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

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23

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

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39 authors edited and approved the final manuscript.

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41 **ABSTRACT**

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

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

49 METHODS: We used high dimensional flow cytometry, unsupervised computational
50 analysis, and functional studies to comprehensively phenotype a range of non antigen-
51 specific immune parameters in a group of well-characterized adolescents with clinically
52 defined single peanut allergy, multi-food allergy and aged-matched non-food allergic
53 controls.

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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/unstimualed] 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

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85 adolescents with clinically defined peanut allergy, multi-food allergy and aged-matched non-
86 food allergic controls.

87 MATERIALS AND METHODS

88 Subjects and study design

89 Peripheral blood mononuclear cell (PBMC) samples from 59 subjects in the SchoolNuts
90 cohort, Melbourne, Australia [4] were used in this study (n=19 non-food allergic (no FA), n=20
91 single peanut allergic (peanut only), n=20 multi-food allergic (multi-FA)). The SchoolNuts
92 study was a questionnaire survey among 10- to 14-year-old adolescents and their parents,
93 followed by clinic evaluation including oral food challenge when food allergy was suspected
94 from questionnaire response [6]. Figure 1 describes the experimental workflow. Clinic-defined
95 food allergy was defined as a positive oral food challenge (OFC) or convincing recent or
96 severe history in the context of IgE sensitisation (skin prick test (SPT) wheal size of ≥ 3mm
97 or sIgE ≥ 0.35KuA/L), as described for the cohort previously [4]. Single peanut allergic
98 children had clinic-defined peanut allergy with no evidence of sensitisation or a negative
99 OFC to all other foods. Multi-food allergic children had clinic-defined peanut allergy with
100 clinic-defined allergy to at least one other food (n=16 had peanut allergy plus ≥ one tree nut
101 allergy, n=4 had peanut allergy plus egg allergy, Table 1). Non-food allergic children had no
102 evidence of sensitisation (< 3mm) to a panel of 15 food allergens by SPT (egg white, cow's
103 milk, soy, peanut, cashew, almond, hazelnut, walnut, pistachio, macadamia, pecan, brazil nut,
104 pine nut, sesame, shellfish). OFCs were performed as described previously [7] and serum-
105 specific IgE was measured using the ImmunoCAP System FEIA (Phadia AB). 13 out of the
106 40 food allergic participants in this study had bloods taken following a peanut or treenut
107 OFC, the remaining 27 food allergic participants had bloods taken on a non OFC day. Where
108 bloods were taken on an OFC day, the sample was collected within 1h of completing the
109 OFC. We have previously reported no differences in cellular activation or plasma cytokine
110 production in food allergic infants who had a blood sample taken on a non-OFC day versus
111 an active OFC day [8,9].

112 Preparation of cells for fluorescence activated cell sorting (FACS)

113 Blood was collected at clinic appointments and PBMCs were isolated by density gradient and
114 cryopreserved in liquid nitrogen as previously described [10]. PBMCs were thawed in 10mL
115 thaw media (complete RPMI supplemented with 10% heat-inactivated FCS with 25U/mL
116 benzonase at 37°C). PBMCs were centrifuged at 300 x g for 10 minutes and washed in media

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117 prior to a viability count. Mean viability, as determined by trypan blue exclusion, was 93%.
118 Following cell count, PBMCs were washed in PBS at 300 x g for 10 minutes and
119 resuspended in PBS at 1x10^6/mL. Fixable viability stain 510 (BD Biosiences) was added at
120 0.5µl per mL of cell suspension. Cells were incubated at room temperature for 15 minutes
121 protected from light, washed in FACS buffer (2% FCS, 2mM EDTA in PBS) and
122 resuspended in 50µl FACS buffer containing human FC-block for five minutes. The antibody
123 cocktail (Table S1) made up at 2X was added 1:1 to the resuspended cells and incubated on
124 ice for 30 minutes. Cells were washed and resuspended in 300µl FACS buffer for cell sorting.
125 Monocytes and naïve CD4 T cells were sorted using a BD FACS-ARIA Fusion according to
126 the gating strategy outlined in Figure S1. One million events per sample were recorded for
127 immune phenotyping analysis, and an average of 3x10^5 and 6.7x10^5 cells were sorted for
128 monocytes and naïve CD4 T cells, respectively. Compensation was performed at the time of
129 sample acquisition using compensation beads (BD Biosciences).

