**ORIGINAL ARTICLE**

Asthma and Rhinitis

**Birch pollen-specific subcutaneous immunotherapy reduces ILC2 frequency but does not suppress IL-33 in mice**

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**Abstract**

**Background:** The underlying mechanism of allergen-specific subcutaneous immunotherapy (SCIT) is not yet fully understood, but suppression of allergen-specific Th2 cells and production of allergen-specific IgG4 antibodies are two hallmarks. The impact on the innate arm of the immune system is far less clear.

**Objective:** The aim of this study was to investigate the effect of birch pollen (BP) SCIT on the innate immune response in a BP SCIT mouse model.

**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 the BP SCIT on innate cytokine levels in lung, the number and the functionality of ILC2s and the airway inflammation was determined.

**Results:** Mice with BP allergy had an increased level of the innate cytokines IL-33, IL-25, GM-CSF and IL-5+ ILC2s in the lungs. BP SCIT suppressed the number of IL-5+ ILC2s, mast cell tryptase release, Th2 cytokine production, eosinophil recruitment and peribronchial inflammatory infiltrates. In contrast, innate cytokine production and collagen deposition in the airways were not affected.

**Conclusion and Clinical Relevance:** BP SCIT is able to suppress the adaptive and part of the innate immune response, but this is not sufficient to inhibit collagen deposition and the IL-33 expression in the airways in mice.

**Keywords**

airway hyper-reactivity, birch pollen allergy, immunotherapy, innate lymphoid cells type 2, mucosal immunity, murine asthma model

1 | INTRODUCTION

Although allergen-specific subcutaneous immunotherapy (SCIT) has been used for over 100 years and has been demonstrated to be an effective disease-modifying treatment for allergic diseases, the underlying mechanism is still not fully understood. The relief of IgE-mediated allergic symptoms has been attributed to a modification of the allergen-specific adaptive immune response: suppression of Th2 responses and induction of protective IgG antibodies.1-3 Although it has been recognized that the innate immune response, and especially innate lymphoid cells type 2 (ILC2), contribute greatly to inducing and maintaining allergic inflammatory responses, the effect of SCIT on the innate immune response has been investigated scarcely. Considering that the innate immune response is not regulated directly by allergen-specific receptors, it can be questioned whether an allergen-specific therapy like SCIT also impacts the innate immune response. Recently, much progress has been made in the elucidation of the interaction between the innate immune response and...
adapting immune response in allergic asthma. It is clear that the innate immune response functions beyond sparking off allergic airway inflammation but that it is also involved in the chronic character of the inflammation. Asthma patients have an increased expression of the innate cytokines IL-33, TSLP and IL-25 in their lungs.4-6 Christiansen et al demonstrated an increased concentration of IL-33 and increased frequency of IL-5+IL-13+ ILC2s in the BALF in asthmatics. In severe asthmatics, ILC2s were even the most predominant source of IL-5 and IL-13 despite CD4+ T cells were more abundant.8,9 From mouse studies, it has been demonstrated that ILC2s are able to induce an eosinophilic airway inflammation and airway hyper-reactivity even in the absence of T cells.10 Together with the observation that genetic variability in IL-33 and the IL-33 receptor is associated with asthma, these findings emphasize the role of IL-33 and ILC2s in the asthmatic airway inflammation.11 This innate lymphoid cell type functions beyond the induction of the primary inflammatory response to the allergen and fulfills a prominent role in the maintenance of chronic asthma. The relative contribution of Th2 cells vs ILC2s is still under debate.7,12 The epithelium secretes innate inflammatory cytokines, like IL-33, IL-25, TSLP and GM-CSF, after an encounter with allergens but possibly also after nonallergenic stimuli (eg cold air).13 Epithelium can respond to allergens by recognizing evolutionarily conserved patterns or it becomes activated by allergens exerting protease activity, like some major house dust mite allergens or pollen allergens.14,15 The secreted innate cytokines IL-33, IL-25, TSLP and GM-CSF are pluripotent and effect many innate and adaptive immune cells that contribute to the Th2 response. Epithelial cells can promote a Th2 response by upregulating OX40L on DCs and can activate innate lymphoid cells type 2 (ILC2s).16 Recently, it has been demonstrated that ILC2s have a crucial role in the leakiness of the asthmatic epithelium by disrupting epithelial tight junctions by IL-13.15 This could facilitate an increased entry of allergens. Oliphant et al demonstrated an allergen-specific feedback mechanism in which Th2 cells can interact with ILC2s via MHCII and activate ILC2s via secreted IL-2. A decrease in allergen-specific Th2 cells can therefore result in dampening the ILC2 population and activation.17 Lao-Araya et al showed that patients treated with a subcutaneous grass pollen-specific immunotherapy did not show the seasonal increase in ILC2s in PBMCs as seen in untreated patients, indicating that SCIT can suppress circulating ILC2s.

