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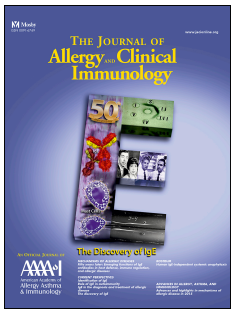

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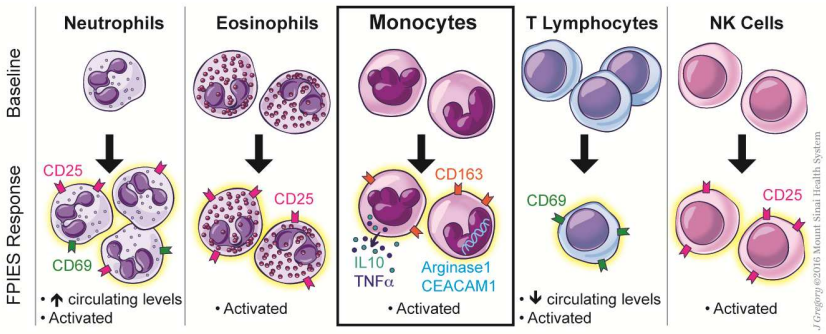

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Systemic Innate Immune Activation in Food Protein Induced Enterocolitis Syndrome (FPIES)

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ABSTRACT

Background: Food protein induced enterocolitis syndrome (FPIES) is a non-IgE-mediated food allergy of infancy whose pathophysiology is poorly understood.

Objectives: We set out to identify and phenotype allergen-responsive cells in peripheral blood of a cohort of subjects undergoing supervised food challenge for FPIES.

Methods: We profiled antigen-responsive cells in peripheral blood mononuclear cells by flow cytometry, and examined cells in whole blood obtained before and after challenge by CyTOF mass cytometry and RNAseq.

Results: Using a CD154-based detection approach, we observed that milk, soy, or rice-responsive T cells, and TNFα-producing CD154+ T cells were significantly lower in those with outgrown FPIES compared to active FPIES. However, levels were within the normal range and were inconsistent with a role in the pathophysiology of FPIES. Profiling of whole blood by CyTOF demonstrated profound activation of cells of the innate immune system after food challenge, including monocytes, neutrophils, NK cells, and eosinophils. Activation was not observed in children with outgrown FPIES. We confirmed this pattern of innate immune activation in a larger cohort by RNAseq. Furthermore, we observed pan-T cell activation and redistribution from the circulation after a positive food challenge but not in those who had outgrown their FPIES.

Conclusions: Our data demonstrate a compelling role of systemic innate immune activation in adverse reactions elicited by foods in FPIES. Further investigation is needed to identify the mechanism of antigen specificity of adverse reactions to foods in FPIES.

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Key Messages

- FPIES reactions after food challenge are associated with a systemic activation of innate cells including neutrophils, monocytes, eosinophils, and NK cells, and a redistribution of T cells from the peripheral circulation.
- Food allergen-specific T cells are detected in peripheral blood of subjects with FPIES, but are within the range found in healthy controls.

Capsule Summary

Using blood obtained before and after food challenges for FPIES, we show that individuals with symptoms in response to challenge have activation of neutrophils, monocytes, eosinophils, and NK cells that is not observed in those who have outgrown FPIES. This novel finding places the innate immune system at the center of FPIES pathogenesis.

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INTRODUCTION

Food protein induced enterocolitis syndrome (FPIES) is a disease of infancy characterized by profuse vomiting and lethargy beginning 2 hours after food ingestion, with a subset experiencing delayed diarrhea [1]. Although most infants outgrow FPIES by school age, a minority retain clinical reactivity into adolescence or adulthood [2]. Foods triggering FPIES are the most common foods introduced early into the infant's diet, including cow's milk, soy, rice, and oats, but a wide range of foods have been reported to induce FPIES symptoms. Reactions are consistent with antigen specificity, and although most individuals react to a single food, multi-food reactivity also occurs [3]. There is growing awareness of FPIES as a clinical entity, highlighted by a number of publications in the last 5 years summarizing clinical experience with FPIES [2, 4-6].

FPIES is classified as a non-IgE-mediated food allergy, although allergen-specific IgE can be found in some patients with FPIES and is associated with persistent disease [2]. There are conflicting reports about levels of food-specific antibodies in FPIES or food protein induced enteropathy [7-9]. We have examined food-specific IgG, and IgA levels in subjects with milk-induced FPIES and found a relative absence of milk-specific immunoglobulins compared to tolerant controls [10, 11]. In patients with food protein induced enteropathy, a disease whose relationship to FPIES is unclear and which has not been reported in recent clinical summaries, chronic antigen exposure leads to diarrhea and vomiting and is associated with villous atrophy and T cell infiltration [3]. PBMCs from patients with non-IgE-mediated cow's milk allergy show increased TNFα production compared to PBMCs from patients with outgrown cow's milk allergy [12], but it is not clear if the patients described in that cohort would fit diagnostic criteria of FPIES. Patients with FPIES have also been described to have a Th2-skewed cytokine profile from antigen re-stimulated PBMCs [13], consistent with our recent findings [11]. It is difficult to reconcile a Th2 skewed T cell profile as underlying such a distinct clinical entity as FPIES. As we recently reviewed [3], there is currently a lack of understanding of the immunologic basis of adverse reactions to foods in FPIES.

Diagnosis of FPIES is based on clinical history and response to food elimination. Patients with a history of FPIES undergo a supervised food challenge when there is reason to believe that the patients may have outgrown their reactivity, usually 12-24 months since their last

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reaction. In our center, food challenges for FPIES are performed in the clinical research center, with full resuscitation facilities for rapid intravenous fluid (IVF) repletion. We collected specimens from patients undergoing a food challenge for FPIES. Blood specimens were collected immediately prior to challenge, and again 4-6 hours after challenge. We found evidence for a profound systemic innate immune activation associated with FPIES reactions, in the absence of an abnormal or pathogenic antigen-specific T cell response. These data point to a critical role of the innate immune system in mediating adverse reactions to foods in FPIES.

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METHODS

Study population

The research protocol was approved by the Icahn School of Medicine at Mount Sinai Institutional Review Board. Written informed consent was obtained prior to enrollment. Patients aged from 1 to 21 years previously diagnosed with FPIES underwent an oral food challenge (OFC) in the inpatient clinical research unit to evaluate for resolution. Table 1 shows the clinical characteristics of the study subjects. A peripheral intravenous line was inserted prior to the OFC. During OFC, the challenge food was administered in three equal portions over 30 minutes. The OFC was considered positive based on the diagnostic criteria defined by Powell [14]: emesis and or diarrhea, and an increase in blood polymorphonuclear leukocyte count > 3,500 cells/mm^3 peaking at 6 hours. Following a negative OFC, children were observed for 4 hours, whereas following a positive OFC they were treated (2 of 14 treated with steroids, the remainder with intravenous fluid with or without Zantac) and observed until stable, usually discharged within 6 hours. Blood samples were obtained immediately before the OFC as well as 4 hours after a negative OFC and 6 hours after a positive OFC. There were gender differences between groups, with positive challenges being overwhelmingly male, while negative challenges were gender balanced. Healthy adult (non-age-matched) controls that were non-food-allergic by self-report were recruited to provide a reference of a healthy CD154 response to foods.

Cell isolation and culture

Blood was obtained in 10 ml heparinized vacutainer tubes. PBMCs were isolated, and cultured in AimV with 5% Human AB serum. 4 x 10^6 cells in 1 ml were plated in 24-well plates. Cells were stimulated for 6 or 18h with milk antigens (a mix of 50 μg/ml each of α, β, and κ caseins) (Sigma Aldrich, St. Louis, MO), or soy or rice extract prepared from flour at 100 μg/ml. Extracts were cleaned of endotoxin using DetoxiGel columns (ThermoFisher, Rockford, IL), verified by Pierce LAL Endotoxin quantification kit (ThermoFisher) prior to use.

4 hours prior to harvest, Brefeldin A (BD Biosciences) was added to cells. Cells were harvested, stained with fixable live/dead stain, followed by surface markers (CD3-APC-Cy7

(eBioscience), CD4-Brilliant Violet 405 (Biolegend), CXCR5-PerCP-Cy5.5 (BD Biosciences), CCR6-PE-Cy7 (BD Biosciences), CCR9-FITC (BD Biosciences)). Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), followed by permeabilization with Permeabilization Buffer (eBioscience), and intracellular staining (CD154-PE (eBioscience), TNFα-AlexaFluor700, IL-13-v450, IL-10-PE-CF594, and IL-9-AlexaFluor647). Cells were acquired on a BD LSRFortessa, and analysis was performed on FlowJo Software (TreeStar, Ashland, OR). In some studies, CD14-PE-Cy7 was used to identify monocytes.

Mass Cytometry Analysis (CyTOF)

Sample Preparation

Whole blood samples were treated BD PhosFlow Lyse/Fix Buffer (BD Biosciences) prior to freezing the sample in 10% DMSO/PBS at -80 C. Thawed samples were first barcoded with Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm, San Francisco, CA), according to the manufacturer's protocol. All the antibodies used in this study were either purchased pre-conjugated from Fluidigm or were conjugated using X8 MaxPar conjugation kits according to the manufacturer's protocol (Table 2). Barcoded samples were combined, treated with heparin to eliminate non-specific binding to eosinophils [15], and subsequently stained with specific antibodies (Table 2) and acquired as one multiplex sample, followed by software de-barcoding and individual sample analysis. After washing, the samples were then incubated with 0.125nM Ir nucleic acid intercalator (Fluidigm) to enable cell identification based on DNA-content, and stored in PBS with freshly diluted 2% formaldehyde (Electron Microscopy Sciences) until acquisition.