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

131 Monocytes were resuspended at 1x10^5/100µl in cell culture media (complete RPMI
132 supplemented with 10% FCS and penicillin streptomycin) and seeded at 1x10^5 per well in 96-
133 well round bottom culture plates. Monocytes were cultured with an additional 100µl of cell
134 culture media (unstimulated) or an additional 100µl of cell culture media containing 20ng/mL
135 LPS (stimulated – final 10ng/mL) (LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich) for
136 24h at 37°C, 5% CO_2. Cell culture supernatants were harvested and frozen at -80°C for later
137 quantification of inflammatory cytokines. Naïve CD4 T cells were resuspended at
138 8x10^4/200µl in T cell activation media (complete RPMI supplemented with 10% FCS,
139 penicillin streptomycin and 200IU/mL of IL2) and seeded at 8x10^4/well in 96-well round
140 bottom culture plates. At least two wells per sample were left unstimulated, and at least two
141 wells were stimulated with anti-CD3/CD28 T cell activator DynaBeads for 72h at 37°C, 5%
142 CO_2. Following the 72h incubation, Dynbeads were removed from the cell culture
143 supernatant using a magnet and supernatants were stored at -80°C for later quantification of
144 cytokines. Cell counts were performed following naïve CD4 T cell culture for assessment of
145 viability (by trypan blue exclusion) and proliferative capacity following activation.

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

147 Supernatants from both monocyte and naïve CD4 T cell cultures were thawed and cytokines
148 were assessed using the Human Soluble Protein Flex Set Cytometric Bead Array (BD

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149 Biosciences) according to the manufacturer's instructions. Cytometric bead array data were
150 acquired on an LSR II X-20 Fortessa and analysed using the FCAP Array Software. Both
151 sample types were assessed for the following 18 cytokines: IL-1α, IL-1β, IL-6, IL-8, IL-10,
152 MCP-1, MIP-1α, RANTES, TNFα, IFNγ, IL-2, IL-4, IL-13, IL-5, IL-9, IL-12p70, IL-17A,
153 MIG. Cytokine levels below the detection range were arbitrarily reported as half the lower
154 limit of detection for each cytokine and included in the analysis. There were six cytokines
155 (IL-4, IL-5, IL-9, IL-12p70, IL-17A and MIG) that were undetectable in all samples and were
156 excluded from further analysis.

157 **Flow cytometry data analysis**

158 Flow cytometry files underwent standard pre-processing to remove debris, doublets and to
159 select for live cells. Live single cells were analysed by manual gating and unsupervised
160 computational methods in parallel. Manual gating strategies are outlined in Figure S1.
161 Unsupervised computational analysis was performed using 3×10^5 randomly selected cells
162 within the pre-gated live single cell population per file. Values were arcsinh transformed with
163 a co-factor of 150 as previously described for flow cytometry data [11]. Unsupervised clustering
164 was performed on the expression values of 13 markers (CD3, CD4, CD8, CD19, CD56,
165 CD14, CD45RA, CCR7, CD25, CD127, CD16, CD11c, HLA-DR) using the FlowSOM
166 algorithm [12] (R Package FlowSOM version 1.18.0) with a predetermined number of 25
167 clusters in the meta-clustering step. The marker expression levels per cluster were visualized
168 in a heatmap using the approach as previously described [13]. Briefly, for each marker, the
169 expression levels for all cells were scaled to values between 0 and 1 using the 1st and 99th
170 percentile as boundaries. Using these scaled values, the median levels of the 13 markers for
171 the 25 meta-clusters were visualized in a heatmap (R package pheatmap, version 1.0.12).
172 Based on expression patterns, clusters were manually annotated and merged if two clusters
173 represented the same cell type. Clusters with < 0.5% of all cells were excluded from further
174 analysis. The final set of FlowSOM clusters were depicted in a heatmap as described above,
175 along with the frequency (% of live cells) of each cluster. Furthermore, to visualize this high-
176 dimensional data in two dimensions, the dimensionality reduction approach Uniform
177 Manifold Approximation and Projection (UMAP)[14] was applied to 9992 randomly selected
178 cells (1724 per file) using the R package UMAP (version 0.2.5.0).

