels of LPS together with allergen induces a Th2-type response with eosinophilic infiltration in the airways. However, inhalation of high levels of LPS led to a Th1 response (characterized by the cytokines IL-12 and interferon (IFNγ)), dampening the Th2-driven inflammation and inducing neutrophilic inflammation. Thus, the effect of LPS exposure on allergic sensitization and asthma appears to depend on the presence or absence of preexisting asthma and the timing and dose of LPS exposure.

Most of the abovementioned studies were performed with concentrations of LPS comparable with LPS levels measured in settled dust samples from floors and mattresses in the homes of allergic and non-allergic subjects, varying from <3000 to >180,000 EU/g dust. These levels are very high compared to the airborne indoor LPS exposure (0.58-1.49 EU/m³) to which the airways of, for example, young children are exposed most of the day. Assuming a daily breathing rate of 3.6 m³/day for a healthy infant aged 0-2 month to 12.1 m³/day for a healthy child aged 5 years, the daily indoor exposure to LPS varies from 2.1 to 18.0 EU/day.

This has led us to propose that antigen-presenting cells from allergic versus non-allergic individuals respond differently to very low concentrations of LPS. We assessed the dose-dependent effect of LPS including a LPS dose that reflects indoor exposure, on the (allergen-induced) cytokine response of peripheral blood mononuclear cells (PBMC) from well-characterized allergic asthmatics and non-allergic healthy volunteers.

**METHODS**

**Subjects**

In a previously described clinical intervention study, we collected blood at baseline from 29 adult allergic patients with intermittent to mild persistent asthma, according to American Thoracic Society criteria. All patients were sensitized to HDM, as determined by skin prick test and IgE to *D. pteronyssinus* > 0.5 kU/L. Blood from 30 non-allergic healthy volunteers, was obtained at the start of another previously described, longitudinal cohort study of apprentice laboratory animal workers. The non-allergic volunteers were matched by age, gender and the presence of pets at
home, had less than 18 months of occupational contact with animals at the start of the study and developed during the 2-year follow-up no sensitization to the animals with which they were working. All subjects gave written informed consent. Both studies were approved by the AMC Medical Ethics Committee.

**Isolation of PBMC from blood samples**

Heparin blood was collected at the start of aforementioned studies. PBMC were isolated within 6 hours by standard density gradient techniques and stored in aliquots in liquid nitrogen until analysis.

**Cell stimulation assays**

We performed ELISPot assays to determine the number of cells producing Th1- and Th2-cytokines and quantified the cytokine production in 24 hours-culture supernatant in a Luminex assay as described in detail earlier\(^17\), with minor modifications. Briefly, cryopreserved PBMC were thawed, washed and incubated in culture medium with phytohemaglutinin (PHA, 0.1 \(\mu\)g/ml) as a positive control, LPS (10 pg/ml or 100 pg/ml), HDM (1 \(\mu\)g/ml) or medium alone. The biological activity of LPS 10 pg/ml (Sigma-Aldrich, St.Louis, MO, USA) was 1 EU/ml (determined in Limulus amebocyte lysate (LAL)-assay) and equalled the amount of LPS contamination in our HDM extract (1 EU/\(\mu\)g). We used cytokine-specific caption and detection antibodies for IL-4, IL-13, IL-10, IL-12p40 and IFN\(\gamma\) from Mabtech AB (Nacka Strand, Sweden), according to the manufacturer’s protocol, and incubated the cells at 37°C and 5% CO\(_2\) for 72 hours in 96-wells PVDF-based membrane plates (Millipore, Billerica, MA, USA). After visualization of the spots with streptavidin-ALP and BCIP/NBT substrate (Sigma-Aldrich), the numbers of cytokine-producing cells were determined by ELISpot analysis software from A.EL.VIS GmbH (Hannover, Germany) and were expressed as positive cells per 1x10\(^5\) stimulated cells.

For quantification of the cytokine production, we stimulated PBMC in round-bottom 96-well plates for 24 hours with the same culture medium and stimuli as used in the ELISpot assays. The amount of several cytokines (IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-13 and IFN\(\gamma\)) was determined in culture supernatant in a Luminex assay with antibodies from Biosource (Camarillo, CA, USA).

Responses from PHA-stimulated cells were treated as positive control and medium-only culture as negative control. All cytokine responses from stimulated cultures were adjusted for background by subtracting the responses from the medium-only culture. Analyses were performed batch-wise to limit inter-assay variation. In addition, we took along control PBMC, which yielded similar results each time.
Flow cytometric analysis

Cryopreserved PBMC were thawed, washed and incubated (10⁶/ml) overnight in culture medium with HDM 1 μg/ml, LPS (10 pg/ml or 100 pg/ml) or medium alone. After washing with cold buffer (PBS containing 0.5% (w/v) bovine serum albumin (Sigma) and 2 mM EDTA (Merck), cells were incubated for 5 min with IL-10-specific catch reagent (IL-10 secretion assay (PE), Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in cold medium, according to manufacturer’s instructions. Subsequently, the cells were resuspended in warm medium and incubated for 45 min in closed tubes at 37°C allowing them to secrete IL-10. After washing with cold buffer, IL-10 detection antibody (PE) and different combinations of the following surface markers were added and incubated for 20 min on ice: CD3 FITC, CD5 APC, CD8 PerC-Cy5.5, CD25 APC, CD16+56 FITC (all from BD), CD4 APC-AF780, CD14 PE-Cy7 and CD19 APC-AF780 (all from eBioscience). Then, cells were washed with cold buffer and analyzed by FACSCanto (BD Biosciences) multicolor flow cytometry and FlowJo software (Tree Star Inc.).

Statistical analysis

SPSS version 15.0.1 was used for statistical analysis (SPSS Inc., Chicago, IL, USA). We used Wilcoxon signed rank test to compare the cytokine responses of different stimuli within subjects. Differences between the allergic and non-allergic group were analyzed with the student’s t-test. When data were not normally distributed, either log transformation was performed (PC₂₀ methacholine, total IgE) to get normal distribution or the non-parametric Mann-Whitney U test was used (cytokine responses). Correlations were determined with the Spearman rank test. Two-sided p-values of <0.05 were considered statistically significant.

RESULTS

Subjects

Thirty healthy non-allergic subjects from the apprentice laboratory animal workers cohort were matched with the 29 subjects from the allergic asthmatic group to minimize differences in daily environmental allergen- and LPS exposure. Subject characteristics are described in table 1. As expected, the forced expiratory volume in 1 second (FEV₁) and provocative concentration of methacholine causing a decline in FEV₁ of 20% from baseline (PC₂₀) of the allergic asthmatic group were significantly lower than those of the healthy group whereas the amount of total immunoglobulin E (IgE) in serum was significantly higher in the allergic asthmatic group.
Table 1. Subject characteristics

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<td>30</td>
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<td>12 / 18</td>
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<td>age (years) #</td>
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<td>24 (18; 40)</td>
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<td>FEV1 (% predicted) ‡</td>
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<td>105.4 (2.0)</td>
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<tr>
<td>PC_{20} methacholine (mg/ml) †</td>
<td>1.38 (1.3)</td>
<td>&gt;8.0</td>
<td>&lt;0.001</td>
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<tr>
<td>Total IgE (kU/L) †</td>
<td>223.2 (1.2)</td>
<td>19.9 (1.2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are expressed as #median (range), †geometric mean (SE), ‡mean (SE). FEV1, forced expiratory volume in 1 second; PC_{20} methacholine, provocative concentration of methacholine causing a decline in FEV1 of 20% from baseline; IgE, immunoglobulin E.

**Impaired induction of cytokines in PBMC from allergic asthmatics in response to indoor airborne concentrations of LPS**

Stimulation of PBMC from allergic asthmatics and non-allergic controls with indoor airborne concentrations of LPS (10 pg/ml) resulted in a significantly lower level of IL-6, IL-10, IL-12p40 and IFNγ in the supernatant of PBMC from the allergic asthmatics compared to those from the non-allergic subjects (p < 0.001, fig. 1A). A ten-fold higher dose of LPS (100 pg/ml) induced a marked increase of these cytokines in both groups and diminished the difference between the groups (fig. 1B). Interestingly, except for the number of IL-10-producing cells after stimulation with the lowest dose of LPS, no difference between the groups was observed for the numbers of cells producing these cytokines (fig.1C, D). The production of IL-4 and IL-5 was not influenced by either dose of LPS (data not shown). In contrast, the highest dose of LPS (100 pg/ml) induced a low number of IL-13-producing cells and very low amounts of IL-13. Both were significantly higher in PBMC from allergic asthmatics compared to those from the non-allergic subjects (mean number of IL-13-positive cells/10^5 cells (SE): 6.7 (1.4) vs. 1.3 (0.5), p < 0.001; mean IL-13 production (SE): 1.31 (0.38) pg/ml vs. 0.53 (0.25) pg/ml, p < 0.05). Overall, stimulation with LPS predominantly induced a Th1-like response in PBMC from both groups.

**LPS reduces HDM-induced Th2 cytokine production in allergic asthmatics**

Addition of a low dose of LPS (100 pg/ml) to HDM-stimulated PBMC from allergic asthmatics, reduced the production of Th2 cytokines (-69%, p < 0.001 for IL-5, -31% for IL-13, p = 0.15, IL-4 levels were below detection limit). At the same time, the production of IL-10, IFNγ and IL-12p40 was significantly increased (fig. 2A). A similar Th2-reducing and Th1- and IL-10-increasing effect of LPS was observed for the number of cytokine-
Differential cytokine responses to low dose LPS in allergic asthmatics

Figure 1. Impaired induction of cytokines in allergic asthmatics in response to indoor concentrations of LPS. PBMC from allergic asthmatics (open bars) and non-allergic healthy subjects (grey bars) were stimulated with LPS 10 pg/ml (A, C) and LPS 100 pg/ml (B, D). Levels of IL-6, IL-10, IL-12p40 and IFNγ, measured in 24 h culture supernatant are shown in figures (A) and (B). Figures (C) and (D) represent the number of cells producing IL-10, IL-12 or IFNγ per 100,000 cells, determined in ELISPOT assays. Mean values (SE) are shown and values are corrected for medium control. ** p<0.01, *** p<0.001.

Figure 2. LPS reduces HDM-induced Th2 cytokine production in allergic asthmatics. (A) Cytokine production after 24 hours by PBMC from allergic asthmatics that were stimulated with HDM (1 μg/ml) in the absence (open bars) or presence (black bars) of LPS (100 pg/ml, grey bars). (B) Number of cytokine-producing PBMC from allergic asthmatics that were stimulated with HDM (1 μg/ml, open bars) in the absence (open bars) or presence (black bars) of LPS (100 pg/ml, grey bars) for 72 hours in an ELISPOT assay. Mean values (SE) are shown and values are corrected for medium control. Significant changes from HDM-stimulated values by addition of LPS are marked with ***p<0.001.
producing cells (fig. 2B). Furthermore, PBMC from allergic asthmatics produced less IL-10, IL-12p40 and IFNγ in response to HDM (1 μg/ml, containing 10 pg LPS), compared to those from non-allergic subjects. Addition of LPS (100 pg/ml) to these HDM-stimulated PBMC abrogated the observed difference in cytokine response and led to an equally pronounced IL-10-, IL-12p40- and IFNγ-production by PBMC from both groups (fig. 3). Because LPS-induced IL-10 production by whole blood cultures of allergic asthmatics has been shown to play a role in the reduction of Th2 cytokines by high doses of LPS²⁰, we looked more closely to the IL-10 producing cells in our study.

**Figure 3.** Addition of LPS to PBMC from allergic asthmatics leads to cytokine responses comparable with those from non-allergic subjects. Cytokine production by PBMC from allergic asthmatics (open bars) and non-allergic healthy volunteers (grey bars) that were stimulated with HDM (1 μg/ml, contaminated with 10 pg LPS) in the absence or presence of LPS (100 pg/ml). Mean values (SE) are shown and values are corrected for medium control.

**Stronger IL-10 response to indoor airborne levels of LPS in non-allergic subjects**

PBMC from non-allergic subjects, stimulated with low (100 pg/ml) and very low (10 pg/ml) doses of LPS, produced significantly more IL-10 than those from allergic asthmatics (mean (se): 37.06 (9.74) pg/ml vs. 3.43 (1.08) pg/ml, p < 0.001 for LPS 10 pg/ml and 368.0 (56.72) pg/ml vs. 164.5 (22.15) pg/ml, p = 0.006 for LPS 100 pg/ml, fig. 4A). Moreover, stimulation with the lowest dose of LPS induced significantly more IL-10-producing cells in PBMC from the non-allergic subjects compared to those from the allergic subjects (mean (se): 148.6 (20.4) /10⁵ cells vs. 81.7 (17.7) /10⁵ cells, p = 0.002, fig. 4B).

The apparent difference between both groups in the HDM-stimulated cultures that can be observed in figure 4, was found to be strongly related to the difference in spontaneous production of IL-10 shown in the medium-only cultures, and was, in contrast to the LPS-induced IL-10 production, non-significant when adjusted for background. Furthermore, plotting the amount of LPS-induced IL-10 against the number of IL-10-producing cells indicated that non-allergic subjects not only had more IL-10 producing cells, but also produced relatively more IL-10 per cell compared with allergic asthmatics, especially
Differential cytokine responses to low dose LPS in allergic asthmatics

Figure 4. Stronger IL-10 response to very low-dose LPS in non-allergic subjects. (A) Production of IL-10 in pg/ml by PBMC of allergic (circles) and non-allergic (triangles) subjects after 24 h incubation with medium alone, HDM (1μg/ml), LPS (10 pg/ml or 100 pg/ml) and a combination of HDM and LPS (100 pg/ml). (B) Number of IL-10-producing PBMC (IL-10-positive spots/10^5 cells in ELISPOT assay) of allergic (circles) and non-allergic (triangles) subjects after 72 h incubation with medium alone, HDM (1μg/ml), LPS (10 pg/ml or 100 pg/ml) and a combination of HDM and LPS (100 pg/ml). Although the data from the stimulated cultures in this figure are not adjusted for medium-control, the observed significant differences between both groups are calculated with values that are corrected for background and are marked with ** p<0.01, *** p<0.001. (C, D): Correlation of the number of IL-10-producing PBMC with the amount of IL-10 produced by PBMC after stimulation with (C) LPS (10 pg/ml) and (D) LPS (100 pg/ml) in allergic (circles, dashed lines) and non-allergic (triangles, solid lines) subjects.

When exposed to indoor airborne levels of LPS (10 pg/ml: Rs = 0.611, p < 0.001 for non-allergics vs Rs = 0.392, p = 0.035 for allergic asthmatics, fig.4C). Thus, PBMC from non-allergic subjects seem to have more IL-10-producing cells that, in addition, produce more IL-10 than those from allergic asthmatics in response to indoor airborne LPS concentrations.

No differences between allergic and non-allergic subjects in IL-10 secreting T- or B- cells
To investigate whether the phenotypes of these IL-10 producing cells were different for both groups, we incubated PBMC from a subgroup of 6 allergic asthmatics and 6 healthy
non-allergic subjects overnight with medium alone, LPS or HDM. The next day, IL-10-positive cells were detected with an IL-10-secretion assay and phenotyped with surface markers for T cells (cytotoxic: CD3−/CD8+, helper: CD3+/CD4+ or T regulatory (Tr)1: CD3+/CD4+/CD25+), B cells (classical: CD3−/CD19+ or B regulatory (Br)1: CD3−/CD19+/CD5+), monocytes (CD3−/CD14+) and NK (CD3−/CD16+/CD56+) cells. No differences between allergic and non-allergic subjects were found with regard to any of these IL-10-secreting phenotypic lymphocytes (data not shown), neither in unstimulated PBMC nor after stimulation with LPS or HDM. Differences in monocytes could not be quantified in this assay, because LPS-stimulated monocytes tended to adhere to the culture plate and were not detectable by flow cytometry.

**DISCUSSION**

In the present study, we show that a very low dose of LPS (10 pg/ml equivalent to 1 EU/ml, determined in LAL-assay), comparable with indoor airborne LPS exposure, can induce cytokines, such as IL-10, IL-12 and IFNγ, in PBMC from non-allergic subjects, but not in PBMC from allergic asthmatics. After stimulation with a 10-times higher dose of LPS the production of these cytokines that can attenuate the production of Th2 mediators, increased in the allergic asthmatic group to levels similar to those of the non-allergic group. Furthermore, after stimulation with HDM (1 μg/ml, containing 10 pg/ml LPS) the production of IL-10, IL-12 and IFNγ by PBMC from allergic asthmatics was less pronounced compared to that by PBMC from non-allergic subjects. Adding LPS (100 pg/ml) to HDM-stimulated PBMC from allergic asthmatics significantly up regulated the production of IL-10, IL-12 and IFNγ, again leading to cytokine levels similar to those of non-allergic subjects. Moreover, as has been shown before with high doses of LPS, addition of this relatively low amount of LPS to HDM-stimulated cells significantly reduced the allergen-induced production of Th2 cytokines in PBMC from allergic asthmatics. The same shift to a non-allergic profile was seen in the number of cells producing these cytokines when LPS was added to HDM-stimulated PBMC from allergic asthmatics.

Interestingly, PBMC from non-allergic subjects not only produced significantly more IL-10, but also had more IL-10-producing cells in response to indoor airborne levels of LPS compared to PBMC from allergic asthmatics. The production of IL-10 by PBMC is facilitated by monocytes, T cells and B cells. IL-10 has been shown to limit immune responses to allergens, inhibit the overproduction of inflammatory cytokines and induce tolerance.