CyTOF data acquisition and analysis

Immediately prior to acquisition, the samples were washed once with PBS, once with deionized water and resuspended at a concentration of 600,000 cells/ml in water containing a 1/20 dilution of EQ 4 element beads (Fluidigm). Following routine autotuning according to the manufacturer's recommendations, the samples were acquired on a CyTOF2 mass cytometer (Fluidigm) equipped with a SuperSample system (VictorianAirships, Alamo, CA) at a flow rate of 0.045ml/min. For quality control, the acquisition event rate was maintained under 400

events/s, and the EQ beads were confirmed to have a median Eu151 intensity of over 1000 to ensure appropriate mass sensitivity. The resulting FCS files were normalized using the bead-based normalization tool in the CyTOF2 software and uploaded to Cytobank for analysis. Cells events were identified as Ir191/193 DNA+ Ce140- events, and doublets were excluded on the basis of higher DNA content and longer event length.

Major immune populations were identified either by manual gating on biaxial plots or by using a spanning-tree progression analysis of density-normalized events (SPADE), which is complementary to existing approaches for analyzing cytometry data by enabling multiple cell types to be visualized in branched tree structure [16].

RNA sequencing and co-expression analysis

Whole blood was collected before and after challenge in PAXgene tubes. Tubes were stored at -80C until RNA isolation. RNA was isolated with PAXgene Blood RNA kit (Qiagen, Valencia, CA) according to manufacturer's protocol. Preparation of libraries and sequencing was performed by the Genomics Core at the Icahn School of Medicine at Mount Sinai. 100 base pair single end reads were generated on an Illumina HiSeq 2500, mapped in Tophat, and converted into read counts via HTseq. Gene expression levels were generated from the read count data using DESeq2 and voom R packages.

Weighted correlation network analysis and module identification was completed using WGCNA R package [17]. Each module may be represented by an eigengene, which is effectively a weighted average expression profile of all the genes in the module. Modules consist of genes whose expression is correlated irrespective of the direction of correlation, so some genes in a module may have an opposite pattern of expression from the eigengene representing the module. The correlation between modules and clinical information was evaluated through linear regression. Functional enrichment of co-expressed modules was done using GSEA resource (http://software.broadinstitute.org/gsea/index.jsp).

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RESULTS

CD154-based detection of allergen-specific T cells

PBMCs from subjects who had undergone food challenge to soy, milk, or rice for assessment of FPIES were incubated with the relevant allergens for 6 or 18h prior to identification of allergen-responsive cells by CD154 expression. Incubation with food extract led to an increase in frequency of CD154 expression on $CD3^{+}CD4^{+}$ T cells at 6 h that continued to increase at 18h (gating and representative dot plots shown in Figure E1, quantification by allergen and time-point in Figure E2). The median frequency of CD154+ cells per million CD4+ T cells was 41, 133, and 164 for cells stimulated with milk for 0, 6, or 18h; 38, 101, and 705 for cells stimulated with soy for 0, 6, or 18h; and 37, 64, and 142 for cells stimulated with rice for 0, 6, or 18h (Figure 1A). CD154+ cells co-expressed TNFα, but did not express IL-13, IL-9, or IL-10. Cells stimulated with anti-CD3/CD28 stimulator beads were used as positive controls. Food-responsive T cells did not express mucosal homing molecules CCR6 or CCR9, nor did they express the follicle homing receptor CXCR5 (not shown).

When comparing samples from subjects who reacted or not to food challenge (termed active FPIES and Outgrown, respectively), subjects who did not react had significantly fewer food-responsive $CD154^{+}$ T cells, and significantly fewer food-responsive $CD154^{+}TNF\alpha^{+}$ T cells compared to subjects with active FPIES who reacted to their food challenge (Figure 1B, Figure 1C). However, when compared to responses observed in healthy control subjects, subjects with active FPIES did not have elevated numbers of food-responsive T cells, indicating that this difference was within the normal range and unlikely to explain the pathophysiology of FPIES. This pattern was observed in samples obtained before as well as 4-6 hours after food challenge.

Positive food challenge is associated with reduced activation of blood monocytes post-challenge

We examined subsets other than CD3+CD4+ lymphocytes as a source of TNFα. We did not observe activation of CD8+ T cells by cytokine production. Unexpectedly, we observed that stimulation of PBMCs with milk or rice antigen induced a significant production of both TNFα and IL-10 from a $CD3^{neg}CD4^{neg}$ population with a higher side scatter consistent with monocytes

(Figure 2A, Figure E3). Monocyte markers were not included in our original CD154 panel, but in additional experiments we observed that casein antigen-induced TNFα and IL-10 production was localized to a CD14+ monocyte population (Figure 2B). BSA as a control antigen did not induce cytokine production (Figure 2B). This response was not unique to FPIES, and was also true for healthy controls (data not shown), demonstrating an innate immune response to these food antigens. Despite the similar innate response to these antigens prior to food challenge, we observed a significant reduction in TNFα and IL-10 production from non-T cell sources in subjects with active FPIES, but not subjects who passed their food challenge, in blood obtained post-challenge (Figure 2C). This was not antigen specific, as we observed the same pattern of decreased responsiveness to milk or rice antigen post-challenge in a subject who reacted to food challenge with banana (data not shown). This reduced responsiveness of monocytes post-challenge suggested that these cells may be activated in vivo during food-elicited reactions in FPIES.

CyTOF-based profiling of the hematopoietic response to food challenge

It is well established that FPIES reactions are associated with an increase in circulating neutrophils, which is part of Powell's diagnostic criteria for FPIES [14]. As shown in Figure 3, we confirmed a significant increase in circulating neutrophils in subjects who had reactions to food challenge, but not in those who had outgrown their FPIES. We also observed a significant decrease in lymphocytes (Figure 3) post-challenge in those with active FPIES, but not in those with outgrown FPIES. This was observed for proportion of cells as shown as well as absolute counts (not shown). Monocytes, basophils, and platelets were unchanged, while eosinophils were significantly lower after food challenge in both active and outgrown FPIES.

To determine if activation status rather than just cell number was altered during a food challenge *in* vivo, we developed a panel (Table 2) for profiling innate immune activation in whole blood by mass cytometry (CyTOF). We employed a barcoding approach that allowed matched pre-and post-challenge samples from multiple subjects to be pooled, stained and analyzed as a single sample, thereby minimizing experimental variability and permitting evaluation of subtle changes in marker expression patterns across cell types [18]. In addition to

markers to identify all major hematopoietic subsets, we included activation markers of monocytes, neutrophils, NK cells, γd T cells, and eosinophils. Pre- and post-challenge blood samples were analyzed using 2 subjects challenged to milk, and 2 subjects challenged to cereals (oat/wheat), with one of each reacting to challenge with typical FPIES symptoms. Sample number was limited because we began to collect specimens for CyTOF analysis (requiring different sample preparation) at the end of the study based on the previous findings from flow cytometry analysis of PBMCs.

We utilized SPADE analysis to examine the change in expression (post-challenge compared to pre-challenge) of a number of activation markers across all hematopoietic populations. Cell populations were identified based on positive and negative expression of surface markers (Figure E4). We observed an expansion of neutrophils and $CD56^{dim}$ NK cells, and a loss of CD4+ and CD8+ T cells and $CD56^{bright}$ NK cells post-challenge in those with active FPIES but not outgrown FPIES (Figure E5). We found evidence for global activation of monocytes, neutrophils, eosinophils, and NK cells in those with active FPIES but not with outgrown FPIES. As shown in Figure 4A (as fold change, with median intensity shown in Figure E6), the monocyte activation marker CD163 was markedly upregulated after challenge in CD16+CD14+ and CD16-CD14+ monocytes in subjects who reacted to food challenge, but not those without symptoms. The activation marker CD25 was upregulated on a number of immune subsets (Figure 4B, Figure E7), most strikingly eosinophils and CD56dim NK cells but also neutrophils. Activated neutrophils downregulate FcγRIII, which was observed after challenge in those with active FPIES (Figure 4D, Figure E9). Neutrophils were also found to upregulate expression of CD69, which has been reported to occur in response to cytokine stimulation [19]. CD69 was also increased throughout the CD3+ cell compartment, which also showed a loss of cell numbers (Figure 4C, Figure E8). These data indicate a broad activation of innate immune cells during adverse reactions to foods in FPIES, as well as a global loss of T lymphocytes from the circulation.

Innate immune activation in peripheral blood Identified by RNA sequencing

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We obtained RNA samples from whole blood of 26 subjects before and 4-6 hours after food challenge, including 14 with outgrown FPIES who did not react on challenge, and 12 with active FPIES who reacted on challenge. We performed RNA sequencing to obtain a transcriptional profile of the whole peripheral blood compartment. Additionally, we identified co-expressed gene modules, and examined correlation of these with parameters including response to food challenge. Three modules had a high positive correlation with challenge outcome and circulating neutrophils and a negative correlation with circulating lymphocytes (Figure 5). Pathway analysis showed significant enrichment for CD14+ monocytes, despite the lack of correlation with circulating monocyte levels. Furthermore, pathway analysis showed a significant enrichment for genes associated with “Defense Response” and “Response to Wounding”.

We then examined expression of innate immunity genes selected from a correlated gene module (Figure 6). We observed significant up and down regulation of a number of key innate immune genes in the active FPIES cohort, but not the outgrown cohort, after challenge (Figure 6). These included arginase 1 (ARG1), CEACAM1, NLRC4, NOD2, TLR5, and BAFF (TNFSF13B). We also observed downregulation of the genes CCL28, CCR4, and GATA3. Several of these genes, such as CEACAM1 (also known as CD66a) are expressed by neutrophils and may reflect changes in neutrophil number. We used the neutrophil marker genes STEAP4, DYSF, and KCNJ15 to adjust for neutrophil number [20]. These genes were significantly upregulated after challenge in active FPIES but not outgrown subjects (not shown). Adjustment of CEACAM1 and ARG1 expression with these neutrophil marker genes resulted in a maintained significant upregulation, indicating that increased expression is due to upregulation upon activation, as well as increased neutrophil cell number.

