

Characterization of plasma cytokines in an infant population cohort of challenge-proven food allergy

T. D. Dang^{1,2}, M. L. K. Tang^{1,2,3}, J. J. Koplin^{1,2}, P. V. Licciardi^{1,2}, J. K. Eckert¹, T. Tan^{1,2},
L. C. Gurrin^{1,4}, A.-L. Ponsonby^{1,2,4}, S. C. Dharmage^{1,4} & K. J. Allen^{1,2,3} for the HealthNuts study

¹Murdoch Childrens Research Institute; ²Department of Paediatrics, University of Melbourne; ³Department of Allergy and Immunology, Royal Children's Hospital; ⁴The Centre for Molecular, Environmental, Genetic and Analytic Epidemiology, University of Melbourne, Parkville, Vic., Australia

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Correspondence

Professor Katrina J. Allen, Department of Allergy and Immunology, The Royal Children's Hospital, Flemington Road, Parkville 3052, Vic., Australia.
Tel.: 613 9345 4870
Fax: 613 9345 4848
E-mail: katie.allen@rch.org.au

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Abstract

Background: Sensitization to food allergens indicates the production of food-specific IgE; however, sensitization is not a definite indicator of allergic reaction upon ingestion (N Engl J Med, 344, 2001, 30; J Allergy Clin Immunol, 120, 2007, 491). Currently, food challenge is the best approach to identify the presence or absence of allergy. While 95% positive predictive values (PPVs) thresholds for sIgE can assist with identifying increased likelihood of allergy among those who are sensitized, there are no specific biological markers that differentiate between allergic and sensitized individuals.

Objectives: To determine whether plasma serum cytokine profiles predict (i) sensitization to peanut and egg and (ii) food allergy among sensitized infants.

Methods: Peanut-sensitized (PT) and egg-sensitized 14-month-old infants and nonsensitized controls enrolled in HealthNuts, a population-based study of food allergy, underwent an oral food challenge (OFC). Blood was collected within 1 h after OFC. Serum levels of Th1, Th2 and regulatory cytokines were determined in allergic ($n = 79$), sensitized ($n = 40$) and nonsensitized, nonallergic ($n = 37$) infants by multiplex assay.

Results: Food-sensitized infants had significantly higher plasma IL-4, IL-13, IL-12p70 and lower IL-10 levels compared to nonsensitized infants. IL-10 and IL-6 levels were significantly higher in sensitized compared with allergic infants. Egg-allergic infants had significantly higher IL-13 and IL-12p70 levels compared to peanut-allergic (PA) infants.

Conclusion: Levels of Th2-related cytokines in plasma are higher in food-sensitized infants, irrespective of clinical food allergy status. In contrast, IL-10 levels appear to predict food allergy among sensitized infants. Differences in IL-13 and IL-12p70 between egg- and peanut-allergic infants could help explain the different resolution rates of the allergies.

The prevalence of IgE-mediated food allergy has continued to rise in recent decades and now affects up to 6–10% of young children and 3–4% of adults (3–7). Although some

individuals who produce food-specific IgE antibodies will react on consumption of the food in question (food allergic), others are sensitized but can consume the food without any adverse consequences (food sensitized). The mechanisms underpinning why some food-sensitized children are tolerant and some are allergic have not been fully elucidated (1, 2). There are currently various *in vitro* mediator release assays, which could be performed to differentiate between sensitization and allergy, including the 'basophil activation test'

Abbreviations

OFC, oral food challenge; SPT, skin prick test; sIgE, specific immunoglobulin E; PA, peanut allergic; EA, egg allergic; PT, peanut sensitized; ET, egg sensitized; NSNA, nonsensitized, nonallergic.

assays (8). However, this is a complex assay that is not widely available and requires separation of peripheral blood mononuclear cells. Biomarkers distinguishing these clinical states would be valuable if easily accessible and cheap and might be used in the prediction of future tolerance development.

IgE-mediated food allergy is characterized by a deregulation of the immune response in which there is increased production of Th2 cytokines, resulting in B-cell class switching and production of food-specific IgE antibodies. Although mechanistic studies have explored the secretion of cytokines from cultured PBMCs (9, 10), to our knowledge analysis of circulating cytokine profiles in the plasma of food-allergic individuals has not been extensively studied. Plasma cytokines are easily obtainable from blood and do not require labour-intensive PBMC isolation and culture and therefore represent a simpler alternative for food allergy diagnosis.

