

# Dysregulated Th1 and Th2 responses in food-allergic children – Does elimination diet contribute to the dysregulation?

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Infants with eczema and sensitization to foods are recommended skin care and, if food allergy is proven, an elimination diet. Although most of these children tolerate foods before 3 yr of age, some children experience prolonged food allergy. To our knowledge, no prospective study has investigated the cytokine profile in food-sensitized eczematous children with prolonged food intolerance. The aim of the study was to prospectively investigate the development of cytokine production induced by food allergen in food-sensitized eczematous children who, at 4½ yr of age, were allergic or tolerant to egg or milk. Twenty-one eczematous infants, [age 5 (3–10) months; median and range], sensitized to egg and/or milk were included, put on elimination diet and followed prospectively. At 4½ yr of age, the children were defined as tolerant or allergic to egg and/or milk based on open or double-blind placebo-controlled food challenges. Peripheral blood mononuclear cells (PBMC) were isolated from the children on inclusion, after 6 wk of elimination diet, and at 3 and 4½ yr of age. Ovalbumin,  $\beta$ -lactoglobulin and tetanus toxoid-induced IL-4, -5, -10, -13 and IFN- $\gamma$  production from PBMC were analyzed with enzyme-linked immunosorbent assay. The IFN- $\gamma$  and IL-5 secretion induced by food allergen at 4½ yr was higher in cell cultures from children who were allergic to egg or milk than in tolerant children. In food-allergic children, the levels of IFN- $\gamma$  and IL-5 were higher at 4½ yr compared with inclusion levels, but this increase was generally not observed in the tolerant children who consumed milk and egg. In conclusion, immune cells from food-allergic children on an elimination diet respond with up-regulated T helper 1 and T helper 2 cytokine secretion induced by food allergen. We hypothesize that allergen elimination may influence the regulatory mechanisms maintaining balanced immune responses to innocuous food antigens.

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The immune functions in children who develop allergy and in those who remain non-allergic differ in several aspects (1). The early IgE responses to allergens are eventually down-regulated in infants who remain non-allergic, whereas they reach higher and more persistent levels in those who develop allergy (2). Immune responses in the neonatal period are of the T helper (Th) 2 type

(3), possibly as a consequence of the Th2-skewed immunologic balance in utero during pregnancy (4). The Th2-like responses that characterize atopic infants with high production of IL-4, -5, -9 and -13 (5) may be due to a defective diversion of the neonatal Th2-skewed immunity. In healthy individuals, Th1-like immune responses dominate, but other factors counteract Th1-like

reactions, e.g., the anti-inflammatory cytokine IL-10, which also regulates Th2-like responses and suppresses IgE production. However, the role of Th1 responses in atopic children is more debated, as decreased (6), similar (5) and increased (7) levels of IFN- $\gamma$  production have been reported. The immune response pattern is thought to be set during infancy, and events occurring during this period are of primary importance in determining responder phenotype later in life.

The first atopic manifestation in childhood is usually eczema, often associated with sensitization to food allergens (8).  $\beta$ -lactoglobulin (BLG) in cow's milk and ovalbumin (OVA) in hen's egg are common allergenic food proteins encountered during infancy. In clinical practice, a temporary elimination of the suspected allergen from the diet is suggested for children with food allergy (9). Although the majority of children will develop clinical tolerance before the age of three, some children show a prolonged period of food intolerance (8, 10). Tolerance to foods might be associated with Th1 responses as high production of IFN- $\gamma$  has been observed in children with eczema but without cow's milk allergy, whereas eczematous children with cow's milk allergy have a Th2-skewed response (11). In a cross-sectional study, Noma et al. (12) have shown that OVA-induced IFN- $\gamma$  production is higher, and IL-4 synthesis lower, in patients outgrowing egg allergy than in patients with persistent allergy. It is not known whether the skewed immunity observed among food allergic individuals is related to the allergic phenotype *per se*, or whether it is a consequence of allergen avoidance in these patients. To our knowledge, no prospective study has investigated the cytokine profile in food-sensitized eczematous children with prolonged food intolerance.

The aim of this study was to monitor prospectively the cytokine profile induced by food allergen in young children with eczema and food sensitization up to 4½ yr of age and to evaluate differences in cytokine production between children who could or could not tolerate egg and milk at 4½ yr of age.

