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Lara Utsch Mendes Gouveia

Cover: Tulip fields, The Netherlands by Lara Utsch M. Gouveia.

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Mouse models of allergic inflammation: from allergic sensitization to immunotherapy

ACADEMISCH PROEFSCHRIFT

Ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. K.I.J. Maex

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Lara Utsch Mendes Gouveia

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Faculteit der Geneeskunde

“Great are the works of the LORD, studied by all who delight in them.” Psalm 111:2

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Chapter 1

Scope of this thesis

According to World Health Organisation (2015), worldwide there has been a rise in prevalence of allergic diseases in the past 50 years. Allergic diseases are the result of an aberrant immune response by T helper type 2 cells to allergens, resulting in the release of cytokines IL-4, IL-5, IL-13 and induction of allergen specific IgE [1;2]. Allergic sensitization is considered to be a multifactorial process that involves among others, environmental factors, genetics and characteristics of allergens. In normal conditions, exposure to non-infectious protein antigens induces a state of tolerance, which is essential to maintain homeostasis under constant exposure to environmental antigens. Antigen-presentation by antigen presenting cells to $CD4^+$ T helper cells in lymphoid organs is a critical step in the induction of T regulatory cells and the establishment of tolerance [3]. A failure in the mechanisms of tolerance, leads to activation of pro-inflammatory pathways and the development of allergen specific T cell responses.

Physical barriers such as epithelial cells are an important mechanism of protection against allergy development restricting the access of allergens to immune cells. In recent years epithelial cells have been recognized as more than just a passive physical barrier to allergens. These cells can actively contribute to allergic sensitization by producing proinflammatory factors [4]. Allergens with proteolytic properties can do more than just disrupt the epithelial physical barrier gaining access to the immune niche in tissues. They can also activate epithelial cells and induce secretion of cytokines such TSLP, IL-33 and IL-25 and release of damage-associated molecular patterns (DAMPs) [4]. These mediators can initiate Th2 responses among others by orchestrating DC maturation and activation.

The development of animal models of various allergies has been beneficial in allowing extensive investigations into mechanisms involved in the allergic pathways. These animal models can be used to predict potential risk factors as well as to test novel treatments and immunotherapy. The risk of anaphylaxis in humans is a limiting factor for the development of allergen-based immunotherapy [5]. In this context, animal models can play an essential role in providing a platform for refining therapeutic treatments and ensuring safety, prior to application in humans.

This thesis focuses on an essential question in understanding allergy: what makes an individual respond to allergens? We have studied this question from different perspectives, to elucidate several mechanisms by which allergens can initiate a Th2 mediated allergic response. We identified a role for (i) the capacity to cope with allergen induced oxidative stress (a characteristic of the individual), (ii) the role of epithelial contact with HDM allergens in the development of an IgE response, (iii) the immunological interplay between allergic immune responses directed against unrelated allergens in different compartments of the body (interplay between a food allergy and a respiratory allergy) and (iv) the immunological interplay between cross-reactive allergens (cross-reactivity to birch pollen Bet v 1 and the apple allergen Mal d 1) in BP allergy and after BP immunotherapy.

Mechanisms of allergic sensitization

The role of oxidative stress on ongoing allergic inflammatory responses and asthma has been extensively investigated. However the role of an excess of reactive oxygen species (ROS) as a trigger mechanism for allergic sensitization has been poorly explored. Several studies are in support for a role of oxidative stress in allergic sensitization. Murine studies show that birch pollen-induced oxidative stress mediated IL-4 and IgE production [6]**.** ROS generation by the protease papain was responsible for its adjuvant effect in an ovalbumin-induced allergic sensitization model [7]**.** In human experimental studies, allergic sensitization was induced when intranasal exposure to allergens was accompanied by exposure to diesel exhaust particles (an oxidizing agent) [8]. In **chapter 2** we showed that a differential capacity to counteract the effects of oxidative stress is associated with the development of allergic sensitization to HDM in mice and with occupational allergy in man. In **chapter 3** we review

and discuss how oxidative stress may prime the immune system for the development of allergen-specific Th2 responses.

It is estimated that 10–20% of the European population is allergic to house dust mite (HDM) (*Dermatophagoides pteronyssinus*) [9]. HDM is a ubiquitous allergen and an important trigger of allergic airway responses, yet the mechanism by which HDM stimulates the innate immune system is still not fully understood. HDM allergens belong to protein families with diverse biological functions that contribute to their allergenicity. HDM-derived cysteine (Der p 1 / Der f 1 [the homologue of Der p 1 in *Dermatophagoides farinae*]) and serine (Der p 3, 6, 9 and Der f 3, 6, 9) proteases have been shown to disrupt tight junctions in lung epithelium causing the release of pro-inflammatory cytokines (such as GM-CSF, IL-6, IL-33, and IL-25) and eotaxin, favouring the induction of an IgE response and a Th2 response by modulating the balance between IL-4 and IFN-γ [10-16]. In **chapter 4** we investigated the role of epithelial contact with HDM in the sensitization. We used an adoptive transfer model with HDM pulsed dendritic cells directly in the airways to circumvent the epithelial response. We demonstrated that a DC-driven sensitization induced a clear Th2-mediated airway inflammation upon a single HDM inhalation, while a humoral immune response was completely absent. Recent evidence demonstrated that IL-33 is crucial for development of allergic airway inflammation to HDM [17-19]. IL-33 has the ability to induce maturation and upregulation of the Th2 skewing costimulatory molecule OX40L on DCs [20]. Administration of IL-33 restored the humoral immune response in our DC transfer model, as seen after direct epithelial contact with HDM.

Immunological interplay between different primary allergic sensitizations and immunological cross-reactivity

Atopic individuals frequently develop sensitization against different allergens over time (polysensitization), resulting in multiple atopic disorders often affecting different organs. Affected organs can range from the skin, the upper and lower airways, the eyes, and the gastro-intestinal tract, to the cardiovascular and neurological systems. A phenomenon usually referred to as the “atopic march” has been described, being the progression from early onset atopic dermatitis with a high comorbidity for food allergy towards allergies of the airways like asthma and rhino-conjunctivitis later in life. Several epidemiological reports

suggest an association between sensitization to food allergens and an increased risk for the development of asthma [21;22]. A possible interplay between different primary sensitizations with their own affected organs has been studied very scarcely. In **chapter 5** we aimed to develop a murine model to study the possible interplay between a food allergy (peanut) and HDM-induced respiratory allergy. Our data showed that a preceding peanut sensitization boosted the IgE and HDM-specific Th2 response in the airways in mice.

In the last part of the thesis we studied how the allergic immune response can be re-directed to a tolerogenic response. Although highly effective drugs are available to suppress allergic symptoms, they do not cure allergy. The development of more efficient immunotherapy strategies are currently a great challenge. Allergen-specific immunotherapy has been used for over 100 years as a treatment for allergic diseases. Subcutaneous immunotherapy (SCIT) consists of a series of subcutaneous injections with increasing doses of allergen, which induces desensitization and relief of allergic symptoms. The clinical efficacy of SCIT is well documented. However the long treatment period (3-5 years) and occurrence of unwanted side effects warrant further improvement. The reduction of allergic symptoms by SCIT has been hypothesised to be dependent on a decrease in Th2 cytokine production. Several mechanisms have been proposed to contribute to this decreased Th2 immunity: T cell anergy, a shift from a Th2 towards a Th1 response and/or the induction of Tregs [23;24] and the induction of allergen-specific IgG antibodies, in particular IgG_4. In **chapter 6** we developed a murine model for birch pollen (BP) specific subcutaneous immunotherapy to suppress BP allergy. Surprisingly, we demonstrated that reduction of Th2 cytokine production by BP SCIT was not sufficient to immediately reduce AHR in mice, but only after continued BP SCIT, possibly by allowing sequential effects on IL-17A production, broncho-alveolar lavage fluid (BALF) IL-5 levels and increased titres of BP IgG_{2a}.

In **chapter 7**, we studied the phenomenon of cross-sensitization to related allergens. It is estimated that approximately 70% of patients with birch pollen (BP) allergy experience allergic symptoms after consumption of apple, celery, hazelnuts, or other BP related foods, the so called PR-10 (pathogenesis-related protein family 10) proteins [25-28]. Respiratory exposure to birch (and other related tree) pollen is responsible for primary sensitization to PR-10 allergens. Bet v 1 and Mal d 1 share 65% amino acid sequence identity, and as a

consequence, IgE antibodies against Bet v 1 cross-react to Mal d 1 [29], leading to (mild) clinical apple allergy in a significant number of BP allergic patients [30;31].

Despite the clear link between Bet v 1-Mal d 1 cross-reactivity and the high prevalence of apple allergy amongst BP allergic patients, there are scarce and conflicting reports as to whether BP immunotherapy is able to relieve allergic symptoms to apple in these patients. In **chapter 7** we studied cross-reactivity to Mal d 1 in BP sensitized mice and evaluated the effect of BP specific immunotherapy on the cross-reactivity and hypersensitivity to Mal d 1. We demonstrated that BP immunotherapy ameliorated the anaphylactic symptoms induced by Mal d 1. This was associated with a shift in the ratios of both Bet v 1 and Mal d 1 specific IgG over IgE and a shift towards IL-10 production over Th2 cytokines IL-5 and IL-13.

In **chapter 8** the main findings of the studies described in this thesis are presented and discussed for their clinical relevance.

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Chapter 2

Allergic sensitization is associated with inadequate anti-oxidant responses in mice and men

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ABSTRACT

Background: Allergies arise from aberrant Th2 responses to allergens. The processes involved in the genesis of allergic sensitization remain elusive. Some allergens such as derived from house dust mites, have proteolytic activity which can induce oxidative stress *in vivo.* A reduced capacity of the host to control oxidative stress might prime for allergic sensitization.

Methods: Two different strains of mice were compared for their anti-oxidant and immune response to HDM. Protease activity of the HDM extract was reduced to investigate its role in oxidative stress induction in the airways and whether this induction could determine allergic sensitization and inflammation. The role of oxidative stress in allergic sensitization was also investigated in humans. An occupational cohort of animal workers was followed for development of sensitization to rodent urinary proteins. Levels of oxidative stress in serum and anti-oxidant responses by PBMCs were determined.

Results: Susceptibility to allergic sensitization to mite allergens in mice was highly dependent on host genetic background and was associated with oxidative stress in the lungs before allergen exposure and poor anti-oxidant response after allergen exposure. Reduction of mite protease activity limited its capacity to induce oxidative stress and allergic inflammation in mice. We showed that also in human subjects, oxidative stress before allergen exposure and poor anti-oxidant responses were associated with predisposition to occupational allergy.

Conclusion: Our study indicates that oxidative stress condition before allergen exposure due to an inadequate anti-oxidant response primes for allergic Th2 responses.

INTRODUCTION

Allergies arise from aberrant Th2 immune responses to allergens [1]. The processes underlying this unwanted response remain elusive. Pattern recognition receptors (PRRs) such as Toll-like receptors are important regulators of immune response to microbial components such as bacterial lipopolysaccharides (LPS) [2]. TLR4 triggering by LPS has been found crucial for the initiation of allergen-specific Th2 responses to house dust mite (HDM) in mice [3;4]. However, Th2 responses to inhaled HDM can also be induced in C3H/HeJ, mice with impaired TLR4 signalling [5]. The development of Th2 allergic responses to HDM in C3H/HeJ mice, independently of TLR4 signalling, argues strongly for an additional mechanism independent of the LPS-TLR4 pathway.

C3H/HeJ mice have been reported as having a natural reduced anti-oxidant response to influenza in the lungs and this is associated with high morbidity to influenza infection [6]. An impaired or reduced capacity to regulate oxidative stress could be related to the development of aberrant immune responses including allergies. Oxidative stress has been described to orchestrate the Th2 response to cysteine proteases such as papain [7]. The role of oxidative stress in HDM induced allergic sensitization is unknown, however several studies with other allergens support its role in allergic sensitization [7-9]. Various HDM derived allergens have distinct biological functions among which proteases [10] that can also induce oxidative stress *in vivo* [7]. Oxidative stress happens when reactive oxygen species (ROS) from local and/or environmental sources overwhelm anti-oxidant responses [11]. ROS, during oxidative stress, can activate the immune system [11] and in the context of allergen exposure, may facilitate allergic sensitization.

We investigated the immune and anti-oxidant responses to HDM in different strains of mice and whether HDM protease activity was important for allergic sensitization via oxidative stress induction. In a cohort of occupational allergy we addressed whether oxidative stress was also associated with susceptibility to allergic sensitization to a HDM unrelated allergen, namely rodent urinary proteins.

METHODS

Reagents. Periodic Acid Schiff's, N-acetyl-L-cysteine (NAC), Xanthine, Xanthine oxidase and Propidium iodide, Sigma-Aldrich Corp. St. Louis, MO, USA; rGM-CSF, Thermo Fisher Scientific, Waltham, MA, USA; MHCII-FITC, CD11c-APC, CD86-PE, CD80-PE, CD40-PE, Rat IgG_{2a}, Ham IgG antibodies and ELISA kit Ready-set-go! eBioscience Inc, San Diego, CA, USA IL4, IL-5, IL-13 and IFNγ, eBioscience Inc via Immunosource, Halle-Zoersel, Belgium; Antibody to FcRγII/III 2.4G2, provided by Louis Boon, Bioceros, Utrecht, The Netherlands; Bicinchoninic acid (BCA) kit, Bio-Rad Laboratories Inc.;Hercules, CA, USA BlueSepharose, AmershamPharmacia, UK; Antibodies to 4-HNE, Nrf2, HO-1, Santa CruzCA, USA; Antibody to β-actina, GeneTexIrvine, CA, USA; IgE, IgG_1 and IgG_{2a}, ELISA kit Opteia, BDSan Diego, CA, USA; Trizol, InvitrogenLife Technologies, USA; First strand cDNA Synthesis Kit, Thermo Fisher Scientific, MA, USA; SYBR Green PCR Master Mix, Applied Biosystems. Warrington, UK.

HDM extracts. A spent mite medium extract (Dermatophagoides pteronyssinus, LoTox^{TM} LTN-DPE-4, lotnr 33019, INDOOR Biotechnologies, Cardiff, UK) and a crushed whole body house dust mite extract (Dermatophagoides pteronyssinus, XPB82D3A2.5, lotnr 136401, Greer Laboratories; Lenoir, NC, USA) was used. The first extract will be referred to as low-toxin HDM (LT-HDM) with an endotoxin level of ≤3 EU per 1 mg protein and the second will be referred to as high-toxin HDM (HT-HDM) containing 199 EU endotoxins per 1 mg of protein as determined by a LAL assay. In all experiments, 1 µg Der p 1, which is equivalent to 6.1 µg protein of LT-HDM and 31 µg protein of HT-HDM was used.

Murine studies

Mice. Female mice C3H/HeJ, C3H/HeN (Harlan, Bicester Oxon, UK) and Balb/c (Harlan, Horst, The Netherlands), 6-8 weeks old, were housed under specific pathogen-free conditions at AMC animal facility. All experiments were approved by the AMC animal ethics committee, The Netherlands.

Sensitization. Mice were anaesthetized with isoflurane and 30 µl of HDM extracts or Phosphate Buffered Saline (PBS) was administrated as previously described [12]. Briefly, mice were exposed intranasally to HDM for 3 cycles of five consecutive days and two days'

rest. Four weeks after the last cycle, mice were rechallenged three times and killed two days later.

Bronchoalveolar lavage fluid (BALF). Cells from the airway lumen were obtained by three subsequent washes with 1 ml PBS and 0.1 mM EDTA, after intratracheal cannulation. Cell differentiation was determined by FACS as previously described [13].

Lymph node restimulation. Cells were plated in 96-well round bottom plates at $2x10^5$ cells per well and restimulated for 4 days with 100 µg ml^{-1} HDM extract. Cytokines in supernatants were analysed by ELISA.

Immunoglobulins. Serum IgE, IgG_1 and $IgG2_a$ were analysed by ELISA (ELISA kit Opteia, BS). For specific immunoglobulin assays, plates were coated overnight with HDM antigens instead of capture antibodies. Standard curves of murine immunoglobulins were used as qualitative reference.

Histology. Frozen lung sections (6 µm) were stained with Periodic Acid Schiff's. Inflammation and mucus-producing goblet cells were semi-quantified as previously described in [14]. Briefly, number of goblet cells were determined in the airways and scored according to the percentage of cells per airway (0 = <5%, 1= 5-25%, 2= 25-50%, 3=50-75%, 4=>75%). Airway inflammation was scored as follows: 0= no inflammation, 1=cuffing of inflammatory cells at 1 or 2 sides of the airway, 2=thin (<5 cells thick) layer surrounding the airway, 3= thick (>5 cells thick) layer surrounding the airway.

Bone-Marrow derived dendritic cells (BMDCs). Bone marrow cells from naïve C3H/HeJ and Balb/c mice were differentiated into dendritic cells *in vitro* as previously described [15]. For Nrf2 determination, on day 9 of culture, differentiated DCs were incubated overnight with or without LT-HDM (1 µg Der p 1/ml). For CD40, CD80 and CD86 determination by FACS, on day 9 of culture, cells were incubated with or without 5 mM of NAC at 37 ^oC in 5% CO_2, 30 min prior to overnight incubation with LT-HDM extract (1 µg Der p 1/ml). Propidium iodide was used for cell viability.

Western blots (WB). Proteins from lung homogenates and BMDCs were extracted with Laemmli buffer: 20% wt/vol sodium dodecyl sulfate (SDS), 30% vol/vol glycerol, 30% vol/vol deionized water in 1 M Tris-base pH 6.8. Proteins were next diluted in 4% wt/vol SDS, 10%

vol/vol 2-mercaptoethanol, 20% vol/vol glycerol, and 0.004% vol/vol bromophenol blue in 125 mM Tris-HCl pH 6.8 and separated on 13% SDS/PAGE. After transfer to polyvinylidene difluoride membranes and blocking with 5% wt/vol skim milk, blots were incubated with primary polyclonal antibodies to Nrf2 or 4-HNE. Subsequently, they were incubated with IRDye 680LT-conjugated secondary antibodies. Blots were visualized using infrared fluorescence detection Odyssey Imager and software (LI-COR Biosciences, Lincoln, NE, USA). Loading was normalized per β-actin and relative optical density (Rel.OD) values were used as quantification units.

Real-time PCR. Total lung RNA was extracted with Trizol according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using first-strand cDNA synthesis kit. PCR was performed in a 10 µl reaction volume including 5 µl of SYBR Green, 200 nM of each FW and RV primers, 2 µl of cDNA and nuclease free water. For each gene, reaction was performed in duplicate. Duplicate standard curves were constructed by serial dilution (1:5) from a concentrated pool of cDNA. All reactions were performed in optical 96-well reaction plates using the ABI Prism 7500 system (Applied Biosystems). mRNA concentrations were calculated based on the standard curve method [16] and normalized to the housekeeping gene HPRT.

Protease activity assay. Heated LT-HDM was serially diluted (1:2) in reaction buffer (0.2 M sodium phosphate, 1 mM EDTA, pH 7.0) and mixed with 20% vol/vol 20 mM cysteine substrate or reaction buffer (50 mM TRIS, 20 mM $CaCl_2$, pH 8.2) and mixed with 50% vol/vol 2 mM trypsin substrate or reaction buffer (0.1 M TRIS, 0.96 M NaCl, 10 mM $CaCl_2$, pH 8) and mixed with 50% vol/vol chymotrypsin substrate in 96-well NUNC plates. For the standard curves, enzymes were serially diluted (1:2) and mixed with respective substrates. Starting enzymes dilutions: papain, 700 µg ml^{-1}; trypsin, 2 µg ml^{-1}, and chymotrypsin, 200 µg ml^{-1}. Absorbance was measured at 415 nm after the development of colour.

Cap inhibition assay. ImmunoCAP component (ThermoFisher Scientific, Thermo Fisher Scientific Inc, Waltham, MA, USA) was performed according the manufacturer's instructions. Prior to incubation in ImmunoCAP, serum from a HDM allergic subject was inhibited for 1 hour with LT-HDM or heated LT-HDM. After inhibition, residual IgE binding was measured

using CAPs coated with mite extract, Der p 1 or Der p 2. Results were expressed in percentage of inhibition.

Human studies

Study design. Study population consisted of 37 temporary laboratory animal workers from a previous study[17]. Briefly, participants were followed for 2 years and occupational allergic sensitization to rodent urinary proteins was monitored. They were seen at the start of their application as animal workers (T0), after 4 months (T4), 1 year (T12) and 2 years (T24) for blood collection and clinical evaluations. Herein, we compared 21 workers who did not develop sensitization to rodents with 16 animal workers who did. 4-HNE-modified proteins and HO-1 were accessed in serum and Nrf2 was accessed in Peripheral Blood Mononuclear Cells (PBMCs were available of five "*de-novo* sensitized" individuals and four "non *de-novo* sensitized" individuals) by WB.

Western blots. Serum samples were treated with BlueSepharose 6B CL to reduce the albumin content. Proteins were treated and blotted as previously described in the murine section. Polyclonal antibodies to Nrf2, HO-1 or 4-HNE were used. Total protein was determined by BCA. Samples were normalized per 50 µg of protein and Optical Density (OD) values were used as quantification units.

PBMC. Cells were cultured overnight with xanthine (0.5 mM): xanthine oxidase (50 mU). Nrf2 protein expression was analysed by WB in total cell lysate.

Statistical analysis. Statistical significance was tested with Mann-Whitney *U* test. Experiments were repeated at least twice unless stated otherwise in Figure Legends. For correlation analysis, Pearson correlation coefficient was calculated. Significance was established at $P < 0.05$.

RESULTS

Allergic sensitization to HDM depends on mice genetic background

C3H/HeJ mice have been reported as having a natural reduced anti-oxidant response in the lungs [6]. We investigated whether this characteristic would influence C3H/HeJ immune response to HDM. The composition of HDM extracts can affect the immune response [18], therefore we used two different commercially available HDM extracts. First, C3H/HeJ and Balb/c mice were exposed to HDM extract with high endotoxin level (HT-HDM). As Balb/c have been described to be dependent on β-glucans/TLR2 and LPS/TLR4 signalling for HDM sensitization [19] and given that β-glucans and LPS are able to induce oxidative stress, we selected a second HDM extract which contains only trace levels of both toxins (LT-HDM). As expected, inhalation of HT-HDM resulted in a strong Th2-type inflammatory response in Balb/c, as reflected by eosinophil recruitment in the airway lumen, Th2 cytokine production, peribronchial inflammatory infiltrates, goblet cell hyperplasia, total IgE and HDM-specific $IgG_{1.}$ IFNγ and HDM-specific IgG_{2a} were also increased (**Fig. 1*a-e***). LT-HDM failed to induce any significant immune response in Balb/c mice, except for a small increase in mucus production (**Fig. 1a-*e***). Despite the mutant nonfunctional TLR4 in C3H/HeJ mice, HT-HDM induced all hallmarks of a robust Th2 inflammation, similar to Balb/c. Also here, IFNγ and IgG_{2a} were increased. However, in contrast to Balb/c, C3H/HeJ mice also developed a full-blown Th2-type immune response to LT-HDM (**Fig. 2*a-e***). This was not accompanied by an increase in IFNγ and IgG_{2a}.

To clarify whether the differential responsiveness to LT-HDM between both strains was due to the different genetic background rather than to the mutation in C3H/HeJ mice, we studied C3H/HeN mice, the wild-type background of C3H/HeJ mice. Similar to the mutant mice, C3H/HeN developed a full-blown Th2 mediated airway inflammation in response to LT-HDM without the induction of IFNγ and IgG_{2a} (**Fig. 3*a-e***). The inflammatory response induced by HT-HDM in C3H/HeN mice, was accompanied by the induction of IFNγ and IgG_{2a} similarly to C3H/HeJ. Together this indicates that sensitization to HDM depends on mice genetic background and is not strictly dependent on TLR4 signalling.

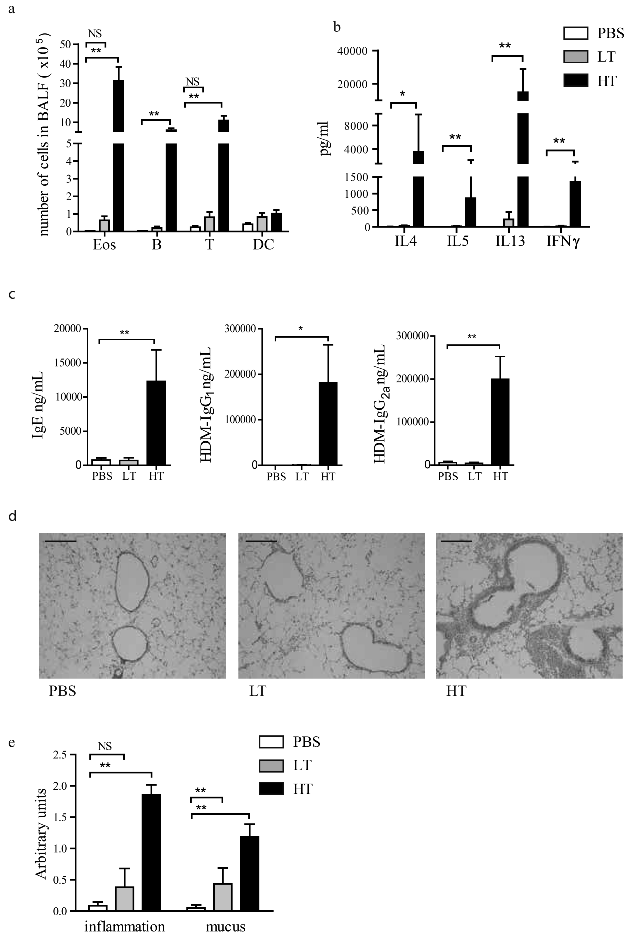

Figure 1. Balb/c immune response to LT-HDM (LT) and HT-HDM (HT). Balb/c mice were intranasally exposed to LT-HDM or HT-HDM or PBS as a control. (**a**) Number of inflammatory cells in bonchoalveolar lavage fluid (BALF). (**b**) Production of Th2 cytokines IL-4, IL-5, IL-13 and IFNγ by lung draining lymph node cells. (**c**) Total IgE, HDM-IgG_1 and HDM-IgG_{2a} in serum. (**d**) Peri-bronchial inflammatory infiltrates and mucus production and (**e**) quantification. Scale bars in **d** represents 200 µm. Data are presented as means ± SD, *P<0.05, **$P<0.01$.

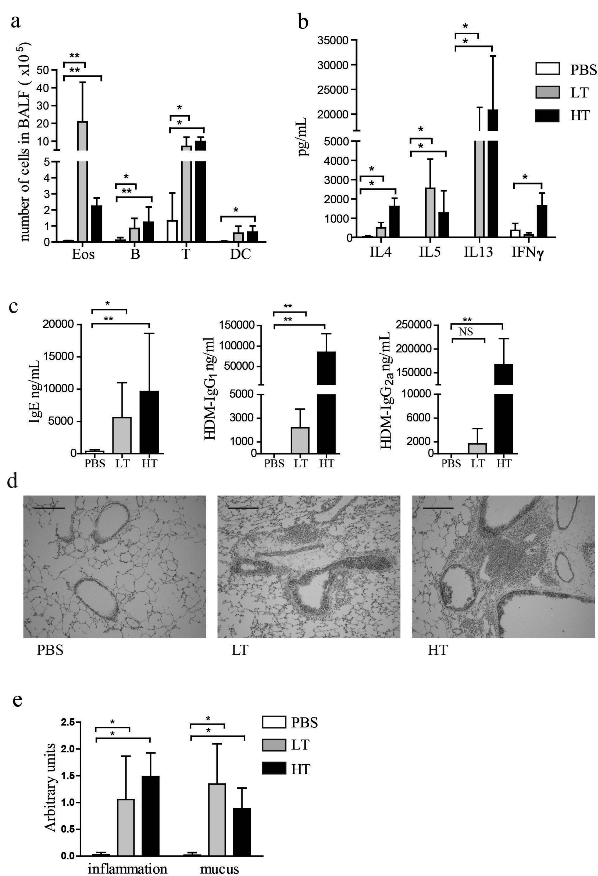

Figure 2. TLR4-mutant mice immune response to LT-HDM (LT) and HT-HDM (HT). C3H/HeJ mice were intranasally exposed to LT-HDM or HT-HDM or PBS as a control. (**a**) Number of inflammatory cells in bronchoalveolar lavage fluid (BALF). (**b**) Production of Th2 cytokines IL-4, IL-5, IL-13 and IFNγ by lung draining lymph node cells. (**c**) Total IgE, HDM-IgG_1 and HDM-IgG_{2a} in serum. (**d**) Peri-bronchial inflammatory infiltrates and mucus production and (**e**) quantification. Scale bars in **d** represents 200 μm. Data are presented as means ± SD, *P<0.05, **$P<0.01$.

Figure 3. *Wild-type* C3H/HeN immune response to LT-HDM (LT) and HT-HDM (HT). C3H/HeN mice were intranasally exposed to LT-HDM or HT-HDM or PBS as a control. (**a**) Number of inflammatory cells in bronchoalveolar lavage fluid (BALF). (**b**) Production of Th2 cytokines IL-4, IL-5, IL-13 and IFNγ by lymph node cells. (**c**) Total IgE, HDM-IgG_1 and HDM-IgG_{2a} in serum. (**d**) Peri-bronchial inflammatory infiltrates and mucus production and (**e**) quantification. Scale bars in **d** represents 200 µm. Experiment performed once with n=5. Data are presented as means ± SD, *P<0.05, **$P<0.01$.

Oxidative stress and anti-oxidant protein expression in response to LT-HDM in C3H/HeJ and Balb/c mice

We hypothesized that susceptibility to LT-HDM allergic sensitization in C3H/HeJ mice could be related to a deficient antioxidant response in the lungs. In order to establish the alternative mechanism for HDM allergic sensitization that seems to be at least partly independent of TLR4-LPS pathway, we performed our further experiments with TLR4-mutant C3H/HeJ mice. We compared the level of oxidative stress before and after a single exposure to LT-HDM in Balb/c and C3H/HeJ mice, resistant and susceptible to LT-HDM sensitization, respectively. Before exposure, the concentration 4-hydroxynonenal-modified proteins (4-HNE), a marker for oxidative stress [20], was markedly lower in lungs of Balb/c mice compared to C3H/HeJ. LT-HDM induced an increase of 4-HNE-modified proteins in the lungs of both strains. However, this increase was significant in Balb/c but not in C3H/HeJ mice, very likely due to its prominent high level of oxidative stress before exposure (**Fig. 4*a*, *b***). Levels of mRNA for anti-oxidant enzymes glutathione peroxidase-1 (GPx-1) and heme oxygenase-1 (HO-1) were increased in response to LT-HDM in lungs of Balb/c, but not in C3H/HeJ mice (**Fig. 4*c***). The levels of 4-HNE-modified proteins inversely correlated with the levels of HO-1 mRNA (**Fig. 4*d***).

We compared the capacity to up-regulate nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of the anti-oxidant response among which HO-1 [21], in response to LT-HDM in bone marrow-derived dendritic cells (BMDCs) from both strains. After 24h of LT-HDM exposure, Nrf2 expression was increased in Balb/c but not in C3H/HeJ BMDCs (**Fig.4*e*, *f***).

As DC activation and subsequent migration to lymph nodes is an important step in the initiation of an adaptive response [22] and subsequent induction of specific-allergen Type 2 cell differentiation [23]**,** we examined whether LT-HDM induced activation of C3H/HeJ DCs and whether this activation could be inhibited by the anti-oxidant N-acetyl-L-cysteine (NAC), a potent ROS scavenger. LT-HDM exposure upregulated CD40, CD80 and CD86 compared to unexposed cells. Treatment of DCs with NAC inhibited up-regulation of CD40 and CD80 after LT-HDM exposure (**Fig. 4*g***).

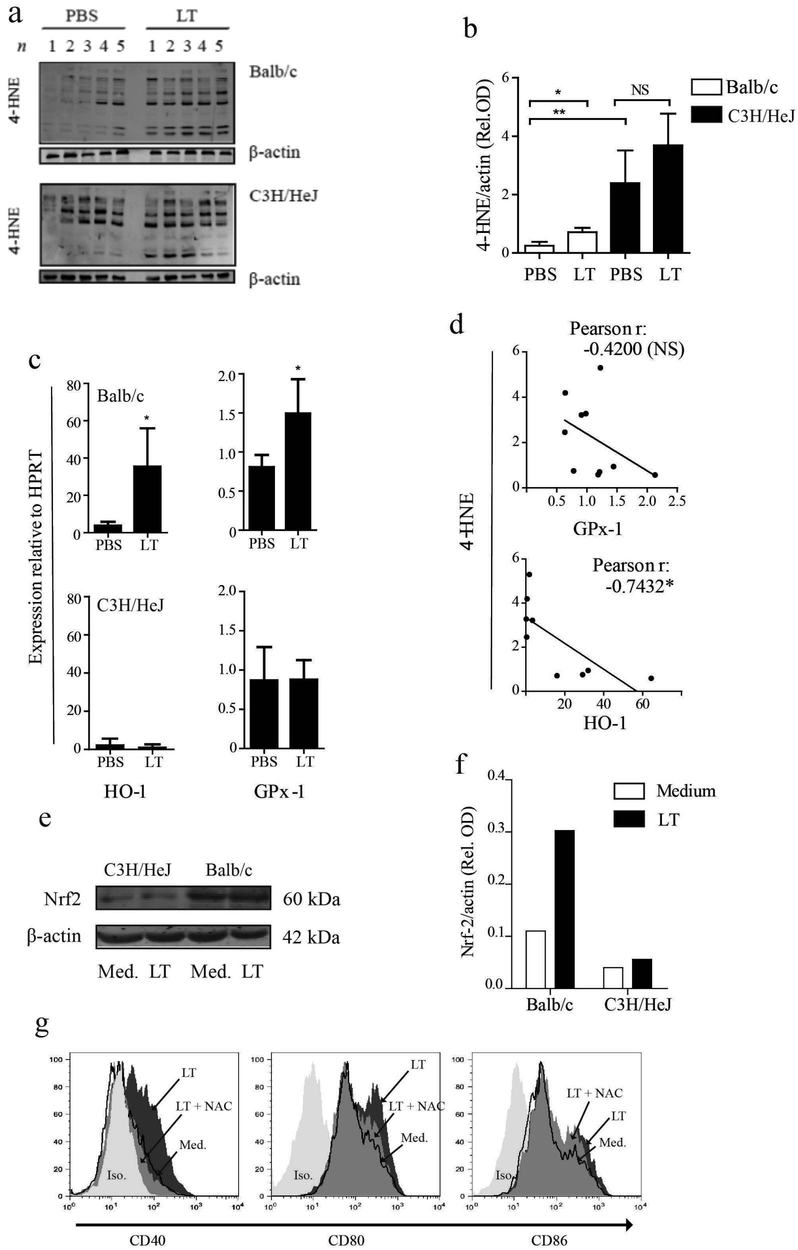
a
PBS
LT
n
1 2 3 4 5
1 2 3 4 5
4-HNE
Balb/c
β-actin
4-HNE
C3H/HeJ
β-actin
b
4-HNE/actin (Rel.OD)
Balb/c
C3H/HeJ
NS
PBS
LT
PBS
LT
c
Expression relative to HPRT
Balb/c
C3H/HeJ
PBS
LT
HO-1
GPx-1
d
Pearson r:
-0.4200 (NS)
4-HNE
GPx-1
Pearson r:
-0.7432*
HO-1
e
C3H/HeJ
Balb/c
Nrf2
60 kDa
β-actin
42 kDa
Med. LT Med. LT
f
Nrf-2/actin (Rel. OD)
Medium
LT
Balb/c
C3H/HeJ
g
LT
LT + NAC
Med.
Iso.
CD40
CD80
CD86

Figure 4. Oxidative stress levels and anti-oxidant protein expression in mice, after HDM exposure. Balb/c and C3H/HeJ (n=5) were intranasally exposed to LT-HDM or PBS as a control and lungs were removed 24h later for analysis. (**a**) Immunoblotting of 4-HNE-modified proteins (4-HNE) in lung homogenate and (**b**) quantification. (**c**) mRNA expression of anti-oxidant enzymes Heme oxigenase-1 (HO-1) and Glutathione Peroxidase-1 (GPx-1) in lung tissue. **(d)** Correlation of 4-HNE-modified proteins (4-HNE) with GPx-1 and HO-1 mRNA expression. (**e**) Immunoblotting of Nrf2 in total bone-marrow derived dendritic cell lysates (blots are representative of two independent experiments) and (**f**) quantification of protein expression. (**g**) Expression of co-stimulatory molecules CD40, CD80 and CD86 on bone-marrow derived dendritic cells from naïve C3H/HeJ mice, cultured with LT-HDM (LT) in the absence or presence of ROS scavenger *N*-acetyl-L-cysteine (NAC). Data are presented as means ± SD, *$P<0.05$, **$P<0.01$.

