Effects of environmental exposures on asthma phenotypes in the mouse

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Chapter 7
Farm dust exposure and development of non-eosinophilic airway inflammation in mice: the role of epithelium, macrophages and B cell follicles

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ABSTRACT

Introduction Farming life can on the one hand protect against development of allergies and allergic asthma, but on the other hand induce airway symptoms like dyspnea, cough and wheezing in non-allergic individuals. We recently showed that farm dust exposure decreases house dust mite (HDM)-induced IgE and eosinophilic airway inflammation and simultaneously induces non-allergic airway inflammation.

Aim To investigate mechanisms underlying the observed decreased allergic airway inflammation and simultaneously increased, possibly TH17 mediated, non-allergic airway inflammation.

Methods Lungs of mice previously exposed to dust from cowsheds were studied in detail for Toll-like receptor (TLR)2 and TLR4 expression in epithelium, thymic stromal lymphopoietin (TSLP) levels, the number of alternatively activated macrophages and the composition of infiltrates. Mice were intranasally exposed 4 times/week for 5 weeks to phosphate buffered saline (PBS) or farm dust (1 mg/ml, 50 µl/day, collected from five different farms in North Germany), followed by PBS or HDM (2.5 mg/ml, 10 µl/day).

Results Farm dust exposed mice had lower TLR2 and TLR4 expression in epithelium and TSLP levels in lung tissue than HDM exposed mice. Expression of YM1 by alternatively activated macrophages was lower in farm dust exposed mice as well. The infiltrates in lung tissue from dust exposed mice consisted of B cells and follicular dendritic cells and IgG1 levels in serum were higher in farm dust exposed mice than in HDM exposed mice.

Conclusions Downregulation of TLR2 and TLR4 expression in epithelium may contribute to the observed dust-induced, non-allergic asthma phenotype, since downregulation of TLR expression associated with lower levels of TSLP. Lack of TSLP may have prevented induction of allergic sensitisation and airway inflammation in farm dust exposed mice. Additionally, we observed formation of B-cell follicles in dust exposed mice, which could be caused by the observed higher numbers of TH17 cells in these mice.

INTRODUCTION

Asthma is defined as a chronic inflammatory disease of the airways, which is characterized by airway hyperresponsiveness and leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing (Global INitiative for Asthma, GINA). Many studies have focussed on allergic asthma, which is characterised by eosinophilic inflammation, a TH2 type immune response and allergen specific IgE production. However, a considerable proportion of asthma, sometimes even more than 50%, is not attributable to allergic sensitisation. Although not much research has focussed on non-allergic asthma specifically, it is nowadays thought that non-allergic asthma does not involve an allergen specific IgE response, tends to display neutrophilic instead of eosinophilic inflammation, is frequently clinically steroid resistant and often more severe.

Several studies report that the incidence of asthma has increased over the past decades worldwide, especially in industrialized western countries with increased hygiene standards. However it is not known whether this increase is attributable to allergic or non-allergic asthma. A hypothesis explaining the increase in asthma prevalence is the hygiene hypothesis, stating that lack of microbial exposure early in life induces a lack of protection against allergies and (allergic) asthma later in life. Epidemiological studies investigating the effect of living in a farming environment, which causes higher microbial exposure than urban living environments, indeed have shown that farming life may confer protection against allergies and allergic asthma in childhood. However, farmers and agricultural workers exposed to farm dust less likely had allergies, but nevertheless had respiratory symptoms such as shortness of breath, cough and wheezing. This indicates that farm dust exposure may protect against allergies, but can induce non-allergic asthma. Indeed, we found in our animal model of HDM-induced allergic airway inflammation, that farm dust exposure decreases allergic airway inflammation (eosinophilic inflammation and TH2 cytokines in lung tissue, total and HDM-specific IgE in serum) but simultaneously induced a non-allergic type of airway inflammation.
inflammation (large inflammatory infiltrates, T\(_{17}\) cells and associated cytokines) and methacholine (MCh) responsiveness. The mechanisms underlying the decreased allergic airway inflammation and simultaneously increased (possibly T\(_{17}\) mediated) non-allergic airway inflammation in our previous study are yet unknown. Recently it was shown that Toll-like receptor (TLR)4 in epithelium is essential for HDM induced allergic airway inflammation.\(^{15}\) Signalling through TLR4 resulted in recruitment and activation of dendritic cells, which can skew naïve T cells to differentiate towards the T\(_{H2}\) type, thereby inducing allergic airway inflammation.

