Altered immune cell profiles and impaired CD4 T cell activation in single and multi-food allergic adolescents

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Short running title: Immunity in single and multi-food allergic adolescents

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ABSTRACT

BACKGROUND: Approximately 5% of adolescents have a food allergy, with peanut and tree nut allergies the most common. Having two or more food allergies in adolescence also doubles the risk of any adverse food reaction, and is associated with increased dietary and social burden. Investigations of immune function in persistently food allergic children are rare.

OBJECTIVE: In the present study, we aimed to investigate the immune mechanisms that underlie food allergy in adolescence.

METHODS: We used high dimensional flow cytometry, unsupervised computational analysis, and functional studies to comprehensively phenotype a range of non antigen-specific immune parameters in a group of well-characterized adolescents with clinically defined single peanut allergy, multi-food allergy and aged-matched non-food allergic controls.
RESULTS: We show that food allergic adolescents have higher circulating proportions of dendritic cells (p=0.0084, FDR-adjusted p=0.087, median in no FA: 0.63% live cells, in FA: 0.93%), and higher frequency of activated, memory-like Tregs relative to non-food allergic adolescents (p=0.011, FDR-adjusted p=0.087, median in no FA: 0.49% live cells, in FA: 0.65%). Cytokine profiling revealed that CD3/CD28 stimulated naïve CD4 T cells from food allergic adolescents produced less IL-6 (p=0.0020, FDR-adjusted p=0.018, median log2 fold change [stimulated/unstimulated] in no FA: 3.03, in FA: 1.92) and TNFα (p=0.0044, FDR-adjusted p=0.020, median in no FA: 9.16, in FA: 8.64) and may secrete less IFNγ (p=0.035, FDR-adjusted p=0.11, median in no FA: 6.29, in FA: 5.67) than naïve CD4 T cells from non-food allergic controls. No differences between clinical groups were observed for LPS stimulated monocyte secretion of cytokines.

CONCLUSIONS: These results have important implications for understanding the evolution of the immune response in food allergy throughout childhood, revealing that dendritic cell and T cell signatures previously identified in early life may persist through to adolescence.

INTRODUCTION

IgE-mediated food allergies are a global concern. Despite a significant disease burden, there are limited treatment options and management relies on allergen avoidance, which can be difficult to achieve and may result in severe reactions upon accidental ingestion. This is particularly true for the adolescent age group, who are at the highest risk of anaphylaxis following allergen exposure.1,2

Despite this, studies investigating the prevalence of food allergy in the adolescent age group are rare. Recent work suggests that between 2-5% of school aged children have food allergy, with peanut and tree nut being the most common.3,4 Interestingly, about half of food allergic 10-14 year old adolescents were shown to have experienced recent adverse reactions to foods and having two or more food allergies doubled the risk of an adverse reaction.5 The immune responses that underly the unique risks associated with food allergy in adolescence, as well as the contribution of the immune system in the persistence of single or multi food allergies, have been scarcely investigated.

In the present study, we used high dimensional flow cytometry and functional studies to comprehensively phenotype a range of immune parameters in a group of well-characterized
adolescents with clinically defined peanut allergy, multi-food allergy and aged-matched non-food allergic controls.

MATERIALS AND METHODS

Subjects and study design

Peripheral blood mononuclear cell (PBMC) samples from 59 subjects in the SchoolNuts cohort, Melbourne, Australia were used in this study (n=19 non-food allergic (no FA), n=20 single peanut allergic (peanut only), n=20 multi-food allergic (multi-FA)). The SchoolNuts study was a questionnaire survey among 10- to 14-year-old adolescents and their parents, followed by clinic evaluation including oral food challenge when food allergy was suspected from questionnaire response. Figure 1 describes the experimental workflow. Clinic-defined food allergy was defined as a positive oral food challenge (OFC) or convincing recent or severe history in the context of IgE sensitisation (skin prick test (SPT) wheal size of ≥3mm or sIgE ≥ 0.35KuA/L), as described for the cohort previously. Single peanut allergic children had clinic-defined peanut allergy with no evidence of sensitisation or a negative OFC to all other foods. Multi-food allergic children had clinic-defined peanut allergy with clinic-defined allergy to at least one other food (n=16 had peanut allergy plus ≥ one tree nut allergy, n=4 had peanut allergy plus egg allergy, Table 1). Non-food allergic children had no evidence of sensitisation (<3mm) to a panel of 15 food allergens by SPT (egg white, cow’s milk, soy, peanut, cashew, almond, hazelnut, walnut, pistachio, macadamia, pecan, brazil nut, pine nut, sesame, shellfish). OFCs were performed as described previously and serum-specific IgE was measured using the ImmunoCAP System FEIA (Phadia AB). 13 out of the 40 food allergic participants in this study had bloods taken following a peanut or tree nut OFC, the remaining 27 food allergic participants had bloods taken on a non OFC day. Where bloods were taken on an OFC day, the sample was collected within 1h of completing the OFC. We have previously reported no differences in cellular activation or plasma cytokine production in food allergic infants who had a blood sample taken on a non-OFC day versus an active OFC day.