Besides the contribution of inflammation to the pathology of asthma, structural changes in the Airways are equally important.19 Asthma patients often show a thickened reticular basement membrane and an increased volume of airway smooth muscle. Interestingly, it was shown that fibroblasts from asthmatic biopsies were able to produce collagen after stimulation with IL-33.20 In a mouse model for chronic asthma with airway remodelling and persistent airway hyper-reactivity, it was demonstrated that elimination of T cells resolved airway inflammation but not remodelling. Elimination of T cells and ILC2s resulted in resolution of airway hyper-reactivity.7 Together, these data underline the involvement of the innate immune response in asthma.

In this study, we investigated in a mouse model whether suppression of the adaptive immune response by birch pollen-specific SCIT treatment is sufficient to decrease the number of airway ILC2s, innate cytokine production and prevent collagen deposition in the Airways.21

2 MATERIALS AND METHODS

2.1 Mice

Female 6-8-week-old BALB/c mice (Envigo, 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 Academic Medical Centre with the University of Amsterdam, the Netherlands under the approval numbers DSK102773, DSK102937, DSK102956 en DSK49AF-1.

2.2 Study design

2.2.1 Sensitisation to birch pollen

BALB/c mice were sensitized intraperitoneally (i.p.) at day 0, 7, 14 with 8.2 μg birch pollen extract (BPE) (BPE provided by HAL Allergy BV, Leiden, The Netherlands: containing 117 μg Bet v 1/mg dry weight extract as determined by Bet v 1 ELISA and an endotoxin level of 2.84 EU/mg as determined by the Limulus Amebocyte Lysate assay (Lonza Verviers, Belgium), using a dose equivalent to 1 μg Bet v 1, adsorbed to 1 mg alum (Imject alum, Pierce Biotechnology, Thermo scientific, Rockford, IL, USA), or as a control with alum alone. Under isoflurane anaesthesia, 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 at day 21, 22, 23.

2.2.2 Birch pollen subcutaneous immunotherapy

At day 35, 42, 49, 56, 63, 70, 77 and 84, mice with a BP induced allergic airway inflammation underwent subcutaneous (s.c.) immunotherapy, administering 0.3 mg BPE adsorbed to 1 mg alum while control mice received PBS. To investigate the effectiveness of the immunotherapy, mice were rechallenged with a 0.1% BPE aerosol or PBS aerosol at day 98, 99, 100. Two days later, mice were sacrificed to evaluate the 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 IgG2a).

2.3 Collection and analysis of tissues

2.3.1 Bronchoalveolar lavage fluid

BAL inflammatory cells were obtained by intratracheal cannulation, and the airway lumen was lavaged 3× with the same millilitre PBS containing 0.1 mmol/L EDTA. Cell differentiation was done by flow cytometric analysis as described before.12,22 BALF was stored at −20°C until IL-5 was determined by ELISA (Ready set go,
2.4 | 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 $2 \times 10^5$ cells per well and were restimulated for 4 days with 100 μg/mL BPE. Supernatants were analysed for IL-2, 4, 5, -13, -10, -17A and IFNγ production by ELISA (Ready-set-go!, eBioscience).

