

Dynamics of cytokine mRNA expression and fecal biomarkers in school-children undergoing a double-blind placebo-controlled food challenge series



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ABSTRACT

Background: There is need for prognostic markers for symptomatic food allergy since current diagnostic methods are insufficient and/or time and labor consuming.

Objective: To estimate the cytokine mRNA profiles in peripheral blood mononuclear cells (PBMC) before and after a double-blind placebo-controlled food challenge series in schoolchildren with suspected allergy to milk, egg or cod and in healthy controls. Analyses of fecal inflammatory biomarkers before and after the challenge were included.

Methods: Twelve-year-old children from a population-based cohort reporting complete avoidance of milk, egg, cod or wheat due to perceived hypersensitivity were clinically examined and those with suspected food allergy were evaluated with a 3-session double-blind placebo-controlled food challenge (n = 18). Seven healthy controls participated in a double-blind challenge with egg. Before and after the challenge series, the cytokine mRNA expression was quantified for 13 cytokines discriminating between humoral Th2-, cytotoxic Th1-, regulatory Th3/Tr1- and inflammatory responses. Fecal calprotectin and eosinophil-derived neurotoxin (EDN) were also analyzed in children with suspected food allergy before and after the challenge series.

Results: Pre challenge, children with suspected food allergy had higher IL-13 and TNF- α expression and lower IFN- γ and IL-15 expression compared to healthy controls (all $p < 0.05$). Children with challenge-proven food allergy had increased IL13 and IL-10 expression compared to the levels seen in negative challenges ($p < 0.05$). Post challenge, IL-1 β and IL-6 mRNA levels were elevated in the food allergic children compared to controls ($p < 0.05$). Fecal calprotectin and EDN levels were higher in challenge-proven food allergy compared to a negative challenge although not statistically significantly.

Conclusion & clinical relevance: Increased baseline mRNA levels of the Th2-related cytokine IL-13 and the regulatory cytokine IL-10 predicted a positive food challenge outcome. These cytokines in combination with fecal calprotectin and EDN might serve as future prognostic markers for symptomatic, IgE-mediated food allergy but need further validation in a larger patient cohort.

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Abbreviations: DBPCFC, double-blind placebo-controlled food challenge; EDN, eosinophil-derived neurotoxin; OLIN, Obstructive Lung Disease In Northern Sweden Studies; PBMC, peripheral blood mononuclear cells; TGF- β , transforming growth factor beta.

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1. Introduction

Allergy to basic foods like cow's milk and hen's egg affects 4–5% of children in Western countries during the first years of life [1,2], however, most children develop tolerance in their preschool years [3]. Cod allergy on the other hand can start in early childhood, but more commonly emerges at school age [4,5]. While egg and cod allergies are predominantly IgE-mediated, IgE and non-IgE

mediated milk allergy is equally common [6]. Since detectable specific IgE in serum and positive skin prick tests (SPT) can only be demonstrated in IgE-mediated allergies and food sensitization often occurs without symptoms [7], double-blind placebo-controlled food challenges (DBPCFC) are considered gold standard in diagnosing food allergy [5,6]. There is, however, an ongoing search for new pertinent, prognostic markers that can predict tolerance without the need to conduct an oral food challenge, as the procedure is time-consuming, labor-intensive and can cause severe allergic reactions [1,5].

Food allergens are mainly presented to the immune system of the gut. During the interactions between the gut microbiota and T-regulatory cells, a tolerogenic interleukin (IL)-10/Transforming growth factor beta (TGF- β) response is induced that seems to be essential for tolerance development [8–10]. As one of the major regulatory cytokine of inflammatory responses, IL-10 appears to play a critical role in tolerance development and outgrowth of food allergy [11–13]. In addition, TGF- β has been shown to down-regulate the immune system by inhibiting proliferation of T-cells and activation of macrophages and thereby enhance tolerance development [14]. Higher levels of IL-10 have been found in sera from infants sensitized to egg and peanut compared to non-sensitized children but also in food-sensitized children with no symptoms compared to children with an ongoing food allergy [11,15]. Similarly to children with asthma and eczema, food allergic children have cytokine response patterns with increased Th-2 associated cytokines e.g. IL-4, IL-5 and IL-13 and reduced interferon gamma (IFN- γ) production. This dysregulated cytokine response is usually detectable already at a very young age [16,17], even before the onset of food allergy symptoms [18].

More than 50% of children with food allergy present with at least one gastrointestinal symptom [19]. Eosinophils are activated in food allergic intestinal inflammation [20] and previous studies have shown that eosinophil-derived neurotoxin (EDN), one of the major proteins released by eosinophils, might serve as a possible marker of ongoing food allergic reactions [20,21]. Others have shown that measurement of the cytosolic, immunomodulatory protein calprotectin in feces can be a helpful tool in the follow-up of response to treatment and determination of reoccurrence of cow's milk allergy [22].

Few studies have investigated the dynamics of cytokine profiles in blood or fecal inflammatory markers before and after food challenges and these analyzed only a few marker cytokines or intestinal biomarkers [21,23]. Here, we aimed at detailing the cytokine mRNA profiles discriminating between a humoral allergy-promoting Th2 response, cellular cytotoxicity-promoting Th1 response, Th3- and Tr1 regulatory responses and inflammatory response in PBMC before and after a DBPCFC series in schoolchildren with suspected allergy to cow's milk, hen's egg and/or cod and in relation to healthy controls. Fecal calprotectin and EDN levels as markers of intestinal inflammation were also analyzed in relation to a positive or negative challenge outcome.