According to Min et al., the induction of IL-10 in particular, but not of IL-12 or IFNγ, was pivotal for the Th2-cytokine inhibiting effect of high doses of LPS. With this in mind, we wondered if the non-allergic subjects had more or different IL-10-secreting cells that were possibly deficient in allergic asthmatics and/or if they just had a lower threshold to LPS.
To determine if the difference in IL-10-producing cells in our study was related to regulatory Tr1 and Br1 cells, recently described by Akdis and Hussaarts, we stimulated the PBMC from both groups with the very low dose of LPS and analyzed the IL-10-secreting cells with flow cytometry. In contrast to previously mentioned studies, we observed no differences in the number of IL-10-secreting Tr1 or Br1 cells between the PBMC from allergic asthmatics and non-allergic subjects.

Also IL-10-producing monocytes have been shown to play an important role in the reduction of Th2-cytokines and induction of tolerance during, for example, allergen-specific immunotherapy (SIT). Unfortunately, a high percentage of the monocytes in our study adhered to the culture plate after stimulation with LPS, which made it impossible to analyze and quantify these cells accurately with flow cytometry. Still, the fact that besides IL-10 also the production of IL-6 and IL-12p40, which are mainly produced by monocytes, significantly increased after stimulation with LPS might indicate that the LPS-induced ‘Th2-reducing’ effect is mainly the result of IL-10-secreting monocytes. Further research is needed to confirm the role of IL-10 and monocytes in this process.

With regard to the reduced responsiveness of allergic asthmatics to LPS, conflicting studies have been published concerning Toll-like receptor (TLR)-4, the major signaling receptor for LPS. In the periphery, TLR-4 is mainly expressed by monocytes and forms a complex with CD14, LPS binding protein (LBP), myeloid differentiation protein (MD)-2 and intracellular components (reviewed in 33 and 34). Polymorphisms of CD14 and TLR-4, have been suggested to be associated with allergy and asthma. However, other studies have not found this association. In our study, the similar cytokine response to LPS 100 pg/ml in PBMC from both groups indicates that it is not likely that there is a marked difference between allergic asthmatics and non-allergic healthy subjects in the overall expression of TLR-4 and CD14 by PBMC. The fact that the cells of the allergic asthmatics failed to respond to the low indoor levels of LPS in contrast to the cells of the non-allergic subjects suggests a different threshold in the signaling cascade. Upon binding to the TLR-4 complex, LPS can activate several signal transduction pathways including the MyD88-dependent pathways leading to downstream activation of nuclear factor kappa B (NFkB) and mitogen-activated protein kinase (MAPK) p38, which is followed by an increased production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukins; the MyD88-independent pathway via TRIF (Toll-interleukin-receptor (TIR)-domain-containing adaptor protein inducing IFN), leads to activation of IFN-regulatory factor 3 (IRF3), which increases the production of IFNβ. Differences in the presence or absence of pivotal proteins in these pathways might explain the higher threshold to LPS in allergic asthmatics. It remains to be determined which proteins are responsible for the higher threshold and whether this difference in threshold also applies to other cells such...
as airway epithelial cells, dendritic cells and alveolar macrophages, which make up the first line of defense against inhaled antigens.

The results of this study are in line with the protective role of LPS during the development of Th2-responses to allergens. Our results suggest that 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 industrialized countries are currently too low to overcome this threshold. The higher LPS-induced IL-10 levels and number of IL-10-producing cells in non-allergic subjects suggest a “regulatory” role for IL-10 in this process. Possible (epi)-genetic differences in the induction of dampening- or activating-proteins of the TLR-4 signaling pathways that might be responsible for this difference in threshold, might also explain the exaggerated response to LPS in established asthma.

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Increase in allergen-specific IgE and ex vivo-Th2 responses after a single bronchial challenge with house dust mite in allergic asthmatics

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ABSTRACT

Background: Airway responsiveness to allergen in patients with allergic asthma is studied by bronchial allergen challenge. Although the typical features of the early and late response on lung function and bronchial inflammation after allergen challenge are well known, little has been reported as yet on any changes in systemic allergic and immunologic parameters after 4-6 weeks.

Methods: In a clinical study, 27 subjects with allergic asthma and house dust mite (HDM) allergy underwent a bronchial allergen challenge with HDM. Blood samples were collected before and 5 weeks after allergen challenge. Serum levels of total IgE and allergen-specific IgE were measured and peripheral blood mononuclear cells were isolated and stimulated ex vivo with HDM to determine the allergen-specific T-cell cytokine response.

Results: Five weeks after bronchial allergen challenge with HDM the amount of circulating IgE against HDM and the major allergenic components Der p1 and Der p2 was significantly increased (10.8% and 8.8%, respectively). IgE antibodies against other environmental allergens decreased (grass pollen) or remained unchanged (cat dander). Allergen-induced Th2-cytokine production was also significantly increased (p < 0.001, p = 0.014 and p = 0.006 for IL-4, IL-5 and IL-13, respectively). The increase in Der p1- and Der p2-specific IgE in serum correlated with increased numbers of Th2-cytokine-producing cells (Rs = 0.56, p = 0.002 and Rs = 0.54, p = 0.004 for IL-4 and IL-13, respectively).

Conclusions: A single bronchial allergen challenge with HDM is accompanied by increased levels of allergen-specific IgE for HDM in serum and an enhanced Th2 response to HDM still detectable 5 weeks after challenge.

INTRODUCTION

Bronchial allergen challenge is an investigative tool to provide insight into the airway inflammatory processes and airway pathology caused by inhaled allergens. The challenge model is well standardized and can be used in clinical studies to evaluate the efficacy of (new) asthma therapies. The airway-related symptoms of the early and late asthmatic response (LAR) after bronchial allergen challenge are transient and return to baseline within weeks. Little is known yet about any systemic effects of the allergen challenge after 4-6 weeks.

Inhalation of allergen into the lower airways by sensitized subjects results in an immunoglobulin (Ig) E-mediated acute bronchoconstriction (early asthmatic response, EAR), which is maximal 15-30 min after exposure and resolves by about 2 h. At the same time many cytokines, such as interleukin (IL)-4, IL-5 and IL-13, are released both locally and systemically. Whereas IL-4 and IL-13 are important for IgE secretion, IL-5 plays a pivotal role in the recruitment, activation and survival of eosinophils in the airways. This allergen-induced eosinophilic airway inflammation is associated with the development of
a second episode of bronchoconstriction (LAR), which occurs 3-8 h after inhalation of allergen, and an increased airway hyperresponsiveness (AHR), which may last for up to several days or weeks.\(^8\)

In several studies, concerning the safety and reproducibility of bronchial allergen challenge, no significant differences were found in EAR, LAR and eosinophilic inflammation between challenges that were repeated after a minimum of 2 weeks\(^4,9-11\). From these results it was concluded that the experimental challenge model is safe and reproducible and that all of the allergen-induced effects of the challenge return to baseline within a few weeks. These results are in contrast with other studies that report a persistent increase in LAR and AHR after repeated bronchial challenges\(^12,13\). Cockcroft et al.\(^12\) mentioned a so-called priming effect of allergen challenge that did not occur in all patients and suggested a possible association with the development of naturally occurring asthma. However, the mechanism underlying this priming response is still unclear.

We hypothesized that a single bronchial allergen challenge, apart from the known early and late responses on lung function and inflammatory parameters, which occur within 24 h, might also enhance longer-lasting systemic B- and T-cell responses to allergen. To test this hypothesis, we assessed the allergen-specific responses in blood samples from house dust mite (HDM)-allergic asthmatics that were collected before and 5 weeks after a single bronchial allergen challenge. Total IgE and allergen-specific IgE levels were measured in serum. Peripheral blood mononuclear cells (PBMC) were isolated and stimulated \textit{ex vivo} with HDM to compare the allergen-specific T-cell responses.

**METHODS**

**Study population and design**

For a clinical intervention study, assessing the effect of synbiotics on allergic asthma, adult patients with intermittent to mild persistent asthma and HDM allergy, as determined by positive skin prick test and ImmunoCAP-system (IgE to \textit{Dermatophagoides pteronyssinus} > 0.5 kU/L), were included. Patients were not allowed to use immunosuppressive medication, long-acting \(\beta_2\)-agonists, oral antihistamines and (inhaled) corticosteroids for 4 weeks prior to and during the study. Usage of short-acting \(\beta_2\)-agonists was allowed until 12 hours before each visit. A detailed description of the study population is published elsewhere\(^14\).

The study had a double-blind, placebo-controlled, parallel design and started with a baseline visit: on day one blood and sputum samples were collected and baseline bronchial hyperresponsiveness was determined with PC\(_{20}\)methacholine according to standardized procedures\(^3\); on day two patients were challenged with HDM, blood was collected at 1, 6 and 24 h (day 3) after HDM challenge, sputum was induced at 6 and 24 h.
(day 3) and PC_{20} methacholine at 24 h (day 3). Subsequently, subjects were randomly allocated to receive a food supplement with or without synbiotics for 4 weeks. After intervention, patients were subjected to the same schedule as for baseline visit. Whenever cosensitization to pollen was observed, the study was performed outside the relevant pollen season.

Subjects were recruited via advertisement on internal billboards in the Academic Medical Center (AMC) in Amsterdam and gave written informed consent. The study was approved by the Medical Ethical Committee of the AMC.

**Bronchial allergen challenge**

All subjects underwent a bronchial allergen challenge, inhaling doubling doses of a standardized *D. pteronyssinus* extract (50,000 BU/ml, containing 98 μg/ml Der p1 and 7 μg/ml Der p2, ALK Abelló, Nieuwegein, the Netherlands) with 10-min intervals via a reservoir aerosol delivery system until a decline in forced expiratory volume in 1 s (FEV1) of 20% from baseline (median of three measurements within 5%) was reached.$^{15}$ Changes in FEV1 were measured every 10 min for the first hour, then hourly for at least 6 h.

**Analysis of blood samples**

Blood was collected on day 1 of the challenge schedule: 24 h before and 5 weeks after baseline allergen challenge. After blood clotting for 30-60 min, samples were centrifuged and serum was stored in aliquots at −80°C until analysis. Serum levels of total IgE and allergen-specific IgE antibodies against HDM, Der p1, Der p2, grass pollen and cat dander were determined using the ImmunoCAP-system (Phadia, Uppsala, Sweden).

Peripheral blood mononuclear cells (PBMC) were isolated within 2 h from heparinized blood by standard density gradient techniques and stored in aliquots in liquid nitrogen until analysis.

**Cell stimulation assays**

We performed ELISpot assays to determine the number of cells producing Th1 and Th2 cytokines after allergen-specific stimulation and quantified the allergen-induced cytokine production by PBMC in a Luminex assay as described in detail earlier.$^{14}$, with minor modifications. Briefly, we first optimized the stimulation assays to ensure the cytokine response was allergen specific and not influenced by, for example, endotoxin contamination of the HDM extract (1 EU/μg). Stimulation of PBMC with 0.2 μg HDM/ml induced a typical allergen-specific cytokine response. Then, cryopreserved PBMC were thawed, washed and incubated in culture medium with phytohemagglutinin (PHA, 0.1 μg/ml) as a positive control, HDM (0.2 μg/ml) or medium alone. We used cytokine-
specific caption and detection antibodies for IL-4, IL-13, IL-10, IL-12 and IFNγ from Mabtech AB (Nacka Strand, Sweden), according to the manufacturer’s protocol, and incubated the cells at 37°C and 5% CO₂ for 72 h in 96-well PVDF-based membrane plates (Millipore, Billerica, MA, USA). After visualization of the spots with streptavidin-ALP and BCIP/NBT substrate (Sigma-Aldrich, St.Louis, MO, USA), the numbers of cytokine-producing cells were determined by ELISpot analysis software from A.EL.VIS GmbH (Hannover, Germany) and were expressed as positive cells per 10⁵ stimulated cells.

For quantification of allergen-induced cytokine production, we stimulated PBMC in round-bottom 96-well plates for 24 h with the same culture medium and stimuli as used for the ELISpot assay. The amount of several cytokines (IL-4, IL-5, IL-10, IL-12, IL-13 and IFNγ) was determined in the supernatant in a Luminex assay with antibodies from Biosource (Camarillo, CA, USA). Responses from PHA-stimulated cells were treated as positive control and medium-only culture as negative control. All T-cell responses from stimulated cultures were adjusted for background by subtracting the responses from the medium-only culture. All paired samples were processed in parallel, and analyses were performed batchwise to limit inter-assay variation. In addition, in every batch we took along control PBMC, which yielded similar results each time.

Statistical analysis
Allergen-induced changes in IgE within patients were analysed using the Student’s t-test. The role of possible confounding factors (including the intervention with synbiotics) that might be related to the changes in IgE or HDM-induced cytokine responses was studied in general linear models (ANOVA and repeated-measures ANOVA). When data were not normally distributed, either log transformation was performed (PC₂₀methacholine and total allergen dose) to obtain normal distribution or the non-parametric Mann-Whitney U-test was used (changes in cytokine-producing cells and cytokine production). Correlations were determined with the Spearman’s rank test. Two-sided p-values of < 0.05 were considered statistically significant. We used SPSS version 15.0.1 (SPSS Inc., Chicago, IL, USA) for statistical analyses.

RESULTS
Subject characteristics
We obtained blood from twenty-seven allergic asthmatic patients who underwent a bronchial allergen challenge with HDM [geometric mean cumulative dose (range): 321 BU (20-1718)] before and 5 weeks after challenge. Subject characteristics of these patients are described in table 1. The challenges were performed year-round and, when co-sensitization to pollen was observed, outside the relevant pollen season. None of the
Table 1. Subject characteristics

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<td>HDM-specific IgE (kU/L) ‡</td>
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Values are expressed as *median (range), †mean (SD), ‡geometric mean (GSD).

FEV1, forced expiratory volume in 1 s; PC<sub>20</sub> methacholine, provocative concentration of methacholine causing a decline in FEV1 of 20% from baseline; Δ<sub>max</sub>, maximum decline during the first hour after allergen challenge (early asthmatic response) and from 2-6 h after allergen challenge (late asthmatic response); IgE, Immunoglobulin E; HDM, house dust mite; EAR, early asthmatic response; LAR, late asthmatic response.

patients posed changes that could affect their environmental allergen exposure, 6 months prior to the start and during the study.

**Allergen challenge-induced changes in serum levels of IgE**

Five weeks after bronchial allergen challenge with HDM, the amount of circulating IgE antibodies against HDM and against the major HDM allergen components Der p1 and Der p2 was significantly increased (+10.8% and +8.8%, respectively, p < 0.05, fig. 1A). In contrast, levels of specific IgE against other environmental allergens to which the subjects were sensitized remained unchanged (cat dander) or decreased (grass pollen, −6.5%, p < 0.05). The difference between changes in IgE to HDM and IgE to control allergens was statistically significant. Serum levels of total IgE were also increased but did not reach statistical significance (+8.1%, p = 0.055). The increase in HDM-specific IgE was associated with PC<sub>20</sub> methacholine (Rs = 0.734, p < 0.001, fig. 1B), and was not influenced by the intervention with synbiotics.

**Allergen-induced cytokine production by PBMC**

Compared with the HDM-induced cytokine response of ex vivo-stimulated PBMC from blood taken before challenge, we found a significant increase in the amount of Th2 cytokines, produced by PBMC that were isolated 5 weeks after challenge (p < 0.001, p = 0.014 and p = 0.006 for IL-4, IL-5 and IL-13, respectively, fig. 2). Moreover, we found a
Figure 1. (A) Five weeks after allergen challenge, the amount of specific IgE against house dust mite (HDM) and the HDM-allergenic components Der p1 and Der p2 in serum was significantly increased (+10.8% and +8.8%, respectively). Serum levels of specific IgE against other environmental allergens did not change (cat dander, ◯) or decreased (grass pollen, -6.5%, ○). Data are expressed as percentage change from baseline. *p < 0.05. (B) The increase in HDM-specific IgE significantly correlated with the PC20methacholine, determined one day before allergen challenge.

Figure 2. Five weeks after allergen challenge, the allergen-induced Th2-cytokine production was significantly increased by peripheral blood mononuclear cells (PBMC) that were stimulated ex vivo with house dust mite for 24 h. This also applied to the IL-10 production. No significant changes were found in Th1-cytokine production. The positive controls by allergen-independent phytohemagglutinin stimulation (open bars) are provided for comparison. Data are corrected for medium control and expressed as picogram per ml supernatant.
significant increase in the production of IL-10 (p = 0.002). There were no changes in the allergen-induced production of Th1 cytokines, such as IL-12 and IFNγ.

Consistent with the enhanced allergen-induced Th2-cytokine production, we found a trendwise increase in the number of cells producing IL-4 or IL-13 (p = 0.079 and p = 0.061, respectively) after stimulation of PBMC with HDM (fig. 3A). This trend was not found for cells producing IL-10, IL-12 or IFNγ. Allergen-independent stimulation with PHA showed that there was no difference in the total number of responsive PBMC at both time points for these cytokines (data not shown). The increase in the number of Th2-cytokine-producing cells correlated with the increase in Der p1- and Der p2-specific IgE in serum (Rs = 0.56, p = 0.002 and Rs = 0.54, p = 0.004 for IL-4 and IL-13 respectively, fig. 3B). The increase in HDM-specific IgE also correlated with the increase in IL-4 production (Rs = 0.455, p = 0.017), but not with the increased production of other cytokines. When corrected for intervention, which we found to reduce Th2-cytokine production, the increase in production of all Th2-cytokines was significantly correlated to the increase in HDM-specific IgE.

