

BRIEF COMMUNICATION

Allergen-specific Th2 responses in young children precede sensitization later in life

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Abstract

Allergic sensitization is initiated by allergen-specific Th2-cell responses. Data on early allergen-specific T-cell responses in allergic children are scarce. We hypothesized that allergen-specific Th2-cell responses can be detected preceding sensitization. Therefore, peripheral blood mononuclear cells (PBMC) of nonsensitized, 'not-yet' sensitized or sensitized children were cultured with highly purified allergens. Cytokine levels in supernatant were determined using multiplex assay and GATA3 expression by flow cytometry. PBMC of sensitized children aged 3 and 5 years showed higher production of IL4, IL5 and IL13 and higher expression of GATA3 in response to purified allergens compared to nonsensitized children. PBMC of children that were 'not-yet' sensitized already showed higher levels of IL5 and IL13 and higher GATA3 expression at age 3 years. This shows that allergen-specific *in vitro* Th2 responses precede the detection of allergen-specific IgE, which can provide a window of opportunity for novel therapeutic interventions.

The role of Th2 cells in allergic disease can be either indirect by driving differentiation of eosinophils and production of immunoglobulin E (IgE), the hallmark of allergic sensitization, or direct through release of inflammatory cytokines. The differentiation of Th2 cells is controlled by the double zinc finger transcription factor GATA3 (1). Although the concept that Th2-cell polarization by allergens precedes B-cell programming towards IgE production is not new, data on early allergen-specific human T-cell responses, supporting this concept, are scarce. Furthermore, many studies have reported on allergen-specific T-cell responses in both allergic and non-allergic individuals, but with conflicting results (2–5). It is important to note that many allergens and allergen extracts contain contaminants (6, 7) and have different intrinsic

properties known to be potent triggers of the innate immune system and to be able to modulate adaptive immune responses (8, 9). This can trouble the study of established allergen-specific immune responses *in vitro*. Therefore, in the current study, we used highly purified allergens. Furthermore, we used samples of a well-defined cohort of early wheezers (Reubsaet et al. *Am J Respir Crit Care Med* accepted for publication) to investigate early T-cell responses in sensitized, nonsensitized and not-yet sensitized children (Table S1 of the online Supporting Information).

Methods

Definitions

Sensitized was defined as the presence of specific IgE for HDM and/or birch and/or cat.

Nonsensitized was defined as the absence of specific IgE for HDM and/or birch and/or cat.

Abbreviations

Bet v 1, *Betula verrucosa* group 1; Der p 1, *Dermatophagoides pteronyssinus* group 1; Fel d 1, *Felis domesticus* group 1; HDM, house dust mite; IgE, immunoglobulin E; TT, tetanus toxoid.