2. Materials and methods

2.1. Study population

The current study is based on a pediatric cohort within the OLIN Studies (Obstructive Lung Disease In Northern Sweden), recruited in 2006. In brief, all 2704 children in first and second grade, aged 7–8 years, in three municipalities in Northern Sweden were invited to a parental questionnaire study on allergic diseases, and 96% participated [24]. At the follow-up in 2010 the participation rate was equally high, when parents of 2612 children (then 11–12 years) completed the questionnaire [25]. In 2010, all children with

reported allergy to the following foods: cow's milk, hen's egg, fish and wheat, were invited to participate in a clinical evaluation including a structured interview, a physical examination by one pediatric allergist (Dr. Anna Winberg) and analyses of food specific IgE. Of those 125 invited, 94 children (75%) participated in the clinical evaluations [26].

Children diagnosed as having a current allergy to milk, egg, cod or wheat were then invited to further evaluation with a DBPCFC series. Of these 25 children, 18 (72% of invited) participated in a total of 20 challenge series with any of the foods: milk, egg or cod. In addition, 7 children with no reported allergy, randomly selected from the same OLIN cohort, participated in a DBPCFC series with hen's egg. All study participants and their parents gave an informed and written consent. The study was approved by the regional Ethics Committee in Umeå.

2.2. Experimental setup of challenge tests

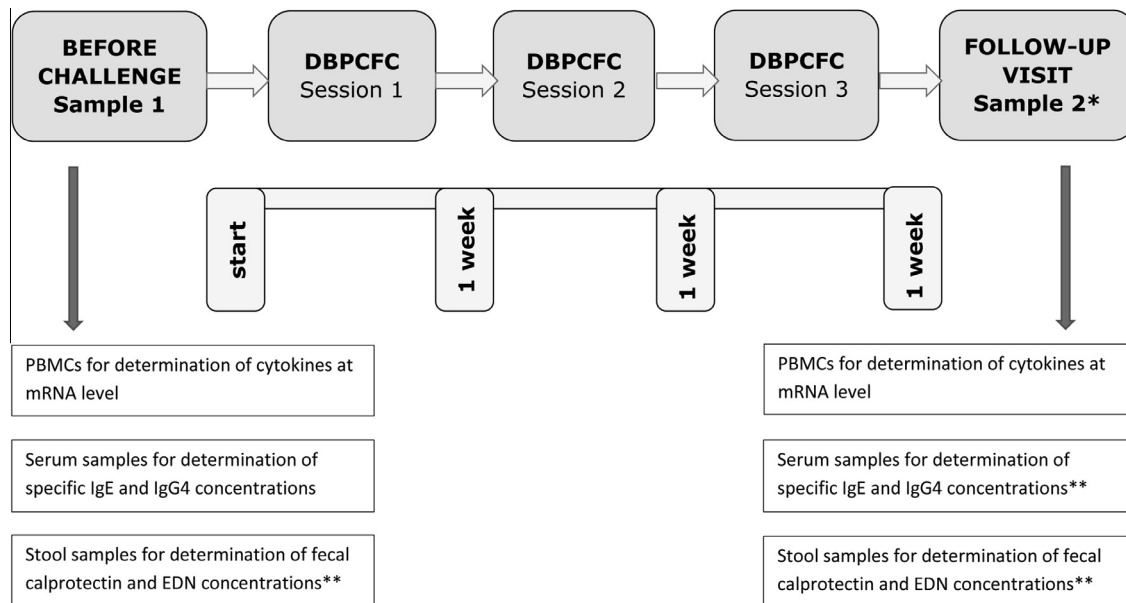
The DBPCFC series included three challenge sessions for each food (cow's milk, hen's egg or cod), two with active substance and one with placebo [26,27]. The challenge sessions were set one week apart, and the serving order was drawn by lot. The doses were given 30 min apart until objective symptoms were elicited or the final challenge dose was completed. The total cumulative dose of food protein given at each session was 2.0–2.4 g. The children were observed for 2 h after each completed challenge session and, after hospital departure, they completed a 3-day symptom diary. If a child had an anaphylactic reaction during the challenge, the challenge series was terminated. The DBPCFC was evaluated at a follow-up visit one week after the last challenge session (Fig. 1). A food challenge was considered positive if the challenge-triggered symptoms were objective and/or reproducible or if the symptoms were severe enough for the challenge series to be terminated [26]. To avoid the pollen season, the DBPCFC series were performed during the periods; February to March and September 2011.

2.3. Collection of blood and stool samples

Blood samples were collected from the 18 children with suspected food allergy and the 7 age-matched healthy controls participating in the DBPCFC series. Samples were taken immediately before (baseline samples) and at the follow-up visit 1 week after the completed challenge series (post challenge samples). If a challenge series was terminated before completion due to a severe reaction, the post challenge sample was collected one week after the challenge reaction. Blood was drawn in EDTA-containing tubes and kept at 4 °C until processing. The blood samples were transported for analysis within 24 h at 4 °C. Stool samples were collected from children with suspected food allergy at baseline and one week after the completed DBPCFC series.