Partial inhibition of protease activity in HDM extract reduced HDM-allergic inflammation in mice

Next, we verified whether the induction of oxidative stress was dependent on the protease activity in LT-HDM. Protease activity was attenuated by heating (30 minutes at 65°C). In heated LT-HDM (ht-LT-HDM), there was significant inhibition of its trypsin and chymo-trypsin protease activity while cysteine protease activity was not affected (**See Figure S1**). Importantly, heating did not compromise the allergenic potency as expressed by unaltered IgE recognition of heated mite allergens (**See Figure S2 and S3**). Heated LT-HDM induced markedly less 4-HNE-modified proteins in C3H/HeJ BMDCs, compared to control LT-HDM (**See Figure S4A,B**). To confirm this finding *in vivo*, mice were exposed to PBS, LT-HDM or heated LT-HDM and the level of 4-HNE modified proteins in lung was determined after 24 hours. In concordance with the *in vitro* assay, heated LT-HDM induced less oxidative stress compared to LT-HDM, although this did not reach significance **(See Figure S4B in the Online Repository)**. Next, we examined the effect of the reduced oxidative capacity of heated LT-HDM *in vivo*. Allergic characteristics were significantly decreased in mice exposed to heated LT-HDM in comparison to LT-HDM. Heat-treatment significantly reduced the recruitment of eosinophils, dendritic cells, and T and B lymphocytes to the airways, IL-4, total IgE and HDM-specific IgG_1 production (**Fig. 5*a-e***). Peri-bronchial inflammatory infiltrates, goblet cell hyperplasia, IL-5 and IL-13 showed a small decrease as well but did not reach statistical significance. This suggests that a reduced capacity to induce allergic inflammation by heated LT-HDM could be attributed to its reduced capacity to generate oxidative stress.

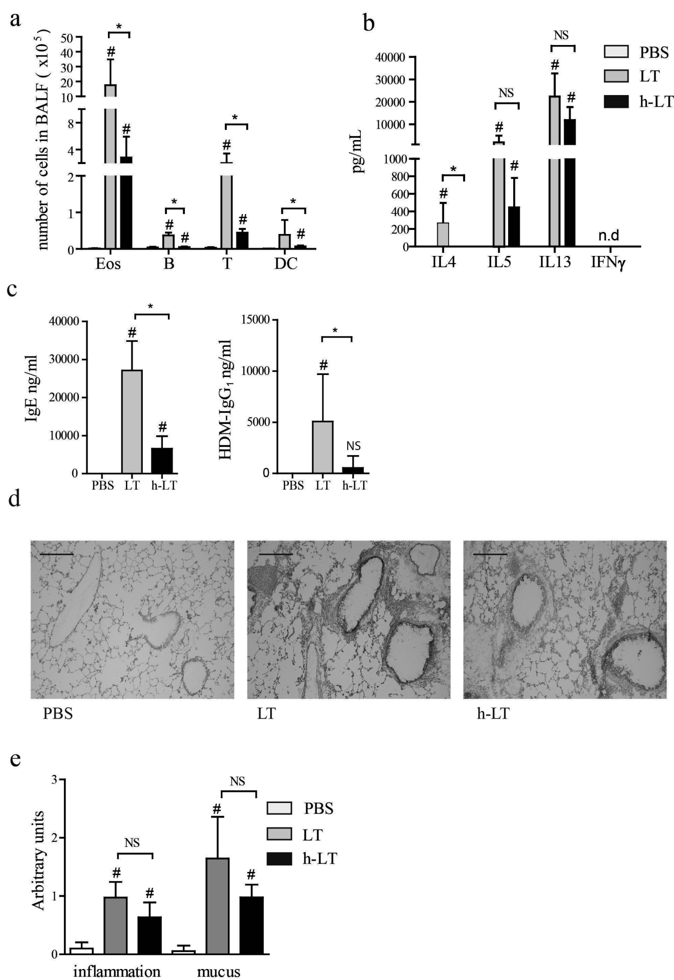

Figure 5. Effect of HDM protease activity inhibition *in vivo*. C3H/HeJ mice were intranasally exposed to LT-HDM or heated LT-HDM (h-LT) or PBS as a control. (**a**) Number of inflammatory cells in bronchoalveolar lavage fluid. (**b**) Production of Th2 cytokines IL-4, IL-5, IL-13 and IFNγ by lung draining lymph node cells. (**c**) Total IgE and HDM-IgG_1 in serum. (**d**) Peribronchial inflammatory infiltrates and mucus production and (**e**) quantification. Scale bars in **d** represents 200 µm. Experiment performed once with five mice per group. Data are presented as means ± SD, $^{\#,*}P<0.05$.

Allergic sensitization to rodent proteins in humans is associated reduced capacity to express HO-1 and Nrf2

Next, we investigated whether an insufficient capacity to cope with oxidative stress also correlates to allergic sensitization in humans. Previously we have determined sensitization to rodents urinary proteins in a cohort of atopic individuals up to 2 years after occupational exposure [17]. Sixteen of 37 atopic individuals became *de-novo* sensitized to rodents urinary proteins during this period as determined by allergic symptoms, the development of allergen-specific IgE and allergen-induced IL-4 production [17]. We accessed 4-HNE-modified proteins in serum collected before occupational exposure started (T0) and after four months (T4), one year (T12) and two years (T24). Those who became *de-novo* sensitized (S) to laboratory animals showed significantly higher levels of 4-HNE-modified proteins in serum before exposure started (T0) and in all subsequent time points, compared to control individuals that did not develop *de-novo* sensitization (Non-S) (**Fig. 6*a*, *b***). Expression of HO-1 in serum was significantly lower in sensitized group (S), indicative of a reduced anti-oxidant capacity (**Fig 6*c*, *d***). At T0, T4 and T12, a significant inverse correlation was observed between 4-HNE-modified proteins and HO-1 expression (**Fig. 6*e***), although this correlation attenuated over time and was lost after two years. In order to analyse the capacity to upregulate Nrf2 during oxidative stress induced by xanthine/xanthine oxidase, which induces ROS superoxide, we evaluated PBMCs collected at T0 from *de-novo* sensitized (n=5) and not sensitized subjects (n=4). Nrf2 expression in PBMCs from *de-novo* sensitized individuals (S) was lower than controls (Non-S). Although this did not reach significance possibly due to the limited number of available samples (**Fig. 6*f*, *g***), the relevance of the differences in Nrf2 expression between both groups was reflected by the strong negative correlation with the level of oxidative stress (4-HNE-modified proteins) (**Fig. 6*h***). Overall, our human data are in support of our murine studies, in which the development of allergic sensitization was associated with an inadequate anti-oxidant response.

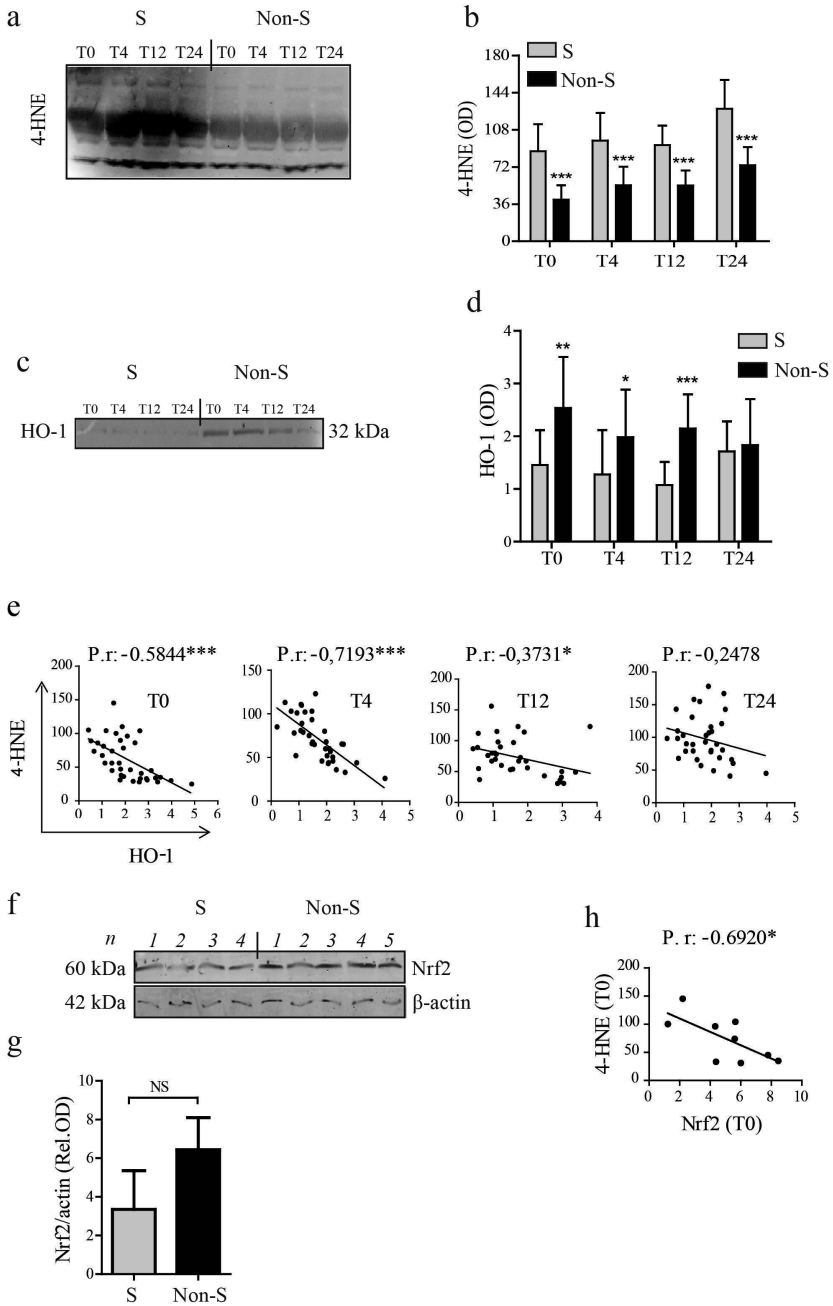
a
S
Non-S
T0
T4
T12
T24
4-HNE
b
S
Non-S
4-HNE (OD)
c
HO-1
32 kDa
d
HO-1 (OD)
e
P.r: -0.5844***
P.r: -0,7193***
P.r: -0,3731*
P.r: -0,2478
4-HNE
HO-1
f
n
60 kDa
42 kDa
Nrf2
β-actin
g
NS
Nrf2/actin (Rel.OD)
h
P. r: -0.6920*
4-HNE (T0)
Nrf2 (T0)

Figure 6. Oxidative stress levels and anti-oxidant protein expression in animal laboratory workers. Human serum samples from 16 *de-novo* sensitized (S) and 21 non-*de-novo* sensitized (Non-S) individuals were analyzed for 4-HNE-modified proteins (4-HNE) and HO-1 expression in different time points (T0=before occupational exposure and T4, T12 and T24=4, 12 and 24 months respectively after occupational exposure). (**a**) Representative immunoblotting of 4-HNE-modified proteins (4-HNE) from one individual per group and (**b**) quantification of protein expression. (**c**) Immunoblotting of Hemoxigenase-1 and (**d**) quantification of protein expression. (**e**) Correlation of 4-HNE-modified proteins (4-HNE) and HO-1 expression in different time points (Pearson r: T0=-0.5844***, T4= -0.7193***, T12= -0.3731* and T24=-0.2478). (**f**) Immunoblotting of Nrf2 in PBMCs from S (n=4) and Non-S (n=5) individuals and (**g**) quantification of protein expression. (**h**) Correlation of 4-HNE-modified proteins (4-HNE) and Nrf2 expression (Pearson r: -0.6920*). Data are presented as means ± SD, *P<0.05, **P<0.01, ***P<0.001.

DISCUSSION

What makes individuals more susceptible than others to allergic sensitization is considered a multifactorial process that involves among others, genetics and types of allergens. In the present study we show that inadequate anti-oxidant responses are strongly associated with allergic sensitization.

In our study, we showed that C3H/HeJ mice which have a natural reduced anti-oxidant response were susceptible to sensitization to LT-HDM, while Balb/c mice with a better anti-oxidant response were resistant. In an occupational cohort of animal laboratory workers, we observed that individuals with higher levels of oxidative stress before allergen exposure were prone to develop *de-novo* sensitization to urinary proteins. In addition, PBMCs of sensitized individuals showed a reduced capacity to upregulate the major regulator of anti-oxidant responses Nrf-2, when subjected to oxidative stress condition *in vitro*. These findings indicate that exposure to allergenic proteins combined with host inadequate anti-oxidant response, dramatically increased the likelihood for the development of allergic sensitization.

The exact mechanism by which ROS, in the context of oxidative stress, can initiate adaptive immune responses to an allergen is unknown. ROS at a relatively low concentration serve as essential second messenger mediating cellular responses to many physiological stimuli for example, by regulating the redox status of transcription factors [24;25]. However, excessive ROS production can contribute to an enhanced immune response [25]. ROS are known for stimulating Th2 like responses [7;26] and to induce maturation and antigen presentation by DCs [27-29], which is an important step in the initiation of adaptive immunity including allergy. In concordance, treatment of LT-HDM exposed DCs with NAC to prevent formation of ROS, decreased LT-HDM induced expression of the costimulatory molecules CD40 and CD80. ROS is able to decrease the thresholds for CD28 activation on T cells by enhancing IL-2 and IL-2R expression [30;31]. ROS can affect DCs also indirectly. Under oxidative stress condition, damaged, dead or activated structural cells, can release Danger-Associated Molecular Patterns (DAMPs) and cytokines, able to promote DC maturation [32-34].

To address whether HDM protease activity was involved in the induction of oxidative stress and allergic inflammation, we reduced protease activity of HDM allergens using mild heating.

The use of heat has the advantage of specific intervention, targeting the allergen alone in contrast to other intervention methods. The systemic use of anti-oxidants could also affect oxidative stress induced by the inflammatory process itself and the baseline redox status of the host. Protease inhibitors can inhibit endogenous proteases activated during HDM induced inflammation which would overestimate effect of protease inhibition. Mild heating of HDM reduced its proteolytic activity without affecting IgE-binding potency. This led to a clear inhibition of oxidative stress in dendritic cells *in vitro* (Suppl. fig 4) and significant attenuation of allergen sensitization and Th2 inflammation *in vivo*. This suggested that the level of allergic airway inflammation was dependent on the level of oxidative stress induced by HDM proteases. However, when the anti-oxidant response was sufficient to counter HDM induced oxidative stress, as observed in Balb/c exposed to LT-HDM, no inflammatory response was induced. In Balb/c, TLR4 signalling via LPS present in the HT-HDM extract was likely strong enough to outcompete the anti-oxidant capacity in Balb/c mice resulting in sensitization. Recently, Hammad et al [3] demonstrated elegantly the dependence on TLR4 for HDM sensitization in Balb/c, herein we show that C3H/HeJ mice with an impaired TLR4 signalling were able to mount an immune response to inhaled HT-HDM. Our results indicate that their natural reduced anti-oxidant response might have bypassed the need for TLR4 signalling. Oxidative stress is known to preferentially lead to the induction of type 2 cytokines (IL-4, IL-13) in $CD4^+$ T cells in favour of type 1 cytokines (IFNγ) [7;26]. Interestingly, LT-HDM induced a polarized type 2 response, with IL-4, IL-5 and IL-13 production, but not IFNγ or IgG_{2a} in wild type C3H/HeN and mutant C3H/HeJ mice, suggestive of a response induced by oxidative stress. HT-HDM however, additionally induced IFNγ and IgG_{2a} in TLR4 mutant C3H/HeJ mice, suggesting that another stimulus provided by HT-HDM, such as LPS and β-glucans able to trigger TLR2, was needed for this mixed Th1/Th2 response without the need of TLR4 signalling.

There is increasing evidence that a deficient antioxidant system may contribute to allergy development. Nrf2 and HO-1 deficiency predisposes mice to more severe allergic inflammation [35-37]. In humans, polymorphisms in genes coding for enzymes that play a role in scavenging ROS have been associated with an increased risk for the development of atopic disorders [38;39]. Anti-oxidant proteins not only provide protection against oxidant injury but are also involved in immune modulation. For example, HO-1 suppresses T cell

function and proliferation *in vitro* and *in vivo* [40] and its expression in DCs, is involved in the induction of $CD4^+CD25^+$ T regulatory cells [41]. Anti-oxidant mechanisms are crucial in the regulation of cellular redox homeostasis. Deficiency of key anti-oxidant components (such as Nrf2) perturbs intracellular redox status, increasing the basal levels of intracellular ROS [26;42] affecting DC phenotype and function [27;28].

In conclusion, our findings indicate that oxidative stress before allergen exposure, due to inadequate anti-oxidant response, primes for allergic Th2 responses. The inability to cope with oxidative stress might represent an underlying mechanism of why certain proteins act as an allergen in some individuals and are harmless to others. More studies are necessary to understand the mechanisms by which oxidative stress initiate a Th2 response to allergens.

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SUPPLEMENTARY FIGURES

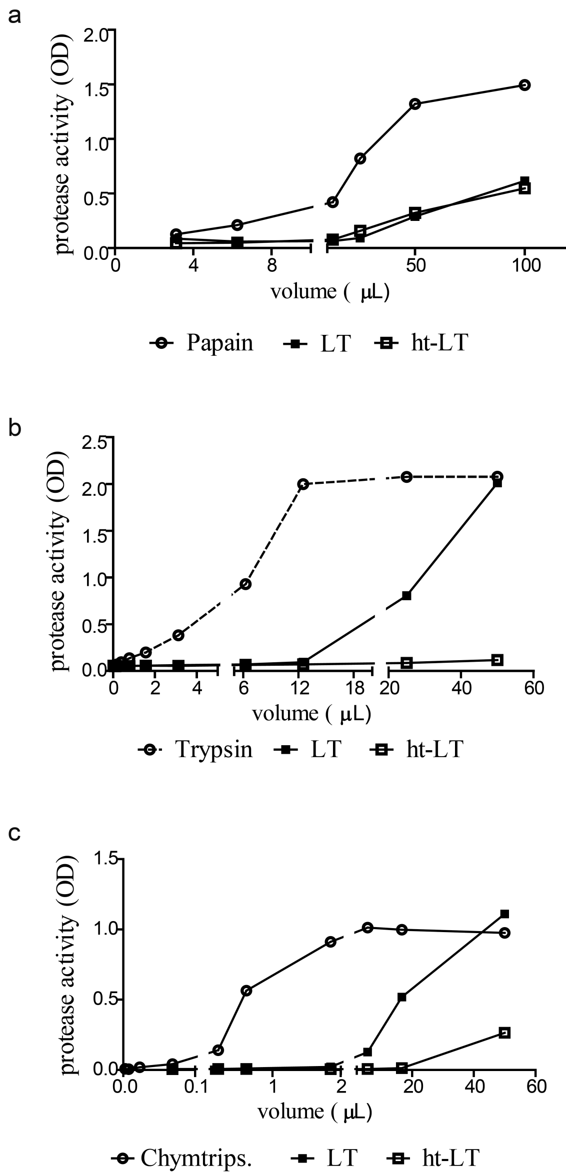

Figure S1. Protease activity of LT-HDM (LT) and heated LT-HDM (h-LT).

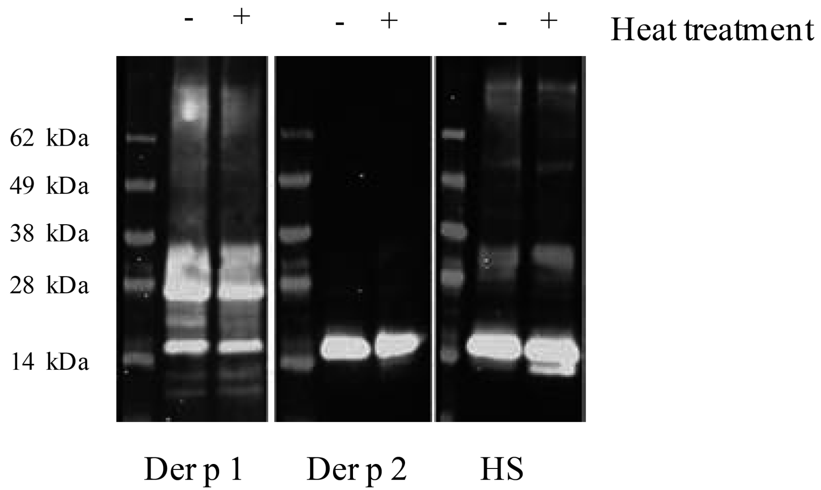

Figure S2. Heating of LT-HDM extract did not compromise antigen recognition by mite allergen specific IgE as determined by immunoblotting.

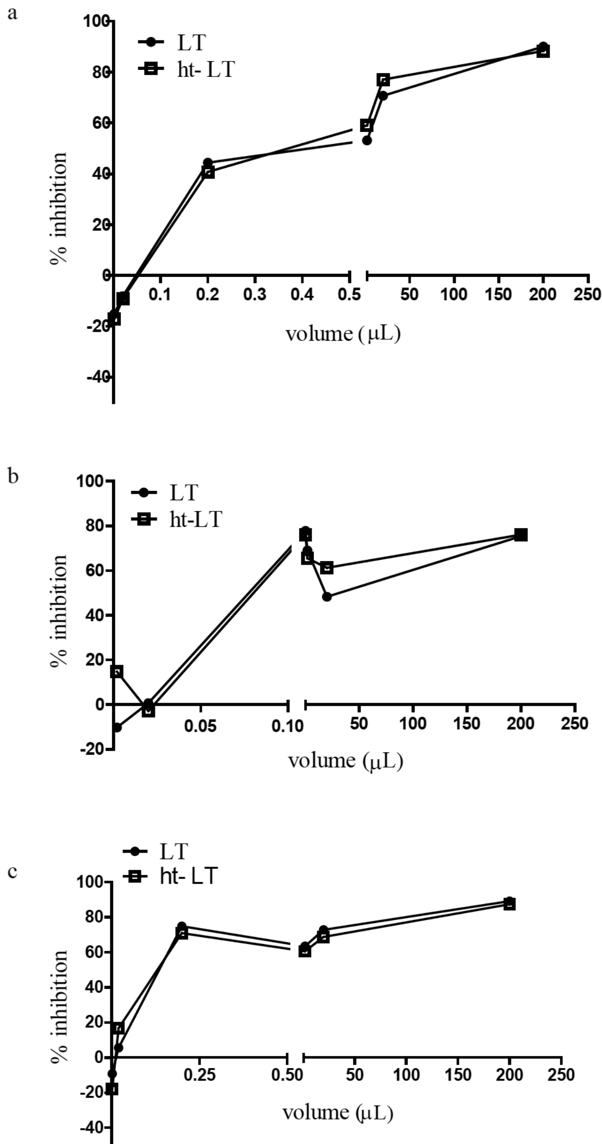

Figure S3. Heating of LT-HDM did not compromise antigen recognition by mite allergen specific IgE as determined by CAP inhibition assay.

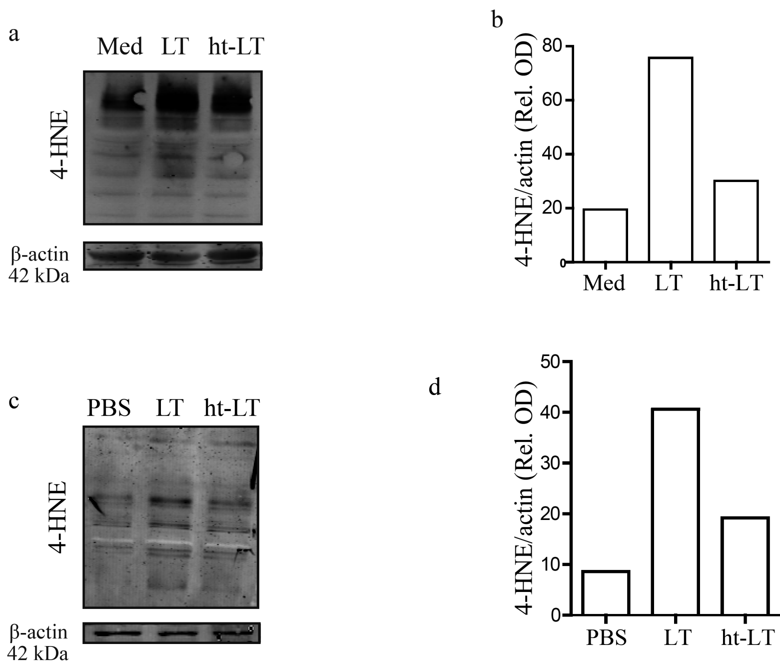

Figure S4. Heat treatment of LT-HDM reduced its capacity to induce oxidative stress *in vitro* (after overnight exposure of C3H/HeJ BMDCs) and *in vivo* (in C3H/HeJ lungs, 24 hr after exposure).

Chapter 3

Oxidative stress: promoter of allergic sensitization?

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Under review

ABSTRACT

Allergies arise from aberrant T helper type 2 responses to allergens. The processes involved in the genesis of allergic sensitization remain elusive. There has been growing interest in the role of oxidative stress in allergic sensitization. Oxidative stress is an imbalance between the anti-oxidant capacity of a cell and its exposure to reactive oxygen species (ROS). ROS are formed due to external stimuli and continously within all cells as a consequence of oxidative biochemical reactions including mitochondrial respiration. They have a role in cell homeostasis and are under strict control. Phagocytic cells and epithelial cells at barrier surfaces in skin, lung and gut produce ROS as a defense mechanism against pathogens but also in response to allergens and pollutants. When the amount of ROS overwhelms the anti-oxidant capacity, damage to macromolecules such as proteins, lipids and DNA may ensue. This imbalance in redox homeostasis can act as a trigger to induce an immune response to an allergen. Herein we discuss how oxidative stress can contribute to allergic sensitization. We review the effect of ROS on DC and T cells, how environmental stimuli (like allergens) can induce ROS, how variations in the cellular anti-oxidant capacity in response to external stimuli may explain differences in susceptibility to allergic sensitization between individuals and how oxidative stress can enhance allergenicity of a protein.

INTRODUCTION

Allergic diseases are the result of an aberrant immune response by T helper type 2 cells to allergens, resulting in the release of cytokines IL-4, IL-5, IL-13 and induction of allergen specific IgE [1;2]. Although many studies have addressed and clarified the underlying complex mechanisms in the pathogenesis of allergic disorders, the circumstances that spark off allergic sensitization are less clear. Allergy is induced by a select group of proteins without clear similarities and not all people are equally susceptible to develop sensitization to the same allergens. There is growing evidence that oxidative stress during allergen encounter may play a role in the allergic sensitization process at different levels, by favoring a Th2 immune response but also by increasing immunogenicity of the allergen.

Oxidative stress is a condition generated when the production of free radicals such as reactive oxygen species (ROS) overwhelms cellular anti-oxidant defenses [3]. Reactive oxygen species are partially reduced and highly reactive metabolites of O_2 that include, amongst others, superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$)[4]. The mitochondrial respiratory chain is a major source of ROS during homeostasis. The primary function of the respiratory chain is to provide energy to the cell. Produced at limited concentrations under strict control of anti-oxidant mechanisms, they have important roles in cell signaling, growth and homeostasis [3;5]. As an example, when stimulated by cytokines, growth factors and hormones, most cell types elicit a discrete oxidative burst generating low concentrations of ROS [5]. ROS then operate as important messengers of signal transduction through the oxidative modification of kinases and phosphatases, which are present in many signaling pathways including MAPK and NF-κB [5;6]. However, large concentrations of ROS caused by external stimuli or by deficiencies in anti-oxidant systems, may lead to activation of signal transduction pathways and also cause permanent changes in gene expression when these concentrations reach abnormal physiological levels resulting in disease and ultimately cell death [3;7-9].

ROS generation is generally a cascade of reactions that starts with the production of superoxide. Apart from the mitochondria respiratory chain, superoxide is generated mainly by xanthine oxidoreductase system (XOR) and NAD(P)H oxidases (NOX) [10;11]. All NOX

family members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide [10]. The physiological function of NOX enzymes is the generation of ROS for host defense, inflammation and various cellular physiological processes [10]. The majority of NOX enzymes require cytosolic activation or subunit assembly that can be triggered for example, by cytokine and chemokine binding on their respective receptors [10]. Allergen exposure can trigger the release of several chemokines and cytokines, like GMCSF, by structural or innate immune cells [12;13] and therefore can potentially trigger cytosolic activation of NOX. DUOX 1 and 2 are isoforms of NOX expressed in the airway epithelium and contrary to other NOX, they do not require cytosolic activation or subunit assembly but instead they are directly activated by intracellular Ca^{2+} [10]. Another important source of ROS is XOR. This enzyme system mostly exists in a form of xanthine dehydrogenase (XDH), but it can be converted to xanthine oxidase (XO) by reversible sulfhydryl oxidation or by irreversible proteolytic modification [11]. Oxidation of xanthine by XOR yields uric acid, superoxide and hydrogen peroxide [11]. In addition, free iron released from iron-containing molecules can participate in the Fenton reaction, generating highly reactive hydroxyl (OH^-) radicals. Therefore heme groups, iron-storage proteins and free iron constitute another potential endogenous source of ROS and oxidative stress [6] (See Figure 1). However the significance of this reaction is under debate and might only occur in organisms with increased iron levels (as in conditions of hemochromatosis). The aim of this review is to discuss how oxidative stress can contribute to the development of allergic sensitization.

Biochemical characteristics of ROS determine biological impact

The biochemical characteristics of each ROS such as stability and reactivity determines their biological impact and the mechanisms by which they are able to trigger molecular pathways involved in the regulation of immune responses.

Superoxide (O2-) is generated by NAD(P)H oxidases and XOR in the mitochondria, phagosomes and extracellular spaces [10;11]. After its generation, superoxide rapidly dismutates to H_2O_2 either spontaneously, particularly at low pH or by the action of superoxide dismutases which are present extracellularly (EC-SOD), in the cytosol (Cu-Zn-SOD), or inside mitochondria (Mn-SOD) [14]. The capacity of superoxide to cross cell

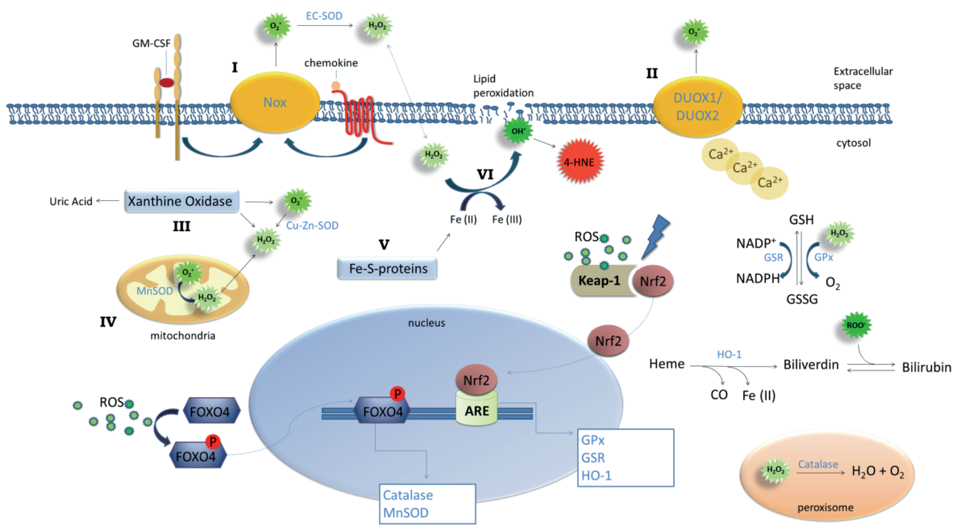

Figure 1. ROS generating and detoxifying mechanisms. NADPH oxidases such as Nox-1, -2 and -3 (I) and DUOX1 and -2 (II), xanthine oxidase (III), mitochondrial respiration (IV), and iron-sulfur clusters (V) are important sources of ROS production by the cell. Activation of these pathways combined with insufficient or inadequate antioxidant mechanisms result in oxidative stress. NADPH complexes on cellular membrane can be activated by ligand binding of GM-CSF and chemokine receptors (I) and DUOX1 and 2 can be activated by increased intracellular concentrations of calcium (II) leading to extracellular generation of superoxide. This radical is desmuted into H_2O_2 spontaneously or by the action of EC-SOD; Xanthine oxidase can be activated by several pathways including proteolysis and TLR4 triggering generating uric acid, superoxide and hydrogen peroxide. Increased intracellular production of superoxide results in the release of free irons from iron-sulfer clusters (V) feeding the Fenton reaction (VI) yielding hydroxyl ions. Hydroxyl lipophilic properties result in the attack of membrane lipids in a process called lipid peroxidation generating another potent oxidant 4-HNE. Oxidative stress provides the condition for the activation and nuclear translocation of transcription factors FOXO4 and Nrf2 initiating the transcription of key antioxidant enzymes.

membranes is limited and it has a high affinity for iron-sulfur clusters in proteins. Interestingly, ROS-induced maturation of DCs is mediated specifically by superoxide and not by H_2O_2 [15].

Hydroxyl ion (OH^-) is a result of iron oxidation by hydrogen peroxide in the Fenton reaction. It has the shortest half-life compared to O_2^- and H_2O_2; it reacts with almost any biological component it encounters, and is therefore the primary source of cell damage [16]. Due to its lack of specificity and its incapacity to freely diffuse through cell membranes, OH^- exhibits no important role on redox cell signaling (See Figure 1).

Among all ROS, H_2O_2 is the most stable. It is a mild oxidant compared to superoxide and hydroxyl ion (OH^-). It is rapidly generated after an extracellular stimulus and can be easily removed by several enzymes, mainly catalase and peroxidases [17]. The uncharged nature and relative stability of H_2O_2 permits free diffusion across cellular membranes to participate as a messenger in signal transduction.

How ROS, and especially H_2O_2, in the context of oxidative stress can prime the immune system for allergic responses

A broader overview of the effect of oxidative stress on the immune system has been detailed elsewhere [3]. Here we focus on some of the early effects of ROS on DCs and T cells, during oxidative stress that may promote allergic sensitization.

Dendritic cells (DCs) play a central role in the initiation of allergen-specific type 2 responses [18;19]. Mature DCs express high surface levels of multiple ligands important for T cell activation including CD40 and CD80/86 [20]. ROS can affect DCs directly but also indirectly. Under oxidative stress conditions, activated, damaged, or dead structural cells, can release danger-associated molecular patterns (DAMPs) and cytokines [21], able to promote DC maturation [22-24]. Besides maturation of DCs, ROS has the capacity to prime DCs and T cells for the induction of T helper type 2 responses. The Th2 skewing properties of ROS include the capacity to inhibit the expression of type 1 instructing cytokine IL-12 by DCs [9] and to induce production of type 2 cytokines by type 1 cells [8;25].

H_2O_2 has special characteristics which makes this ROS a potent immune modulator and a well-known second messenger. H_2O_2 targets cysteine residues in diverse proteins [17] and is capable of activating molecules involved in immune cell activation such as phosphatases and NF-kB [17;26;27]. CD28 activation on T lymphocytes can result in a strong increase of H_2O_2 levels [28], indicating that this ROS may be involved in the signaling pathway of CD28 co-stimulation. Exogenous H_2O_2 can induce activation and proliferation of T cells by enhancing IL2 and IL2R expression [29] facilitating the development of the adaptive response. We speculate that mild oxidative stress induced by certain allergens on DCs, can enhance H_2O_2 production, which can cross the T cell membrane during DC-T cell interaction. Inside T cells, diffused H_2O_2 can induce IL2/IL2R expression [29]. In this way, an elecated level of H_2O_2 can lower the threshold expression of CD28 ligands (CD80 and CD86) by non-fully mature DCs to induce an adaptive response.

H_2O_2 is also produced during TCR stimulation by MHCII on DCs [30]. TCR activation is possible by the phosphorylation of tyrosine-based activation motifs (ITAMs) on the cytosolic side of the TCR/CD3 complex by lymphocyte protein tyrosine kinase (Lck). The CD45 receptor tyrosine phosphatase modulates the phosphorylation and activation of Lck and other Src family tyrosine kinases. H_2O_2 mediated oxidation can inactivate phosphatases, resulting in an increased phosphorylation [26]. Interestingly, it has demonstrated that H_2O_2 can induce TCR stimulation, by enhancing the activation of MAP kinases [30-33]. This suggests that H_2O_2 can favor antigen-specific responses by enhancing the TCR signal on T cells.

Environmental oxidative stress-inducing agents and allergy development

Certain allergens and pollutants are able to trigger ROS production and, consequently, oxidative stress. We will discuss here the capacity of these agents to induce oxidative stress and how ROS production during this first encounter with an allergen or pollutant can act as a danger signal priming the immune system to a Th2 response.