2.5 | Serum BP-specific Immunoglobulins

Serum was analysed for the level of BP-specific IgE, IgG1 and IgG2a by ELISA (IgE and IgG1: Opteia, BD, San Diego, CA, USA, IgG2a: eBioscience) as previously described.21 In short, Maxisorp plates were coated overnight with 100 μg BPE. After blocking with 10% FCS, serum samples were incubated for 2 hours and 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, IgG1 and IgG2a, respectively, while sera of PBS control animals were diluted 30x, 100x and 50x. A standard curve of murine IgE, IgG1 or IgG2a, respectively, was used as a quantitative reference.

2.6 | Airway histology

Frozen sections (6 μm) were stained with Periodic Acid Schiff’s reagent (Sigma-Aldrich). Degree of inflammation and mucus production was semi-quantified as described before.23 The collagen deposition was determined on frozen sections stained with Martius Scarlet Blue staining and was quantified via image analysis as described before.23 In short, threshold Red Green Blue (RGB) values were specified for collagen staining, and the area collagen deposition per μm basement membrane was determined using an image analysing system (LEICA, Wetzlar, Germany). The determined area of pixels within the selected colour setting was divided by the length of BM of each airway, from each animal ten airways were analysed.

2.7 | Cytokine detection in lung tissue

Lungs were harvested and lung homogenate was lysed by adding 1 mL Greenberger’s buffer (300 mmol/L NaCl, 15 mmol/L Tris-HCl, 2 mmol/L MgCl2, 2 mmol/L, Triton (X-100), pepstatin A, leupeptin, aprotinin (all 20 ng/mL), pH 7.4) after 30 minutes incubating on ice. Then, the lysate was centrifuged at 500xg for 10 minutes 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 Fisher Scientific, Rockford, USA). IL-33, IL-25, TSLP and GM-CSF protein concentration (μg/mg total protein) was measured by ELISA (Ready-SET-Go ELISA KIT, eBioscience).

For IL-5 production by lung ILC2s, lung tissue was minced in small fragments and digested with collagenase IV (100 U/mL) for 60 minutes at 37°C. Red blood cells were lysed with Ammonium chloride lysis buffer. Cells were stained for surface markers to identify ILC2 and stained for intracellular IL-5 as described above for BALF ILC2s (Figure S1).

2.8 | Tryptase activity

Tryptase activity was determined in BALF as described before.24 In short, 10 μL 6-mmol/L chromogenic tryptase substrate S-2288 (Chromogenix Instrumentation Laboratory, Milan, Italy) was added to 70 μL 57 mmol/L Tris-HCl (pH 8.3) in a 96-well microtiter plate. Undiluted BALF sample (40 μL) was added to start the reaction. The use of the substrate was followed for one hour at 450 nm at 37°C. The difference in optical density (OD) at each time point was calculated compared to the OD at the zero time point.

2.9 | Statistical analysis

For statistical analysis, differences between groups were analysed with the Mann-Whitney U test. Association between parameters was determined by Pearson Correlation Coefficient r. Differences were considered to be significant at a $P$ value of less than 0.05.

3 | RESULTS

3.1 | BP SCIT does not inhibit the increased IL-33, IL-25 and GM-CSF production in lung

We demonstrated before that in mice with BP induced asthma, the adaptive immune response to BP can be suppressed by treatment with subcutaneous immunotherapy with a high dose of BP. In the present study, we explored the effect on the innate immune response to BP. First, we confirmed that BP SCIT treatment strongly decreased the Th2 cytokine production and the related eosinophilia, and that the BP-specific immunoglobulin response shifted from IgE towards an IgG1/G2a response (Figure 1A-D and Figure S2).21 To investigate whether BP SCIT would also have an effect on the non-allergen-specific innate response after BP aerosol exposure of BP
sensitized mice, we determined the level of the innate cytokines IL-33, IL-25, GM-CSF and TSLP in lung. BP sensitized and challenged mice that were not treated (“No SCIT”) showed an increased concentration of the innate cytokines IL-33, IL-25 and GM-CSF, but not TSLP, in lung tissue compared to PBS controls. BP SCIT treatment was not able to suppress the increase in these innate cytokines (Figure 2A-D). Eotaxin-2 is considered to be an important chemottractant for eosinophils and is released by monocytes among others. In concordance with the decreased eosinophilia, the level of eotaxin-2 in BALF was decreased as well as the IL-5 concentration in lung tissue (Figure 2E,F).

