IL-9 is a key component of memory Th cell peanut-specific responses from peanut allergic children

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**Abbreviations:**

- B7: \( \alpha_4 \beta_7 \) integrin
- CISH: Cytokine-inducible SH2-containing protein
- CLA: Cutaneous lymphocyte antigen
- FACS: Flow-cytometry
- ICCS: Intracellular cytokine staining
- IQR: Interquartile range
- NA: non-peanut allergic
- OVA: ovalbumin
- PA: peanut allergic
- PBMCs: Peripheral blood mononuclear cells
- PBS: Phosphate buffered saline
- PS: Peanut sensitized
- RF: Random Forrest algorithm
- RT-qPCR: Real time quantitative polymerase chain reaction
- TGF\( \beta \): Transforming Growth Factor beta
- Th: T helper

**Word count: 3501**
Abstract:

Background: Differentiation between peanut allergic (PA) and peanut sensitized (PS) individuals who tolerate peanut but have peanut-specific IgE and/or positive skin prick tests represents a significant diagnostic difficulty. Previously, gene expression microarrays were successfully used to identify biomarkers and explore immune responses during peanut allergy immunotherapy.

Objective: Characterization of peanut-specific responses from PA, PS and atopic non-peanut-allergic (NA) children.

Methods: A preliminary, exploratory microarray investigation of gene expression in peanut-activated memory Th subsets from 3PA and 3NA children identified potential peanut allergy diagnostic biomarkers. Microarray findings were confirmed using RT-qPCR in 30 individuals (12PA, 12PS and 6NA). Flow cytometry was used to identify the Th subsets involved.

Results: Amongst 387 differentially expressed genes, IL9 showed the greatest difference between PA and NA (45.59-fold change, p<0.001) followed by IL5 then IL13. Notably, IL9 allowed the most accurate classification of PA and NA using a machine learning approach using recursive feature elimination and the Random Forest algorithm. Skin- and respectively gut-homing Th cells from PA donors expressed similar Th2 and Th9-associated genes. RT-qPCR confirmed that IL9 was the highest differentially expressed gene between PA and NA (23.3-fold change, P<0.01) and PS children (18.5-fold change, P<0.05). Intracellular cytokine staining showed that IL9 and the Th2-specific cytokine IL5 are produced by distinct Th populations.
**Conclusion:** In this study, IL9 best differentiated between PA and PS (and atopic NA) children. Mutually exclusive production of IL9 and the Th2-specific IL5 suggests that the IL9-producing cells belong to the recently described Th9 subset.

**Clinical applicability:** IL9 may be a useful biomarker to distinguish between peanut allergic and peanut sensitized children. Further prospective studies are required to validate these findings and determine whether IL9 responses could be targeted in treating and preventing peanut allergy.

**Capsule summary:** In vitro peanut-specific responses of memory Th cells from peanut allergic children include IL9 production in addition to the well-characterized Th2 cytokines.

**Key words:** Peanut allergy, peanut sensitization, gene expression, RT-qPCR, microarray, IL9, Th9, IL5, IL13, Th2

**Abstract word count:** 246
**Introduction**

Peanut allergy has increased over the last decades so that its prevalence in childhood is currently estimated at 1.4%\(^1\) in the USA and reaches 1.8% in the UK\(^2,3\). Since peanut allergy is rarely outgrown and is responsible for a significant proportion of severe anaphylactic reactions to foods, it represents a major population health concern\(^4\).

Mechanistically, peanut allergy is driven by Th cells whose determinant role was confirmed by the observation that allergy can be transferred from peanut allergic (PA) donors to non-peanut allergic (NA) recipients via solid organ transplantation which entails Th cell transfer\(^5\). Conversely, peanut allergy resolution has been observed after bone marrow transplantation\(^6\). We\(^7\) and others\(^8,9\) showed that the in vitro peanut-specific Th cell response in PA individuals is dominated by a Th2-polarised population characterized by the production of Th2 cytokines such as IL4, IL5 and IL13. However, we also observed that a significant number of in vitro peanut-responding Th cells did not produce Th2 (nor Th1-specific) cytokines even when restimulated with phorbol ester and calcium ionophore, suggesting that they may belong to distinct Th cell subsets\(^10\).