179 Frequencies of each cluster were compared between no FA controls and food allergy groups
180 using Wilcoxon rank sum tests. The p values were adjusted for multiple comparisons using

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181 the Benjamini and Hochberg approach to control the false discovery rate (FDR). FDR-
182 adjusted $p < 0.1$ were considered significant. To perform the differential abundance analysis
183 for all three groups, the Kruskal-Wallis rank sum test was used (2 degrees of freedom [df]),
184 and p values were FDR-adjusted. Only for clusters showing a significant (FDR-adjusted $p <$
185 0.1) difference between the three groups, subsequent Wilcoxon rank sum tests were to be
186 performed to compare all sets of two groups. However, in order to look further into the
187 FlowSOM clusters that were significantly different in the comparison of the no FA with FA
188 participants, for these clusters Wilcoxon rank sum tests were performed between all sets of
189 two groups among the three groups, even though the FDR-adjusted p from the Kruskal-
190 Wallis rank sum tests were ≥ 0.1. Cell types that were identified by the unsupervised analysis
191 as being significantly different were validated by manual gating.

192 **Analysis of cytokines in cell culture supernatant**

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

201 **Statistical analysis**

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

208 **Ethics**

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

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

213 **RESULTS**

214 **Study participants**

215 A subset of 59 adolescents from the SchoolNuts cohort[4] were used in this study. Table 1
216 describes the demographics and clinical characteristics of the participants included in this
217 study. 57% of the non-food allergic (no FA), 60% of the peanut-only allergic (peanut only),
218 and 50% of the multi-food allergic (multi-FA) particiants were male. The median age in each
219 of the three groups was 13 years. We compared the no FA controls with the total food allergy
220 (FA) group, which included both the peanut only and the multi-FA participants, as well as the
221 three groups with each other.

222 **Cell populations identified by unsupervised clustering**

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

240 **Altered circulating immune cell profiles in food allergic adolescents**

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241 We next compared the frequencies of circulating immune cell populations between food
242 allergic and non-food allergic adolescents. Food allergic adolescents have higher proportions
243 of conventional DCs ($CD3^{-}CD19^{-}CD56^{-}HLADR^{+}CD11c^{+}CD14^{-}$) (p=0.0084, FDR-adjusted
244 p=0.087, median in no FA: 0.63% live cells, in FA: 0.93%, Wilcoxon rank sum test) and
245 activated ($CD3^{+}CD4^{+}CD127^{low}CD25^{+}$CD45RA-CCR7-) Tregs (p=0.011, FDR-adjusted
246 p=0.087, median in no FA: 0.49% live cells, in FA: 0.65%, Wilcoxon rank sum test) relative
247 to non-food allergic adolescents. When investigating these subsets across the 3 participant
248 groups, significantly higher proportions of DCs were seen in both the peanut only (median
249 0.94% live cells) and multi-FA (median 0.88%) groups relative to no-FA controls (p=0.050
250 and p=0.0099, Wilcoxon rank sum test, respectively) (Figure 2e, Figure S4). Significantly
251 higher activated Treg cell proportions were observed between the peanut only (median 0.67%
252 live cells) and no-FA controls (p=0.0026, Wilcoxon rank sum test), but not between the
253 multi-FA (median 0.63%) group relative to non-food allergic adolescents (p=0.17, Wilcoxon
254 rank sum test). These trends were also confirmed by manual gating analysis (Figure S5 and
255 S6). The frequencies of cell clusters that were not significantly different between the groups
256 are presented in Figure S7.

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

258 Our previous studies involving the HealthNuts cohort have revealed key differences in the
259 response capacity of both naïve CD4 T cells and monocytes in food allergic infants relative to
260 non-allergic controls. In the current study, proliferation across all three groups was similar –
261 an average of 3.8-fold increase in CD4 T cell count (with average of 95% viability) was
262 observed following stimulation. We were able to detect nine cytokines in the cell culture
263 supernatants of anti-CD3/CD28 stimulated naive CD4 T cells. These were IL-2, IL-6, TNFα,
264 IL-10, MIP-1α, IFNγ, RANTES, IL-13 and IL-8 as depicted in the heatmap in Figure 3a.
265 Naïve CD4 T cells from food allergic adolescents showed lower median IL-6 (p=0.0020,
266 FDR-adjusted p=0.018, Wilcoxon rank sum test) and TNFα (p=0.0044, FDR-adjusted
267 p=0.020, Wilcoxon rank sum test) responses compared to the non-food allergic controls
268 (Figure 3b). Median IFNγ responses also appeared to be lower in the food allergic group,
269 however this did not remain significant after correction for multiple comparisons (p=0.035,
270 FDR-adjusted p=0.11, Wilcoxon rank sum test) (Figure 3b). When investigating naïve CD4 T
271 cell cytokine responses in our three groups, both the single and multi-FA groups showed
272 significantly lower median levels of both IL-6 and TNFα compared to controls (Figure 3c).
273 The IL-6 response was significantly lower in peanut only (median log2 fold change

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[stimulated/unstimualed]: 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 naive 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

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306 allergic adolescents. Naïve CD4 T cells from both single and multi-FA adolescents were
307 hypo-responsive to stimulation, producing less IL-6 and TNFα following T cell receptor
308 engagement relative to healthy controls. Monocytes from both food allergic and non-food
309 allergic adolescents responded to endotoxin stimulation with rapid inflammatory cytokine
310 production, however no statistically significant difference in monocyte responsiveness was
311 observed between the clinical groups.