2.3.1. Specific IgE and IgG4

Sera were stored frozen until analysis of specific IgE and specific IgG4 to the whole allergen extracts egg and cod and to the allergen components α -lactalbumin (Bos d 4), β -lactoglobulin Bos d 5), casein (Bos d 8), ovalbumin (nGal d 2), ovomucoid (nGal d 1), and parvalbumin (rGad c 1) (ImmunoCAP, ThermoFisher Diagnostics, Uppsala, Sweden). Specific IgE to the whole allergen extract milk was also analyzed, but there was no available test for IgG4 to the crude milk allergen. In addition, the ImmunoCAP fx5 food screen test including: cow's milk, hen's egg, cod, soy, wheat, and peanut and the ImmunoCAP inhalant screen test including: birch, timothy, mugwort, cat, dog, horse, dust mites (Dermatophagoides pteronyssius and farinae), and *Claudosporium* (ImmunoCAP, ThermoFisher Diagnostics, Uppsala, Sweden) were also analyzed. For all IgE-tests a level of >0.35 kU/L was considered positive.



*In case of an anaphylactic reaction the DBPCFC-series was aborted and Sample 2 was taken at a follow-up visit 1 week after the last challenge session.

** Samples only collected from children with suspected food allergy

Fig. 1. Challenge schedule.

2.3.2. Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation as previously described [28,29]. In brief, after centrifugation at 2000 rpm/min during 10 min, the inter-phase containing lymphocytes and macrophages was collected, washed in phosphate buffered saline (PBS) and cell concentration was determined. About 3×10^6 PBMC were pelleted by centrifugation at 8000g, resuspended in 10 μ L of PBS, resolved in 350 μ L of RLT lysis buffer (RNeasy Mini kit, QIAGEN, Tübingen, Germany) and kept at -80°C until total RNA extraction was performed.

2.3.3. Total RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

We performed a cytokine gene expression analysis by real-time RT-qPCR according to the MIQE requirements [30]. Total RNA was isolated using RNeasy Mini kit (QIAGEN, Tübingen, Germany) according to the manufacturer's protocol. The RNA yield (average yield 2574 ng in a total volume of 30 μ L) and purity (average A_{260}/A_{280} ratio 1.7) was assessed by NanoDrop N-1000 spectrophotometer (ThermoFisher Scientific Inc. USA). Reverse transcription was done as previously described [28,29,31] followed by multiplex qPCR with 18s rRNA as endogenous control (Eukaryotic 18S rRNA endogenous control, VIC®/MGD probe, primer limited, Catalog # 4319413E, Life Technologies) for evaluation of RNA integrity, efficiency of reverse transcription and cDNA stability [32]. We used premade TaqMan® assays (Applied Biosystems), official gene symbol and assay ID numbers were as follows: IL1B (Hs01555410_m1), IL2 (Hs00174114_m1), IL4 (Hs00174122_m1), IL5 (Hs00174200_m1), IL6 (Hs00985639_m1), IL8 (Hs00174103_m1), IL10 (Hs00961622_m1), IL13 (Hs00174379_m1), IL15 (Hs01003716_m1), IL17A (Hs00174383_m1), IFNG (Hs00989291_m1), TNF (Hs00174128_m1) and TGFBI (Hs99999918_m1). The cytokine assessment was chosen to discriminate between a humoral allergy-promoting Th2 response, cellular cytotoxicity-promoting Th1 response, Th3- and Tr1 regulatory responses and inflammatory response. Total RNA from PMA/ionomycin-stimulated PBMC was used in the positive assay controls and templates were omitted in the negative assay

controls. The PCR reactions were run on a 7900HT instrument (Life Technologies/Applied Biosystems) with factory default settings for 40 cycles and the results were analyzed with a relative quantification method.

2.3.4. Fecal markers

Stool samples were stored frozen before analysis of fecal markers. Two fecal markers, calprotectin (PhiCal®Calprotectin) and Eosinophil-derived neurotoxin (EDN) were quantified using ELISA methods (Immundiagnostik, Bensheim, Germany) according to the manufacturer's instructions.

2.4. Statistical analyses

Data were analyzed using IBM SPSS Software version 21 (New York, USA). Since data were not normally distributed, medians were used as measures of central tendency and quartiles as measures of range. For comparison between groups, the non-parametric Mann-Whitney *U* test was applied. When there was no detectable cytokine mRNA expression, a value below the detection limit of the equipment ($n = 0.0000000001$) was given in order to be able to calculate differences in cytokine mRNA expression between groups. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Baseline characteristics

Baseline characteristics of the study participants are presented in Table 1. Blood samples collected for measurement of cytokine mRNA expression were analysable in 17 out of 18 children with suspected food allergy. Of these, 8 had at least one positive DBPCFC series outcome whereof 3 had anaphylactic reactions, and 9 had negative challenge outcomes. In addition, cytokine mRNA expression could be analyzed in blood samples from 6 out of 7 controls.

Table 1
Baseline characteristics of (a) children with suspected food allergy and (b) controls, including challenge outcome, food specific IgE and IgG4, and total IgE for food and inhalant screen tests.