Indeed, the discovery of Treg, Th17, Th22 and more recently Th9 shows that Th cell responses go beyond the binary Th1/Th2 paradigm\(^11\). Treg cells suppress IgE production\(^12\) and block mast cell, basophil and eosinophil activation\(^13\), whereas in mouse models of asthma, Th17 cells increase eosinophilia and IgE production\(^14\). Th9 was recently identified as a distinct Th cell subset induced by the combination of IL4 and TGFβ\(^15\). IL9, which is the Th9 subset-defining cytokine, is a mast cell growth factor which increases cytokine production in activated mast cells and enhances IL4-driven IgE production by B cells. IL9 is highly expressed in the lungs of asthmatic humans\(^16\) and in an experimental mouse model, neutralizing anti-IL9 antibodies were shown to ameliorate Th9-mediated asthma\(^17,18\).
In the present study, we aimed to further dissect the peanut-responding Th cell subsets by separating the skin-homing Th cells which express the Cutaneous Lymphocyte Antigen (CLA)\(^{(19)}\) and the gut-homing memory Th cells (which express the B7 gut-homing marker) respectively.\(^{(20)}\) Skin exposure to peanut antigens following skin stripping to mimic an eczema phenotype leads to strong Th2-skewed responses in mice, supporting the concept of epicutaneous sensitization.\(^{(21)}\) Furthermore, we recently showed that peanut-specific proliferation is quantitatively higher in the skin-homing than in gut-homing Th cells in PA individuals.\(^{(22)}\) Conversely, oral exposure to antigens underlies oral tolerance and is associated with higher levels of antigen-specific proliferation of the gut-homing T cells.\(^{(20)}\) This has led to the Dual Allergen Exposure Hypothesis being proposed, in that the timing and balance of cutaneous versus oral exposure to an allergen may determine whether the child develops allergy or tolerance.\(^{(23)}\)

In this study we aimed to assess differential gene expression in peripheral blood mononuclear cells (PBMCs) cultured with peanut from PA and atopic NA children, in both skin and gut homing Th cells using gene microarray, RT-qPCR and flow-cytometry (FACS) analysis of intracellular cytokine production.
Materials and methods

Study participants

PA, peanut sensitized (PS) and atopic NA donors were recruited from a tertiary referral allergy center at St. Thomas Hospital Children’s Allergy Unit, London UK. Informed consent was obtained prior to participation. Ethical approval for this study was obtained from St Thomas Research Ethics Committee (ref.10/H0802/45). Three PA and three atopic NA children were recruited for gene expression analysis and 36 microarrays were carried out on various T cell populations derived from their peanut-stimulated and unstimulated PBMCs (as negative controls). Forty participants recruited for the gene microarray work were all males in order to avoid differential gene expression linked to gender. Patients recruited for subsequent reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and FACS analysis did not have gender as a selection criterion. Peanut allergy was defined as having a convincing history of an immediate hypersensitivity reaction following exposure to peanut and a skin prick test to peanut extract of ≥8mm or peanut-specific (s)IgE ≥15kU/L. Peanut tolerance was defined as regular peanut consumption, i.e. eating an age-appropriate amount of peanut in one meal without any allergic symptoms within the last month; of which some were PS (defined by SPT≥3mm or peanut (s)IgE ≥0.35kU/L. If there was no history of peanut consumption, children underwent a double-blind placebo controlled food challenge (DBPCFC). The demographic and clinical characteristics of all study participants are described in Table E1.

Details of the methodology are described in the online repository, with an overview below:

1) PBMC isolation and peanut stimulation in vitro
2) Cell labeling and flow-cytometry sorting
3) RNA extraction
4) cDNA synthesis
5) cDNA fragmentation, biotin-labeling and hybridization
6) Microarray data normalization and quality checks
7) Partek Suite analysis of microarray
8) Microarray gene expression classification approach
9) RT-qPCR confirmation of gene expression
10) FACS analysis of intracellular cytokine production