To date, no existing studies have explored differences in food-sensitized infants who are either tolerant or allergic. Establishing key immunological differences in these individuals may help in the development of a diagnostic tool for distinguishing allergy *vs* tolerance without the need to perform an oral food challenge (OFC) and ultimately new therapeutic targets to promote the resolution of food allergy.

We aimed to study the plasma cytokine profiles of allergic, sensitized and nonsensitized, nonallergic (NSNA) infants in our population-based study of 5276 infants (the HealthNuts cohort) (11).

Methods

Subjects

Subjects were randomly selected from the previously described HealthNuts study (11). In brief, 5276 11- to 15-month-old infants were recruited as they attended 131 council-run immunization sessions across Melbourne, Australia, between September 2007 and August 2011. Infants with a detectable skin prick test (SPT) wheal to any of the following: egg white, peanut, sesame, and either cow's milk or shrimp, were invited to a formal food challenge 4–8 weeks later at Melbourne's Royal Children's Hospital. A random sample of infants with negative SPTs to all four foods were also invited to undergo a food challenge (negative controls for the clinic study sample).

Predefined criteria for a positive OFC

Food challenges were performed as previously described (11). In brief, the criterion for a positive reaction was at least one of the following signs present during OFC: three or more concurrent noncontact hives (urticarial lesion) lasting for more than 5 min, perioral or periorbital angioedema, vomiting (excluding immediate postingestion gag/vomits) or evidence of anaphylaxis as defined by the Australian Society of Clinical Allergy and Immunology (evidence of circulatory or respiratory compromise) within 2 h of the last dose of the OFC (12).

Definitions

Peanut- or egg-allergic (PA or EA) infants were defined as having positive SPT ≥ 2 mm and an unequivocal allergic reaction during OFC. Peanut- or egg-sensitized (PT or ET) infants had a positive SPT ≥ 2 mm and a negative OFC. Nonsensitized, nonallergic infants had a negative SPT (wheal 0 mm) to peanut, egg, sesame and cow's milk together with a negative peanut or egg challenge outcome ($n = 37$).

To allow investigation of differences between peanut- and EA/sensitized infants, primary analyses were restricted to single-sensitized individuals. In other words, infants classified as PT ($n = 14$) and PA ($n = 14$) were allergic/sensitized to peanut, but not to egg, sesame, shrimp or cow's milk. Infants classified as ET ($n = 15$) and EA ($n = 49$) were allergic/sensitized to egg, but not to peanut, sesame, shrimp or cow's milk.

Plasma collection and detection of cytokine levels

Blood was collected into a sodium heparin tube (Sarstedt) 1–2 h after the last dose of the OFC. The blood was centrifuged at 700 *g* for 10 min within 2 h after the blood was taken and the plasma was collected and frozen at -20°C until use. To determine whether taking blood immediately after an OFC affected cytokine measurement, we compared the results from blood samples taken on another day with the remaining bloods taken on the day of challenge. There were no significant differences between the bloods that were taken on the day of the food challenge *vs* the bloods that were taken on a different day ($n = 13$; Fig. S1). These results showed that there were no differences between cytokine levels in relation to whether bloods were taken on a nonchallenge day *vs* after an active challenge day, consistent with a previous study that shows no changes between the placebo and the active group 4 h after an OFC (13).

Plasma levels of IL-4, IL-6, IL-10, IL-12p70, IL-13, TNF- α and IFN- γ were measured by multiplex assay (Millipore MILLIPLEX[®] MAP kit, high-sensitivity HSCTYO-60SK (Merck Millipore, Billerica, MA, USA)). Assays were performed undiluted in duplicate. The standard curve included two samples at 1 in 2 dilution starting at 1000 pg/ml and subsequently 1 in 5 dilution with the last point of the limit at 0.13 pg/ml. Concentrations were calculated using a weighted five-parameter logistic curve. The sensitivity, intra-assay variability and spike recovery of this assay have been validated by the issuer on their website. Also, they observed no or negligible cross-reactivity between the antibodies and any of the other analytes investigated in the plasma matrix.