DISCUSSION
To test our hypothesis that a single bronchial allergen challenge might enhance systemic B- and T-cell responses to allergen at a time point when the local allergen-induced effects on lung function and bronchial inflammation are normally resolved, we analyzed the allergen-specific responses in blood samples, taken before and 5 weeks after a bronchial allergen challenge during a clinical intervention study. Here, we show that a single bronchial allergen challenge with HDM in sensitized patients slightly, but significantly increased the HDM-specific IgE levels in serum. This increase was paralleled by an enhanced Th2 response of PBMC to HDM.

Because patients did not move house, change the interior of their houses or change pets 6 months prior to and during the study, and participated year-round, a significant change in environmental allergen exposure is an unlikely cause of the changes in HDM-specific IgE. Moreover, Der p1 exposure during the challenge [geometric mean cumulative dose (range): 629 ng (39-3367)] was relatively high compared with the expected natural exposure. The nightly exposure to Der p1 is approximately 6 ng as suggested by Gore et al. As an intra-individual control we measured specific IgE levels against allergens that were not used for challenge. The lack of an increase in the IgE levels against these control allergens strongly argues for an HDM-specific effect. Therefore, we conclude that the increase in HDM-specific IgE was most likely the result of the bronchial allergen challenge.

There was a positive association between the increase in HDM-specific IgE and the baseline PC_{20}methacholine. The PC_{20}methacholine has been shown to be pivotal for the
Figure 3. (A) Five weeks after allergen challenge, we found an increased number of peripheral blood mononuclear cells (PBMC) that produced Th2 cytokines after stimulation for 72 h with house dust mite (HDM). (B) The increase in HDM-specific IgE (Der p1 + Der p2) in serum 5 weeks after allergen challenge with HDM significantly correlated with the increase in number of Th2-cytokine-producing PBMC after stimulation with HDM in an ELISpot assay. No changes were found for IL-10- and IFN-γ-producing cells. Data are corrected for medium control and expressed as number of cytokine-producing cells (spots) per 100,000 ex vivo-stimulated PBMC.

dose of HDM needed to reach a 20% decline in FEV1 during the challenge (the correlation between PC_{20} methacholine and the total allergen dose delivered was Rs = 0.609, p = 0.001). Therefore, we assume that the total dose of allergen, which was highest in patients with relatively little AHR, is the main factor to determine the increase in specific IgE.

In an animal study, repeated intratracheal inoculation of Dermatophagoides farinae (Der f) in mice significantly increased both the total and Der f-specific IgE antibody concentrations in BALF and serum. After repeated challenges, the authors also observed chronic airway inflammation, characterized by increased numbers of lymphocytes and eosinophils and increased levels of IL-5 and IFN-γ in BALF.

Although we did find an increase in allergen-specific IgE and in the allergen-induced production of Th2 cytokines, this enhanced systemic sensitivity to HDM neither caused a detectable increase in baseline airway responsiveness at 5 weeks after challenge nor was
it accompanied by an enhanced allergen-induced response in airway inflammation and airway responsiveness 24 h after a second allergen challenge in the intervention study (table 2 in reference 14).

This is consistent with the aforementioned studies that investigated the safety and reproducibility of allergen challenges in asthmatics.\textsuperscript{4,9,10} Apparently, the persistent small changes in systemic allergic parameters, caused by the single allergen challenge, do not lead to a detectable aggravation of the increase in airway responsiveness and bronchial inflammation within the 24 h after a second allergen challenge. However, accumulation of such long-term effects by chronic or repeated experimental allergen exposure might resemble the effects of natural repeated exposure to allergen that results during, for example, the grass pollen season in the induction of bronchial hyperresponsiveness and inflammatory responses involving T cells, mast cells, and eosinophils.\textsuperscript{19-21} In a 2-year follow-up study of starting laboratory animal workers, inhalation of a comparable cumulative dose of allergen resulted in the development of allergen-specific IgE antibodies that was associated with the development of an allergen-specific IL-4 response.\textsuperscript{22} These changes in allergic and immunologic parameters also did not result in detectable changes in bronchial hyperresponsiveness, but may help to understand the mechanism of increasing airway responsiveness after repeated or prolonged allergen exposure.

To our knowledge, this is the first study to show effects of a single bronchial allergen challenge on systemic allergic and immunologic parameters 5 weeks after allergen challenge at a time point when bronchial responsiveness and inflammation already have returned to baseline levels. The increased levels of HDM-specific IgE and the enhanced sensitivity of PBMC to HDM at 5 weeks after a high-dose inhalation of HDM emphasize the role of B and T cells in the development and maintenance of allergy. It remains to be established how long the systemic sensitivity to allergen will be enhanced and whether multiple experimental challenges would lead to aggravation or induction of allergic diseases (resembling natural exposure). Moreover, it has to be confirmed whether these results only apply for HDM or also to other allergens. The enhanced systemic sensitivity to allergen after a single challenge should be taken into consideration when repeated allergen challenges are performed and when these challenges are used in clinical studies. The effect appears to be dependent on the total dose of allergen delivered during allergen challenge. Therefore, patients with relatively little airway hyperreactivity are at greatest risk to experience an increase in allergen-specific IgE as they will receive the highest dose of allergen in standard provocation protocols.
Enhanced allergen-specific immune response after allergen challenge

Acknowledgements
The authors wish to thank Serge Versteeg for measuring the levels of IgE in serum, Barbara Smids and Tamara Dekker for their assistance with the stimulation assays, and Astrid van Leeuwen (Sanquin, Amsterdam) for providing the house dust mite extract. Dr. A.S. Groenstichting is acknowledged for providing laboratory equipment.

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Early activation of coagulation after allergen challenge in patients with allergic asthma

Marcel Schouten, Marianne A. van de Pol, Marcel Levi, Tom van der Poll, Jaring S. van der Zee

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Asthma is characterized by allergic airway inflammation which is associated with bronchial hyperresponsiveness and airway obstruction\(^1\). Recent evidence indicates that activation of coagulation within the airways in asthma may aggravate inflammation\(^2\). Asthma patients were found to have elevated concentrations of thrombin, thrombin-antithrombin complexes (TATc) and soluble tissue factor and reduced activated protein C (APC)/thrombin ratios in induced sputum\(^3,4\). However, knowledge on coagulation activation in the lower airways in asthma in humans is limited, especially with regard to the acute impact of an allergen challenge. We therefore determined activation of coagulation in the bronchoalveolar space and the acute effect of a segmental allergen challenge hereon in asthma patients as compared to healthy controls.

Our study population has been described previously\(^5\). In short, thirteen allergic asthmatic subjects and nine healthy volunteers were included. Patients had a positive skin prick test for house dust mite allergens, grass pollen or both. Patients had not experienced an exacerbation of asthma during at least 2 months and had not used bronchodilators for at least 8 h before the investigations. None of the subjects had experienced recent airway infection or used anti-inflammatory or anticoagulant drugs. The study was approved by the Internal Review Board of the Academic Medical Center Amsterdam and written informed consent was obtained from all participants. Intracutaneous dose-response series with house dust mite or grass pollen (ALK Abello, Nieuwegein, The Netherlands) were performed to determine the concentration that produced a 10 mm wheal response 15 minutes after injection. Asthmatic subjects underwent an intrabronchial challenge with 1 mL of this allergen concentration – brought to a final volume of 5 mL with saline – whereas controls were challenged with the highest concentration applied in the patient group. Levels of lipopolysaccharide in the allergen solution were < 1.3 pg mL\(^{-1}\) in all subjects.

Bronchoscopy and bronchoalveolar lavage (BAL) were performed as described previously\(^6\). Directly preceding allergen challenge, a BAL of the lingula was performed. After this lavage, allergen was administered in the lateral or medial segment of the right middle lobe. Four hours after allergen challenge, a BAL was performed of the segment challenged with allergen. Total cell numbers in BAL fluid (BALF) were determined by manual counting. For differential cell counts, cells were centrifuged and stained with Romanovsky and Jenner-Giemsa. All other measurements were performed in cell free supernatants obtained after centrifugation of BALF at 500 g and 4°C. TATc, soluble tissue factor and soluble thrombomodulin were measured using commercially available ELISAs (TATc: Behringwerke AG, Marburg, Germany; soluble tissue factor: American Diagnostics, Greenwich, CT, USA; soluble thrombomodulin: Diagnostica Stago, Asnières-sur-Seine,
Figure 1. Activation of coagulation and cell influx. Levels of (A) thrombin-antithrombin complexes (TATc), (B) soluble tissue factor (sTF), (C) activated protein C (APC) and (D) soluble thrombomodulin (sTM), (E) total cell counts and (F) eosinophils in bronchoalveolar lavage fluid obtained before and 4 h after an intrabronchial allergen challenge in controls (white) and asthma patients (grey). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. *, ** and *** indicate statistical significance as compared to controls (p<0.05, p<0.01 and p<0.001 respectively, Mann-Whitney U Test) and *, ** and *** indicate statistical significance as compared to t = 0 (p<0.05, p<0.01 and p<0.001 respectively, paired t test).

Activated protein C (APC) was measured with an enzyme capture assay as described earlier.7

Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-8, IL-10 and interferon (IFN)-γ were measured by multiplex bead array (Luminex, Austin, TX, USA).
At baseline, asthma patients had elevated TATc and soluble tissue factor levels in BALF as compared to controls (fig. 1A, B). After allergen challenge, asthma patients showed a substantial rise in TATc, whereas control subjects displayed only a small increase. Soluble tissue factor levels increased in both patients and controls. Since the activation of PC is mediated by cell-bound thrombomodulin and previous studies had shown that shedding of thrombomodulin during pulmonary inflammation impairs the activation of PC, we in addition to APC measured soluble thrombomodulin levels. At baseline, there were no significant differences in APC and soluble thrombomodulin levels between patients and controls (fig. 1C, D). However, upon allergen challenge, APC levels markedly decreased in patients as compared to controls. Moreover, allergen challenge induced a rise in soluble thrombomodulin concentrations in patients only.

At baseline and after allergen challenge there were no differences in total cell counts (fig. 1E) or differentials (not shown) in BALF between patients and controls. Notably, there were no differences in eosinophil count between patients and controls either at baseline or after allergen challenge (fig. 1F). There were no differences in cytokine and chemokine levels between patients and controls at baseline (not shown). Upon allergen challenge, levels of TNF-α and IL-1β increased modestly in both patients and controls, but in controls this was not significant. After allergen challenge, levels of TNF-α and IL-1β were not different between patients and controls: TNF-α median 0.13 (interquartile range 0.12-3.5) vs. 0.13 (0.12-6.4) pg mL⁻¹; IL-1β 0.19 (0.06-0.63) vs. 0.13 (0.06-0.57) pg mL⁻¹. Levels of IL-6 and IL-8 increased in both patients and controls, but were not different between groups after challenge: IL-6 6.2 (5.3-21.5) vs. 8.3 (4.8-14.6) pg mL⁻¹; IL-8 84 (37-529) vs. 68 (8.0-512) pg mL⁻¹. Levels of IL-2, IL-4, IL-10 and IFN-γ were below detection in both groups.

Our findings indicate that airway inflammation is associated with a procoagulant state in the bronchoalveolar space in asthma. This extends previous investigations in asthma patients: Gabazza et al.³ reported increased concentrations of thrombin, TATc and soluble tissue factor in induced sputum in a study population comparable with our population. Terada et al.⁹ found increased thrombin activity in BALF of asthma patients 48 h after a bronchial allergen challenge. Of note, in this study cell counts increased more than 4-fold and eosinophil counts increased to 44% of the total count, whereas we saw no increase in cell counts and no alterations in eosinophil counts. This difference most likely is due to the different sampling time after allergen challenge. Importantly, eosinophils express substantial amounts of tissue factor, the main inducer of coagulation in blood and the lung.¹¹ However, our results demonstrate pulmonary activation of coagulation in asthma and on allergen challenge independent of eosinophil influx.

APC has been implicated as an important regulator of coagulation and inflammation and earlier evidence has suggested insufficient generation of APC in the upper airways of
asthma patients: Hataji et al.\textsuperscript{4} reported decreased APC/thrombin and APC/PC ratios in induced sputum in asthma patients. In accordance, mice with allergic airway inflammation demonstrated reduced APC/thrombin ratios in BALF\textsuperscript{12}. In this model, inhalation of APC inhibited not only coagulation activation but also inflammation and airway hyperresponsiveness\textsuperscript{12}. We here show that APC levels are reduced in BALF of asthma patients 4 h after a bronchial allergen challenge. Soluble thrombomodulin levels were increased only in patients, which may have contributed to a reduced capacity to generate APC. Reduced APC levels in turn could at least partially explain the observed rise in thrombin generation. Remarkably, inflammatory responses were not different between patients and controls.

Our study has limitations: We studied only patients with mild asthma. Moreover, we studied a small number of subjects and the effect of a bronchial allergen challenge was studied after only one time point. We cannot exclude that the bronchoscopy and BAL procedure contributed to the inflammatory response. Conceivably the allergen itself contributed to the inflammatory response. Since controls were challenged with the highest allergen dose applied in patients they received a higher amount of allergen than most of the patients. This probably made them more susceptible to aspecific effects of the challenge, which could have masked a relatively larger inflammatory response in asthma patients to a comparable stimulus. Moreover, this could have influenced coagulation measurements, which would then have resulted in an underestimation of the differences found between patients and controls.

In conclusion, we show that coagulation is activated in the bronchoalveolar compartment in asthma patients and that intrabronchial allergen challenge aggravates coagulation activation and induces a decrease in APC concentrations in asthma patients as early as within 4 h. Although animal studies have suggested a pathogenetic role for activation of coagulation and downregulation of APC in asthma, it remains to be established in a larger study whether the procoagulant state as observed in our study contributes to disease and whether restoring the balance between coagulation and anticoagulation in asthma patients would impact on inflammation and disease activity.

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Synbiotics reduce allergen-induced T-helper 2 response and improve peak expiratory flow in allergic asthmatics

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ABSTRACT

Background: Previous studies suggest that pre-/probiotics can be used in prevention and treatment of early allergic disease in newborns and young children.

Objective: To determine effect of treatment with synbiotics (90% short-chain galacto-oligosaccharides, 10% long-chain fructo-oligosaccharides: Immunofortis® and Bifidobacterium breve M-16V) on allergic responses in adults with established allergic asthma. Primary outcome was allergen-induced bronchial inflammation as represented by eosinophil counts.

Methods: Twenty-nine patients with asthma and house dust mite (HDM) allergy were randomized in a double-blind parallel design to receive placebo or synbiotics for 4 weeks. At study entry and after treatment a bronchial allergen challenge with HDM was performed, followed by lung function tests, collection of blood (in/ex vivo IL-5) and induced sputum (inflammatory parameters). During treatment a diary was kept with peak expiratory flow (PEF) and asthma scores.

Results: Treatment did not affect the allergen-induced increase in sputum eosinophils at 6 and 24 hrs after challenge. Likewise, other parameters for bronchial inflammation and early and late changes in lung function did not differ upon treatment. Both the morning and evening PEF, however, significantly increased during synbiotics treatment (morning p = 0.003, evening p = 0.011). Also, the increase in serum IL-5 after allergen challenge was significantly inhibited by synbiotics (p = 0.034), as was ex vivo allergen-induced Th2-cytokine (IL-5 and IL-4 + IL-13) production by PBMCs (p = 0.046). In vivo (24 hrs) and ex vivo IL-5 production were associated.

Conclusion: Four-week treatment with synbiotics had no effect on bronchial inflammation and LAR, but did significantly reduce systemic production of Th2-cytokines after allergen challenge and improved PEF.

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways in which many inflammatory and immune cells and mediators are implicated. The chronic airway inflammation is associated with airway hyperresponsiveness (AHR) which leads to recurrent episodes of shortness of breath with variable airflow obstruction. Activated mast cells, eosinophils and T-helper 2 lymphocytes (Th2), producing inflammatory mediators like IL-4, IL-5 and IL-13, contribute to persistent airway inflammation, leading to the pathology characteristic of asthma. Nowadays, treatment is based mainly on suppression of airway inflammation with (inhaled) corticosteroids and relief of symptoms with bronchodilators. Nevertheless, these therapies do not consistently abrogate airway inflammation in patients with...
asthma. Other approaches to modulate the immune-mediated inflammatory responses are under development, including treatment with probiotics. The composition of the intestinal microbiota may contribute to the genesis of immunological diseases, including allergy and asthma. The bacterial composition has been implicated in the development of gut-associated lymphoid tissue (GALT), which largely regulates Th1- and Th2-type immunity and immune tolerance from early life. In line herewith, the composition of the intestinal microbiota differs between healthy infants in countries with a low and high prevalence of allergy and between allergic and non-allergic infants in both environments. Therefore, modulation of the gut microbiota through the use of probiotics may, directly or indirectly, modulate the immune system. The reported modes of action of pre/probiotics are strain dependent and range from modulating local innate and adaptive mucosal immune responses to systemic effects. The latter may comprise modulation of systemic cytokine responses and of regulatory T cells. Together these multiple actions may explain why effects of pre/probiotics are not limited to the developing immune responses in the gut, and also why mucosa other than that of the gut can be affected.

Some clinical studies suggested that certain probiotic strains, mostly Lactobacilli and Bifidobacteria, promote early immune system maturation and may alleviate symptoms of (IgE-associated) eczema in young children. Conversely, studies with Lactobacilli in older individuals with already established respiratory disease have failed to show improvement in symptoms of asthma or allergic rhinitis. In addition, although some studies have reported benefit in the treatment and prevention of atopic eczema, none has had any clear effects on the development of Th2-mediated allergic responses.