Overview of methods
PBMCs from 3 PA and 3 NA children were cultured with peanut antigens for 18h. Subsequently activated (CD69+) skin-homing (CLA+) memory Th cells (CD4+CD45RO), and respectively activated (CD69+) gut-homing (B7+) memory Th cells were sorted by FACS into Trizol LS. mRNA was extracted using the miRNeasy Mini kit (Qiagen, UK) and cDNA was synthesized using the NuGEN Ovation® Pico WTA system v2 kit (NuGEN, California, USA). Following data normalization and quality checks, differential gene expression between PA and NA donors was determined by Affymetrix GeneChip® Human Gene 1.0 ST Array. Microarray results were analyzed using Partek Genomics Suite and using an automatic classification approach, as currently used in data mining / machine learning fields. Microarray findings were subsequently confirmed at a gene level using RT-qPCR on whole PBMCs from 30 children (12 PA, 12 PS and 6 NA) and at a protein level using FACS intracellular cytokine staining (ICCS) in 5 PA and 5 PS individuals.
Results

Microarray analysis of gene expression in peanut-activated memory Th cells from PA vs. NA donors.

The level of gene expression in peanut-activated memory Th cells from PA and atopic NA donors is displayed as a volcano plot using Partek Genomics Suite (FIG. 1); the horizontal axis shows the fold-change difference of gene expression between PA and NA whereas the vertical axis displays the statistical significance of the difference of gene expression between PA and atopic NA, taking into account gene expression variability within the PA and the NA groups respectively. The genes appearing in the top right area were upregulated in PA versus NA; the most statistically significant differently expressed gene was IL9 (45.59 fold change, p<0.001) followed by the Th2 gene cluster containing IL5 and IL13. Conversely, the genes appearing in the top left area (e.g. SULT1B1, RGS18) were upregulated in atopic NA donors.

Peanut specific memory T-helper cells from PA donors express typical Th2-specific genes as well as Th9 associated genes

A hierarchical clustering heatmap of the dataset (FIG. 2) consisting of 48 differentially expressed genes, selected through a filter criteria of at least two-fold changes of expression with P<0.05 was produced using Partek Genomics Suite. There was notable differential expression between peanut stimulated activated (CD69+) Th cells from PA and NA donors for Th2 signature genes (IL-4, IL-5, IL-13) and Th9 associated genes (IL-9, IRF4, IL17RB). CCL1 (Chemokine (C-C motif) ligand 1), IL31 and IL3 were preferentially expressed in skin-homing Th cells from PA donors. Other genes differentially expressed in PA versus NA and their functions and involvement in allergy are described in Table I. Most genes preferentially expressed in NA vs. PA were as yet not functionally understood and only SULT1B1, GCNT4 and RGS18 could be identified (Table I).
ANOVA of genes expressed in activated skin and gut-homing peanut-responding memory Th cells from PA donors vs. non-activated PA Th cells, non-antigen PA Th cells and NA Th cells

In order to compare the basal, non-stimulated level of gene expression in skin and gut homing peanut-activated memory Th cells, we performed 36 microarrays (displayed in Table EII) in three types of Th cell subsets isolated from PA and NA donors respectively:

I) Peanut activated: CD69+ Th cells from PBMCs cultured in peanut protein

II) Peanut non-activated: CD69- Th cells that were not activated isolated from the same PBMCs cultured with peanut protein (internal negative controls)

III) No peanut (unstimulated): CD69- Th cells isolated from the PBMCs where no peanut antigen was added (external negative controls)

Differential gene expression of peanut stimulated (P+) activated (CD69+) Th cells between PA and atopic NA is displayed in FIG. 3; we also include results for internal negative controls (peanut stimulated, non-activated (CD69-)) and external negative controls (unstimulated, non-activated (CD69-)) in the online repository FIG. E4. There was differential gene expression of IL9 and associated genes IL17RB and IRF4 as well as classical Th2 cytokines (IL5, IL13 and IL4) and CISH (Cytokine-inducible SH2-containing protein) in PA versus NA donors. CCL1 and IL31 were preferentially increased in skin-homing CD69+ memory Th2 cells from PA children; however, Th2/Th9 cytokines were not expressed preferentially in skin- or gut-homing Th cells. All external negative controls (unstimulated CD69- Th cells) in PA and NA donor had low expression of all genes assessed. There was very little background expression of IL9, IL4, IL5, IL13, IL31 and CCL1 in internal negative controls (P+ CD69-) in PA and NA donors; however, there was some background expression of IL17RB, IRF4 and CISH in P+ CD69- Th cells (FIG. E4).
IL9 allows the differentiation between PA and NA individuals when used in the frame of a Random Forest (RF) classifier.