Statistics

All statistical analyses were performed using STATA software (release 12.0; StataCorp, College Station, TX, USA). The data were log-transformed to normalize the data for analysis and are presented as the geometric mean $\pm$ 95% CI. Comparisons between groups were made on the log-transformed data by two-tailed unpaired *t*-test. Data were plotted using Graphpad Prism 5.03 software (GraphPad Software, La Jolla, CA, USA).

Ethics

Ethics approval was obtained for the HealthNuts study from the Victorian State Government Office for Children (reference no. CDF/07/492), the Victorian State Government Department of Human Services (reference no. 10/07) and the Royal Children's Hospital Human Research Ethics Committee (reference no. 27047).

Results

Cytokine profiles of food-sensitized vs non-food-sensitized infants

A total of 173 plasma samples from HealthNuts patients were analysed. An initial analysis comparing single-sensitized to multiple-sensitized infants was performed to determine whether they had differing cytokine profiles (results below). We established that infants with multiple sensitization had different cytokine profiles compared to single-sensitized infants; hence, the following analyses were restricted to only single-sensitized infants. Of the infants with cytokine data available, 129 could be categorized as one of the following: single-sensitized PA, EA, peanut tolerant, egg tolerant or NSNA (Table 1).

Consistent with our primary hypothesis, our data showed that both food-allergic and food-sensitized infants had significantly higher levels of the Th2 cytokines IL-4 ($P < 0.001$) and IL-13 ($P < 0.001$) compared to the NSNA group (Fig. 1A). However, surprisingly, IL-12 ($P < 0.001$) levels were also higher in the allergic group compared to the NSNA (Fig. 1A). We also found that the plasma levels of IL-10 and IL-6 were significantly lower in food-allergic infants compared to the NSNA group (Fig. 1A).

Compared with sensitized infants, food-allergic infants only had significantly lower levels of plasma IL-10 ($P < 0.001$, Fig. 1A). However, we were unable to detect any differences in IL-4, IL-6, IL-12, IL-13, TNF- α and IFN- γ levels between sensitized and allergic infants.

Overall, the largest differences were observed between sensitized infants and nonsensitized infants (Fig. 1B). Cytokine

profiles are distinctly different for sensitized and nonsensitized infants, with sensitized infants having significantly higher IL-4, IL-13 and IL-12 levels and lower IL-10 and IL-6 levels.

The role of egg and peanut sensitization status in cytokine outcomes

We also examined whether there were differences between egg sensitization and peanut sensitization. We found that ET infants had significantly higher plasma levels of IL-13 and IL-12 and lower levels of IL-10 compared to PT infants (Fig. 2). No differences were detected for IL-6 (Fig. 2) or IL-4, IFN- γ and TNF- α (data not shown).

Overall, serum cytokine profiles for PT infants resembled those of nonsensitized infants, with no significant differences in levels of any of the cytokines examined except that PA infants had lower levels of IL-10 compared with nonsensitized infants.

Multiple-food-sensitization plasma cytokine profiles

We compared plasma cytokine profiles between infants with a single-food sensitization to egg or peanut and those with multiple-food sensitizations. Infants sensitized to both egg and peanut had significantly higher concentrations of a number of cytokines including IL-12, IL-10 and IL-6 compared to single-sensitized infants (Fig. 3). The IL-12p70 plasma cytokine concentrations were markedly higher ($P < 0.05$) in the PA infants with multiple sensitization (13.3 ± 2.4 pg/ml) compared to PA infants with single peanut sensitization (0.8 ± 1.9 pg/ml). It was observed that IL-10 was significantly higher ($P < 0.01$) in the multiple-sensitized group (121.8 ± 1.3 pg/ml) compared to single-sensitized group (60.8 ± 1.2 pg/ml) of PA infants. Also, the IL-6 concentration was also trending towards significance ($P = 0.08$) in infants with multiple-sensitized group (20.9 ± 1.4 and 22.4 ± 1.5 pg/ml) compared to the single-sensitized group (2.4 ± 1.8 and 1.8 ± 2.1 pg/ml) of PA infants. The same trend was also observed for the Th2 cytokine IL-4, with multiple-sensitized infants having higher concentrations compared to single-sensitized infants, irrespective of their clinical