Prebiotics are indigestible oligosaccharides that promote the growth and activity of commensal bacteria, mainly of Bifidobacteria. It has been demonstrated that a specific mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) in a ratio of 9:1 (Immunofortis®) normalized the intestinal microbiota of bottle-fed infants to that of breast-fed infants. In a recent double-blind, placebo-controlled multicentre trial with 90 infants with atopic dermatitis, it was shown that Bifidobacteria with the specific mixture of scGOS/lcFOS affected the microflora during the 12 weeks of treatment. In addition, in a murine model for allergic hypersensitivity, using ovalbumin as allergen, dietary scGOS/lcFOS reduced the allergic inflammation.

In an ovalbumin allergic mouse model, oral administration of Bifidobacterium breve M-16V significantly decreased the influx of inflammatory cells in lungs after allergen challenge (lymphocytes (-61%) and eosinophils (-46%)), and reduced airway hyperresponsiveness (AHR) and the level of Th2-type cytokines (IL-4, IL-5 and IL-10) in
bronchoalveolar lavage fluid (BALF). A combination of this probiotic strain and Immunofortis® (synbiotics) demonstrated synergistic effects in suppressing the AHR.

In a double-blind, placebo-controlled study, we determined whether supplementing these synbiotics for four weeks had similar results, as observed in the animal model, on the allergic asthmatic responses of adult patients with house dust mite (HDM)-IgE-mediated allergic asthma. Primary outcome was change in allergen-induced bronchial inflammation as determined by eosinophil counts in sputum after allergen challenge. Secondary outcomes included changes in lung function (FEV1) during the late asthmatic reaction (LAR) and allergen-induced bronchial inflammation as determined by neutrophil counts and the level of myeloperoxidase (MPO) and eosinophil cationic protein (ECP) in sputum after allergen challenge. In addition, bronchial hyperresponsiveness, determined by PC_{20}methacholine, peak expiratory flow (PEF), measured during intervention, and immunological parameters, determined in serum and ex vivo HDM-stimulated peripheral blood mononuclear cells (PBMCs) were evaluated as secondary outcomes.

This is the first study to show that treatment with synbiotics reduces the allergen-induced systemic Th2 response and improves the PEF in patients with allergic asthma.

METHODS
Patients
We included 29 adult allergic patients with intermittent to mild persistent asthma, according to American Thoracic Society criteria. All patients were sensitized to HDM, as determined by skin prick test and IgE to *D. pteronyssinus* > 0.5 kU/L, and were able to stop short-acting β_{2}-adrenoceptor agonists for ≥12 h before each visit, and long acting β_{2}-agonists, oral antihistamines and inhaled corticosteroids for 4 weeks prior to and during the study.

Patients with a history of allergen-specific immunotherapy or a respiratory tract infection within 6 weeks prior to the study were excluded. Other exclusion criteria were current smoking, within 6 months prior to the study or a smoking history of ≥10 pack years, and significant changes in environmental allergen exposure 6 months prior to and during the study. Patients co-sensitized to pollen participated only outside the relevant pollen season. In addition, patients were not allowed to use probiotic bacteria, antibiotics and immunosuppressive medication 4 weeks prior to and during the study. Subjects were recruited via advertisement in the Academic Medical Center (AMC) in Amsterdam and gave written informed consent. The study was approved by the AMC Medical Ethics Committee.
Effect of synbiotics in allergic asthmatics

Figure 1. Study schedule. After a medication wash-out period of 4 weeks, baseline values were obtained during 3 consecutive days: on day one, blood and sputum samples were collected and PC\textsubscript{20} methacholine was determined; on day two, patients were challenged with HDM and blood was collected after 1, 6 and 24 h (day 3), induced sputum samples were collected after 6 and 24 h (day 3) and PC\textsubscript{20} methacholine was repeated after 24 h (day 3). Then, subjects were randomized to receive placebo or synbiotics twice daily for 4 weeks, followed by a second period of 3 days involving the same procedures.

Study design
The intervention study had a double-blind, placebo-controlled, parallel design (fig. 1). If necessary, a medication-wash-out period of ≥4 weeks was included. The study started with a baseline visit: on day one, blood and sputum samples were collected and baseline bronchial hyperresponsiveness was determined with PC\textsubscript{20} methacholine; on day two, patients were challenged with HDM, blood was collected at 1, 6 and 24 h (day 3) after HDM challenge, induced sputum samples at 6 and 24 h (day 3) and PC\textsubscript{20} methacholine at 24 h (day 3). Subsequently, subjects were randomly allocated to receive a food supplement with or without synbiotics (probiotic strain: *Bifidobacterium breve* M-16V (10\textsuperscript{10} cfu) combined with a specific prebiotic scGOS/lcFOS mixture (7.2:0.8 gram) (Immunofortis®), Danone Research, Wageningen, The Netherlands) twice daily for a period of 4 weeks. The placebo was also produced by Danone, consisted of maltodextrin only and was indistinguishable from the verum. During the intervention period, study subjects measured their PEF twice daily and kept a diary, including asthma scores, questions on tolerance and acceptance of the product, gastrointestinal complaints, possible illnesses and medication. After intervention, patients were subjected to the same schedule as for baseline visit. During the study, patients were provided with rescue salbutamol (Airomir, TevaPharma, Haarlem, The Netherlands). Safety parameters included tolerance of the product as recorded by patients in diaries, haemocytometric values (hemoglobin and hematocrit), liver function parameters (alanine aminotransferase and aspartate aminotransferase in plasma) and renal function (urea, creatinine and albumine in plasma).
Lung function and Allergen challenge
Spirometry, PC\textsubscript{20} \textit{methacholine} and bronchial allergen challenge were performed according to standardized procedures\textsuperscript{26}. Forced expiratory volume in 1 second (FEV\textsubscript{1}) and forced vital capacity (FVC) were measured with a Vmax 22 spirometer (SensorMedics) and with a portable spirometer (Micromedical diarycard, Sensor Medics). Peak expiratory flow was measured with a portable peak flow meter (Respironics HealthScan Inc., NJ, USA).

Allergen challenge was performed as described earlier\textsuperscript{29}. The LAR was defined by maximal drop in FEV\textsubscript{1} and area under the curve (AUC) from 2-6 h after allergen challenge.

Sputum induction, processing and analysis.
Sputum induction and processing, with DTT to liquefy the whole sample, was performed with minor modifications as described elsewhere\textsuperscript{30}. Differential cell counts were expressed as number and percentage of cells excluding squamous epithelial cells. Sputum samples containing >80% squamous cells on differential cell counting were excluded from analysis. Sputum ECP and MPO were determined as described\textsuperscript{31,32}.

Processing and analysis of blood
Total and differential leukocyte counts were determined in EDTA-blood. Serum samples (for IgE and IL-5) were stored at -80°C until analysis. IgE was determined by ImmunoCAP (Phadia AB, Uppsala, Sweden) and IL-5 was determined by ELISA\textsuperscript{33}. Haemoglobin, hematocrit, ALAT, ASAT, urea, creatinine and albumin were assessed as safety parameters in heparinized blood. Peripheral blood mononuclear cells (PBMCs) were isolated within 2 h after collection from heparinized blood by standard density gradient techniques and stored in liquid-N\textsubscript{2} until analysis.

ELISpot assay
Numbers of cytokine-producing cells after HDM-specific stimulation were determined in ELISpot assay (Mabtech AB, Sweden). Briefly, cryopreserved PBMCs were thawed, washed and preincubated for 4 h in round-bottom tubes (Micronic, McMurray, PA, USA) at 4x10\textsuperscript{5} per ml culture medium (RPMI 1640 (Gibco, Invitrogen Ltd, Paisley, UK), containing 10% heat-inactivated FCS (Gibco), antibiotics and L-glutamine (Gibco) in the absence or presence of HDM (5 \textmu g/ml, kindly provided by Sanquin, Amsterdam, The Netherlands). Culture medium and phytohemaglutinin (PHA, 0.1 \textmu g/ml) were used respectively as negative and positive control. Then, cells were diluted to a final 2x10\textsuperscript{5} (for IL-4), 1x10\textsuperscript{5} (for IL-12, IL-13), 5x10\textsuperscript{4} (for IL-10) and 2.5x10\textsuperscript{4} (for IFN-\gamma) cells per well and incubated in duplicate at 37°C and 5% CO\textsubscript{2} for 72 h in 96-well PVDF-based membrane plates (Millipore, Billerica, MA, USA), which were coated with monoclonal antibodies to either IL-4, IL-10, IL-
After 72 h, plates were washed and cytokine production by individual cells was visualized using biotinylated detection antibodies, streptavidin-ALP and BCIP/NBT substrate (Sigma-Aldrich, St. Louis, MO, USA) successively. Numbers of cytokine-producing cells were determined by software from A.EL.VIS GmbH (Hannover, Germany) and were expressed as positive cells per 1x10^5 stimulated PBMCs. Number of positive cells in stimulated cultures was adjusted for background by subtracting number of positive cells from medium-only culture. Analyses were performed batchwise to limit inter-assay variation. In addition, we took along control PBMC, which yielded similar results each time.

**Luminex assay**

For cytokine production by PBMCs after HDM-specific stimulation, PBMCs were cultured for 24 h in round-bottom microtiter plates with the same culture medium and stimuli used in the ELISpot assay. IL-4 (lower limit of detection in pg/ml: 0.56), IL-5 (0.19), IL-6 (0.26), IL-10 (0.4), IL-12p40/p70 (1.51), IL-12p70 (0.42), IL-13 (0.98), IL-17 (0.21) and IFN-γ (0.13) were determined in Luminex assay (Biosource, Camarillo, CA, USA). Apart from the IL-17 data, none of the values were below the lower limit of detection. The production of each cytokine was adjusted for background by subtracting the production in the medium-only supernatant. All samples were analysed in parallel, and for repeat measurements (above higher limit of detection), samples that were determined in the first run were taken along and yielded similar results.

**Immunofluorescent staining and flow cytometry**

To determine percentage of CD4^+^/CD25^+^/IL7R^-^ regulatory T cells, 500,000 PBMCs were incubated for 30 min at 4°C with anti-CD3-FITC, anti-CD4-PerCP, anti-CD25-APC and anti-CD127-PE (BD Biosciences, San Jose, CA, USA; according to manufacturer’s protocols). For intracellular FoxP3 staining, PBMCs were first stained with anti-CD3-FITC, anti-CD4-PerCP and anti-CD25-PE (BD Biosciences), then fixed, permeabilized and stained with anti-FoxP3-APC (eBioscience, San Diego, CA, USA). Cells were washed and analyzed using a FACSCalibur flow cytometer and FlowJo software.

**Statistical analysis**

The sample size was based on the outcome of an intervention study with the probiotic strain *B. breve* M16-V in an ovalbumin allergic mouse model, that resulted in a consistently lower AHR to methacholine and a 50% reduction in number of eosinophils in BALF after allergen challenge. With an average eosinophil count of 15.1 ± 6.6 x10^4 per gram sputum in asthmatic patients after allergen challenge aiming to detect a 50%
reduction, testing two sided with 5% significance and 80% power, a group size of minimal 12 persons per arm was required.

Randomization was carried out prior to the study by the logistics manager of Danone Research, using a 4-block design. Subjects who were not-compliant, i.e. used study medication less than 2 weeks, terminated the study early or violated the protocol, were excluded from statistical analyses.

When data were normally distributed and the variance was homogeneous, differences between treatment groups were analysed using ANOVA, including the factor treatment. Special attention was given to confounding factors and covariates that could be related to primary efficacy parameters such as baseline values. When data were not normally distributed, either data transformation was performed to get normal distribution or the non-parametric Mann-Whitney test was used. Differences, which occurred over time between treatment groups were analysed using a mixed model. In the mixed linear model evaluating the effect on PEF the factor “day” was included as a repeated measure and baseline PEF was included as a covariate. Treatment and the interaction term treatment by day were included in the model. Two-sided p-values of less than 0.05 were considered statistically significant. The statistical analysis was performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Subject characteristics

At baseline, subject characteristics were not significantly different between both study groups (table 1). From 29 randomized patients, 3 subjects were excluded from all analyses: 2 subjects in the synbiotics group were considered drop-outs, as they stopped in the first week of intervention for personal reasons; 1 subject from the placebo group was not-compliant, as she did not consume the study product for at least 2 weeks of intervention period, because of intestinal side-effects. Post hoc analysis with inclusion of the data of this patient did not change the results for the primary and secondary outcomes. From another subject in the placebo group, sputum, serum and lung function data obtained after the second HDM challenge were excluded from analyses as she accidentally received half the cumulative dose of allergen given during the first challenge. Mild gastrointestinal complaints (diarrhoea or obstipation) were reported most frequently and slightly more in the placebo group than in the verum group (3/14 in the verum group and 7/15 in the placebo group respectively). No long-term adverse effects were experienced. All blood safety parameters showed normal values before and after intervention (not shown).
Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Synbiotics</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (drop-outs/not-compliant)</td>
<td>15 (0 / 1)</td>
<td>14 (2 / 0)</td>
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<tr>
<td>Gender (male/female)</td>
<td>3 / 11</td>
<td>2 / 10</td>
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</tr>
<tr>
<td>Age (years) *</td>
<td>25.0 (18; 51)</td>
<td>28.0 (21; 51)</td>
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<tr>
<td>FEV1 (%) predicted †</td>
<td>93.9 (2.5)</td>
<td>97.1 (2.8)</td>
<td>0.226</td>
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<tr>
<td>PC_{20} methacholine (mg/ml) ‡</td>
<td>1.72 (1.4)</td>
<td>1.27 (1.4)</td>
<td>0.504</td>
</tr>
<tr>
<td>AUC EAR (ΔFEV1*hour) †</td>
<td>20.9 (1.9)</td>
<td>18.8 (1.3)</td>
<td>0.390</td>
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<tr>
<td>AUC LAR (ΔFEV1*hour) †</td>
<td>39.0 (4.8)</td>
<td>38.7 (10.5)</td>
<td>0.980</td>
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<tr>
<td>PEF_{morning} (L/min) † / [median]</td>
<td>425 (24) / [399]</td>
<td>382 (20) / [383]</td>
<td>0.195</td>
</tr>
<tr>
<td>PEF_{evening} (L/min) † / [median]</td>
<td>445 (24) / [396]</td>
<td>398 (19) / [402]</td>
<td>0.149</td>
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<tr>
<td>Total IgE (kU/L) ‡</td>
<td>182 (1.4)</td>
<td>257 (1.3)</td>
<td>0.537</td>
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<td>HDM-specific IgE (kU/L) ‡</td>
<td>20.8 (1.5)</td>
<td>33.3 (1.4)</td>
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<td>Previous use of LABA</td>
<td>3/15</td>
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<td>Previous use of ICS</td>
<td>6/15</td>
<td>5/14</td>
<td>1.0</td>
</tr>
<tr>
<td>Diagnosis of asthma (years)§</td>
<td>11.1 (7.5)</td>
<td>18.5 (14.6)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Values are expressed as *median (range), †mean (SE), ‡geometric mean (SE), §mean (SD).

FEV1, forced expiratory volume in 1 second; PC_{20} methacholine, provocative concentration of methacholine causing a decline in FEV1 of 20% from baseline; AUC, area under the curve during the first hour after allergen challenge (early asthmatic reaction) and from 2-6 hours after allergen challenge (late asthmatic reaction); PEF, peak expiratory flow; IgE, Immunoglobulin E; LABA, long-acting beta-agonist; ICS, inhaled corticosteroids.

**Primary outcome**

**HDM-induced bronchial inflammation determined by eosinophil counts in sputum**

We observed a significant increase at 6 and 24 h after HDM challenge in sputum eosinophil counts (table 2). The increase in sputum eosinophils during the LAR did not differ significantly between both groups after intervention.