We used RF to classify the peanut stimulated, activated (CD69+) Th cell responses in PA vs. NA children. We built 250, 500 and 750 trees (analysis pathways); the number of analysis pathways is dictated by a trade-off between the efficiency (computing speed and memory consumption) of the classifier and the quality of its predictions. Using this approach RF was able to differentiate between PA vs. NA children with 100% accuracy even when the gene selection was limited to sets of 10, then 5, then only 2 genes. In this final analysis, the two genes that are able to accurately distinguish between PA and NA samples were: IL9 and HSPA5 for 250 trees, IL9 and 8069610 for 500 trees, and IL9 and HUWE1 for 750 trees (FIG. E4). From these sets of genes IL9 expression was the best discriminating gene between memory Th cells from PA and NA children as shown in FIG. 4.

Differential gene expression of Th2 and Th9 cytokines between PA and NA individuals is confirmed by RT-qPCR

The expression of IL5 and IL13 (signature genes for Th2 responses) and respectively IL9 and IRF4 (reflecting Th9 responses) was higher in PBMCs isolated from PA donors than NA donors (FIG. 5). Relative quantitation (RQ) for IL9 was 25.40 [Interquartile range [IQR] 4.09-65.33] in PA versus 1.09 [IQR 0.96-2.10] in NA; which equated to a 23.30-fold change in IL9 expression. RQ for IL5 was 61.73 [IQR 4.29-167.87] in PA versus 2.86 [1.80-4.97] in NA (21.6-fold change); RQ for IL13 was 53.96 [IQR 31.68-121.14] in PA versus 4.54 [IQR 1.80-7.15] in NA (11.89-fold difference). Thus IL9 had the highest fold differential gene expression between PA and NA donors. Significant differential gene expression (RQ) was also observed for other genes known to be involved in allergic responses such as IL31, CISH and CCL1 (P<0.01) and the Th2/Th9-specific transcription factor IRF4 (P<0.05). On RT-qPCR analysis there was no significant differential IL4 and IL17RB expression between PA and NA donors.
Differential gene expression of Th2 and Th9 cytokines between PA and PS individuals by RT-qPCR

Following the finding that IL9 had the highest differential expression in PA versus NA we sought to determine whether this would also be a useful biomarker to differentiate between PA and PS children (FIG. 5). RQ for IL9 was 25.40 [IQR 4.09-65.33] in PA versus 1.37 [IQR 1.21-3.88] in PS (18.54-fold change). RQ for IL5 was 61.73 [IQR 4.29-167.87] in PA versus 3.40 [1.14-14.29] in PS (18.16-fold change) and for IL13 was 53.96 [IQR 31.68-121.14] in PA versus 7.56 [IQR 4.08-22.86] in PS (7.14-fold difference). Thus IL9 also had the highest fold differential gene expression between PA and PS donors. CCL1, CISH and IL31 were also differentially expressed between PA and PS donors. One PS child who was eating whole peanuts 1-2 times/month had raised IL9, IL5 and IL31 and represents the outlier for the PS RQ box-plots.

Different Th populations secrete IL9 and IL5 cytokines

ICCS of peanut stimulated memory T-helper cells demonstrated that IL5 and IL9 are produced by distinct Th-cell subsets (FIG. 6A and 6B); memory Th cells from all PA donors produced more IL9 and IL5 than PS children and the pattern of cytokine expression was mutually exclusive (Table E3).
Discussion

We employed the well-established Affymetrix microarray method to determine gene expression profiles of memory T cells responding to peanut antigens in vitro in order to characterize differential gene expression and the different Th cell subsets involved in peanut allergy. Microarray gene expression data found evidence of high IL9 expression in activated memory Th cells from PA donors in addition to the expected Th2 gene signature. Applying a Random Forrest algorithm we found that IL9 was the best gene classifier that allows differentiation between PA and NA children with 100% accuracy. Using RT-qPCR and FACS we confirmed the presence of IL9 at a gene and protein level, and showed that IL9 was the best gene to distinguish between PA and NA children (23.3-fold difference) as well as between PA and PS children (18.5-fold difference); thus, making IL9 a good biomarker for clinical peanut allergy in this study. The differential expression of Th2 signature genes (IL5 and IL13) and Th9 associated genes (IL9 and the transcription factor IRF4) in PA versus NA allergic children as well as the dichotomous pattern of IL5 and IL9 production in peanut-stimulated activated memory T cells suggests the involvement of both Th2 and a distinct Th9 subset in peanut allergy.