I. Allergens

Some allergens have an intrinsic enzymatic activity, such as proteases (e.g. house dust mites, cockroach and pollen grains) [34;35] or NAD(P)H oxidase (e.g. pollen grains)[36]. The capacity of proteases to induce oxidative stress is well documented [37], however, a causal

relationship between their capacity to induce oxidative stress and to promote allergic sensitization has only been addressed recently. Tang et al. [9] showed that the cysteine protease papain, when given in combination with OVA, induced an OVA-specific allergic response in mice via the induction of oxidative stress. In our lab, we recently demonstrated that HDM proteases are responsible for the capacity of HDM allergens to induce oxidative stress. Partial inhibition of protease activity dramatically decreased the capacity of HDM to induce oxidative stress and the subsequent allergic inflammation in mice [38].

In addition to proteases, some allergen sources such as pollen also possess intrinsic NAD(P)H oxidase activity [36]. Although NAD(P)H oxidase has the capacity to generate ROS and to contribute to allergic inflammation [39;40], the participation of pollen-intrinsic NAD(P)H oxidase activity in the process of pollen-induced allergic sensitization has not yet been addressed *in vivo*. *In vitro* studies however, suggest that pollen-intrinsic NAD(P)H oxidase activity can contribute to primary allergic sensitization through its capacity to modulate the function of antigen-presenting cells via induction of ROS. Csillag et al [41] demonstrated that treatment with ragweed pollen grains induced maturation of DCs with upregulation of CD80, -83 ,-86 and HLA-DR. The pollen-treated DCs induced the differentiation of naive T lymphocytes toward effector T cells with a mixed profile. Anti-oxidants inhibited both the phenotypic and functional changes of DCs, underlining the importance of oxidative stress in these processes.

The ways by which allergens can trigger ROS-generating pathways such as XOR and NOX in host cells are not clear. Proteolytic allergens such as derived from mite and cockroach can induce intracellular calcium oscillations through the PAR2-involved pathway [42] suggesting another potential mechanism by which proteolytic allergens can activate NOX. Recently Hristova and colleagues demonstrated in human airway epithelium cells that exposure to HDM resulted in an increased DUOX-1 expression, but not DUOX-2, which correlated with the level of formed H_2O_2 and with an increased secretion of the Th2 skewing, innate cytokine IL-33[43]. In this study, the secretion of IL-33 could be inhibited by silencing DUOX-1. The relevance of this mechanism was underlined by the observation by these authors, that asthmatic patients expressed more DUOX-1 in their nasal epithelium cells after exposure to allergens in comparison to healthy controls, whereas no difference in DUOX-2 expression

was found. Allergen exposure leads to uric acid production [44], which initiates XOR activation, however the molecular pathways of XOR activation in this context have not yet been elucidated (see Figure 1).

II. Pollutants

Ambient air contains a range of pollutants that includes gases (e.g. nitrogen dioxide and ozone) and particulates. Epidemiological studies reveal that exposure to particulate air pollution is associated with, elicitation, exacerbation, and increased likelihood for the development of allergic diseases in humans [45;46]. There is substantial evidence from human and mouse studies for the role of oxidative stress as an underlying mechanism linking pollution and allergy [47-56]. One of the well-studied particulate pollutants, diesel exhaust particles (DEP) have been shown to exert a strong adjuvant effect in allergic sensitization. Devouassoux et al [57] demonstrated that blood basophils from allergic and non-allergic subjects released more IL-4 and histamine after DEP exposure. DEP adjuvant potential was also demonstrated in a human study in which atopic individuals developed *de-novo* allergic sensitization to a neo-allergen only when previously exposed to DEP [58]. Several mouse studies demonstrated that DEP can contribute to the induction of allergen-specific IgE and Th2 responses [59-63]. The fact that the effects of DEP can be reverted by treatment with anti-oxidants such as ROS scavenger n-acetylcysteine [56;57;64] indicate that its adjuvant activity is linked to its capacity to induce oxidative stress. Li et al [65] also demonstrated that the enhanced *in vivo* adjuvant effects of ultra-fine particulate matter correlated with its *in vitro* oxidant potential.

Oxidative stress generation due to inadequate anti-oxidant responses

In addition to the characteristics of certain allergens to induce ROS, the (in-)capacity to reduce these allergen induced ROS by the anti-oxidant machinery might represent a key role in allergic sensitization. Anti-oxidant mechanisms are crucial in the regulation of cellular redox homeostasis. To counteract the deleterious effects of oxidative stress, cells have developed an elaborate anti-oxidant defense to maintain redox equilibrium [66]**.** Anti-oxidants may be enzymatic or non-enzymatic. Catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, thioredoxin and heme oxigenase-1 (HO-1) are examples

of anti-oxidant enzymes. The non-enzymatic anti-oxidants include glutathione, vitamin E and C, uric acid, albumin, bilirubin and melatonin [67]. Glutathione (GSH) is the major cellular redox buffer contributing to maintain the intracellular redox homeostasis (see Figure 1). The ratio of reduced and oxidized glutathione (GSH/GSSG) is a good measure of oxidative stress in an organism [6], particularly when ROS are predominantly formed in phagocytes. Deficiency of anti-oxidant mechanisms perturbs intracellular redox status, increasing the basal levels of intracellular ROS affecting cell phenotype and function [15;41;68-71;71-74].

The contribution of inadequate anti-oxidant mechanisms for the development of allergies is slowly being recognized. Polymorphisms in genes involved in ROS detoxification are associated with atopy in humans. The Glutathione S-transferase P1 (GSTP1) gene polymorphic variants are associated with altered catalytic function of this enzyme. The frequency of GSTP1 genotype Val/Val was shown to correlate with lower risk of atopy while the genotypes lle/lle and lle/Val correlated with increased risk of atopy [75;76]. In support, Mapp and colleagues [77] also showed that subjects with occupational asthma to isocyanates were predominantly carriers of the GSTP1 alleles lle/lle or lle/Val while asymptomatic individuals carried the alleles Val/Val.

The transcription of anti-oxidant enzyme genes such as catalase and MnSOD are regulated by the forkhead box transcription factor O (FoxO) subfamily, FOXO4 [78;79], while glutathione reductase (GSR), glutathione peroxidase (GPX), thioredoxin (Trx), thioredoxin reductase (TrxR), and heme oxigenase 1 (HO-1) transcription is regulated by Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2)[80]. In response to oxidative stress FOXO4 is phosphorylated and subsequently translocate to the nucleus [79]; cytosolic Nrf2 detaches from its inhibitor, Kelch-like ECH-associated protein 1 (Keap 1) and also translocate to the nucleus where it binds to the Anti-oxidant Response Elements (AREs) [80](see Figure 1).

The regulation of numerous key anti-oxidant related genes makes Nrf2 a master orchestrator of anti-oxidant responses. The effect of Nrf2 absence in allergic sensitization is well documented. Nrf2 deficiency has been shown to increase the sensitivity to develop allergic sensitization and to promote a Th2 bias phenotype in antigen-presenting cells [72]. In mice, Nrf2 deficiency enhanced the adjuvant effect of ambient ultrafine particles (UFP) [81-83] and increased susceptibility for the development of a severe airway allergic response to ovalbumin [84]. Un-stimulated Nrf2 deficient cells, contrary to Nrf2 wild type cells,

exhibited a pro-type 2 phenotype characterized by a higher baseline level of type 2 related cytokine IL6, and no production of type 1-related cytokine IL12p70 [82].

HO-1, one of the enzymes regulated by Nrf-2 has the capacity to increase the levels of reduced glutathione and to degrade heme into biliverdin that later turns into bilirubin (see Figure 1). Both sub-products of heme breakdown have potent anti-oxidant properties [85]. HO-1 is expressed at low levels in basal conditions and is rapidly and vigorously induced by oxidative stress and inflammatory stimuli. HO-1 has an essential role in the regulation of inflammation and immune modulation. Both HO-1 knockout mice and human cases of HO-1 gene deficiency exhibit a significantly enhanced pro-inflammatory state [86;87]. HO-1 can modulate inflammation in many ways: by the inhibition of adhesion molecules on endothelial cells by preventing endothelial cells activation and interaction with leukocytes [88]; by suppression of neutrophil rolling, adhesion and migration, thereby preventing the entry of neutrophil at the site of inflammation [89]; HO-1 is capable of suppressing the function and proliferation of T effector cells [90]; and when its expression is enhanced on DCs, HO-1 is involved in the induction of $CD4^+CD25^+$ T regulatory cells [91].

Allergic sensitization is associated with a decreased anti-oxidant response in human and in mice

The relationship between oxidative stress and allergic sensitization is complex because allergen-induced oxidative stress can be at the origin of the Th2 response but, at the same time, inflammation will generate endogenous oxidative stress. We recently observed in our lab that susceptibility for sensitization was associated with an inadequate anti-oxidant response in human. Newly employed animal workers were followed for two years for the development of allergy to rodent urinary proteins [38]. Individuals who became *de novo*-sensitized to urinary proteins had higher serum levels of 4-HNE modified proteins and a lower expression of HO-1 at baseline before allergen exposure. Both parameters are indicative of a reduced anti-oxidant capacity. A functional test with PBMCs, which were exposed to X/XO *in vitro*, showed a lower upregulation of Nrf2 in *de novo*-sensitized individuals compared with the resistant controls. Although this study underlines that the ability to cope with oxidative stress was a risk factor for the development of allergic

sensitization, the underlying mechanisms as to how oxidative stress initiates a Th2 response to allergens remain enigmatic.

To explore this further, we studied the susceptibility to HDM sensitization in a mouse model with two strains of mice, which differed in their capacity to cope with oxidative stress. Sensitization to house dust mite (HDM) in mice was associated with an incapacity to upregulate Nrf2 and HO-1 upon induction of oxidative stress by mite proteases [38]. In Balb/c mice, exposure to a HDM extract with trace levels of endotoxins (LT-HDM) was not sufficient to induce a Th2 immune response [38]. This is in accordance with several studies showing that TLR4 triggering by LPS is crucial for the initiation of allergen-specific Th2 responses in Balb/c mice [92;93]. However, in mice with a C3H background, the LT-HDM extract induced robust allergic inflammation. Susceptibility of C3H/HeJ to LT-HDM was associated with an inadequate anti-oxidant response in the lungs after LT-HDM inhalation, while Balb/c resistance to allergic sensitization to LT-HDM was associated with an intact anti-oxidant response.

Interestingly, we observed a differential threshold for DC maturation in the induction of a Th2 immune response to HDM in these two strains. Intranasal exposure to LT-HDM induced much less maturation of airway DCs compared to a HDM extract containing high levels of endotoxin (HT-HDM) ([38;94] and unpublished data). Remarkably, C3H/HeJ mice were capable to exert a full-blown allergic type 2 inflammation after intranasal exposure to LT-HDM, despite a low-immunogenic profile of airway DCs (low levels of co-stimulatory surface proteins CD40, CD80, and CD86 compared to HT-HDM). This suggests that a fully mature phenotype of DCs was not necessary for the induction of an allergen-specific Th2 response in C3H/HeJ mice, while in Balb/c this seemed to be a requirement. Because C3H/HeJ showed a defective anti-oxidant response in the lungs, which was demonstrated by the high level of oxidative stress in lungs at baseline and defective anti-oxidant protein induction after LT-HDM inhalation [38], we hypothesized that oxidative stress played a key role in the induction of the allergen-specific response to LT-HDM, possibly by decreasing the threshold for T cell activation (see Figure 2).

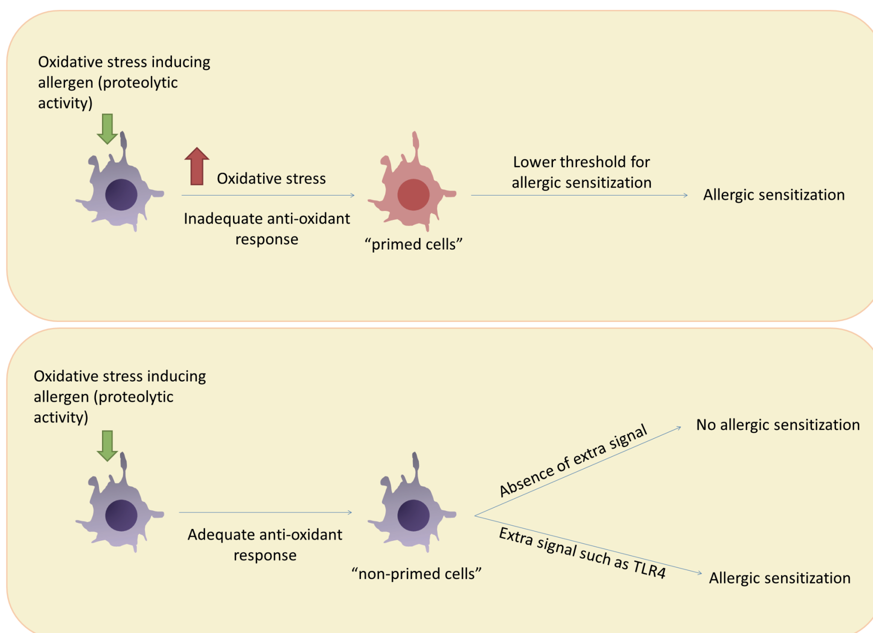

Figure 2. Schematic representation of how an inadequate anti-oxidant response may favour priming of the immune system for allergic sensitization. In the upper panel, allergen-induced oxidative stress in a host with an inadequate antioxidant response can prime immune cells such as T cells and DCs towards a Th2 response by decreasing the threshold for T cell activation. In the lower panel allergen-induced oxidative stress in a host with an adequate antioxidant response will not result in allergic sensitization unless a stronger signal such ligation of TLR4 is provided.

Although the level of H_2O_2 was not determined in DCs and T cells after LT-HDM exposure, preliminary data from our lab showed indeed evidence for H_2O_2 as a candidate mediator of allergic sensitization. LT-HDM exposure was followed by an increase in the expression of MnSOD (unpublished data) but not of GPx-1 [38] in the lung cells of C3H/HeJ mice. In Balb/c mice an opposite pattern was observed after LT inhalation; no MnSOD induction (unpublished data) and enhancement of GPx-1 expression [38]. MnSOD is the main mechanism for H_2O_2 generation by the cell while GPx-1 is one of the main systems for H_2O_2 removal. Increased MnSOD activity and decreased GPx-1 activity may promote H_2O_2 accumulation. This suggests that in C3H/HeJ mice after LT-HDM inhalation generation of

H_2O_2 was possibly not followed by proper removal and this ROS accumulated. As H_2O_2 is able to decrease the threshold for T cell activation, this can compensate for the lack of co-stimulation provided by non-fully mature DC. This hypothesis is in line with the growing body of evidence placing mitochondria, a major generator of physiological H_2O_2 via MnSOD (31), as being critical in T cell activation [31].

Uric Acid: oxidative-stress mediated alarmin effect?

Uric acid (UA) is a product of the oxidation of xanthine by xanthine oxidase and it has been suggested as an adjuvant in the process of allergic sensitization. UA is undoubtedly a potent activator of the immune system, however, its mechanisms of action are not clear. Although in its soluble form, UA has anti-oxidant properties and it can be found in nasal secretions of healthy individuals [95], in crystal forms they behave as Danger Associated Molecular Pattern (DAMP), which will act as an alarmin on the immune system. UA crystals have pro-inflammatory properties and may clinically be manifested as gout [96] but also have been implicated in the pathogenesis of other diseases such as allergies [44;97;98]. The main mechanism described for UA crystals immunogenicity is its ability to activate inflammasome NALP3, increasing caspase-1 activity resulting in the release of pro-inflammatory cytokine IL-1β [97;99]. However, inflammasome activation is not the only mechanism which is underlying the immunogenicity of UA, as other studies demonstrated that UA can both enhance the expression of MHCII, CD86, CD80 and OX40L on DCs and induce release of IL-1β, independently of caspase-1 activation [44;98].

Like ROS, UA seems to have the capacity not only to induce maturation of DCs but also to skew towards a Th2 response. Intranasal administration of UA crystals together with allergens in mice triggered the production of type 2 cytokines IL-5 and IL-13 but not IFN-γ, a Th1 cytokine [97]. Moreover, DCs stimulated with ovalbumin in presence of UA did not produce the Th1 instructive cytokine IL-12 [44]. UA has been shown to be able to induce upregulation of CD86 and OX40L on DCs independently of Toll-2 and -4 activation and induce Th2 cell immunity in a Syk- and PI3-kinase dependent manner [44;98]. Syk kinase and PI3-kinase dependent signaling have been shown to be sensitive to oxidative stress stimuli [100]. We propose that a potential mechanism by which UA activates the immune system could be through oxidative stress. The capacity of UA to generate oxidative stress has been

demonstrated in vascular smooth muscle cells [101] however, in allergic disease models this has been overlooked.

Studies showing a dramatic decrease in allergic inflammation after UA inactivation have not considered the inhibition of oxidative stress as a possible mediator for the observed suppression [44;97;98]. The role of UA in the allergic response has been identified by experimental inhibition. The most commonly used method is the systemic use of uricase and xanthine oxidase inhibitors such as allopurinol and febuxostat (see Figure 3). However, the result of UA inhibition via oxidation by uricase leads to the formation of allantoin, a molecule with anti-inflammatory properties. Allantoin-treated ovalbumin-allergic mice showed a significant decrease in the inflammatory response [102]. In other words, the use of uricase decreased UA but at the same time increased the allantoin levels, which is known to dampen inflammation. Moreover, inhibiting UA production by blocking XO with allopurinol or febuxostat can also decrease the production of ROS superoxide and H_2O_2 but also decreases the formation of peroxynitrite, which is a potent oxidizing agent also generated via XOR pathway [103]. Peroxynitrite generation can greatly contribute to the amplification of oxidative stress events by triggering cytotoxic processes including lipid peroxidation [104], which result in the generation of other reactive oxidative stress by-products such as 4-HNE and may contribute to inflammation. In addition, allopurinol is a non-specific XO inhibitor, which also has strong ROS scavenging properties that could contribute to the inflammation dampening effect via dampening of oxidative stress [11]. The xanthine oxidase inhibitors will not only inhibit UA but will also dampen the level of ROS.

In conclusion, the mechanisms by which UA crystals act as an alarmin in the allergic sensitization process and the magnitude of its role in allergic inflammation are not clear. A few crucial questions remain unanswered: whether the production of UA during allergen exposure involves a simultaneous significant increase in oxidative stress due to activation of XOR; and whether part of the pro-inflammatory effect of UA is due to oxidative stress induction.

Effect of oxidative stress on protein immunogenicity

An intriguing question related to the causes of the development of allergies is which intrinsic properties of an allergen (physicochemical/biochemical) and which extrinsic factors (e.g. patient-related) make proteins potential allergens. Oxidative stress could play a role in this,

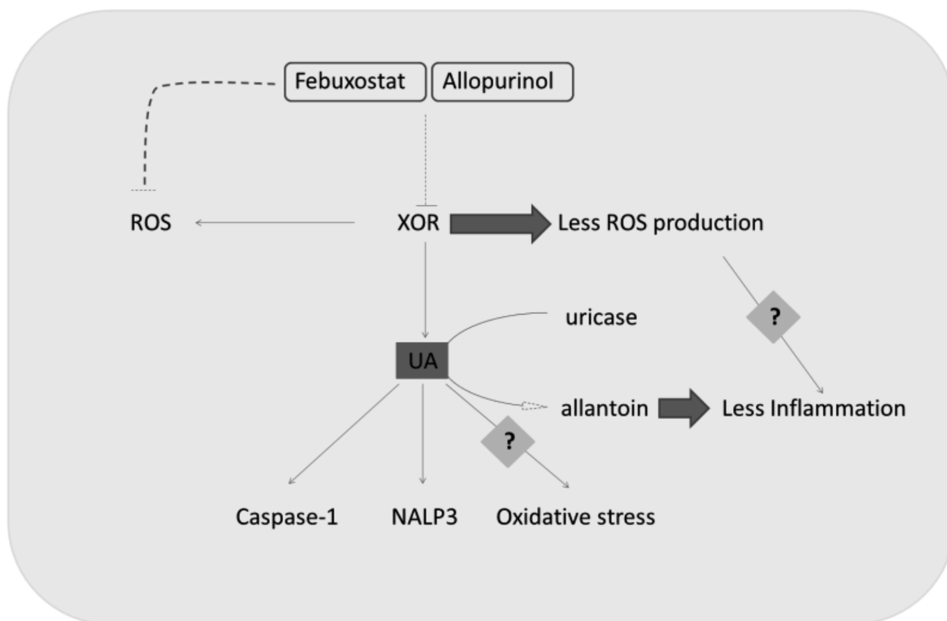

Figure 3. Effect of uric acid (UA) inhibitors in allergic inflammation. Both Febuxostat and Allopurinol inhibit xanthine oxidoreductase (XOR). Febuxostat also acts as a ROS scavenger. Both inhibition of XOR and the ROS scavenging properties could contribute to a decrease in ROS production and inflammation, besides the reduction in UA. Oxidation of UA via uricase, also a common method used to inhibit UA *in vivo* yields the formation of allantoin, a potent anti-inflammatory molecule. The mechanisms by which UA activates the immune system can be via activation of Caspase-1 and inflammasome NALP3 but also unknown mechanisms.

both as an intrinsic factor (protein properties promoting oxidative stress and down-stream effects) and an extrinsic factor (anti-oxidant capacity of exposed subject). Not only some inherent characteristics of proteins, which make them capable of inducing oxidative stress (e.g. protease activity) are involved, but there is also evidence that oxidative stress can enhance the immunogenicity of a protein by tagging it for immune recognition. A study by Allison et al in 2000 [105] showed that glycolaldehyde is a potential mediator of innate immunity by tagging antigens for immune recognition. Glycolaldehyde is an aldehyde formed by ROS attack on carbohydrates, a process called glycoxidation. Glycolaldehyde introduces aldehydes into protein antigens creating reactive carbonyl moieties that make them more immunogenic. Similar to glycolaldehyde, 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are among the most common bioactive aldehyde products of

oxidative stress [106], generated as a result of a free radical chain breakdown of polyunsaturated fatty acid residues in cholesterol esters, phospholipids, and triglycerides in the process called lipid peroxidation [107;108]. 4-HNE and MDA like glycolaldehyde also modulate proteins by creating reactive carbonyl adducts [109;110]. The occurrence and implication of reactive carbonyl modified proteins in a wide range of oxidation-driven pathologies [111] demonstrate the profound impact of these reactive compounds on the immune response and it is tempting to speculate about their role in the sensitization to allergens.

Moghaddam et al [112], demonstrated that reactive carbonyl adduction on antigens can enhance antigen presentation and T cell proliferation with a Th2 bias, in the absence of conventional co-stimulation. Recently we showed that, as soon as 24hr after inhalation of HDM, 4-HNE reactive carbonyls adducts on proteins could be detected in the lungs of two distinct strains of mice. Interestingly, mice displaying higher levels of 4-HNE protein adducts in lung homogenates both at basal levels and after allergen exposure, developed a robust airway allergic inflammation to HDM while another strain, with lower levels of reactive carbonyl adduction and a better anti-oxidant response, was protected against the development of allergic inflammation [38]. Higher levels of 4-HNE protein modification in these mice indicate an excess in the production of this highly reactive radical. It is possible that allergens administered to these mice in such conditions could undergo aldehyde modifications becoming more allergenic. We were not able to analyze whether the administered allergens were chemically modified by this mechanism. In support of this murine study, atopic individuals with higher basal levels of 4-HNE adducts in serum proteins were also more susceptible to allergic sensitization to a neo-allergen [38]. It would be interesting to investigate whether within the host, in conditions of elevated oxidative stress allergens will suffer modification by aldehydes, and become (more) immunogenic and therefore increase the likelihood for allergic sensitization.

The development of strategies and technologies to detect exogenous protein “tagging” by oxidative stress by-products in vivo will shed light on the question why antigens are allergens in some individuals but not in others.

Summary and concluding remarks

Oxidative stress induction either through oxidizing environmental agents and allergens or through deficiencies in key anti-oxidant systems may play an important role in the mechanism of allergic sensitization by affecting immune cell function and phenotype as well as in DC-T cell interaction. In this review we shed some light on the old question why a certain protein can cause allergic reactions in some individuals but not in others. Unraveling the ways by which allergens and pollutants trigger ROS-generating systems resulting in oxidative stress and the effects of ROS and other oxidative stress by-products on the immune system will help us design strategies to prevent development of allergies.

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Chapter 4

IL-33 promotes the induction of immunoglobulin production after inhalation of HDM in mice

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ABSTRACT

Background: The initial immune response to house dust mite (HDM) is orchestrated by an interplay between epithelial cells (ECs) and dendritic cells (DCs). Innate cytokines released by HDM exposed ECs, activate airway DCs and effector inflammatory cells, which together induce a HDM-specific Th2 cell response. Here we investigate the respective roles of DCs and IL-33 in sensitization to HDM.

Method: Balb/c mice were exposed via the airways to different HDM extracts, differing in at least endotoxin levels (Lotox (LT) and HiTox (HT)). Alternatively, HDM pulsed DCs in the presence or absence of additional LT-HDM, or administration of LT-HDM plus recombinant IL-33 was intratracheally (i.t.) administered to induce allergic airway inflammation. Eosinophil recruitment, cytokine production, serum immunoglobulins and airway histology were analyzed.

Results: Direct exposure of airways with HT-HDM induced an eosinophilic airway inflammation, Th2 cytokine production and an increase in total IgE and HDM IgG_1 while LT-HDM was not able to do so. In contrast, i.t. instillation of LT-HDM pulsed DCs induced a similar airway inflammation, mucus production and cytokine production but IgE or HDM IgG_1 was not induced. Administration of HDM pulsed DCs together with LT-HDM, to supply B cells with unprocessed antigen, was not sufficient to induce antibody production. Simultaneous administration of recombinant IL-33 with LT-HDM induced an antibody response, besides a cellular immune response.

Conclusion: These results demonstrate that HDM pulsed DCs were able to drive a Th2 response but that IL-33 was needed to induce a humoral immune response to a single inhalational challenge to HDM.

INTRODUCTION

Allergic asthma is a Th2-driven disease, which causes bronchoconstriction, airway hyper-responsiveness, mucus cell hyperplasia, elevated IgE production and eosinophilic airway inflammation. Exposure to allergens derived from house dust mite (HDM) is a risk factor for this severe lung disease. It is estimated that 10-20% of the population is allergic to HDM[1]. The underlying immunological mechanisms which are involved in the development of house dust mite induced airway inflammation have been the focus of many recent studies.

The interplay between dendritic cells (DCs) and epithelial cells (ECs) has been shown to be crucial for the initial immune response to HDM[2]. TLR4 signaling in ECs by lipopolysaccharide (LPS) originating from the gastro-intestinal tract of the house dust mite, has been demonstrated to contribute to Th2-type adaptive immune responses via activation of mucosal DCs[2-4]. Activation of ECs by exposure to HDM results in the release of innate cytokines like IL-33, TSLP, GM-CSF and IL-25[2]. Recent evidence demonstrated that IL-33 is crucial for development of allergic airway inflammation to HDM[5-10]. IL-33 has a superior ability to induce maturation and up-regulation of the Th2 skewing co-stimulatory molecule OX40L on DCs[8]. IL-33-activated DCs were shown to be more potent in inducing eosinophilic airway inflammation and mucus production in an OVA DC-driven asthma model[11]. However, IL-33 is a pluripotent cytokine which activates other inflammatory cells like eosinophils, mast cells, basophils, Th2 cells, and expands type 2 innate lymphoid cells (ILC2s) which are able to secrete IL-5 and IL-13 via ST2 receptor signaling[12].

Interestingly, administration of IL-33 is sufficient to induce eosinophil recruitment, mucus production and airway hyper-reactivity in an immune system without T and B cells[9]. Genome wide studies investigating asthma-associated alleles identified only 8 alleles, among which the IL-33 receptor, IL-33 and IL-13[13;14]. Increased levels of IL-33 are found in the plasma of asthma patients and in the lungs of allergic mice[15;16]. The respective roles of DCs and IL-33 in the immune response to inhaled HDM still need to be further elucidated.

Here we compared a DC-driven immune response to HDM with an IL-33-driven immune response in a mouse model for HDM-induced allergic airway inflammation. We demonstrate that a DC-driven immune response induced a clear Th2-mediated airway inflammation, but

that IL-33 was needed to induce a humoral immune response after a single HDM inhalational exposure.

METHODS

Mice

Female Balb/c mice (6-8 weeks, Harlan, Horst, The Netherlands) were housed under specific pathogen-free conditions at the animal care facility of the AMC. All experiments were approved by the AMC Animal Ethics Committee, The Netherlands.

HDM extracts

A spent mite medium extract (Dermatophagoides pteronyssinus, LoTox^{TM} LTN-DPE-4, lotnr 33019, INDOOR Biotechnologies, Cardiff, UK) and a crushed whole body house dust mite extract (Dermatophagoides pteronyssinus, XPB82D3A2.5, lotnr 136401, Greer Laboratories; Lenoir, NC, USA) was used. The first extract will be referred to as LoTox HDM (LT-HDM) with an endotoxin level of ≤0.061 EU/µg Der p 1 and the second will be referred to as HiTox HDM (HT-HDM) containing 1.2 EU endotoxins/µg Der p 1, according to the manufacturer's datasheet. In all experiments, 1 µg Der p 1, which is equivalent to 6.1 µg protein of LT-HDM and 31 µg protein of HT-HDM was used.

Generation, HDM pulsing and phenotyping of bone marrow-derived DCs

Bone marrow-derived DCs were obtained as described earlier[17]. On day 9 of culture, cells were pulsed overnight with 31 µg protein/ml HT-HDM or 6.1 µg protein/ml LT-HDM (equivalent to 1 µg Der p 1/ml for both extracts). At day 10 of DC culture, cells were stained with MHCII-FITC, CD11c-APC, in combination with PE-labeled CD80, CD86, CD40, OX40L or Rat IgG_{2a} and Ham IgG (eBioscience Inc, San Diego, CA, USA). To prevent non-specific antibody binding, anti-FcRγII/III antibody (2.4G2, provided by Louis Boon, Bioceros, Utrecht, The Netherlands) was added to the monoclonal antibody mixture. Propidium iodide (Sigma Aldrich Corp. St. Louis, MO, USA) was used to distinguish between live and dead. Airway DCs were phenotyped as described before[18]. Expression of co-stimulatory molecules were analysed using FlowJo software (Tree Star Inc, Ashland, OR). IL-6 and IL-10 production by HDM pulsed DCs was determined in supernatants obtained at day 10 (stored at -20°C) by ELISA (Ready-SET-Go ELISA KIT, eBioscience, San Diego, CA) according to manufacturer's instructions.

Mouse models of HDM induced eosinophilic airway inflammation

Mice were intra-tracheally (i.t.) exposed at day 0 to 31 µg (protein) HT-HDM or 6.1 µg (protein) LT-HDM (equivalent to 1 µg Der p 1 in both extracts) and challenged intranasally (i.n.) at day 10 with the same concentration HDM extract. As a control, mice received i.t. PBS at day 0 and 31 µg HT-HDM or PBS at day 10 (Fig 1A). Alternatively, mice were instilled i.t. with $1x10^6$ *in vitro* HT-HDM (Fig 1B) or LT-HDM pulsed DCs (Fig 1C) or as a control unpulsed DCs at day 0. One group received additionally to LT-HDM pulsed DCs, 6.1 µg LT-HDM i.t. (Fig 1C). To investigate the effect of IL-33, mice received i.t. either (i) LT-HDM pulsed DCs + LT-HDM, (ii) LT-HDM pulsed DCs + LT-HDM + 0.5 µg IL-33 (eBioscience, San Diego, CA), (iii) LT-HDM + 0.5 µg IL-33 or (iv) PBS (Fig 1D). At day 10, all mice were challenged i.n. with 31 µg HT-HDM or 6.1 µg LT-HDM, accordingly to the extract used for sensitization. At day 13, mice were killed.

Collection and analysis of cells and tissues

Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid inflammatory cells were obtained by airway lumen lavage with 3x1 ml PBS supplemented with 0.1 mM EDTA. Cell differentiation was made by flow cytometric analysis as described earlier[19].

Ex vivo restimulation of lung-draining lymph node cells

Lung draining lymph node cell suspensions were plated in 96 well round bottom plates at a density of $2x10^5$ cells per well and were restimulated for 4 days with 31 µg/ml HT-HDM or 6.1 µg/ml LT-HDM (equivalent to 1 µg Der p 1/ml). Supernatants were analysed for IL-4, IL-5, IL-13, IFNγ and IL-17A production by ELISA (Ready-SET-Go ELISA KIT, eBioscience, San Diego, CA) according to manufacturer’s instructions.

Serum immunoglobulins

Serum was analysed for the level of total and HDM specific IgE and IgG_1 by ELISA (Opteia; BD, San Diego, CA, USA). A standard curve of murine IgE or IgG_1 was used as a quantitative reference.

Airway histology

Frozen sections (5 µm) were stained with periodic acid Schiff's reagent (Sigma-Aldrich). Degree of inflammation and mucus producing goblet cells were semi-quantified as described earlier [18].

Real Time PCR on lung tissue

Mice were exposed i.t. to HT-HDM, LT-HDM, HT-HDM pulsed DCs or PBS and 24 hours later lung tissue was macerated in Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) to isolate total RNA. After the synthesis of cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Netherlands), real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 200 nM of each primer:
IL-33 FW: 5'CTGCTTGCTTTCCTTATGCACACGT3';
IL-33 RV: 5'AAGAACCAAAGAAGGGGCAGAAGCT3';
HPRT FW: 5'AGTCCTGTGGCCATCTGCCTAGT3';
HPRT RV: 5' CTGGGGACGCAGCAACTGACA3').
All reactions were performed in optical 96-well reaction plates using StepOne Plus RealTime PCR System (Applied Biosystems, US). IL-33 mRNA concentrations from each sample were calculated based on the standard curve as previously described and were normalized to the concentration of the housekeeping gene HPRT[20].

Protein detection in lung tissue

Mice were exposed i.t. to HT-HDM, LT-HDM (equivalent to 1 µg Der p 1 of both extracts), $1x10^6$ HT-HDM pulsed DCs or PBS and 24 hours later lungs were harvested and lung homogenate was lysed by adding 1 ml Greenberger's buffer (300 mM NaCl, 15 mM Tris HCl, 2 mM MgCl2, 2 mM, Triton (X-100), pepstatin A, leupeptin, aprotinin (all 20 ng/ml), pH 7.4) after 30 min incubating on ice. Then, the lysate was centrifuged at 500xg for 10 min and the supernatant was stored at −20°C until further use. The concentration (mg/ml) of total lung protein was measured by using BCA Protein Assay Kit according to manufacturer's

instruction (Pierce-Thermo Scientific, Rockford, USA). IL-33 protein concentration (μg/mg total protein) was measured by ELISA (Ready-SET-Go ELISA KIT, eBioscience, San Diego, CA).

Statistical analysis

For statistical analysis, the Mann-Whitney U-test was performed using GraphPad Prism 5 software. All experiments were performed at least 2 times with 4–6 animals per group. Differences were considered to be significant at a P value of <0.05.

RESULTS

Differential capacity of HDM extracts to induce an immune response after inhalational challenge

The immune response of Balb/c mice upon inhalation of two different HDM extracts was compared. A crushed whole body extract (HT-HDM) and a spent mite medium extract (LT-HDM) were used at an equal concentration of Der p 1 (1 µg) but HT-HDM contained a 20-fold higher endotoxin level. As shown by us and others before [21], mice exposed at day 0 (i.t.) and 10 (i.n.) to HT-HDM (HT/HT) developed eosinophilic airway inflammation, Th2 cytokine production, mucus production and elevated levels of HDM specific IgG_1, total IgE and HDM specific IgE production, although the last did not reach significance (Fig 2). In contrast, exposure of the airways to LT-HDM (LT/LT) induced a milder inflammation (Fig 2) without visible peribronchial inflammatory infiltrates, and no detectable IL-4 or antibody production. IL-5 and IL-13 production, eosinophil recruitment and mucus production were detectable, but significantly less compared with HT-HDM exposure. A single airway exposure (i.n.) to HT-HDM at day 10 (PBS/HT) induced a limited recruitment of eosinophils to the BAL compartment, induced IL-4, IL-5 and IL-13 production and increased IFNγ production. PBS/HT did not result in inflammatory infiltrates, nor in mucus and antibody production (Fig 2).