Challenge food	Sex	Main reported food induced symptoms before challenge	Challenge reaction	IgE (kU/L)				IgG4 ^a (kU/L)			Food IgE screen (kU/L)	Inhalant IgE screen (kU/L)
				Milk	Lactalbumin	Lactoglobulin	Casein	Lactalbumin	Lactoglobulin	Casein		
(a)												
Milk	M	Eczema, diarrhea	None	0.00	0.01	0.01	0.02	0.05	0.43	0.27	0.04	0.04
	M	Vomiting, diarrhea	None	0.00	0.01	0.01	0.02	0.00	0.00	0.16	0.04	0.04
	F	Flatulence	None	0.01	0.02	0.03	0.03	2.29	5.91	1.11	0.06	0.13
	F	Diarrhea	None	0.08	0.04	0.12	0.05	0.15	3.08	0.53	0.20	0.29
	F	Eczema, diarrhea	Mild	0.00	0.01	0.01	0.02	0.82	3.61	0.53	0.03	0.08
	F	OAS ^b , vomiting	Mild	2.45	0.55	1.08	1.33	1.40	2.16	3.27	33.60	29.50
	F	OAS ^b , flatulence	Mild	0.51	0.09	0.09	0.50	0.23	0.25	0.74	2.96	25.80
	M	Eczema, urticaria	Severe	3.27	0.93	1.48	3.57	2.28	0.20	1.39	36.10	22.00
			Egg	Ovomucoid	Ovalbumin		Egg	Ovomucoid	Ovalbumin			
Egg	M	Vomiting	None	0.52	0.00	0.61		0.08	0.00	0.05	0.91	17.00
	F	Urticaria	None	0.21	0.02	0.29		0.04	0.00	0.00	1.98	80.70
	F	Diarrhea	Mild	0.80	0.23	0.54		22.30	7.73	17.90	1.11	2.43
	M	OAS ^b , stomach pain	Severe	13.1	6.64	5.15		0.22	0.15	0.06	13.40	0.36
	F	Eczema	Severe	7.81	0.07	0.84		0.30	0.00	0.32	8.19	68.00
			Cod	Parvalbumin			Cod	Parvalbumin				
Cod	F	Flatulence	None	0.00	0.00			0.00	0.02		0.14	0.06
	M	Eczema	None	0.00	0.00			0.00	0.00		0.40	20.20
	M	OAS ^b	None	0.00	0.01			0.00	0.00		0.11	29.90
	F	Eczema	Mild	8.03	3.94			0.02	0.00		13.40	0.36
			Egg	Ovomucoid	Ovalbumin		Egg	Ovomucoid	Ovalbumin			
(b)												
Egg	F	None	None	0.09	0.02	0.08		6.54	0.31	7.73	0.33	0.20
	F	None	None	0.00	0.00	0.00		0.09	0.09	0.00	0.02	0.03
	M	None	None	0.00	0.00	0.00		1.06	0.12	0.00	0.03	0.03
	M	None	None	0.00	0.00	0.00		0.22	0.00	0.25	0.05	0.02
	M	None	None	0.00	0.00	0.01		2.41	0.00	3.07	0.05	0.03
	F	None	None	0.03	0.02	0.03		6.35	3.73	5.87	0.11	0.12

^a No crude extract available for IgG4 milk.

^b OAS: oral allergy symptoms.

3.2. Specific IgE and IgG4 to crude allergens and allergen components

Detected specific IgE and IgG4 levels to the whole food allergen extracts and allergen components at baseline are presented in Table 1. Out of 8 children with a positive challenge outcome, 7 children had specific IgE levels to the challenged food above 0.35 kU/L. The food specific IgE and IgG4 levels were similar in the baseline and post challenge samples, independent of challenge outcome, except in one child with an anaphylactic reaction to egg, where specific IgE to egg increased from 7.81 kU/L at baseline to 11.8 kU/L post challenge.

3.3. Cytokine mRNA expression in peripheral blood mononuclear cells (PBMC)

3.3.1. Circulating PBMCs showed higher spontaneous IL-13 and TNF- α , and lower IFN- γ and IL-15 mRNA expression in children with suspected food allergy

At baseline, we measured cytokine mRNA levels in freshly isolated PBMCs to monitor spontaneous cytokine expression by circulating mononuclear immune cells. At that sampling, children with suspected food allergy expressed higher IL-13 ($p = 0.003$) and TNF- α ($p = 0.016$), and lower IFN- γ ($p = 0.024$) and IL-15 (0.036) mRNA levels compared to controls (Fig. 2a). No significant differences were seen for the other investigated cytokines.

In children with suspected food allergy, those with a positive challenge outcome expressed higher IL-13 mRNA levels at baseline compared to those with a negative food challenge ($p = 0.027$). When compared to controls, the difference in IL-13

mRNA expression was higher in children with a positive food challenge ($p = 0.001$) while the difference did not quite reach statistical significance for children with a negative challenge outcome ($p = 0.05$). Of note, IL-13 mRNA was only expressed in 1 out of 6 healthy controls. Children with a positive challenge outcome also expressed higher IL-10 mRNA levels at baseline compared to children with a negative food challenge ($p = 0.036$). Furthermore, the baseline IL-15 mRNA level was twice as high in children with a positive compared to those with a negative food challenge outcome, although this difference did not reach statistical significance ($p = 0.059$) (Supplementary Table 1).

3.3.2. The cytokine mRNA profile post challenge was characterized by increased expression of pro-inflammatory cytokines in children with suspected food allergy

Post challenge, we assessed the type of adaptive immune response that was generated following *in vivo* activation with a food allergen. Children with suspected food allergy showed significantly higher mRNA expression levels of the pro-inflammatory cytokines IL-6 ($p = 0.001$) and IL-1 β ($p = 0.036$) compared to controls (Fig. 2b). No significant differences in cytokine mRNA profiles were seen between children with positive and negative food challenge outcomes in the post challenge measurements (Supplementary Table 2).