**Secondary outcomes**

**Changes in lung function (FEV1) after HDM challenge**

Bronchial exposure to HDM (geometric mean cumulative dose (range): 313 BU (20-1718)) caused an immediate decline in FEV1 of ≥ 20% (EAR) in all subjects (fig. 2A). Most subjects also experienced a LAR several hours after inhalation of the last dose of HDM (mean maximum decline in FEV1 from 2 to 6 h after challenge (SE): -18.9 % (2.3)). We found no significant differences in lung function between both groups during
Table 2. Sputum inflammatory parameters before and after treatment

<table>
<thead>
<tr>
<th></th>
<th>Before treatment</th>
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<th>After treatment</th>
<th></th>
<th>p-value</th>
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<tr>
<td></td>
<td>hr</td>
<td>Placebo</td>
<td>Synbiotics</td>
<td>p-value</td>
<td>Placebo</td>
<td>Synbiotics</td>
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<tr>
<td>Eosinophils (x10⁴/gr)</td>
<td>24</td>
<td>0.8 (1.5)</td>
<td>2.0 (1.5)</td>
<td>0.103</td>
<td>1.8 (1.5)</td>
<td>1.4 (1.9)</td>
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<tr>
<td></td>
<td>6</td>
<td>14.2 (1.3)**</td>
<td>14.3 (1.5)**</td>
<td>0.981</td>
<td>19.3 (1.5)**</td>
<td>10.7 (1.7)**</td>
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<td></td>
<td>24</td>
<td>8.5 (1.6)**</td>
<td>7.0 (2.1)*</td>
<td>0.826</td>
<td>9.5 (1.7)**</td>
<td>9.5 (1.7)**</td>
</tr>
<tr>
<td>Neutrophils (x10⁴/gr)</td>
<td>24</td>
<td>15.8 (1.3)</td>
<td>17.0 (1.3)</td>
<td>0.839</td>
<td>20.6 (1.3)</td>
<td>18.8 (1.4)</td>
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<td></td>
<td>6</td>
<td>38.7 (1.3)*</td>
<td>36.7 (1.4)**</td>
<td>0.894</td>
<td>42.3 (1.4)*</td>
<td>32.2 (1.4)*</td>
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<td></td>
<td>24</td>
<td>29.0 (1.4)</td>
<td>35.2 (1.5)*</td>
<td>0.724</td>
<td>26.4 (1.3)</td>
<td>44.0 (1.5)**</td>
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<td>ECP (ng/gr)</td>
<td>24</td>
<td>7 (1.6)</td>
<td>18 (1.5)</td>
<td>0.114</td>
<td>15 (1.6)</td>
<td>19 (1.5)</td>
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<tr>
<td></td>
<td>6</td>
<td>83 (1.7)**</td>
<td>114 (1.7)**</td>
<td>0.667</td>
<td>87 (1.7)**</td>
<td>73 (1.7)**</td>
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<tr>
<td></td>
<td>24</td>
<td>62 (1.9)**</td>
<td>114 (2.0)**</td>
<td>0.526</td>
<td>65 (1.9)**</td>
<td>138 (1.9)**</td>
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<tr>
<td>MPO (ng/gr)</td>
<td>24</td>
<td>261 (1.8)</td>
<td>367 (1.4)</td>
<td>0.637</td>
<td>356 (1.8)</td>
<td>323 (1.4)</td>
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<tr>
<td></td>
<td>6</td>
<td>621 (1.5)</td>
<td>756 (1.4)**</td>
<td>0.718</td>
<td>636 (1.6)</td>
<td>562 (1.5)</td>
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<tr>
<td></td>
<td>24</td>
<td>541 (1.6)</td>
<td>889 (1.6)**</td>
<td>0.479</td>
<td>582 (1.6)</td>
<td>884 (1.4)**</td>
</tr>
</tbody>
</table>

Values are expressed as geometric mean (SE) per gram sputum. ECP, eosinophil cationic protein; MPO, myeloperoxidase. Significant changes from prechallenge baseline values (-24 hr) are marked with *p<0.05, **p<0.01, ***p<0.001.
the LAR after intervention (difference in mean AUC from 2 to 6 h after challenge (SE): +2.3 (7.9) after placebo vs -3.5 (7.8) after synbiotics, p = 0.603, fig. 2B). Likewise, the calculated provocative dose of allergen for causing an early phase 20% decline in FEV1 and the maximal fall in FEV1 during the late phase did not differ between groups.

HDM-induced bronchial inflammation
We observed a significant increase at 6 and 24 h after HDM challenge in sputum neutrophil numbers as well as amounts of ECP and MPO (table 2). The increase in sputum inflammatory parameters during the LAR after intervention did not differ significantly between both groups.
Lung function parameters

The peak expiratory flow (PEF), measured twice daily during 4 weeks of intervention, significantly improved over time in the synbiotics group (morning p = 0.003, evening p = 0.011), compared to placebo (fig. 3). The change in morning and evening PEF differed significantly between both groups from week 3 on. No significant differences between both groups were observed in asthma symptom scores, short-acting $\beta_2$-agonist usage and $PC_{20}$methacholine (pre- and post-challenge), during and after intervention.

HDM-induced cytokine production

Serum IL-5 was significantly elevated at 6 and 24 h after baseline HDM challenge (fig. 4A). The use of synbiotics, however, was associated with a significant lower increase in serum IL-5 at 24 h after HDM challenge compared to that for placebo (mean increase in serum IL-5 at 24 h after challenge (SE): from 2.5 (1.5) to 74.1 (28.4) pg/ml after placebo vs from 1.4 (0.4) to 36.3 (18.7) pg/ml after synbiotics, $p = 0.034$; fig. 4B). At 6 h after challenge we found trendwise reduced serum IL-5 levels in the synbiotics group ($p = 0.092$).

In line with serum IL-5, ex vivo production of IL-5 was increased after placebo ($p = 0.055$), but not after synbiotics (mean increase in IL-5 (SE): 16.5 (7.8) pg/ml after placebo vs 2.4 (3.3) pg/ml after synbiotics; fig. 4C). Interestingly, serum IL-5 at 24 h after HDM challenge correlated with IL-5 production by HDM-stimulated PBMCs ($Rs = 0.845, p < 0.001$; fig. 4D).

Consistent with changes for IL-5, ex vivo-production of other Th2 cytokines (IL-4 and IL-13; fig. 4E) by HDM-stimulated PBMCs after placebo was also enhanced as opposed to that after synbiotics ($p = 0.046$). The number of IL-4 and IL-13-producing cells showed a small, but non-significant increase after intervention in both groups (data not shown). No significant differences were found for numbers of IL-10-, IL-12- and IFN-$\gamma$-producing cells nor for the production of these cytokines by HDM-stimulated PBMCs (fig. 4F).

FACS analysis

The percentage of regulatory T cells, determined by $CD4^+/CD25^+/IL7R^-$ T cells (mean percentage (SE): 4.9 (0.3)% of $CD3^+$-lymphocytes) or the expression of FoxP3 in $CD4^+/CD25^{high}$ T cells (mean MFI (SE): 12.3 (0.4)), did not change after intervention for both study groups (not shown).
**DISCUSSION**

Modulation of the intestinal microbiota by prebiotics, probiotics or a combination (synbiotics) is being investigated as a potential anti-allergic treatment strategy. In the present double-blind, placebo-controlled clinical study, we investigated the potential therapeutic effect of a specific synbiotic mixture to target established allergic asthma. We show that treatment with synbiotics for four weeks did not significantly reduce the allergen-induced eosinophilic airway inflammation in allergic asthmatics during the LAR after bronchial challenge with HDM. Interestingly, treatment with synbiotics is associated with a significant improvement in the PEF and with a reduced production of IL-5 after allergen exposure, both *in vivo* and *ex vivo*. Moreover, the increased *ex vivo* production of
other Th2-type cytokines (IL-4 and IL-13) by HDM-stimulated PBMCs in the placebo group is absent in the synbiotics group.

Based on the suppression of HDM-induced Th2-cytokine production by synbiotics, a reduction in allergic airway inflammation after allergen challenge as observed in the mouse model was expected, but not found. The chronology of sensitization and subsequent airway allergen challenge in the mouse model is different from that in allergic asthmatics and this may explain observed differences. Also, the reduction in airway inflammation by synbiotics in allergic asthmatics may have been less and therefore the power of the study insufficient. In addition, the large variation of inflammatory parameters in induced sputum samples may have hindered the detection of small changes in inflammation in asthmatics.

The finding that both morning and evening PEF improved significantly during treatment with synbiotics when compared to that with placebo may indicate that the change in HDM-induced systemic Th2 response did ameliorate the clinical course of asthma. In concordance with the mechanism proposed by Kukkonen, synbiotics may modulate the course of sensitization to clinical disease by suppressing the production of Th2-type cytokines.

After placebo treatment, the HDM-induced production of IL-5 in vivo and production of IL-4, IL-5 and IL-13 by PBMCs ex vivo was increased. This may be because of the baseline HDM challenge in the absence of treatment with anti-inflammatory drugs. As we observed no significant differences in the number of Th2-cytokine-producing cells between both treatment groups, the augmented Th2-cytokine production in the placebo group may relate to enhanced activation of PBMCs by HDM, which is suppressed by treatment with synbiotics. Van der Veen et al. reported significant increases of serum IL-5 at 6 and 24 hours after bronchial allergen challenge which correlated with the allergen-specific proliferative response of peripheral T lymphocytes in vitro. The association of these systemic Th2 phenomena with the magnitude of the LAR emphasizes the relevance for the clinical course of allergic asthma.

In contrast to several human studies, suggesting an increase in Th1-cytokines (IFN-γ) or regulatory cytokines (IL-10) or regulatory T cells after probiotic treatment, the immune-modulatory effect of synbiotics was not mediated by the induction of or a change in Th1- or regulatory T-cell cytokines or cell numbers.

To determine whether reduction in Th2-cytokines by treatment with synbiotics will eventually lead to a clinical relevant reduction in bronchial inflammation and improvement in asthmatic symptoms, this study should be repeated with a larger number of patients to increase the power of the study. Moreover, prolonged administration of
Effect of synbiotics in allergic asthmatics

Synbiotics should be investigated to study the long-term effect on Th2 suppression and allergen-induced inflammatory responses.

To our knowledge, this is the first study to show that synbiotics improve the peak expiratory flow and reduce the systemic production of Th2-type cytokines like IL-4, IL-5 and IL-13 in allergic asthmatics. Thus, treatment with synbiotics may gradually decrease the allergen-induced immunological response in allergic patients with established asthma.

Acknowledgements

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Improved validity of cellular and molecular biomarkers for induced sputum in asthma and COPD

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* These authors contributed equally to this study.

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ABSTRACT

Background: Induced sputum biomarkers are important for phenotyping and monitoring of patients with asthma and COPD, but contamination with saliva enhances biomarker variability.

Methods: Squamous cell counts were tested as a measure of dilution by saliva for various biomarkers (total and differential cell count, eosinophil cationic protein, myeloperoxidase, interleukin-8 and alpha2-macroglobulin) in whole sputum samples from 29 COPD patients (247 samples) and 25 asthma patients (235 samples). Further, we determined repeatability of these biomarkers between sputum plugs that were removed from sputum to limit dilution with saliva (8 asthma patients).

Results: Whole sputum samples with ≤90% squamous cells, showed inverse log-linear relationships between absolute cell counts and levels of soluble parameters with percentage squamous cells. These log-linear relationships enabled correction of whole sputum data for saliva contamination, reducing variability and improving repeatability of sputum parameters. Interleukin-8, alpha2-macroglobulin and % neutrophils were heterogeneously distributed between selected plugs. Soluble biomarkers were found to interact dynamically with sputum plug matrix, leading to rapid equilibration with the surrounding fluid phase.

Conclusions: Analyzing multiple plugs and limiting exposure of plugs to fluid reduces biomarker variation in the selected plug method. Analysis of whole sputum with ≤90% squamous cells is improved by correction for the percentage squamous cells. These improvements will further advance the implementation of cellular and molecular monitoring in airway disease.

INTRODUCTION

Analysis of induced sputum, which is produced by subjects after being exposed to nebulized saline, is a safe and relatively non-invasive method to assess airway inflammation and effects of interventions in a range of airway diseases\(^\text{1-5}\). Standardization of methods for sputum induction, collection and analyses has consolidated and extended its use in experimental studies and to a major extent in clinical trials\(^\text{6-10}\). The analysis of sputum, however, is frustrated by the contamination with secretions from the upper airways and oral cavity, such as saliva. In studies comparing cell numbers and inflammatory parameters in paired sputum and saliva samples, saliva was found to contain low numbers of inflammatory and immune cells (predominantly squamous epithelial cells), a low protein content and low amounts of soluble inflammatory markers\(^\text{11,12}\). Contamination of sputum with saliva thus primarily leads to dilution, but to which extent is unknown.
Two distinct methods have been advocated to deal with this variable dilution of sputum. In the selected plug method, sputum plugs are selected and physically removed from the sputum and processed. In the whole sputum method, all of the sputum sample is processed in which the relative presence of squamous epithelial cells is considered an indicator of contamination with saliva, labeling the sputum valid or not. Both the selected plugs and whole sputum method are being used, although there appears to be a preference for the selected plug method.

Having performed multiple studies using both methods, we have re-evaluated and performed additional studies aimed at further reducing the variability for sputum analyses. For the whole sputum analysis we assessed whether the presence of squamous cells indeed can serve as a quantitative marker of dilution for soluble and cellular parameters. For the selected plug method we determined the variation of soluble and cellular inflammatory parameters between plugs within a sputum sample and also whether soluble parameters in plugs are affected by surrounding fluid, such as occurs during processing the sample. Implementation of our findings should lead to attenuation of the variation of sputum parameters and thus enhance the discriminative power of sputum parameters in experimental and clinical studies.

**METHODS**
To determine whether squamous cells can serve as a quantitative marker of dilution for soluble and cellular parameters in whole sputum we analyzed data from 482 sputum samples. To assess the variation of soluble and cellular inflammatory parameters in selected plugs we analyzed plugs from patients with mild asthma.

**Whole sputum method: Subjects and data sets**
Data sets from two prospective clinical studies, one with asthma and one with chronic obstructive pulmonary disease (COPD) patients, were analyzed. COPD patients participated in a 16-months study and sputum samples (maximally 14) were obtained after placebo or systemic and inhaled corticosteroid treatment and some during exacerbations, referred to as ‘different study conditions’ in the text. Patients were between 40 and 75 years, with middle-age onset of symptoms, a cigarette consumption of ≥ 15 pack-years, a forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) ratio ≤ 0.70 and FEV1 reversibility ≤ 11% of predicted.

Asthma patients participated in a 6-weeks study and sputum samples (maximally 10) were obtained after treatment with an inhaled corticosteroid and a long-acting beta-agonist or placebo, before and after allergen provocation, also referred to in the text as ‘different study conditions’. Patients were between 18 and 50 years, never- or ex-smokers.
with ≤ 10 pack-years, with a FEV\textsubscript{1} ≥ 80% of predicted and a provocative concentration causing FEV\textsubscript{1} to fall 20% (PC\textsubscript{20}) histamine ≤ 8 mg/ml. Both studies were approved by the medical ethics committee of the Academic Medical Center; written informed consent was obtained. The induction of sputum was identical to the procedure for the selected plugs described below, and the processing has been described earlier\textsuperscript{21,22}.

**Selected plug method: Subjects**

Asthma patients (n=8) were selected among outpatients visiting the Academic Medical Centre, Amsterdam. The diagnosis of asthma was based on the Global Initiative for Asthma (GINA) Guidelines\textsuperscript{23} and asthma severity was defined based on the 2007 consensus\textsuperscript{24}. Patients had episodic chest symptoms, pre-bronchodilator FEV\textsubscript{1}>75% predicted, documented reversibility in FEV\textsubscript{1} >200 ml by 400 µg inhaled salbutamol and/or airway hyperresponsiveness (PC\textsubscript{20} methacholine <4 mg/mL) during the past 12 months. Patients were non-smokers and were treated with low doses of inhaled corticosteroids (< 1000 µg/day inhaled beclomethasone equivalent) and/or long-acting bronchodilators. The study was approved by the medical ethics committee of the Academic Medical Center and all patients gave their written informed consent.

**Selected plug method: Induction and processing of sputum for selected plugs**

Sputum induction was performed according to ERS recommendations\textsuperscript{25}. In short, patients received pre-treatment with 400 µg inhaled salbutamol. FEV\textsubscript{1} was measured by standardized spirometry before three episodes of 5 min inhalation of aerosolized 4.5% hypertonic saline solution generated by an ultrasonic nebulizer (KLAVAmed, Bielefeld, Germany). Prior to sputum production, subjects were asked to rinse their mouth with water, swallow, and blow their nose to minimize contamination of sputum with saliva and postnasal drip fluid. Patients were encouraged to cough and expectorate sputum. Sputum was collected in a sterile container and transported on ice to the lab and processed immediately.

Sputum plugs were selected from sputum dispersed in a petri-dish on a black background using a fine forceps, transferred to the lid of the petri-dish where plugs were moved with small circular motions to spread saliva across the lid and keep the plugs in one mass. To determine variability of inflammatory parameters between plugs from one sputum sample multiple (between 3-6) plugs were distributed over 3 to 4 tubes with 100-400 mg of plugs per tube. Per tube, 4 volume-equivalents (with 100 mg of sputum = 100 µl volume) of 0.1% of freshly prepared sputolysin (Calbiochem) were added, vortexed and incubated on a roller bank at 4°C for 15 min. Cells were pelleted (10 minutes, 4°C, 470 x g), and supernatant was stored in aliquots at -80°C till analyses. Cell pellets were re-
suspended in 1 ml in phosphate-buffered saline (PBS). The total cell number was determined by counting manually in a Bürker counting chamber. To determine whether soluble biomarkers associated with sputum plugs were exchanged with a fluid phase, plugs were treated with 8 volume-equivalents of Dulbecco’s PBS (D-PBS) on a roller bank at 4°C for 15 min. after which plugs were collected by centrifugation (10 min, 470 x g, at 4°C) and supernatant was aliquoted for further analyses. The pelleted plugs were treated with sputolysin as indicated above.

Cells were cytocentrifuged for 2 min at 550 rpm in Shandon Cytocentrifuge and stained with Romanovsky (Diff-Quick) and May-Grunwald-Giemsa (MGG). Differential cell counts were based on 500 non-squamous cells. Sputum samples containing > 80% non-squamous cells on differential cell counted were excluded for differential analysis. Squamous and non-squamous epithelial cells, macrophages, lymphocytes, neutrophils and eosinophils were identified. Differential cell counts were expressed as the percentage of non-squamous cells. Absolute cell numbers were calculated as (% cell x total cell count)/sputum weight. Sputum cell counts were performed by one experienced and qualified technician blinded to the details. As an extra control 10% of the samples were analyzed by a second technician.

Levels of eosinophil cationic protein (ECP)\textsuperscript{26}, interleukin (IL)-8\textsuperscript{27}, myeloperoxidase (MPO)\textsuperscript{27} and alpha2-macroglobulin (A2M)\textsuperscript{28} were measured in sputum with enzyme-linked immuno-sorbent assays (ELISAs).