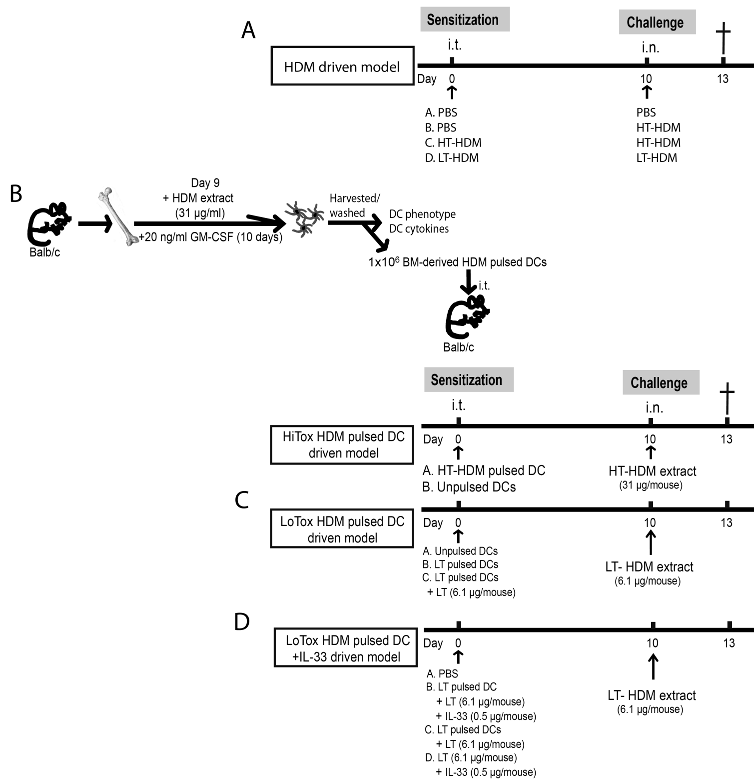


Figure 1: Study designs to investigate the immune response to house dust mite (HDM) after A) inhalation of HT-HDM (crushed whole body extract) or LT-HDM (spent mite medium extract), B) after adoptive transfer into the airways of HT-HDM pulsed BMDCs, C) after adoptive transfer of LT-HDM pulsed BMDCs with or without additional LT-HDM extract and D) adoptive transfer of LT-HDM pulsed BMDCs with additional LT-HDM extract with or without IL-33 in comparison with inhalational exposure to LT-HDM and IL-33 or with PBS as a control. In all models, mice were challenged with the corresponding HDM extract at day 10 and killed at day 13.

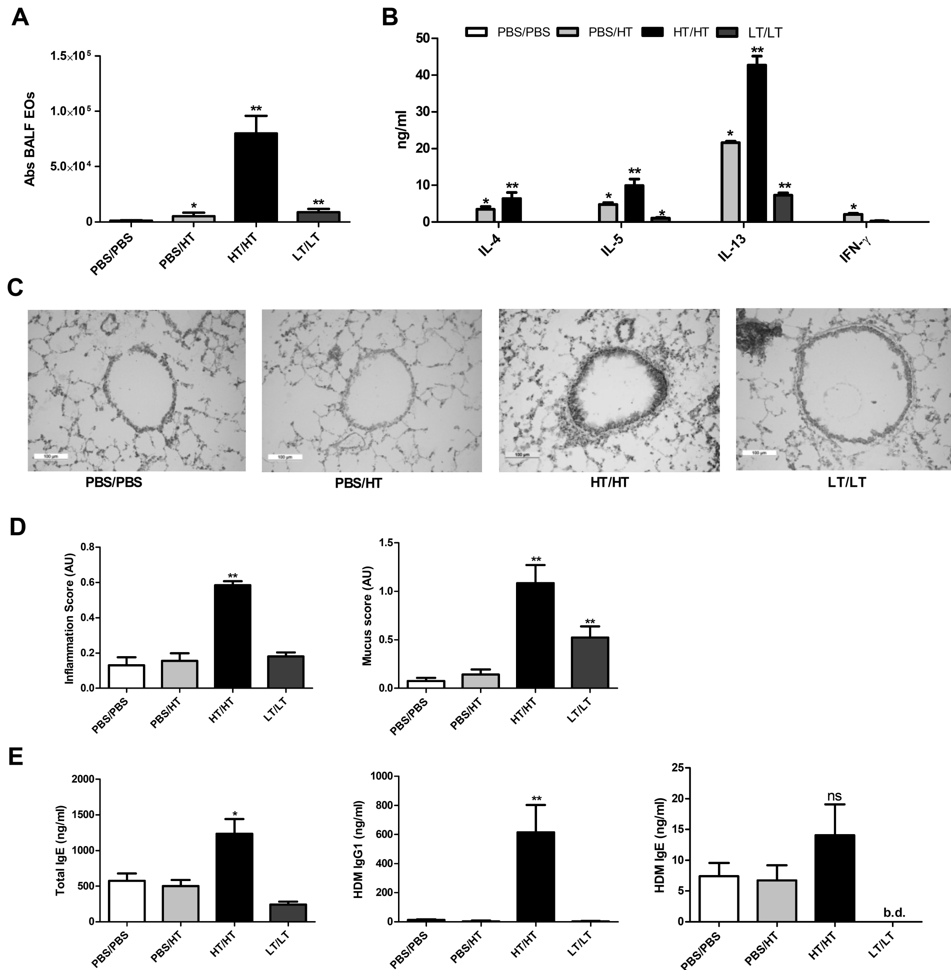


Figure 2: Mice were exposed via the airways to HT-HDM (HT/HT), LT-HDM (LT/LT) or as a control to PBS (PBS/PBS) at day 0 and 10 or as an additional control to a single intranasal exposure to HT-HDM (PBS/HT). At day 13 the recruitment of eosinophils to the BALF compartment was analysed (A), production of Th2 cytokines IL-4, IL-5, IL-13 and IFNg was determined in supernatants of *ex vivo* restimulated lung-draining lymph node cells with HDM (B). Lung slides were stained with PAS to determine peribronchial inflammatory infiltrates and mucus production (C). Peribronchial infiltrates (left graph) and mucus production (right graph) were quantified (D). Total IgE, HDM IgG_1 and HDM IgE were determined in serum by ELISA (E). Data are presented as means ± SEM. B.d.: below detection limit, *P≤0.05, **P≤0.01 vs PBS/PBS.

Different capacity of HDM extracts to activate BMDCs

To determine the role of DCs in the immune response to inhaled HDM, *in vitro* HDM pulsed bone marrow-derived DCs (BMDCs) were administered i.t. to mice. First, we determined expression of co-stimulatory molecules by BMDCs after pulsing with HT-HDM or LT-HDM. The expression of the co-stimulatory molecules CD80, CD86, CD40 and to a lesser extent OX40L was increased in both LT-HDM pulsed and HT-HDM pulsed BMDCs compared to unpulsed BMDCs. The induction by LT-HDM was however less pronounced than seen in HT-HDM-pulsed DCs (Fig 3A). In concordance, LT-HDM pulsed DCs produced 13 fold less IL-6 and 30 fold less IL-10 compared with HT-HDM DCs (Fig 3B).

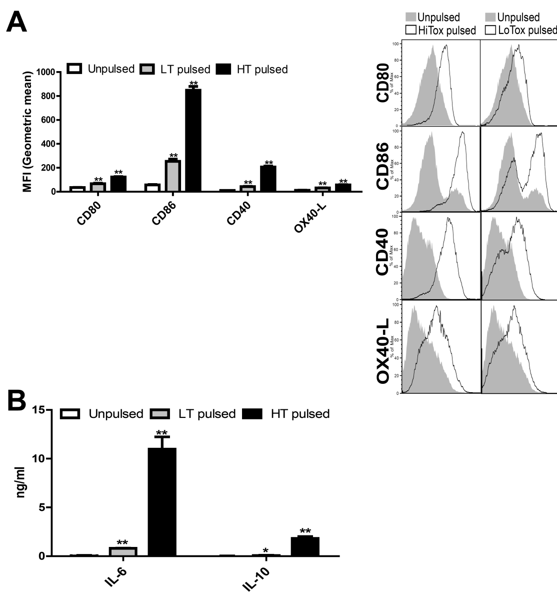


Figure 3: Bone marrow derived DCs were pulsed overnight at day 9 with two different HDM extracts, HT-HDM and LT-HDM, and were analysed on day 10 for the expression of costimulatory molecules CD80, CD86, CD40 and OX40L (A). The concentration IL-6 and IL-10 was determined in the supernatant by ELISA (B). Data are presented as means ± SEM. *P≤0.05, **P≤0.01 vs unpulsed DCs and LT-HDM pulsed DCs.

Exposure of HDM pulsed DCs induced a cellular but not a humoral immune response to HDM

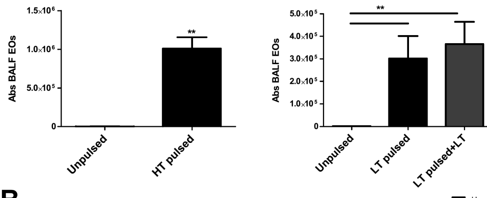
Next, we administered HT-HDM-pulsed BMDC, LT-HDM-pulsed BMDCs or, as a control, unpulsed DCs to the airways of naïve Balb/c mice to induce an immune response to HDM. After 10 days, mice were challenged with a single i.n. exposure with the corresponding HT-HDM or LT-HDM extract.

As shown before by Zhang et al. and Plantinga et al.[22;23], i.t. administration of HT-HDM-pulsed BMDCs followed by a single i.n. challenge with HT-HDM recruited more eosinophils to the bronchoalveolar compartment compared to mice instilled with unpulsed DCs (Fig 4A, left panel). In concordance, IL-4, IL-5, IL-13 and IL-17A production in lung-draining lymph node cells was increased (Fig 4B, left panels) and airway histology analysis showed an increase in mucus production and peribronchial inflammatory infiltrates (Fig 4C and D [upper panels]).

Although i.t. administered DCs pulsed with LT-HDM were less mature and produced less inflammatory cytokines (Fig 3), a clear eosinophilic recruitment, mucus production and IL-4, IL-5, IL-13 and IL-17A production was observed after a single i.n. challenge with LT-HDM (Fig 4 A-D).

Although exposure of LT-HDM DCs or HT-HDM DCs both induced a strong IL-4 production, the induction of total IgE, HDM specific IgE (data not shown) or HDM specific IgG was completely absent (Fig 4E), in contrast to mice sensitized through direct airway exposure to HT-HDM extract (Fig 2E). To investigate whether the absence of soluble unprocessed HDM, needed by B-cells to be activated towards antibody production, could explain the lack of an antibody response, we co-administered LT-HDM with LT-HDM-pulsed DCs. Exposure to unprocessed soluble LT-HDM did, however, not result in induction of HDM-specific IgG_1, increase of total IgE (Fig 4E) or induction of HDM specific IgE (data not shown). The immunological parameters that were induced by LT-HDM-pulsed DCs such as eosinophils and Th2 cytokines were not affected by the addition of soluble LT-HDM (Fig 4A-D).

A



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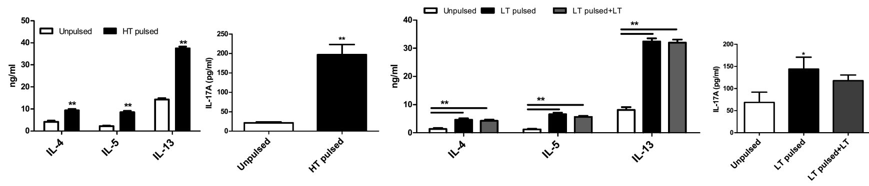

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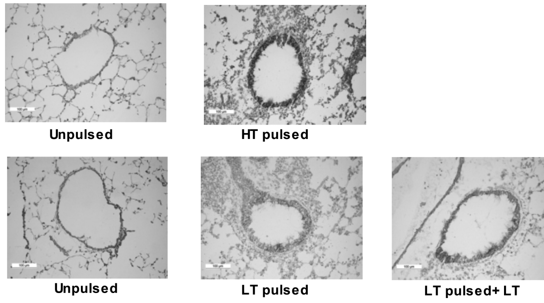

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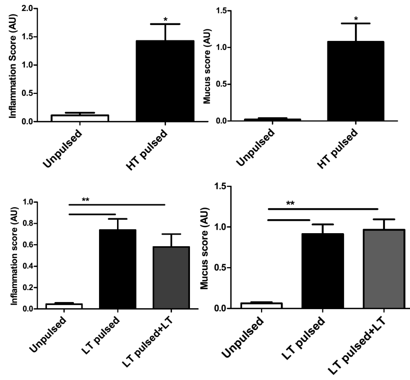

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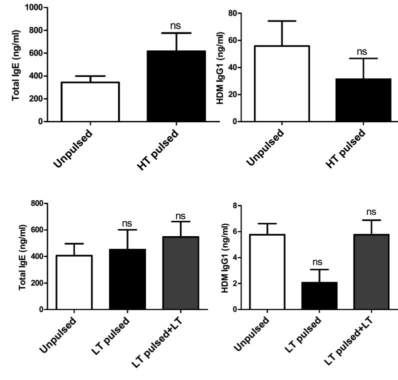


Figure 4: Mice were intratracheally instilled with HT-HDM pulsed or LT-HDM pulsed BMDCs. To investigate their potential to induce an immune response to inhaled HDM, mice were challenged with, respectively, HT-HDM or LT-HDM at day 10. The need for free HDM for immunoglobulin induction was investigated by the simultaneous administration of LT-HDM and LT-HDM pulsed DCs at day 0. Eosinophil recruitment to the airways was examined at day 13 (A). Production of Th2 cytokines IL-4, IL-5, IL-13 and IL-17A was determined in supernatants of *ex vivo* restimulated lung-draining lymph node cells with HDM (B). Lung slides were stained with PAS to determine peribronchial inflammatory infiltrates and mucus production (C). Peribronchial infiltrates (left graph) and mucus production were quantified (right graph) (D). Total IgE and HDM IgG_1 were determined in serum by ELISA (E). Data are presented as means ± SEM. $^*P \leq 0.05$, $^{**}P \leq 0.01$ *vs* unpulsed DCs.

IL-33 promotes a humoral immune response to HDM

Activation of airway epithelium by HT-HDM has been reported to induce IL-33(3), a central innate cytokine for the development of HDM induced inflammation(23). As airway epithelium is not directly exposed to HDM by instillation of HDM pulsed DCs, we compared IL-33 release upon exposure to HT-HDM to HT-HDM-pulsed DCs, to LT-HDM or to PBS. Only exposure of airway epithelial cells to soluble HT-HDM enhanced IL-33 mRNA expression and IL-33 protein production in lung tissue. Neither HT-HDM-pulsed DCs nor LT-HDM did affect IL-33 expression (Fig 5A).

To investigate whether the lack of an IL-33 response could explain the absence of an antibody response, we investigated whether simultaneous administration of IL-33 together with soluble LT-HDM (LT/IL-33) or with LT-HDM-pulsed DCs plus soluble LT-HDM (LTDC/LT/IL-33) would result in the induction of an antibody response. Exogenously administered IL-33 together with LT-HDM pulsed DCs (LTDC/LT/IL-33) induced HDM IgG_1 and total IgE (but not HDM IgE, data not shown), which was accompanied by an enhanced IL-4 production, increased recruitment of eosinophils, more severe peribronchial infiltration and mucus production, in comparison with LTDC/LT sensitized mice (Fig 5B-F). Unexpectedly, LTDC/LT sensitized mice had an increased total IgE compared to PBS control mice (Fig 5F, left panel), but not in comparison to mice which received unpulsed DCs (Fig 4E, lower left panel). This indicates that the adaptive transfer of DCs, independent of pulsing with HDM, was capable of inducing total IgE. However, HDM specific IgG_1 was not induced in LTDC/LT sensitized mice compared with PBS control mice. Remarkably, simultaneous administration of IL-33 and LT-HDM, without administering LT-HDM-pulsed DCs, was as efficient in inducing HDM IgG_1 and total IgE, although IL-4, IL-5, IL-13 production and airway inflammation were lower than induced by a LTDC/LT/IL-33 sensitization (Fig 5C-E).

Finally, we investigated whether the IL-33-facilitated immunoglobulin responses upon exposure to LT-HDM would be mediated via maturation of airway DCs. Mice were exposed i.t. to HT-HDM, LT-HDM, LT-HDM/IL-33 or as a control to PBS. Twenty-four hours later, airway $MHCII^{hi}CD11^{hi}CD3^{-}CD19^{-}$DCs were analyzed for CD80, CD86, CD40 and OX40L. In airways exposed to HT-HDM, all measured co-stimulatory molecules, except OX40L, were upregulated on airway DCs, while DCs in LT-HDM and in LT-HDM/IL-33 exposed lungs were

not different from PBS-exposed airways (Fig 6A). However, the percentage of DCs of total lung cells was increased after exposure to LT-HDM/IL-33 in a similar degree as seen after HT-HDM exposure but not in LT-HDM or PBS exposed lungs (Fig 6B).

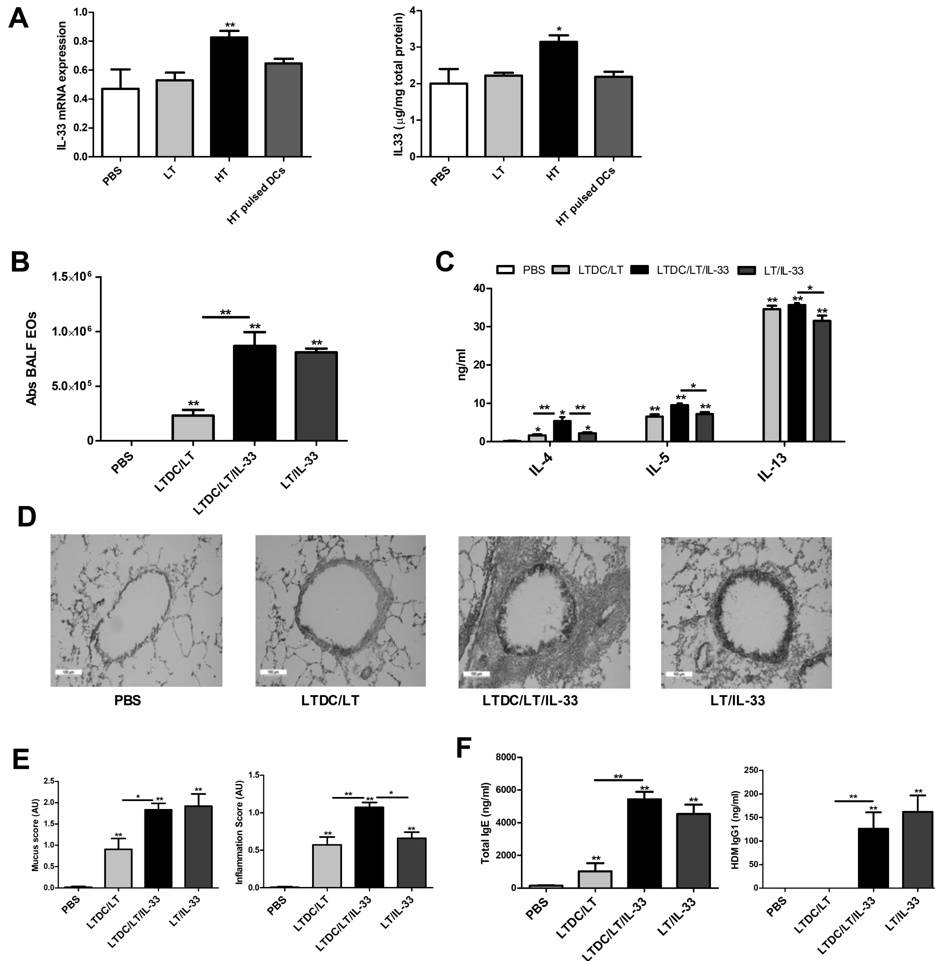
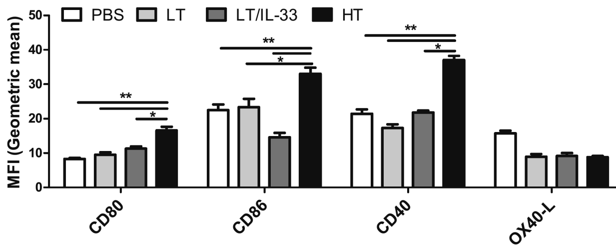


Figure 5: Mice were exposed intratracheally to PBS, LT-HDM, HT-HDM or HT-HDM pulsed DCs and after 24 hr., IL-33 mRNA expression and protein concentration were analysed in the lung tissue by real-time PCR and ELISA, respectively (A). Mice were exposed at day 0 by (1) PBS, (2) LT-HDM pulsed DCs with additional LT-HDM (LTDC/LT), (3) LT-HDM pulsed DCs with additional LT-HDM and IL-33 (LTDC/LT/IL-33) or (4) LT-HDM and IL-33 (LT/IL33). All mice were challenged i.n. at day 10 with LT-HDM. Eosinophil recruitment to the airways was examined at day 13 (B). Production of Th2 cytokines IL-4, IL-5 and IL-13 was determined in supernatants of *ex vivo* restimulated lung-draining lymph node cells with HDM (C). Lung slides were stained with PAS to determine mucus production and peribronchial inflammatory infiltrates (D). Mucus production (left graph) and peribronchial infiltrates (right

graph) were quantified (E). Total IgE and HDM IgG_1 were determined in serum by ELISA (F). Data are presented as means ± SEM. *$P \leq 0.05$, **$P \leq 0.01$ vs PBS.

A



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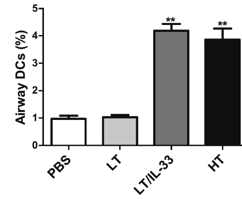

Figure 6: Mice were exposed intratracheally to PBS, LT-HDM (LT), LT-HDM with IL-33 (LT/IL-33) or HT-HDM (HT) and 24 hours later, airway DCs were analysed for expression of costimulatory molecules CD80, CD86, CD40 and OX40L (A) and the percentage of airway DCs of total lung cells was determined (B). Data are presented as means ± SEM. **P≤0.01 vs PBS.

DISCUSSION

In the present study we report an important role for IL-33 in the induction of immunoglobulin responses upon inhalation of HDM. An IL-33 driven, but not a HDM DC driven, immune response resulted in increased levels of IgE and HDM specific IgG_1.

Recently, much progress has been made in understanding the underlying mechanisms for IL-33 in the development of an allergic response[12]. Several reports have shown a role for IL-33 via recruitment and activation of DCs[5;8;11]. It was therefore of interest to investigate whether DCs were sufficient to induce a humoral immune response or whether IL-33 was needed.

Our study revealed that HDM pulsed DCs were capable of inducing a Th2 mediated airway inflammation, as seen after direct exposure to HDM, but this was not accompanied by HDM specific IgG_1 and total IgE. Intratracheal administration of HT-HDM-pulsed-DCs bypasses the direct interaction of ECs with HT-HDM. In contrast to direct exposure to HT-HDM, no IL-33 was detected in lung tissue after exposure to HT-HDM pulsed DCs (Fig 5A). HDM pulsed DCs were also not likely to be a source for IL-33 because IL-33 could not be detected in either LT- or HT-HDM pulsed DC culture supernatants (data not shown). We demonstrated that simultaneous exposure to IL-33, one of the innate cytokines secreted by the epithelium after exposure to HT-HDM[2], elevated total IgE and HDM IgG_1 in serum.

Other groups have demonstrated that HDM pulsed bone marrow or lymph node derived DCs are able to induce Th2 immunity and in several studies this was accompanied by a humoral immune response. However, in these studies, mice were exposed to multiple mucosal challenges[6;22-27]. The repeated stimulation of airway epithelium might have induced secretion of inflammatory cytokines, like IL-33, which was sufficient to induce an antibody response.

In contrast to our results, Zhang et al showed an increase of total IgE after HDM pulsed DC sensitization followed by a single HDM challenge but did not investigate the HDM specific IgG_1 levels[22]. Interestingly, we observed that transfer of LT-DCs was able to increase the level of total IgE compared to PBS transferred mice but not in comparison to mice which received unpulsed DCs (fig 4E and 5F). This indicates that “natural” (not HDM related) IgE response was boosted by the DC transfer itself. We speculate that recruitment/activation of

IL-4 producing innate cells, like basophils, by release of chemokines by the transferred DCs might have been involved. However, sensitization with HDM-pulsed DCs did not result in a HDM specific IgG_1 response in comparison to unpulsed DCs or in comparison to PBS control mice.

Absence of an antibody response after DC transfer could not be explained by the lack of free antigen, which is needed to stimulate B-cells via their Ig-receptors. Simultaneous administration of LT-HDM and LT-HDM pulsed DCs did not facilitate a humoral immune response, despite a strong IL-4 production. LT-HDM extract was used because, in contrast to HT-HDM, it does not induce sensitization after exposure and did not enhance IL-33 production in lung (Fig 5A). This mite spent culture extract is likely to differ greatly in its properties from HT-HDM because mainly excretions of the mite, like the allergenic feces, are present instead of crushed whole HDM bodies and has a lower endotoxin content.

Several reports are in support of a direct role of IL-33 in antibody production[5;8;28-31]. These models use a relatively weaker stimulus compared to our direct exposure model in which we challenge mice with 100 µg HDM. These models used either OVA, an innocuous protein without proteolytic activity in contrast to several HDM allergens, or they used a lower dose (1 µg) of HDM or they blocked IL-33 signaling during the challenge phase as well. In our HDM direct exposure model, blockade of IL-33 by local (10 µg i.t.) and systemic (50 µg i.p.) anti-IL-33 treatment at the time of sensitization did not affect the antibody response (Suppl Fig 1). This blocking experiment indicates that at least the antibody response is not exclusively regulated by IL-33. The strength of the stimulus used, 100 µg HDM, might have activated complementary pathways which have compensated for the loss of IL-33 signaling in contrast to the above mentioned reports.

IL-33 has been described to affect the inflammatory milieu in many ways, like recruitment and activation of airway DCs potentiating Th2 help for B cells [8;11]. Simultaneous exposure of IL-33 and LT-HDM induced an increased recruitment of DCs to the airways and enhanced IL-4 production compared to exposure to LT-HDM alone. This might have contributed to the induction of the humoral immune response by IL-33. As an adoptive transfer of HDM pulsed DCs was not sufficient to induce a humoral immune response, despite a strong IL-4 production, IL-33 might have activated an additional pathway. IL-33 has also been described to increase CD40L expression on $CD4^+$ T cells which could have contributed to the induction

of immunoglobulin production by B cells[32]. An alternative pathway for IL-33 enhanced Th2 help for B cells, could be via IL-33 recruited and activated innate cells, like ILC2s, eosinophils and mast cells, which can provide a source of IL-4 and in case of ILC2s can interact with B cells via ICOS-ICOSL expression[8;32-34]. The rapid immune response that we observed after a single exposure to HT-HDM suggests that the epithelial release of several chemo attractants[35] like IL-33, recruited innate inflammatory cells which might have been crucial for the induction of immunoglobulins during the secondary immune response to inhaled HT-HDM. The involvement of innate cells in the immunoglobulin production by B cells will have to be elucidated in future experiments.

In conclusion, our data indicate that a DC mediated Th2 response was not able to induce a humoral immune response after a single HDM exposure but that IL-33 was sufficient.

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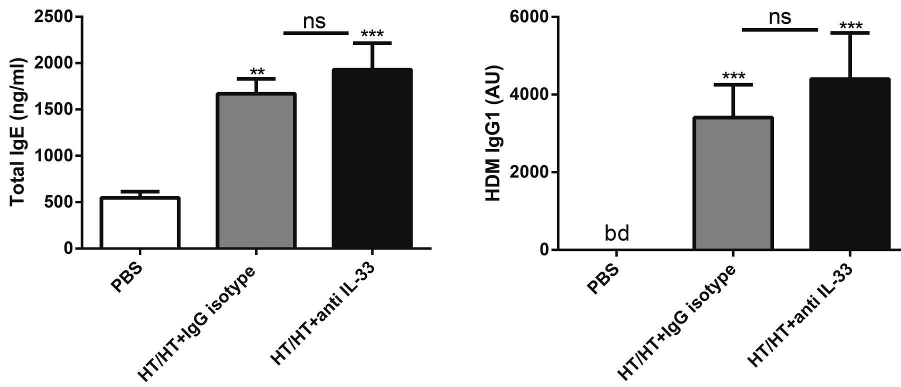

Supplementary figure 1: Mice were treated at day 0, with 50 µg anti-IL-33 (or isotype control) i.p. 30 minutes before i.t. administration of 100 µg HT-HDM and 10 µg anti-IL-33 (or isotype control). Ten days later, mice were rechallenged with 100 µg HT-HDM. Serum levels of total IgE and HDM specific IgG_1 were determined by ELISA at day 13. Data are presented as means ± SEM. **P≤0.01, ***P≤0.001 vs PBS.

Chapter 5

Experimental food allergy to peanut enhances the immune response to house dust mite in the airways of mice

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ABSTRACT

Background: Food allergy has been associated with an increased risk for the development of allergic asthma. Asthma is a risk factor for the development of an anaphylactic response to food allergens. An immunological interplay between sensitization to different allergens in different compartments of the body might be involved.

Objective: To evaluate the immunological interplay between intragastrical peanut (PE) sensitization and respiratory sensitization to house dust mite (HDM) allergens.

Methods: BALB/c mice were intra-gastrically sensitized to peanut or sham-sensitized and challenged systemically to PE. Between sensitization and challenge, mice were intranasally exposed to HDM extract or PBS, as a control. The response to HDM (eosinophil recruitment, cytokine response, HDM specific immunoglobulins, and airway hyper-reactivity) and to PE (cytokine response, mast cells in gut, mMCP-1 in serum, body temperature) was assessed.

Results: A preceding PE sensitization increased HDM induced production of IL-4, IL-5, IL-13 and IFNγ in lung draining lymph nodes and total IgE levels in HDM sensitized mice. However, recruitment of inflammatory cells to the airways or airway hyper-reactivity was not aggravated in PE/HDM double-sensitized mice. Alternatively, HDM induced airway inflammation did not significantly affect the immune response or the anaphylactic response to a systemic challenge with peanut.

Conclusion and Clinical Relevance: Our data show that a preceding peanut sensitization boosted the IgE and HDM-specific Th2 response in the airways in mice. It contributes to the understanding of the underlying immunological mechanism of polysensitization which often occurs in allergic individuals over time.

INTRODUCTION

Atopic disorders, including food allergy and allergic asthma, have increased tremendously in the last four decades. Studies in experimental animal models have brought significant progress in the elucidation of the immunological mechanisms of the process of sensitization and allergy. Where animal models have been mainly used to study a single sensitization and associated clinical phenotype, it is evident that humans frequently suffer from multiple atopic disorders, often affecting different organs. A possible interplay between different primary sensitizations with their own affected organs has been studied very scarcely.

Affected organs can range from the skin (eczema, urticaria), the upper (runny and/or stuffed nose, sneezing) and lower (wheezing, shortness of breath) airways, the eyes (conjunctivitis, itching), and the gastro-intestinal tract (vomiting, diarrhoea, cramps, angioedema), to the cardiovascular and neurological systems (dizziness, drop in blood pressure, loss of consciousness). The organ of entry does not necessarily overlap with the organ that is affected (e.g. food can cause symptoms of skin), and multiple organs can be affected at the same time (e.g. food can induce allergic symptoms of skin, gastro-intestinal tract and cardiovascular system). Allergic individuals often display symptoms in multiple organs and are sensitive to multiple allergen sources. It is not uncommon that allergic patients have rhino-conjunctivitis and/or asthma caused by house dust mite but also food allergy to e.g. peanut.

The underlying immunological mechanism for polysensitization remains unclear. A phenomenon usually referred to as the “atopic march” has been described, being the progression from early onset atopic dermatitis with a high comorbidity for food allergy towards allergies of the airways like asthma and rhino-conjunctivitis later in life. Several epidemiological reports suggest an association between sensitization to food allergens and an increased risk for the development of asthma. A longitudinal study in cow’s milk sensitized children at the age of 7 months, showed an increased airway inflammation and higher bronchial responsiveness to histamine at 8 years of age compared to control children without cow’s milk sensitization [1]. Patelis et al. [2] showed in a population-based study that simultaneous sensitization to food allergens and respiratory allergens strongly increased the risk for development of asthma compared to sensitization to respiratory allergens

exclusively (OR=18.3 vs OR=5.6). Tariq et al. [3] demonstrated that egg allergy in infancy predicts respiratory allergic disease at 4 years of age. The prevalence of food IgE sensitization is up to 45% in asthmatics [4;5] suggesting an immunological interplay. It is well established that asthma accompanying food allergy is a risk factor for the occurrence of anaphylaxis [6;7], but the mechanism by which asthma contributes to this systemic response has not been elucidated.

In the present study we aimed to develop a murine model to study the possible interplay between a non-cross-reactive food and respiratory allergy. To that end, mice were intra-gastrically sensitized with peanut and after one month, systemically challenged i.p. with peanut. Between sensitization and challenge, mice were exposed to house dust mite (HDM) via the airways or to PBS. This order was chosen because food allergy often precedes the development of asthma in humans. Cellular and humoral immune responses to peanut and HDM were examined as well as the subsequent clinical manifestations.

METHODS

Animals. Female Balb/c mice (Harlan, Horst, The Netherlands), were housed under specific pathogen-free conditions at the animal facility of the AMC. All experiments were approved by the animal ethics committee of the AMC, The Netherlands.

Peanut extract (PE) preparation. Whole roasted peanuts (O'Lacy International B.V., The Netherlands) were ground and protein was extracted by mixing 50 g of peanuts with 500 mL 20mM Tris buffer. After overnight incubation under agitation at 4°C, the insoluble particulate matter was removed by centrifugation (10.000xg for 20 minutes), and the supernatant was centrifuged again (2.460xg for 30 minutes) to remove residual particles. The protein concentration (16,6 mg/ml) was determined using a Pierce BCA protein Assay kit, using bovine serum albumin (BSA) as a standard following the manufacturer's instruction (Pierce, Thermo Scientific). The extract contained 0.32 mg Ara h 1/mg total protein and 32.6 µg Ara h 2/mg total protein, as determined by ELISA (Ara h 1 and Ara h 2 ELISA kit, Indoor Biotechnologies, Cardiff, UK).

Sensitization protocol. To co-sensitize mice to peanut and house dust mite (PE/HDM mice), BALB/c mice were first sensitized intra-gastrically with 100 µg of PE mixed with 10 µg of Staphylococcal enterotoxin B (SEB), from Staphylococcus aureus (Sigma-Aldrich, Zwijndrecht, The Netherlands) as previously described [8], on days 0, 3, 7, 10, 14, 17 followed by intranasal administration of 100 µg HDM (Greer Laboratories, Lenoir, USA) on days 35, 42, 49, 50 and 51. As controls, one group of mice was sensitized to PE but not to HDM (PE/PBS mice), one group was exposed to HDM and not to PE (PBS/HDM mice), and one group was not sensitized to PE or HDM (PBS/PBS mice). On day 52, all mice were challenged intra-peritoneally with 500 µg of PE and core body temperature was determined. On day 53 airway hyper-reactivity was assessed. For the *ex vivo* restimulation of splenocytes with PE (fig 3b), mice were i.g. sensitized to PE and challenged with PE at day 52 and sacrificed at day 54, as described above.

Core body temperature. The core body temperature was measured with a hand-held device with a mouse rectal probe (Physitemp Instruments Inc, Clifton, NJ, USA) every 10 minutes after intraperitoneal administration of 500 µg PE.

Airway hyperresponsiveness (AHR). AHR to inhaled methacholine was measured by barometric plethysmography in conscious mice (EMKA Technologies, Paris, France). Nonspecific responsiveness was measured by exposing mice to aerosolized PBS to set a baseline value, followed by increasing concentrations of aerosolized methacholine (3.125, 6.25, 12.5, 25 and 50 mg/mL in PBS for 3 min; Sigma-Aldrich) using ultrasonic nebulizers. PenH (enhanced pause) values were measured during 5 min after each methacholine aerosol.

Broncho-alveolar lavage fluid (BALF). Inflammatory cells were obtained from the airway lumen by three subsequent washes with 1 mL PBS containing 0.1mM EDTA after intra-tracheal cannulation. Cell differentiation was done by FACS analysis as described elsewhere [9].

Serum Immunoglobulins and mouse Mast Cell Protease-1. Serum was analysed for the level of total and HDM or peanut specific IgE and IgG_1 by ELISA (IgE: Opteia, BD, San Diego, USA, IgG_1: Ready-Set-Go, Ebioscience via Immunosource, Halle-Zoersel, Belgium). In short, Nunc maxisorp plates were coated with 50 µg PE or 100 µg HDM (equivalent to 1 µg/mL Der p 1) mL^{-1} at 4°C overnight. After blocking with 10% fetal calf serum (FCS) in PBS, serum samples were incubated for two hours. Total and allergen specific immunoglobulins were determined following the manufacturer's instructions. We used a standard curve of murine IgE and IgG_1 as reference. MMCP-1 in serum was determined by ELISA (MMCP-1: Ready-SET-Go kit, eBioscience Inc., Immunosource, Halle-Zoersel, Belgium).