Preparation of cells for fluorescence activated cell sorting (FACS)

Blood was collected at clinic appointments and PBMCs were isolated by density gradient and cryopreserved in liquid nitrogen as previously described. PBMCs were thawed in 10mL thaw media (complete RPMI supplemented with 10% heat-inactivated FCS with 25U/mL benzonase at 37°C). PBMCs were centrifuged at 300 x g for 10 minutes and washed in media.
prior to a viability count. Mean viability, as determined by trypan blue exclusion, was 93%.

Following cell count, PBMCs were washed in PBS at 300 x g for 10 minutes and
resuspended in PBS at 1x10^6/mL. Fixable viability stain 510 (BD Biosciences) was added at
0.5µl per mL of cell suspension. Cells were incubated at room temperature for 15 minutes
protected from light, washed in FACS buffer (2% FCS, 2mM EDTA in PBS) and
resuspended in 50µl FACS buffer containing human FC-block for five minutes. The antibody
cocktail (Table S1) made up at 2X was added 1:1 to the resuspended cells and incubated on
ice for 30 minutes. Cells were washed and resuspended in 300µl FACS buffer for cell sorting.
Monocytes and naïve CD4 T cells were sorted using a BD FACS-ARIA Fusion according to
the gating strategy outlined in Figure S1. One million events per sample were recorded for
immune phenotyping analysis, and an average of 3x10^5 and 6.7x10^5 cells were sorted for
monocytes and naïve CD4 T cells, respectively. Compensation was performed at the time of
sample acquisition using compensation beads (BD Biosciences).

**Stimulation of purified monocytes and naïve CD4 T cells**

Monocytes were resuspended at 1x10^5/100µl in cell culture media (complete RPMI
supplemented with 10% FCS and penicillin streptomycin) and seeded at 1x10^5 per well in 96-
well round bottom culture plates. Monocytes were cultured with an additional 100µl of cell
culture media (unstimulated) or an additional 100µl of cell culture media containing 20ng/mL
LPS (stimulated — final 10ng/mL) (LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich) for
24h at 37°C, 5% CO_2_. Cell culture supernatants were harvested and frozen at -80°C for later
quantification of inflammatory cytokines. Naïve CD4 T cells were resuspended at
8x10^4/200µl in T cell activation media (complete RPMI supplemented with 10% FCS,
penicillin streptomycin and 200IU/mL of IL2) and seeded at 8x10^4/well in 96-well round
bottom culture plates. At least two wells per sample were left unstimulated, and at least two
wells were stimulated with anti-CD3/CD28 T cell activator DynaBeads for 72h at 37°C, 5%
CO_2_. Following the 72h incubation, Dynbeads were removed from the cell culture
supernatant using a magnet and supernatants were stored at -80°C for later quantification of
cytokines. Cell counts were performed following naïve CD4 T cell culture for assessment of
viability (by trypan blue exclusion) and proliferative capacity following activation.