DISCUSSION

The immune mechanisms responsible for severe vomiting and shock-like symptoms in response to specific food ingestion in FPIES remain poorly understood. Our data do not provide an immunologic explanation for the antigen specificity of reactions to foods in FPIES as we do not find evidence for either antibodies[11] or T cell responses specific to foods that are outside of the normal range. Clinical responses to challenge with purified milk allergens, including caseins, have been reported [21]. This indicates that reactions are not triggered by a non-protein source. Our lack of detection of an increased frequency or altered phenotype of allergen-responsive T cells does not necessarily rule out a role of T cells in FPIES. One explanation is that antigen-responsive cells with a pathogenic phenotype are localized to the gastrointestinal tract and not found in circulation. In celiac disease, circulating antigen-specific T cells have been identified only 6 days after antigen provocation [22, 23] and therefore 6 hours post-challenge may be insufficient time for expansion or mobilization of food-specific T cells with a unique pathogenic phenotype into the circulation after activation within the gastrointestinal mucosa. However, in IgE-mediated food allergy and eosinophilic gastroenteritis, food-specific T cells can be readily identified in peripheral blood after ex vivo stimulation [24, 25]. Another explanation for our lack of detection of increased food-specific T cells is that antigens must be modified in the gastrointestinal tract in order to elicit recognition, similar to gluten peptides in celiac disease [26]. However, elicitation of lower gastrointestinal symptoms has been described in response to rectal administration of cow's milk [27], indicating that there is some level of immune activation in the absence of exposure to enzymes from stomach or small intestine. Further studies are clearly needed to identify the mechanism of recognition of food antigens by the immune system in FPIES.

In our previous report, we used proliferation-based assays and measurement of cytokines in culture supernatants to profile the immune response in FPIES [11]. We previously observed an increase in IL-9 and a decrease in IL-10 from milk-restimulated PBMCs from active versus outgrown FPIES. Using CD154-based approaches, we were unable to identify milk, soy, or rice-responsive T cells expressing these cytokines. This suggests that the source of these cytokines may be non-T cells. We show that IL-10 is produced by CD14+ monocytes in response

to milk or rice allergen stimulation *in vitro*, and that IL-10 as well as TNFα production was suppressed after reactions to food challenge. We observed a broad systemic innate immune activation associated with positive food challenges. We first observed that monocytes were less responsive to allergen stimulation when obtained after a positive food challenge, and based on this observation we used CyTOF profiling to identify immune activation that occurred in vivo during a positive food challenge. This was observed not just on monocytes, but also on neutrophils, eosinophils, and CD56dim NK cells. We also observed changes in gene expression consistent with innate immune activation, with CD14+ monocytes being identified through pathway analysis. This systemic innate immune activation may contribute to shock-like symptoms of FPIES including hypotension and pallor. Interestingly, innate immune activation featuring NK cell activation and enrichment of pathways consistent with TLR4- and TREM1-activated monocytes has been identified through transcriptional profiling of IgE-mediated food allergy. Stone et al identified these pathways as upregulated *in* vivo in patients presenting to the emergency department with anaphylaxis [28], and we recently found the same pathways to be induced by egg stimulation in patients with IgE-mediated egg allergy [29]. The mechanism triggering this innate immune activation in FPIES is not clear. Although we observed direct activation of monocytes by milk and rice allergens *in vitro*, this was not a response that was unique to FPIES subjects. Activated cells in vivo could potentially be responding to systemic release of a cytokine. In a previous report, we had measured cytokines in serum of subjects undergoing food challenge for FPIES. In blood obtained 4 hours after symptom onset, we did not observe a significant increase in pro-inflammatory cytokines such as TNFα, IL-1, or IL-6, but we did observe a significant increase in serum IL-10 as well as IL-8 in those who had FPIES reactions [11], likely reflecting the innate cell activation in vivo that we report here. A more detailed kinetic analysis of systemic mediators, including cytokines, is needed to understand the relationship between systemic mediator release, systemic immune cell activation, and symptoms. Of particular importance is to identify immune activation that precedes the onset of symptoms.

Although we could not identify an antigen-specific T cell response in FPIES, we observed a global loss of lymphocytes from the peripheral circulation following a positive food challenge.

353 Using CyTOF analysis, we observed a broad activation of CD4+, CD8+, and γδ lymphocytes in
354 addition to innate cell activation. The mechanism of this global lymphocyte activation may be in
355 response to cytokines or other circulating mediators. It has been shown in mice that pan- T cell
356 activation using anti-CD3 antibodies leads to gastrointestinal homing of Th17 cells resulting in
357 intestinal damage [30], suggesting a potential link between acute reactions in FPIES and
358 enteropathy observed in response to chronic allergen exposure.
359 A limitation of these studies is the use of peripheral blood to study immune mechanisms
360 of adverse reactions to foods. Because FPIES manifestations are systemic, leading to shock-like
361 symptoms, it is reasonable to think that peripheral blood would be an appropriate site for
362 examination of immune activation. However, important eliciting events may be restricted to
363 the gastrointestinal tract. Intestinal biopsy is not required for clinical care, and therefore there
364 is a need for minimally invasive approaches of profiling the gastrointestinal mucosa during
365 FPIES challenges. Other limitations include the difference in timing of blood draw between
366 those who passed versus failed food challenge (4 versus approximately 6 hours). We cannot
367 rule out a contribution of this difference in timing. The imbalance in gender between groups is
368 also a factor that we were not able to control for and could contribute to differences found.
369 In summary, we show that adverse reactions to foods in subjects with FPIES are
370 associated with a broad systemic innate activation as well as activation and redistribution of
371 lymphocytes from the circulation. We have not yet identified the upstream triggering
372 mechanism of specific recognition of foods, as there is a marked absence of abnormal or
373 elevated antigen-specific B or T cell responses. Further studies are needed to study whether
374 innate immune activation precedes symptom onset, as well as to identify the mechanism of
375 food antigen recognition in the gastrointestinal mucosa.
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Table 1: Subject Characteristics

	FPIES	***Outgrown***
N	14	*16*
Age in years, median (range)	7.5 (1.3-21)	*4.6 (1-21)*
Gender	*13M/1F*	*7M/9F*
Foods Used for Challenge	*Milk (4)* *Rice (4)* *Soy (3)* *Oat (1)* *Banana (1)* *Beef (1)*	*Milk (7)* *Rice (3)* *Soy (1)* *Egg (2)* *Wheat (1)* *Salmon (1)* *Bean (1)*
Food-specific IgE	*1/14*	*0/16*

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380 ***Table 2: Antibody panel for CyTOF analysis***

Channel	Target	Category
In113	CD57	NK Cell
In115	CD45	Pan-Leukocyte
Nd142	CD19	B cells
Nd143	CD45RA	T cell subset
Nd144	CD69	Activation
Nd145	CD4	T cell subset
Nd146	CD8	T cell subset
SM147	CD49d	Homing
Nd148	CD16	Granulocytes, Monocytes
Sm149	CD127	IL-7 receptor
Nd150	CD1C	DCs
Eu151	CD123	Basophils, pDCs
Sm152	CD66B	Neutrophils, Eosinophils
Eu153	PD-1	Activation
Sm154	CD163	Activation
Gd155	CD27	Differentiation
Tb159	CD103	Homing
Gd160	CD14	Monocytes
Dy161	CD56	NK cells
Dy162	CD64	Activation
Er166	CD25	Activation
Er168	CD3	T cells
Tm169	Beta 7	Homing
Er170	CD38	Differentiation/Activation
Yb171	CD161	Activation
Yb174	HLADR	APCs, Activation

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FIGURE LEGENDS

Figure 1: CD154-based detection of food-specific T cells. A) Quantification of CD154+ CD4+ T cells after 0, 6, or 18h of stimulation with milk, soy, or rice. B) Frequency of CD154+ CD4+ T cells in the absence (-) or presence (+) of stimulation with milk, rice, or soy extract (extract matched to challenge food) in unchallenged healthy controls, or in those who reacted (FPIES) or not (outgrown) to a food challenge. C) Frequency of TNFα+CD154+ CD4+ T cells as in (B). *$p < 0.05$ compared to media control, #$p < 0.05$ compared to FPIES.

Figure 2: Monocyte hypo-responsiveness after FPIES reactions. A) TNFα and IL-10 secretion from $CD3^{neg}$ $CD4^{neg}$ SSC^{Med} cells 6 h after stimulation with casein, rice, or anti-CD3/CD28 stimulator beads. B) Expression of TNFα in CD14+ monocytes after stimulation with casein, but not BSA as control. C) Quantification of TNFα and IL-10 expression in $CD3^{neg}$ $CD4^{neg}$ SSC^{Med} cells 6h after stimulation with casein or rice in those who reacted to challenge (FPIES) and those who did not (Outgrown), before and 4-6h after food challenge. *$p<0.05$, ns = non-significant.

Figure 3: Impact of FPIES reactions on peripheral blood cells. Frequency of neutrophils (PMN), lymphocytes, monocytes, platelets, eosinophils, and basophils before (pre) and 4-6 hours after (post) food challenge. FPIES indicates subjects with reactions to foods, Outgrown indicates subjects who did not react to challenge. *$p<0.05$, ****$p<0.0001$. Bottom graphs summarize the change in circulating neutrophil and lymphocyte number (post-pre) in the two groups.