Table 1 Characteristics of the study group

Allergy sensitisation Allergic status	Peanut		Egg		Non sensitized, non-allergic Non allergic (NSNA)
	Allergic (PA)	Tolerant (PT)	Allergic (EA)	Tolerant (ET)	
Total number	26	24	63	23	37
Multiple sensitisation	12 (46%)	10 (31%)	14 (22%)	8 (34%)	N/A
Blood taken on the day of the oral food challenge	21 (80%)	20 (83%)	39 (62%)	15 (65%)	37 (100%)
IgE (kUA/ml) mean $\pm$ SD	38.9 $\pm$ 43.9	23.5 $\pm$ 28.9	38.8 $\pm$ 43.7	28.6 $\pm$ 23.9	25.5 $\pm$ 28.7
SPT wheal size (mm) mean $\pm$ SD	8.8 $\pm$ 3.1	4.2 $\pm$ 1.8	4.4 $\pm$ 1.6	4.3 $\pm$ 2.0	0 $\pm$ 0
Eczema	16 (66%)	13 (54%)	17 (27%)	4 (17%)	11 (30%)

EA, egg allergic; ET, egg sensitized; PA, peanut allergic; PT, peanut sensitized; SPT, skin prick test; NSNA, nonsensitized, nonallergic.

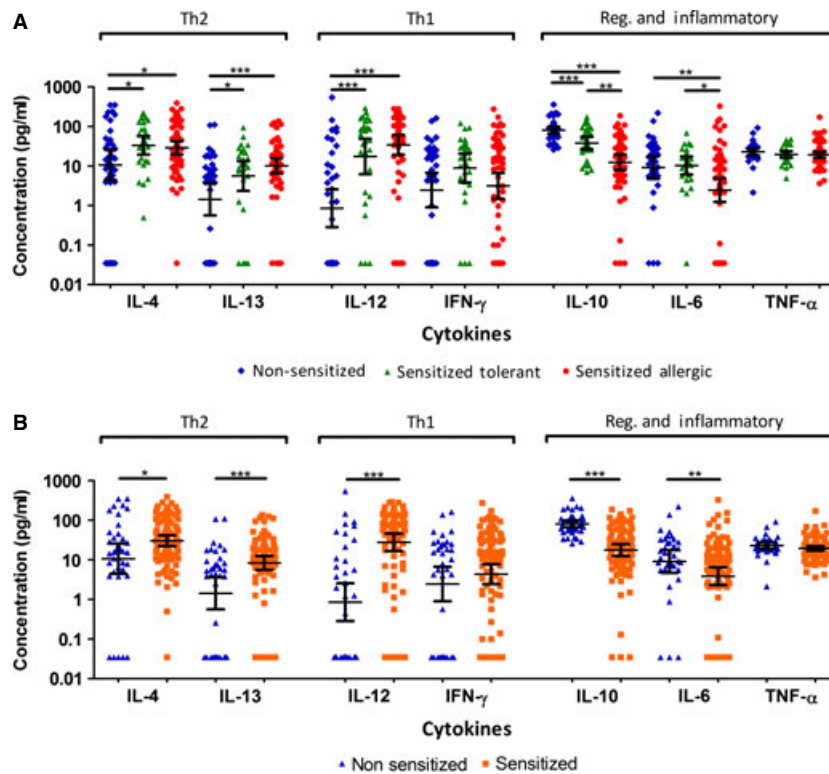


Figure 1 (A) Geometric mean $\pm$ 95% CI of Th2 IL-4, IL-13, Th1 IL-12, IFN- γ , Reg IL-10, and proinflammatory IL-6 and TNF- α cytokine concentrations from: single sensitized allergic (red) $n = 63$, single sensitized tolerant (green) $n = 29$, and non-sensitized non allergics (blue) $n = 37$. Comparisons between groups were performed on geometric mean data by two-tailed unpaired t -test. * P -value < 0.05 , ** P -value < 0.01 , *** P -value < 0.001

(B) Geometric mean $\pm$ 95% CI of Th2 IL-4, IL-13, Th1 IL-12, IFN- γ , Reg IL-10, and proinflammatory IL-6 and TNF- α cytokine concentrations from: single food sensitized (orange) $n = 92$, and non-sensitized non allergics (blue) $n = 37$. Comparisons between groups were performed on geometric mean data by two-tailed unpaired t -test. * P -value < 0.05 , ** P -value < 0.01 , *** P -value < 0.001 .