## Methods

### Subjects

This study is based on 21 eczematous infants who were food-skin prick tests (SPT) positive, selected from a center participating in a prospective multicenter study of immunologic development in children with eczema and suspected food allergy (13). All 21 children were under 2 yr of

age on inclusion and were treated with a temporary egg and/or milk elimination diet, and emollients and/or topical steroids. They were examined four times: on inclusion, after a treatment period of 6 wk and at 3–4½ yr of age. The diagnosis of eczema was established using the Hanifin-Rajka (14) criteria, and eczema severity was assessed at each visit using the SCORAD scoring system (15). On inclusion and at the 4½-yr examination, SPT to fresh hen's egg white and cow's milk were performed. On inclusion, four children had positive SPT ( $\geq 3$  mm) to egg only, whereas seven were SPT positive to milk, and 10 were SPT positive to both egg and milk. The children with positive SPT to egg and/or milk were treated by eliminating the offending food for at least 6 wk. After that, they re-introduced the food items when it was considered safe and without risk for anaphylactic reactions. At 4½ yr of age, six children were classified as food allergic; one to egg, and five to both egg and milk. The diagnosis of food allergy was based on either a positive double-blind placebo-controlled food challenge, or a recent allergic reaction after an accidental exposure to the offending foods, i.e., an inadvertent open challenge (16). Fifteen children tolerated both egg and milk at the 4½-yr follow-up, and egg and milk had been included in their diet at least since they were 3 yr of age. One child did not tolerate egg at the 4½-yr follow-up, but had consumed milk from 4 yr of age. On inclusion and at the 4½-yr follow-up, serum levels of egg- and milk-specific IgE antibodies were analyzed with UniCAP®, according to the recommendations of the manufacturer (Phadia, Uppsala, Sweden). Test results at values  $\geq 0.35$  kUA/l were considered positive. All children were immunized to tetanus at least once when they were included in the study.

### Blood sampling and cell cultures

Sera were collected after allowing blood to clot at room temperature and were then stored at  $-20^{\circ}\text{C}$  until analysis of specific IgE antibodies. Venous blood was drawn into heparin-treated tubes. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll Paque density gradient (Pharmacia, Uppsala, Sweden), washed three times with RPMI-1640 (Life Technologies AB, Täby, Sweden) including 2% fetal calf serum (Life Technologies) and thereafter cryopreserved according to standard methodology in 10% dimethyl sulfoxide (Sigma-Aldrich, Stockholm, Sweden), 50% fetal calf serum and 40% RPMI-1640, and stored in liquid N<sub>2</sub> until analysis.

After thawing, the viability of the cells was controlled with trypan blue, and  $1 \times 10^6$  viable PBMC were cultured in 1 ml AIM-V serum free medium (Life Technologies AB) with  $20 \mu\text{M}$   $\beta$ -mercaptoethanol (Sigma-Aldrich) only, or with  $100 \mu\text{g/ml}$  BLG (Sigma-Aldrich),  $100 \mu\text{g/ml}$  OVA (grade VI, Sigma-Aldrich) or  $100 \text{ ng/ml}$  tetanus toxoid (TT) (R&D systems Europe Ltd, Oxon, UK), with or without  $2 \mu\text{g/ml}$  of  $\alpha$ -IL4R (Calbiochem, VWR International AB, Stockholm, Sweden) for 24 h or 6 days in  $37^\circ\text{C}$  with  $5\%$   $\text{CO}_2$  (Forma  $\text{CO}_2$ -incubator model 3862, Forma Scientific Inc., Marietta, OH, USA). Cell suspensions were centrifuged and supernatants were removed and stored at  $-70^\circ\text{C}$  until analysis.

#### Cytokine analysis

The levels of IL-4, IL-10, IL-13 and IFN- $\gamma$  in the cell supernatants were analyzed in duplicate with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Peli-Pair, CLB Amsterdam, The Netherlands) according to the manufacturer's recommendations. An in-house ELISA was used to determine IL-5 levels, (5). For analysis of IL-10, supernatants from PBMC cultured for 24 h were used, whereas all other cytokines were measured in the 6 day cultures. IL-4 analyzes were performed in cultures with added  $\alpha$ -IL4R antibody. The sensitivity limits for quantitative determinations were  $6.2 \text{ pg/ml}$  (IL-4),  $6.3 \text{ pg/ml}$  (IL-5),  $4.6 \text{ pg/ml}$  (IL-10),  $2.0 \text{ pg/ml}$  (IL-13) and  $15.6 \text{ pg/ml}$  (IFN- $\gamma$ ). Samples with a value below the limit for quantitative determinations were assigned a value corresponding to half of that value. Unstimulated responses (cultures with medium only) were subtracted from responses from stimulated cultures. One sample did not respond to any allergen and was excluded from the analysis. As a result of limited amount of material, some cytokine analyzes are missing in a few samples.