Figure 4: Innate cell activation in peripheral blood measured by CyTOF. Peripheral blood was obtained before and after food challenge in 4 subjects, 2 with active FPIES and 2 with outgrown FPIES. Shown are SPADE plots indicating fold change (post challenge versus pre challenge) in CD163 (A), CD25 (B), CD69 (C) and CD16 (D) expression across immune subsets. The size of the bubbles represents the cell number in each node, while the color intensity indicates fold change in expression of the respective marker relative to pre challenge baseline. The cell populations are labeled as identified by pattern of surface marker expression. Note the increased expression of CD163 in the monocyte populations, and increased CD25 in CD56dim NK cells,

498 neutrophils, and eosinophils in FPIES, but not outgrown subjects. CD69 expression shows
499 upregulation across multiple immune compartments including the T lymphocyte populations.
500
501 **Figure 5: Relationship between co-expressed gene modules and clinical and immunologic**
502 **characteristics. A)** Association between gene modules and clinical characteristics, including
503 clinical response to challenge, CBCs, and treatment. Red indicates positive correlation, blue
504 indicates negative correlation, and text in each box indicates correlation with p value in
505 parentheses. **B)** Table showing selected enrichment analysis results for modules 12 and 14.
506
507 **Figure 6: Challenge-induced changes in innate immune gene expression. A)** Fold change in
508 gene expression from whole blood (post-challenge vs. pre-challenge) for candidate immune
509 genes selected from Module 14 in Figure 5. Only candidate genes with significant differences
510 (with FDR correction of 0.01 for multiple comparisons) are shown. Mean + SEM of n=14
511 (Outgrown) and 11 (FPIES). **B)** Adjustment of gene expression using neutrophil specific genes.
512 ** $p < 0.01$, *** $p < 0.001$.
513
514

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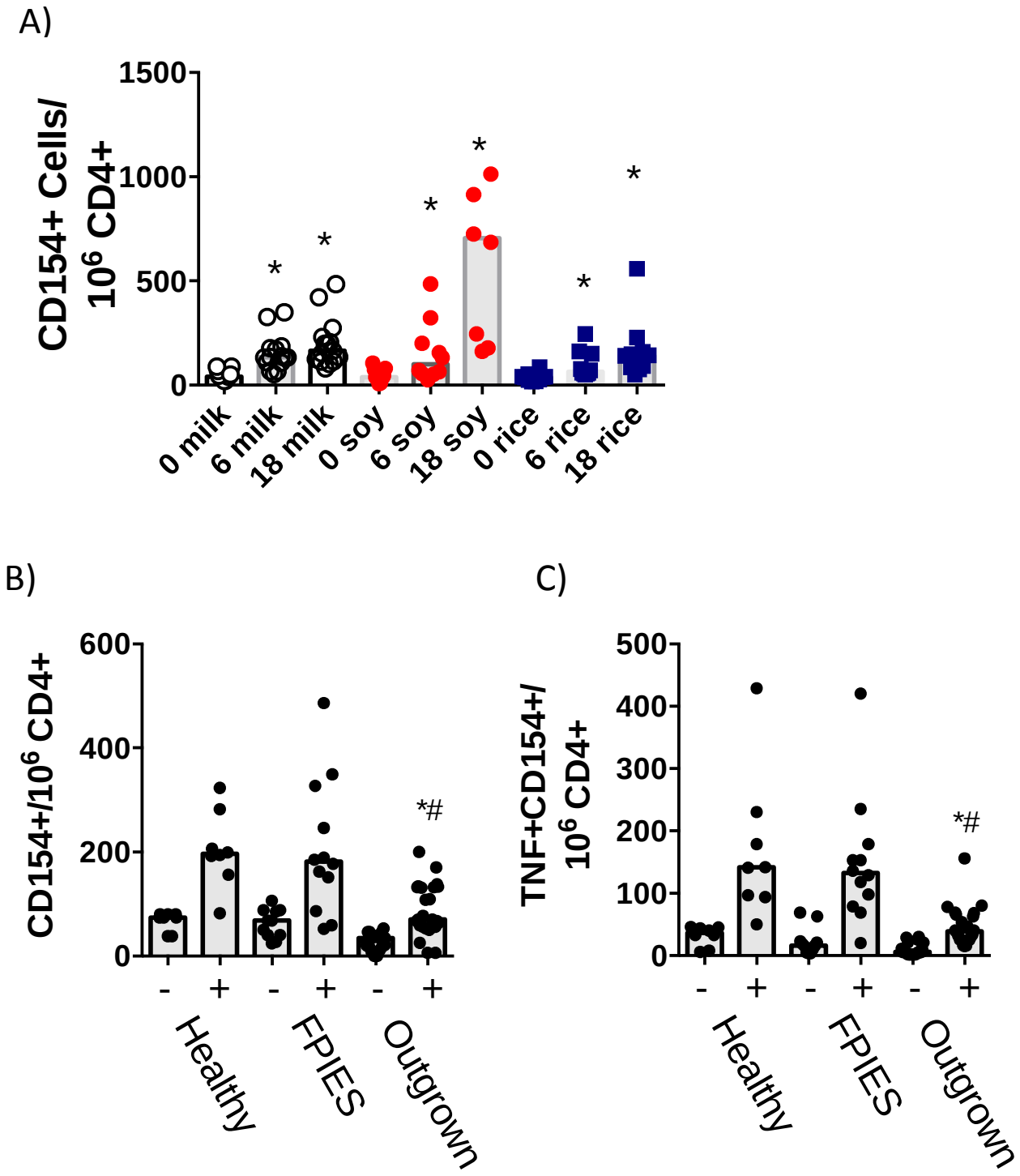

A)
CD154+ Cells/ 10^6 CD4+
1500
1000
500
0
0 milk
6 milk
18 milk
0 soy
6 soy
18 soy
0 rice
6 rice
18 rice
B)
CD154+/10^6 CD4+
600
400
200
0
*#
- + - + - +
Healthy
FPIES
Outgrown
C)
TNF+CD154+/ 10^6 CD4+
500
400
300
200
100
0
*#
- + - + - +
Healthy
FPIES
Outgrown

Figure 1

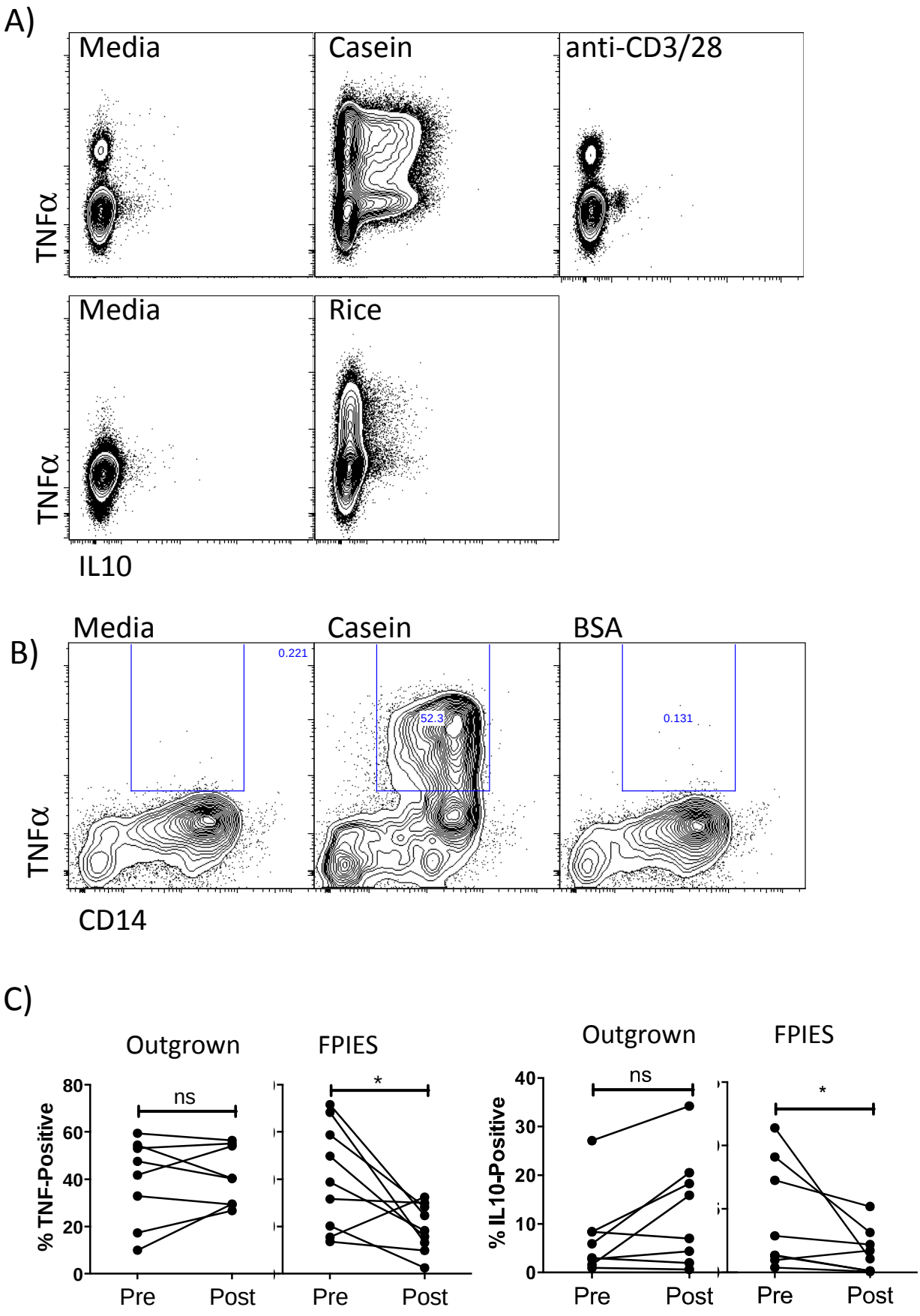

A)
Media
Casein
anti-CD3/28
TNFα
Media
Rice
TNFα
IL10
B)
Media
Casein
BSA
0.221
52.3
0.131
TNFα
CD14
C)
Outgrown
FPIES
Outgrown
FPIES
% TNF-Positive
% IL10-Positive
ns
*
ns
*
Pre
Post
Pre
Post
Pre
Post
Pre
Post