**Statistical analyses**

**Whole induced sputa:** Sputum data (except % neutrophils and % squamous cells) were base 10 log-transformed prior to all analyses in order to obtain normal distributions. Values of 0% eosinophils were arbitrarily assigned 0.05% before log-transformation. Non-squamous Total Cell Count in 9 whole sputum samples with 100% squamous cells were arbitrarily assigned 0.01x10\textsuperscript{6}/g. Differences in the presence of squamous cells and albumin content under different study conditions (for explanation see ‘Subjects and data sets: For the whole sputum method’) were checked by one-way analysis of variance of % squamous cells and of albumin levels over the study conditions within each patient group. Pearson’s test was used to correlate inflammatory parameters, linear regression was performed on the data (log-transformed when appropriate) versus percentage squamous cells and versus log-transformed albumin levels. Bonferroni’s correction was applied to compensate for multiplicity when analyzing 8 parameters from the same sputum sample. P-values below 0.00625 were considered statistically significant. An explorative Mixed Models analysis was performed post hoc to investigate whether incorporating multiple samples from the same patient (despite sampling under different study conditions) had a
significant impact on the correlation between non-squamous total cell count and % squamous cells. Similarly, an explorative analysis was performed with only one sample, i.e. the first sample, per patient.

On basis of the observed correlations we were able to correct sputum parameters for dilution by saliva. A regression coefficient was calculated for each parameter. We used the formula $y = a + b \cdot x$ to calculate the theoretical (log-transformed) value of the parameter in non-diluted sputum at 0% squamous cells ($y$), from the (log-transformed) measured value of the parameter ($a$), the (positive) regression coefficient for that parameter ($b$) and the % of squamous cells ($x$). The extent of dilution was calculated with $10^{b \cdot x}$. Regression coefficients were also calculated for the relation between log-transformed inflammatory parameters and log-transformed albumin levels and the magnitude of dilution was determined relative to log-transformed albumin levels over the range from the 5th to the 95th percentile (2.5 to 628 μg/g sputum). For graphic display, data is also shown within ten subsets of data points of equal size for increasing % squamous cells (cut-off values 4.0, 8.7, 14.4, 23.0, 30.0, 45.0, 63.0, 78.4 and 90.0%) and for increasing albumin content (cut-off values 3.3, 7.1, 14.9, 26.3, 41.8, 61.1, 88.3, 142.0 and 281.7 μg/g).

In the post-hoc analyses, repeatability (intraclass correlation coefficient), within-patients variability (standard deviation of the absolute difference between the two samples of the log-transformed data) and the between-patients variability (standard deviation of log-transformed data in the first sample) before and after correction for dilution on the basis of % squamous cells were tested by t-test.

Selected plugs: To express the variation of soluble and cellular sputum parameters between 2 or 3 tubes with selected plugs for 8 distinct sputa, we determined the repeatability (intraclass correlation coefficient (ICC); two-way mixed, consistency) using SPSS 20. An ICC of ≥ 0.80 was taken to indicate good repeatability.

RESULTS

Whole sputum analyses

Patients
Data of 247 and 235 induced sputum samples, from 29 patients with COPD and 25 with asthma respectively, were analyzed. Patient characteristics and baseline sputum data are given in supplemental Table 1.

Percentage squamous epithelial cells
The median % squamous epithelial cells was higher in samples from asthma patients than from COPD patients (48% versus 20%, $p < 0.001$): 22% of asthma samples and 15% of COPD samples contained ≥80% squamous cells. In 9 samples (all from 4 patients with
Whole sputum and selected plugs

COPD) there was a surplus of non-discernible, mainly squamous cells, for which % squamous cells was arbitrarily set at 100%. The % squamous cells in subsequent samples from most patients differed widely, but for some patients the % squamous cells were similar in all samples (see supplemental figs. 1 and 2). There were no significant differences in % squamous cells in samples obtained under different study conditions (see ‘Subjects and data sets’ in Methods; p = 0.38 and p = 0.48 for asthma and COPD, respectively; see supplemental figs. 3 and 4).

**Absolute and relative cell counts**

Non-squamous Total Cell Count (TCC) decreased significantly in a log-linear mode with increasing % squamous cells, both for COPD samples (r = −0.82, p < 0.001; fig. 1A) and asthma samples (r = −0.85, p < 0.001, fig. 1B). The regression lines for log-transformed TCC versus % squamous cells showed regression coefficients of −0.020 (95% Confidence Interval (CI): −0.022, −0.018) for COPD samples and −0.017 (95% CI: −0.018, −0.016) for asthma samples (see Methods). These regression coefficients allowed us to calculate the theoretical TCC at 0% squamous cells (i.e. no contamination with saliva), and the extent of dilution of the samples (see Methods). For TCC we calculated a 100-fold dilution (95% C.I. 63 – 126-fold) in samples with 100% squamous cells relative to samples with 0% squamous cells for COPD samples and a 50-fold dilution (95% C.I. 40 – 63-fold) for asthma samples (supplemental table 2). For an ‘average’ sample, i.e. a sample at the median value of % squamous cells, there was an 8-fold dilution of TCC in asthma samples and a 2.5-fold dilution of TCC in COPD samples compared to samples with 0% squamous cells.

A post hoc Mixed Model analysis showed that correction for taking multiple samples from the same patient yielded similar log-linear relationships as shown in figures 1A and B, with a regression coefficient of −0.0189 (p < 0.001), corresponding with a 78-fold dilution at 100% squamous cells and non-significant differences in regression coefficients between patients with COPD or asthma (p = 0.21). Similar significant relationships as shown in figures 1A and B were found when the analysis was restricted to the parameter values for the first sputum sample of each patient (r = −0.70 for COPD and r = −0.90 for asthma, both p < 0.001, see supplemental fig. 5). The box-plot figure indicates that variation of TCC data per subset is similar for all decades with increasing % squamous cells (see Methods) except for the subset with % squamous cells above 90%, both in COPD and asthma (fig. 1C).

The % neutrophils did not significantly change with increasing % squamous cells, both for COPD (r = 0.002, p = 0.97) and asthma samples (r = 0.05, p = 0.48). The % eosinophils slightly decreased with increasing % squamous cells (r = −0.25 and −0.34, for COPD and asthma samples respectively, both p < 0.001, see supplemental fig. 6). However, this was
Figure 1. Non-squamous Total Cell Count (10^6/g sputum, log-transformed) expressed as a function of % squamous cells in sputum samples as individual data from patients with COPD (A, top) and from patients with asthma (B, middle), and grouped in ten equal sized subsets with increasing % squamous cells (C, bottom).

Figure 2. Eosinophil cationic protein (ECP) levels (μg/g sputum, log-transformed) expressed as a function of % squamous cells in sputum samples as individual data from patients with COPD (A, top) and from patients with asthma (B, middle), and grouped in ten equal sized subsets with increasing % squamous cells (C, bottom).
mainly due to samples with >90% squamous cells containing no eosinophils at all. Excluding these samples, % eosinophils marginally, but still significantly decreased with increasing % squamous cells ($r = -0.16, p = 0.017$ and $r = -0.14, p = 0.039$, respectively for COPD and asthma).

**Soluble inflammatory markers**
Log-transformed data for myeloperoxidase (MPO), interleukin-8 (IL-8), eosinophil cationic protein (ECP), α-2 macroglobulin (A2M) and albumin (Alb) showed linear decreases with increasing % squamous cells. Data for ECP and MPO are shown in figure 2 and supplemental figure 7 and are representative of other markers. Fold-dilution for soluble parameters at 100% squamous cells relative to 0% squamous cells is shown in supplemental table 2. We could not calculate fold-dilution for IL-8 for asthma as in half of these samples the IL-8 level was below the detection limit. Samples with undetectable IL-8, however, had significantly higher % squamous cell counts than samples with detectable IL-8 (mean 55% versus 45%, t-test $p < 0.01$). At median squamous cell counts the fold-dilution for soluble parameters ranged between 1.4 and 3.2-fold in asthma, in COPD between 1.3 and 2.1-fold.

**Correction using % squamous cells**
In line with the negative correlation between inflammatory markers and % squamous cells for dilution by saliva we found a positive correlation between inflammatory parameters and albumin levels. This indicates that albumin may too be taken as a surrogate marker of dilution with saliva. We argued that correction for dilution on basis of % squamous cells (see Methods) would make parameters independent of albumin levels, as was found. (fig. 3, top versus bottom). In contrast to our findings for % squamous cells, albumin levels tended to differ between study conditions ($p = 0.066$ and $p = 0.095$ for COPD and asthma samples, respectively).

**Post-hoc analyses**
To determine the effect of correcting sputum data for contamination with saliva we reanalyzed data from a previously published study on repeatability of sputum data. In that study, sputum was obtained twice within one week in 21 clinically stable COPD patients. The total number of sputa in this sub-analysis comprised 17% of the COPD sputa that were used for the correlation studies. Nine inflammatory parameters were studied: non-squamous TCC, numbers of neutrophils, eosinophils and macrophages per g sputum, levels of MPO, IL-8, ECP, A2M and Alb. Data were corrected for % squamous cells with a mean regression coefficient of 0.0155 (see Methods). Correction resulted in a markedly
Figure 3. Effect of correction for dilution on the relationship between non-squamous total cell count (TCC, $10^6$/g sputum, log-transformed, left), eosinophil cationic protein (ECP, μg/g sputum, log-transformed, middle), and myeloperoxidase (MPO, μg/g sputum, log-transformed, right) with albumin level (μg/g, log-transformed, split in ten equal subsets of equal size with increasing albumin level), in sputum samples from patients with COPD (open bars) and asthma (filled bars). Top: without correction, bottom: with correction using % squamous cells (see Methods).
improved repeatability (higher intraclass correlation coefficients), and in smaller within-
patients and between-patients variability (all p < 0.01) (supplemental table 3).

A second post-hoc analysis was done on data from an intervention study on an
allergen challenge in allergic asthma patients\textsuperscript{21}. Data were obtained from 43% of the
asthma sputa of the correlation study. The effect of intervention on the number of
eosinophils and ECP levels per g sputum were analyzed before and after correction.
Previously we observed no difference (p = 0.3) after single-dose pretreatment with
salmeterol/fluticasone propionate compared to fluticasone alone\textsuperscript{21}. After correcting the
data for dilution we observed a tendency (p = 0.06) towards a reduced increase of sputum
eosinophil counts at 24 hours following the allergen provocation in patients treated with
salmeterol/fluticasone as compared to with fluticasone alone.

\textit{Selected plug analyses}

For each of 8 patients with asthma, multiple sputum plugs were obtained from the
sputum sample and distributed over 2 or 3 tubes (100-400 mg of plugs per tube) and
subsequently analyzed for soluble and cellular parameters. The repeatability of biomarker
data between plugs from the same sample is shown in table 1. In parallel, selected plugs in
1 to 3 tubes were exposed for 15 minutes to isotonic protein-free PBS, after which it was
determined whether soluble components from the plugs equilibrated with PBS. Forty to
60\% of the soluble parameters that were originally present in the plugs ended up in PBS
(table 2). This indicates that the interaction of soluble parameters with the sputum plug is
dynamic.

\textbf{DISCUSSION}

Contamination of sputum samples with unknown amounts of saliva increases the variation
of sputum biomarkers and thus reduces the discriminative power of these biomarkers in
e.g. clinical trials. Sputum processed according to the whole sputum method and
contaminated with ≥80\% squamous cells are considered to be contaminated markedly
with saliva and therefore taken as invalid. We found inverse log-linear relationships for
both non-squamous absolute cell numbers (TCC) and soluble inflammatory parameters
with \% squamous epithelial cells, indicating that the percentage of squamous cells can
serve as a quantitative measure for contamination of sputum samples with saliva.
Furthermore, we found that sputum samples with ≤90\% squamous epithelial cells are still
valid for analyses, increasing the number of valid samples. For the selected sputum plug
method we showed that biomarkers are distributed heterogeneous between plugs and
thus analyzing few plugs will add to the variability. Further, soluble biomarkers were found
to interact dynamically with sputum plug constituents, leading to leakage of biomarkers
Table 1. Repeatability of soluble and cellular inflammatory parameters between selected sputum plugs from asthmatics.

<table>
<thead>
<tr>
<th>parameter</th>
<th>ICC</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>0.37</td>
<td>8</td>
</tr>
<tr>
<td>MPO</td>
<td>0.78</td>
<td>6</td>
</tr>
<tr>
<td>ECP</td>
<td>0.95</td>
<td>8</td>
</tr>
<tr>
<td>A2M</td>
<td>0.22</td>
<td>8</td>
</tr>
<tr>
<td>% neutrophils</td>
<td>0.31</td>
<td>5</td>
</tr>
</tbody>
</table>

ICC, intraclass correlation coefficient; n, number of patients from whom plugs were analyzed. Sputum plugs from 8 patients were analyzed, but for two patients MPO could not be determined reliably. For three patients % neutrophils were not taken along; for two patients % squamous cells was ≥ 90%, and for one patient the % neutrophils was ≥ 90%.

Table 2. Relative amounts (%) of soluble parameters associated with selected plugs that end up in the surrounding medium.

<table>
<thead>
<tr>
<th>IL-8</th>
<th>MPO</th>
<th>ECP</th>
<th>A2M</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.2</td>
<td>50.2</td>
<td>45.7</td>
<td>42.0</td>
<td>mean</td>
<td>SD</td>
</tr>
</tbody>
</table>

into the surrounding fluid phase. Thus, plugs should be selected and separated from a fluid phase as soon as possible after sampling.

For the whole sputum method we carefully analyzed data from a large number of sputum samples from COPD and asthma patients. Variation of sputum parameters depends on variable dilution by saliva, on biological variation over time in individual subjects and on differences related to the condition of the patient, such as (corticosteroid) treatment, stable disease and exacerbation. Another important source of variation are inter-individual differences. To limit variation by inter-individual differences we analyzed data from multiple sputum samples from a limited number of patients rather than one sputum sample from many patients. With respect to the variation due to differences in the condition of the patient, retrospective analysis indicated that these different conditions did not underlie the observed relationship between sputum parameters and % squamous epithelial cells. Furthermore, we also showed that a similar correlation was found when only single samples for each patient were analyzed. Therefore, we propose that the observed relationships of biomarkers with the % squamous epithelial cells are genuine and are not biased by inclusion of multiple samples from each patient.
Apart from dilution by saliva, saline used to induce sputum expectoration may also contribute to dilution. Our data do not allow an estimation of the extent of dilution by saline. Previously, however, it was shown that chloride content of sputum samples obtained after induction by hypertonic saline was only slightly higher than in samples obtained after induction by isotonic saline, indicating that only a small proportion of the fluid phase of the sputum sample originated from the nebulized saline. Moreover, dilution with saline would lead to decreased levels of inflammatory parameters with an unaltered % squamous cells. Thus, we propose that the inverse relationship between % squamous cells and inflammatory markers reflects dilution of induced whole sputum samples predominantly by saliva.

Previously albumin content has been taken as a measure of dilution of airway secretions. Indeed, we found that lower values of sputum albumin were correlated with lower values of other inflammatory parameters in sputum. And further, when TCC was corrected for dilution using % squamous cells, the positive relationship of TCC with albumin content was largely reduced (fig. 3), indicating that increasing % squamous cells and decreasing albumin content can serve both as measures of dilution. Correction for albumin in sputum, however, is controversial since albumin permeation may differ with disease severity and treatment. Indeed, we found a tendency for albumin content to vary with study conditions (see ‘Subjects and data sets’ in Methods). Therefore, we propose that % squamous cells provides a better measure of dilution of whole sputum samples by saliva than albumin content.

In sputum samples with >90% squamous cells the variation of sputum parameters, in particular that of cellular parameters, was larger than in samples with ≤90% squamous cells. This indicates that, for samples with >90% squamous cells, parameters are not only affected by dilution but also by other factors, which enhance variation. Previously a cut-off level of 80% squamous cells was proposed as the presence of large amounts of squamous cells physically obscure other cells and thus reduce the accuracy. We report a higher, 90% cut-off level, which may relate to our extended procedure for counting cells (see Methods). The validity of a cut-off value of 90% is reinforced by cell differentials that remain virtually unaffected in sputum samples with up to 90% squamous cells. Therefore we propose that, following our cell count protocol, samples with ≤90% squamous cells are valid for statistical analyses. By adopting 90% as cut-off level, the number of samples in our study deemed invalid was reduced markedly in comparison to the widely used cut-off level of 80% squamous cells: from 14.6 % to 8.9 % excluded samples for COPD and from 21.7 % to 11.5 % respectively for asthma.
The dilution of an ‘average’ sputum sample by saliva is far smaller than that calculated for a sputum sample with 100% squamous cells (supplementary table 2), but still is considerable: for a sample with median % squamous cells a 8-fold and 2.5-fold dilution in total cell counts was calculated for asthma and COPD, respectively, and a maximal 3-fold dilution for soluble markers both for asthma and COPD. Overall, the extent of dilution of sputum in an “average” sample at median % squamous cells was larger in asthma than in COPD samples even though maximal dilution, calculated to occur at 100% squamous cells, was larger within COPD than in asthma samples.