312 Previous studies have revealed alterations in T cell function in food allergy. Most work
313 indicates that multi-functional Th2 cells are strongly associated with food allergic immune
314 responses, and that elimination of pathogenic Th2 cells is indicative of clinical responses to
315 oral allergen immunotherapy [15,16]. Deficits in T cell function have also been observed at birth
316 and in the first year of life, with two studies reporting a marked deficit in naïve CD4 T cell
317 proliferative capacity following *in vitro* T cell receptor activation in egg allergic infants [17,18].
318 Here, we show that naïve CD4 T cells from food allergic and healthy adolescents had similar
319 proliferative capacity following anti-CD3/CD28 T cell receptor activation, however naïve
320 CD4 T cells from food allergic adolescents showed marked reductions in the capacity to
321 produce cytokines including IL-6, TNFα and IFNγ. This demonstrates that whilst the
322 lympho-proliferative deficit observed in early life in food allergic infants may be restored by
323 adolescence, dysfunction of multipotent precursor naïve CD4 T cells may continue to drive a
324 food allergy phenotype in later life.

325 We also show that food allergic adolescents have increased circulating levels of activated
326 Tregs, with the most significant increase observed in the single peanut allergic participants.
327 Previous studies investigating Treg proportions in food allergy have focused on food allergic
328 infants or young children and have generated conflicting results, showing decreases in
329 circulating Tregs in food allergic children relative to healthy controls [19,20] or no change at all
330 [21]. Vitamin D insufficiency, a proposed risk factor for the development of food allergy, has
331 also been associated with a deficit in circulating Tregs in food allergic infants [19,22].
332 Additional functional studies of Tregs in allergic children undergoing peanut oral
333 immunotherapy showed that the development of immune tolerance was associated with
334 hypomethylation of FOXP3 CpG sites in antigen-induced Treg populations [23]. Another study
335 showed impaired Treg cell regeneration following in vivo allergen exposure in food allergic
336 infants [24]. Whether the differences in activated Treg cell proportions observed in our study
337 are associated with functional changes warrants further investigation.

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338 Work from our group and others has revealed early life innate immune hyper-responsiveness
339 as a unique signature in the development of food allergy. Elevated production of
340 inflammatory cytokines in the cord blood of children who subsequently develop food allergy
341 has been shown to correlate with the propensity for Th2 responses at birth and during the first
342 year of life [25,26]. A skew towards higher myeloid cells in the circulation, and a hyper-
343 activated inflammatory response in monocytes following *in vitro* stimulation, has also been
344 described in egg and peanut allergic infants [8,10]. These features are remarkably similar to
345 those that define trained immunity – whereby innate immune cells retain a memory of earlier
346 microbial encounters and elicit an enhanced response upon secondary exposure [27]. Innate
347 immune responses in food allergic adolescents have not been previously described. In this
348 study, we show that both single peanut and multi-food allergic adolescents have increased
349 circulating levels of dendritic cells relative to non-food allergic controls, a signature observed
350 previously in egg allergic 12 month old infants [8]. Monocytes from food allergic adolescents
351 also tended to produce more inflammatory cytokines following endotoxin stimulation relative
352 to non-food allergic controls, however this response did not reach significance following
353 multiple comparison correction. Collectively, these findings suggest that some aspects of
354 innate immune dysfunction observed in early life (increased proportions of circulating
355 dendritic cells) persist into adolescence in food allergic individuals.