**Quantification of cytokines in monocyte and naïve CD4 T cell culture supernatants**

Supernatants from both monocyte and naïve CD4 T cell cultures were thawed and cytokines
were assessed using the Human Soluble Protein Flex Set Cytometric Bead Array (BD
Biosciences) according to the manufacturer’s instructions. Cytometric bead array data were acquired on an LSR II X-20 Fortessa and analysed using the FCAP Array Software. Both sample types were assessed for the following 18 cytokines: IL-1α, IL-1β, IL-6, IL-8, IL-10, MCP-1, MIP-1α, RANTES, TNFα, IFNγ, IL-2, IL-4, IL-13, IL-5, IL-9, IL-12p70, IL-17A, MIG. Cytokine levels below the detection range were arbitrarily reported as half the lower limit of detection for each cytokine and included in the analysis. There were six cytokines (IL-4, IL-5, IL-9, IL-12p70, IL-17A and MIG) that were undetectable in all samples and were excluded from further analysis.

Flow cytometry data analysis

Flow cytometry files underwent standard pre-processing to remove debris, doublets and to select for live cells. Live single cells were analysed by manual gating and unsupervised computational methods in parallel. Manual gating strategies are outlined in Figure S1. Unsupervised computational analysis was performed using 3x10^5 randomly selected cells within the pre-gated live single cell population per file. Values were arcsinh transformed with a co-factor of 150 as previously described for flow cytometry data. Unsupervised clustering was performed on the expression values of 13 markers (CD3, CD4, CD8, CD19, CD56, CD14, CD45RA, CCR7, CD25, CD127, CD16, CD11c, HLA-DR) using the FlowSOM algorithm (R Package FlowSOM version 1.18.0) with a predetermined number of 25 clusters in the meta-clustering step. The marker expression levels per cluster were visualized in a heatmap using the approach as previously described. Briefly, for each marker, the expression levels for all cells were scaled to values between 0 and 1 using the 1st and 99th percentile as boundaries. Using these scaled values, the median levels of the 13 markers for the 25 meta-clusters were visualized in a heatmap (R package pheatmap, version 1.0.12). Based on expression patterns, clusters were manually annotated and merged if two clusters represented the same cell type. Clusters with < 0.5% of all cells were excluded from further analysis. The final set of FlowSOM clusters were depicted in a heatmap as described above, along with the frequency (% of live cells) of each cluster. Furthermore, to visualize this high-dimensional data in two dimensions, the dimensionality reduction approach Uniform Manifold Approximation and Projection (UMAP) was applied to 9992 randomly selected cells (1724 per file) using the R package UMAP (version 0.2.5.0).

Frequencies of each cluster were compared between no FA controls and food allergy groups using Wilcoxon rank sum tests. The p values were adjusted for multiple comparisons using
the Benjamini and Hochberg approach to control the false discovery rate (FDR). FDR-
adjusted \( p < 0.1 \) were considered significant. To perform the differential abundance analysis
for all three groups, the Kruskal-Wallis rank sum test was used (2 degrees of freedom [df]),
and \( p \) values were FDR-adjusted. Only for clusters showing a significant (FDR-adjusted \( p < 
0.1 \)) difference between the three groups, subsequent Wilcoxon rank sum tests were to be
performed to compare all sets of two groups. However, in order to look further into the
FlowSOM clusters that were significantly different in the comparison of the no FA with FA
participants, for these clusters Wilcoxon rank sum tests were performed between all sets of
two groups among the three groups, even though the FDR-adjusted \( p \) from the Kruskal-
Wallis rank sum tests were \( \geq 0.1 \). Cell types that were identified by the unsupervised analysis
as being significantly different were validated by manual gating.

**Analysis of cytokines in cell culture supernatant**

For the naïve CD4 T cell cytokine data, fold changes between the stimulated and
unstimulated cytokine concentrations were compared between no FA and all FA using the
Wilcoxon rank sum test and among the three groups using the Kruskal-Wallis rank sum test
(with 2 df) with subsequent Wilcoxon rank sum tests between two groups for cytokines with
significant results. For the monocyte data, the cytokine concentrations after stimulation were
compared between no FA and FA using the Wilcoxon rank sum tests and the Kruskal-Wallis
rank sum test (with 2 df) to compare all three groups. All \( p \) values were FDR-adjusted and
FDR-adjusted \( p < 0.1 \) were considered significant.