Jabeen et al. reviewed the recently discovered Th cell subset Th9 that produces IL9 but not the other Th2-defining cytokines such as IL5 and IL13.\(^{41}\) Th9 cells have been described in the inflamed skin of patients with atopictic eczema\(^ {42}\) and in the bronchial mucosa in mouse models of allergic asthma,\(^ {16}\) and Th9 is emerging as an important T cell subset in human respiratory allergic disease.\(^ {15;41}\) IL9 has previously been described as the top ranking gene (from 1482 differentially expressed genes) for discriminating between atopic and non-atopic responses to house dust mite.\(^ {37}\) IL9 production is also important for in vivo allergic responses in seasonal allergic rhinitis, as successful specific immunotherapy with grass pollen led to the decrease of IL9 mRNA and IL9 protein in the patients’ nasal mucosa.\(^ {43}\) IRF4 is necessary for the differentiation of Th9 cells,\(^ {29;30}\) and has previously been described
using gene expression microarrays in allergen-stimulated PBMCs from patients with allergic rhinitis.\textsuperscript{(44)}

In mice, oral antigen induced anaphylaxis to ovalbumin (OVA) is IgE mediated and predominantly IL9 and IL9 receptor pathway dependent.\textsuperscript{(45)} IL9\textsuperscript{−/−} and IL9R\textsuperscript{−/−} mice developed OVA-specific IgE levels following intraperitoneal OVA/alum immunisation but did not develop anaphylaxis after OVA oral gavage and had reduced intestinal mast cell proliferation and degranulation. This might explain the role of IL9 in IgE mediated food allergy versus only IgE specific sensitization. In a recent study in humans, IL9 was the best gene to discriminate between peanut stimulated PBMCs from PA versus NA (28-fold difference on RT-qPCR); however, IL9 expression was not assessed in PS individuals.\textsuperscript{(46)} Other differences between their manuscript and ours were (i) they assessed adults rather than children, (ii) their peanut tolerant group were generally non-atopic whereas the majority of our peanut tolerant group were atopic thus accounting for genes upregulated due to atopy rather than peanut allergy perse (iii) exploratory microarray analysis was not used to determine candidate genes thus other important differentiation genes might have been excluded (iv) IL9 gene expression was initially investigated in the entire PBMC population rather than in different T cells subsets to determine which cells were producing IL-9; however, importantly, they showed significantly raised IL5 and IL9 levels in the 5-day peanut culture supernatants of PA versus NA individuals (using ELISA).

We confirmed differential gene expression between PA and NA for IL9 and its transcription factor IRF4, signature Th2 genes (IL5, IL13) and other genes important for allergen specific responses (CISH, IL31 and CCL1) by RT-qPCR. Confirmation of microarray findings was performed using RT-qPCR of whole PBMCs rather than FACS sorted cells because, if IL9 is to be useful as a diagnostic biomarker then it should be able to distinguish between PA and NA individuals in unseparated cells rather than in very small Th cell subsets that involve cumbersome and highly skilled experimental procedure. The IL25 receptor (IL17RB) and IL4
were no longer significantly differentially expressed between PA and NA on RT-qPCR. The IL25 receptor (IL17RB) is expressed in Th9 cells generated in vitro in the presence of TGFβ and IL4. The difference between findings in the microarray and RT-qPCR may be because the microarray was performed on peanut cultured activated memory Th cells, whereas the RT-qPCR was performed on whole PBMCs thus the signal may not be as strong in a mixed cell culture; furthermore, on microarray analysis IL17RB and IL4 were not as highly differentially expressed as IL9, IL5 and IL13 in PA versus NA (FIG. 1). CISH was upregulated in PA versus NA and PS children and has been shown to be differentially expressed in house dust mite-stimulated T cells from atopic individuals vs. non-atopic individuals and in ovalbumin-stimulated PBMCs from egg allergic vs. non-egg allergic patients.