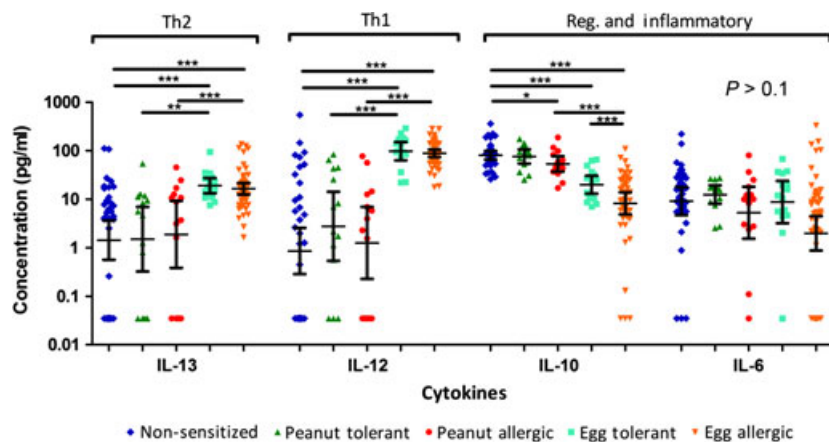


Figure 2 Geometric mean $\pm$ 95% CI of Th2 IL-13, Th1 IL-12, and Reg IL-10, and proinflammatory IL-6 cytokine concentrations from: peanut sensitized allergic (red) $n = 14$, tolerant (green) $n = 14$; egg sensitized allergic (orange) $n = 49$, tolerant (aqua) $n = 15$, and non

sensitized non allergics (blue) $n = 37$. Comparisons between groups were performed on geometric mean data by two-tailed unpaired t -test. * P -value < 0.05 , ** P -value < 0.01 , *** P -value < 0.001 .

status, although the difference did not reach a statistical significance.

Cytokine profiles of infants with and without eczema

We examined the plasma cytokine profiles of infants with and without eczema on the day of food challenge, irrespective of their food sensitization status and allergic outcome. No differences were detected in any of the cytokines between the eczema and the noneczema samples (Fig. 4).

Discussion

We present the first study to define and compare plasma cytokine profiles of food-allergic, food-sensitized and non-sensitized infants from one of the largest population-based cohort studies investigating epidemiology of food allergy to date. We confirmed that OFC-proven food-allergic infants

have significantly higher plasma IL-4 and IL-13 than non-sensitized infants, cytokines that have been shown to be involved with allergic inflammation (14, 15). We also found that food-allergic infants were deficient in both plasma IL-10 and IL-6 compared to both sensitized and non-sensitized infants. Although it has been previously published that the balance between Th1 and Th2 responses is important in the development of food allergy (15, 16), we found Th1 plasma IL-12 levels to be elevated in food-allergic infants compared to non-sensitized infants. Although IL-12 has been reported to be capable of inhibiting allergic inflammation (17), it has also been recognized as a key driver in the pathogenesis of other immune-inflammatory diseases such as psoriasis, multiple sclerosis and Crohn's disease (18, 19).

Previous studies investigating the role of IFN- γ in allergic disease have shown inconsistent results. It has previously been demonstrated that IFN- γ and IL-12 are dysregulated in atopic dermatitis (20). However, our study shows that

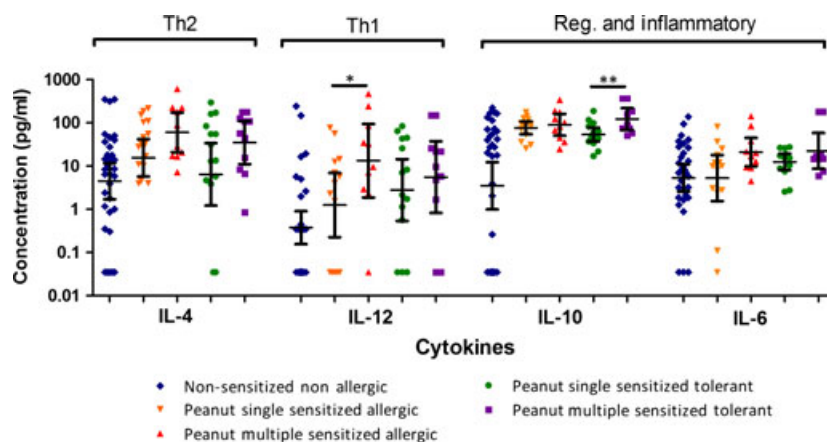


Figure 3 Geometric mean $\pm$ 95% CI of Th2 IL-4, Th1 IL-12, Reg IL-10, and Inflammatory IL-6 cytokine concentrations from: donors who are non sensitized non-allergic (blue) $n = 37$; peanut allergic and single sensitized to peanut (orange) $n = 14$ or have multiple food sensitizations (red) $n = 12$; peanut tolerant and single