**Statistical analysis**

All statistical analysis was performed in R (version 3.6.2) and statistical tests were performed
two-sided. Plotting of data was done using the ggplot2 R package (version 3.3.0) unless
otherwise stated. Boxplots show the medians, the 1\(^{st}\) and 3\(^{rd}\) quartile as well as the smallest
and largest values after exclusion of outliers (greater than the 3\(^{rd}\) quartile plus 1.5 times the
interquartile range (IQR), or less than the 1\(^{st}\) quantile minus 1.5 times the IQR) as whiskers.
Individual data points are shown.

**Ethics**

Ethics approval to conduct the SchoolNuts study was obtained from the Royal Children’s
Hospital Research Ethics Committee (no. 31079), the Department of Education and Early

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Childhood, and the Catholic Education Office. Written and informed consent was obtained from the parents of participants in this study.

RESULTS

Study participants

A subset of 59 adolescents from the SchoolNuts cohort were used in this study. Table 1 describes the demographics and clinical characteristics of the participants included in this study. 57% of the non-food allergic (no FA), 60% of the peanut-only allergic (peanut only), and 50% of the multi-food allergic (multi-FA) participants were male. The median age in each of the three groups was 13 years. We compared the no FA controls with the total food allergy (FA) group, which included both the peanut only and the multi-FA participants, as well as the three groups with each other.

Cell populations identified by unsupervised clustering

The 25 meta-clusters that were identified by unsupervised clustering using FlowSOM were manually annotated and small clusters representing < 0.5% of analysed cells excluded (Figure S2), leaving 16 clusters for the final analyses (Figure 2a). Based on marker expression, the clusters were classified as naïve CD4 T cells (25.5% of live cells), central memory CD4 T cells (10.3%), naïve CD8 T cells (13.6%), central memory CD8 T cells (1.3%), effector memory CD8 T cells (4.3%), naïve regulatory T cells (Tregs) (1.4%), activated Tregs (0.6%), B cells (13.5%), classical monocytes (8.9%), non-classical monocytes (1.1%), dendritic cells (DCs) (0.9%), CD56+ CD16+ NK cells (7.3%), CD56bright CD16- NK cells (0.7%) (Figure 2a). Three clusters could not be confidently annotated and were recorded as undefined cluster 1 and 2, and unspecified CD3+ T cells. The frequencies of the major cell types identified in this analysis were comparable to those obtained by manual gating (Figure S3). The frequency of each cluster for each participant is shown in Figure 2b. To further visualise these data, the non-linear dimensionality reduction technique UMAP was applied to a randomly selected subset of cells as shown in Figure 2c. The cells were colour highlighted by their respective FlowSOM cluster. CD4 T cell (naïve, memory, Tregs), CD8 T cell (naïve, effector, memory), B cell, NK cell and myeloid cell populations (DCs, monocytes) form distinct clusters that separate within the two-dimensional space.

Altered circulating immune cell profiles in food allergic adolescents
We next compared the frequencies of circulating immune cell populations between food allergic and non-food allergic adolescents. Food allergic adolescents have higher proportions of conventional DCs (CD3-CD19-CD56-HLADR+CD11c+CD14-) (p=0.0084, FDR-adjusted p=0.087, median in no FA: 0.63% live cells, in FA: 0.93%, Wilcoxon rank sum test) and activated (CD3+CD127lowCD25+CD45RA-CCR7-) Tregs (p=0.011, FDR-adjusted p=0.087, median in no FA: 0.49% live cells, in FA: 0.65%, Wilcoxon rank sum test) relative to non-food allergic adolescents. When investigating these subsets across the 3 participant groups, significantly higher proportions of DCs were seen in both the peanut only (median 0.94% live cells) and multi-FA (median 0.88%) groups relative to no-FA controls (p=0.050 and p=0.0099, Wilcoxon rank sum test, respectively) (Figure 2e, Figure S4). Significantly higher activated Treg cell proportions were observed between the peanut only (median 0.67% live cells) and no-FA controls (p=0.0026, Wilcoxon rank sum test), but not between the multi-FA (median 0.63%) group relative to non-food allergic adolescents (p=0.17, Wilcoxon rank sum test). These trends were also confirmed by manual gating analysis (Figure S5 and S6). The frequencies of cell clusters that were not significantly different between the groups are presented in Figure S7.