To gain more insight into the mechanisms involved in down regulation of allergic airway inflammation by farm dust exposure, we studied TLR2 and TLR4 expression in airway epithelium from dust exposed mice. Little is known about the characteristics of non-allergic airway inflammation in non-allergic asthma patients. Since our farm dust exposed mice provide an opportunity to study non-allergic asthma in more detail, we aimed to better characterize the airway inflammation in this setting. Therefore, we analysed the cellular composition of farm dust induced infiltrates as well as the antibody levels in these mice in more detail.

**MATERIALS AND METHODS**

**ANTIGENS**

Dust was collected from five farms in North Germany and extracted according to the published protocol\(^ {16}\). Hereafter, dust extract is referred to as ‘dust’. Dust exposure: 1mg/ml PBS, 50 μl/day. House dust mite (HDM) consisted of crushed whole bodies from dermatophagoides pteronyssinus (Greer Laboratories, Lenoir, USA). HDM exposure: 2.5 mg/ml PBS, 10 μl/day.

**STUDY DESIGN**

To induce allergic airway inflammation, 10-week old female BALB/c mice were anesthetized with isoflurane and intranasally exposed to dust, or PBS as a control. Mice were exposed four times per week, during five consecutive weeks. To investigate the protective effect of dust exposure, mice were anesthetized and intranasally exposed to dust, or PBS as a control, 1 min before every exposure to HDM (or PBS) (figure 1). Experimental groups: PBS+PBS exposure (n=8), PBS+HDM exposure (n=10), dust+PBS exposure (n=9), dust+HDM exposure (n=10). Twenty-four hours after the last allergen exposure MCh responsiveness was assessed as described previously\(^ {17}\).

All animal protocols were approved by the local Committee on Animal Experimentation and were performed under strict governmental and international guidelines on animal experimentation.

**TISSUE COLLECTION**

Twenty-four hours after the MCh assessment, mice were anaesthetized and sacrificed. Blood was taken by a heart punction. The two smallest right lung lobes were snap frozen; the remaining lobes were carefully inflated with 0.9 ml 50% Tissue Tek, O.C.T. (Sakura, Zoeterwoude, The Netherlands) in PBS. The left lung lobe was formalin fixed and embedded in paraffin, the two smaller right lung lobes were snap frozen and stored at -80°C until use.

**MEASUREMENT OF THYMIC STROMAL LYMPHOPOIEtin (TSLP) IN LUNG TISSUE**

Snap frozen lung tissue was homogenized and TSLP in lung tissue was measured by Enzyme Linked Immuno Sorbent Assay (ELISA) as described by the manufacturer (R&D systems, Minneapolis, USA).

**MEASUREMENT OF IgG1, IgG2a AND IgM IN SERUM**

IgG1, IgG2a and IgM levels in serum were measured by ELISA. Flat bottomed 96-wells plates were coated overnight with anti-mouse IgG1 (BD Biosciences, San Jose, USA), a monoclonal anti mouse IgG2a antibody (BD Biosciences, OPTEIA kit) or goat anti mouse Ig (Southern Biotech Associates (SBA), Birmingham, USA) respectively. Plates were blocked with assay diluents (IgG1 and IgG2a ELISA) or 0.2% ELK/PBS (IgM ELISA). Serum samples were added and incubated for 2h. A monoclonal anti-mouse IgG1\(^ {BD}\) antibody (BD Biosciences), a monoclonal anti-mouse
IgG2a antibody (BD Biosciences, OPTEIA kit) or a polyclonal goat anti-mouse IgM antibody (SBA) were added and incubated for 1h (IgG1 and IgG2a ELISA) or 2h (IgM ELISA). Horseradish peroxidase conjugated streptavidin (DAKO, Glostrup, Denmark) or avidin-biotin complex (DAKO (IgM ELISA)) was added for 30 min. Plates were developed using tetramethyl benzidine (TMB) substrate (Sigma Aldrich, St Louis, USA), stopped and optical densities were read at 450 nm using a Varioscan ELISA reader (Thermo Scientific, Waltham, USA).