sensitized to peanut (green) $n = 14$ or have multiple food sensitizations (purple) $n = 10$. Comparisons between groups were performed on geometric mean data by two-tailed unpaired t -test. * P -value < 0.05 , ** P -value < 0.01 .

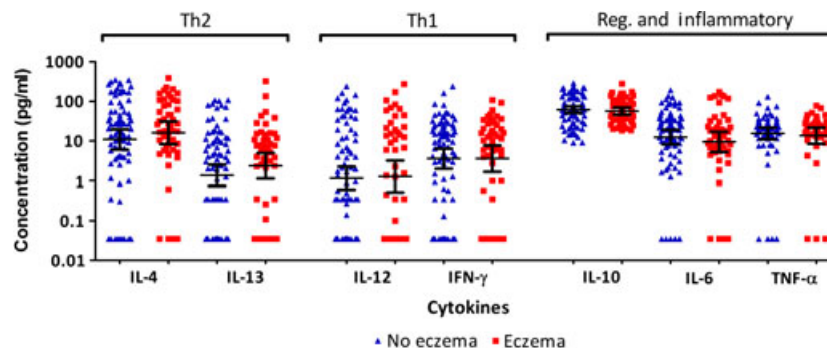


Figure 4 Geometric mean $\pm$ 95% CI of Th2 IL-4, IL-13, Th1 IL-12, IFN- γ , Reg IL-10, and Inflammatory IL-6 and TNF- α cytokine concentrations from: eczema infants (red) $n = 49$, non-eczema infants

(blue) $n = 80$. Comparisons between groups were performed on geometric mean data by two-tailed unpaired t -test.

the IFN- γ levels in the allergic group do not significantly differ when compared to the sensitized group or the nonsensitized controls, in line with a number of other studies (21, 22). However, no previous studies have investigated the role of IL-12 in food sensitization and food allergy, and contrary to our hypothesis, food-allergic infants had an elevated level of plasma IL-12 compared to nonallergic infants. The role of IL-12 in suppressing the development of the allergic phenotype has been well documented, including suppression of differentiation of naive T cells into Th1 cells (23, 24). Our findings of high IL-12 levels in sensitized infants are consistent with the Tulic et al., (25) demonstrating allergic infants produce exaggerated Th1 responses that fail to return to levels which normal healthy infants produce during early life.

We established that the cytokine profiles of allergic infants are different to the proposed allergic paradigm of a Th1–Th2 balance (25, 26) and more consistent with general immune-inflammatory upregulation with Th1 IL-12 and Th2 IL-4 and IL-13 cytokines at high levels while deficient in IL-10. The role of IL-10 in immune regulation has been well documented (27), and the low levels seen in food-allergic infants are likely to contribute to the inability of allergic infants to regulate the inflammatory response upon exposure to the food. Deficient plasma IL-10 levels in our food-allergic infants compared to both sensitized and nonallergic infants suggest that a failure to activate and regulate tolerance could contribute to this clinical phenotype. Similarly, reduced levels of IL-6 were found in food-allergic infants compared to sensitized and nonsensitized infants, although its precise role in food allergy is not fully understood. It has previously been shown that IL-6 is required for IgG1 production by B cells (28), providing a possible explanation for the reduced levels of circulating IL-6 seen in our food-allergic infants. It is plausible that reduced IL-6 levels may increase the likelihood of developing food allergy as a consequence of the inability to effectively class-switch from an allergic IgE phenotype to nonallergic IgG phenotype (28). Therefore, the fine balance between these circulating cytokines may be one of several factors that determine whether an allergic reaction develops in a susceptible individual.