The log-linear regression coefficients and thus also the fold-dilution differed between sputum parameters (supplementary table 2). These differences between sputum parameters may be explained by assuming that saliva contains different levels of sputum parameters. In addition, but not excluding the previous explanation, sputum parameters may display differential interactions with mucin structures, influencing the diffusion rate of components during transport of sputum in the airways. Diffusion of A2M, a large protein with a molecular mass of 725 kDa, is restricted by the tight mucin network, as a consequence of which the fold-decrease is relatively low. In fact, A2M levels were markedly higher in the gel phase as opposed to the soluble phase of spontaneous sputum in COPD\textsuperscript{27,28}. Also the charge of the protein may affect its diffusion rate, like positively charged proteins interacting with negative (sulphur groups) charges on mucins. Interestingly, cationic ECP shows a steeper decline with increasing % squamous cells in sputum samples from COPD patients than in that from asthma patients (supplementary table 2). Non-squamous cell counts decreased markedly with increasing % squamous cells, which may indicate that the larger part of squamous cells may be associated with the surface of sputum plugs allowing their easy removal.

We applied this novel approach to re-evaluate two clinical studies from our institution. Sputum data from samples with ≤90% squamous cells were included and data were corrected for dilution. In the parent COPD study assessing repeatability of sputum parameters in two subsequent sputum samples from clinically stable patients\textsuperscript{29}, correction for dilution increased repeatability and decreased both within-patients variability and between-patients variability (supplementary table 3). In the parent asthma study\textsuperscript{21}, investigating the effects of two therapeutic interventions on allergen-induced inflammation, correction led to a reduced variability of parameters and improved the power of the study, revealing a tendency (p = 0.06) for a difference between treatments, which was not found before (p = 0.3). Taken together, we have shown that when the % squamous cells is taken as a measure of dilution by saliva such a correction enhances the discriminative power of sputum parameters.
An important finding in relation to the selected sputum plug method was the rapid exchange of soluble biomarkers in the sputum plugs with the surrounding fluid. In our experimental set up we took several precautions to not promote this exchange. Plugs were incubated with an isotonic buffer to not disturb ionic interactions between soluble biomarkers and the mucin network in the plugs. The incubation was performed at 4°C, which prevents degranulation of associated cells. And finally, the plugs were incubated for 15 min only, which is relatively short compared to the 1 to 2 hours that is usually allowed between sampling and processing of sputum. Despite these conditions, 40 to 60% of the plug-associated soluble biomarkers ended up in the fluid phase. In part this marked exchange may be due to the 8 volume-equivalents used to incubate the plugs with. Nevertheless, this finding reflects that soluble biomarkers interact dynamically with the matrix of the plugs. Taken together these findings imply that selected sputum plugs should be separated as soon as possible after sampling. To limit exchange, additional techniques such as washing of the plugs should be kept to a minimum.

We also found that biomarkers were distributed heterogeneously between the selected plugs. Values for MPO and ECP were relatively consistent between the various plugs, but the variation between the plugs for A2M, IL-8 and % neutrophils was large. This variation is not explained by variation of the employed quantitative assays (ELISAs etc.). The samples were measured in parallel and the intra-assay variation for the assays was below 10%. This heterogeneity underlines the need to analyze multiple if not all plugs for the analyses. We used between 100-400 mg of plugs that revealed this heterogeneity, indicating that more plugs are required to reduce the variation.

In conclusion, we have identified means to reduce variation of sputum biomarkers for both the selected plug and the whole sputum method. For the selected plug method, analyses of a substantial number of plugs and minimizing the interaction with surrounding fluid are crucial to limit variation. For the whole sputum method, the % of squamous cells can be taken as a quantitative measure of contamination with saliva, allowing to correct for dilution. In addition, samples with up to 90% squamous cells can be analyzed, which can provide a significant increment in the number of valid sputa.

Acknowledgements
We gratefully acknowledge Petra Teiwes for lung function tests and collection of sputum. The studies on the selected plug method were facilitated by a grant from ‘Stichting Astmabestrijding’ (2008/003). For the whole sputum method, the parent studies received unrestricted grants from AstraZeneca (the COPD study, BN-00P-0087) and from GlaxoSmithKline (the asthma study, SER98010).
REFERENCES


**SUPPLEMENT**

**Table S1.** Demographic and baseline sputum data of patients at enrolment into the study.

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male / female</td>
<td>21 / 8</td>
<td>8 / 17</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>64.7 (51 - 76)</td>
<td>25.2 (19 - 35)</td>
</tr>
<tr>
<td>FEV(_1) (% predicted)</td>
<td>61.1 (29 - 97)</td>
<td>102.0 (79 - 120)</td>
</tr>
<tr>
<td>Total Cell Count (10(^6)/g sputum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- including squamous cells</td>
<td>1.90 (0.30 – 21.8)</td>
<td>1.04 (0.25 – 3.64)</td>
</tr>
<tr>
<td>- excluding squamous cells</td>
<td>0.98 (0.11 – 21.5)</td>
<td>0.58 (0.02 – 3.48)</td>
</tr>
<tr>
<td>Squamous cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>19.6 (1.2 – 91.3)</td>
<td>48.0 (3.6 – 95.1)</td>
</tr>
<tr>
<td>- 10(^6)/g sputum</td>
<td>0.35 (0.02 – 4.15)</td>
<td>0.33 (0.10 – 1.12)</td>
</tr>
<tr>
<td>Neutrophils (% of non-squamous cells)</td>
<td>70.6 (31.1 – 96.5)</td>
<td>30.0 (4.1 – 83.5)</td>
</tr>
<tr>
<td>Eosinophils (% of non-squamous cells)</td>
<td>1.2 (0.1 – 21.6)</td>
<td>5.2 (0 – 72.2)</td>
</tr>
<tr>
<td>MPO (µg/g sputum)</td>
<td>9.4 (0.4 – 188)</td>
<td>0.74 (0.16 – 10.5)</td>
</tr>
<tr>
<td>IL-8 (ng/g sputum)</td>
<td>5.0 (0.4 – 174)</td>
<td>0.25 (0.10 – 1.73)</td>
</tr>
<tr>
<td>ECP (µg/g sputum)</td>
<td>322 (10 – 38260)</td>
<td>57.0 (4.8 – 322)</td>
</tr>
<tr>
<td>Alb (µg/g sputum)</td>
<td>59.2 (1.6 – 2379)</td>
<td>30.6 (0.89 – 97.4)</td>
</tr>
<tr>
<td>A2M (µg/g sputum)</td>
<td>1.10 (0.34 – 50.4)</td>
<td>1.35 (0.21 – 8.92)</td>
</tr>
</tbody>
</table>

Data expressed as absolute numbers or mean (range) for age and FEV\(_1\), and for all sputum data as median (95% Confidence Interval), obtained in a stable state without corticosteroid treatment. FEV\(_1\), post-bronchodilator forced expiratory volume in the first second; MPO, myeloperoxidase; ECP, eosinophil cationic protein; IL-8, interleukin 8; Alb, albumin; A2M, alpha-2-macroglobulin.

**Table S2.** Calculated maximal dilution of inflammatory parameters in sputum samples with 100% squamous cells.

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-squamous Total Cell Count</td>
<td>100 (63 – 158)</td>
<td>50 (40 – 63)</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>4.0 (2.0 – 10)(^*)</td>
<td>7.9 (4.0 – 16)(^*)</td>
</tr>
<tr>
<td>Albumin</td>
<td>10 (6.3 – 20)</td>
<td>3.2 (2.0 – 6.3)</td>
</tr>
<tr>
<td>alpha-2-macroglobulin</td>
<td>4.0 (2.5 – 7.9)</td>
<td>2.5 (1.6 – 5.0)</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>20 (13 – 40)</td>
<td>2.0 (1.6 – 3.2)</td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>25 (10 – 50)</td>
<td>N/A</td>
</tr>
<tr>
<td>Eosinophil Cationic Protein</td>
<td>40 (20 - 79)</td>
<td>10 (6.3 – 16)</td>
</tr>
</tbody>
</table>

Data from 247 COPD samples and 235 asthma samples, dilution expressed as n-fold decrease (95% Confidence Interval) at 100% squamous cells relative to 0% squamous cells; % neutrophils and % eosinophils as % of non-squamous cells. N/A, not applicable, as there is no significant correlation with % squamous cells; \(^*\) data expressed as absolute decrease and 95% Confidence Interval; \(^\#\) relation became non-significant (N/A) when restricted to samples with <90% squamous cells.
Table S3. Repeatability of sputum data without and with correction for dilution using % squamous cells.

<table>
<thead>
<tr>
<th></th>
<th>Ri</th>
<th>Within-patient variability†</th>
<th>Between-patients variability‡</th>
<th>Ri after correction§</th>
<th>Within-patient variability after correction§</th>
<th>Between-patients variability after correction§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-sq. TCC (10^6/g)</td>
<td>0.52</td>
<td>0.287</td>
<td>0.556</td>
<td>0.70</td>
<td>0.156</td>
<td>0.391</td>
</tr>
<tr>
<td>Neutrophil count (10^3/g)</td>
<td>0.52</td>
<td>0.306</td>
<td>0.580</td>
<td>0.70</td>
<td>0.193</td>
<td>0.438</td>
</tr>
<tr>
<td>Eosinophil count (10^3/g)</td>
<td>0.35</td>
<td>0.549</td>
<td>0.773</td>
<td>0.62</td>
<td>0.390</td>
<td>0.685</td>
</tr>
<tr>
<td>Macrophage count (10^3/g)</td>
<td>0.51</td>
<td>0.369</td>
<td>0.630</td>
<td>0.57</td>
<td>0.303</td>
<td>0.494</td>
</tr>
<tr>
<td>MPO (μg/g)</td>
<td>0.47</td>
<td>0.544</td>
<td>0.754</td>
<td>0.63</td>
<td>0.354</td>
<td>0.653</td>
</tr>
<tr>
<td>IL-8 (ng/g)</td>
<td>0.34</td>
<td>0.676</td>
<td>0.781</td>
<td>0.46</td>
<td>0.384</td>
<td>0.611</td>
</tr>
<tr>
<td>ECP (ng/g)</td>
<td>0.52</td>
<td>0.552</td>
<td>0.804</td>
<td>0.74</td>
<td>0.323</td>
<td>0.705</td>
</tr>
<tr>
<td>Alb (μg/g)</td>
<td>0.70</td>
<td>0.367</td>
<td>0.737</td>
<td>0.86</td>
<td>0.211</td>
<td>0.722</td>
</tr>
<tr>
<td>A2M (μg/g)</td>
<td>0.60</td>
<td>0.315</td>
<td>0.533</td>
<td>0.81</td>
<td>0.211</td>
<td>0.593</td>
</tr>
</tbody>
</table>

Data from sets with paired samples from 21 COPD patients obtained within one week. Ri, intraclass correlation coefficient; † within-patients variability is the standard deviation of the absolute difference of the base-10 logarithmic transformed data; ‡ between-patients variability is the standard deviation of the log-transformed data from the first sample; § correction for dilution by adding to the log-transformed data the value of “ % squamous cells x 0.0155 ”; ¶ gram of sputum. Non-sq.TCC, non-squamous total cell count; MPO, myeloperoxidase; IL-8, interleukin 8; ECP, eosinophil cationic protein; Alb, albumin; A2M, alpha-2-macroglobulin.
Figure S1. Non-squamous Total Cell Count ($10^6$/g sputum, log-transformed) expressed as a function of % squamous cells in sputum samples in individual patients with COPD who had at least two samples.
Figure S2. Non-squamous Total Cell Count ($10^6$/g sputum, log-transformed) expressed as a function of % squamous cells in sputum samples in individual patients with asthma who had at least two samples.
Figure S2 (continued). Non-squamous Total Cell Count ($10^6/g$ sputum, log-transformed) expressed as a function of % squamous cells in sputum samples in individual patients with asthma who had at least two samples.
Figure S3. Squamous Cell count (%) Boxplots in sputum samples from COPD patients collected under different study conditions. At entry (n = 31), after Run-in (n = 67), after prednisolone (n = 42), after inhaled budesonide (n = 49), after inhaled placebo (n = 43) or immediate before or after an exacerbation (n = 15). Differences between conditions not significant (p = 0.48).

Figure S4. Squamous Cell count (%) Boxplots in sputum samples from asthma patients collected under different study conditions. At entry (n = 25), after Run-in (n = 63), 6 or 24 hours after allergen challenge (n = 99) or 1 week after allergen challenge (n = 47). Differences between conditions not significant (p = 0.38).
Figure S5. Non-squamous Total Cell Count ($10^6$/g sputum, log-transformed) expressed as a function of % squamous cells in sputum samples from 29 patients with COPD (triangles) and from 25 patients with asthma (circles), obtained at enrolment in the study. Pearson’s $r = 0.79$, $p < 0.001$. 
Figure S6. Eosinophil count (%, log-transformed) expressed as a function of % squamous cells in sputum samples as individual data from patients with COPD (A, top) and from patients with asthma (B, middle), and grouped in ten equal sized subsets with increasing % squamous cells (C, bottom). Values of 0% eosinophils have been assigned arbitrarily the value of 0.05%.

Figure S7. Myeloperoxidase (MPO) levels (µg/g sputum, log-transformed) expressed as a function of % squamous cells in sputum samples as individual data from patients with COPD (A, top) and from patients with asthma (B, middle), and grouped in ten equal sized subsets with increasing % squamous cells (C, bottom).
Summary and General discussion
Chapter 8

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 IFNγ 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 (IgE) 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>0.018</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 literature⁵,⁶. 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 ratspecific 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. 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. Recently, asthmatic patients were also found to have an altered microbial composition in the airways compared with non-asthmatics. 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. 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. 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. 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. 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, it might take a longer period of diet change or probiotic intake to shift from an ‘atopic’ gut microbiota to a healthy one. 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. 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 *Acinetobacter Iwoffii* F78, a bacterium from farming environment with possible allergy-protective properties, for activation of a Th1-polarizing program in human dendritic cells. 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. 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. 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. 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.
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. Similar exaggerated Th2 responses were also found in mice that were treated with antibiotics to alter the composition of commensal microbiota. 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
Summary and General discussion


Nederlandse samenvatting
Curriculum vitae
PhD portfolio
List of publications
Dankwoord
Inleiding

Allergisch astma is een chronische ontstekingsziekte van de luchtwegen. De luchtwegen van astmapatiënten zijn overgevoelig voor prikkels in de inademingslucht, waardoor slijmvorming optreedt en de onderliggende spierlaag geïrriteerd raakt en samentrekt. Klachten als piepende ademhaling, hoesten en benauwdheid worden meestal afgewisseld met klachtenvrije periodes. Kenmerkend voor de chronische ontsteking in de luchtwegen van patiënten met milde tot matige astma is de verhoogde aanwezigheid van eosinofiele cellen, mestcellen en T helper (Th) type 2 lymfocyten. Deze laatste cellen produceren onder andere de cytokines interleukine (IL)-4, IL-5 en IL-13, mediatoren die een belangrijke sturende rol spelen bij allergische ontstekingsprocessen. De mate waarin al deze cellen in de luchtwegen aanwezig zijn verschilt per patiënt en de ernst van de astma.

Continue blootstelling aan allergenen in de lucht zorgt ervoor dat de luchtwegen van allergische astmapatiënten chronisch geïrriteerd en ontstoken zijn, waardoor de bekleding van de luchtwegen, het slijmvlies, beschadigd raakt en nog gevoeliger wordt voor prikkels van buitenaf. Verder verandert ten gevolge van de chronische ontstekingsprocessen de weefselopbouw van de luchtwegen, hetgeen ook bijdraagt aan een grotere gevoeligheid voor prikkels in de inademingslucht.

Piepende ademhaling bij virusinfecties en de ontwikkeling van allergie op jonge leeftijd zijn twee belangrijke kenmerken die gekoppeld zijn aan een verhoogd risico op het ontwikkelen van astma. De laatste decennia is in landen met een Westerse levensstijl tegelijk met de verstekeling en bijbehorende veranderingen in voeding en leefomstandigheden een enorme toename gezien in het aantal mensen met allergisch astma en andere allergische ziekten. Sinds Strachan eind jaren tachtig suggereerde dat de toename in allergie samenhangt met een afname in infecties door betere hygiëne (hygiëne hypothese), wordt veel onderzoek gedaan naar de rol van omgevingsfactoren gedurende de ontwikkeling van astma en andere allergische aandoeningen. Alhoewel bekend is dat blootstelling aan allergenen samenhangt met het ontwikkelen van allergisch astma, weet men nog steeds niet precies welke mechanismen ten grondslag liggen aan deze allergische ontsteking. In dit proefschrift wordt onder andere ingegaan op deze mechanismen en de invloed van omgevingsfactoren zoals allergenen, een bacteriële cellwand component (endotoxine) en bacteriën op de ontwikkeling en het voortduren van de allergische ontstekingsactiviteit bij astma.