#### Statistics

As the results were not normally distributed, non-parametric tests were used. Paired analyzes were performed with the Wilcoxon signed-rank test and unpaired analyzes with the Mann-Whitney  $U$ -test. The chi-square test was used for categorical variables, and Fisher's exact test was used when the expected frequency for any cell was less than five. A probability level of  $<5\%$  was considered to be statistically significant. The calculations were performed with a statistical package, StatView 5.0 for PC (SAS Institute Inc., Cary, NC, USA).

#### Ethics

The study was approved by the Human Research Ethics Committee at the Faculty of Health Sciences in Linköping. Written informed consent was obtained from the children's parents. To minimize the discomfort for the children, topical analgesic cream was used prior to the collection of blood samples.

#### Results

Among 21 eczematous infants with suspected food allergy, 15 could tolerate egg and milk at the age of  $4\frac{1}{2}$  and had been able to include the foods into their diet at least since 4 yr of age. One child could tolerate milk but not egg. The children who were egg-allergic and on an egg elimination diet at  $4\frac{1}{2}$  yr of age, reacted more frequently with up-regulated OVA induced IL-5 secretion than did egg-tolerant children ( $4/6$  vs.  $0/9$ ,  $p < 0.01$ ), and the levels of IL-5 were higher in the egg-allergic children (Fig. 1a). The trend was similar for OVA-stimulated IL-5 production at 3 yr of age (Fig. 1a). After BLG stimulation, IL-5 production was higher in milk-allergic children on a milk elimination diet, than in milk-tolerant children at  $4\frac{1}{2}$  yr of age (Fig. 1b). The levels of IFN- $\gamma$  induced by OVA (Fig. 2a) and BLG (Fig. 2b) were significantly higher in allergic children than in tolerant children (Fig. 2) at  $4\frac{1}{2}$  yr of age. The levels of OVA-, BLG- and TT-induced IL-4, IL-10 and IL-13, TT-induced IL-5 and IFN- $\gamma$  as well as unstimulated cytokine production were similar in allergic and tolerant children at all ages (data not shown).

The secretion of IL-5 (Fig. 1) and IFN- $\gamma$  (Fig. 2) from PBMC activated with OVA and BLG was higher at the  $4\frac{1}{2}$ -yr follow-up compared with the levels on inclusion in both the egg- and milk-allergic children. These differences were not observed in the egg- and milk-tolerant children, except for BLG-induced IL-5 production, which increased from inclusion to the  $4\frac{1}{2}$ -yr follow-up (Fig. 1b). No changes over time could be observed in children who were allergic or tolerant at  $4\frac{1}{2}$  yr with respect to OVA-, BLG- and TT-induced IL-4, IL-10 and IL-13 production, TT-induced IL-5 and IFN- $\gamma$ , or unstimulated cytokine secretion (data not shown).

The elimination of egg and/or milk from the diet of SPT positive infants was associated with increased OVA- and BLG- induced IL-5 and IFN- $\gamma$  responses (Table 1). These increased food-allergen-specific cytokine responses were not observed in infants who, during the same time period, ingested egg and milk, respectively (data not shown).