3.2 | BP SCIT reduces the number of ILC2s

As IL-33 and IL-25 are important activators of ILC2s,25-27 and ILC2s have been recognized as an important innate cell type involved in Th2 cell sensitisation and propagation of Th2 driven disease,28 we determined the effect of BP SCIT on the number of ILC2s and their production of IL-5. BP SCIT treatment greatly reduced the number of BALF and lung ILC2s (Figure 3A, identified as Lin–[CD3–CD19–B220–DX5–FcrR–GR–1–CD11c–;CD45–, ST2+, CD127+, CD25+ in BALF and in lung). Li et al12 demonstrated that accumulation of ILC2s is dependent on the presence of Th2 cells. BP SCIT decreased the Th2 cytokine production in the lung draining lymph nodes (Figure 1A) and largely inhibited the accumulation of T cells to the bronchoalveolar compartment (Figure 3B). A strong positive correlation between the number of Th2 cells and ILC2s in BALF was observed. A similar correlation was also found for the presence of eosinophils and ILC2s (Figure 3B,C).

Because innate cytokines like IL-33 and IL-25 have been reported to induce IL-5 production by ILC2s in parasitic infection models, the percentage of IL-5 producing ILC2s was determined. BP induced inflammation enhanced the proportion of IL-5 producing ILC2 within the ILC2 population. Both the number of IL-5 producing ILC2s and the percentage of IL-5+ ILC2 increased sharply (Figure 3D, 5.8% in controls vs 19.3% in “No SCIT” mice, P = 0.0002) and Figure S1). Interestingly, although BP SCIT suppressed the number of ILC2s, the percentage of IL-5 producing ILC2s within the remaining population did not change. The effect of BP SCIT on the local production of IL-5 and IL-13 was determined in BALF. BP SCIT largely reduced the concentration IL-5 and IL-13 in BALF (Figure 3E).

3.3 | BP SCIT suppresses mast cell protease-1 and tryptase activity

IL-33 has been reported to be involved in the adhesion, maturation, cytokine secretion and degranulation of mast cells. This innate cell type plays an important role in the allergic immune response. Mast cells degranulate after cross-linking of bound allergen-specific immunoglobulins type E by the allergen and secrete pro-inflammatory cytokines.29 BP SCIT treatment changed the Bet v 1 IgG2a/IgE ratio in favour of IgG2a (Figure 1D). To investigate whether BP SCIT

**Figure 1** BP SCIT inhibited the adaptive immune response. Production of IL-4, IL-5 and IL-13 in supernatants of lung draining lymph node cells after ex-vivo restimulation with BP (A). Recruitment of eosinophils to the BALF compartment (B). The degree of inflammatory infiltrates in the lungs was determined on lung slides stained with PAS and quantified (C). The shift in the ratio of BP-specific IgE and BP-specific IgG2a in serum (D). Data are presented as means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 vs PBS
resulted in a suppressed degranulation of mast cells, we determined Mouse Mast Cell Protease-1 (MMCP-1) in serum and the tryptase activity locally in the BALF. Untreated BP sensitized and challenged mice had an increased level of MMCP-1 and tryptase activity, which were suppressed by the BP SCIT treatment (Figure 4A,B).

3.4 | BP SCIT does not reduce collagen deposition or mucus production

BP SCIT is able to reduce the adaptive immune response, the accumulation of ILC2s and the degranulation of mast cells. As both ILC2s and mast cells have been thought to be involved in remodelling of the airways, we analysed mucus production and collagen deposition after BP SCIT. BP sensitization and challenge induced mucus production and collagen deposition in the lungs of mice. BP SCIT was able to inhibit the inflammatory infiltrate and the mucus production (Figure 5A), but the residual mucus production was still substantial and did not return to baseline (Figure 5A,C). On the other hand, BP SCIT did not reduce the degree of collagen deposition. BP sensitized/challenged mice and BP SCIT treated mice had a similar collagen deposition (Figure 5D and quantified in B).