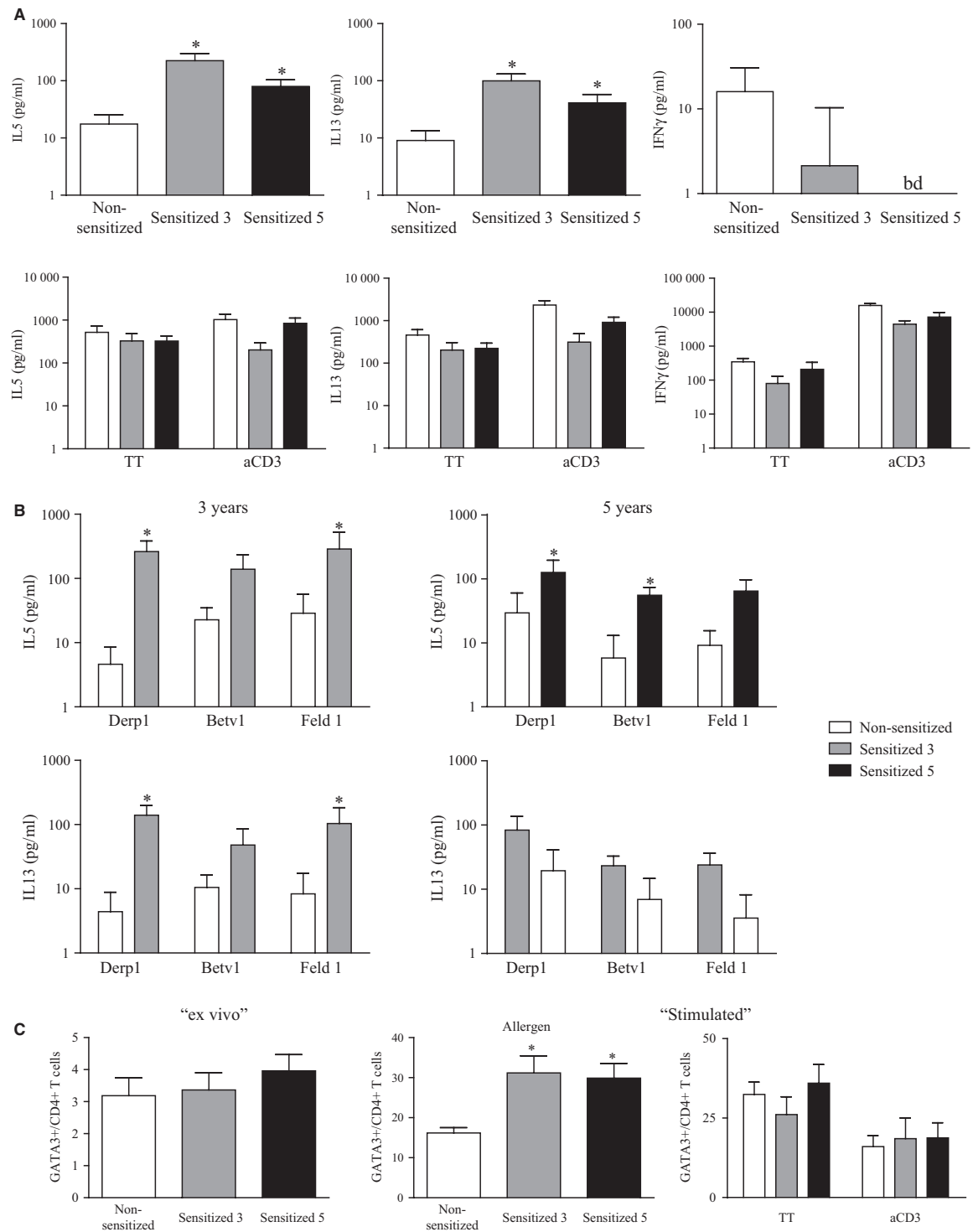


Figure 1 Allergen-sensitized children show specific Th2 cytokine response at age 3 and 5 years. (A) IL5, IL13 and IFN γ levels in the supernatant of peripheral blood mononuclear cells from house dust mite ($n = 7$ at 3 years, $n = 8$ at 5 years), birch ($n = 6$ at 3 years, $n = 8$ at 5 years) and/or cat ($n = 3$ at 3 years, $n = 9$ at 5 years) sensitized (data for all allergens combined at 3 years: grey bars; data for all allergens combined at 5 years; black bars) and non-

sensitized (white bars; combined data from $n = 14$ at 3 years and $n = 11$ at 5 years) children after culture with Der p 1, Bet v 1, Fel d 1, tetanus toxoid and anti-CD3. (B) IL5 and IL13 levels in supernatant according to stimulating allergen. (C) GATA3 expression in CD4 $^{+}$ T cells *ex vivo* (left) and after culture (middle and right). Data shown are mean + SEM. * $P < 0.05$ compared to nonsensitized; bd, below detection.