3.3.3. Dynamics of cytokine mRNA expression during anaphylaxis

As three children developed anaphylaxis during the challenge series, this gave us the unique opportunity to describe the cytokine

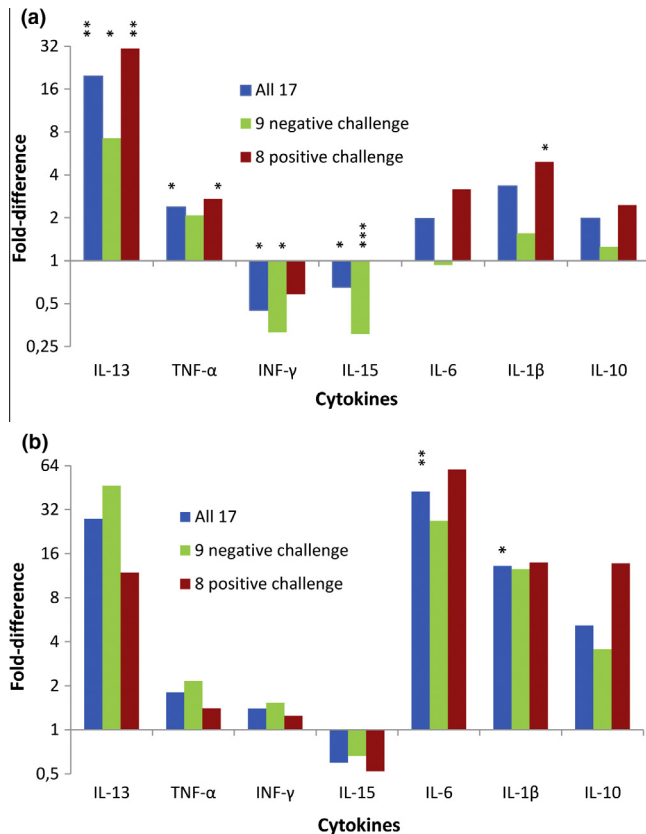


Fig. 2. Relative cytokine mRNA expression in PBMC at (a) baseline and (b) post challenge. Fold-difference between median expression levels in allergy group ($n = 17$, blue bars), negative challenge outcome subgroup ($n = 9$, green bars) and positive challenge outcome subgroup ($n = 8$, red bars) – all in comparison with control group ($n = 6$, reference line with fold-difference = 1). The data were analyzed with a ddCt method resulting in relative quantities (RQ) corresponding to the reference expression level in stimulated PBMC that is equal to 1. For each group, the average RQ value was calculated. Fold-change value is a result of division the average RQ after challenge by the average RQ before challenge. Student's paired t -Test was used to assess variation in each group and fold-change with p -values < 0.05 were considered statistically significant.

mRNA profile at baseline and post challenge (i.e. one week after anaphylaxis). Of those who reacted this severely, two were challenged with egg and one with milk. The spontaneous cytokine mRNA profile in children who reacted with anaphylaxis was characterized by higher expression of the Th2-related cytokines IL-4 and IL-13 compared to controls. For both IL-4 and IL-13, there

was a 10-fold difference in the mRNA levels between children with anaphylaxis and the healthy controls (Fig. 3).

One week after the anaphylactic reaction, the adaptive immune response was characterized by higher expression of the pro-inflammatory cytokines: IL-1 β , IL-6 and TNF- α compared to the post-challenge samples of the controls. This was particularly evident for IL-6 and IL-1 β , that increased a 100-fold and 30-fold respectively, in children with anaphylaxis compared to the healthy controls. Further, the expressed mRNA level of the regulatory cytokine IL-10 was 10-fold higher among children with anaphylaxis compared to controls after the completed DBPCFC series. In contrast, the post challenge mRNA level of the regulatory cytokine TGF- β 1 in children with anaphylaxis was decreased and there was no detectable difference in the mRNA levels of Th2-related cytokines expressed post challenge between children with anaphylaxis and controls (Fig. 3).

3.4. Inflammatory markers in feces

Paired stool samples at baseline and post challenge were available from 12 children with suspected food allergy, 6 with a positive food challenge and 6 with a negative challenge outcome (Table 2). Both at baseline and post challenge, the fecal calprotectin level in children with a positive food challenge outcome was almost twice as high as in children with a negative challenge outcome although the difference in calprotectin levels did not reach statistical significance ($p = 0.150/p = 0.078$). In addition, the median level of detected fecal EDN at baseline was almost twice as high in children with a positive challenge compared to a negative challenge outcome, though again, the difference was not statistically significant ($p = 0.262$) (Table 2).

4. Discussion

Here we explored the dynamics of cytokine mRNA expression in children with suspected allergy to milk, egg or cod before and after a double-blind placebo-controlled food challenge series, and in comparison with a healthy control group. At baseline, the circulating PBMCs showed higher spontaneous IL-13 and TNF- α , and lower INF- γ and IL-15 mRNA expression in children with suspected food allergy compared with controls. When comparing children with a positive challenge outcome versus children with a negative outcome, it became evident that the baseline mRNA level of the Th2-related cytokine IL-13 and also the regulatory cytokine IL-10, were higher in children with challenge-proven food allergy. Post challenge, children with suspected food allergy expressed significantly higher mRNA levels of the pro-inflammatory cytokines