Ex vivo re-stimulation of spleen and lymph node cells. Spleens and lung draining lymph node cell suspensions were plated in 96 well round bottom plates at a density of $2x10^5$ cells per well and were re-stimulated for 4 days with 100 µg/mL HDM or 200 µg/mL peanut extract. Supernatants were analysed for IL-4, IL-5, IL-13 and IFNγ production by ELISA (Ready-set-go!, eBioscience Inc via Immunosource, Halle-Zoersel, Belgium).

Histology. Frozen lung sections (6 µm) were stained with Periodic Acid Schiff's reagent (Sigma-Aldrich). Degree of inflammation and mucus producing goblet cells were semi-quantified as described before [10]. Both small and large intestines were flushed with PBS before they were inflated with OCT Tissue Tek (Sakura Finetek Europe, Zoeterwoude, The

Netherlands) before they were snap frozen in liquid nitrogen. Frozen intestinal sections (6 µm) were stained with Toluidine Blue O (Sigma-Aldrich). Number of mast cells (Toluidine Blue stained cells) was determined per cm^2 of tissue using light microscope and software Leica Application Suite (LAS), V3, Heerbrugg, Switzerland.

Statistical analysis. For statistical analysis, Mann-Whitney U test was performed and represented as +/- SEM. All experiments were performed 2-4 times with 5 animals per group. Results from all experiments were pooled for statistical analysis. Differences were considered to be significant at $P < 0.05$.

RESULTS

Prior sensitization to peanut enhanced subsequent Th2 response to HDM

To investigate whether sensitization to peanut via the gastrointestinal mucosa, would prime sensitization to HDM via the lung mucosa or vice versa, mice were exposed to both allergens as shown in fig. 1a. Two validated models for respiratory allergy to HDM and gastrointestinal allergy to peanut were applied in the same mouse [8, 11]. Mice sensitized to both HDM and PE had a higher level of total IgE compared with single sensitized mice. However, no significant increase was observed in allergen specific immunoglobulins in HDM/PE sensitized mice compared with mono-sensitized mice, except for a non-significant increase in PE specific IgE response (fig. 1b and c). To determine whether the HDM specific T cell response was altered by the preceding PE sensitization, lung-draining mediastinal lymph node (LLN) cells were re-stimulated *in vitro* with HDM. Production of IL-4, IL-5, IL-13 and IFNγ by LLN cells of mice sensitized to peanut (PE/HDM) was significantly increased in comparison with mice without a peanut allergy (PBS/HDM) (fig. 2a).

As the mediastinal lymph nodes drain both the lung and the peritoneum, we restimulated LLN cells *ex vivo* with PE to investigate the T cell response to the systemical challenge with PE. As expected, HDM sensitized mice (PBS/HDM) did not show any cytokine production, while both PE/PBS and PE/HDM mice showed an increased in IL-4, IL-5 IL-13 and IFNγ production compared with PBS/PBS mice (fig. 2b). Double sensitized mice (PE/HDM) showed a similar trend for an increase in the level of cytokines induced by PE, as was seen for HDM, in comparison with single sensitized PE/PBS mice, albeit in much lesser extent and significance was not reached. We hypothesized that the intraperitoneal challenge with PE at day 52 activated circulating PE specific memory T cells in the LLN, after the i.g. priming to PE. To confirm the presence of circulating PE specific T cells, mice were sensitized i.g. to PE or with PBS as a control and challenged with PE i.p. at day 52. Spleen cells were harvested at day 54 (fig 1a) and restimulated with PE. The cytokine production of IL-4, IL-5, IL-13, IFNγ was significantly increased in PE sensitized mice in comparison with PBS mice (fig. 3), indicative of circulating memory T cells after i.g priming and subsequent i.p. challenge.

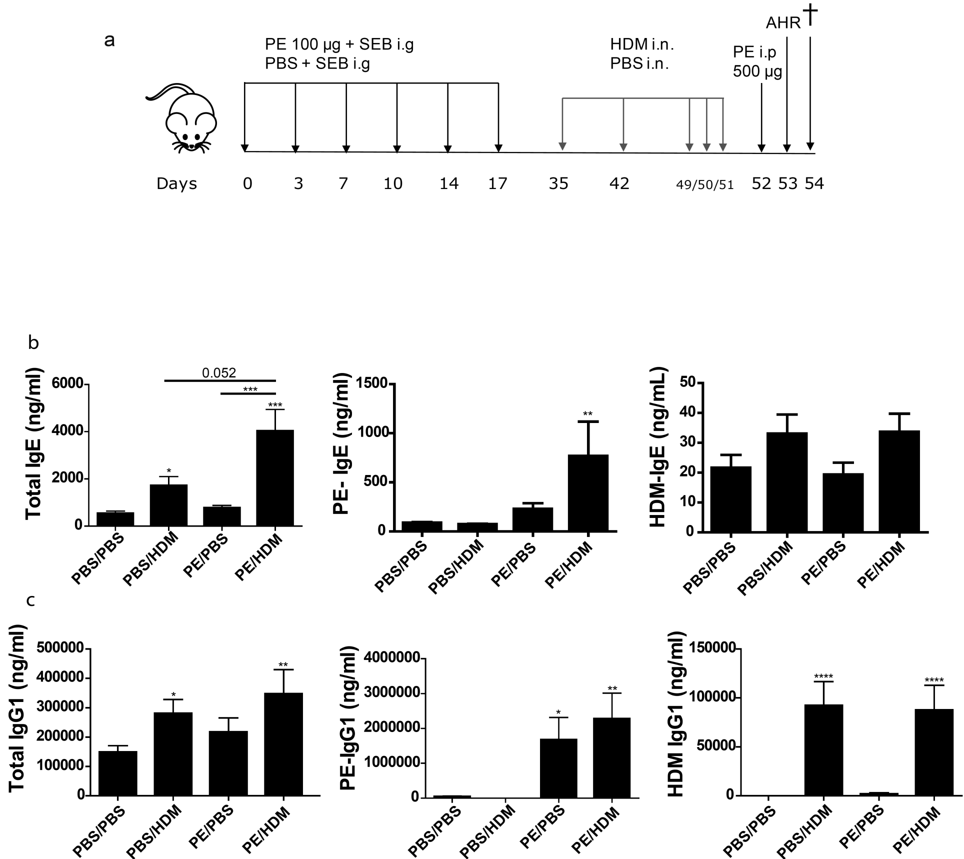

Figure 1. Immunoglobulin levels in serum after peanut and HDM exposure. BALB/c mice were sensitized i.g. with 100 µg/mouse of PE or PBS mixed with 10 µg of Staphylococcal enterotoxin B (SEB). Mice were challenged i.p. with 500 µg of PE at day 52. In between PE sensitization period and PE challenge, the same mice received intranasally, 100 µg/mouse of HDM or PBS (a). Mice were killed at day 54 and total and allergen specific IgE (b) and IgG_1 (c) were determined. Data are presented as means ± SEM, **P<0.05, **P<0.01, ***P<0.001 vs PBS/PBS.*

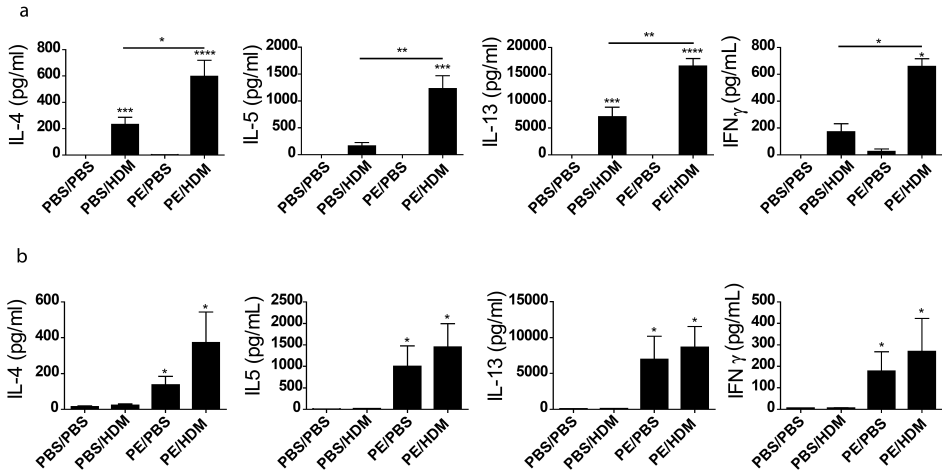

Figure 2. Allergen induced cytokine production in LLN. Production of IL-4, IL-5, IL-13 and IFNγ in supernatants of lung draining lymph node cells (LLN) after a) *ex vivo* restimulation with HDM and b) after *ex vivo* restimulation with PE. Data are presented as means ± SEM, **P<0.05, **P<0.01, ***P<0.001 vs PBS/PBS.*

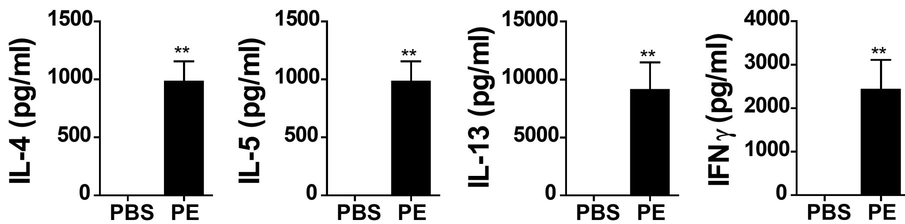

Figure 3. Peanut induced cytokine production by splenocytes. Production of IL-4, IL-5, IL-13 and IFNγ in supernatants of splenocytes after *ex vivo* restimulation with PE. Data are presented as means ± SEM, ***P<0.01*

Effect of peanut sensitization on HDM-induced airway inflammation and AHR

Both PBS/HDM and PE/HDM mice showed a clear recruitment of eosinophils, B and T cells to the broncho-alveolar compartment in response to inhaled HDM while PBS/PBS or PE/PBS mice did not (fig. 4a). A small and not significant increase was seen in the recruitment of eosinophils in HDM challenged mice with a peanut allergy compared to HDM sensitized mice (PBS/HDM) without a peanut allergy. Although in the PE/PBS group, cell counts in BALF did not show an increase in cell recruitment, analysis of the airway histology revealed a moderate, but significant, increased influx of inflammatory cells and a weak increase of mucus production in the lungs (fig. 4b and c) compared with PBS/PBS control mice.

To investigate whether airway hyper-reactivity would be increased in PE/HDM mice in comparison to PBS/HDM mice, AHR to methacholine was determined. Despite the increased Th2 cytokine production, preceding peanut sensitization and challenge did not increase PenH values (fig. 4d).

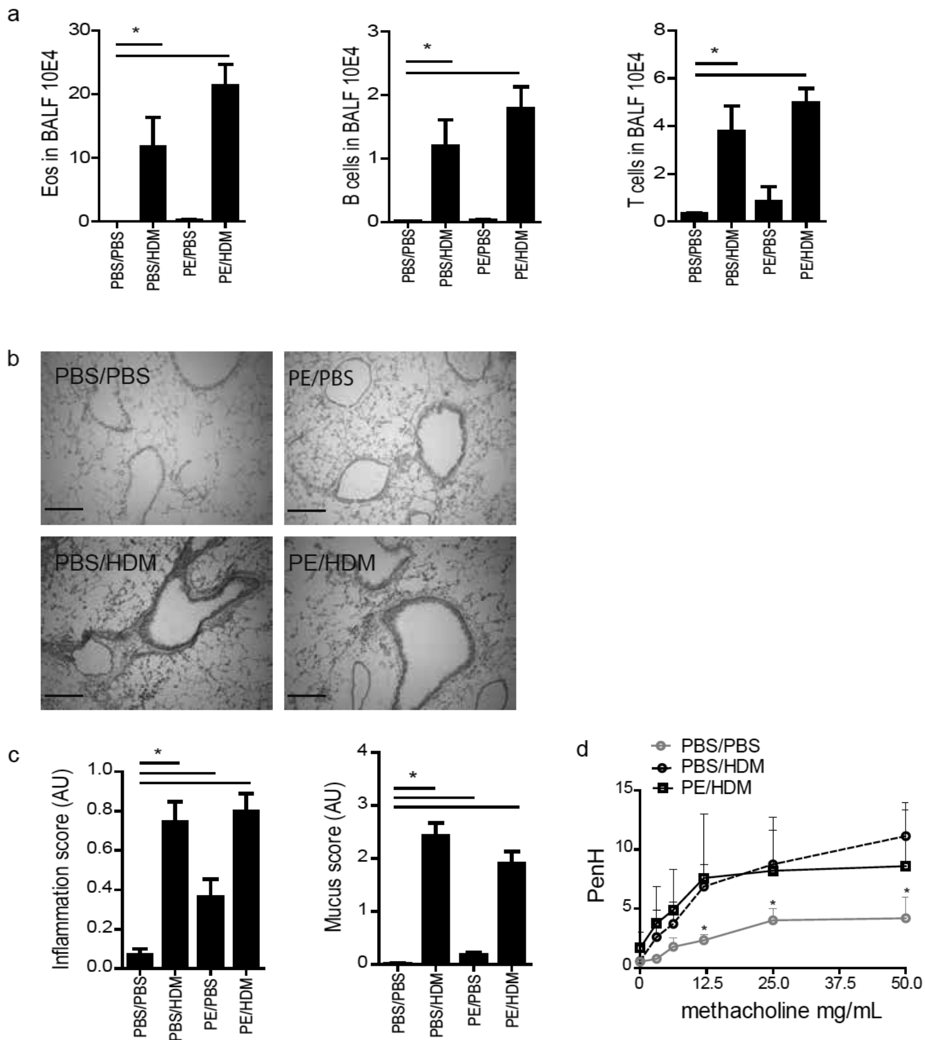

Figure 4. Lung inflammation and airway hyper-reactivity. Inflammatory cell recruitment to the airways (a). Lung slides shown at x10 were stained with Periodic Acid Schiff's reagent to determine peri-bronchial inflammatory infiltrates and mucus production (b) and quantified (c). Airway hyper-reactivity to methacholine was determined on day 53 (d). Data are presented as means ± SEM, *P<0.05.

No effect of HDM-induced airway inflammation on the anaphylactic response to peanut

Because asthma is a risk factor for the development of an anaphylactic response to food which manifests as life-threatening bronchospasm, we investigated in our model whether PE/HDM mice would react more severely to a systemic challenge with 500 µg PE i.p. compared with PE/PBS mice. PE/PBS mice developed a hypersensitivity reaction which is displayed by a severe drop in body temperature reaching an almost fatal temperature (towards 30°C). PE/HDM mice showed a similar drop in temperature (fig 5a).

As mast cells in the gut mucosa have been associated with an anaphylactic response [12], the number of mast cells in the gut was determined. No significant differences could be observed, but in PE/HDM mice, mast cell numbers were slightly increased compared with PE/PBS mice (fig. 5b). As a marker for mast cell activation, the concentration of murine mast cell protease 1 (mMCP-1) in serum was determined (fig 5c). PE/HDM mice had higher levels of mMCP-1 in serum compared with PE/PBS mice. In PE/HDM mice a significant positive correlation between the level of total IgE and the number of mast cells in the gut was observed, indicating that polysensitization might be associated with an increased number of mast cells in the gut mucosa (fig. 5d).

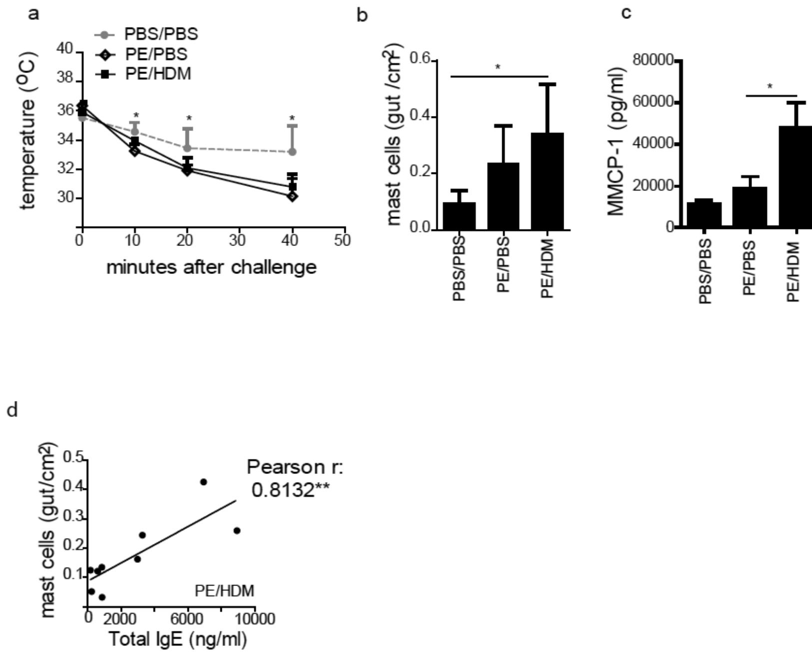

Figure 5. Anaphylactic response and numbers of mast cells in gut tissue. Core body temperature was measured after i.p. PE challenge on day 52 (*P<0.05: PE/PBS and PE/HDM vs PBS/PBS) (a). Number of mast cells in the gut was determined by Toluidine blue staining of intestinal tissue sections and represented as number of cells/per cm^2 of tissue (b). mMCP-1 levels in serum (c). Correlation between serum level of total IgE and mast cell numbers (number of cells/cm^2) in the gut of PE/HDM mice (d). Data are presented as means ± SEM , **P*<0.05, ***P*<0.01.

DISCUSSION

Several epidemiological studies have suggested that food allergy is associated with an increased risk to develop allergic airway disease [3;13;14]. The present study experimentally demonstrated interplay between the adaptive immune response evoked by exposure to peanut via the gastrointestinal mucosal tract and the immune response evoked by HDM via the airway mucosa.

We developed a model in which peanut allergic sensitization was followed by sensitization to HDM. Our model showed that the main hallmarks for both peanut allergy and HDM allergic asthma were successfully induced in the same mouse. PE and HDM co-sensitized mice developed a clear Th2 response to PE and HDM and showed increased PE-specific and HDM specific IgE and IgG_1 in serum. In addition, mice developed anaphylaxis when peanut was systemically injected. At the same time they also displayed all characteristics of allergic asthma, such as increased airway hyper-reactivity to methacholine, eosinophilic airway inflammation, and increased mucus production and peri-bronchial infiltrates. Interestingly, mice with PE and HDM allergy produced higher levels of Th2 cytokines in response to HDM compared to HDM-mono-sensitized mice.

These findings raise the question of how PE allergy might have boosted the HDM-specific T cells. We could detect peanut specific T cells in LLN and spleen after intragastric sensitization to peanut. It is tempting to speculate that unspecific bystander activation of HDM specific T cells by these circulating PE specific T cells, has led to a higher cytokine production by the HDM-specific T cells. The mechanism of bystander stimulation is a phenomenon primarily described for $CD8^+$ T cells [15;16], but which has also been clearly demonstrated for $CD4^+$ T cells [17;18]. Factors produced during the primary and secondary antigen-specific response, such as IL-2 and IL-15, activate unrelated effector $CD4^+$ T cells [18]. As the mediastinal lymph nodes drain both the lung as well as the peritoneum [19], the i.p. injection with peanut can have activated circulating peanut specific T cells in the LLN which then consequently contributed to an increased cytokine production by HDM specific T cells. These data suggest that activation of T cells by exposure to multiple allergens at the same time might have a synergistic effect, which results in an increased cytokine response. Alternatively, we cannot rule out that PE-specific T cells might have been induced in LLN due to unwanted peanut

sensitization via the lungs caused by an accidental delivery of PE during gavage. Translating our experimental data to real life, we can speculate that in peanut allergic humans circulating peanut specific Th2 cells might trigger the activation/expansion of Th2 cells which are specific for a second unrelated allergen, like derived from house dust mite.

Although preceding peanut sensitization increased HDM-specific Th2 cytokine production, this did not translate into aggravated lung inflammation or AHR. Based on the current dataset we can only speculate why the clinical phenotype does not follow the immunological changes. Perhaps the increase in Th2 cytokines is too small to have a clinical impact. Brandt et al. [21] and Bihouée et al. [22] both demonstrated that mice with an OVA alum induced gastrointestinal allergy developed an increased AHR and eosinophil recruitment after intranasal HDM exposure compared with mice without gastrointestinal allergy. The route of sensitization to the food allergen (gastrointestinal versus i.p.) and the choice of food allergen (peanut vs OVA) as well as the type of adjuvant (SEB vs alum) might have played a role. In contrast, we demonstrated an enhanced Th2 cytokine production after *ex vivo* restimulation of lung draining lymph nodes cells with HDM while Brandt et al. did not observe an enhanced Th2 cytokine production. In support of our data, Bihouée et al. observed an increase in IL- 4 and IL-5 in BALF but did not determine HDM induced Th2 cytokines in lymph nodes. The lack of an aggravation of eosinophilia could be explained by our high dose HDM model. This might have induced such a strong innate immune response resulting in recruitment of inflammatory cells and AHR [22] which overwhelmed the boosting effect of the preceding peanut sensitization on the adaptive immune response to HDM. It is important to note that AHR is multifactorial process which does not have a linear correlation with IL-5 or the IgE level. However, future experiments might reveal an effect on AHR when a suboptimal dose of HDM would be used. The preceding peanut allergy might lower the threshold for the induction of a HDM induced inflammation, allowing to envisage an effect at AHR.

Asthma is a risk factor for the development for an anaphylactic response to food allergens. In our model, PE/HDM mice did have a slight increase in mast cells in the gut which correlated with the level of total IgE. The intestinal recruitment and activation of mast cells has been reported to be dependent on IgE [24;25]. In support of an enhanced mast cell

degranulation also the level of MMCP-1 in serum was increased compared with PE/PBS mice but this did not result in a more severe drop in temperature. We speculate that the maximal drop in temperature was yet reached and could not be aggravated any further. An alternative explanation might be that hypothermia in mice is not solely dependent on IgE but also on IgG_1 [26]. The PE/HDM mice did not show an increased PE-IgG_1 level compared to PE/PBS mice.

In conclusion, our data show that allergy to peanut boosted the HDM-specific Th2 cytokine response in the airways. Further research is warranted to clarify the underlying mechanisms of T cell boosting in mice expressing concomitant food and respiratory allergy and whether secondary allergic sensitization in allergic individuals is facilitated by the mechanism of bystander activation of $CD4^+$ T cells.

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Chapter 6

Birch pollen immunotherapy in mice: inhibition of Th2 inflammation is not sufficient to decrease airway hyper-reactivity

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ABSTRACT

Background: Suppression of Th2 cytokine production by allergen specific Th2 cells is considered to be critical for the suppression of allergic symptoms by subcutaneous immunotherapy. The aim of this study was to develop a mouse model for birch pollen (BP) immunotherapy to elucidate underlying mechanisms that contribute to improvement of clinical symptoms.

Methods: Mice with birch pollen induced allergic airway inflammation received weekly subcutaneous immunotherapy injections with birch pollen extract (BPE) adsorbed to alum. The effect of an increasing dose of BPE adsorbed to a fixed concentration of alum on the suppression of airway inflammation and airway hyperresponsiveness (AHR) was determined. After 2, 4, 6, or 8 immunotherapy injections mice were rechallenged with the same allergen and all hallmarks of allergic asthma were evaluated.

Results: Suppression of the immunological parameters by immunotherapy was dependent on the dose BPE. Two injections were sufficient to suppress IL-4, -5, -13, -10 and IFNγ production, eosinophil recruitment and peribronchial inflammatory infiltrates. Also BP specific immunoglobulins were upregulated, but this was not sufficient to reduce AHR. Eight injections were needed to suppress AHR. The gradual reduction in AHR was inversely associated with the increase of BP IgG_{2a}.

Conclusions: BP SCIT induces an early suppression of Th2 mediated eosinophilic airway inflammation, but AHR is only effectively reduced after continued SCIT conceivably by allowing IgG_{2a} antibody titres to build up.

INTRODUCTION

Allergen specific immunotherapy has been used for over 100 years as treatment for allergic diseases and represents the only causal treatment. It consists of a series of subcutaneous (s.c.) injections with increasing doses of allergen, which induces desensitization and relief of allergic symptoms. Sublingual immunotherapy is currently evaluated as a safe, non-invasive alternative, but meta-analyses suggest that subcutaneous immunotherapy (SCIT) has greater efficacy when both are compared to placebo[1]. The clinical efficacy of SCIT is well documented, and much progress has been made in elucidating underlying immunological responses, but it is still a matter of debate as to which immune changes are decisive in reaching the beneficial effect [2-4].

The reduction in allergic symptoms by SCIT has been hypothesized to be dependent on a decrease in Th2 cytokine production. Several mechanisms have been proposed to contribute to this decreased Th2 immunity: T cell anergy, a shift from a Th2 towards a Th1 response and/or the induction of regulatory T cells [5;6]. The induction of allergen specific IgG antibodies, in particular IgG_4, during SCIT is another prominent observation [4]. This led to the formulation of the so-called blocking antibody hypothesis, stating that allergen-specific IgG antibodies compete with IgE for allergen-binding, thereby neutralizing allergens and preventing IgE cross-linking and degranulation of mast cells. By a similar mechanism, allergen-specific IgG has been shown to inhibit IgE-facilitated antigen-presentation by antigen-presenting cells to T helper cells[7]. Interestingly, in humans treated by bee sting immunotherapy, a decreased tendency for anaphylaxis is observed very early, but the change in the IgE/IgG_4 ratio, the inhibited release of mediators from eosinophils and tissue mast cells and the decrease in late phase skin test reactivity are observed only after months [6]. This suggests that there are sequential effects of immunotherapy on the immune system preventing different clinical symptoms. It is important to establish the sequence of events for the immunological and clinical changes that occur in the path towards effective treatment[7]. To study this in detail, we used a murine model for birch pollen (BP) allergy to determine the sequential effects of BP SCIT. Most BP immunotherapy models use mice which are sensitized with recombinant Bet v 1, this being the major allergen in BP allergic humans. In this study, Bet v 1 sensitization induced a mild inflammation and no airway

hyper-responsiveness (Suppl fig 1) in contrast to mice sensitized with BP [8-10]. We show here that reduction of Th2 cytokine production by BP SCIT was not sufficient to immediately reduce AHR in mice, but was rather associated with the sequential effects on IL-17A production, broncho-alveolar lavage fluid (BALF) IL-5 levels and increased titres of BP IgG_{2a}.

METHODS

Mice

Female BALB/c mice, 6-8 weeks of age (from Harlan, Horst, the Netherlands) were housed under specific pathogen-free conditions at the animal facility of the Academic Medical Center. All experiments were approved by the animal ethics committee of the AMC, the Netherlands.

Study design

Sensitization to birch pollen:

BALB/c mice were sensitized intraperitoneally (fig. 1A) on days 0, 7, 14 with 8.2 µg birch pollen extract (BPE; HAL Allergy BV, Leiden, The Netherlands) containing 117 µg Bet v 1/mg dry weight extract (determined by Bet v 1 ELISA) and an endotoxin level of 2,84 EU/mg (determined by the limulus amebocyte lysate assay; Indoor Biotechnologies Inc., Cardiff, UK), using a dose equivalent to 1 µg Bet v 1, adsorbed to 1 mg alum (Imject alum, Pierce, Rockford, Ill., USA) or as a control with alum alone. Under isoflurane anaesthesia, the mice were challenged intranasally (i.n.) with 100 µg BPE (equivalent to a dose of 11.7 µg Bet v 1) in 30 µl PBS or PBS as a control on days 21-23. One day later, AHR to methacholine was assessed. On day 25, the mice were sacrificed for the analysis of eosinophil recruitment to the airways, IL-4, -5, -13, -10, -17A and IFNγ production, airway histology and the humoral immune response (BP-specific IgE, IgG1 and IgG_{2a}).

BP SCIT:

First, a dose response experiment was performed. On days 35, 42, 49, 56, 63, 70, 77 and 84, mice with a BP induced allergic airway inflammation underwent SCIT, adapted from a protocol for grass pollen immunotherapy, described by Mondoulet et al[11], administering 0.01, 0.1, 0.3 or 3 mg BPE adsorbed to 1 mg alum; control mice received PBS. To investigate the effectiveness of the immunotherapy, the mice were rechallenged with a 0.1% BPE aerosol at day 98-100 and on day 101, AHR was assessed by methacholine challenge. One day later, the mice were sacrificed to evaluate the same immunological parameters as before the start of immunotherapy (fig. 1A).

Second, a kinetic experiment was performed. The mice with a BP-induced allergic airway inflammation received 2, 4, 6 or 8 SCIT injections containing 0.3 mg alum-adsorbed BPE or PBS as a control. The concentration of 0.3 mg was found to be effective, though sub-optimal compared to a 3 mg dose, but was chosen so as to limit distress to the animals caused by the severe drop in core body temperature upon injection of 3 mg. Two weeks after the last immunotherapy injection, mice received a 30-min administration of 0.1% BPE aerosol on three consecutive days. AHR upon methacholine challenge was determined 24 hours after the last BPE aerosol application and mice were sacrificed one day later for analysis of immunological parameters (fig. 1B).

Collection and analysis of tissues

Bronchoalveolar lavage fluid (BALF)

BALF inflammatory cells were obtained by intratracheal cannulation and the airway lumen was lavaged 3 times with the same 1 ml PBS containing 0.1 mM EDTA, except for fig 2B when mice were lavaged with 3 times with 1 ml. Cell differentiation was done by flow cytometric analysis as described before[12]. The BALF was stored at -20°C until IL-5 and eotaxin-2 were determined by ELISA (Ready set go!, eBioscience Inc., Immunosource, Halle-Zoersel, Belgium).

Restimulation of lung-draining lymph node cells ex vivo

Lung-draining lymph node cell suspensions were plated in 96 well round-bottom plates at a density of 2 x 10^5 cells per well and were restimulated for 4 days with 100 µg/ml BPE. Supernatants were analysed for IL-4, -5, -13, -10, -17A and IFNγ production by ELISA.

Serum BP-specific immunoglobulins

Serum was analysed for the level of BP specific IgE, IgG_1 (Opteia, BD, San Diego, Calif., USA) IgG_{2a} (eBioscience) by ELISA, as previously described [13]. In short, maxisorp plates were coated overnight with 100 µg BPE. After blocking with 10% FCS, serum samples were incubated for two hours, followed by an HRP detection step, according to the

manufacturer's instructions. Serum samples of BP allergic animals were diluted 200x, 200.000x, 50x for IgE, IgG_1 and IgG_{2a} respectively, while the sera of PBS control animals were diluted 30, 100 and 50 times, respectively. A standard curve of murine IgE, IgG_1 or IgG_{2a} respectively was used as a quantitative reference.

Airway histology

Frozen sections (6 µm) were stained with periodic acid Schiff (Sigma-Aldrich). The degree of inflammation and mucus production was semi-quantified, as described before [13].

Airway hyper-responsiveness (AHR)

AHR to inhaled methacholine was measured by barometric plethysmography in conscious mice (EMKA Technologies). Non-specific responsiveness was measured by exposing mice to aerosolized PBS to set a baseline value, followed by increasing concentrations of aerosolized methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml in PBS for 3 min; Sigma-Aldrich) using ultrasonic nebulizers. PenH (enhanced pause) values were measured during 5 min after each methacholine aerosol. For each individual animal, the area under the dose-response curve of PenH to increasing concentrations of inhaled methacholine was determined.

Core body temperature

The core body temperature was measured with a hand-held device with a mouse rectal probe (Physitemp Instruments Inc, Clifton, NJ, USA) 30 min after administration of each SCIT injection.

Statistical analysis

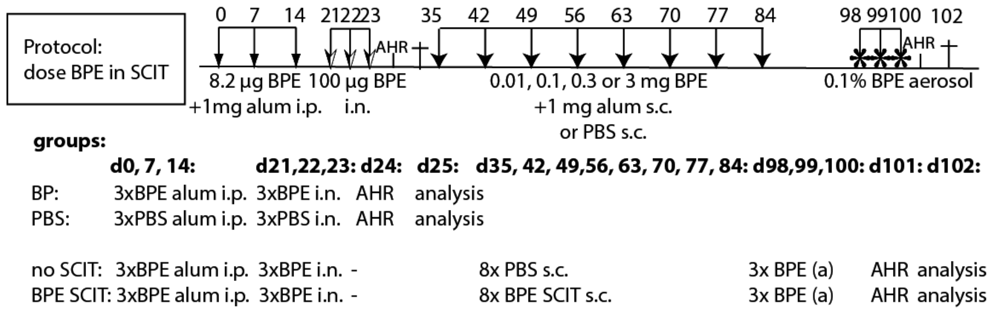
For statistical analysis, differences between groups were analysed with the Mann-Whitney U test using IBM SPSS Statistics 20 software. Association between parameters was determined by Pearson Correlation Coefficient r. Differences were considered to be significant at a p value <0.05.

RESULTS

Airway inflammation preceding BP SCIT

BP-induced inflammation was induced in BALB/c mice as described in the Materials and Methods section (fig. 1A). Compared to the PBS-sensitized and challenged control mice, the BP-sensitized and challenged mice developed AHR (fig. 2A). This was accompanied by recruitment of eosinophils to the airways and increased levels of IL-5 in the BALF (fig. 2B), inflammatory infiltrates around the bronchi (fig. 2C, left panel), increased mucus production (fig. 2C, right panel), the induction of BP-specific IgE, IgG_1 and IgG_{2a} (fig. 2D) and BP-specific IL-4, -5, -13, -10, and IFNγ production by lung-draining lymph node cells. IL-17A was detected at slightly higher levels in the BP exposed mice, but this did not exceed the background level measured in the controls (fig. 2E).

A



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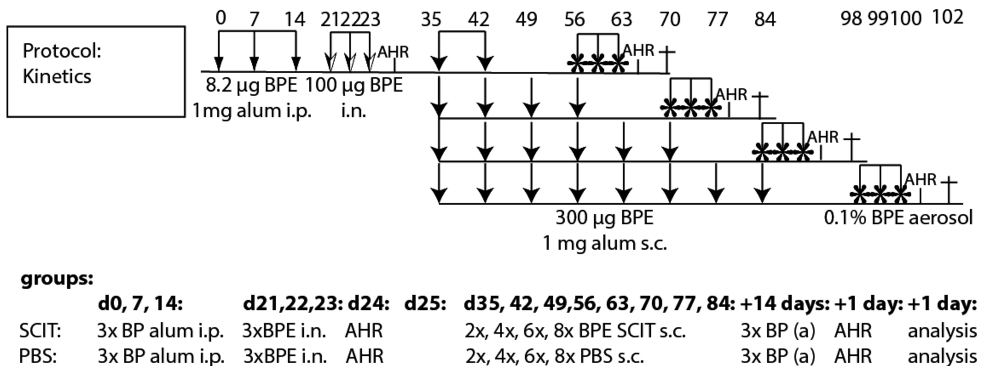
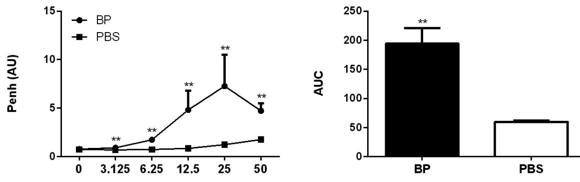


Fig 1. Experimental designs. Mice were sensitized intraperitoneally (i.p.) by BP absorbed to alum on days 0, 7, 14, and were challenged with BP intranasally (i.n.) on days 21-23. AHR was determined 24 hours after the last BP challenge, and 1 day later, a subgroup of mice were sacrificed for analysis of immunological parameters on day 25. Ten days later, the mice received a weekly SCIT (BP adsorbed to 1 mg alum) injection with 0.01, 0.1, 0.3 or 3 mg of BP. They were rechallenged 2 weeks after the last injection with three daily aerosol applications of 0.1% BPE. One day later, AHR was determined, and 24 hours later, the mice were sacrificed to examine the immunological parameters (A). In the kinetic experiment, the mice with a BP-induced allergic airway inflammation received 2, 4, 6, 8 BP SCIT injections (BP/alum containing 0.3 mg BP adsorbed to 1 mg of alum). Two weeks after the last BP SCIT injection, they were rechallenged with a daily BPE aerosol application for 3 consecutive days. One day later, AHR was determined, and 24 hours later, the mice were sacrificed to examine the immunological parameters (B).