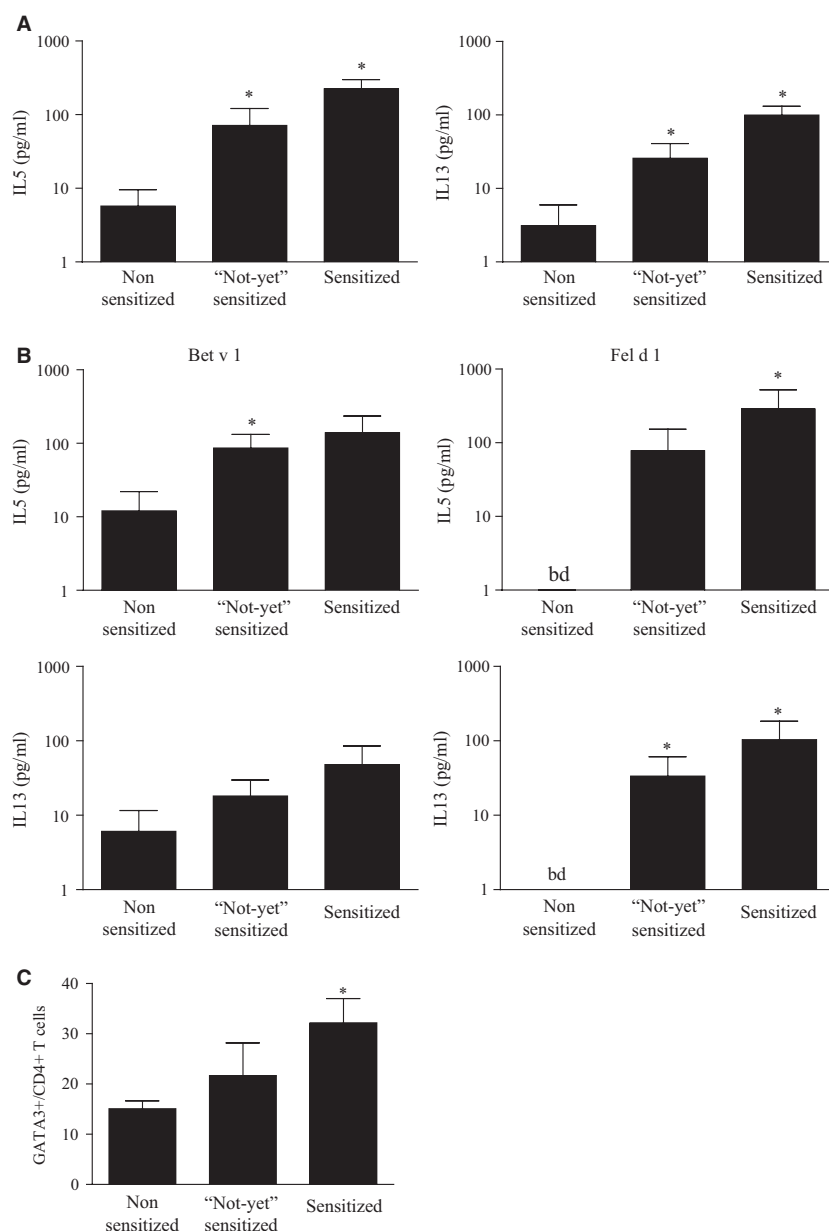


Figure 2 Th2 cytokines precede IgE. Peripheral blood mononuclear cells from children aged 3 years sensitized for house dust mite (HDM) ($n = 6$), birch ($n = 5$) and/or cat ($n = 3$) at both 3 and 5 years (black bars), at 5 years only ($n = 1, 2$ and 6 for HDM, birch and cat, respectively; grey bars) or nonsensitized ($n = 12, 12$ and 10 , respectively; white bars) cultured with Der p 1, Bet v 1 or Fel d 1.

'Not-yet' sensitized was defined as the absence of specific IgE for HDM and/or birch and/or cat at age three with the presence of specific IgE for HDM and/or birch and/or cat at age 5 years.

Culture and cytokine analysis

For stimulation experiments, cells were cultured in the presence of $10 \mu\text{g/ml}$ LoTox Der p 1 (endotoxin level 0.01

(A) Supernatant IL5 and IL13, data for all allergens combined. (B) Supernatant IL5 and IL13 after Bet v 1 (left) and Fel d 1 (right) stimulation. (C) GATA3 expression in CD4^+ T cells determined by flow cytometry, data for all allergens combined. Data shown are mean $\pm$ SEM. * $P < 0.05$ compared to nonsensitized.

EU/ μg), Bet v 1 (*Betula verrucosa* group 1; endotoxin level $0.01\text{--}0.019 \text{ EU}/\mu\text{g}$), Fel d 1 (*Felis domesticus* 1; endotoxin level $0.012 \text{ EU}/\mu\text{g}$) (Indoor Biotechnologies, Charlottesville, VA, USA), $1 \mu\text{g/ml}$ plate bound anti-CD3 (clone OKT3 eBioscience, San Diego, CA, USA) or 0.5 Lf/ml tetanus toxoid (TT) (NVI, Bilthoven, the Netherlands). Cytokine levels were measured in supernatants of PBMC cultures by multiplex assay (Luminex), as previously described (10).