A notable finding in this study was the lack of differential expression of Th2 and Th9-related genes between skin and gut-homing memory Th cell subsets in PA individuals given our previous findings. Additionally, there was no significant difference in the number CLA+ versus B7+ activated memory Th cells sorted from peanut stimulated culture in PA versus NA children, although there was a trend towards higher responses to peanut from the gut-homing versus skin-homing memory Th subset in NA donors \( (P=0.05) \). One potential explanation for this is that the PA individuals whom we investigated had long-term, well-established peanut allergy; it is therefore plausible that whilst the original Th2 and Th9 gene expression profile may have been limited only to the skin-homing Th cell subset, in the long term the Th2 and Th9 responses became dominant in all lymphocyte homing compartments as a consequence of allergic individuals’ repeated exposure to peanut, either through eczematous skin or through accidental oral exposure. Future experiments could further identify the differences between peanut-specific immune responses of skin and gut-homing subsets in PS individuals, if this was carried out longitudinally, in a prospective approach, as children progress from PS to PA.
Nonetheless, other genes were differentially expressed in skin vs. gut homing Th cells; CCL1 (Chemokine C-C motif ligand 1), IL31 and IL3 were upregulated in skin homing Th cells from PA donors compared with gut-homing Th cells from PA and gut- and skin-homing Th cells from NA donors. CCL1 is secreted by activated T cells and IgE-activated human mast cells and binds to the CCR8 receptor expressed by Th2 cells, dendritic cells, monocytes, NK and immature B cells. Notably, CCR8 is expressed by approximately 70% of Th cells recruited in the asthmatic airways, which is why oral forms of the small molecule CCR8 antagonists are currently being developed for therapeutic purposes. IL31 is produced mainly by activated Th2 cells and is increased in skin biopsies of patients with eczema and contact dermatitis. IL3 increases the activation and release of mediators from eosinophils and basophils in response to IgE FcεRI cross-linking. The differential expression of these genes reflects the contrast between the skin and gut homing Th cell subsets observed in the PCA analysis (FIG. E3); however, the differential expression of homing-related genes was not mirrored by a differential expression of Th2 and Th9 subset-defining cytokine genes. The lack of such difference suggests that in patients with well-established peanut allergy, allergen-specific T cell populations comprise Th2 and Th9 cells regardless of their homing phenotype.

Another unexpected finding was the absence of an identifiable Treg gene signature in the peanut-responding memory Th cells from NA donors; however, the microarray did identify three genes SULT1B1, GCNT4 and RGS18 and other as yet unidentified genes which were differentially expressed in NA vs. PA children. The absence of classical Treg biomarkers suggests that a suppressor cytokine environment is not actively induced following only short-term (18h) in vitro stimulation with antigens from tolerated foods. Another potential explanation for this finding is that the peanut specific (CD69+) Treg cells in NA do not express CLA or B7 homing markers and were thus not gated and isolated on FACS analysis.
Conclusion

In this study, IL9 was the best discriminator for PA children versus NA children and also importantly PS children. Mutually exclusive production of Th9 specific (IL9) and Th2 specific (IL5) cytokines suggest that IL9-producing cells belong to the distinct Th9 subset population. The use of gene expression microarrays to generate hypotheses by evaluating the overall immune response to an allergen in a small number of patients, followed by further in-depth investigations has previously been successfully applied to elucidate the mechanisms underlying peanut oral immunotherapy,\(^{(49)}\) and for predicting the efficacy of venom immunotherapy.\(^{(50)}\) Future research into the interplay between the Th9 and Th2 subsets may clarify whether the success of preventative therapeutic approaches aimed at peanut allergy resolution could be evaluated on the basis of IL9 secretion and/or Th9 suppression in peanut-specific in vitro responses. Prospective studies should further evaluate IL9 as a biomarker for PA and as a potential target for the prevention and treatment of PA.

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Table I. List and outline of the known roles in allergic responses of genes which are significantly different (p<0.05 between PA and NA). Genes are listed in order of statistical significance. All genes were upregulated in PA vs. NA except the SULT1B1, GCNT4 and RGS18 which were upregulated in NA vs. PA (grey fill).