4 | DISCUSSION

Here, we show that BP allergen-specific immunotherapy not only suppressed the adaptive immune response to inhaled BPE but also reduced the accumulation of IL-5+ ILC2s in the BALF and in lung. However, the collagen deposition was not inhibited. A plausible explanation is that BP SCIT did not dampen the production of the innate cytokines IL-33, GM-CSF and IL-25 in the lung after inhalation of BPE.

Innate cytokines like IL-33, TSLP and IL-25 are known to be involved in mucus production and remodelling of the airways. Exposure of nasal epithelium to IL-33 in vitro, results in an upregulated expression of the mucin gene, enhanced mucus production and goblet hyperplasia. This indicates that IL-33 is sufficient to induce mucus production, even in the absence of other cells of the immune system, like ILC2s or Th2 cells. This might explain why the mucus production after BP SCIT was not inhibited completely (Figure 5), although it was significantly decreased. Vannella et al demonstrated a redundant role for these cytokines in fibrosis in a Th2 cytokine-driven inflammation. In an ongoing chronic HDM inflammation, the blockade of these innate cytokines could not dampen the immune response or the remodelling. These data indicate that in the presence of Th2 cells, these innate cytokines are not necessary for the maintenance of remodelling. Our data complement these studies, showing that despite a strong inhibition of Th2 cells remodelling of the airways persisted, possibly due to elevated levels of IL-33 and IL-25. Our finding that IL-33 was not affected after BP SCIT is similar to the findings, in some extend, as published by Christianson et al. She showed that in a chronic asthma model depletion of T cells in an experimental manner (by anti-CD3 treatment or by irradiation followed by a transfer of bone marrow to restore naive T cells) resolved airway inflammation while the increased IL-33 expression in the airways persisted after this
immune ablation. The depletion of T cells dampened the airway inflammation while the remodelling of the airways persisted in this mouse model. However, Christianson demonstrated that ILC2s provide a positive feedback to epithelial cells to produce IL-33 via secretion of IL-13 and are crucial for the maintenance of asthma. In contrast, in our study, the suppression of the number of ILC2s by BP...
**FIGURE 4**  BP SCIT decreased MMCP-1 in serum and BALF tryptase activity. The concentration MMCP-1 in serum (A) and the tryptase activity in BALF (B). Data are presented as means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 vs PBS

**FIGURE 5**  BP SCIT did not inhibit remodelling of the airways. Mucus production and peribronchial infiltrates were quantified on PAS stained lung slides (A). Collagen deposition per μm basal membrane was quantified on MSB stained lung slides (B). Representative lung slides stained with PAS from each experimental group (C). Representative lung slides stained with MSB staining from each experimental group (D). Data are presented as means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 vs PBS
SCIT was not sufficient to reduce IL-33. Further research is needed to explore which alternative pathways may be involved in the maintenance of IL-33 in the lungs. For therapeutic interventions, it should be taken into account that remodelling and inflammation are interrelated but seem to be, at least partly, independent processes. A therapy targeted at suppressing the inflammation might not resolve remodelling of the airways sufficiently. A complementary therapy aimed at resolving remodelling might be necessary.