A



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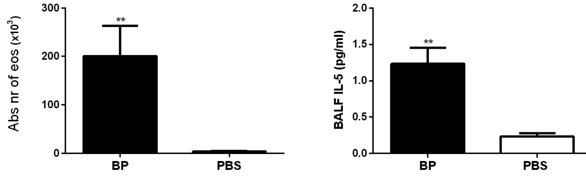

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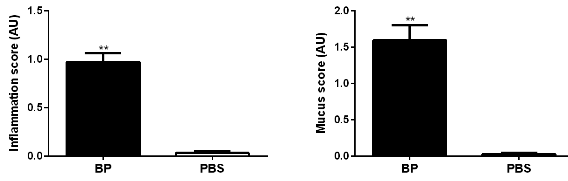

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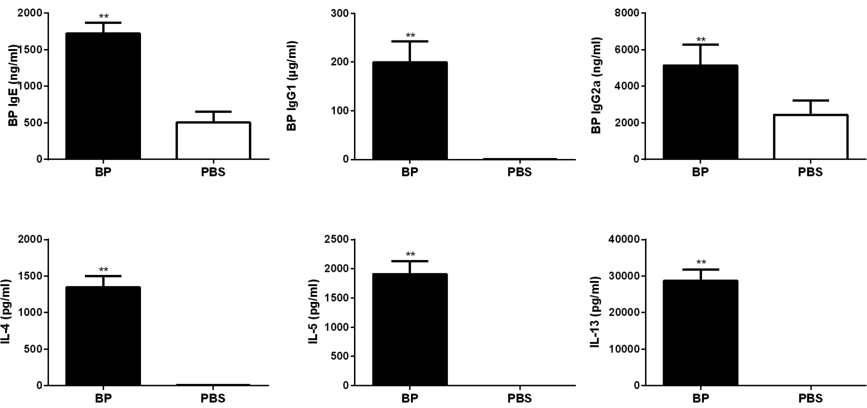

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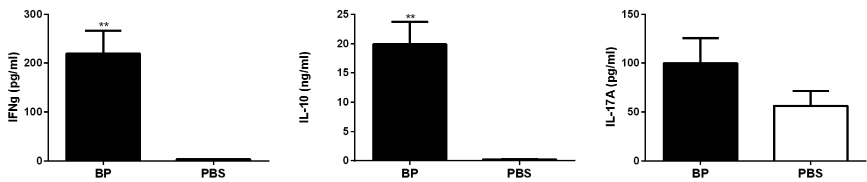


Fig 2. Mice developed BP-induced airway inflammation and AHR before starting BP SCIT. The mice were sensitized and challenged with BP (according to Fig 1). AHR (A; left panel: PenH dose-response curves, right panel: average area under the curve of individual PenH dose-response curves) were determined 24 hours after the last challenge (on day 24) and mice were sacrificed one day later (day 25) for analysis of eosinophil recruitment and IL-5 concentration in the BALF (B), peribronchial infiltrates (C, left panel), mucus production (C, right panel), BP-specific IgE, IgG_1 and IgG_{2a} (D) and cytokine production after *ex vivo* restimulation of lung draining lymph node cells with BP (E). *P<0.05, **P<0.01 vs PBS.

Dose-dependent modulation of immune responses by BP SCIT

To assess the efficacy of BP SCIT to reduce the immune response to inhaled BP, the BP sensitized mice were treated weekly with 0.01, 0.1, 0.3 or 3 mg BPE adsorbed to 1 mg alum (s.c.) and the immune response was evaluated after inhalational challenge with BP. To determine whether injection with BP SCIT induced an anaphylactic response, the core body temperature was measured 30 minutes after each injection [14]. BP SCIT induced a drop in body temperature after the first injection in a dose dependent manner, but subsequent injections led to much less pronounced effects on body temperature, except in mice receiving the highest (3 mg) allergen dose (fig. 3A).

The efficacy of BP SCIT to suppress cytokine production by BP-specific Th2 cells was analysed. BP SCIT inhibited Th2 cytokine production of IL-4, IL-5 and IL-13 but also of Th1 related IFNγ, Treg related IL-10 and Th17 related IL-17A in a dose-dependent way (fig. 3B). The recruitment of eosinophils, the local concentration of IL-5 in BALF (fig. 3C) and the induction of peribronchial inflammatory infiltrates were suppressed in a dose-dependent way (fig. 3D, left panel). Mucus production in response to exposure to BPE aerosols was only inhibited after the 3-mg BP SCIT treatment (fig. 3D, right panel). The induction of BP-specific IgE was inversely correlated with BP SCIT dose, whereas BP-specific IgG_1 showed a positive dose-response relation (fig. 3E). BP-specific IgG_{2a} was increased but did not show a clear dose-response. BP SCIT did not suppress AHR in a similar dose-dependency as was seen for the other parameters. Although a modest decrease in AHR was observed, significance was not reached in this particular experiment as in the other experiments (fig. 5D), likely because of a lack of statistical power due to the insufficient number of mice left in the “no SCIT” group for AHR measurement (n=5) (fig. 3F).

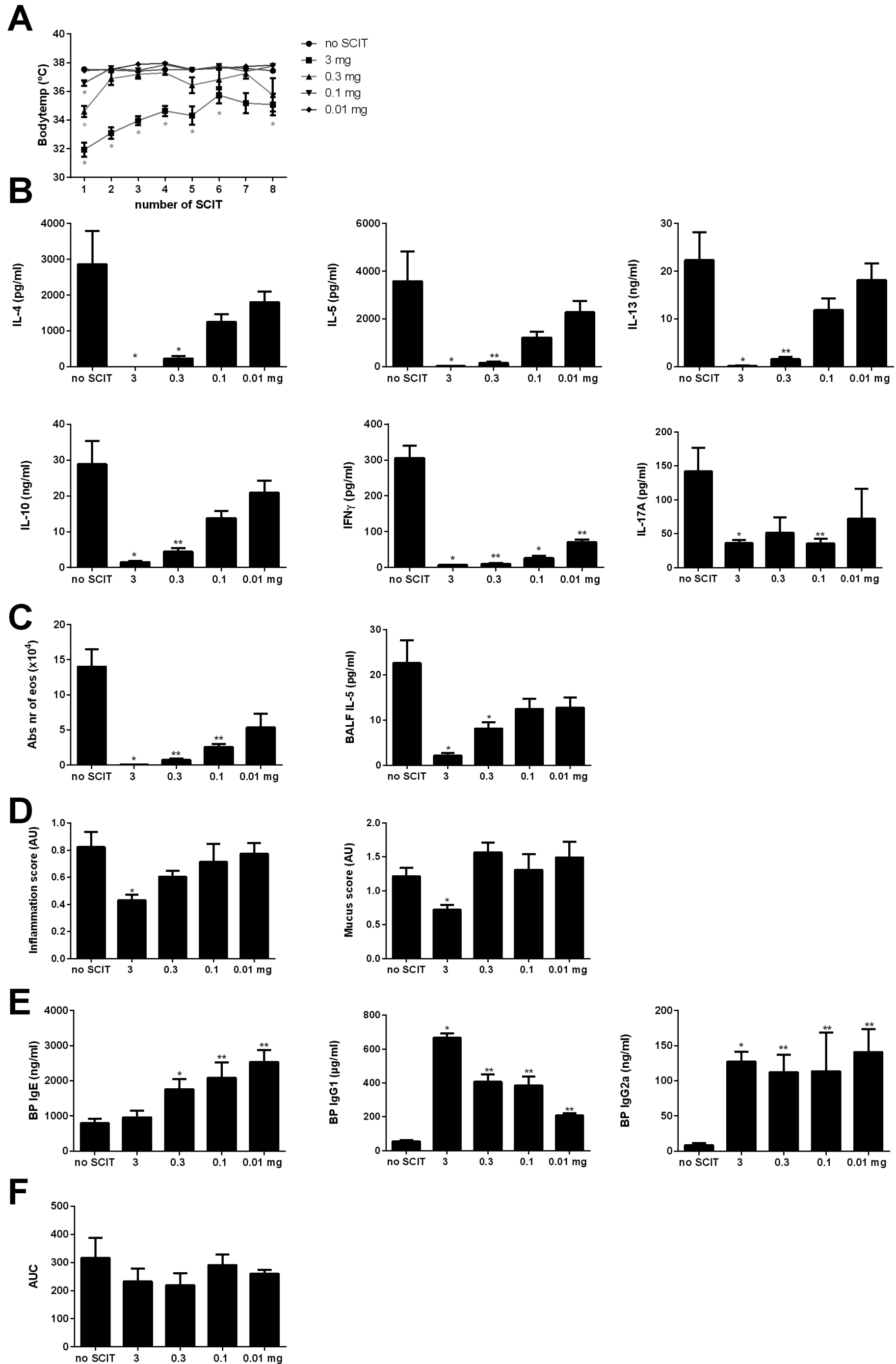
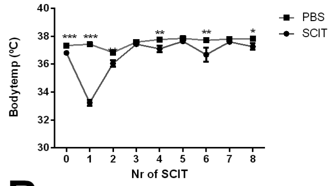

A
B
C
D
E
F
no SCIT
3 mg
0.3 mg
0.1 mg
0.01 mg
Bodytemp (°C)
number of SCIT
IL-4 (pg/ml)
IL-5 (pg/ml)
IL-13 (ng/ml)
IL-10 (ng/ml)
IFNγ (pg/ml)
IL-17A (pg/ml)
Abs nr of eos (x10^4)
BALF IL-5 (pg/ml)
Inflammation score (AU)
Mucus score (AU)
BP IgE (ng/ml)
BP IgG1 (μg/ml)
BP IgG2a (ng/ml)
AUC
no SCIT 3 0.3 0.1 0.01 mg

Fig 3. BP SCIT effects are dose dependent. Mice with BP-induced allergic airway inflammation were treated with 8 SCIT injections of 0.01, 0.1, 0.3 or 3 mg of BP adsorbed to 1 mg alum. The effect of the different concentrations of BP on the drop in core body temperature was assessed 30 min after each injection (A).The mice were rechallenged with 3 BPE aerosols. Cytokine production after *ex vivo* restimulation of lung-draining lymph node cells (B), eosinophil recruitment and IL-5 concentration in the BALF (C), mucus production (D, left panel) and peribronchial infiltrates (D, right panel), and BP-specific IgE, IgG_1 and IgG_{2a} levels in serum (E) were analysed. AHR was determined 24 hours after the last aerosol application(F). *P<0.05, **P<0.01 vs No SCIT.

Kinetics of the inhibition of Th2 inflammation and amelioration of AHR.

It was determined how many BP SCIT injections were needed to suppress the immunological parameters and AHR. Mice were rechallenged with BPE aerosols after 2, 4, 6 or 8 BP SCIT injections. As in the previous experiment, the first SCIT injection induced a sharp decrease in body temperature which was not observed after following injections, although a small but significant drop was still observed at 4, 6 and 8 injections (fig. 4A). Two SCIT injections suppressed IL-4, IL-5, IL-13, IFNγ and IL-10 production (fig. 4B), eosinophil recruitment (fig. 4C) and peribronchial inflammation (fig. 4D, left panel) to a level similar to that seen after 8 BP SCIT injections. IL-17A suppression was observed after 4 and 8 BP SCIT injections (fig. 4B). Interestingly, BALF IL-5 was not inhibited until the 8th BP SCIT injection (fig. 4C, right panel). In contrast to the cellular response, additional SCIT injections did have an effect on the humoral immune response. An increasing number of SCIT injections induced a rise in BP-specific IgE, IgG_1 and IgG_{2a} in the serum. The increase of BP-specific IgE levelled out after 6 injections, and that of BP-specific IgG_1 levelled out after the 4th injection (fig. 4E). In contrast, BP-specific IgG_{2a} steadily increased up to the 8th injection. Interestingly, AHR was only inhibited significantly after 8 injections compared with mice receiving PBS (fig 5. A-D, F). Both groups of BP-sensitized mice (receiving either 8 PBS or 8 SCIT injections) had a similar AHR before starting therapy (day 24) and mice receiving PBS had a similar increased AHR at day 102 after BP rechallenge compared with day 24 (fig. 1B and 5E). The level of BP-specific IgG_{2a} correlated strongly with the suppression of AHR (fig. 5G: r^2=0.78 and p=0.0034), while BP-specific IgE and BP-specific IgG_1 were less strongly correlated with the suppression of AHR (both r^2=0.64, $p<0.05$). Eight times BP SCIT decreased the BP IgE/BP IgG_{2a} ratio compared to 2x and 4x SCIT and, although not significantly, to 6x SCIT (fig. 5h).

A



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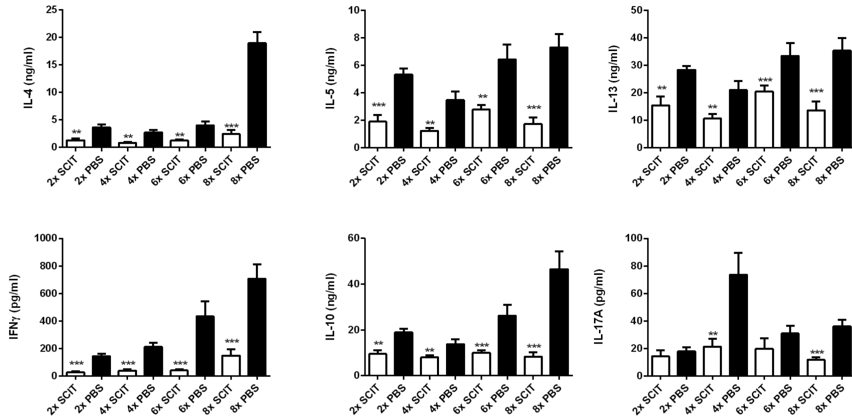

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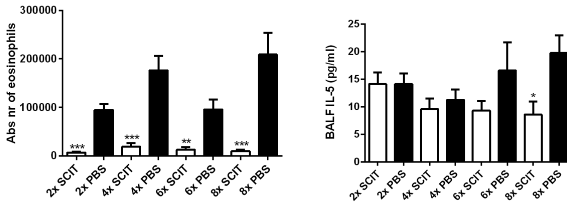

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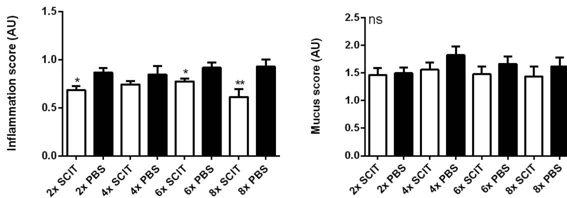

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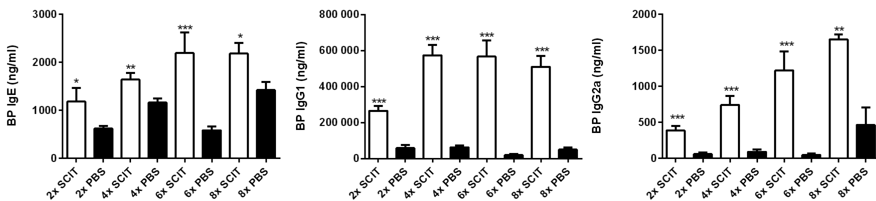


Fig 4. Kinetics of suppression of allergic airway inflammation during BP SCIT. Mice with BP-induced allergic airway inflammation were treated with 2, 4, 6, or 8 SCIT injections consisting of 0.3 mg BP absorbed to 1 mg alum, and were rechallenged 2 weeks later with 3 BPE aerosols. The core body temperature was assessed 30 min after each BP SCIT injection (A). The effects of BP SCIT were determined on cytokine production of lung-draining lymph node cells (B), eosinophil recruitment (C, left panel) and IL-5 concentration in the BALF (C, right panel), peribronchial inflammation (D, left panel) and mucus production (D, right panel) and BP-specific IgE, IgG_1 and IgG_{2a} levels in serum (E) were analysed. *P<0.05, **P<0.01, ***P<0.001 vs mice receiving corresponding number of PBS injections instead of BP SCIT.

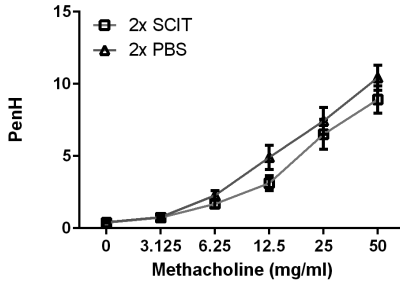
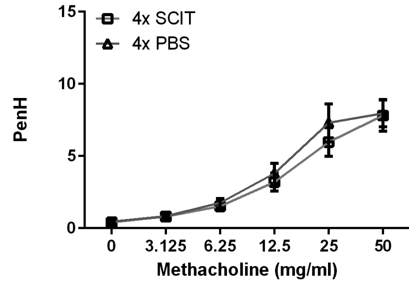
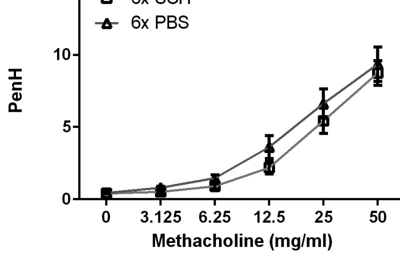
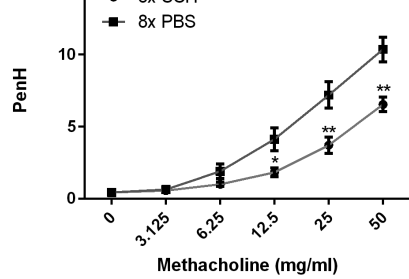
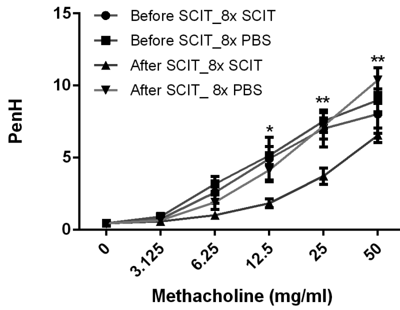
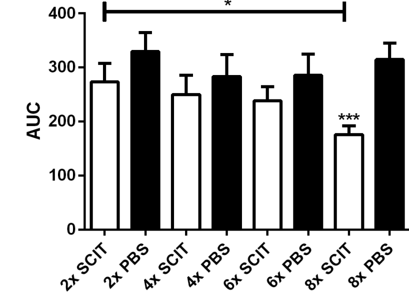
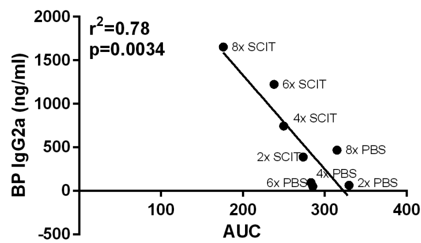
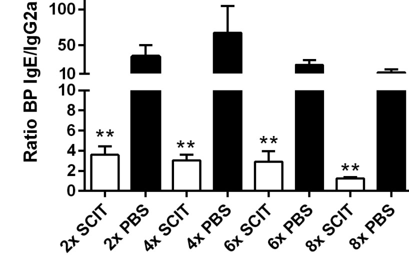

A
2x SCIT
2x PBS
PenH
Methacholine (mg/ml)

B
4x SCIT
4x PBS
PenH
Methacholine (mg/ml)

C
6x SCIT
6x PBS
PenH
Methacholine (mg/ml)

D
8x SCIT
8x PBS
PenH
Methacholine (mg/ml)

E
Before SCIT_8x SCIT
Before SCIT_8x PBS
After SCIT_8x SCIT
After SCIT_ 8x PBS
PenH
Methacholine (mg/ml)

F
AUC
2x SCIT
2x PBS
4x SCIT
4x PBS
6x SCIT
6x PBS
8x SCIT
8x PBS

G
r2=0.78
p=0.0034
BP IgG2a (ng/ml)
AUC
8x SCIT
6x SCIT
4x SCIT
8x PBS
2x SCIT
4x PBS
6x PBS
2x PBS

H
Ratio BP IgE/IgG2a
2x SCIT
2x PBS
4x SCIT
4x PBS
6x SCIT
6x PBS
8x SCIT
8x PBS

Fig 5. Inhibition of AHR correlated with induction of BP IgG_{2a}. Mice treated with 2 (A), 4 (B), 6 (C) or 8 BP SCIT injections (D) were rechallenged with 3 BPE aerosols, and 24 hours later, AHR was determined. AHR was also determined before starting BP SCIT or PBS treatment and compared with AHR after 8 BP SCIT or 8 PBS treatments (E). The average area under the curve of the PenH values depicted in A-D were determined (F) and were plotted against the average level of BP IgG_{2a} in serum found in mice receiving 2, 4, 6 or 8 BP SCIT injections or PBS as a control. Correlation was assessed by determining the correlation coefficient (G). The BP IgE/BP IgG_{2a} ratio was compared between the mice in the BP SCIT group (2, 4, 6, or 8 injections) and those in the PBS control group and between the different number of SCIT injections (H). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs mice receiving corresponding number of PBS injections instead of BP SCIT.

DISCUSSION

In this study, it was shown that suppression of Th2 cytokine production and eosinophil recruitment was not sufficient to reduce AHR, but that a prolonged period of BP SCIT was needed.

Interestingly, although BP SCIT induced an early suppression of Th2 cytokine production and eosinophil recruitment not sufficient to inhibit AHR, the anaphylactic response, as demonstrated by a drop in body temperature after the first SCIT injection, was almost completely absent after the second BP SCIT injection, except for with the highest BP concentration. In human immunotherapy, this early protective effect against anaphylaxis has been explained by desensitization of mast cells and basophils [6;15]. Although AHR showed a trend to decrease with the number of injections (fig 5F&G), for significant suppression of AHR compared to "no SCIT" treatment, 8 injections were needed. Interestingly, the gradual decrease in AHR was inversely correlated with an increase of BP-specific IgG_{2a}. A similar gradual increase of allergen-specific IgG_{2a} during SCIT treatment was reported in an ovalbumin (OVA) SCIT model [16]. Whether BP-specific IgG_{2a} antibodies have a functional role or whether they are more an epi-phenomenon of another pathway still has to be explored. A possible mechanism could be via competition for IgE-mediated basophil release, as recently described in BP-sensitized mice, in which treatment with rabbit anti-Bet v 1 IgG inhibited IgE mediated basophil degranulation [17]. In support of a functional role for BP-specific IgG_{2a}, intratracheal instillation of OVA-specific IgG_{2a} or IgG_1 in mice with OVA-induced allergic asthma was sufficient to suppress AHR after OVA aerosol challenge [18]. Likewise, in a Fel d 1 immunotherapy model, it was demonstrated that allergen-specific IgG_1 and IgG_{2a}, but not $CD4^+$ T cells, were critical for alleviation of local and systemic symptoms [19]. In humans, the induction of allergen-specific IgG antibodies, especially of the IgG_4 subclass, is a hallmark of SCIT, and its bioactivity to block IgE-allergen interactions has been associated with clinical improvement [20-22]. In contrast, Shirinbak et al. showed that, in mice, the suppression of a Th2 driven airway inflammation after OVA SCIT was not dependent on the induction of IgG or IgA responses, FcγRIIB signalling or B cell function [23], but the effect on AHR was not examined. Our study suggests that induction of

immunoglobulins might play a role in suppression of AHR following suppression of a Th2 cytokine response.

The serum IgE concentration was inversely correlated with the concentration of allergen used in the BP SCIT formulation, in contrast to the dose-dependent suppression of IL-4. We cannot exclude that this result might have been caused by binding of the coated BPE by BP specific IgG_1 in the ELISA. This needs further investigation.

Both eosinophil recruitment and IL-5 production in lung-draining lymph nodes was suppressed early in BP SCIT, whereas concentrations of IL-5 in the BALF decreased only after 8 SCIT injections. This did not hamper suppression of eosinophil recruitment because eotaxin-2 was suppressed; this is considered to be the main chemoattractant for eosinophils while IL-5 is involved in mainly activation and survival of eosinophils [24] (Supplementary fig. 1). Eosinophils have been thought to be instrumental in AHR, but in this study, the inhibition of eosinophilic airway inflammation after 2 SCIT injections was not accompanied by reduced AHR. Eosinophils can be dispensible for AHR as recently shown in eosinophil-deficient mice that developed AHR in response to house dust mite extract [25]. Hamelmann et al. described 15 years ago that IgE is important for AHR development especially when there is a limited number of eosinophils [26]. It is therefore tempting to speculate about a dependency on an eosinophil/BP-specific IgE/IgG_{2a} axis for the amelioration of AHR. Although BP SCIT suppressed eosinophil recruitment very early, BP-specific IgE did not decrease (or even increased), as seen in humans after starting SCIT[27], and BP-IgG_{2a} might had to increase sufficiently to compete with the BP-specific IgE in order to result in the amelioration of AHR.

As increased IL-17A levels have been associated with an increase in AHR in both mice and humans, the levels of IL-17A were analysed before and after BP SCIT [28;29]. Although IL-17A production was only slightly, but not significantly, enhanced in BP-sensitized mice before starting BP SCIT, 8 injections of BP SCIT did suppress IL-17A production significantly. This suggests that suppression of IL-17A contributed to the observed amelioration of AHR.

A critical issue for interpretation of the kinetic experiment is whether AHR in PBS-treated mice was stable during the full 8 weeks of treatment. In fact, AHR was identical after 2, 4, 6 and 8 weeks of PBS treatment. It can therefore be ruled out that the inhibition of AHR

observed in mice receiving 8 weeks of active immunotherapy could be explained by 'natural' loss of AHR. It was due to the BP SCIT treatment. Interestingly, the PBS-treated control mice demonstrated increased IL-4, -5, -13, -10, -17A and IFNγ production, eosinophil influx and IgE production after BP re-challenge after 8 weeks compared with after 2 weeks ($p<0.05$), suggesting that the increased interval without exposure to the allergen increased the Th2 inflammation after the inhalation of BPE aerosols. The mechanism underlying this aggravation of Th2 inflammation is unclear. Our study shows that BP SCIT was capable of suppressing this expansion of Th2 inflammation to levels similar to those observed in mice receiving 2 BP SCIT injections.

One goal of clinical research into allergen-specific SCIT is to identify a marker for successful SCIT. Our study suggests that a decrease in Th2 cytokine production in the T cells after SCIT may confirm that the immune system is being redirected in a desirable manner. This, however, does not automatically result in an improved clinical outcome. In human SCIT, the functional activity of IgG_4 seems to be one of the most useful markers at the moment [7;22]. Mice do not produce IgG_4 and murine IgG_1 is considered to be its murine homologue [30;31]. In our mouse model, we found that BP-specific IgG_1 was the most abundant subclass after BP SCIT, however BP IgG_{2a} was more strongly correlated to the amelioration of AHR and may play a similar role as human IgG_4.

In conclusion, we showed, for the first time, that BP SCIT induced sequential effects in mice. The suppression of IL-4, -5, -10, -13, and IFNγ production, peribronchial inflammatory infiltrates and induction of allergen specific immunoglobulins was established before AHR was suppressed. The suppression of AHR was accompanied by the suppression of IL-5 levels in the BALF, the suppression of IL-17A and the induction of BP IgG_{2a}. In contrast, mice became resistant to anaphylactic responses to SCIT injections, measured by a drop in body temperature, after only 1 injection. This model may be helpful as a preclinical model to improve immunotherapy for BP allergy and to identify therapeutic targets to ameliorate clinical symptoms.

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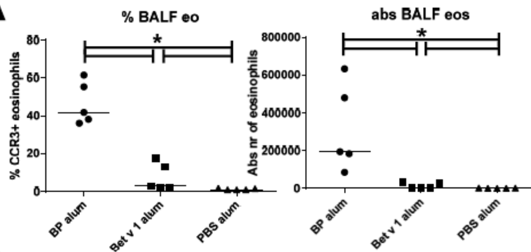

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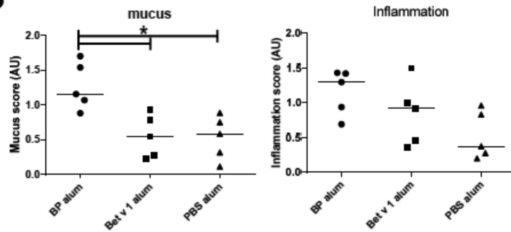

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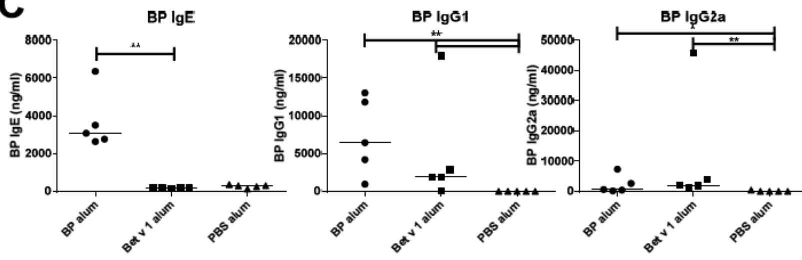

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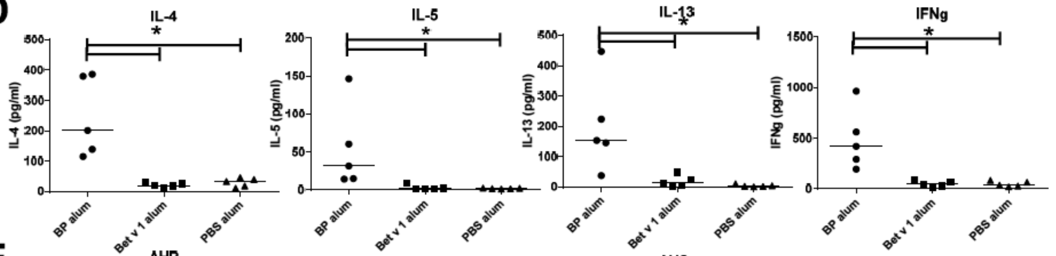

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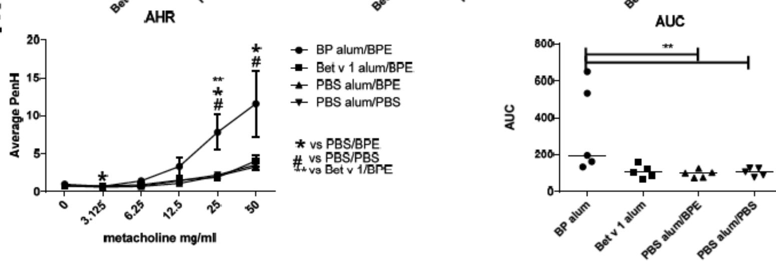
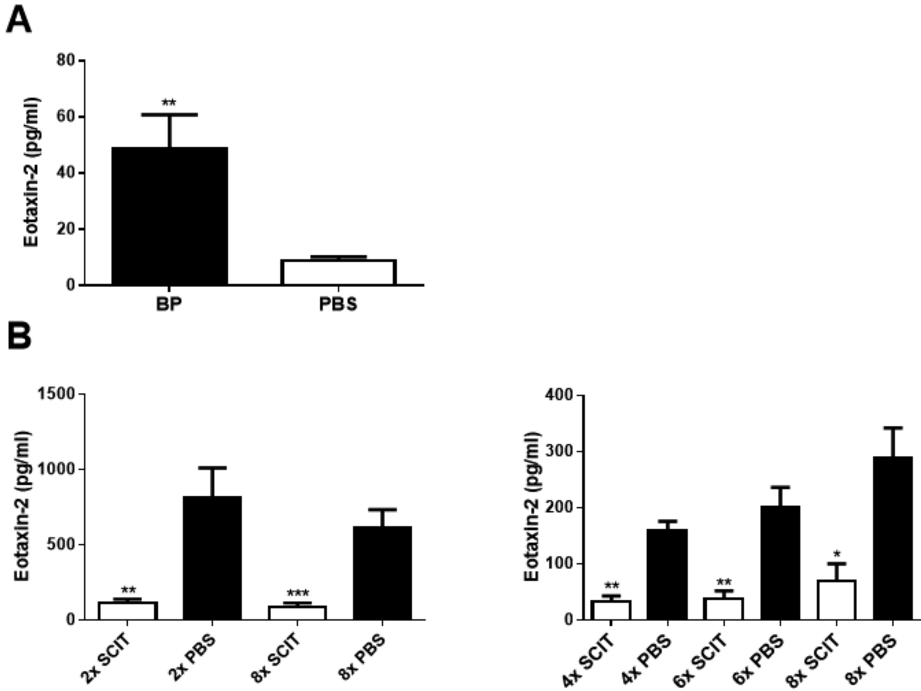


Supplementary figure 1. The mice received on days 0, 7, 14 an i.p. injection with Bet v 1 (10 µg)/alum (1 mg) or BPE (8.2 µg BP containing 1 µg Bet v 1)/alum(1 mg). They were challenged on days 21-23 with 100 µg BP intranasally. AHR was determined on day 24 and mice were sacrificed on day 25 for analysis of the immunological parameters.



Supplementary figure 2. Eotaxin-2 levels were determined in individual BALF samples of mice with a BP induced eosinophilic airway inflammation (A), or in BALF of mice which received 2 BP SCIT or 8 BP SCIT injections (left panel) or in pooled BALF samples of 2-3 animals after treatment with 4, 6 or 8 BP SCIT injections (right panel). The pooled samples were obtained from two independent experiments and were analysed together to achieve a sufficient number of data points for statistical analysis (B). BP SCIT treated mice were rechallenged with 3 BPE aerosols and 48 hours later the airways were lavaged to determine Eotaxin-2 levels. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs mice receiving a corresponding number of PBS injections instead of BP SCIT.

Chapter 7

Birch pollen immunotherapy inhibits anaphylaxis to the cross-reactive apple allergen Mal d 1 in mice

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ABSTRACT

Background: Cross-reactive apple allergy is a common co-morbidity of birch pollen allergy, caused by the presence of a Bet v 1 homologue allergen in apple, Mal d 1. Treatment of tree pollen hay fever by immunotherapy is well-established, but its effect on the accompanying apple allergy is debated.

Objective: To establish a mouse model of birch pollen induced cross-reactivity to Mal d 1 and investigate the effect of birch pollen immunotherapy on the cross-reactivity to Mal d 1.

Methods: Respiratory allergy was induced in Balb/c mice by intraperitoneal exposure to alum-adsorbed birch pollen extract (BPE) in combination with short or prolonged intranasal exposure to BPE. To evaluate the response to Mal d 1, mice were exposed intraperitoneally to Mal d 1. Immunoglobulin responses and cytokine production by splenocytes were measured by ELISA. Allergic symptoms were evaluated by measuring airway hyper-reactivity and hypothermia as a surrogate marker for anaphylaxis. Immunotherapy was performed subcutaneously with alum-adsorbed BPE.

Results: Mice exposed to BPE develop cross-reactive IgE to Mal d 1. Early after exposure to BPE, this response is still weak and does not yet translate into anaphylaxis. Interestingly, later re-challenge with BPE increased cross-reactivity to a level where Mal d 1 exposure induced anaphylaxis. Cross-sensitization can also be induced by systemic Mal d 1 exposure. Birch pollen immunotherapy significantly reduced the anaphylactic response of mice to Mal d 1.

Conclusion & Clinical Relevance: A mouse model mimicking birch pollen induced cross-reactivity to Mal d 1 was successfully established. In this model, birch pollen immunotherapy significantly ameliorated the anaphylaxis induced by Mal d 1. Our experimental data suggest that boosting of Mal d 1 recognizing immunoglobulins by BP SCIT is important for the amelioration of apple allergy in human.

INTRODUCTION

Pollen-related food allergy is the most common food allergy in individuals in Northern and Central Europe [1]. It is estimated that approximately 70% of patients with birch pollen (BP)

allergy experience allergic symptoms after consumption of apple, celery, hazelnuts, or other BP-related foods [2-5]. However, sensitization to Bet v 1 usually does not trigger allergic reactions to all foods that contain Bet v 1-related allergens but is associated with the presence of IgE recognizing the specific homologue [6]. It is well-established that this co-morbidity is caused by IgE cross-reactivity between the major BP allergen Bet v 1 and its homologues in fruits, tree nuts and some vegetables, all belonging to the so-called PR-10 (pathogenesis-related protein family 10) proteins. This family of cross-reactive food allergens, such as for example Mal d 1 from apple, is very sensitive to gastro-intestinal digestion and is therefore considered not to be capable of inducing primary sensitization via the gut immune system. For similar reasons systemic reactions are extremely rare with patients almost exclusively reporting mild oral symptoms. Respiratory exposure to birch (and other related tree) pollen is responsible for primary sensitization to PR-10 allergens. Bet v 1 and Mal d 1 share 65% amino acid sequence identity, and as a consequence, IgE antibodies against Bet v 1 cross-react to Mal d 1 [7], leading to (mild) clinical apple allergy in a significant number of BP allergic patients [8;9].