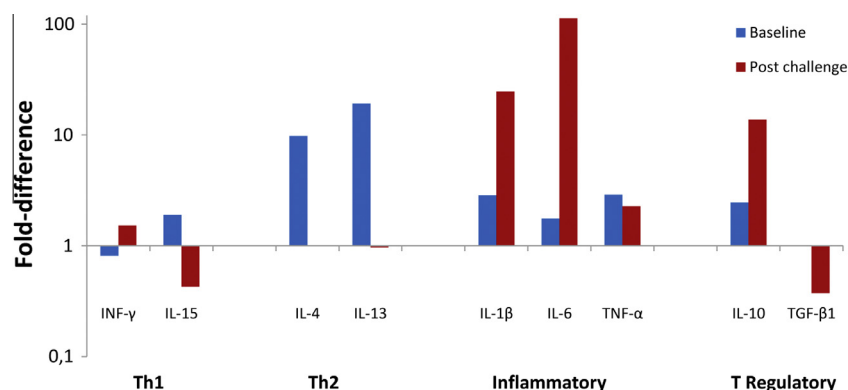


Fig. 3. Relative cytokine mRNA expression in peripheral blood mononuclear cells (PBMC) during anaphylaxis. Fold-difference in cytokine expression levels between anaphylaxis and control group (=1) at baseline (dark grey) and post challenge (light grey).

Table 2

Fecal markers calprotectin and EDN at baseline and post challenge in children with positive and negative challenge outcomes.

Marker	Positive challenge outcome (n = 6)		Negative challenge outcome (n = 6)		p-value
	Median ng/mg	Percentiles (25–75)	Median ng/mg	Percentiles (25–75)	
<i>Baseline</i>					
Calprotectin	25.8	(12.4–64.1)	16.45	(6.8–22.3)	0.150
EDN	110.50	(53.6–929.0)	57.50	(35.9–165.1)	0.262
<i>Post challenge</i>					
Calprotectin	24.10	(13.9–63.2)	8.82	(3.8–26.8)	0.078
EDN	132.80	(76.70–1177.6)	106.10	(58.5–168.1)	0.423

IL-6 and IL-1 β compared to healthy controls, while there were no differences between children with challenge-proven food allergy and those with a negative challenge outcome. In addition, the fecal inflammatory markers calprotectin and EDN was higher in children with challenge-proven food allergy compared to children with a negative challenge outcome, though the differences did not reach statistical significance.

In our study, the spontaneous cytokine mRNA expression in PBMCs differed significantly between children with suspected food allergy and controls both at baseline and post challenge. Before the food challenge, children with suspected food allergy expressed higher mRNA levels of the Th2-related cytokine IL-13, while controls expressed higher levels of the Th1-related cytokine IFN- γ and IL-15, a cytokine associated with T-cell proliferation [33]. This could be consistent with previous findings, showing both higher expressed [16] and secreted [15] levels of Th2-related cytokines, IL-13 in particular, in food allergic children compared to controls. For comparison, the single study that has assessed spontaneous cytokine expression in food allergic adults, showed higher levels of IFN- γ in PBMCs from healthy controls compared to subjects with milk allergy [34]. In the present study, cytokine expression following a food challenge series, showed higher levels of the pro-inflammatory cytokines IL-1 β and IL-6 in children with suspected allergy to the challenge food compared to controls. This is consistent with previous finding showing elevated IL-6 levels in food allergen stimulated PBMCs from challenge-proven milk and egg allergic children compared to children who have developed tolerance [35].

At baseline, the Th2-related cytokine IL-13 was hardly expressed at all in healthy controls, marginally expressed in children with a negative challenge outcome though significantly higher in children with challenge-proven food allergy. However, no significant differences in the IL-13 mRNA levels could be detected between children with a positive or negative challenge outcome or in controls after the completed challenge-series. Previous data show high levels of IL-13 mRNA expression following stimulation of PBMCs from food allergic patients with relevant food allergens and that the expressed level of IL-13 decreases after tolerance development [15,36]. In addition, Metcalfe et al. recently published data showing that an elevated egg-specific Th2 cytokine response – of IL-13 and IL-5 in particular – at 4 months of age could predict egg allergy at 12 months [37]. Collectively, these findings suggest a possible use of IL-13 as a prognostic biomarker for an ongoing food allergy.

Before the DBPCFC, the mRNA expression of the regulatory cytokine IL-10 was higher among children with challenge proven food allergy compared to those with a negative challenge outcome, who expressed IL-10 mRNA levels similar to that of healthy controls. Post challenge, no significant differences in IL-10 were detected between any of the groups. IL-10 is a major regulatory cytokine [38] that inhibits the production of IgE and up-regulates the secretion of IgG4 [39]. Higher levels of IL-10 have been shown in cow's milk protein stimulated PBMCs from children with IgE-mediated

milk allergy compared to children with non-IgE mediated milk allergy [36,40]. Further, higher levels of IL-10 has been detected in children during tolerance development compared to children with symptomatic food allergy [12,15]. Due to its active role in tolerance development, IL-10 has been discussed as a possible biomarker for symptomatic IgE-mediated food allergy [11]. However, since data are not univocal and study results vary with study populations and investigated food allergens [15,17,41], it has been suggested that analyzing the regulatory cytokine IL-10 in addition to a broad Th2-profile would be preferable [13,42,43]. Also, there is a need for further studies investigating the specificity of elevated Th2-related cytokines in differentiating immunological reactivity to food versus other allergens.