Figure 2

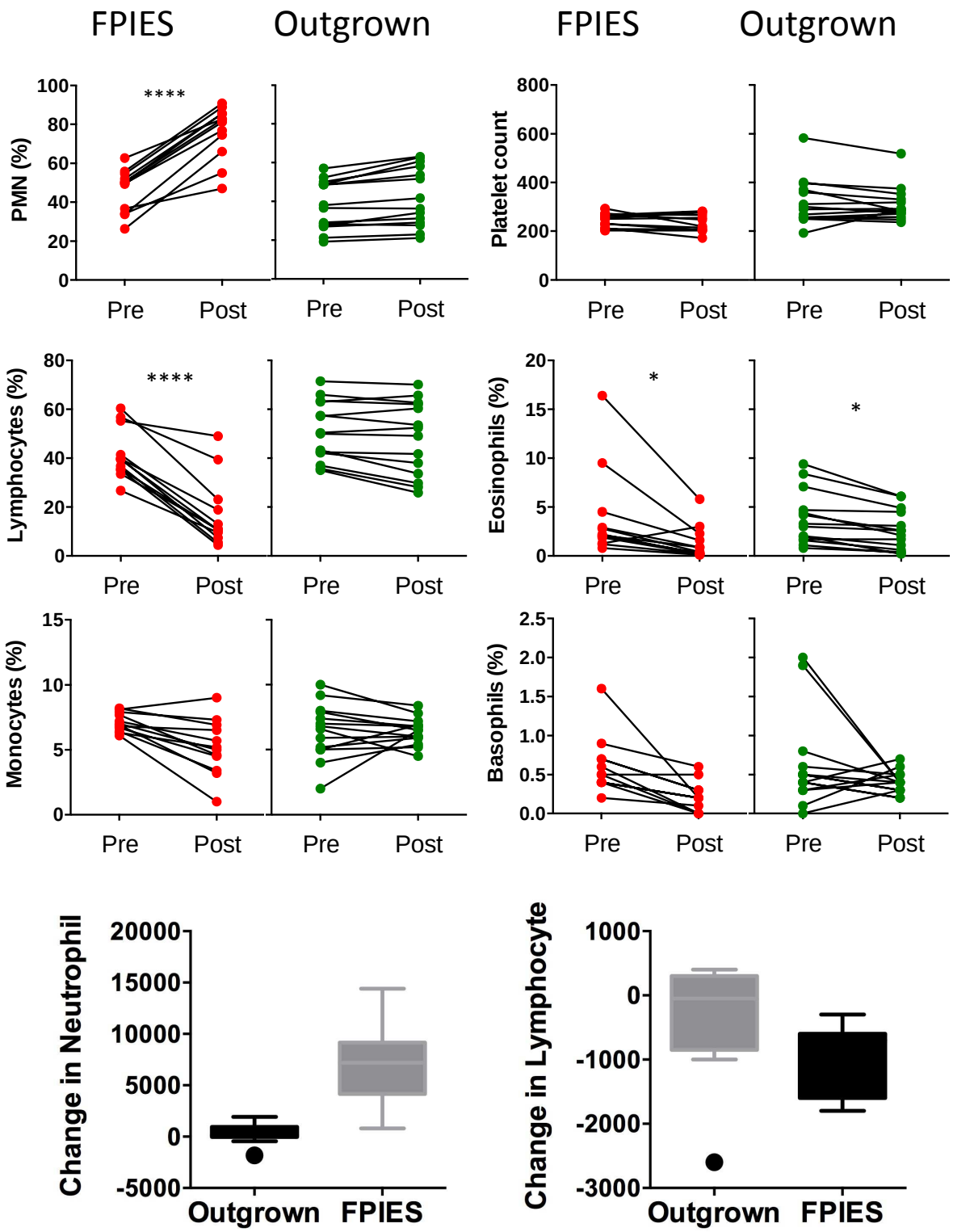
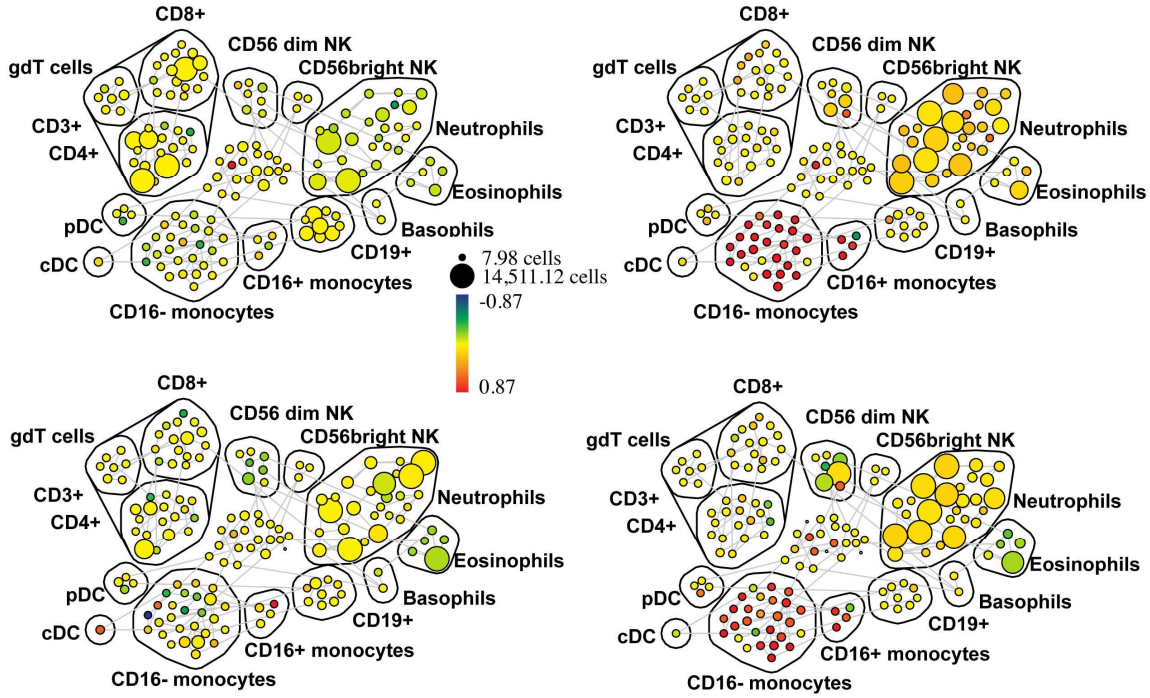

FPIES
Outgrown
FPIES
Outgrown
PMN (%)
****
Platelet count
Lymphocytes (%)
****
Eosinophils (%)
*
*
Monocytes (%)
Basophils (%)
Pre
Post
Change in Neutrophil
20000
15000
10000
5000
0
-5000
Outgrown
FPIES
Change in Lymphocyte
1000
0
-1000
-2000
-3000
Outgrown
FPIES

Figure 3

Outgrown FPIES

A) CD163



B) CD25

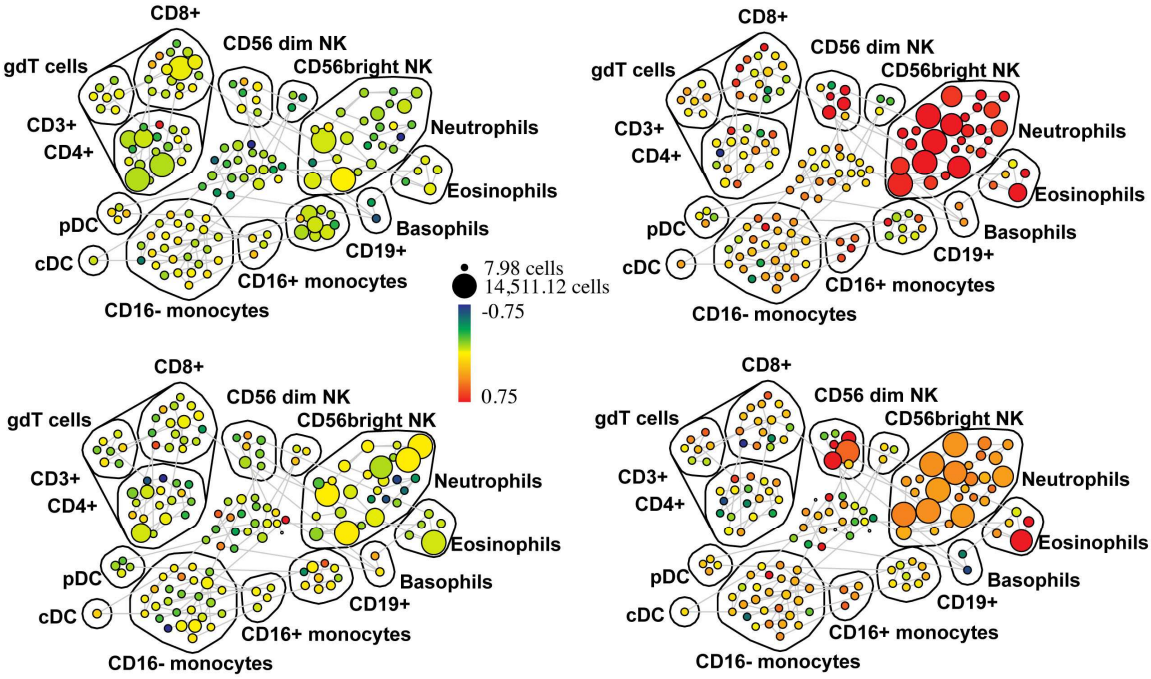
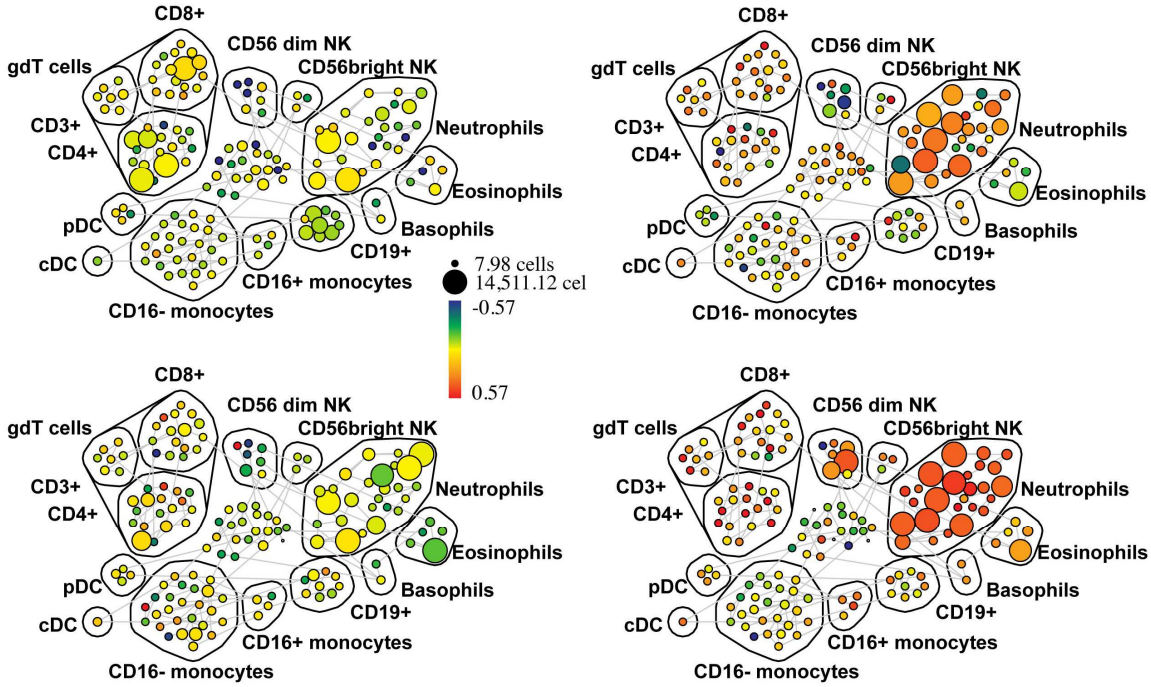