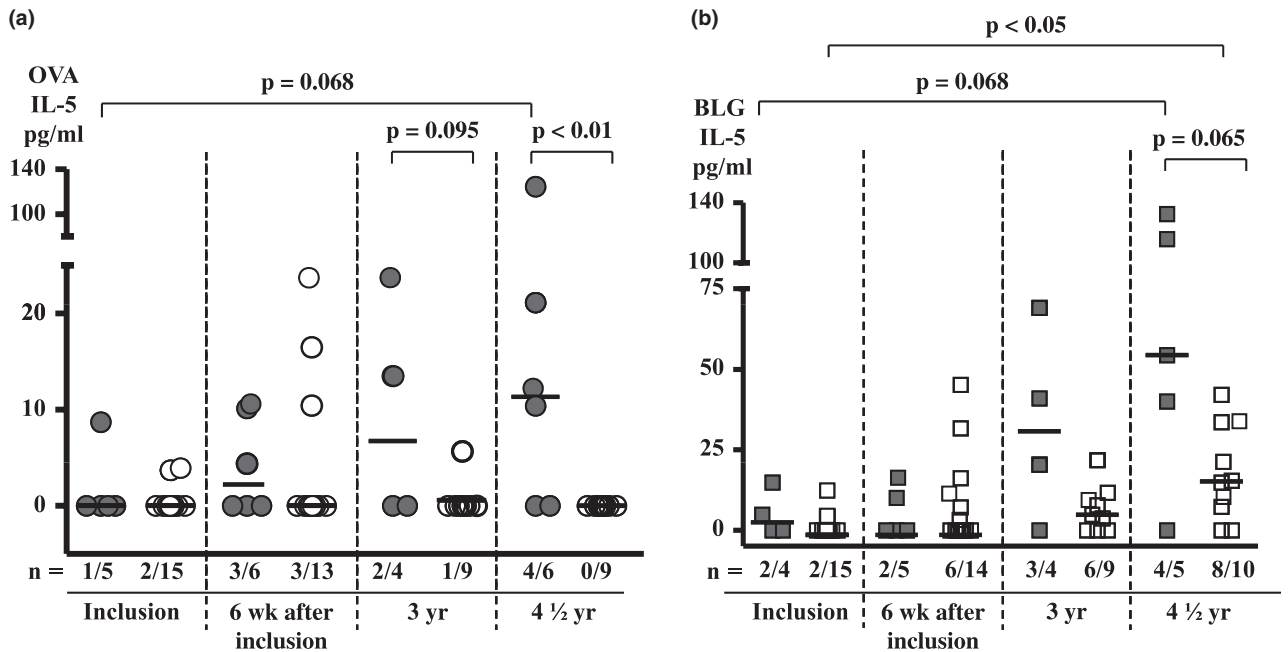


Fig. 1. The levels of IL-5 after (a) Ovalbumin stimulation in children who at 4 1/2 yr of age are allergic to egg (filled dots) and are on an egg elimination diet, or children who are tolerant and have ingested egg at least since 4 yr of age (open dots), and (b) BLG stimulation in children who at 4 1/2 yr of age are allergic to milk (filled squares) and are on a milk elimination diet, or children who are tolerant and have ingested milk at least since 4 yr of age (open squares). All children had eczema and food sensitization to egg and/or milk on inclusion (< 2 yr). n = numbers of positive samples (above cut-off concentration) in relation to the total number of analyzed samples. The median levels are indicated (black line).

At 4 1/2 yr of age, the levels of specific IgE to egg and milk were significantly higher in the food-allergic groups than among tolerant chil-

dren (Table 2). However, on inclusion there were no differences between the groups regarding food-specific IgE antibody levels (Table 2).