Lower naïve CD4 T cell activation in single and multi-food allergic adolescents

Our previous studies involving the HealthNuts cohort have revealed key differences in the response capacity of both naïve CD4 T cells and monocytes in food allergic infants relative to non-allergic controls. In the current study, proliferation across all three groups was similar – an average of 3.8-fold increase in CD4 T cell count (with average of 95% viability) was observed following stimulation. We were able to detect nine cytokines in the cell culture supernatants of anti-CD3/CD28 stimulated naïve CD4 T cells. These were IL-2, IL-6, TNFα, IL-10, MIP-1α, IFNγ, RANTES, IL-13 and IL-8 as depicted in the heatmap in Figure 3a. Naïve CD4 T cells from food allergic adolescents showed lower median IL-6 (p=0.0020, FDR-adjusted p=0.018, Wilcoxon rank sum test) and TNFα (p=0.0044, FDR-adjusted p=0.020, Wilcoxon rank sum test) responses compared to the non-food allergic controls (Figure 3b). Median IFNγ responses also appeared to be lower in the food allergic group, however this did not remain significant after correction for multiple comparisons (p=0.035, FDR-adjusted p=0.11, Wilcoxon rank sum test) (Figure 3b). When investigating naïve CD4 T cell cytokine responses in our three groups, both the single and multi-FA groups showed significantly lower median levels of both IL-6 and TNFα compared to controls (Figure 3c). The IL-6 response was significantly lower in peanut only (median log2 fold change...
[stimulated/unstimulated]: 2.02) relative to no FA (median log2 fold change: 3.03, p=0.037, Wilcoxon rank sum test), as well as multi-FA (median log2 fold change: 1.82) relative to no FA (p=0.0016, Wilcoxon rank sum test). TNFα was significantly lower in peanut only (median log2 fold change: 8.74) relative to no FA (median log2 fold change: 9.16, p=0.027, Wilcoxon rank sum test), as well multi-FA (median log2 fold change: 8.54) relative to no FA (p=0.0087, Wilcoxon rank sum test). Almost all naïve CD4 T cell samples showed no IL-6 or TNFα expression without stimulation (Supplemental Figure S8). Consequently, as seen for the fold change responses, IL-6 and TNFα concentrations in naïve CD4 T cells after stimulation were also significantly lower in FA participants (median IL-6: 3.1 pg/ml, median TNFα: 253.9 pg/ml) relative to no FA controls (IL-6: median: 8.3 pg/ml, p=0.00022; TNFα: median: 345.5 pg/ml, p=0.0073, Wilcoxon rank sum test). When investigating our three groups, IL-6 and TNFα concentrations in naïve CD4 T cells after stimulation were significantly lower in peanut only (median IL-6: 3.9 pg/ml, median TNFα: 256.5 pg/ml) relative to no FA (IL-6: p=0.028; TNFα: p=0.027, Wilcoxon rank sum test), as well as multi-FA (median IL-6: 2.8 pg/ml, median TNFα: 244.3 pg/ml) relative to no FA (p<0.0001, p=0.019, respectively, Wilcoxon rank sum test, Supplemental Figure S8). All other naïve CD4 T cell cytokine responses are presented in Figure S9, revealing no significant difference in IL-10, IL-13, IL-2, IL-8, MIP-1α and RANTES between groups. In LPS-stimulated monocyte cultures, we detected IL-10, RANTES, IL-1α, MCP-1, IL-6, IL-8, TNFα, IL-1β and MIP-1α as depicted in the heatmap in Figure 4a. However, there were no statistically significant differences in these cytokines between groups (Figure 4b, Supplemental Figure S10).

For the key observations of increased DCs and activated Tregs, as well as reduced CD4 T cell production of IL-6 and TNFα, no significant difference was detected between samples that were taken from a food allergic participant following OFC or on a non-OFC day (Figure S11).