Figure 4

Outgrown FPIES

C) CD69



D) CD16

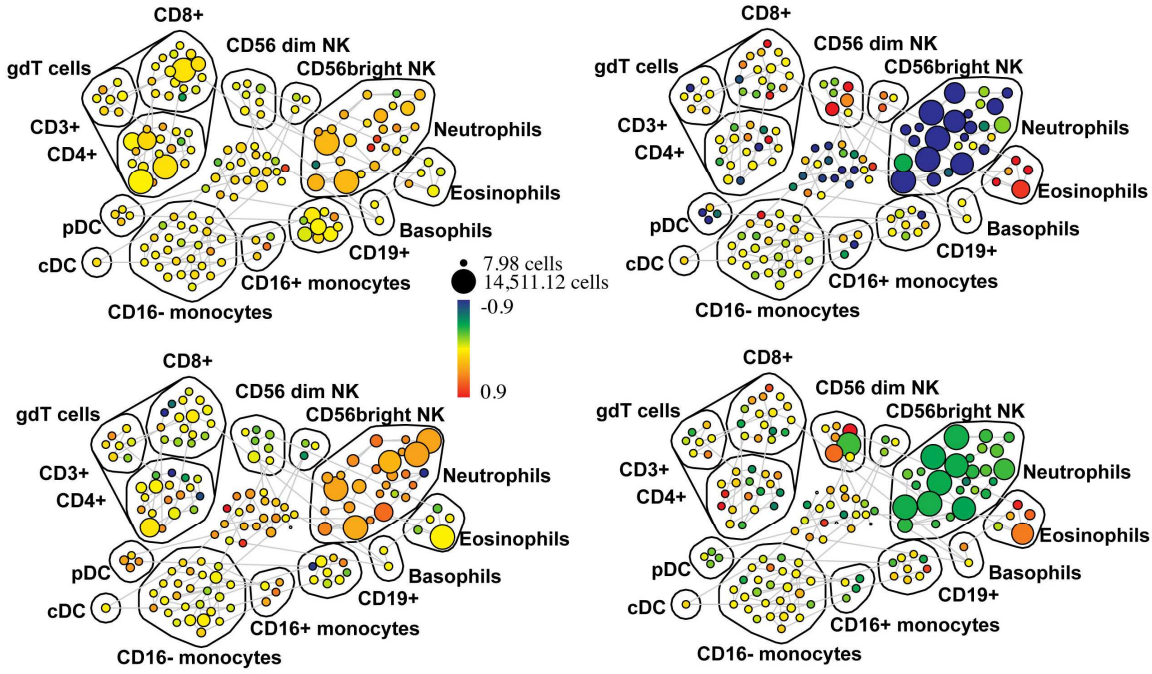
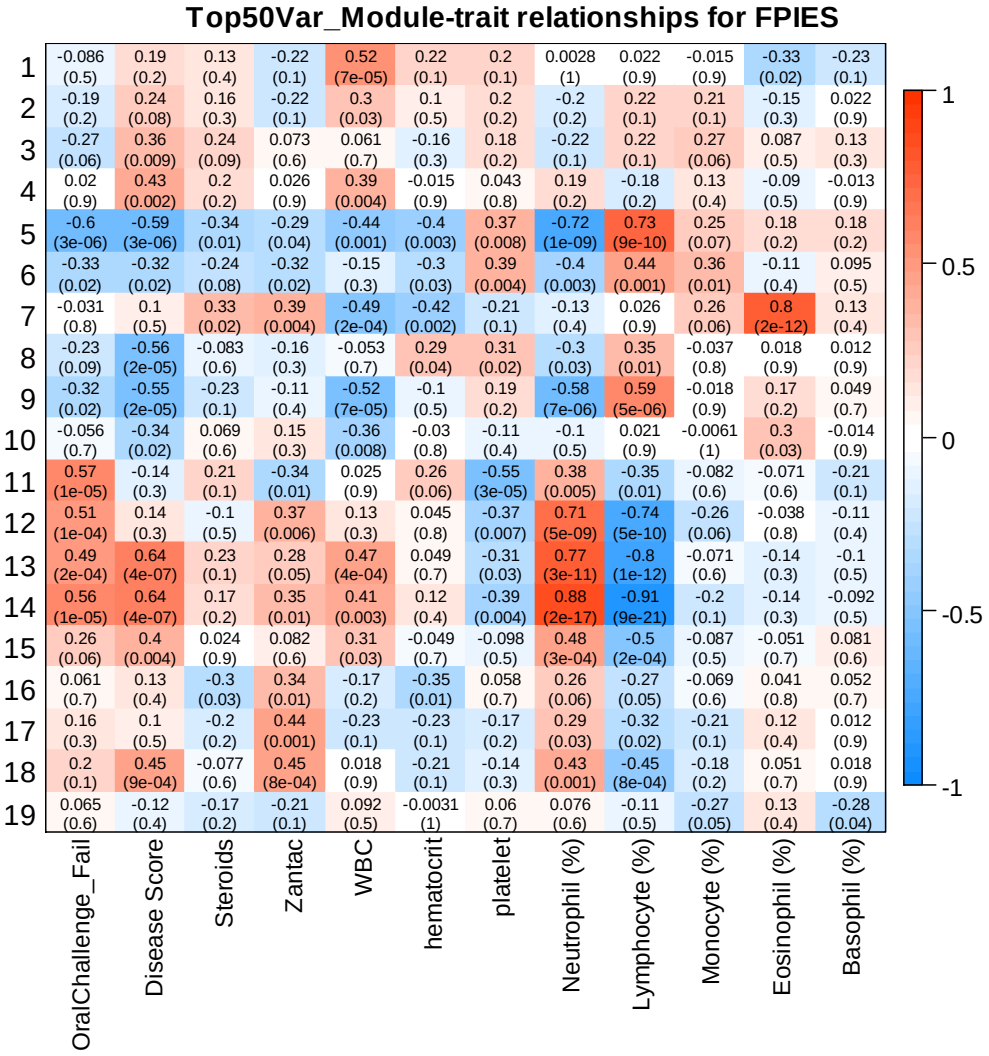


Figure 4

A)



B)

Module	Category	Description	P Value
14	Human Gene Atlas	CD14+ Monocytes	3.3×10^{-5}
14	Go Biological Process	Defense Response	4.1×10^{-6}
14	Go Biological Process	Response to Wounding	4.3×10^{-5}
12	Human Gene Atlas	CD14+ Monocytes	7.5×10^{-9}