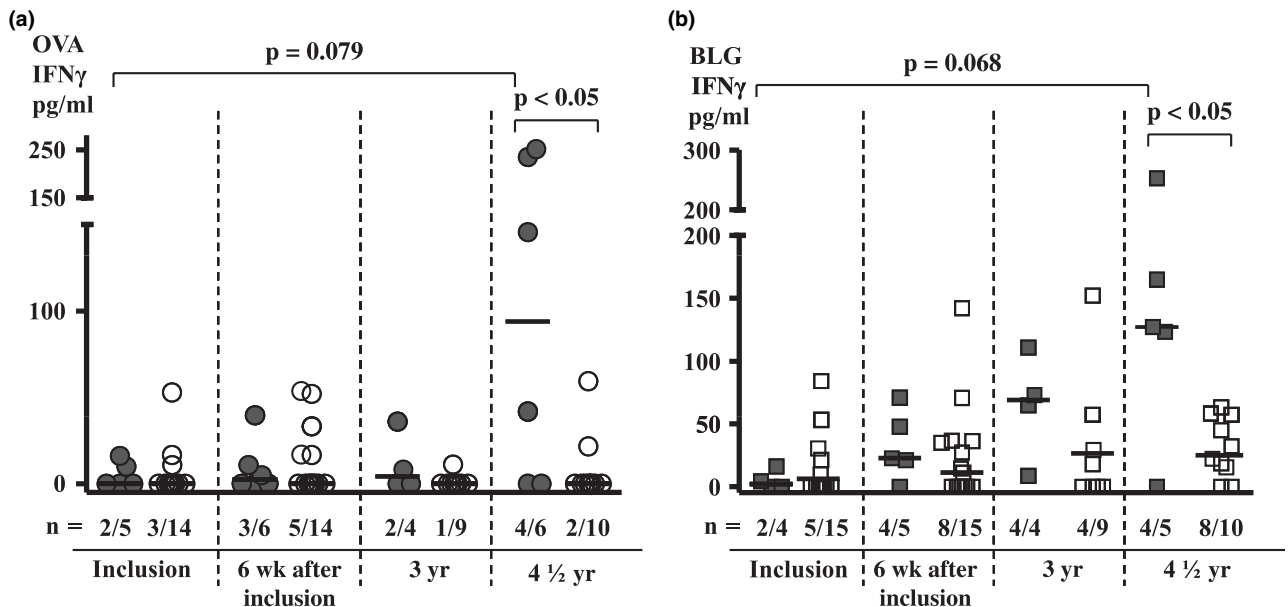


Fig. 2. The levels of IFN-γ after (a) Ovalbumin stimulation in children who at 4 1/2 yr of age are allergic to egg (filled dots) and are on an egg elimination diet, or children who are tolerant and have ingested egg at least since 4 yr of age (open dots) and (b) β-lactoglobulin stimulation in children who at 4 1/2 yr of age are allergic to milk (filled squares) and are on a milk elimination diet, or children who are tolerant and have ingested milk at least since 4 yr of age (open squares). All children had eczema and food sensitization to egg and/or milk on inclusion (< 2 yr). n = numbers of positive samples (above cut-off concentration) in relation to the total number of analyzed samples. The median levels are indicated (black line).

Table 1. OVA and BLG induced IFN- $\gamma$  and IL-5 production [median and (range)] in PBMC from eczematous infants before (inclusion) and after 6 wk (Visit 2) of treatment with skin care and egg and milk elimination diet. The p-values are indicated for differences between inclusion and visit 2

|               | SPT+          |                  | p-value |
|---------------|---------------|------------------|---------|
|               | Inclusion     | Visit 2          |         |
| OVA           |               | <i>Egg SPT+</i>  |         |
| IFN- $\gamma$ | 0.0 (0.0–48)  | 0.0 (0.0–52)     | <0.05   |
| IL-5          | 0.0 (0.0–8.7) | 0.0 (0.0–28)     | <0.05   |
| BLG           |               | <i>Milk SPT+</i> |         |
| IFN- $\gamma$ | 0.0 (0.0–84)  | 24 (0.0–140)     | <0.01   |
| IL-5          | 0.0 (0.0–14)  | 0.0 (0.0–45)     | <0.05   |

OVA, ovalbumin; BLG,  $\beta$ -lactoglobulin; PBMC, peripheral blood mononuclear cells; SPT, skin prick test.

## Discussion

In this study we have shown that children with food allergy at 4½ yr on a prolonged elimination diet responded with higher food-allergen-induced production of both Th1- and Th2-related cytokines *in vitro* than did children who tolerated the food at that age. No such differences were observed on inclusion, i.e., before diagnosis and food elimination. The levels of both OVA- and BLG-induced IFN- $\gamma$  and IL-5 were higher at the 4½-yr follow-up compared with inclusion levels in food-allergic children, whereas this was generally not observed in children who tolerated egg and milk at the 4½-yr follow-up.

A higher degree of immune activation has previously been associated with food allergy. *In vitro*-generated cow's milk protein-specific T cells from milk-allergic children express higher levels of activation cell-surface markers such as CD25 and CD30 than those from children who have developed tolerance (17), suggesting a strong food-specific T cell response in children with persistent food allergy. This may be due to a diminished ability among the allergic children to maintain T regulatory (Treg) cells. This assumption is supported by feeding experiments in mice, in which the role of CD4 + CD25+ Treg cells has been observed in mediating oral tolerance

(18). Treg cells have been shown to suppress both Th1 and Th2 cells (19). However, the defective response in food-allergic children may be caused by the protracted elimination diet and not by the allergic status *per se*. Children with cow's milk allergy who were able to perform a 1-wk challenge with increasing doses of milk had higher numbers of CD4 + CD25+ Treg cells after the challenge period than did children who had to discontinue the milk challenge (20). Notably, before the challenge the percentage of CD4 + CD25+ cells was similar in both groups when all the children were on an elimination diet. These results imply that allergen avoidance may cause defective immune tolerance mechanisms and that continuous exposure is needed for sustained immune regulation. This might be one explanation for the increased food-allergen-specific cytokine responses in the food-allergic children on an elimination diet in our study.