356 Immune and epidemiological studies on food allergy in adolescence are limited. Peanut and
357 tree nuts are the most common food allergies in this age group [4,5,28-30]. In our SchoolNuts
358 cohort, having a nut allergy was associated with a three-fold increased risk of a severe
359 adverse food reaction consistent with anaphylaxis when compared to those without nut
360 allergy. Additionally, having more than two food allergies doubled the risk of any adverse
361 food reaction compared to those with a single food allergy [5]. This supported the need for us to
362 explore immune responses in both single peanut allergic and multi-food allergic adolescents.
363 All of the multi-food allergic participants included in this study had peanut allergy plus at
364 least one other food allergy, with 80% having co-existent peanut and tree nut allergies. Whilst
365 we did not observe significant differences between the multi and single food allergic
366 individuals, a synergetic effect of multi-FA was observed on several key immune signatures.
367 Similar results have been observed in a study comparing gene expression levels in children
368 with allergic diseases (asthma, atopic dermatitis, rhinitis) when considered as a single disease
369 or multimorbidity (at least two diseases) [31]. A unique signature of eight genes identified
370 multimorbidity, characterised by enrichment of eosinophil and innate immune associated

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

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

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

391 **References**

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492

493 **Data availability**

494 The data that support the findings of this study are available from the corresponding author
495 upon reasonable request.

496

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

	Non-food	Peanut-only	Multi-food

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	allergic[a]	allergic[b]	allergic[c]
Total number	19	20	20
Sex: male, n (%)	11 (57%)	12 (60%)	10 (50%)
Age at blood collection (years), median (min-max)	13 (10-15)	13 (11-16)	13 (11-14)
Current asthma[ǂ], n (%)	10 (53%)	8 (47%) [3 ND]	6 (33%) [2 ND]
Family history of food allergy*, n (%)	4 (42%)	5 (29%) [3 ND]	6 (30%) [3 ND]
Both parents born in Australia, n (%)	17 (89%)	15 (75%)	12 (60%)
Peanut SPT (mm), median (min-max)	0 (0-2)	10 (6-35)	10 (3-30)
Peanut sIgE (kUA/L), median (min-max)	ND	5.4 (0.5-68)	3.9 (0.37-101)
Peanut Allergy + ≥ one Tree Nut allergy	0 (0%)	0 (0%)	16 (80%)
Peanut Allergy + egg allergy	0 (0%)	0 (0%)	4 (20%)

498

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

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

507 [*]parent or sibling history of food allergy

508 [ǂ] doctor diagnosed asthma requiring medication in the last 12 months

509 ND: data not available

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511

512 **Figure Legends**

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

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

540 **Figure 3. CD4 T cell cytokine responses following anti-CD3/CD28 stimulation in food**
541 **allergic adolescents. (a)** Heatmap representing log2 fold change of cytokines in naïve CD4 T
542 cell culture supernatants following 72h of anti-CD3/CD28 stimulation. IL-2, IL-6, TNFα, IL-

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543 10, MIP-1α, IFNγ, RANTES, IL-13 and IL-8 were detectable. **(b)** Fold-change of IL-6, TNFα
544 and IFNγ in non-food allergic (n=18) and food allergic (n=36) adolescents. **(c)** Fold-change
545 of IL-6, TNFα and IFNγ in non-food allergic (n=18), single peanut allergic (n=18) and multi
546 food allergic (n=18) adolescents. P values by Kruskal-Wallis rank sum test (three groups) and
547 Wilcoxon rank sum test (two groups compared). In the boxplots, the medians are shown. The
548 "hinges" represent the first and third quartile. The whiskers are the smallest and largest
549 values after exclusion of outliers (greater than the 75th percentile plus 1.5 times the IQR, or
550 less than 25th percentile minus 1.5 times the IQR).