**DISCUSSION.**

This study reveals circulating immune cell signatures and functional responses associated with single- and multi-food allergy in adolescence. Using unsupervised analysis of multi-parameter flow cytometry data, we show that both single and multi-FA adolescents have higher circulating proportions of dendritic cells, and that single peanut allergic adolescents have significantly higher frequency of activated, memory-like Tregs relative to non-food...
allergic adolescents. Naïve CD4 T cells from both single and multi-FA adolescents were hypo-responsive to stimulation, producing less IL-6 and TNFα following T cell receptor engagement relative to healthy controls. Monocytes from both food allergic and non-food allergic adolescents responded to endotoxin stimulation with rapid inflammatory cytokine production, however no statistically significant difference in monocyte responsiveness was observed between the clinical groups.

Previous studies have revealed alterations in T cell function in food allergy. Most work indicates that multi-functional Th2 cells are strongly associated with food allergic immune responses, and that elimination of pathogenic Th2 cells is indicative of clinical responses to oral allergen immunotherapy. Deficits in T cell function have also been observed at birth and in the first year of life, with two studies reporting a marked deficit in naïve CD4 T cell proliferative capacity following in vitro T cell receptor activation in egg allergic infants. Here, we show that naïve CD4 T cells from food allergic and healthy adolescents had similar proliferative capacity following anti-CD3/CD28 T cell receptor activation, however naïve CD4 T cells from food allergic adolescents showed marked reductions in the capacity to produce cytokines including IL-6, TNFα and IFNγ. This demonstrates that whilst the lympho-proliferative deficit observed in early life in food allergic infants may be restored by adolescence, dysfunction of multipotent precursor naïve CD4 T cells may continue to drive a food allergy phenotype in later life.

We also show that food allergic adolescents have increased circulating levels of activated Tregs, with the most significant increase observed in the single peanut allergic participants. Previous studies investigating Treg proportions in food allergy have focused on food allergic infants or young children and have generated conflicting results, showing decreases in circulating Tregs in food allergic children relative to healthy controls or no change at all. Vitamin D insufficiency, a proposed risk factor for the development of food allergy, has also been associated with a deficit in circulating Tregs in food allergic infants. Additional functional studies of Tregs in allergic children undergoing peanut oral immunotherapy showed that the development of immune tolerance was associated with hypomethylation of FOXP3 CpG sites in antigen-induced Treg populations. Another study showed impaired Treg cell regeneration following in vivo allergen exposure in food allergic infants. Whether the differences in activated Treg cell proportions observed in our study are associated with functional changes warrants further investigation.
Work from our group and others has revealed early life innate immune hyper-responsiveness as a unique signature in the development of food allergy. Elevated production of inflammatory cytokines in the cord blood of children who subsequently develop food allergy has been shown to correlate with the propensity for Th2 responses at birth and during the first year of life. A skew towards higher myeloid cells in the circulation, and a hyper-activated inflammatory response in monocytes following *in vitro* stimulation, has also been described in egg and peanut allergic infants. These features are remarkably similar to those that define trained immunity – whereby innate immune cells retain a memory of earlier microbial encounters and elicit an enhanced response upon secondary exposure. Innate immune responses in food allergic adolescents have not been previously described. In this study, we show that both single peanut and multi-food allergic adolescents have increased circulating levels of dendritic cells relative to non-food allergic controls, a signature observed previously in egg allergic 12 month old infants. Monocytes from food allergic adolescents also tended to produce more inflammatory cytokines following endotoxin stimulation relative to non-food allergic controls, however this response did not reach significance following multiple comparison correction. Collectively, these findings suggest that some aspects of innate immune dysfunction observed in early life (increased proportions of circulating dendritic cells) persist into adolescence in food allergic individuals.

Immune and epidemiological studies on food allergy in adolescence are limited. Peanut and tree nuts are the most common food allergies in this age group. In our SchoolNuts cohort, having a nut allergy was associated with a three-fold increased risk of a severe adverse food reaction consistent with anaphylaxis when compared to those without nut allergy. Additionally, having more than two food allergies doubled the risk of any adverse food reaction compared to those with a single food allergy. This supported the need for us to explore immune responses in both single peanut allergic and multi-food allergic adolescents. All of the multi-food allergic participants included in this study had peanut allergy plus at least one other food allergy, with 80% having co-existent peanut and tree nut allergies. Whilst we did not observe significant differences between the multi and single food allergic individuals, a synergetic effect of multi-FA was observed on several key immune signatures. Similar results have been observed in a study comparing gene expression levels in children with allergic diseases (asthma, atopic dermatitis, rhinitis) when considered as a single disease or multimorbidity (at least two diseases). A unique signature of eight genes identified multimorbidity, characterised by enrichment of eosinophil and innate immune associated...