Histology and Immunohistochemistry

To determine B cells, dendritic cells, follicular dendritic cells (fDC), alternatively activated macrophages, TLR2 and TLR4, 4 µm cryosections of lung tissue were stained with a rat anti CD19 antibody (BD Biosciences), a rat anti mouse CD11c<sub>pe</sub> antibody (BD Biosciences), a rat anti mouse fDCm2 antibody (BD Biosciences), a goat anti mouse YM1<sub>BIO</sub> antibody (R&D systems, Minneapolis, USA), a goat anti mouse TLR2 or TLR4 antibody (SantaCruz, Santa Cruz, USA) respectively. Expression of TLR2 and TLR4 in epithelium was scored visually for each airway separately (1=low expression, 2=intermediate expression, or 3=high expression) and the mean expression for all airways in the total lung section is shown. Since B cells in infiltrates are tightly packed together, individual cells were difficult to discern and therefore the volume percentage of B cells in infiltrates was calculated by morphometric analysis using Leica Qwin image analysis software (Leica Microsystems, Wetzlar, Germany). Alternatively activated macrophages were counted manually in whole lung sections and corrected for the total area of lung section.

Statistical analysis

To determine 1) the effect of HDM exposure, 2) the effect of dust exposure, and 3) the interaction of the effect of dust exposure on the effect of HDM exposure, we performed a multiple linear regression analysis (SPSS 14.0 software, SPSS Inc). When residuals were not normally distributed, appropriate log10 or 1/x transformation was performed. A significant HDM effect means that HDM exposed mice differ from PBS exposed mice. A significant dust effect means that dust+PBS exposed mice differ from PBS exposed mice. A significant interaction between the effect of dust exposure and the effect of HDM exposure means that the effect of HDM exposure is different in dust+HDM exposed mice compared with PBS+HDM exposed mice.

Results

Farm dust exposure decreases TLR2 and TLR4 expression in epithelium

Figure 1 shows that HDM exposed mice had higher expression of TLR2 and comparable expression of TLR4 in epithelium compared with PBS exposed mice (for representative pictures of immunohistochemical stainings, see figure 4). Dust+PBS exposure decreased TLR2 expression compared with PBS exposed mice and in comparison with dust+HDM exposed mice, while TLR4 expression was not significantly changed. The effect of HDM exposure was different in dust+HDM exposed mice compared with PBS+HDM exposed mice.
Exposed mice had a lower expression of TLR2 and TLR4 in airway epithelium compared with PBS exposed mice. Dust+HDM exposure decreased the response to HDM, inducing a lower expression of both TLR2 and TLR4 in epithelium compared to PBS or HDM exposed mice (interaction).

**Farm dust exposure decreases levels of thymic stromal lymphopoietin (TSLP) in lung tissue**

Since downregulated TLR2/4 expression may affect TSLP production, we measured TSLP levels in lung tissue (figure 2). HDM exposed mice showed higher levels of TSLP than PBS exposed mice. Dust+PBS exposure induced no increase in TSLP compared with PBS exposure and dust+HDM exposure decreased the response to HDM (interaction), resulting in TSLP levels that were equal to TSLP levels in PBS exposed mice.

**Figure 2:** Relative levels of thymic stromal lymphopoietin (TSLP) in lung tissue of PBS, HDM, dust+PBS and dust+HDM exposed mice. The median TSLP level of the PBS group was set to 1. Individual data points and medians are shown. “Interaction” indicates a smaller effect of HDM exposure in dust exposed mice as compared to mice not exposed to dust. p values in the graph are from multiple linear regression analyses.

**Figure 3:** A) Numbers of YM1+ macrophages in lung parenchyma, B) numbers of CD68 cells beneath the epithelium, C) CD68 spindle cell beneath the epithelial basement membrane and D) volume % of CD19 in infiltrates in PBS, HDM, dust+PBS or dust+HDM exposed mice. Individual data points and medians are shown. “Interaction” indicates a smaller effect of HDM exposure in dust exposed mice as compared to mice not exposed to dust. p values in the graph are from multiple linear regression analyses.
Farm dust exposure decreases YM1 production by alternatively activated alveolar macrophages (AAMΦ).