<table>
<thead>
<tr>
<th>Gene</th>
<th>p-value</th>
<th>Biological functions of genes and involvement in allergy</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL9</td>
<td>0.0002</td>
<td>IL9 is produced by Th9, mast cells and eosinophils; its main targets are T cells, B cells and mast cells. TGFβ in the presence of IL4 reprograms Th2-cell differentiation and leads to the development of Th9 cells that produce IL-9 and IL-10. Thus IL-4 blocks the generation of TGFβ–induced FoxP3+ Treg cells and induces Th9 cells. IL-9 is involved in Th2 inflammatory reactions, promotes the production of IL4–induced IgE, induces chemokine and mucus secretion by bronchial epithelial cells, and leads to mast cell proliferation. (25)</td>
</tr>
<tr>
<td>IL5</td>
<td>0.0024</td>
<td>IL5 is an eosinophil and B-cell growth factor mainly produced by Th2 cells, activated eosinophils, mast cells, CD8+Tc2 cells, NK cells and NKT cells. IL5 promotes proliferation, activation, differentiation, survival and adhesion of eosinophils. (25)</td>
</tr>
<tr>
<td>SULT1B1</td>
<td>0.0029</td>
<td>Sulfotransferase family cytosolic 1B member 1 enzyme catalyzes the sulfate conjugation of hormones, neurotransmitters and drugs. (26)</td>
</tr>
<tr>
<td>IL13</td>
<td>0.0086</td>
<td>IL13 is secreted by activated Th2 cells, mast cells, basophils, eosinophils and NKT cells. IL13 targets are B cells, mast cells, epithelial cells, eosinophils, smooth muscle cells and macrophages. IL-13 contributes to asthma and allergic rhinitis late-phase responses. (25)</td>
</tr>
<tr>
<td>IL4</td>
<td>0.0134</td>
<td>IL4 is mainly produced by Th2 cells, basophils, eosinophils, mast cells and a subset of NK T cells. The main IL4 targets are T and B cells to induce the differentiation of antigen-stimulated naïve T cells into Th2 cells, increase the expression of class II MHC molecules in B cells, enhance expression of CD23, induce IgE class switch and upregulate expression of IL4R. (25)</td>
</tr>
<tr>
<td>LIMA1</td>
<td>0.0135</td>
<td>LIM domain and actin binding 1 is a cytoskeleton-associated protein that inhibits actin filament depolymerization. (27)</td>
</tr>
<tr>
<td>IL31</td>
<td>0.0137</td>
<td>IL31 is a cytokine related to the IL6–type cytokines. IL31 is produced mainly by activated Th2 cells and CD8+ T cells. IL31 expression is increased in skin biopsies of patients with eczema and allergic contact dermatitis. Serum IL31 levels is correlated with eczema severity. (25)</td>
</tr>
<tr>
<td>IL3</td>
<td>0.0149</td>
<td>IL3 is expressed by T cells, macrophages, stromal cells, NK cells, mast cells, and eosinophils. Amongst other functions IL3 also increases the activation and release of mediators from eosinophils and basophils in response to IgE FcE cross-linking. (25)</td>
</tr>
<tr>
<td>CD200R1</td>
<td>0.0153</td>
<td>Cell surface glycoprotein CD200 receptor 1 is a receptor for the OX-2 membrane glycoprotein. Chronic infection drives expression of the inhibitory receptor CD200R, and its ligand CD200, by mouse and human CD4 T cells. (36)</td>
</tr>
<tr>
<td>Gene</td>
<td>FDR</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>RGS18</td>
<td>0.0176</td>
<td>Regulator of G-protein signaling 18 is a protein implicated in lymphocyte function by modulating signaling through 7-transmembrane receptors, in chemokine signaling and homing, and in differentiation and transformation.</td>
</tr>
<tr>
<td>FAM115A</td>
<td>0.0237</td>
<td>Family with sequence similarity 115, member A is a protein with unknown function.</td>
</tr>
<tr>
<td>IRF4</td>
<td>0.0243</td>
<td>Interferon regulatory factor 4 regulates a large number of genes relevant to Th cell differentiation. IRF4 is also essential for the developmental program of Th9 cells.</td>
</tr>
<tr>
<td>CEACAM1</td>
<td>0.0277</td>
<td>Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a cell-cell adhesion molecule detected on leukocytes, epithelia and endothelia. Multiple cellular activities including apoptosis, tumor suppression and modulation of innate and adaptive immune responses.</td>
</tr>
<tr>
<td>IL4R</td>
<td>0.0281</td>
<td>There are 2 types of IL4 binding receptors: type I IL4R, which predominates in hematopoietic cells and which is responsible for IL4 signaling in T cells, and type II IL4R, which is expressed on hematopoietic and non-hematopoietic cells but not T cells.</td>
</tr>
<tr>
<td>GCNT4</td>
<td>0.0289</td>
<td>Glucosaminyl (N-acetyl) transferase 4 is an enzyme which mediates core 2 O-glycan branching, important in mucin-type biosynthesis.</td>
</tr>
<tr>
<td>MAL</td>
<td>0.03</td>
<td>Myelin and lymphocyte protein has been localized to the endoplasmic reticulum of T-cells and is a candidate linker protein in T-cell signal transduction. It was found increased in house-dust mite-stimulated Th cells from atopic individuals.</td>
</tr>
<tr>
<td>CCL1</td>
<td>0.0337</td>
<td>Chemokine (C-C motif) ligand 1 is secreted by activated T cells and IgE-activated human and mast cells and binds to the CCR8 receptor. CCR8 is expressed by dendritic cells, monocytes, NK and immature B cells and is associated with Th2 cells. CCR8 is expressed by approximately 70% of Th cells recruited in the asthmatic airways and oral small molecule CCR8 inhibitors are being developed.</td>
</tr>
<tr>
<td>CISH</td>
<td>0.0385</td>
<td>Cytokine-inducible SH2-containing protein controls interleukin-2 signaling; and can be induced by IL2, IL3, GM-CSF and EPO in hematopoietic cells. CISH is increased in HDM-stimulated T cells from atopic individuals and ovalbumin-stimulated PBMC from egg allergic patients.</td>
</tr>
<tr>
<td>IL17RB</td>
<td>0.041</td>
<td>IL17RB binds IL17B and IL17E (IL25). IL17 amplifies delayed-type reactions by increasing chemokine production to recruit monocytes and neutrophils to the site of inflammation.</td>
</tr>
<tr>
<td>MS4A3</td>
<td>0.0486</td>
<td>Membrane-spanning 4-domains subfamily A member 3 likely plays a role in signal transduction in lymphoid cells. MS4A3 was shown to be increased in patients with latex and/or vegetable food allergy.</td>
</tr>
</tbody>
</table>
**Figure legends:**