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signalling pathways. A limitation of our work is that we did not assess allergen-specific immune responses, which have been reported previously by us and others, particularly in the context of peanut allergy \textsuperscript{10,32-34}.

A key strength of our study is the systems level approach, utilising a comprehensive immune phenotyping and functional protocol from a single peripheral blood sample in association with advanced unsupervised analytical tools, to understand food allergy in both a high risk and understudied age group. Whilst comprehensive for the well characterized lymphoid and myeloid markers, a limitation of our immune phenotyping panel is that it does not include surface markers for innate-like and unconventional T cell populations, or subsets of immunoglobulin switched/non-switched B cells, which may be relevant in the food allergic immune response \textsuperscript{35,36}. Another limitation in our study is our sample size, particularly given the immune heterogeneity observed within the food allergic groups. This highlights that there are multifactorial pathways likely contributing to differences in food allergy phenotypes that will need to be unraveled in larger studies.

This study provides a detailed characterization of the functional immune response in single and multi-food allergic adolescents relative to aged-matched non-food allergic controls. These results have important implications for understanding the evolution of the immune response in food allergy throughout childhood, revealing that myeloid DC and T cell signatures previously identified in early life are also associated with altered immune responses in adolescence.

References


**Table 1.** Demographics and clinical characteristics of study cohort

<table>
<thead>
<tr>
<th></th>
<th>Non-food</th>
<th>Peanut-only</th>
<th>Multi-food</th>
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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.
<table>
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<th></th>
<th>allergic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>allergic&lt;sup&gt;b&lt;/sup&gt;</th>
<th>allergic&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>Total number</td>
<td>19</td>
<td>20</td>
<td>20</td>
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<tr>
<td>Sex: male, n (%)</td>
<td>11 (57%)</td>
<td>12 (60%)</td>
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<td>Age at blood collection (years), median (min-max)</td>
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<td>13 (11-16)</td>
<td>13 (11-14)</td>
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<td>6 (30%)</td>
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<td>15 (75%)</td>
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<td>10 (3-30)</td>
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<td>3.9 (0.37-101)</td>
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<td>Peanut Allergy + ≥ one Tree Nut allergy</td>
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<td>0 (0%)</td>
<td>16 (80%)</td>
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<td>Peanut Allergy + egg allergy</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4 (20%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Non-food allergic defined as no evidence of sensitisation (<3mm) to a panel of 15 food allergens by SPT (egg white, cow’s milk, soy, peanut, cashew, almond, hazelnut, walnut, pistachio, macadamia, pecan, brazil nut, pine nut, sesame, shellfish).

<sup>b</sup><sup>-</sup><sup>c</sup>. Current clinic-defined food allergy was defined as a positive OFC or convincing recent or severe history in the context of IgE sensitization (SPT wheal size of ≥ 3 mm or sIgE ≥ 0.35), or highly sensitized (SPT wheal size of ≥ 8 mm). <sup>b</sup> Current clinic-defined peanut allergy with no evidence of sensitisation or negative OFC to all other foods. <sup>c</sup> Current clinic-defined peanut allergy with current-clinic defined allergy to at least one other food.

<sup>*</sup> Parent or sibling history of food allergy

<sup>†</sup> Doctor diagnosed asthma requiring medication in the last 12 months

ND: data not available
Figure Legends

Figure 1. Experimental workflow for multiplex flow cytometry analysis and cell culture stimulations of PBMCs. Cryopreserved PBMCs from single peanut allergic (peanut only; n=20), multi food allergic (multi FA; n=20) and non-food allergic controls (no FA; n=19) were thawed for 14-parameter flow cytometry analysis and cell sorting. Monocytes and naïve CD4 T cells (nCD4 T cells) were sorted for in vitro culturing. Monocytes underwent a 24h stimulation with LPS (10ng/mL) or media alone, naïve CD4 T cells underwent a 72h stimulation with antiCD3/CD28 activator beads or media alone. Cell culture supernatants were collected at the end of culturing and assessed for 18 cytokines using a custom multiplex bead array. For data analysis, unsupervised (clustering and visualization) and manual gating analyses were performed in parallel, along with statistical analyses to identify immune signatures that differed between the clinical groups.