For additional methods, see the Methods section of the online Supporting Information.

Results

PBMC of both 3- and 5-year-old children sensitized to HDM, birch and/or cat cultured with Der p 1, Bet v 1 or Fel d 1 showed significantly higher levels of IL5 and IL13 in the supernatant compared to PBMC from nonsensitized children (Fig. 1A). IL4 and IL9 levels, although low, showed a similar trend, whereas there was no difference in IFN γ levels (data not shown). For all three allergens, the allergen-specific Th2 response was increased in children sensitized for the respective allergen (Fig. 1B). The expression of Th2-related transcription factor GATA3 after allergen stimulation was significantly higher in CD4⁺ T cells of sensitized children at age 3 and 5 years compared to nonsensitized children (Fig. 1C middle), whereas there was no difference in *ex vivo* GATA3 expression (Fig. 1C left) (see online Figure S1 for representative FACS plots). Cytokine production and GATA3 expression after TT or anti-CD3 stimulation were not increased in sensitized compared to nonsensitized children (Fig. 1A bottom and 1C right). We detected no significant differences in FOXP3 expression between sensitized and nonsensitized children both *ex vivo* and after stimulation with allergen, TT or anti-CD3 (data not shown).

Next, PBMC of sensitized, 'not-yet' sensitized and non-sensitized 3-year-old children were stimulated with Der p 1, Bet v 1 and Fel d 1. In samples of the 'not-yet' sensitized children, significantly increased IL5 and IL13 levels were found compared to nonsensitized children following specific allergen stimulation (Fig. 2A). One child was 'not-yet' sensitized to HDM at age 3 years. For children 'not-yet' sensitized to birch and cat, we observed the same trend for IL5 and IL13 after culture with Bet v 1 and Fel d 1, respectively (Fig. 2B). In addition, GATA3 expression in CD4⁺ T cells was also higher in the allergen-stimulated cultures of children at age 3 years that were 'not-yet' sensitized compared to nonsensitized children (Fig. 2C).

Discussion

This study demonstrates that PBMC from sensitized children respond to highly purified major allergens with the production of Th2 cytokines IL5 and IL13 as well as upregulation of GATA3 expression. We could not show an allergen-specific response in nonsensitized children in contrast to the IFN γ or IL10-prone responses to allergens reported in other studies (2, 3, 11). Interestingly, the Th2 T-cell response could already be detected before IgE sensitization to a specific allergen was present, indicating that allergen-specific Th2 responses are primed in early life. Although the number of individual patients sensitized to one specific allergen was low, the total number of allergen-sensitized children was sufficient to clearly establish allergen-specific Th2 responses *in vitro*.

Importantly, the allergen-induced Th2 response in the PBMC from sensitized children was not due to a general tendency to generate a Th2-skewed response. Moreover, in contrast to the study of Tulic (12), but in agreement with the study by Weber-Chrysochoou (13), the PBMC from sensitized children were able to generate a normal IFN γ response. This implicates that the underlying mechanism in our population is an allergen-specific signal directly driving Th2 skewing of the antigen-presenting cells and/or T cells and not via a diminished Th1 response. In the current study, we examined the response to a single and highly purified allergenic protein, independently of other allergenic molecules that do possess potent intrinsic innate immunity signals such as HDM and peanut (8, 9, 14). It is clear that in the era of component-resolved diagnostics in allergy (15), also studies of T-cell activation by individual allergenic proteins are important.

In conclusion, we are the first to show that PBMC from young children show allergen-specific Th2 responses that precede the detection of allergen-specific IgE. The allergen-specific *in vitro* Th2 response might provide a window of opportunity for novel therapeutic interventions.

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Author contributions

LLR, BA, BJP, FW, EK helped in study concept and design; LLR, JM, RS helped in acquisition of data; LLR, FW, EK helped in analysis and interpretation of data; LLR, BA, FW, EK helped in drafting of the manuscript; all authors performed critical revision of the manuscript for important intellectual content and gave final approval of the version to be published.