Despite the clear link between Bet v 1-Mal d 1 cross-reactivity and the high prevalence of apple allergy amongst BP allergic patients, there are scarce and conflicting reports as to whether BP immunotherapy is able to relieve allergic symptoms to apple in these patients. Whereas in some studies there is a beneficial effect [10-13], there is limited or no evidence for such an improvement in other studies [14-17]. Evidence in favour or against improvement after immunotherapy for cross-reactive food allergies is both present. An animal model to study the immunological cross-reactivity between Bet v 1 and Mal d 1, and in particular of the impact of birch pollen immunotherapy thereon, may contribute to a better understanding of the conflicting human reports. To that end, we adapted our recently published murine model of birch pollen allergy and immunotherapy to accommodate studying cross-reactivity and hypersensitivity to apple Mal d 1 and the effect of immunotherapy. In this model, mice are exposed to birch pollen extract (BPE) resulting in all the hallmarks of BP allergy, like eosinophilia, BPE- and Bet v 1-specific IgE, airway inflammation and airway hyper-responsiveness. More importantly, without exposure to Mal d 1, these mice developed a cross-reactive IgE response to Mal d 1 over time. In addition, we could demonstrate a clinical (anaphylactic) response (temperature drop) upon exposure to

Mal d 1. In this murine model of birch pollen cross-reactivity and hypersensitivity to Mal d 1, we established that immunotherapy ameliorates these anaphylactic symptoms induced by Mal d 1. This was associated with a shift in the ratios of both Bet v 1 as Mal d 1 specific IgG over IgE and a shift towards IL-10 production over Th2 cytokines IL-5 and IL-13.

METHODS

Animals. Female Balb/c (Harlan, Horst, The Netherlands), were housed under specific pathogen-free conditions at the animal facility of the AMC. All experiments were approved by the animal ethics committee of the AMC, The Netherlands.

Sensitization protocols. Sensitization of Balb/c mice to birch pollen was performed as described earlier [18]. Mice were sensitized intraperitoneally (i.p.) with 8.2 µg (dry weight) birch pollen extract (BPE) (kindly provided by HAL Allergy BV, Leiden, The Netherlands), containing 1 µg Bet v 1 and an endotoxin level of 2.84 EU/mg (determined by the limulus amebocyte lysate assay; Indoor Biotechnologies Inc., Cardiff, UK), adsorbed to 1 mg alum (Imject alum, Pierce) on days 0, 7, 14. Under isoflurane anaesthesia (to avoid unwanted swallowing of the intranasally administered fluid, and instead facilitate it to effectively reach the airways), mice were subsequently challenged intranasally (i.n.) with 100 µg BPE (equivalent to 12 µg Bet v 1) in 30 µl PBS or PBS as a control on days 21, 22 and 23. To assess potential cross-reactive immunological and clinical responses to Mal d 1, mice were challenged i.p. with 120 µg recombinant Mal d 1 (rMal d 1.0108 was produced as previously described with an endotoxin level of <1 EU/mg [19]) or as a positive control with 1 mg BPE (containing 120 µg Bet v 1) at day 31. The intraperitoneal route of exposure to Mal d 1 was chosen because intragastric challenge would not result in a clinical response due to the immediate digestion of Mal d 1 in the gut. At day 41, all mice were challenged i.p. with 60 µg Mal d 1. Serum was collected at day 30 and 38 (fig. 1A). To establish that allergen-induced inflammation in general is not sufficient for the development of an IgE response (cross-) reactive to Mal d 1, mice were sensitized with 10 µg house dust mite (HDM) extract (Greer Laboratories, Lenoir, NC, USA) with 1 mg alum i.p. and challenged with 100 µg HDM for the intranasal challenges. BP-sensitized mice and PBS control mice (according to the above described protocol) were used as a positive and negative control, respectively. All mice received 25 µg Mal d 1 i.p. at day 31 and day 41. Serum was collected at day 30 and 42.

To establish whether multiple BPE challenges, mimicking prolonged seasonal exposure, would result in a Mal d 1 IgE response, mice were sensitized i.p. with 8.2 µg BPE adsorbed to 1 mg alum at day 0, 7, 14 but were exposed fifteen times (instead of 3 times) to 100 µg BPE i.n. on days 3,4, 7-11, 14-18, 21-23. A subgroup of mice was sacrificed for analysis two days

later at day 25, while another group was rechallenged after a period of rest with 3 consecutive daily i.n. exposures of 100 µg BPE at day 57, 58, 59. Serum was collected two days later at day 61 (Experimental schedule in fig. 5A). PBS control mice received the same treatment but without BPE.

BP immunotherapy protocol. Mice were sensitized and challenged with BPE as described above. At day 35 mice started subcutaneous BP immunotherapy (BP SCIT) [18], that is nine weekly subcutaneous (s.c.) injections with 0.3 mg BPE adsorbed to 1 mg alum or PBS. Mice were either re-challenged two weeks after the last s.c. injection with (i) a 30 min 1% (w/v) BPE aerosol for three consecutive days (aerosols rather than i.n. instillations were applied because of enhanced decrease in AHR by BP SCIT as observed in previous experiments) or (ii) an i.p. injection with 120 µg Mal d 1 followed by a second injection of 60 µg Mal d 1 ten days later. Mice were sacrificed 1 day later.

***Ex vivo* restimulation of splenocytes.** Splenocyte suspensions were plated in 96-well round bottom plates at a density of $2x10^5$ cells per well and were re-stimulated for 4 days with 12 µg/ml Bet v 1 (Biomay, Vienna, Austria) or 12 µg/ml Mal d 1. Supernatants were analysed for IL-4, IL-5, IL-10, IL-13 and IFNγ production by ELISA (Ready-set-go!, eBioscience Inc via Immunosource, Halle-Zoersel, Belgium).

Measurement of MMCP-1, serum immunoglobulins and cross-reactivity. Serum was analysed for MMCP-1 (Mouse Mast Cell Protease-1), Bet v 1- and Mal d 1-specific IgE, IgG_1 and IgG_{2a} antibodies by ELISA (MMCP-1: Ready-SET-Go kit, eBioscience Inc., Immunosource, Halle-Zoersel, Belgium, IgE, IgG_1: Opteia, BD, San Diego, USA, IgG_{2a}: eBioscience), following the manufacturers' instructions. To determine allergen specific immunoglobulins, 96-well NUNC plates were coated overnight at 4ºC with 100 µl of 1 µg/ml of either Bet v 1 or Mal d 1. As reference, a standard curve of MMCP-1, total murine IgE, IgG_1 or IgG_{2a} was used. To evaluate cross-reactivity of IgE between Bet v 1 and Mal d 1, pooled serum from five Mal d 1-positive BP-sensitized/Mal d 1-exposed mice was incubated for 30 minutes at room temperature with 20 µg/ml of recombinant Bet v 1 or Mal d 1 (or PBS for uninhibited control), prior to adding the mixture to Mal d 1 or Bet v 1-coated plates. The rest of the ELISA was performed according to the manufacturer's instructions.

Airway hyper-reactivity (AHR). AHR to inhaled methacholine was measured by barometric plethysmography in conscious mice (EMKA Technologies). Non-specific responsiveness was measured by exposing mice to aerosolized PBS to set a baseline value, followed by increasing concentrations of aerosolized methacholine (3.125, 6.25, 12.5, 25 and 50 mg/mL in PBS for 3 min; Sigma-Aldrich, Zwijdrecht, The Netherlands) using ultrasonic nebulizers. PenH (enhanced pause) values were measured during 5 min after each methacholine aerosol.

Core body temperature. The core body temperature was measured with a hand-held device with a mouse rectal probe (Physitemp Instruments Inc, Clifton, NJ, USA) every 10 minutes after i.p. challenge with BPE or Mal d 1 as a measure for allergic anaphylaxis [20].

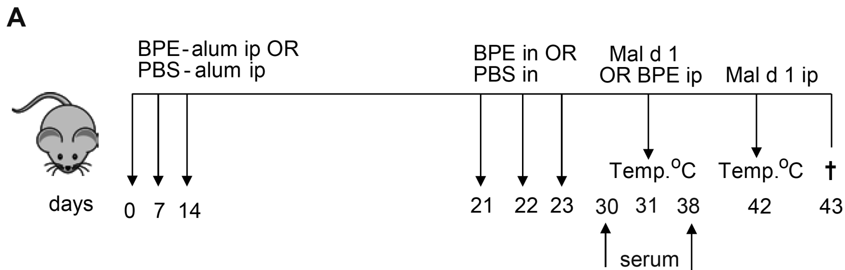
Statistical analysis. For statistical analysis, Mann-Whitney U test was performed for all analyses, except Wilcoxon signed rank test was used to analyse the drop in core body temperature in relation to the baseline measurement (0 min) in fig 4C. All experiments were performed at least 2 times with 5 or more animals per group. Differences were considered to be significant at $p < 0.05$.

RESULTS

Systemic Mal d 1 exposure enhances Mal d 1 cross-reactivity in birch pollen allergic mice

An established model to induce birch pollen sensitization and airway hyper-reactivity [18] was used to evaluate whether cross-reactivity to Mal d 1 was observed and whether this was accompanied by an anaphylactic hypothermia after systemic challenge (fig. 1A). Sensitization to BP resulted in a significant IgE response to Bet v 1 and a very weak but not significant IgE response to Mal d 1 (fig. 1B). The limited degree of cross-reactivity to Mal d 1 was in concordance with the lack of hypothermia upon i.p. exposure to Mal d 1 (BP-Mal d 1), whereas injection with the experimental positive control BPE (BP-BP) did induce such a clinical response (fig. 1C). Subsequent analysis of serum for Mal d 1-recognizing IgE showed that only BP-sensitized mice exposed to Mal d 1 (BP-Mal d 1) had a clear increase in Mal d 1 IgE while sham-sensitized mice (PBS-Mal d 1) did not (fig. 2A). A systemic challenge with BPE, containing a similar level of Bet v 1 in comparison to Mal d 1 challenge, did not boost the Mal d 1 IgE response in BP-sensitized mice (BP-BP) nor was the level of Bet v 1 IgE increased (fig. 2B). A second challenge with Mal d 1 in all mice, induced hypothermia exclusively in the BP-sensitized mice which had developed Mal d 1 IgE after the first Mal d 1 injection (fig. 2C). The rapid induction of Mal d 1 IgE in BP-sensitized mice after a single systemic Mal d 1 injection suggested a cross-reactive phenomenon, rather than a primary sensitization to Mal d 1. Cross-reactivity was confirmed by ELISA inhibition: pre-incubation of serum with Bet v 1 completely inhibited IgE binding to Mal d 1, while Mal d 1 incubation could only partially inhibit Bet v 1 IgE (fig. 2D). Together these inhibition assays indicate that a part of the Bet v 1 IgE repertoire was cross-reactive to Mal d 1.

To determine whether the induction of Mal d 1 IgE was allergen-dependent or rather inflammation-dependent, mice sensitized to BP, HDM or PBS were challenged i.p. to Mal d 1 twice. Serum analysis before challenge with Mal d 1 showed no Mal d 1 IgE in all groups. BP- and HDM-sensitized mice had an increased total and allergen-specific IgE compared with PBS control mice but only mice sensitized to BP developed Mal d 1 recognizing IgE (fig. 2E and suppl. fig. 1).



Groups	Sensitization D 0-7-14	Challenge D21-22-23	First systemic challenge D31	Second systemic challenge D42
PBS-BP/Mal d 1	3X PBS alum	3X PBS in	BPE ip	Mal d 1 ip
PBS/2x Mal d 1	3X PBS alum	3X PBS in	Mal d 1 ip	Mal d 1 ip
BP-BP/Mal d 1	3X BPE alum	3X BPE in	BPE ip	Mal d 1 ip
BP/2x Mal d 1	3X BPE alum	3X BPE in	Mal d 1 ip	Mal d 1 ip

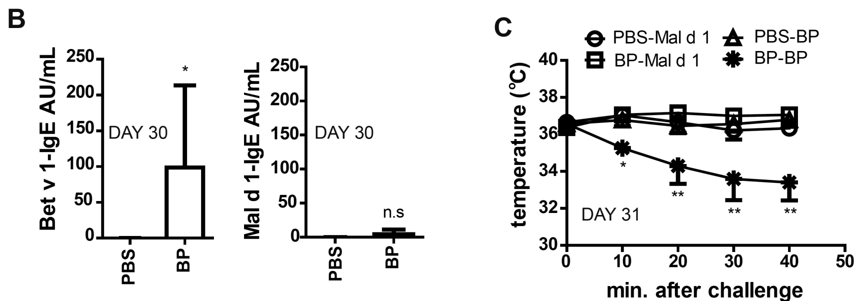


Figure 1. (A) Experimental design BP allergy model and Mal d 1 exposure. BP-sensitized/ challenged and PBS control mice were challenged i.p. with either BPE or Mal d 1 at day 31. All groups were challenged i.p. with Mal d 1 at day 42. Table summarizes treatment received by each group. (B) Serum level of IgE antibodies that recognize Bet v 1 or Mal d 1 antigen (at day 30), one day before Mal d 1 exposure. (c) Core body temperature measurements after the first Mal d 1 or BPE i.p. challenge (day 31). Data are presented as means ± SEM, *P<0.05, **P<0.01 vs PBS control.

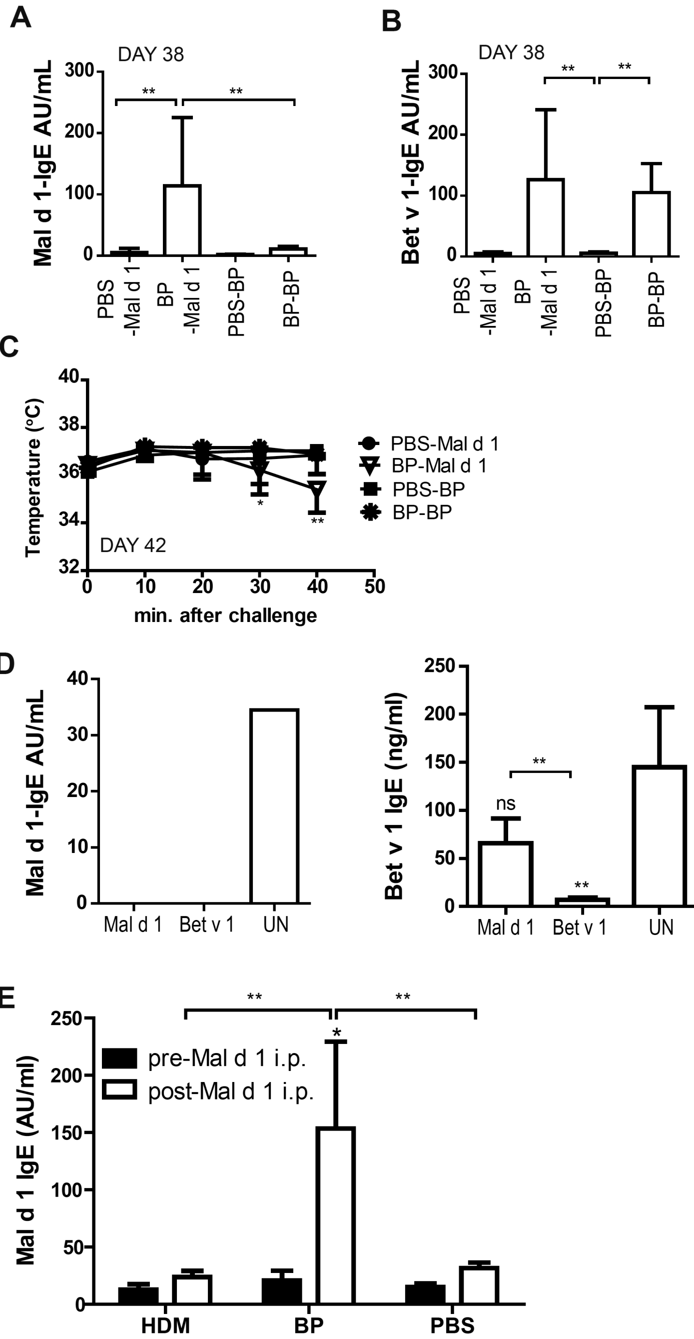

A
DAY 38
Mal d 1-IgE AU/mL
PBS -Mal d 1
BP -Mal d 1
PBS-BP
BP-BP
B
DAY 38
Bet v 1-IgE AU/mL
C
Temperature (°C)
DAY 42
min. after challenge
PBS-Mal d 1
BP-Mal d 1
PBS-BP
BP-BP
D
Mal d 1-IgE AU/mL
Mal d 1
Bet v 1
UN
Bet v 1 IgE (ng/ml)
ns
E
Mal d 1 IgE (AU/ml)
pre-Mal d 1 i.p.
post-Mal d 1 i.p.
HDM
BP
PBS

Figure 2. (A) Serum levels of IgE antibodies that recognize Mal d 1 at day 38, one week after first Mal d 1 injection. (B) Bet v 1 specific IgE in serum at day 38. (c) Core body temperature measurements after Mal d 1 second i.p. challenge (day 42). (D) Assay for detection of cross-reactivity between Mal d 1- and Bet v 1-specific IgE antibodies. A pooled serum sample containing Mal d 1-recognizing IgE was pre-incubated with Mal d 1 or Bet v 1 or not pre-incubated (UN) before detection of Mal d 1-recognizing IgE or Bet v 1-specific IgE by ELISA. (E) Mal d 1-recognizing IgE levels in serum of HDM-sensitized, BP-sensitized and control PBS mice, before (pre-Mal d 1 ip) and after (post-Mal d 1 ip) injection with Mal d 1 at day 31 and day 41 respectively. Data are presented as means ± SEM, *P<0.05, **P<0.01 vs pre-Mal d 1 i.p. control or as indicated.

No cross-reactive T cell response to Mal d 1 in birch pollen allergic mice

The cytokine production in response to Bet v 1 was determined by *ex vivo* re-stimulation of splenocytes. As expected, BP-sensitized/challenged mice had a significant increased IL-4, -5, -13, -10 and IFN-γ production after *ex vivo* re-stimulation with Bet v 1 compared with their PBS control groups (fig. 3, black bars). To investigate the cross-reactive T cell response, the T cell response to Mal d 1 was determined in BP-sensitized mice in comparison to sham-sensitized mice. In mice systemically exposed to Mal d 1 once or twice, either in sham or BP sensitized mice, no significant levels of IL-4, IL-5, IL-13 and IL-10 with the exception of IFN-γ, were induced in response to Mal d 1 *in vitro* restimulation (fig 3, white bars). This indicates that Mal d 1 was not able to induce a significant cross-reactive T cell response in BP sensitized mice and excludes the possibility of primary sensitization to Mal d 1.

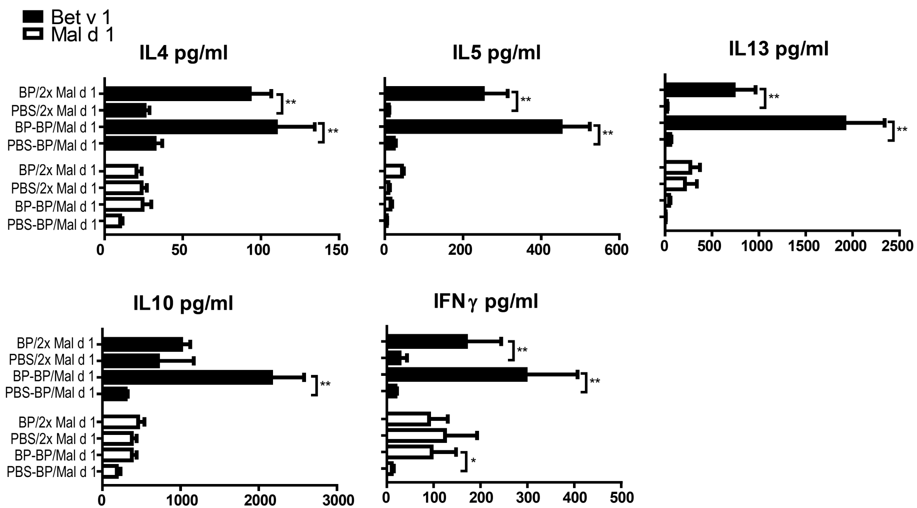


Figure 3. BP-sensitized/challenged and PBS control mice were challenged i.p. with either BPE or Mal d 1 at day 31. All groups were challenged i.p. with Mal d 1 at day 42. One day later splenocytes were restimulated *ex vivo* with (A) Bet v 1 or (B) Mal d 1. Data are presented as means ± SEM, ns: non-significant, *P<0.05, **P<0.01.

BP SCIT protects against an anaphylactic response to systemic challenge with Mal d 1

We questioned whether subcutaneous birch pollen immunotherapy (BP SCIT) is not only effective in down-regulating airway hyper-reactivity after BPE exposure [18] but also in down-regulating Mal d 1 induced anaphylaxis in our BP allergy mouse model. BP-sensitized mice were treated with “BP SCIT” (1x BPE/alum s.c./wk for 9 weeks) or “No SCIT” (1x PBS s.c./for 9 weeks), before they were either challenged with (i) three consecutive daily BPE aerosols or (ii) with two Mal d 1 systemic injections (fig. 4A). As reported before [18], BP SCIT treatment inhibited development of methacholine-induced airway hyper-reactivity (fig. 4C). In BP SCIT mice, exposure to BPE aerosols induced BP-specific IgG_1 and IgG_{2a} in favour of IgE, and vice versa in No SCIT mice (suppl. fig. 2). In response to BPE aerosols, also serum IgE against Mal d 1 was significantly inhibited in the therapy group compared to the sham (“No SCIT”)-treated group ($p<0.001$), whereas IgG_1 and IgG_{2a} had been effectively induced by the therapy and aerosol exposure (fig. 4B). To investigate the effect of BP SCIT on the suppression of Mal d 1 induced hypothermia, mice were injected with Mal d 1 instead of BPE aerosols. BP SCIT- and No SCIT-treated mice both experienced a significant drop in body temperature upon their first i.p. challenge with Mal d 1 (fig. 4D/left panel). Upon a second challenge with Mal d 1 a week later, none of the BP SCIT-treated mice developed hypothermia anymore whereas 3/5 sham-treated mice still developed a clear anaphylactic reaction (fig. 4D/right panel and suppl. fig. 3). Serum Mal d 1-recognizing IgE, IgG_1 and IgG_{2a} levels did not differ significantly between BP SCIT- and No SCIT-treated mice, although Mal d 1-specific IgE tended to be higher in the No SCIT-treated and IgG_1 and IgG_{2a} in the BP SCIT-treated mice. When analysed as ratios of IgG over IgE, for both IgG subclasses, ratios were significantly higher in BP SCIT-treated mice (fig. 4E). As a marker of mast-cell degranulation, the Mouse Mast Cell Protease-1 (MMCP-1) level in serum showed a clear trend ($p=0.055$) to be higher in No SCIT-treated than in BP SCIT-treated mice (fig. 4F). Splenocytes stimulated with Mal d 1 from BP SCIT-treated mice produced slightly less IL-4, IL-5 and IL-13 and more IL-10, but none reached a significant difference (fig. 4G, left panel). When expressed as ratios (IL-10/IL-4, IL-10/IL-5 and IL-10/IL-13), these were significantly higher in case of IL-5 and IL-13 in the BP SCIT-treated group (fig. 4G, right panels). Overall, in a mouse model of BP-immunotherapy, a Mal d 1-specific anti-inflammatory immune response including

blocking antibodies was induced, which was associated with an inhibition of Mal d 1-induced anaphylaxis.

Figure 4

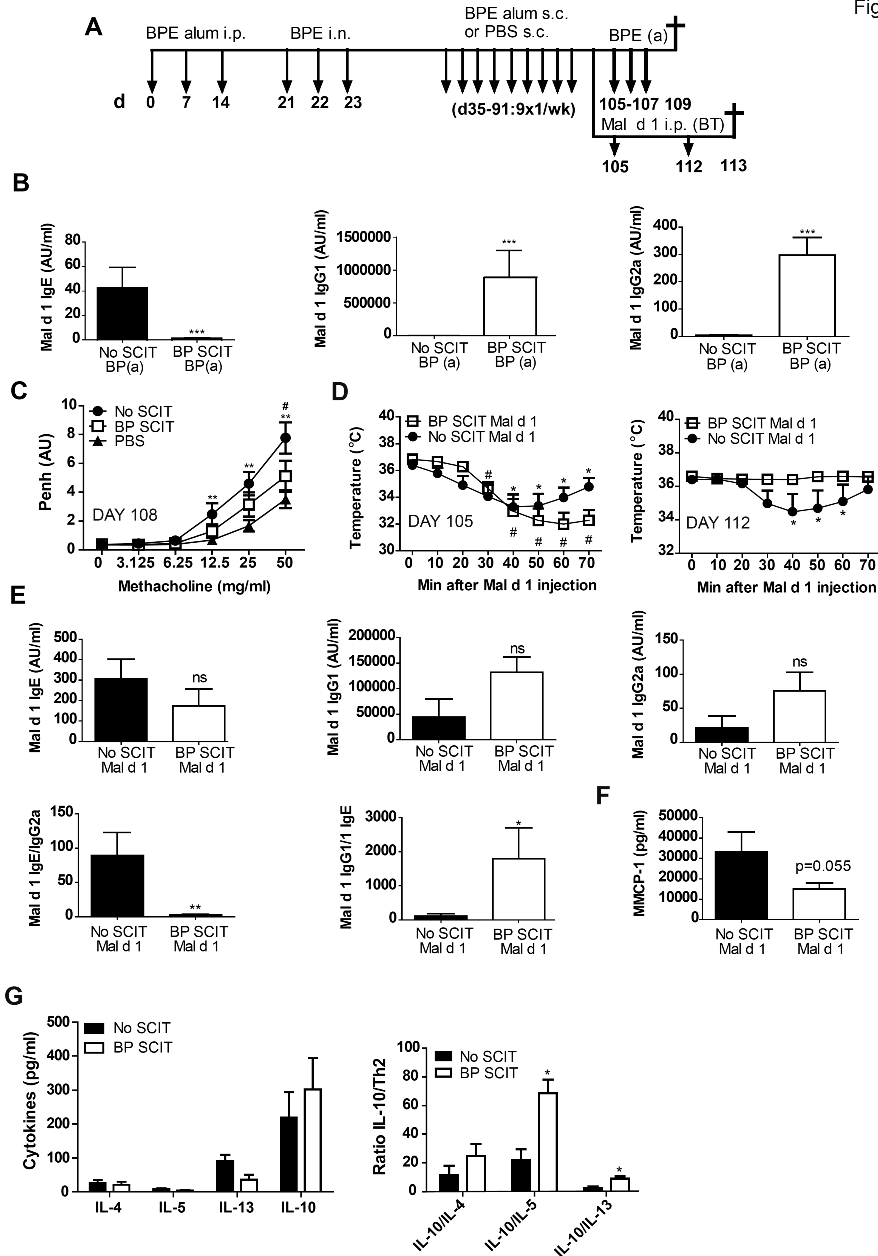


Figure 4. (A) Experimental design for Mal d 1 exposure after BP SCIT (9 x 0.3 mg BPE/alum s.c.) treatment. (B) Mal d 1-recognizing IgE, IgG_1 and IgG_{2a} levels in serum in BP-sensitized/challenged mice treated with BPE alum s.c. (BP SCIT) or PBS s.c. (No SCIT) and challenged with 3 BPE aerosols. (C) Airway hyper-reactivity to methacholine. (D) Core body temperature of BP SCIT- or No SCIT-treated mice exposed to Mal d 1 i.p. at day 105 (left panel) and day 112 (right panel). (E) Mal d 1 IgE, IgG_1 and IgG_{2a} in serum (upper panels). Ratios between Mal d 1 recognizing IgE vs IgG_1 and IgG_{2a} (lower panels) (F) MMCP-1 in serum. (G) Cytokine production by splenocytes after *ex vivo* re-stimulation with Mal d 1 (left panel) and calculated ratios between IL-10 vs IL-4, IL-5 and IL-13 (right panel). Data are presented as means ± SEM, *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs “No SCIT” mice or PBS (fig. C) or vs 0 min (fig. D).

A repeated exposure to BPE after a period of rest results in an increased cross-reactivity to Mal d 1

Surprisingly, we observed that “No SCIT” mice developed Mal d 1 IgE after exposure to BPE aerosols (fig. 4B). This seemed to be in contrast with the lack of Mal d 1 IgE in serum seen in BP-allergic mice, shortly after the last BP challenge (fig. 1B). It is speculated that in pollen-sensitized individuals cross-reactivity occurs naturally after long term exposure to several birch pollen seasons. To explore whether an increased number of intranasal challenges or a period of rest before re-challenge would increase the cross-reactivity, BP-sensitized mice received 15 intranasal challenges with BPE i.n. (5x/wk) and were re-challenged thirty-four days later with 3 BPE i.n. challenges (fig. 5A). The level of Bet v 1 and Mal d 1 recognizing IgE was analysed after the 15xBPE exposure (at day 25) and one month later, after the re-challenge (at day 61). Directly after 15x intranasal challenges, the level of Bet v 1 IgE was significantly different from the PBS group while there was no significant increase in Mal d 1 recognizing IgE (fig. 5B/C). However, after re-challenge with BP after a period of rest, Mal d 1 recognizing IgE was induced. This response was accompanied with a clear trend ($p=0.0632$) for an increased Bet v 1 IgE response (fig. 5C).

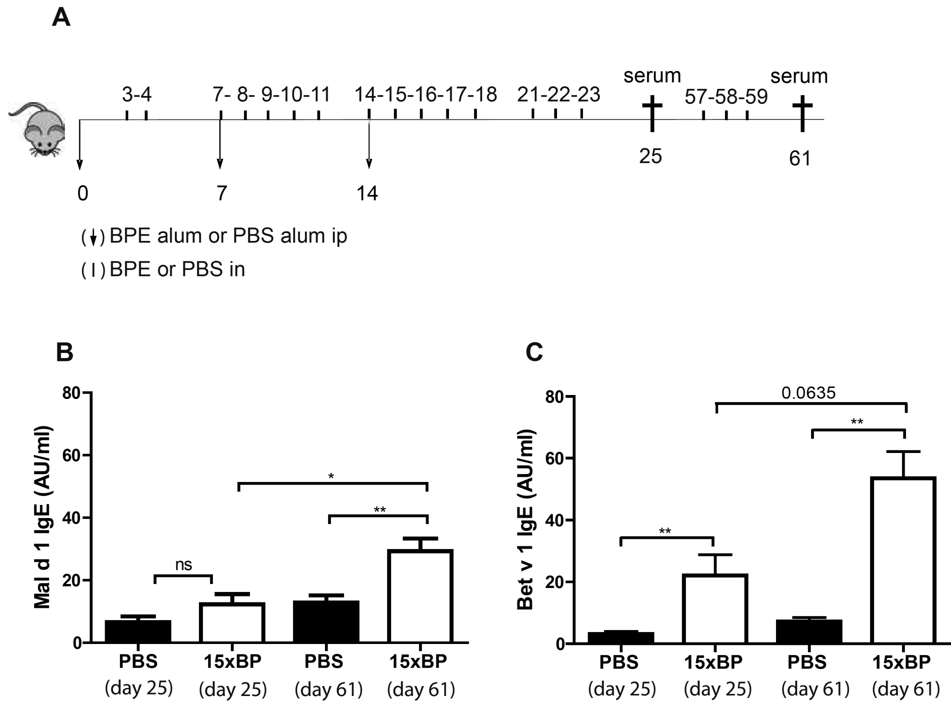


Figure 5. (A) Experimental design for prolonged i.n. BPE exposure. Arrows indicate i.p. injections and bars (I) indicate i.n. treatment. (B) Mal d 1-specific antibodies in serum at day 25 and day 61. (C) Bet v 1-specific IgE in serum at days 25 and 61. Data are presented as means ± SEM, *P<0.05, **P<0.01, ns: non-significant.

DISCUSSION

In this study, we demonstrate that cross-reactivity to Mal d 1 can be induced in a mouse model for BP allergy in the absence of exposure to Mal d 1. The induced Bet v 1 IgE response was initially not accompanied by a Mal d 1-recognizing IgE response but developed when mice were re-challenged with BP after a month rest. Also a systemic exposure to Mal d 1 in BP allergic mice induced a rapid induction of Mal d 1 IgE and mice acquired sensitivity to a Mal d 1 induced anaphylactic hypothermia. BP SCIT treatment induced a shift in antibody ratio of Mal d 1 IgG over Mal d 1 IgE and inhibited this anaphylactic response to a systemic challenge with Mal d 1.

The detection of Mal d 1 IgE in our BP allergy model demonstrated that cross-reactivity of Bet v 1 IgE to Mal d 1 can develop in the absence of exposure to Mal d 1. However, exposure to a single Mal d 1 injection boosted cross-reactivity of Bet v 1 IgE in BP allergic mice rapidly but not in PBS control mice or HDM allergic mice. Primary sensitization to Mal d 1, rather than cross-reactivity, is not likely, because the increase in Mal d 1 IgE was not accompanied by a Mal d 1 specific T cell response. In addition, cross-reactivity was confirmed by the *in vitro* inhibition of 100% of Mal d 1 reactive IgE antibodies found in BP sensitized mice exposed to Mal d 1, by Bet v 1 protein. We speculate that Mal d 1 exposure boosted specifically the Mal d 1 recognizing Bet v 1 IgE response within the Bet v 1 IgE repertoire, recognizing different epitopes of the Bet v 1 allergen. In support, pre-incubation of serum with Mal d 1 inhibited detection of Bet v 1 specific IgE only partially [21;22]. Surprisingly, systemic injections with either Mal d 1 or BPE (containing a corresponding concentration Bet v 1) were equally capable of inducing Bet v 1 IgE, but only Mal d 1 specifically boosted Mal d 1-reactive IgE. Perhaps the Bet v 1 isoform composition is less optimal to boost the cross-reactive IgE response than a similar quantity of Mal d 1 which is for 100% containing the cross-reactive epitopes. We used BPE rather than Bet v 1 for sensitization to mimic human exposure to BP. In earlier experiments, we demonstrated that sensitization with BPE was more efficient in the induction of IgE and IgG_1 against Bet v 1 compared with sensitization with Bet v 1 [18]. To study Bet v 1-specific cross-reactive immunoglobulins to Mal d 1, we therefore chose to sensitize with BPE. It is tempting to speculate that the composition (adjuvant activity, proteolytic activity among other factors) of whole birch pollen extract

might facilitate effective sensitization to the allergen Bet v 1. In this model we used an i.p. injection with Mal d 1 to study the anaphylactic response, which is not the common route for a food allergen. Unfortunately, the oral allergy syndrome cannot be assessed in mice and Mal d 1 is rapidly degraded in the gastric route, which prohibits the use of the physiological path. The development of hypothermia as a marker for an anaphylactic response is often used in food allergen models [20]. Two different pathways of anaphylaxis have been characterised in mice: a classical pathway consisting of antigens, IgE and histamine and an alternative pathway consisting of IgG_1-antigen immune complexes and platelet-activating factor [24]. We did not investigate in depth the relative contribution of these pathways in the anaphylactic hypothermia observed after systemic Mal d 1 exposure but the decreased level of MMCP-1 in BP SCIT-treated mice is indicative of a suppressed Mal d 1-recognizing IgE-mediated mast cell degranulation [25]. Protection of BP SCIT mice by tachyphylaxis of IgE-mediated signals on mast cells, resulting in depletion of the histamine pool does not seem likely [26]. “No SCIT” mice developed a similar drop in temperature after the second injection, in which mast cells also should have undergone tachyphylaxis as well after the first injection.

Remarkably, BP SCIT-treated mice were not immediately protected, as they developed hypothermia after the first Mal d 1 injection. We speculate that the first systemic challenge was needed to boost the protective Mal d 1 cross-reactive IgG_1 and IgG_{2a}. In support, in a previous experiment we observed that in BP-sensitized and -challenged mice, which were not exposed to BP for a month, intranasal exposure to BPE boosted the immunoglobulin levels in serum (Suppl. fig 4). We speculate that BPE aerosols induced the different immunoglobulin pattern in BP SCIT mice in comparison to the No SCIT mice, which was associated with resistance to airway hyper-reactivity. In analogy to that, we hypothesize that the first Mal d 1 injection was needed to boost the Mal d 1 recognizing Bet v 1 specific immunoglobulins, which were induced by the BP SCIT treatment. This needs to be confirmed in a future kinetic experiment following the Ig expression before and after Mal d 1 injection in BP SCIT-treated mice. In support of our experimental findings, also in human BP SCIT studies the induction of Mal d 1 cross-reactive immunoglobulins in addition to the induced Bet v 1 IgE and IgG_4 seems to be related to the efficacy to improve OAS to apple [11;16;17]. It is tempting to speculate that an additional injection of Mal d 1 could potentiate the

protective effect of BP SCIT on apple allergy by boosting the Mal d 1 recognizing immunoglobulins in the Bet v 1 repertoire. An explanation for the inconsistent effect of BP SCIT on cross-reactive apple allergy in human might be found in the allergenic epitopes in the used BP SCIT formulation. We hypothesize that the degree of induction of Mal d 1 recognizing (and protective) IgG_4 within the Bet v 1 IgG_4 repertoire is likely to be involved in the amelioration of apple allergy. However, it is important to note that besides induction of Mal d 1-recognizing IgG_4 other pathways are likely to be involved in suppressing OAS. BP allergic patients, which were exposed to increasing amounts of fresh apple by daily oral intake, became desensitized, albeit transiently. No differences could be detected in Mal d 1 specific IgE/IgG_4 ratios in the desensitized patients [27]. In a BP SCIT study, OAS to apple decreased very rapidly after starting BP SCIT treatment, even before Mal d 1 IgG_4 was induced [11]. These unknown underlying mechanisms might contribute to the quite variable outcome on apple allergy by BP SCIT in human as well. In conclusion, we developed a mouse model to study cross-reactivity to Mal d 1 by Bet v 1 specific B and T cells in birch pollen allergy and during birch pollen specific immunotherapy. Our results suggest that systemic exposure to Mal d 1 boosts the Mal d 1 recognizing Bet v 1 immunoglobulins within the Bet v 1 Ig repertoire. This study contributes to the insight in the underlying mechanism involved in the efficacy of BP SCIT to ameliorate birch pollen related apple allergy.