As we showed previously (chapter 6), HDM exposed mice had higher numbers of alveolar (CD68 expressing) macrophages and dust+PBS and dust+HDM exposure increased the number of alveolar macrophages even further. Since the HDM-induced \( T_{h2} \) response was downregulated in dust exposed mice and AAMΦ are known to induce \( T_{h2} \) responses, we investigated the presence of the AAMΦ marker YM1. HDM exposed mice had higher numbers of YM1 expressing macrophages (figures 3A and 4) but dust+PBS and dust+HDM exposure did not result in lower numbers of AAMΦ. A striking difference between HDM exposed and dust exposed mice was that HDM exposed mice had more YM1 deposition in the lung parenchyma than dust+PBS and dust+HDM exposed mice (figure 4), indicating that AAMΦ were less active in dust exposed mice.
Since the number of alternatively AAMΦ was not different for HDM, dust+PBS and dust+HDM exposed mice, the decreased allergic airway inflammation in dust exposed mice compared with HDM exposed mice could not be attributed to lower numbers of AAMΦ. Therefore we investigated whether decreased antigen presentation in dust exposed mice contributed to decreased allergic airway inflammation in these mice. Both macrophages and dendritic cells can stimulate naive T cells but a discriminative marker for these cells does not exist. To specifically assess the phenotype of the macrophages and/or dendritic cells in lung tissue of farm dust exposed mice an extensive array of markers should be investigated. Since this was not the purpose of this study, we first studied the presence of the CD68+ cells in more detail. Interestingly, HDM exposed mice had more CD68+ spindle cells beneath the epithelial basement membrane than PBS exposed mice (figures 3B, 3C and 4), whereas the cell numbers were lower in dust+PBS and dust+HDM exposed mice compared to HDM exposed mice. These cells turned out to be YM1 negative and since CD68 is found on both macrophages and some dendritic cells, expression of CD11c was investigated to see whether these cells were dendritic cells. However, no CD11c was present on these cells beneath the epithelial layer (data not shown).

Farm dust exposure increases the number of B cells and follicular dendritic cells in infiltrates

There were no B cells in PBS exposed mice whereas the volume percentage of B cells in infiltrates was higher in dust+PBS and dust+HDM exposed mice compared to HDM exposed mice (figures 3D and 4). Furthermore, dust+PBS and dust+HDM exposed mice had more follicular dendritic cells in infiltrates than HDM exposed mice (figure 4). PBS exposed mice had no follicular dendritic cells, since there were no infiltrates in lung tissue present at all.

Farm dust exposure increased IgG1 production

In our previous paper (chapter 6) we showed that dust exposure prevented production of HDM-specific IgE and total IgE. Since the volume percentage of B cells in infiltrates of dust exposed mice were higher than in the HDM and PBS groups, we investigated which type of immunoglobulin was produced by these B cells, since it obviously was not IgE. HDM exposed mice displayed higher levels of IgG1 (figure 5A), IgG2a (figure 5B) and IgM (figure 5C) than PBS exposed mice. Dust+PBS exposed mice had higher levels of IgG1 compared with PBS and HDM exposed mice, but no difference in IgG2a and IgM compared with PBS exposed mice.

Figure 5: Levels of A) IgG1, B) IgG2a and C) IgM in serum of PBS, HDM, dust+PBS or dust+HDM exposed mice. Individual data points and medians are shown. "Interaction" indicates a smaller effect of HDM exposure in dust exposed mice as compared to mice not exposed to dust. p values in the graph are from multiple linear regression analyses.
Dust+HDM exposure surprisingly resulted in a lower level of IgG1 compared to dust+PBS exposed mice. Altogether, dust exposure resulted in an increase in IgG1 levels ("dust effect") but no difference in IgG2a and IgM levels were found.

**DISCUSSION**

We aimed to gain more insight into the mechanisms causing downregulation of HDM-induced airway inflammation in farm dust exposed mice. Our results show that farm dust decreased the expression of TLR2 and TLR4 in airway epithelium, associated with decreased TSLP levels in lung tissue. In addition, we show that farm dust exposure increased the number of B cells and follicular dendritic cells in cellular infiltrates and induced marked production of IgG1 in Balb/c mice.

TLR4 has been shown essential for HDM-induced allergic airway inflammation 15. The observed decreased expression of TLR2 and TLR4 in this study could abolish the recruitment and activation of dendritic cells that are necessary to skew the Th2 immune response. However, it was shown recently that innate immune responses of cultured epithelial cells to HDM are mediated via CCL20 secretion, which is not TLR2 or TLR4 dependent, but relies on beta-glucan receptors 19. Therefore down-regulated expression or blocking of other pattern recognition receptors (PRRs), such as dectin-1, by farm dust exposure may have contributed to downregulation of the HDM-induced allergic airway inflammation in our study as well. These data seem to be in contrast with studies indicating that TLR2 and TLR4 signalling attenuates allergic airway inflammation 20-22. However, these studies were all performed in mouse models using ovalbumin (OVA) as an allergen, requiring sensitisation via intraperitoneal injections of OVA and not via mucosal/epithelial exposure as in our study design. Therefore, different functional involvements of TLRs can be expected in these models. To investigate whether decreased TLR expression by dust exposure could indeed abolish Th2 skewing via decreased dendritic cell activation, we measured TSLP levels in lung tissue. TSLP is an epithelium derived cytokine, involved in development of allergic airway inflammation 23-26. Hence, the lower levels of TSLP observed in farm dust exposed mice in our study may very well be involved in downregulation of the HDM-induced allergic Th2 driven airway inflammation.