Studies of airborne allergens suggest that allergen exposure favours immunologic tolerance (21, 22). Furthermore, allergen-specific immunotherapy is associated with increased levels of IL-10 and/or TGF- $\beta$ , indicating a shift in T-cell responses from Th2 to Treg cells during allergen exposure (23). In a murine model, repeated respiratory exposure to allergens influenced dendritic cells to induce the development of IL-10-secreting Treg cells in a process dependent on both IL-10 and inducible costimulator (ICOS)-ICOS-ligand interaction (24). Moreover, during sublingual immunotherapy the frequency of CD4 + CD25+ T cells increased in combination with increased expression of IL-10 and decreased expression of IL-4 and IFN- $\gamma$  mRNA (25). The 4½-yr-old food-allergic children in our study have been on allergen elimination diet during a long period. This allergen avoidance might thwart the induction of regulatory mechanisms, resulting in an up-regulation of both Th1 and Th2 responses after *in vitro* stimulation with food allergens. This suggestion is further supported by the increased OVA- and BLG-induced IFN- $\gamma$  and IL-5 responses observed after the first 6 wk of egg and milk elimination diet, respectively. Such an increase was not seen in SPT negative infants who were exposed to egg and cow's milk during the same period.

There is an increased risk of acute allergic reactions after accidental re-exposure to cow's milk after a longer cow's milk elimination diet (26). Our study indicates a possible explanation for this increasing risk. The current treatment of food allergy with strict allergen avoidance has recently been questioned (27) and, instead, specific oral tolerance induction based on limited

Table 2. Egg- and milk-specific IgE antibody levels in sensitized eczematous children at inclusion (children younger than 2 yr) or at 4 yr of age. The children are at 4 yr of age tolerant or allergic to egg or milk

|           | Tolerant        | Allergic        | p-value |
|-----------|-----------------|-----------------|---------|
| Egg       |                 |                 |         |
| Inclusion | 1.5 (0.18–26)   | 0.53 (0.18–9.2) | ns      |
| 4.5 yr    | 0.18 (0.18–2.8) | 2.3 (0.18–19)   | 0.017   |
| Milk      |                 |                 |         |
| Inclusion | 0.67 (0.18–33)  | 3.3 (0.18–23)   | ns      |
| 4.5 yr    | 0.18 (0.18–1.0) | 0.99 (0.18–2.1) | 0.009   |

ns, non-significant.

oral administration of the offending food has been suggested as an approach to food-allergy therapy in selected cases (27). Interestingly, the beneficial effect of food-allergen exposure has been indicated in several recent studies, showing increased tolerance to foods after treatment with oral immunotherapy (28, 29).

Considering the anti-inflammatory properties of IL-10, differences could be expected between tolerant and allergic children with respect to this cytokine. Unfortunately, due to our limited number of patients and to the fact that few children responded with IL-10 levels above the cut-off value, we cannot draw any conclusions in respect of IL-10.

In contrast to our results, Noma et al. (12) showed decreased IFN- $\gamma$  and increased IL-4 synthesis after OVA stimulation in children allergic to hen's egg. That study used different methods of analysis and does not indicate whether an elimination diet was instituted. Therefore, it is difficult to evaluate the differences between the two studies. How the immune system develops in young children with eczema who are sensitized to food and why some of them develop persistent food allergy whereas others do not are poorly investigated. To our knowledge our study is the first prospective study that evaluates the cytokine profile in eczematous children with persistent food allergy.

In conclusion, our study shows that food allergy at 4½ yr in children suffering from eczema early in life is associated with high secretion of both Th1 and Th2 cytokine responses after *in vitro* food-allergen stimulation. This dysfunction is not observed in food-tolerant children, perhaps due to their continuous exposure to the allergens.

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