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

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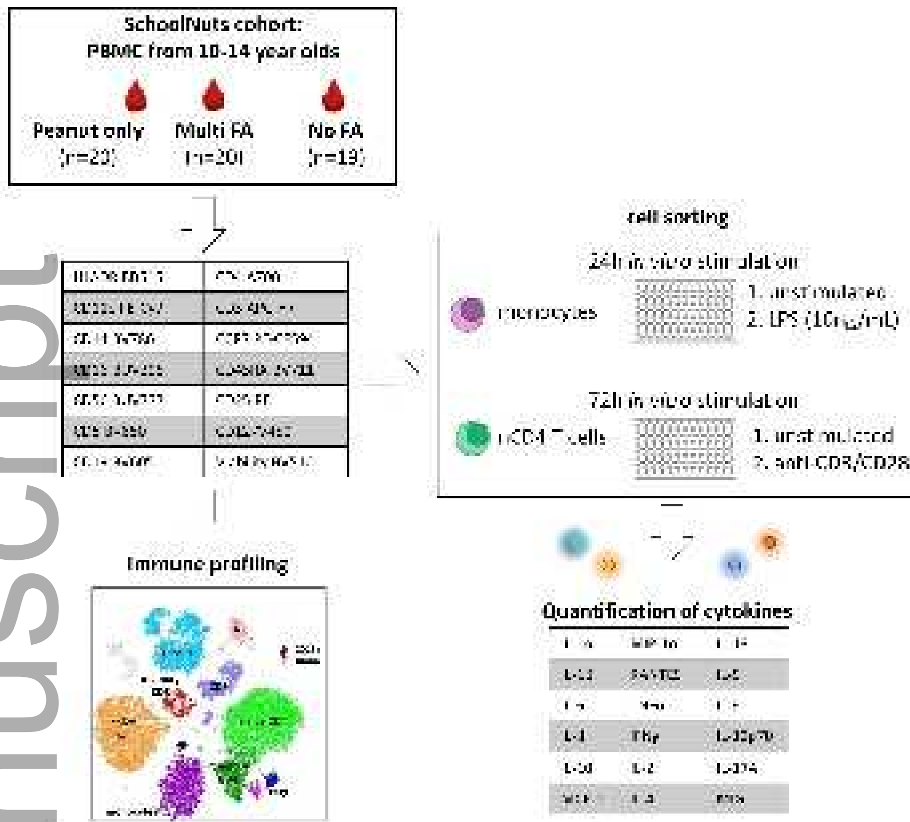

Figure 1

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Figure 2

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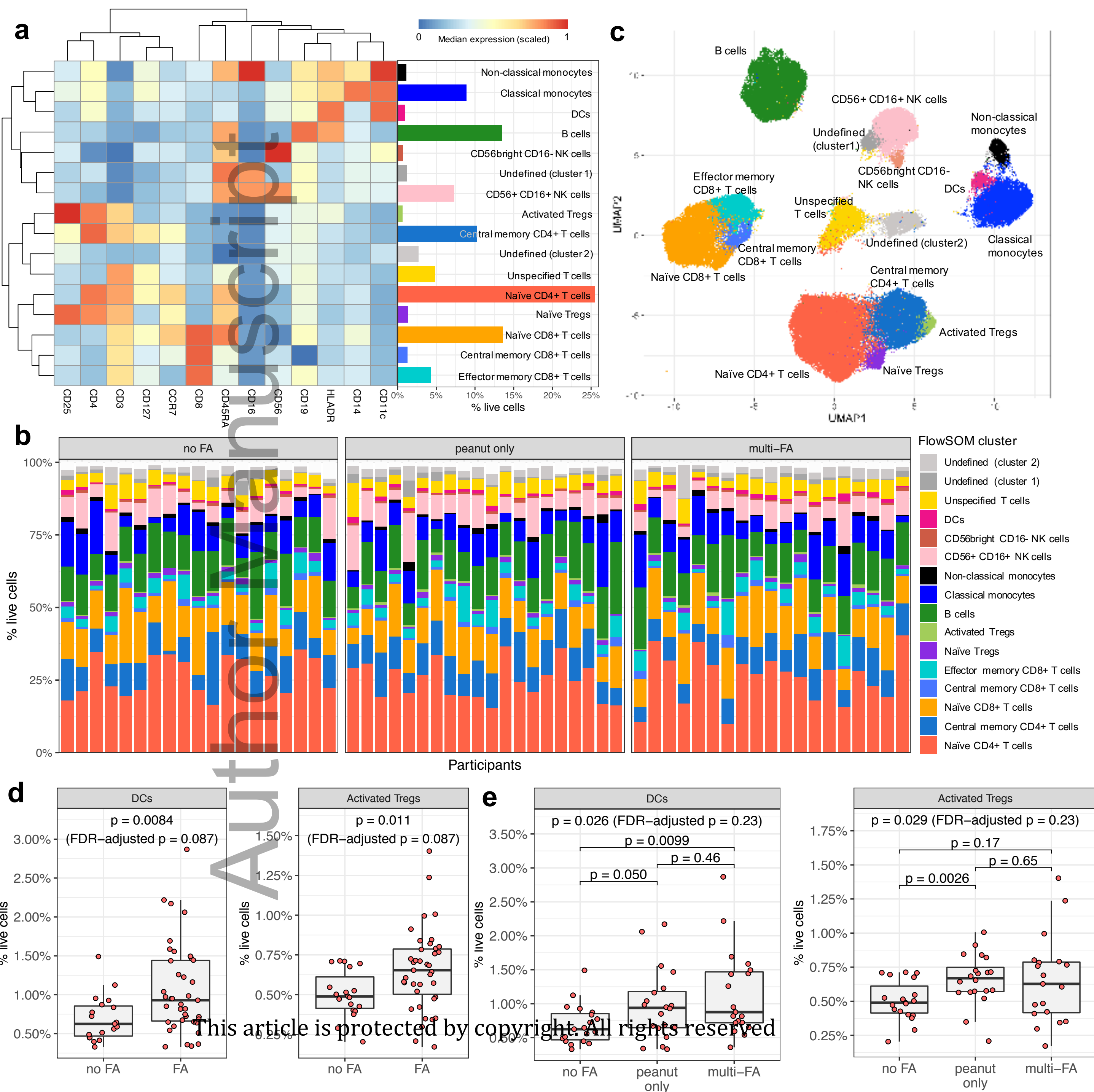