The recruitment of antigen presenting cells driving the Th2 response can be decreased via lower TSLP production as well. Indeed, our data are suggestive of a decrease in antigen presenting cells beneath the epithelial layer in farm dust exposed mice, since we observed CD68+ spindle cells (in contrast to the round CD68+ alveolar macrophages) with long cytoplasmic extensions beneath the epithelial layer in HDM exposed mice and decreased numbers of these cells in dust exposed mice. Although it is known that this phenotype can be attributed to dendritic cells 27, we did not find expression of the epithelial cell marker CD11c on these cells. As was recently shown by Poole et al, organic dust exposure prevents maturation of dendritic cells, illustrated by e.g. decreased CD11c expression 28. Whether HDM and farm dust exposure indeed have differential effects on maturation of dendritic cells remains to be studied in more detail, using more dendritic cell maturation markers. Therefore, the exact phenotype of the CD68 expressing spindle cells remains unknown as well as whether these cells are the Th2 skewing cells in HDM exposed mice.

Another mechanism that could have contributed to downregulation of eosinophilic airway inflammation in farm dust exposed mice is decreased activity of alternatively activated macrophages (AAMΦ). YM1, a lectin binding protein, is a cell marker for AAMΦ 29 and is known to attract eosinophils 30. Although we did not find differences in the numbers of AAMΦ between HDM exposed mice and farm dust exposed mice, we did find that the level of YM1 expression by these AAMΦ was much higher in HDM exposed mice. This indicates that farm dust exposure downregulated the activity of AAMΦ, which can have contributed to decreased Th2 responses in these mice.

We were interested in the phenotype of the cellular infiltrates in lungs of farm dust exposed mice and showed a remarkable increase in the number of B cells and fDCs in these infiltrates. This indicates that farm dust exposure induced B cell follicle formation. Since Th2 cells, previously observed to be increased in farm dust exposed mice
Asthma development in farm dust exposed study mechanisms involved in non-allergic could prove an interesting new model to are directed remains to be studied, but this may be orchestrated by the previously observed increased numbers of $T_{h17}$ cells.

Since IgE levels were lower in farm dust exposed mice than in HDM exposed mice, but the number of B cells in infiltrates was much larger in these mice, we were interested in the immunoglobulin type produced by these B cells. We show that HDM exposure increased IgG1 levels, but that the increase in IgG1 in farm dust exposed mice was far more pronounced. IgG2a and IgM were not altered by farm dust exposure. IgG is known to bind to FC receptors on macrophages, thereby activating them to better internalize the pathogens. Since farm dust exposed mice recruited more macrophages into the parenchyma, this may have contributed to increased clearance of pathogen associated microbial patterns (PAMPs) present in farm dust. Another role of IgG antibodies was already suggested in the 1990s, when asthma patients with negative skin prick tests appeared to have IgG autoantibodies directed against endothelial proteins. Two more recent studies showed increased presence of IgG antibodies against epithelial cell antigens in non-allergic asthma patients compared to allergic asthma patients and healthy controls and in severe asthma patients IgG autoantibodies directed against the epithelial protein alpha enolase were found. This indicates that an autoimmune response involving IgG antibodies, may play a role in non-allergic asthma. To which antigen the IgG1 antibodies in our farm dust exposed mice are directed remains to be studied, but this could prove an interesting new model to study mechanisms involved in non-allergic asthma development in farm dust exposed individuals.

Additionally, this study suggests that farm dust exposure induces a non-eosinophilic asthma phenotype via down regulation of TLR2 and TLR4 expression in epithelium associated with lower TSLP production on the one hand and on the other hand induction of B cell follicle formation and production of IgG1 antibodies by B cells which may be orchestrated by the previously observed increased numbers of $T_{h17}$ cells.

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