Figure 5

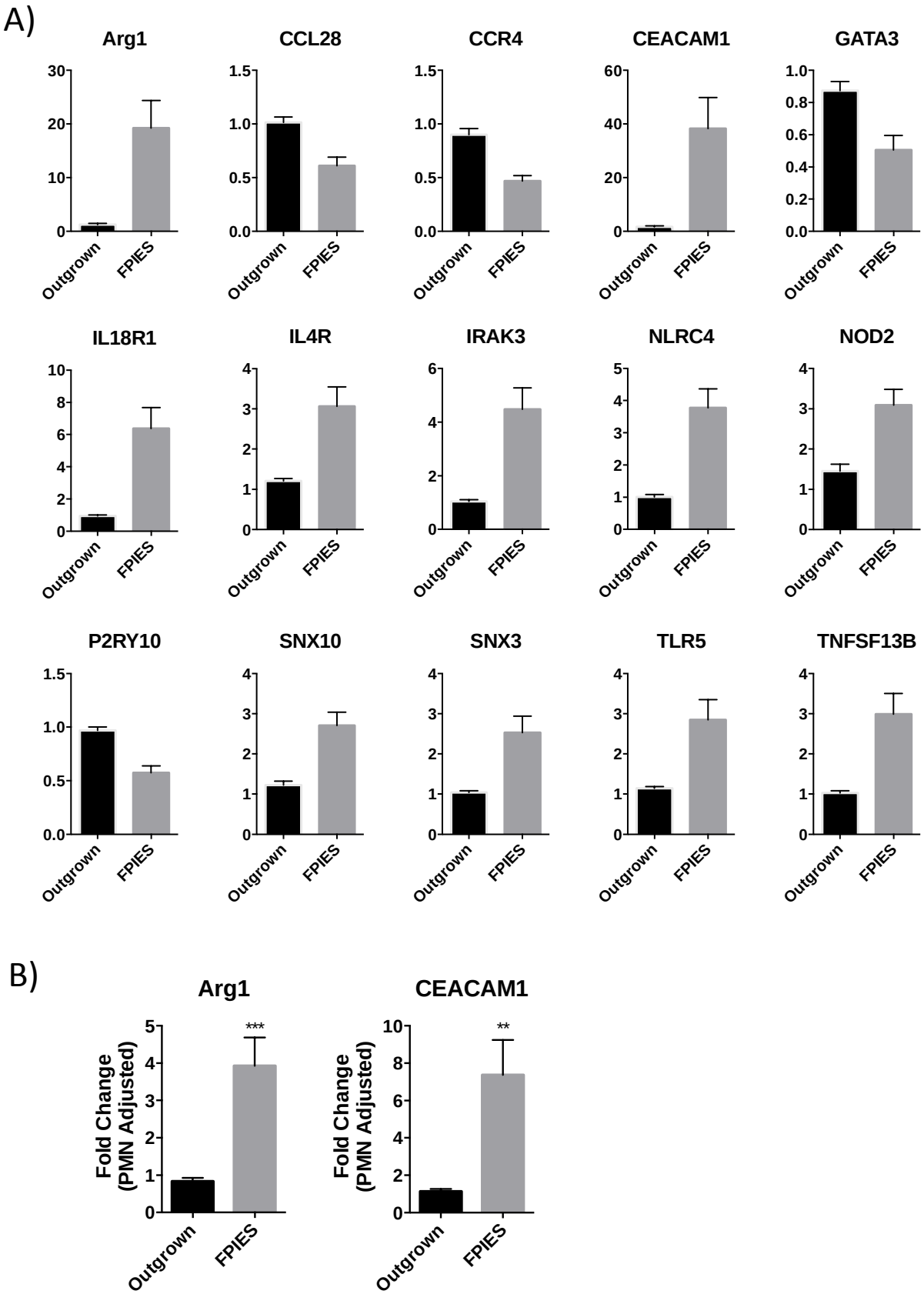

A)
Arg1
CCL28
CCR4
CEACAM1
GATA3
IL18R1
IL4R
IRAK3
NLRC4
NOD2
P2RY10
SNX10
SNX3
TLR5
TNFSF13B
Outgrown
FPIES
B)
Arg1
CEACAM1
Fold Change (PMN Adjusted)
***
**

Figure 6

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Supplemental Figures

Figure E1: Representative Gating. PBMCs were acquired and gated on lymphocyte scatter, CD3+CD4+ cells, and dead cells were excluded (left panels, top to bottom). Right panels: CD154 (y axis) vs TNFa (x axis) in response to stimulation with media alone, milk (casein allergens) for 6 or 18h, or stimulation with anti-CD3/CD28 as positive control for 6h. Top right quadrant indicates the number of CD154+TNFa+ cells per million CD3+CD4+ cells.

Figure E2: CD154 expression by food and timepoint. Scatter plots of CD154 expression on CD4+ T cells after stimulation with media or each food extract for 6 or 18h, in samples obtained pre-challenge or post-challenge.

Figure E3: Representative flow cytometry plots showing scatter properties of TNFα-producing cells, and with an overlay of the scatter of monocytes (CD14+HLADR+).

Figure E4: Identification of cell populations by surface staining. Shown are SPADE plots demonstrating median intensity of staining for each of the markers indicated (red indicating increased intensity). Cell populations (indicated by the larger bubbles drawn around smaller clusters) were identified on the basis of both presence and absence of surface markers. Other markers used for SPADE clustering included CD19, CD56, and CD11b, and for population identification after clustering included CD123 and CD1c.

Figure E5: Change in cell populations after oral food challenge. Shown are SPADE plots for two subjects with outgrown FPIES, and two subjects with active FPIES. The size of the bubbles represents the cell number in each node, while the color intensity indicates relative change in population frequency compared to pre challenge baseline. Note the increase in neutrophils and $CD56^{dim}$ NK cells, and the decrease in CD4+, CD8+, and $CD56^{bright}$ NK cells after challenge in those with active FPIES.

Figure E6: CD163 shown as median intensity in all populations in representative subjects (top panels), and in CD16+ and CD16- monocytes from 4 subjects. T1= Pre-challenge, T2 = Post-challenge.

Figure E7: CD25 shown as median intensity in all populations in representative subjects (top panels), and in neutrophils, CD56dim NK cells, and eosinophils (bottom panels) for all 4 subjects. T1= Pre-challenge, T2 = Post-challenge.

Figure E8: CD69 shown as median intensity in all populations in two representative subjects (top panels), and in neutrophils, and CD3+ T cells for all 4 subjects (bottom panels). T1= Pre-challenge, T2 = Post-challenge.

Figure E9: CD16 shown as median intensity in all populations in two representative subjects (top panels), and in neutrophils (bottom panels) in all 4 subjects. T1= Pre-challenge, T2 = Post-challenge.

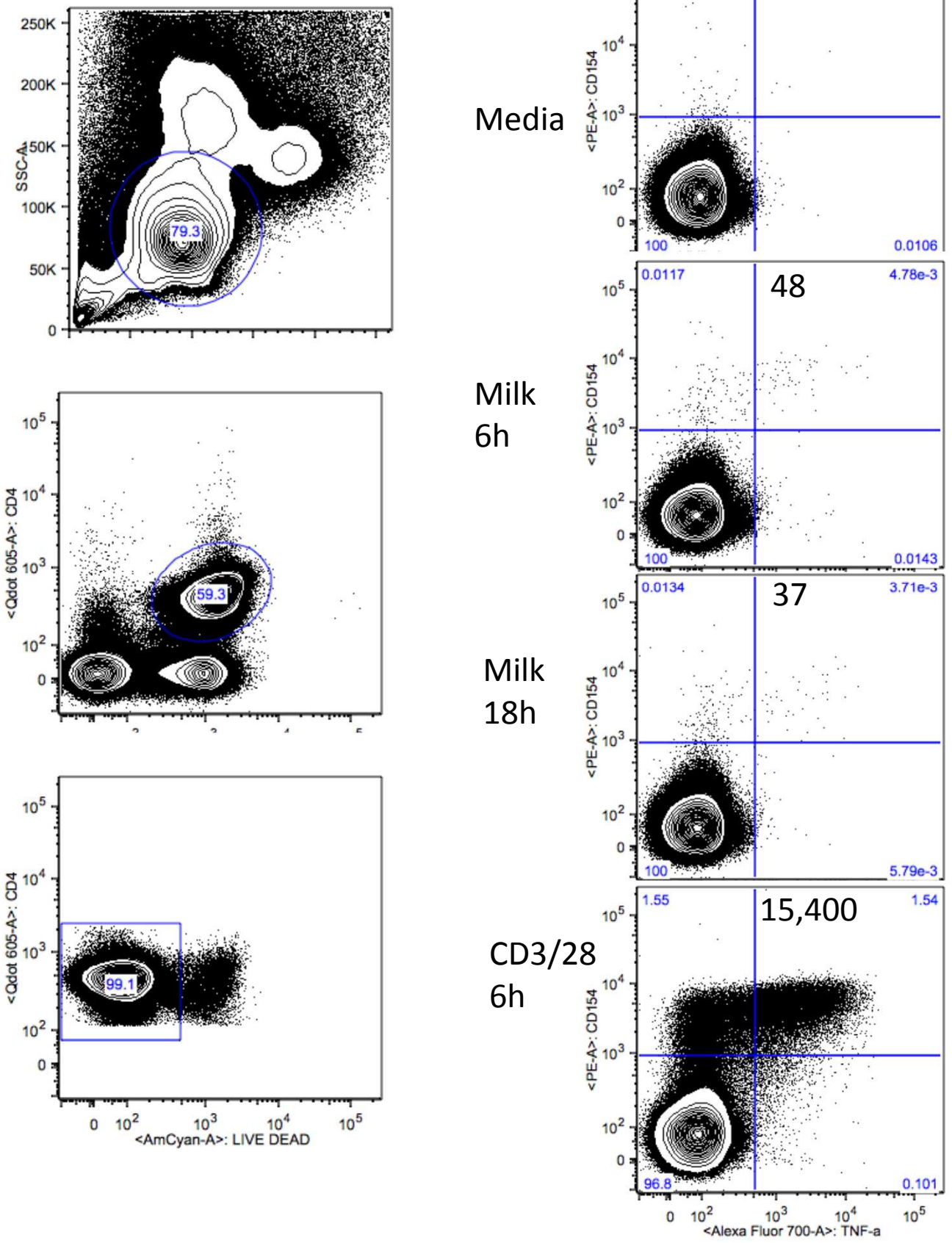
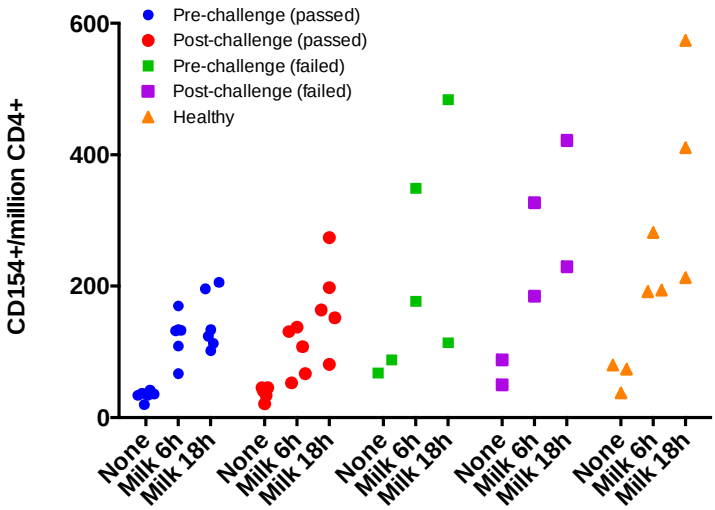
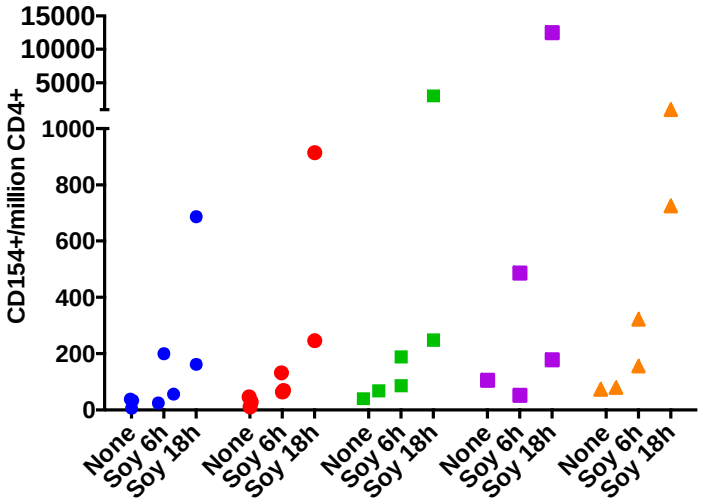