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Figure 3

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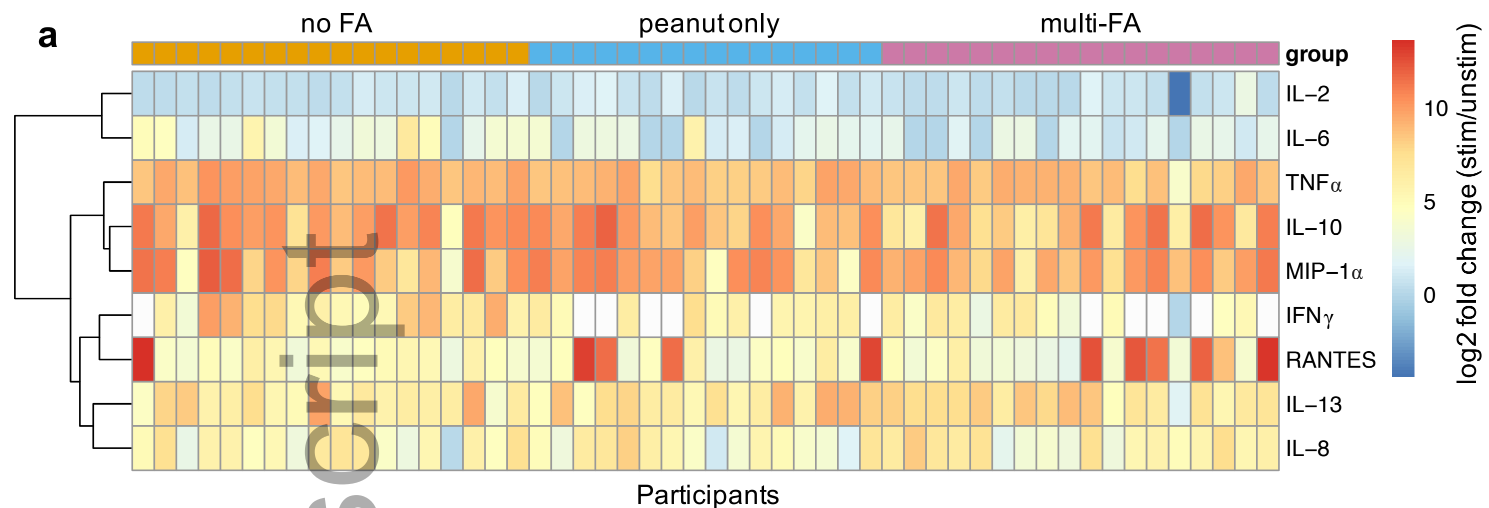