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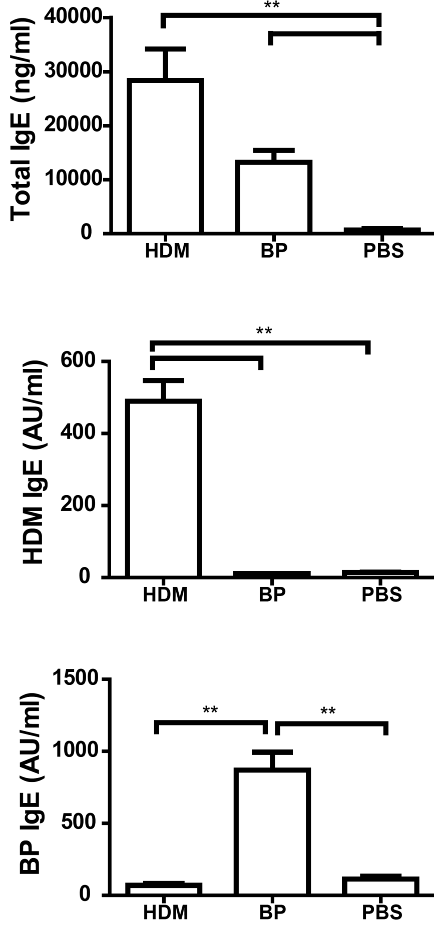
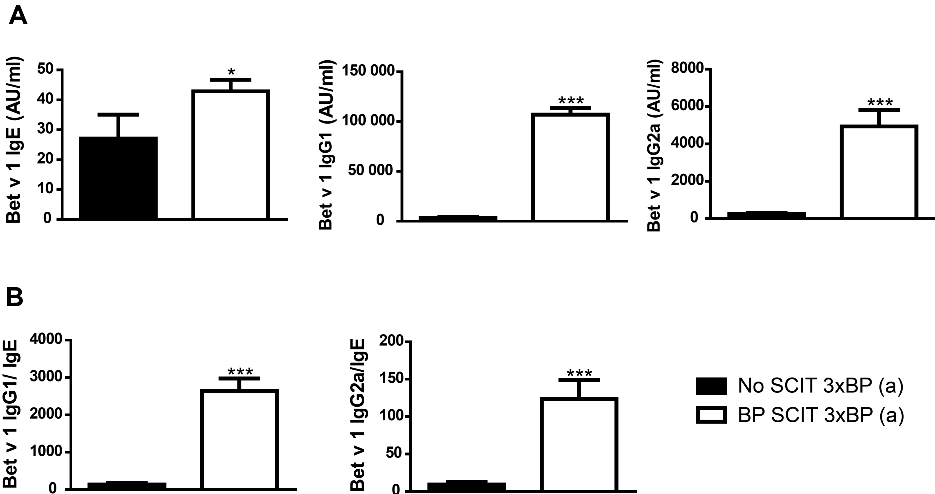
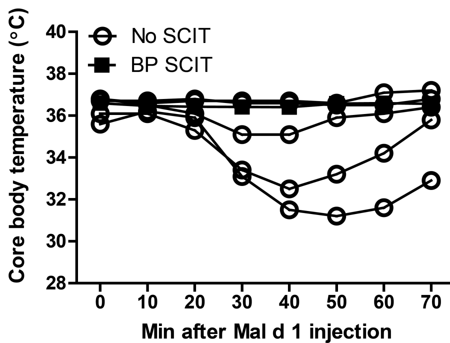


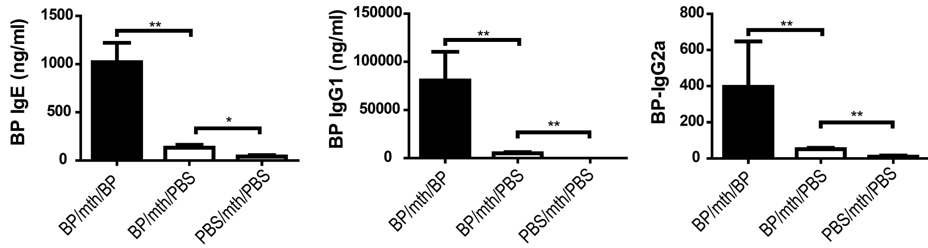
Supplementary Figure 1. Total, HDM-specific and BP-specific IgE in serum of HDM-, BP-sensitized/challenged or PBS control mice after two Mal d 1 i.p. injections. Data are presented as means ± SEM, $*P<0.05$, $**P<0.01$.



Supplementary Figure 2. (A) Bet v 1-specific IgE, IgG_1 and IgG_{2a} levels in serum in BP SCIT-treated mice and No SCIT-treated mice after exposure to 3 BPE aerosols. (B) Calculated ratios of Bet v 1 specific IgE over IgG_1 or IgG_{2a}.



Supplementary Figure 3. Individual core body temperature curves of BP SCIT- or No SCIT-treated mice after a second injection with Mal d 1 i.p.



Supplementary Figure 4. BP-specific IgE, IgG_1 and IgG_{2a} in serum of BP-sensitized/challenged mice which were re-challenged one month later with three intranasal challenges with BPE or PBS. As a control, sham sensitized/challenged mice were re-challenged with three intranasal challenges with PBS. Serum was analysed two days later. Data are presented as means ± SEM, *P<0.05, **P<0.01.

Chapter 8

Discussion and conclusions

Allergic sensitization is a complex process that involves diverse mechanisms and immune pathways that can be redundant or complementary. It is impossible to elect a sole key mechanism in such a complex and heterogeneous phenomenon that is dependent on multiple individual genetic differences and diverse external factors. The use of mouse models is indispensable to study allergic diseases, providing an important complementary tool in providing clues to the advance of human studies. One of the challenges with the development of such mouse models is the need for mimicking human-like clinical symptoms that can be used as readouts. In this thesis, we used well-established mouse models but we also developed murine models to study the mechanisms involved in the initiation of allergic responses, the immunological interplay between allergies in polysensitized individuals, and the mechanisms of protection elicited by immunotherapy. The main findings of this thesis are (i) the indication that proteins involved in anti-oxidant protective pathways such as HO-1 and Nrf2 can be used as indicators of susceptibility to atopy development; (ii) we showed for the first time that HDM-induced IL-33, an innate cytokine produced by airway epithelial cells and inflammatory cells is critical for IgE production; (iii) we developed/reported for the first time mouse models mimicking polysensitization and cross-reactivity, which are allergic conditions frequently observed in humans. In the polysensitization model, a clear distinct allergic response to two unrelated and clinically relevant allergens (peanut and HDM) in different compartments of the body was induced in mice; and in the cross-reactivity model, a prolonged exposure protocol to birch pollen allergens induced a cross-reactive allergic response to apple allergen Mal d 1.

In our experimental HDM allergy model, we demonstrated an interesting association between allergic sensitization and the inability to induce antioxidant proteins [1]. This represents a rapid and protective mechanism in response to oxidative stress [2]. In different strains of mice we demonstrated that mice with a very low antioxidant response upon HDM exposure were susceptible to sensitization to HDM, while a mouse strain with a strong antioxidant response was protected against HDM sensitization. Also in a human cohort studying the induction of an occupational allergy, a similar pattern was observed. Individuals with a poor capacity to upregulate the expression of molecules involved in the antioxidant defence mechanisms such as Nrf2 and HO-1 combined with high basal levels of oxidative stress had an enhanced susceptibility for the development of atopy (**Chapter 2**). This study draws the attention to the role of ROS as potential early pathway in the sparking off a cascade of events that leads to allergic sensitization. Although our data strongly indicate that ROS can prime the immune system for allergic sensitization, the exact mechanisms and pathways by which ROS activate the immune system in the context of allergic responses remain to be elucidated. Recent studies provide evidence that ROS have a direct effect on T cells and DCs, favouring a Th2 response [3-5]. However, also allergens can become more immunogenic after endogenous exposure to ROS. Reactive carbonyls, produced as a result of oxidative stress, can covalently tag inert endogenous but also exogenous proteins alerting the immune system [6]. Among a multitude of oxidation related adducts, only reactive carbonyls have been demonstrated to enhance the Th2 response by increasing allergen uptake by DCs [6]. This process can contribute to the immunogenicity of allergens that are able to induce oxidative stress and which are subsequently labelled with reactive carbonyls. The supporting studies for a role of ROS in allergic sensitization have been reviewed in **Chapter 3**.

An important alarmin involved in the inflammatory response to inhaled allergens is the innate cytokine IL-33 [7-12]. This cytokine is recognized via the ST2-receptor on many effector cells in the allergic response. One of the most important mechanisms is the skewing of allergen presenting DCs to induce differentiation of naïve T cells into Th2 cells. In an adoptive transfer model with HDM allergens pulsed DCs, we were able to circumvent direct exposure of the airways to HDM. HDM exposure in the airways results in IL-33 release, but this was not induced in this model. Surprisingly, mice which were sensitized by transferring

allergen-pulsed DCs directly into the airways were capable of inducing a full-blown inflammation and Th2 response but did not have an immunoglobulin response. Administration of recombinant IL-33 was sufficient to restore the antibody response as seen after direct exposure to HDM (**Chapter 4**). This study shows the importance of the IL-33/Th2 axis for the allergen-induced humoral immune response. The release of IL-33 by the airway epithelium or by other innate immune cells in response to HDM allergens seems to be a crucial factor for immunoglobulin production. In the context of allergen specific immunotherapy with the purpose of tolerance induction in respiratory allergies, our study underlines the importance of the local innate response to allergens in the allergic response. The underlying mechanisms which cause these innate responses to allergens should be considered in the development of therapy. The genome-wide association studies for asthma revealed an important association with IL-33 and the IL-33 receptor, which might form an important therapeutic target [13].

After sensitization to one allergen, it is not uncommon to develop sensitization to a second allergen over time. Is it an overall susceptibility to develop an aberrant immune response to allergens or could an existing allergy prime the immune system to be more prone to develop an allergic response in different organs? Several epidemiological reports suggest an association between sensitization to food allergens and an increased risk for the development of asthma [14;15]. We developed a mouse model of a combined respiratory (HDM) and gastrointestinal (peanut) allergy in order to study a possible immunological interplay between these allergies. We found an interesting enhancement at the level of T cell cytokine response to HDM in case of a preceding peanut allergy but not at the clinical level **(Chapter 5)**. This seems to be a discrepancy. However, airway hyperreactivity is not linearly associated with Th2 cytokine levels. In our birch pollen immunotherapy model, we demonstrated that a suppression of the Th2 cytokine production was not (immediately) accompanied by a decrease in AHR (**Chapter 6**). A shift in the immunoglobulin response seemed to be necessary before the AHR could be suppressed. This indicates that AHR is not solely dependent on the T cell cytokine response, but on different components of the immune response. In addition, the high dose HDM used in this model might have induced such a strong innate immune response resulting in recruitment of inflammatory cells and airway hyperreactivity [16] which overwhelmed the boosting effect of the preceding peanut

sensitization on the adaptive immune response to HDM. Future research in this model with titrating threshold levels of allergen and intervening with different components of the immune response (blocking innate cytokines like IL-33, or effector cytokines like IL-5, or using µMt-/- mice which lack immunoglobulin production) would be of great value to determine the effects on the clinical parameters.

After sensitization to a primary allergen, cross-reactivity to related allergens is a common feature. Certain allergens are structurally related. The birch pollen allergen Bet v 1 has homologous food allergens in apple, celery and hazelnut. IgE directed against a primary allergen (for example Bet v 1) can recognize these related allergens [17-20]. A well-known example is allergy to the apple allergen Mal d 1, caused by respiratory sensitization to birch pollen allergen Bet v 1. Interestingly, not all birch pollen allergic people experience apple allergy or have Mal d 1 recognizing IgE [21]. To gain more insight in the cross-reactivity between allergens, we used a mouse model for birch pollen allergy. We observed that mice which were exposed to birch pollen developed Mal d 1 cross-reactivity. This cross-reactivity was boosted by a single injection to Mal d 1 in Bet v 1 sensitized mice but not in non-sensitized mice (**Chapter 7**). These data suggest that the Bet v 1 specific IgE repertoire can be boosted by exposure to Mal d 1, increasing the Mal d 1 cross-reactive IgE. An important outcome of successful birch pollen immunotherapy is the increase of protective Bet v 1 specific IgG_4 and IgG_1. The underlying protective mechanism is that these antibodies can capture allergens and thereby prevent allergen binding to effector cell bound IgE or can inhibit IgE facilitated allergen presentation to T cells [22]. There is evidence in favour and against improvement after immunotherapy for cross-reactive food allergies. We therefore used our mouse model mimicking cross-reactive apple allergy to investigate the effect of birch pollen immunotherapy. In our model we demonstrated a mechanism by which birch pollen immunotherapy decreased anaphylactic symptoms induced by cross-reactive allergen Mal d 1. Birch pollen immunotherapy induced a Bet v 1 specific IgG_1 and IgG_{2a} response. Boosting the Mal d 1 cross-reactive Bet v 1 specific immunoglobulin response by an injection with Mal d 1 suppressed the anaphylactic response. Our birch pollen immunotherapy model suggests that boosting of the Mal d 1 recognizing Bet v 1 specific antibodies contributed to the protection against a Mal d 1 induced anaphylactic response. Our results can help

clarifying the possible protective mechanisms that can be elicited in immunotherapy in this context.

What makes a protein an allergen and what makes individuals more susceptible than others to a certain allergen are two major questions in allergy research. In this thesis, we believe to have contributed to the knowledge of the mechanisms by which allergens can trigger the immune system and some of the factors that can predict individual susceptibility to atopy (fig. 1). And finally, we believe that despite its challenges and limitations, the development of reliable mouse models is of great value and interest in allergy research.

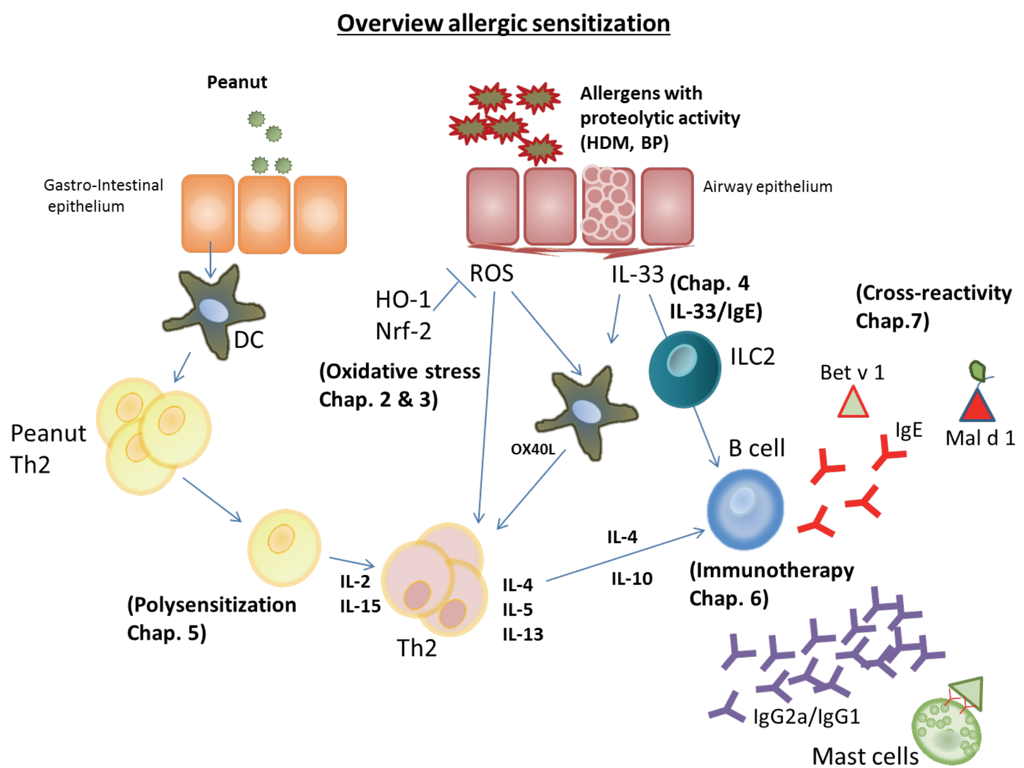


Figure 1: Overview of the studied mechanisms in mouse models for sensitization and immunotherapy

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Appendix

Summary

Samenvatting

Portfolio

Acknowledgements

Summary

BACKGROUND

Allergy is defined as an aberrant immune response to a substance that should be harmless but gives rise to a hypersensitivity reaction. Although a Th2 immune response is normally induced to protect the host against parasite infection, in allergy this Th2 cell mediated immunity is directed against proteins (allergens) present in among others birch pollen, house dust mite faeces and food (like peanuts). The Th2 response drives class switching of antibodies to IgE, which is called allergic sensitization. The IgE molecules will bind to the surface of effector cells like mast cells and upon re-exposure to the allergen, these effector cells will release inflammatory mediators responsible for allergic symptoms. Worldwide sensitization rates to one or more common allergens among school children are currently approaching 40%-50% according to the World Health Organisation. Sensitization to allergens is a strong risk factor for the development of allergic disorders, like allergic asthma, rhinitis, food allergy and atopic eczema. The prevalence of allergic diseases has increased in the industrialized world over the last 50 years and a sharp increase continues in the low- and middle-income countries. Allergic sensitization is a complex multifactorial process that involves both the innate and the adaptive immune response to allergens together with the response of structural cells. The intrinsic traits of the allergen play a distinctive role, as allergy is not directed to random proteins. It is impossible to identify one sole mechanism that is crucial in the induction of this complex phenomenon that is very much determined by individual genetic differences and diverse external factors. Mouse models have contributed greatly to the understanding of the pathogenesis of allergic diseases. It is an important complementary tool in providing clues to the advancement of human studies. The aim of this thesis was to gain insight in the process of sensitization to allergens, using several different mouse models. We investigated the process of sensitization in different situations, outlined below.

Sensitization and oxidative stress

Some allergens have a proteolytic activity and have been reported to induce reactive oxygen species (ROS). In all biological processes where oxygen is metabolized, ROS are formed as a natural byproduct. They have an important role in cell-signalling but these molecules can be potentially cytotoxic and are under strict control of anti-oxidant mechanisms. However, when the amount of ROS exceeds the controlling machinery, significant damage to cell structures can occur. This is known as oxidative stress. In **chapter 2** & **3** we explored our hypothesis that the induction of oxidative stress by allergen exposure could contribute to allergic sensitization. In our mouse study, we showed that the capacity to counteract the effects of oxidative stress is associated with the susceptibility to develop allergic sensitization to house dust mite (HDM). This was confirmed in a study investigating sensitization to rodent urinary proteins in an occupational cohort of animal workers.

Sensitization and IL-33

IL-33 is an important alarmin. It is released when cells are necrotic. However, recent evidence indicates that IL-33 is released after exposure to proteolytic allergens and that it plays a crucial role in the induction of the inflammatory response to HDM. IL-33 has the ability to induce maturation and upregulation of the Th2 skewing costimulatory molecule OX40L on DCs. In **chapter 4** we investigated the individual roles of dendritic cells (DC) and IL-33 in sensitization to HDM allergens. We demonstrated that the humoral immune response was dependent on IL-33, while the cellular response could be induced in the absence of IL-33. This underlines the role of the innate cytokine IL-33 in the sensitization to HDM allergens.

Sensitization in poly-sensitization

It is a common phenomenon that allergic individuals acquire IgE against multiple allergens over time and often show multiple allergic disorders (like combined eczema, rhinitis and asthma). In childhood, food allergy is considered to be a risk factor for the development of allergic asthma later in life. In **chapter 5** we developed a murine model to study the possible interplay between a non-cross-reactive food (peanut)-induced allergy and HDM-induced respiratory allergy. Our data show that a preceding peanut sensitization boosts the IgE and

HDM-specific Th2 response in the airways of mice. This study suggests that a preceding food allergy might lower the threshold of the stimulus needed to induce a Th2 cell response for a subsequent sensitization to HDM.

Sensitization and cross-reactivity

Cross-reactivity in allergic reactions occurs when allergens are structurally related. An individual is sensitized to one allergen but the allergen-specific antibodies also recognize homologous allergens. Apple allergy is a common comorbidity of birch pollen (BP) allergy. Specific IgE to the major birch pollen allergen Bet v 1 can also recognize the homologous apple allergen Mal d 1. Illustrative for the cross-reactivity is that sensitization to Mal d 1 does not occur in the absence of a primary Bet v 1 sensitization. Despite the strong association between Bet v 1 IgE and apple allergy, a successful immunotherapy for birch pollen allergy does not always result in an amelioration of the symptoms of apple allergy. In **chapter 6** we used a murine model for BP allergy to determine the effects of BP subcutaneous immunotherapy (SCIT). We showed that reduction of Th2 cytokine production by BP SCIT was not sufficient to immediately reduce airway hyper-responsiveness (AHR) in mice, but was associated with sequential effects on IL-17A production, IL-5 levels in the broncho-alveolar lavage fluid (BALF) and increased titres of BP IgG_{2a}. In **chapter 7** we used this model to evaluate the effect of BP immunotherapy on cross-reactivity and hypersensitivity to Mal d 1. Birch pollen immunotherapy ameliorated the anaphylactic symptoms induced by Mal d 1. This was associated with a shift in the ratios of both Bet v 1 and Mal d 1 specific IgG over specific IgE to these allergens. Additionally, we noted a shift from production of the Th2 cytokines IL-5 and IL-13 towards IL-10 production. This study indicated that the induction of (protective) Mal d 1 recognizing immunoglobulins by birch pollen immunotherapy contributed to the suppressed anaphylactic response to Mal d 1.

Conclusion

The results described in this thesis provide new insights in the underlying mechanisms in allergic sensitization. Different mechanisms are important during primary sensitization, in poly-sensitization and in cross-reactive sensitization. In primary sensitization, we identified a role for oxidative stress in a HDM allergy model and confirmed our findings in an occupational cohort in which animal laboratory workers were followed for sensitization to rodent urinary proteins. Additionally, we discovered a new role for the innate cytokine IL-33 in the induction of IgE after inhalational exposure to HDM. In the phenomenon of poly-sensitization, our data suggest an immunological interplay between immune responses to unrelated allergens in different compartments of the body. Our data showed that the HDM specific Th2 cytokine response was increased in mice with a preceding peanut allergy, possibly due to a bystander effect of peanut specific T cells. Sensitization via cross-reactivity to a second allergen is caused by recognition of immunoglobulins specific for the first allergen. Our data suggest that cross-reactivity can be boosted by exposure to the second allergen. This can have consequences for the progression of cross-reactive sensitization to cross-reactive allergy but also for the efficacy of immunotherapy directed at a primary allergen, on concomitant cross-reactive allergens. A better understanding of sensitization to different allergens is warranted to design the most appropriate immunotherapy and will yield more specific immunological targets.

Samenvatting

ACHTERGROND

Allergie wordt veroorzaakt door een misplaatste immuunrespons tegen een eiwit dat onschadelijk zou moeten zijn, hetgeen resulteert in een overgevoeligheidsreactie. Normaal gesproken beschermt een T helper 2 reactie tegen een infectie met parasieten. In het geval van een allergie wordt er echter een T helper 2 reactie opgewekt tegen eiwitten afkomstig van bijvoorbeeld berkenpollen, huisstofmijtuitwerpselen of van voedingsmiddelen zoals pinda's. Deze Th2 reactie leidt tot de aanmaak van type E antilichamen (IgE). Dit wordt allergische sensitisatie genoemd. Deze antilichamen binden aan mestcellen en andere effector cellen van het immuunsysteem en na een tweede blootstelling aan het specifieke allergeen worden deze effector cellen geactiveerd, wat leidt tot verschillende allergische symptomen. Volgens de Wereld Gezondheids Organisatie is de wereldwijde prevalentie van sensitisatie tegen één of meerdere veelvoorkomende allergenen onder schoolkinderen veertig tot vijftig procent. Sensitisatie tegen allergenen is een sterke risicofactor voor het ontwikkelen van allergische ziektes, zoals allergisch astma, hooikoorts, voedselallergie en atopisch eczeem. De laatste vijftig jaar is de prevalentie van allergische ziekten enorm toegenomen in de geïndustrialiseerde wereld en deze stijgt nog steeds sterk in de lage- en middelhoge lonen landen. Allergische sensitisatie is een complex multifactorieel proces waarbij zowel het aangeboren als specifieke (verworven) immuunsysteem en structurele cellen (zoals het epitheel) betrokken zijn. De specifieke eigenschappen van de allergenen spelen een belangrijke rol, aangezien allergie niet ontstaat tegen elk willekeurig eiwit. Het is onmogelijk om één bepaald mechanisme aan te wijzen dat allesbepalend is in het ontstaan van dit complexe fenomeen dat afhangt van zowel individuele genetische verschillen als van diverse externe factoren. Muizenmodellen voor allergie hebben veel bijgedragen aan het begrip van het mechanisme van allergische aandoeningen. Het is een belangrijke ondersteuning bij het vinden van aanwijzingen ten behoeve van studies in de mens. Het

doel van dit proefschrift was om meer inzicht te verkrijgen in het proces van allergische sensitisatie in verschillende omstandigheden met behulp van verschillende muizenmodellen.

Sensitisatie en oxidatieve stress

Sommige allergenen hebben proteolytische activiteit en zijn in staat om de aanmaak van reactieve zuurstofverbindingen (ROS) in cellen te induceren. In alle biologische processen waarin zuurstof wordt verbruikt, worden ROS gevormd als natuurlijk bijproduct. Deze verbindingen zijn potentieel schadelijk voor de cel en de concentraties ROS worden door middel van antioxidanten onder controle gehouden. Wanneer dit niet meer lukt, kan er ernstige schade optreden aan celstructuren. Dit wordt “oxidatieve stress” genoemd. In **hoofdstuk 2 en 3** hebben we de hypothese onderzocht dat de aanwezigheid van oxidatieve stress door blootstelling aan allergenen kan bijdragen aan de allergische sensitisatie. In onze muizenstudies hebben we aangetoond dat een verminderde capaciteit om oxidatieve stress tegen te gaan geassocieerd was met de gevoeligheid van muizen om een allergische sensitisatie tegen huisstofmijtallergenen te ontwikkelen. Een dergelijke associatie vonden we ook in een cohort van nieuwe proefdiermedewerkers die gevolgd werden op het ontwikkelen van een sensitisatie tegen urine-eiwitten van knaagdieren.

Sensitisatie en IL-33

IL-33 komt vrij uit cellen als ze necrotisch zijn, en is dus een belangrijk signaal voor weefselschade. Echter, recent onderzoek wijst uit dat IL-33 ook vrijkomt na blootstelling aan allergenen met een proteolytische activiteit en dat het een cruciale rol speelt in de inductie van de allergische respons tegen huisstofmijtallergenen. IL-33 kan dendritische cellen activeren en de expressie van OX40L induceren. OX40L stimuleert het ontstaan van een Th2 respons tijdens de allergeen presentatie. In **hoofdstuk 4** onderzochten we de individuele rol van de dendritische cel en van IL-33 in de sensitisatie tegen huisstofmijtallergenen. We toonden aan dat de humorale immuunrespons afhankelijk was van IL-33, terwijl de cellulaire immuunrespons zich ook in de afwezigheid van IL-33 kon ontwikkelen. Dit onderstreept de rol van IL-33 en daarmee de bijdrage van het niet-specifieke (aangeboren) immuunsysteem in de sensitisatie tegen huisstofmijtallergenen.

Sensitisatie in polysensitisatie

Het komt vaak voor dat allergische individuen in de loop van de tijd een IgE respons tegen verschillende allergenen ontwikkelen. Ze vertonen ook vaak verschillende allergische aandoeningen (zoals een combinatie van allergisch eczeem, hooikoorts en allergisch astma). Tijdens de kinderjaren wordt voedselallergie als een sterke risicofactor gezien voor het ontwikkelen van allergisch astma op latere leeftijd. In **hoofdstuk 5** hebben we een muizenmodel ontwikkeld om de mogelijke interactie tussen een voedselallergie tegen pinda en een niet-gerelateerde inhalatieallergie tegen huisstofmijt te onderzoeken. Onze resultaten lieten zien dat een bestaande pinda-allergie de IgE respons en de specifieke T cel respons tegen huisstofmijt in de luchtwegen van muizen versterkt. Deze immunologische interactie laat zien dat een voorafgaande voedselallergie de sensitisatie tegen huisstofmijtallergenen zou kunnen bevorderen.

Sensitisatie en kruisreactiviteit

Allergische kruisreactiviteit komt voor bij allergenen die qua eiwitstructuur op elkaar lijken. Een individu kan gesensitiseerd zijn voor één allergeen maar de daar tegen gerichte antilichamen kunnen ook een homoloog allergeen herkennen, dat afkomstig is van een andere bron. Zo komt appelallergie komt vaak voor als gevolg van een berkenpollenallergie. Het IgE antilichaam gericht tegen het berkenpollen (BP) allergeen Bet v 1, kan ook het appelallergeen Mal d 1 binden. Illustratief voor de kruisreactiviteit is dat sensitisatie tegen Mal d 1 niet voorkomt zonder een primaire sensitisatie tegen Bet v 1. Ondanks dat er een zeer sterke associatie is tussen Bet v 1 specifiek IgE en de kruisreactieve appelallergie, is een succesvolle immuuntherapie tegen berkenpollen niet automatisch even effectief tegen de appelallergie. In **hoofdstuk 6** hebben we de effecten van subcutane immunotherapie met berkenpollen (BP SCIT) op de immuunrespons onderzocht in een muizenmodel voor berkenpollenallergie. We toonden aan dat de verlaging van de Th2 cytokine productie niet voldoende was om direct de luchtweghyperreactiviteit te remmen maar dat het ook geassocieerd leek te zijn met een verlaging van IL-17A, de IL-5 concentratie in de longspoelvloeistof en een verhoogde concentratie BP specifiek IgG_{2a}. In **hoofdstuk 7** hebben we het effect van de BP SCIT geëvalueerd op de kruisreactiviteit en gevoeligheid voor Mal d 1. Na BP SCIT zagen we een vermindering van de anafylactische reactie na blootstelling aan

Mal d 1. Dit was geassocieerd met een verhoging van de concentratie IgG moleculen ten opzichte van de concentratie IgE. Dit gold zowel voor de antilichamen gericht tegen Bet v 1 als voor Mal d 1. Er was ook een verschuiving zichtbaar in de cytokine productie; er werd relatief meer IL-10 aangemaakt ten opzichte van IL-5 en IL-13. Deze studie toonde aan dat het verhogen van de concentratie beschermende antilichamen tegen Mal d 1 door de berkenpollen specifieke immunotherapie geassocieerd was met een verminderde anafylactische reactie op de Mal d 1 blootstelling.

Conclusie

De resultaten die beschreven staan in dit proefschrift geven nieuwe inzichten in de onderliggende mechanismes die ten grondslag liggen aan het ontwikkelen van een allergische sensitisatie. Verschillende mechanismes zijn belangrijk tijdens een primaire sensitisatie, tijdens polysensitisatie en in een kruisreactieve sensitisatie. Voor een primaire sensitisatie tegen huisstofmijtallergenen hebben we een rol voor oxidatieve stress kunnen aantonen in een muizenmodel voor huisstofmijtallergie en we konden onze bevindingen bevestigen in een cohort studie waarin proefdierlaboratorium medewerkers werden gevolgd voor de ontwikkeling van een sensitisatie tegen urine-eiwitten van knaagdieren. We ontdekten ook een niet eerder beschreven rol voor IL-33 in de IgE respons. Onze resultaten laten een immunologisch samenspel zien tussen allergische reacties in verschillende compartimenten van het lichaam. De huisstofmijt specifieke Th2 cytokine respons was verhoogd in muizen met een pinda-allergie, wellicht doordat aanwezige pinda specifieke T helper 2 cellen een activerende werking hadden (het “bystander” effect). Sensitisatie door kruisreactiviteit voor een tweede, homoloog, allergeen wordt veroorzaakt door de al aanwezige immunoglobulines gericht tegen het eerste allergeen. Onze studie liet zien dat deze respons versterkt kan worden door blootstelling aan het tweede allergeen. Dit heeft consequenties voor het ontwikkelen van een kruisreactieve allergie maar ook voor het effect van een allergeen-specifieke immuuntherapie op vermindering van klachten veroorzaakt door kruisreactieve allergenen. Een beter begrip van de mechanismes die betrokken zijn bij sensitisatie voor verschillende allergenen is nodig om de meest geschikte immuuntherapie te ontwikkelen en dit zal bijdragen aan de ontdekking van nieuwe, allergeen-specifieke therapeutische strategieën.

PhD PORTFOLIO

Name PhD student: Lara Utsch Mendes Gouveia PhD period: January 2010-July 2014 Name PhD supervisor: Prof. Dr. Ronald van Ree Co-supervisor: Dr. ir. Leonie S. van Rijt		
PhD training	**Year**	**Workload (Hours/ECTS)**
General and Specific courses		
- Laboratory animal course Article 9	2010	60hrs
- Advanced Immunology	2013	80hrs/2.9ECTS
Seminars		
- Weekly department seminars	2010-2014	150 hrs
- Weekly AMC lectures	2010-2014	100 hrs
- Bi-weekly ”Allergy” journal club	2012-2014	30 hrs
- Bi-weekly “General” journal club	2010-2012	30 hrs
Presentations at scientific conference (poster)		
- Gouveia, L.; van Rijt, L.; Logiantara, A. TLR4 signalling is not crucial for the development of Th2 responses to HDM. European Academy of Allergy and Clinical Immunology Congress (EAACI) 2011, Istanbul, Turkey.	2011	0,5
- Gouveia, L.; van Rijt, L.; Logiantara, A. TLR4 signalling is not crucial for the development of Th2 responses to HDM. 9th EAACI-GA2LEN Immunology Winter School Basic Immunology Research in Allergy and Clinical Immunology, Davos, Switzerland, 2011.	2011	0,5
- Utsch L, Folisi C, Akkerdaas JH, Logiantara A, van de Pol MA, van der Zee JS, Krop EJ, Lutter R, van Ree R, van Rijt LS. Inadequate antioxidant response is associated with allergic sensitization in mice and man. European Academy of Allergy and Clinical Immunology Congress (EAACI) 2014, Copenhagen, Denmark.	2014	0,5
Presentations (oral)		
- Department meetings EXIM (bi-annual)	2010-2014	8

Teaching		
- Elective course "Allergy: immune-pathogenesis and progression". Supervision bachelor students in practical training in airway histology.	2010-2014	12

Publications	
Peer reviewed	**Year**
- Allergy. 2012 Nov;67(11):1383-91. House dust mite allergic airway inflammation facilitates neosensitization to inhaled allergen in mice. van Rijt LS, Logiantara A, Utsch L, Canbaz D, Boon L, van Ree R.	2012
- Int Arch Allergy Immunol. 2014;165(2):128-39. Birch pollen immunotherapy in mice: inhibition of Th2 inflammation is not sufficient to decrease airway hyper-reactivity. van Rijt LS, Gouveia L, Logiantara A, Canbaz D, Opstelten DJ, van der Kleij HP, van Ree R.	2014
- Allergy. 2015 Oct;70(10):1246-58. Allergic sensitization is associated with inadequate antioxidant responses in mice and men. Utsch L, Folisi C, Akkerdaas JH, Logiantara A, van de Pol MA, van der Zee JS, Krop EJ, Lutter R, van Ree R, van Rijt LS.	2015
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- Clinical and Experimental Allergy. 2016. *Accepted for publication*. Experimental food allergy to peanut enhances the immune response to house dust mite in the airways of mice. Utsch L, Logiantara A, van Ree R, van Rijt LS.	2016
Other **(manuscripts submitted)** - Oxidative stress: promoter of allergic sensitization?	

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