Samenvatting van de belangrijkste bevindingen

Om inzicht te krijgen in de immunologische veranderingen tijdens het ontwikkelen van een allergie (sensibilisatie) hebben we proefdierwerkers, die recent met proefdierwerk waren begonnen, gedurende 2 jaar gevolgd (start, 4 maanden, 1 jaar en 2 jaar). Gedurende deze 2 jaar ontwikkelde 20% van de proefdierwerkers een nieuwe
sensibilisatie tegen proefdierallergenen. Binnen dit cohort vergeleken we de aller geen-specificieke en aspecificieke ex-vivo cytokine respons in bloed van proefdierwerkers die een ratallergene-specificieke sensibilisatie ontwikkelden met die van vergelijkbare proefdierwerkers die geen nieuwe sensibilisatie ontwikkelden. Dit staat beschreven in hoofdstuk 2. Wij vonden dat sensibilisatie tegen ratallergene gepaard ging met een toename in ratallergene-specificieke, IL-4-producerende witte bloedcellen. Het aantal IL-4 producerende witte bloedcellen na stimulatie met ratallergene was kwantitatief geassocieerd met werk-gerelateerde allergische klachten en met de hoeveelheid ratallergene-specifiek immunoglobuline(Ig)E in het bloed. In tegenstelling tot eerdere suggesties in de literatuur dat aller geen-specificieke productie van IL-10 en interferon(IFN)γ, een Th1 cytokine, beschermend zou werken tegen het ontwikkelen van allergie, zagen wij op geen enkel tijdspunt verschil in het aantal ratallergene-specificieke IL-10- en IFNγ-producerende cellen tussen proefdierwerkers die wel of geen ratallergene-specificieke sensibilisatie ontwikkelden. Wel zagen we dat de gemiddelde IL-10 productie in volbloed, afgenomen tijdens de vervolgbezoeken, na aspecifieke stimulatie met Staphylococcus aureus Cowan I strain (SAC) negatief correleerde met de concentratie van ratallergene-specificie IgE in serum na 2 jaar.

Behalve sensibilisatie door blootstelling aan allergenen, kan ook de aan- of afwezigheid van andere omgevingsfactoren een rol spelen in het ontwikkelen van een allergie. Voor het overal aanwezige endotoxine (lipopolysaccharide (LPS), een onderdeel van de celwand van Gramnegatieve bacteriën) geldt bijvoorbeeld dat de richting van de immuunrespons (door productie van Th1- ofwel Th2 cytokinen) afhankelijk is van de concentratie van endotoxine tijdens de sensibilisatie of blootstelling aan allergenen. In hoofdstuk 3 vergelijken we de cytokine respons van witte bloedcellen van allergische en niet-allergische vrijwilligers na stimulatie met lage concentraties LPS (10 pg/ml), vergelijkbaar met de dagelijkse blootstelling aan endotoxine binnenshuis. De witte bloedcellen van de patiënten met allergisch astma bleken na stimulatie met 10 pg/ml LPS significant minder IL-10, IL-12 en IFNγ te produceren dan die van de niet-allergische vrijwilligers. Dit verschil verdween bij stimulatie met een 10 keer hogere concentratie LPS (100 pg/ml). Stimulatie met 100 pg/ml LPS bracht de productie van bovengenoemde cytokines in de allergische groep niet alleen op gelijke hoogte met die van de niet-allergische groep, maar remde ook de productie van Th2 cytokines door met huisstofmijt gestimuleerde witte bloedcellen van huisstofmijt-allergische astma patiënten. Deze resultaten suggereren dat witte bloedcellen van allergische astmatici blootstelling aan een hogere concentratie LPS nodig hebben om de richting van de immuunrespons van Th2 naar Th1 te verschuiven en dat de hoeveelheid LPS binnenshuis in Westerse landen tegenwoordig te laag is om deze drempel te bereiken. Wij denken daarom dat deze drempel voor LPS bijdraagt aan het ontwikkelen van allergieën.
In het onderzoek naar de cellulaire mechanismen, die betrokken zijn bij de ontstekingsprocessen in de luchtwegen van allergische astma patiënten, is de bronchiale allergeneprovocatie een veel gebruikte methode. Bij deze methode ademen vrijwilligers met allergisch astma oplopende concentraties van allergenen in tot een bepaalde graad van benauwdheid optreedt, waarna door middel van sputum inductie of een bronchoalveolaire spoeling de ontstekingscellen en mediatoren uit de luchtwegen worden verzameld en geanalyseerd. Uit onderzoek naar de veiligheid en herhaalbaarheid van bronchiale allergeneprovocaties is gebleken dat de luchtweg-gerelateerde symptomen na ongeveer 2 weken weer verdwenen zijn. Over eventuele systemische effecten is echter nog niet veel bekend. In hoofdstuk 4 tonen we aan dat 5 weken na een allergeneprovocatie met een redelijk hoge dosis huisstofmijt de concentratie van huisstofmijt-specifiek IgE in het bloed nog steeds is verhoogd, terwijl de longfunctiewaarden en de bronchiale ontstekingsparameters na een paar weken alweer terug waren op het niveau van voor de provocatie. Behalve deze toename in huisstofmijt-specifiek IgE zagen we ook een toegenomen productie van Th2 cytokinen door de witte bloedcellen na stimulatie met huisstofmijt. Alhoewel de gevoeligheid van de luchtwegen voor prikkels uit de inademingslucht 5 weken na deze ene allergeneprovocatie niet verhoogd was, zou een opeenstapeling van de gevonden systemische effecten door dagelijkse blootstelling aan allergenen wél kunnen leiden tot de ontwikkeling of verergering van de allergische reactie. De langdurig toegenomen systemische gevoeligheid voor huisstofmijt na eenmalige blootstelling benadrukt de rol van B en T cellen in de ontwikkeling van en de aanhoudende ontstekingsactiviteit bij allergisch astma.

De eosinofiele cellen die zich na inhalatie van allergenen in de luchtwegen verzamelen spelen mogelijk ook een rol bij de lokale activatie van de bloedstolling die is waargenomen in de luchtwegen van astmatische patiënten. Aangezien stolling en ontstekingsactiviteit elkaar beïnvloeden, zou een verhoogde stollingsactivatie de allergische ontsteking in de luchtwegen kunnen verergeren. Om de rol van lokale stollingsactivatie op de ontstekingsprocessen en vice versa in de luchtwegen van allergische astmapatiënten te onderzoeken, hebben we in hoofdstuk 5 de stollingsactivatie die plaats vindt in de luchtwegen van allergische astmapatiënten, en het effect van segmentele allergeneprovocatie hierop, vergeleken met de stollingsactivatie in de luchtwegen van gezonde controles. Zowel vóór de allergene provocatie als 4 uur daarna vonden we significant hogere concentraties van stollingsactivatie parameters in de bronchoalveolaire spoelingsvloeistof van patiënten met allergisch astma vergeleken met die van gezonde individuen. Hierbij ging de toename in trombine-anti-trombine complexen (TATc) en in tissue factor (TF), wat vrijkomt bij weefselschade, gepaard met een afname in de stollingsremmer proteïne C. Hoewel de toename in stollingsactivatie door allergeneprovocatie bij allergische astmapatiënten duidelijk groter was dan bij de gezonde
controles, was dit niet gerelateerd aan de aanwezigheid van eosinofiele cellen in de luchtwegen. Opvallend in deze studie was dat de allergische astmapatiënten na segmentale allergenprovocatie evenveel pro-inflammatoire cytokines produceerden als de gezonde controles, terwijl ze een veel lagere dosis allergen in de luchtwegen toegediend hadden gekregen. Deze resultaten suggereren dat de luchtwegen van allergische astmatici niet alleen een afwijkende reactie hebben op allergenen, maar ook meer stollingsactiviteit vertonen en gevoeliger zijn voor aspecifieke ontsteking.

De huidige behandeling van astma bestaat hoofdzakelijk uit het remmen van de chronische ontsteking in de luchtwegen met (inhaalatie)corticosteroiden en het verlichten van de symptomen met luchtwegverwijdende geneesmiddelen. Deze medicatie vermindert echter alleen de symptomen van astma, maar geneest de ziekte niet. De hygiëne hypothese heeft ertoe geleid dat nieuwe therapiën worden onderzocht die de afwijkende Th2 immuunrespons kunnen veranderen in een niet-allergische Th1 respons of de aanwezigheid en/of werkzaamheid van regulerende T cellen kunnen verhogen. Een van deze nieuwe therapiën is gebaseerd op het direct of indirect beïnvloeden van de immuunrespons door verandering van de samenstelling van de darmflora. In hoofdstuk 6 beschrijven we de resultaten van een dubbelblind, placebo-gecontroleerd klinisch interventie onderzoek naar het effect van synbiotica (een specifieke combinatie van prebiotische oligosacchariden met een probiotische *Bifidobacterium breve* bacteriestam) op de allergische respons bij patiënten met allergisch astma. Voor dit onderzoek werd bij de start van het onderzoek en na 4 weken inname van de synbiotica een bronchiale allergenprovocatie uitgevoerd om uit te zoeken of inname van synbiotica een positief effect zou hebben op de bronchiale ontstekingsactiviteit, de longfunctie en immunologische parameters na inademing van allergenen. Behandeling met synbiotica gedurende 4 weken was geassocieerd met een verbetering in de piekstroom en een verminderde allergen-geïnduceerde productie van de Th2 cytokine IL-5, zowel *in vivo* als *in vitro*. Dit betekent dat inname van synbiotica de toename in gevoeligheid van witte bloedcellen voor allergenen na blootstelling aan deze allergenen, zoals beschreven in hoofdstuk 4, kan onderdrukken, waardoor op langere termijn de allergen-geïnduceerde Th2 immuunrespons bij patiënten met allergisch astma zou kunnen afnemen.

In hoofdstuk 7 stellen we een nieuwe methode voor om analyses van geïnduceerd sputum te corrigeren voor speeksel bijmenging. De analyse van geïnduceerd sputum is een belangrijke en veel gebruikte methode om inzicht te krijgen in de aan- of afwezigheid van verschillende ontstekingscellen en hun mediatoren bij longziekten zoals astma en COPD. Helaas zorgt bijmenging van speeksel vaak voor grote variatie in de uitkomstmaten. Aangezien het sputum, dat vanuit de longen wordt opgehoest, via de mond in een buisje wordt verzameld is het bijna onmogelijk om bijmenging met speeksel te voorkomen. De
nieuwe methode verbetert de validiteit van de cellulaire en moleculaire biomarkers in geïnduceerd sputum.

Conclusie
Ondanks vele onderzoeken naar de ontwikkeling en behandeling van allergisch astma en andere allergische aandoeningen, zijn de mechanismen die ten grondslag liggen aan de ontwikkeling en het voortduren van de allergische ontstekingsactiviteit bij astma nog steeds niet helemaal opgehelderd. De onderzoeken beschreven in dit proefschrift laten zien dat patiënten met allergisch astma blootstelling aan een hogere dosis LPS nodig hebben en misschien een andere TLR-signal route gebruiken om IL-10 te kunnen produceren en de Th2 respons te kunnen terugdraaien. Een interessant gegeven is dat het bij patiënten met bestaande allergieën nog steeds mogelijk was om de allergenspecifieke Th2 respons te moduleren met LPS. Toevoeging van LPS in *in vitro* experimenten en inname van probiotica door patiënten met allergisch astma resulteerden beide in een afname van de allergische respons. Deze resultaten suggereren dat een juiste activatie van het niet-specifieke, aangeboren immuunsysteem de afwijkende Th2 respons zou kunnen remmen. Verder onderzoek naar de mogelijkheden van probiotica om de ontwikkeling van allergie te voorkomen en misschien zelfs bestaande allergische ontstekingsactiviteit terug te draaien is dan ook aan te bevelen.

Een andere opvallende uitkomst van de in dit proefschrift beschreven onderzoeken is dat het bloed vele aspecten weergeeft van de allergische ontstekingsactiviteit in de luchtwegen. Zelfs na enige weken zijn nog steeds de gevolgen van blootstelling aan allergenen in het bloed te meten, terwijl de lokale effecten in de luchtwegen alweer verdwenen zijn. Aan de andere kant zien we ook dat na blootstelling aan allergenen gelijk met de allergische ontstekingsactiviteit ook aspecifieke ontstekingsactiviteit en stollingsactivatie in de luchtwegen plaatsvinden. Het lijkt er zelfs op dat aspecifieke ontsteking en stollingsactivatie veel meer en reeds bij lagere concentraties van allergenen voorkomen in de luchtwegen van allergische astmapatiënten dan in die van gezonde controles. Ook uit recent onderzoek dat is uitgevoerd op onze afdeling is een link gevonden tussen de bloedcirculatie en de lokale responsen in de luchtwegen (van der Sluijs et al.). Bovenstaande resultaten vragen om verder onderzoek naar de regulatie van allergische ontstekingsactiviteit door cellen en mediatoren in de circulatie.
CURRICULUM VITAE

# PhD Portfolio

## General courses:
- Practical biostatistics
  
- Clinical Data Management
  
- Scientific writing in English for publication
  
- BROK (Basiscurius Regelgeving Klinisch Onderzoek)
  
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## Specific courses:
- Clinical skills (spirometry, bronchial challenge procedures, sputum induction, blood collection)
  
- Advanced Immunology
  
- Respiratory Research School Davos-Amsterdam
  
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## Presentations and Conferences:
- Increase in allergen-specific IgE and *in vitro*-Th2 responses after a single bronchial challenge with house dust mite (HDM) in allergic asthmatics, poster presentation at ATS conference Toronto
  
- Endotoxin contamination of allergen extracts suppresses the allergen-induced Th2-cytokine production by *in vitro* stimulated PBMCs, poster presentation at ATS conference Toronto
  
- Syntbiotics improve peak expiratory flow rate and reduce systemic IL-5 after allergen challenge in patients with allergic asthma, poster presentation at EAACI conference Barcelona and at NVVI
  
- Threshold to endotoxin might hamper the development of tolerance in allergic subjects, poster discussion at Longdagen Utrecht and oral presentation at EAACI conference Genève:
  
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## Other:
- ALIFI PhD Retreat
  
- Weekly department seminars
  
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LIST OF PUBLICATIONS

Increase in allergen-specific IgE and ex vivo Th2 responses after a single bronchial challenge with house dust mite in allergic asthmatics.
van de Pol MA, Lutter R, van Ree R, van der Zee JS.

Synbiotics reduce allergen-induced T-helper 2 response and improve peak expiratory flow in allergic asthmatics.
van de Pol MA, Lutter R, Smids BS, Weersink EJ, van der Zee JS.

Dynamics in cytokine responses during the development of occupational sensitization to rats.
Krop EJ, van de Pol MA, Lutter R, Heederik DJ, Aalberse RC, van der Zee JS.

Early activation of coagulation after allergen challenge in patients with allergic asthma.
Schouten M, van de Pol MA, Levi M, van der Poll T, van der Zee JS.
J Thromb Haemost. 2009 Sep;7(9):1592-4

Long-term effects of budesonide on inflammatory status in COPD.
Boorsma M, Lutter R, van de Pol MA, Out TA, Jansen HM, Jonkers RE.
COPD. 2008 Apr;5(2):97-104

Repeatability of inflammatory parameters in induced sputum of COPD patients.
Boorsma M, Lutter R, van de Pol MA, Out TA, Jansen HM, Jonkers RE.
List of publications
DANKWOORD

Eindelijk... het is af! Na jaren van “bijna af..nee, maar het schiet lekker op.. nog 1 artikel.. alleen de discussie nog..nee nog steeds niet, maar wel bijna..”, kan ik nu eindelijk zeggen: HET IS AF!!! Wat een opluchting! In mijn eentje had ik dit nooit kunnen doen en ik wil daarom op deze laatste bladzijden een aantal mensen bedanken die op wat voor manier dan ook hebben geholpen met het tot stand komen van dit proefschrift.

Ten eerste wil ik alle proefpersonen bedanken die bereid waren om allergeen provocaties te ondergaan, klonterige studievoeding in te nemen en bloed en sputum af te staan. Zonder hen had ik een groot deel van de in dit proefschrift beschreven analyses en ex-vivo experimenten niet kunnen uitvoeren.


En natuurlijk mijn paranimfen Barbara en Tamara; eindelijk samen op de voorgrond. Wat een fijn idee dat jullie straks naast mij zitten. Of het nou om het opwerken van sputum, bloed of een stuk long gaat, ELISA’s, luminex of elispot, het is altijd een feest om met jullie samen te werken. Hopelijk komen er nog voldoende projecten om dit nog een tijdje voort te zetten.

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Dankwoord

Collega (arts)onderzoekers van de afdeling Longziekten, jullie zijn niet echt betrokken geweest bij de inhoud van dit proefschrift, maar jullie morele steun bij de afronding ervan en het feit dat er altijd iemand klaar staat voor anamnese en lichamelijk onderzoek bij de huidige projecten heeft mij veel geholpen.

In de afgelopen 10 jaar waarin ik op de afdelingen Experimentele Immunologie en Longziekten heb gewerkt heb ik vele mensen zien komen en gaan. Alle namen opnoemen is onmogelijk, dus voor iedereen die niet bij naam genoemd is, maar mij wel met woord of daad geholpen heeft: Bedankt!

Kwikjes, Meso’s, vrienden, dank voor jullie steun en niet aflatende interesse in de vorderingen van mijn proefschrift. Na 2 juli heb ik eindelijk weer tijd om af te spreken en bij te praten.

Lieve familie en schoonfamilie, zonder jullie hulp was dit boekje nog steeds niet af geweest. In de wekeinden dat Gijs en Lotte bij jullie konden spelen of logeren heb ik uiteindelijk de laatste stukken af kunnen schrijven. Ook jullie bedankt voor de morele steun en het vertrouwen dat het ooit eens af zou zijn. Lieve Jacqueline, Martin en Jeanette, jullie zijn alle drie op je eigen manier een groot voorbeeld voor mij en ik hoop dat jullie er op 2 juli alle drie bij kunnen zijn.

Lieve pap en mam, jullie hebben mij altijd gestimuleerd om mijn kansen te grijpen en geleerd dat je hard moet werken en goed je best moet doen als je echt iets wilt. Bedankt dat jullie altijd voor mij klaar staan.

Allerliefste Rob, Gijs en Lotte, nog even en ik heb weer alle tijd voor jullie! Dank voor alle tijd en geduld. Jullie maken mij erg gelukkig.