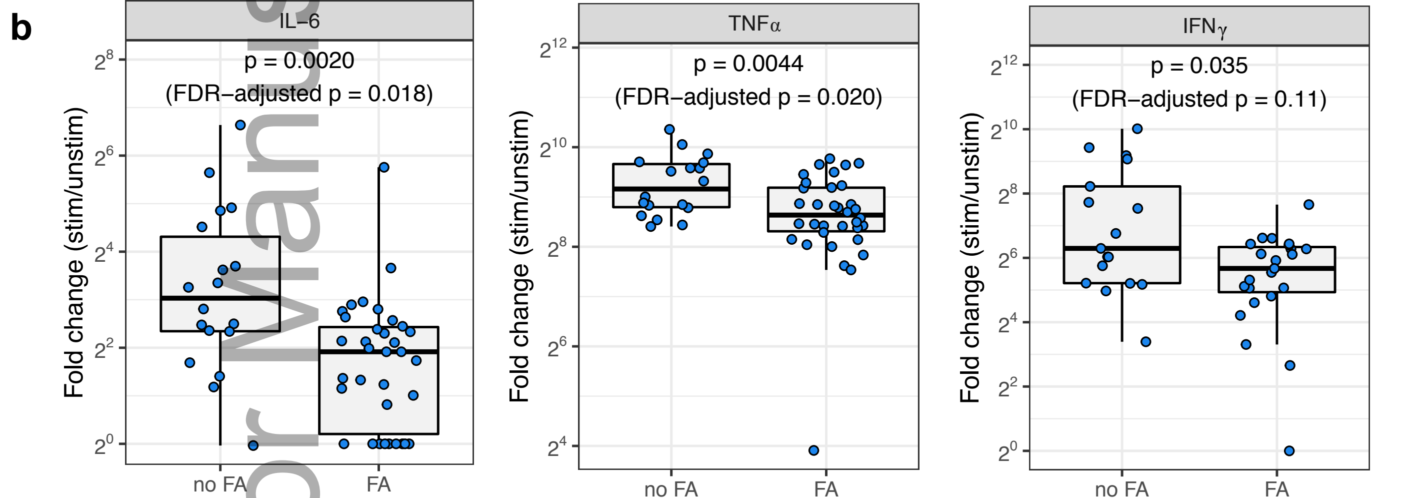

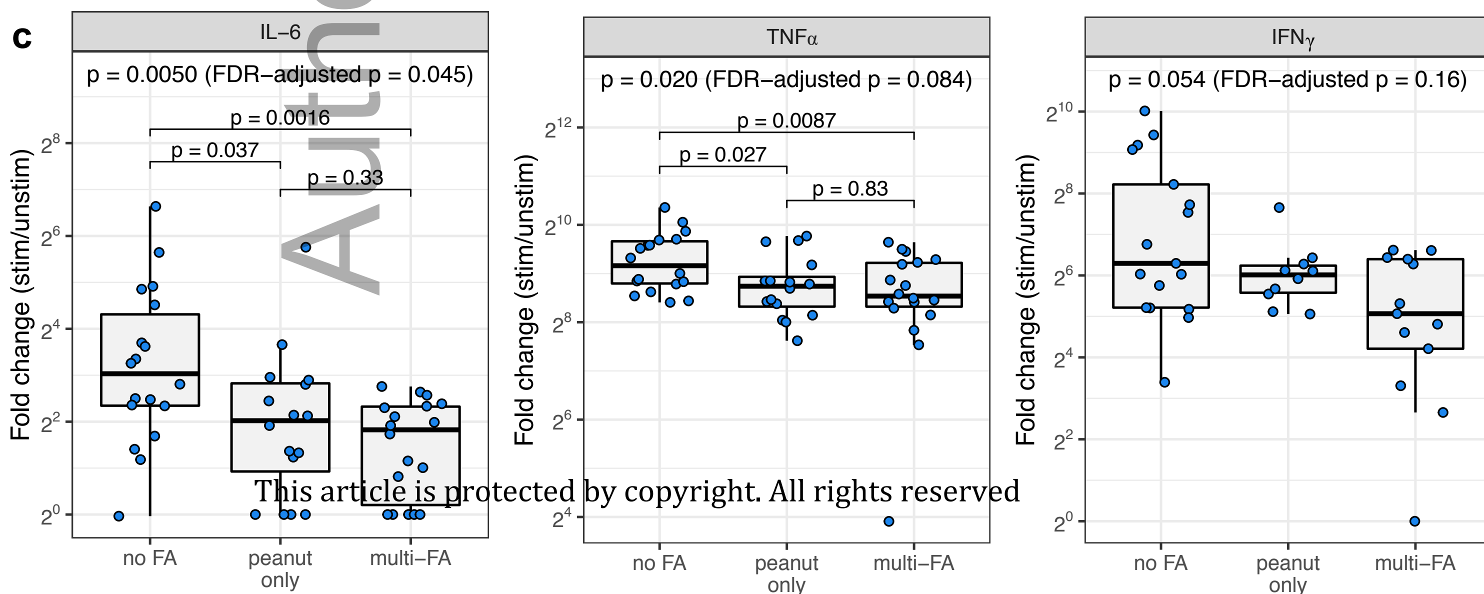

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Figure 4

a

no FA peanut only multi-FA

group

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

Participants

log10 cytokine concentration after stimulation
4
3
2
1
0
−1

b

Cytokine concentration after stimulation (pg/ml)

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)

no FA FA no FA FA no FA FA no FA FA no FA FA

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