ILC2s play an important role in the immunopathology of allergic asthma, both in the Th2 immune response but also in airway hyperreactivity and airway remodelling. As IL-33 and IL-25 are involved in the activation of ILC2s, we explored the effect of BP SCIT on the number and the functionality of ILC2s. Although BP SCIT did not reduce IL-33 and IL-25, less ILC2s were present and produced less IL-5. The effect of allergen-specific immunotherapy on ILC2s has been scarcely investigated. Grass pollen SCIT has been demonstrated to suppress the increase in circulating ILC2s within the pollen season. Outside the pollen season, SCIT did not reduce the increased levels of circulating ILC2 in grass pollen allergic asthmatic rhino conjunctivitis patients and did not lower IL-5 and IL-13 production by ILC2s. This can be explained by the absence of active grass pollen induced inflammation in the lung. It has never been studied whether ILC2s decreased locally at the site of inflammation, due to technical limitations. In the present murine study, we show that BP SCIT decreased ILC2s locally, both in lung and BALF. The underlying pathway how allergen-specific immunotherapy can suppress this innate subset is still unclear. This decrease can be explained by a reduced recruitment from the blood and bone marrow, a decreased local proliferation or increased apoptosis. All these mechanisms could act simultaneously and result together in a decreased number of ILC2s in the tissue. Recently, Karta et al showed in an Alternaria exposure model with increased numbers of ILC2s in the airways that ILC2s are recruited from the bone marrow, via the blood to the lungs. Also, the above described human study showed a reduction in circulating ILC2s in blood after grass pollen SCIT. In support, Christianson showed that after irradiation of mice with a chronic airway inflammation, the number of depleted ILC2s was quickly restored by a transfer with naïve donor bone marrow. Importantly, they also observed that the number of restored (donor derived) ILC2s was higher in mice which had a chronic airway inflammation before they were irradiated compared with irradiated PBS control mice. This was suggested to be dependent on the persistent IL-33 expression in the lung. IL-33/IL-25 induced proliferation is recognized to contribute to extension of ILC2s in the gut and in lung. In addition, Oliphant et al demonstrated that T cell-derived IL-2 can directly induce proliferation of ILC2s. Considering the fact that the number of T cells in the BALF was significantly reduced after BP SCIT, it is tempting to speculate that T cell-induced proliferation of ILC2s will be reduced after BP SCIT. However, the capacity of T cells in lung draining lymph node cells to produce IL-2 after ex vivo restimulation with BPE was not suppressed significantly (Figure S3). Thirdly, ILC2s will go into apoptosis when less T cells are present to deliver IL-2, as demonstrated by Roediger et al. Fourthly, an allergen-specific feedback mechanism has been described via MHCI expression by ILC2s which can interact with allergen-specific Th2 cells. This could regulate the ILC2 population in an allergen-specific manner. In pilot experiments, we have analysed MHCI expression, but in our hands MHCI expression on BALF and lung ILC2s did not exceed control staining, making this explanation unlikely.

In addition, eosinophils can contribute to the proliferation and IL-5/IL-13 cytokine production by ILC2s. An important chemoattractant for eosinophils is eotaxin-2, which is under the control of Th2 cytokine IL-13. Both the concentration of eotaxin-2 and IL-13 were significantly decreased in the BALF after BP SCIT. A suppression of BP-specific Th2 cells in the IL-13 production can limit the IL-5+ ILC2 population via this feedback system. In support of these proposed interactions, the decrease in T cells and eosinophils was accompanied by a decrease in ILC2s.

As IL-33 is involved in the degranulation of mast cells, we determined both MMCP-1 in serum and tryptase activity in BALF. Both were significantly decreased in mice treated with BP SCIT. These are markers for degranulation, but further research is needed to determine whether the number of mast cells is reduced or whether degranulation of mast cells is truly inhibited. In the most recent international consensus about allergen-specific immunotherapy, it was stated that effective therapy should control chronic events leading to remodelling. Although allergen-specific immunotherapy is often used as a treatment for allergic asthma, there is still a matter of debate whether it is effective. Most controlled clinical trials were not designed specifically for asthma. However, both the inflammation-dependent as the independent pathways involved in remodelling will need further elucidation to design an effective therapy.

In conclusion, this study shows that though BP SCIT did not decrease the ILC2 activating cytokines IL-33, IL-25 and GM-CSF, it did suppress the innate IL-5+ ILC2 population. This suppression of both the adaptive immune response and this recently identified important innate cell type in the BP induced airway inflammation were not sufficient to reduce remodelling of the airways. This study underlines the important role of structural cells like the epithelium in the pathogenesis of allergic asthma.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.
AUTHOR CONTRIBUTION
Conception and design: LvR, RvR, acquisition of data: LvR, AL, DC.

FINANCIAL DISCLOSURE
RvR is consultant for HAL Allergy BV, Leiden, The Netherlands and for Citeq BV, Groningen, The Netherlands.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.