FIG. 1. Volcano plot of gene expression fold-change of significantly differentially expressed genes in peanut-activated memory Th cells from 3 PA and 2 NA donors. Horizontal axis: Fold-change differential gene expression between PA and NA (positive values indicate higher expression in PA, negative values reflect higher expression in NA). Vertical axis: Statistical significance of differential gene expression between PA and NA, adjusting for gene expression variability within PA and NA groups respectively. Points represent individual genes.

FIG. 2. Heatmap of 48 differentially-expressed genes in skin and gut-homing peanut-activated memory Th cells from PA (n=3) and NA donors (n=3) (≥2 fold changes of expression, p<0.05). Each row represents Th cell gene expression from an individual donor and each column shows a single gene. Upregulated genes are red (’hot’) whereas downregulated genes are blue (’cold’).

FIG. 3. Expression of Th2/Th9 subset-specific genes and other genes known to be involved in allergic responses in skin (CLA+) and gut-homing (B7+) peanut-activated memory Th cells from PA (n=3) and NA (n=3) donors.

FIG. 4. Heatmap of gene expression in skin and gut-homing peanut-activated memory Th cells for genes selected by random forest classifiers. There are 4 possible 2-gene combinations which each classified Th cell responses between 3 PA and 3 NA samples with 100% accuracy: HSPA5 and IL9, IL9 and 8069610, and IL9 and HUWE1.

FIG 5: Differential RT-qPCR gene expression in PBMCs from peanut allergic (PA, n=12), peanut sensitized (PS, n=12) and atopic non allergic (NA, n=6) children comparing target gene relative to the endogenous gene 18s (ΔCT) in peanut stimulated versus unstimulated cultures (ΔΔCT) converted into relative quantitation (RQ) in log transformed arbitrary units (AU).
FIG. 6. Box-plot of FACS sorted memory Th cells and intracellular cytokine staining of IL5 and IL9 expressed in unstimulated versus peanut-stimulated memory Th cells from 5 PA and 5 PS donor (A) and example of FACS analysis of peanut stimulated memory Th cells from the first PA and PS donor (B).
References


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