This study is the first to directly compare cytokine profiles in egg and peanut allergy at 1 year of age, revealing small differences between the ET profiles and the PT profiles. Those differences were increases in IL-13, IL-12 and a reduced IL-10 concentration for ET infants compared to PT infants. It is possible that the immunological mechanisms involved with egg allergy are different to those with peanut allergy. Egg allergy is reported to resolve before adulthood in over 80% of patients (29), while peanut allergy has a lower resolution rate of only 20% by adulthood (30). Our data suggest that EA infants have a higher Th1 (IL-12) baseline, which may allow the immune system to be more equipped to deal with the high Th2 cytokine levels present. While the EA infants had significantly lower IL-10 concentrations compared to nonallergic infants, these levels have been reported to increase and be maintained as their immune system matures, suggesting a degree of plasticity compared to PA

individuals (31). While the plasma IL-13 and IL-12 cytokine profiles of PT infants differ significantly to ET infants, both the PA and peanut-tolerant infants have similar levels in all the cytokines we measured compared to nonallergic infants. While it has been demonstrated that infant T cells have a higher capacity to produce the regulatory IL-10 (32), we found that PA infants have a significantly reduced IL-10 concentration compared to the nonallergic infants. However, in line with the finding by Lotoski et al., (33) there were no significant differences in plasma IL-10 concentrations between PA and PT infants in our cohort. Hence, while both egg allergy and peanut allergy exhibit similar clinical phenotypes, here we demonstrate that immunologically they have differing plasma cytokine profiles which may underpin their contrasting resolution rates.

It has previously been presented in a small study cohort that children with atopic dermatitis produced significantly lower IL-10 in PBMC cultures compared to healthy individuals (34). However, we found no significant differences in any of the plasma cytokines we measured between infants with nurse-observed eczema on the day of OFC compared to infants without eczema. Hence, differences in cytokine profiles between food-sensitized and nonsensitized children do not appear to be due to differences in eczema prevalence between these groups.

The strengths of this study include the large cohort of clearly phenotyped peanut- and EA and tolerant infants, with allergic status determined through both SPT and an OFC. True population-negative controls were used to evaluate the differences between the plasma cytokine profiles of sensitized and nonsensitized infants. It must be taken into account that the food challenge can possibly induce an increase in some plasma cytokines within 1–3 h. Krogulska et al. have shown that an OFC increased IL-4 levels; however, more importantly, they found that other cytokines such as IL-10 and TNF- α levels were not affected by the OFC 4 h later (13, 35, 36). It is also possible that we have missed differences between food-sensitized and food-allergic infants if the food challenge can increase levels measured at 4 h, for example may be higher in allergic compared to sensitized patients, but this difference was lost as a result of IL-4 being increased postchallenge. Also, another limitation of the study was that only seven major cytokines were measured. There were a number of other cytokines that were not assessed in this cohort, which may have demonstrated more clear differences based on previous reports. Cytokines such as IL-5 and TGF- β have been implicated in allergic inflammation and could be potential biomarkers.

Taken together, we were able to separate the clinical phenotypes of allergy and sensitization by the differences in plasma IL-10 and IL-6, which may be useful as a plasma biomarker for determining food allergy. Moreover, we found different IL-13 and IL-12 levels in the ET cohort compared to the PT cohort, which may relate to mechanistic differences between the resolution of egg and peanut allergy. Further analysis of more patients is required to validate these findings, and a number of additional cytokines may be useful. Identification of a plasma biomarker may provide valuable

information in the diagnosis and management of food allergy.

Author contributions

TD, MT and KA co-conceived the experimental design. TD, TT and PL undertook the experiments in measuring the plasma cytokines. JE, JK and LG contributed with the statistical analysis. TD, PL, JK, MT, SD, ALP and KA drafted and provided the intellectual input to the manuscript.

Conflict of interest

The authors have no conflict of interest with the publication of this article.

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Supporting Information

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

Figure S1. Geometric mean $\pm$ 95% CI of Th2 IL-4, IL-13, Th1 IL-12, IFN- γ , Reg IL-10, and Inflammatory IL-6 and TNF- α cytokine concentrations from: infants with blood taken before OFC (purple) $n = 13$, infants with blood taken after OFC (red) $n = 61$. Comparisons between groups were performed on geometric mean data by 2-tailed unpaired t-test.

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