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Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Representative FACS plots for GATA3 staining.

Table S1. Patient characteristics.

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Online Data Supplement

Allergen-specific Th2 responses in young children precede sensitization later in life

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Methods

Participants and Study design.

The participants described in this paper were 22 preschool children part of a prospective cohort study of early wheezers (Reubsaet et al. Am J Respir Critical Care Medicine 2013, accepted for publication). In short participants were included at the age of 3 years and followed up to at least age 6 years. Every year they were seen at the out patient clinic of our hospital for clinical check up. At inclusion and at the 2nd follow up visit 2 years later, at a time without apparent respiratory symptoms or infections, total and specific IgE were determined from serum and at inclusion a full blood count was performed.

IgE measurement

To determine sensitization to inhalation and food allergens total and specific IgE in serum was measured using the ImmunoCAP[®] method with Pharmacia differential atopy (Phadiatop[®]) test (Phadia AB, Uppsala, Sweden). Specific IgE > 0.35 kU/L was considered positive.

Cell isolation and culture

Peripheral Blood mononuclear cells (PBMC) were isolated using Ficoll Isopaque density gradient centrifugation (Amersham Biosciences, New Jersey, USA). PBMC were frozen in Fetal Calf Serum (FCS) containing 10% DMSO and stored in a -150°C freezer until use.

Flow cytometry

Cultured cells were first incubated with surface antibodies. After surface staining cells were washed twice in FACS buffer, fixed, permeabilized and stained intracellular for GATA3

using the FOXP3 transcription factor staining buffer set (eBioscience, according to manufacturer's instructions).

All mAbs were obtained from Becton Dickinson (San Jose, CA, USA). Stained cells were diluted in FACS buffer and run on a FACSCanto (BD). Data analysis was performed using FlowJo software (Treestar inc. Oregon, USA).

Statistical analysis

For baseline characteristics chi-square test was used to compare frequencies. Cytokine levels were log transformed before statistical analysis. The independent samples *t*-test or Mann-Whitney U test was used to compare normally distributed and non-parametric continuous parameters, respectively for sensitized versus non-sensitized individuals. Differences were considered significant at 2-tailed $P < 0.05$. Statistical analysis was performed using GraphPad Prism Version 5.03 (Graphpad Software) and SPSS 19.0 for windows (Chicago, IL, USA).

Table E1. **Patient Characteristics**

	Non sensitized 3 years N = 12	Sensitized for HDM, birch and/or cat at 3 years N = 10	“not yet” sensitized for HDM, birch and/or cat N = 8	Non sensitized 5 years N = 9	Sensitized for HDM, birch and/or cat at 5 years N = 13
Total IgE (kU/L) mean $\pm$ SD	87 $\pm$ 107	212 $\pm$ 171*	229 $\pm$ 174*	80 $\pm$ 108	377 $\pm$ 480*
Eosinophils (x 10 ⁹ /L) mean $\pm$ SD	0.41 $\pm$ 0.40	0.63 $\pm$ 0.36	0.52 $\pm$ 0.36	nd	nd
Sensitization to: - HDM - Birch - Cat (number (%))	0 (0) 0 (0) 0 (0)	6 (60) 6 (60) 3 (30)	1 (13) [¶] 2 (25) [¶] 6 (75) [¶]	0 (0) 0 (0) 0 (0)	7 (54) 8 (62) 9 (69)
Specific IgE for: - HDM - Birch - Cat mean $\pm$ SD (kU/L)	na na na	15 $\pm$ 30 27 $\pm$ 42 7 $\pm$ 18	7 $\pm$ 16 19 $\pm$ 35 1.2 $\pm$ 2.4	na na na	18 $\pm$ 33 20 $\pm$ 26 10 $\pm$ 27

HDM = house dust mite; nd = not determined; SD = standard deviation; na = not applicable;

* P = < 0.05 compared to non-sensitized; [¶] sensitized at 5 years but not at 3 years.

Figure legend

Figure E1. Representative FACS plots for GATA3 staining. PBMC were cultured for 7 days with (below) or without allergen (above) and stained for CD3, CD4 and GATA3. Showing representative plots for the CD4+GATA3+ population for both conditions within the CD3+ T cell population (middle) and the CD4+ T cell population (right).