During the DBPCFC, three of the children in our study reacted with an anaphylactic reaction. Compared to healthy controls participating in the DBPCFC series, children with anaphylaxis expressed 10-fold higher levels of the Th2-related cytokines IL-4 and IL-13 at baseline and 30–100 fold higher levels of the pro-inflammatory cytokines IL-1 β and IL-6 post challenge. In addition, children with anaphylaxis expressed a 10-fold higher post challenge level of the regulatory cytokine IL-10 compared to controls. As opposed to this finding, the post challenge level of the regulatory cytokine TGF- β 1 was decreased among children with anaphylaxis compared to healthy controls. A possible explanation for this finding, in addition to the absent difference in expressed mRNA levels of Th2-related cytokines between children with anaphylaxis and controls post challenge, could be an exhaustion of the cytokine secretion of Th2 and T-regulatory cytokine producing cells following the severe systemic allergic reaction [44]. To the best of our knowledge, this is the single study that has shown cytokine mRNA expression patterns in children with anaphylaxis at baseline and following *in vivo* stimulation with relevant food allergens. However, due to the low number of children with anaphylaxis, no statistical analyses were performed on this subgroup.

In food allergy, the allergen is usually presented to the immune system through the mucosa in the gastrointestinal tract and the presence of gastrointestinal symptoms is common, especially in children with non-IgE mediated food allergies [45]. This has resulted in an ongoing search for immunological biomarkers detectable in feces [21,22,46,47]. In this study we analyzed calprotectin and EDN in stool samples from children with suspected food allergy, collected before and one week after a completed DBPCFC-series. At baseline the fecal calprotectin and EDN levels were twice as high among children with a challenge proven food allergy compared to children with a negative challenge outcome. These differences did, however, not reach statistical significance. A likely reason for this could be the small study groups rendering low statistical power and the large natural variance in the level of fecal calprotectin [22,48].

In this study we chose to measure cytokine mRNA expression in PBMCs in lieu of measuring levels of biologically active cytokines at the protein level. The rationale for this decision was the high sensitivity and specificity of RT-qPCR as a method and access to a

well-established RT-qPCR for measurement of cytokine mRNA expression. Importantly, a previous study by our group, showed an excellent correlation between the levels of cytokine mRNA measured by this method and detected levels of corresponding cytokines at the protein level [49]. Further, the method of measuring cytokine mRNA expression is less vulnerable to long transport and time delays compared to measurements of cytokine proteins. Since the food challenges were performed in hospitals 250–500 km from the laboratory that performed the cytokine analyses, the transport time was an important issue in our study. Due to practical and logistic reasons, the post challenge samples in our study were sampled one week after the completed food challenge series. The optimal time for measuring post challenge biomarkers is a matter of discussion [17,23]. Previous studies have shown that the mRNA expression of different cytokines peaks at different time intervals and that the time to peak might be different depending on the detection method used [50]. A Polish study investigating serum cytokine protein levels, showed a significant increase in the levels of IL-4 and IL-5 following double-blind challenges with relevant food allergens but not with placebo in children with asthma and IgE-mediated food allergy. The maximum secretion for these cytokines was seen after 24 h [23]. On the contrary, the levels of fecal inflammatory markers and specific IgE and IgG4 are likely to peak weeks after allergen exposure [7,22].

A major strength of this study was the well-defined study participants, including children with suspected food allergy with both positive and negative food challenge outcomes as well as age-matched healthy controls. Another strength is the broad panel of cytokines and fecal biomarkers investigated before and after *in vivo* stimulation with relevant food allergens [23]. The study groups were, however, small and heterogeneous according to type of food allergy, challenge food and the serving order of active and placebo substances during the DBPCFC series [15,40]. The limited number of participants was due to a low prevalence of allergy to basic foods, even though we initially invited all children with reported allergy to milk, egg, cod and wheat from a large population-based cohort with a high prevalence of reported hypersensitivity to these foods [25,26,51]. Since this was a small pilot study describing possible alterations in a number of immunologic markers during a double-blind food challenge series, correction for multiple testing was not performed to sharpen the statistics.

5. Conclusion

In this study, cytokine mRNA profiles were analyzed in 12-year old children with suspected food allergy and age-match healthy controls participating in a DBPCFC series. At baseline, children with a suspected food allergy expressed higher levels of the Th2-related cytokine IL-13 compared to controls. The baseline levels of IL-13 as well as the regulatory cytokine IL-10 were higher among children with challenge-proven food allergy compared to children with a negative challenge outcome. Post challenge, children with a suspected food allergy expressed higher mRNA levels of the pro-inflammatory cytokines IL-1 β and IL-6 compared to healthy controls. Further, fecal calprotectin and EDN levels tended to be higher among children with a challenge-proven food allergy compared to children with a negative challenge outcome, although the difference was not statistically significant. Even if in line with the findings of others [21,37,52], future studies using larger cohorts or more homogenous study groups will be necessary to disentangle whether the cytokines IL-13 and IL-10 or the fecal biomarkers calprotectin and EDN could act as possible prognostic biomarkers for an ongoing food allergy.

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Conflict of interest

None of the authors listed (AW, ON, IN, CL, IA, LMN, ER, CW) declares any conflict of interest related to this work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cyto.2016.09.014>.

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Supplementary table 1. Comparison of cytokine levels from unstimulated cells at baseline between children with positive challenge outcome, negative challenge outcome and controls.