Figure 2. Unsupervised immune cell profiling of PBMCs from non-food allergic, single peanut allergic and multi food allergic adolescents. (a) Heatmap depicting the median expression of 13 surface markers of 16 cell clusters identified using FlowSOM. Bars represent each cluster as proportion of live cells along with their annotated cell phenotype. (b) Stacked bar graph representing all clusters identified in each participant stratified by study group: no food allergy (no FA) (n=19), single peanut allergic (peanut only) (n=20) and multi food allergic (multi FA) (n=19). (c) Uniform Manifold Approximation and Projection (UMAP) representation of 99,992 randomly selected cells (1724 per file) with clusters from the FlowSOM analysis overlaid. (d) Proportions of cell types (DCs and activated Tregs) identified as significantly different between the non-FA and all food allergy groups (FDR-adjusted p<0.1, p-values by Wilcoxon rank sum test). (e) Proportions of DCs and activated Tregs stratified by the three study groups. P values by Kruskal-Wallis rank sum test (three groups) and Wilcoxon rank sum test (two groups). In the boxplots, the medians are shown. The “hinges” represent the first and third quartile. The whiskers are the smallest and largest values after exclusion of outliers (greater than the 75th percentile plus 1.5 times the IQR, or less than 25th percentile minus 1.5 times the IQR).

Figure 3. CD4 T cell cytokine responses following anti-CD3/CD28 stimulation in food allergic adolescents. (a) Heatmap representing log2 fold change of cytokines in naïve CD4 T cell culture supernatants following 72h of anti-CD3/CD28 stimulation. IL-2, IL-6, TNFα, IL-
10, MIP-1α, IFNγ, RANTES, IL-13 and IL-8 were detectable. (b) Fold-change of IL-6, TNFα and IFNγ in non-food allergic (n=18) and food allergic (n=36) adolescents. (c) Fold-change of IL-6, TNFα and IFNγ in non-food allergic (n=18), single peanut allergic (n=18) and multi food allergic (n=18) adolescents. P values by Kruskal-Wallis rank sum test (three groups) and Wilcoxon rank sum test (two groups compared). In the boxplots, the medians are shown. The “hinges” represent the first and third quartile. The whiskers are the smallest and largest values after exclusion of outliers (greater than the 75th percentile plus 1.5 times the IQR, or less than 25th percentile minus 1.5 times the IQR).

Figure 4. Monocyte cytokine responses following endotoxin exposure in food allergic adolescents. (a) Heatmap representing log10 cytokine concentration of nine detectable cytokines from monocyte cell culture supernatants following 24h of LPS stimulation in each individual. (b) Concentration of IL-1α, IL-1β, TNFα, IL-8 and IL-6 in monocyte cell culture supernatants following stimulation in food allergic (n=26) and non-food allergic (n=17) adolescents. P values by Wilcoxon rank sum test. In the boxplots, the medians are shown. The “hinges” represent the first and third quartile. The whiskers are the smallest and largest values after exclusion of outliers (greater than the 75th percentile plus 1.5 times the IQR, or less than 25th percentile minus 1.5 times the IQR).
Figure 2

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Participants log10 cytokine concentration after stimulation

- IL-10
- RANTES
- IL-1α
- MCP-1
- IL-6
- IL-8
- TNFα
- IL-1β
- MIP-1α

**Figure 4**

(a) Heatmap showing the log10 cytokine concentration after stimulation for different groups.

(b) Box plots for cytokines IL-1β, IL-1α, IL-6, IL-8, and TNFα, comparing no FA and FA groups.

- IL-1β: p = 0.28 (FDR-adjusted p = 0.36)
- IL-1α: p = 0.14 (FDR-adjusted p = 0.32)
- IL-6: p = 0.15 (FDR-adjusted p = 0.32)
- IL-8: p = 0.18 (FDR-adjusted p = 0.32)
- TNFα: p = 0.16 (FDR-adjusted p = 0.32)
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