Cytokine	Positive challenge outcome (n=8)		Negative challenge outcome (n=9)		Controls (n=6)	
	median	(percentiles 25-75)	median	(percentiles 25-75)	median	(percentiles 25-75)
IL-2	0.0000000001	(0.0000000001-0.0028387623)	0.0002490160	(0.0000000001-0.0014162935)	0.0000000001	(0.0000000001-0.0000000001)
IL-4	0.0000000001	(0.0000000001-0.0595261545)	0.0000000001	(0.0000000001-0.0327815076)	0.0000000001	(0.0000000001-0.0000000001)
IL-5	0.0000000001	(0.0000000001-0.0041786348)	0.0000000001	(0.0000000001-0.0000000001)	0.0000000001	(0.0000000001-0.0000000001)
IL-10	0.1364201240	(0.0377721788-0.1854757343)	0.0264932200	(0.0120258361-0.0857822925)	0.0343691755	(0.0227141111-0.0521111111)
IL-13	0.0057769925	(0.0012596520-0.0253494470)	0.0002426620	(0.0000951031-0.0021860855)	0.0000000001	(0.0000000001-0.0000000001)
IFN- γ	0.0076527410	(0.0030830313-0.0125824050)	0.0052334100	(0.0020976540-0.0099571785)	0.0158044990	(0.0100439024-0.0211111111)
TGF β 1	1.3335973985	(0.8123563548-2.0238730720)	0.6052101130	(0.5225926230-1.1889943685)	1.1770165050	(0.8908499074-1.5555555556)
IL-1 β	0.0619208785	(0.0297221723-0.1737855043)	0.0366829380	(0.0076865890-0.1465431245)	0.0164204385	(0.0081782108-0.0277777778)
IL-6	0.0297511135	(0.0124649218-0.1223425358)	0.0125950090	(0.0030839695-0.0406110495)	0.0210546515	(0.0146256250-0.0333333333)
IL-8	0.1908182395	(0.1327410805-0.7181101350)	0.1343927300	(0.0217115510-0.2229265915)	0.1264881210	(0.0461682236-0.2222222222)
IL-15	2.5205981275	(1.0806408025-7.1585590743)	1.1367649680	(0.7662794940-1.3502140680)	3.9031426530	(2.6921729167-5.5555555556)
IL-17	0.0000000001	(0.0000000001-0.0038358855)	0.0052645540	(0.0000000001-0.0132133345)	0.0154568950	(0.0000000001-0.0333333333)
TNF α	0.1232355075	(0.1136355495-0.2295840878)	0.0876923010	(0.0487285030-0.2633725765)	0.0514083350	(0.0232329113-0.1111111111)

* p-value 1 : comparison between positive and negative challenge outcomes

** p-value 2 : comparison between positive challenge outcomes and controls

*** p-value 3 : comparison between negative challenge outcomes and controls

Bold font **denotes** statistical significance

The value n=0.0000000001 (below equipment detection level) was given if no cytokine mRNA expression could be detected in the unstimulated cells

Supplementary table 2. Comparison of cytokine mRNA levels from unstimulated cells post challenge between children with positive challenge outcome, negative challenge outcome and controls.

Cytokine	Positive challenge outcome (n=8)		Negative challenge outcome (n=9)		Controls (n=6)	
	median	(percentiles 25-75)	median	(percentiles 25-75)	median	(percentiles 25-75)
IL-2	0.0000000001	(0.0000000001-0.0041045803)	0.0007950220	(0.0000000001–0.0031827725)	0.0000000001	(0.0000000001–0.0000000001)
IL-4	0.0000000001	(0.0000000001-0.0000000001)	0.0723512120	(0.0000000001–0.1683896580)	0.0000000001	(0.0000000001–0.0000000001)
IL-5	0.0000000001	(0.0000000001-0.0316836533)	0.0000000001	(0.0000000001–0.0051287111)	0.0000000001	(0.0000000001–0.0000000001)
IL-10	0.0473843030	(0.0240015638-0.4190911598)	0.0633806820	(0.0373678285–0.2333889885)	0.0204038540	(0.0068720100–0.0687201000)
IL-13	0.0012993355	(0.0000748688-0.0074975390)	0.0003512290	(0.0000000001–0.0031387375)	0.0000000001	(0.0000000001–0.0000000001)
IFN- γ	0.0048452875	(0.0033200343-0.0345441230)	0.0045984360	(0.0034914700–0.0084715530)	0.0098145835	(0.0034779000–0.0347790000)
TGF β 1	0.7405817715	(0.5419185563-0.9916403565)	0.7478117270	(0.6320657280–1.3342264390)	1.3540720680	(0.5953309000–1.3540720680)
IL-1 β	0.0215438455	(0.0112078205-0.3367158395)	0.0232431430	(0.0141709640–0.0444832585)	0.0102304710	(0.0062139000–0.0102304710)
IL-6	0.0187543100	(0.0063788690-0.2947223450)	0.0154165530	(0.0075444150–0.0463292915)	0.0022676035	(0.0015069000–0.0022676035)
IL-8	0.2254329210	(0.1076324773-0.7119949885)	0.2258321830	(0.1181838490–0.7560882955)	0.0838872045	(0.0402919000–0.0838872045)
IL-15	1.8512382990	(1.0848025255- 3.5887255790)	2.8002604160	(2.1924364880–3.8627071875)	3.0189032025	(1.7244857000–3.0189032025)
IL-17	0.0018071336	(0.0000000001-0.0179971990)	0.0041823920	(0.0000000001–0.0066081220)	0.0000000001	(0.0000000001–0.0000000001)
TNF α	0.0550818325	(0.0473340288-0.2090072090)	0.1354668730	(0.0662681220–0.1844165955)	0.0731061205	(0.0406480000–0.0731061205)

* p-value 1 : comparison between positive and negative challenge outcomes

** p-value 2 : comparison between positive challenge outcomes and controls

*** p-value 3 : comparison between negative challenge outcomes and controls

Bold font **denotes** statistical significance

The value n=0.0000000001 (below equipment detection level) was given if no cytokine mRNA expression could be detected in the unstimulated cells