Figure E1

CD154 vs. TNFa


Pre-challenge (passed)
Post-challenge (passed)
Pre-challenge (failed)
Post-challenge (failed)
Healthy
CD154+/million CD4+
600
400
200
0
None
Milk 6h
Milk 18h


CD154+/million CD4+
15000
10000
5000
1000
800
600
400
200
0
None
Soy 6h
Soy 18h

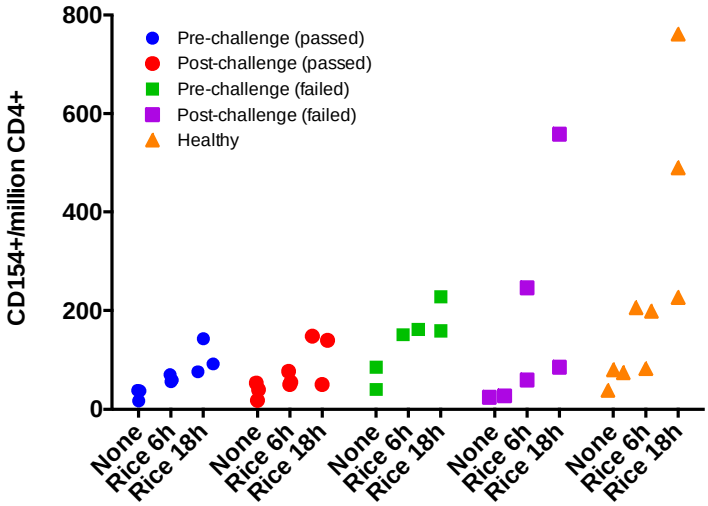

Pre-challenge (passed)
Post-challenge (passed)
Pre-challenge (failed)
Post-challenge (failed)
Healthy
CD154+/million CD4+
800
600
400
200
0
None
Rice 6h
Rice 18h

Figure E2

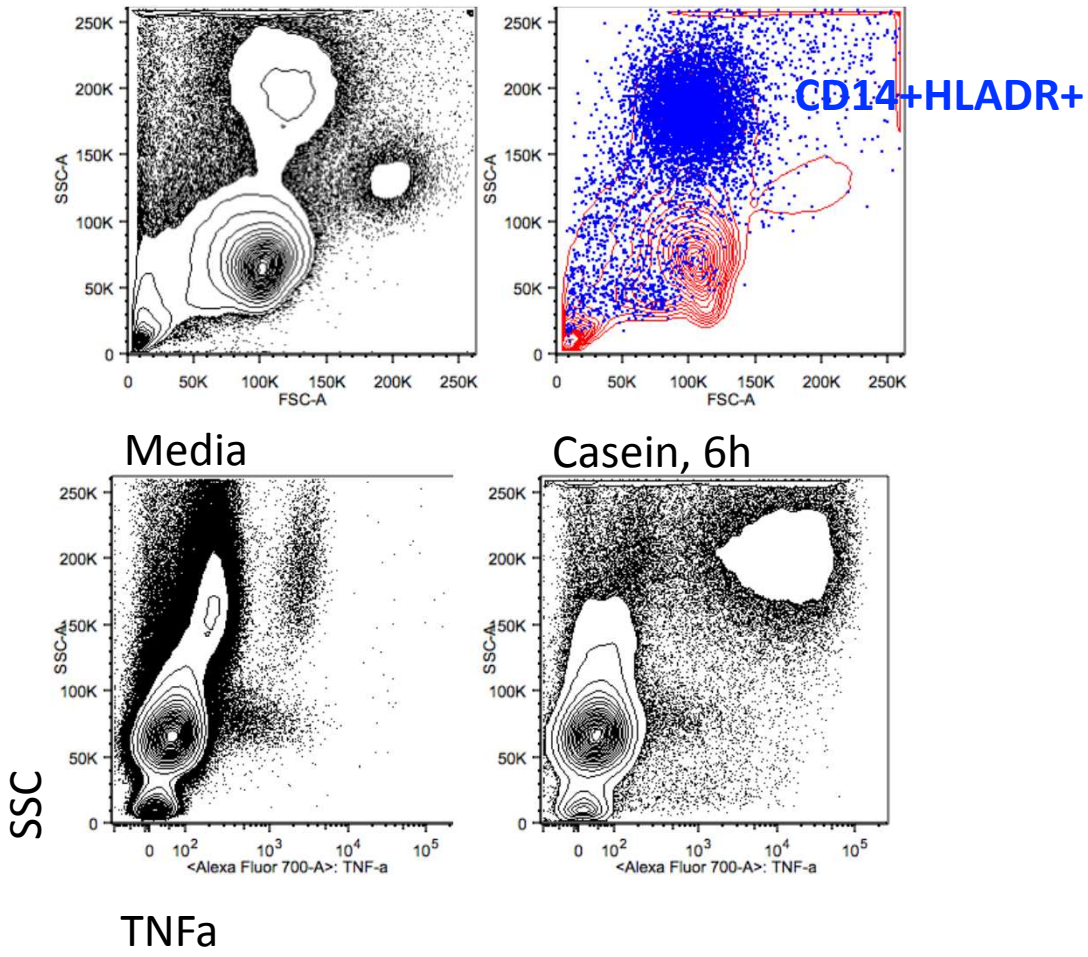

CD14+HLADR+
Media
Casein, 6h
SSC
TNFa
SSC-A
FSC-A
<Alexa Fluor 700-A>: TNF-a

Figure E3

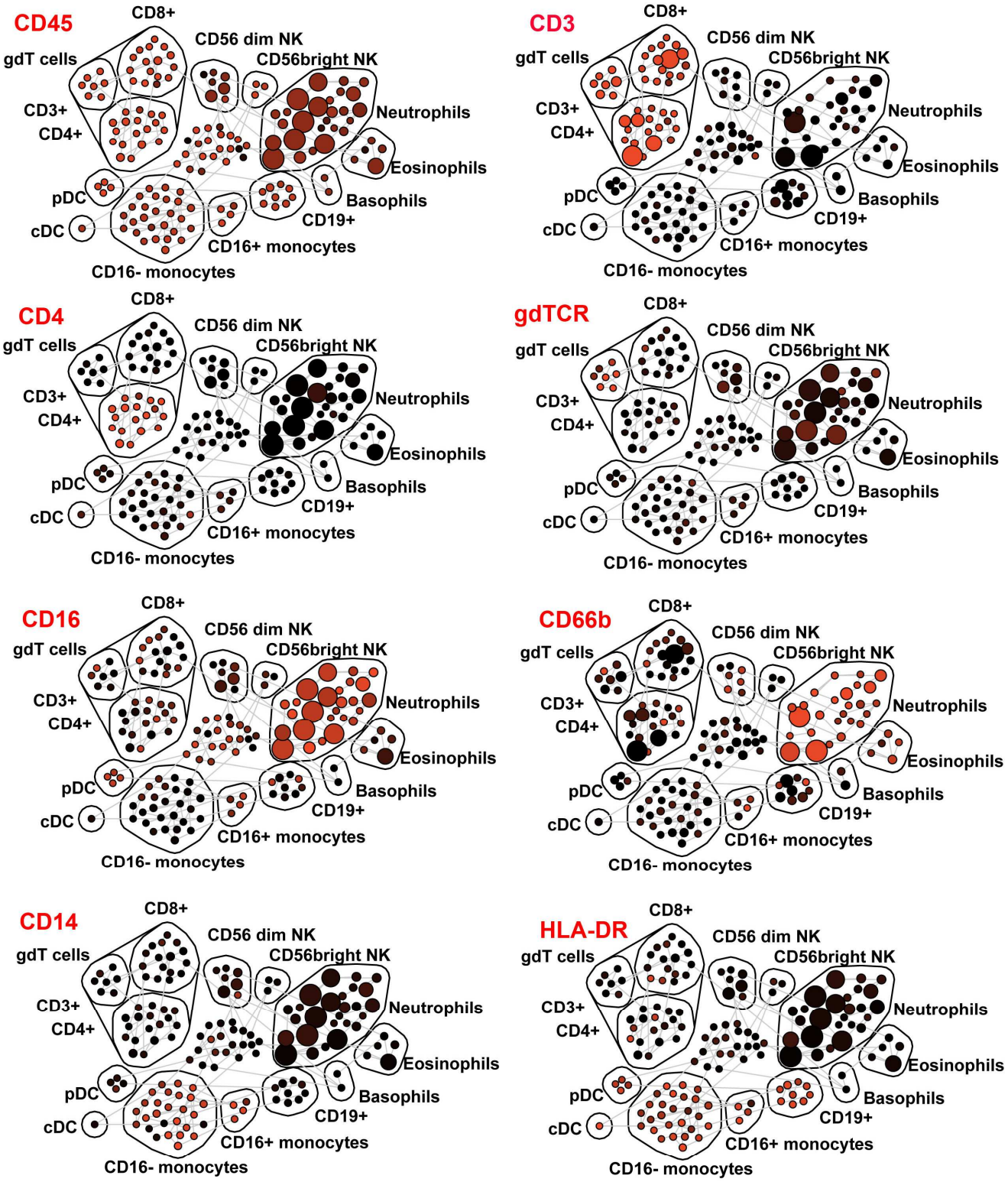

CD45
CD8+
CD56 dim NK
CD56bright NK
gdT cells
CD3+
CD4+
Neutrophils
Eosinophils
pDC
Basophils
CD19+
cDC
CD16+ monocytes
CD16- monocytes
CD3
CD4
gdTCR
CD16
CD66b
CD14
HLA-DR

Figure E4

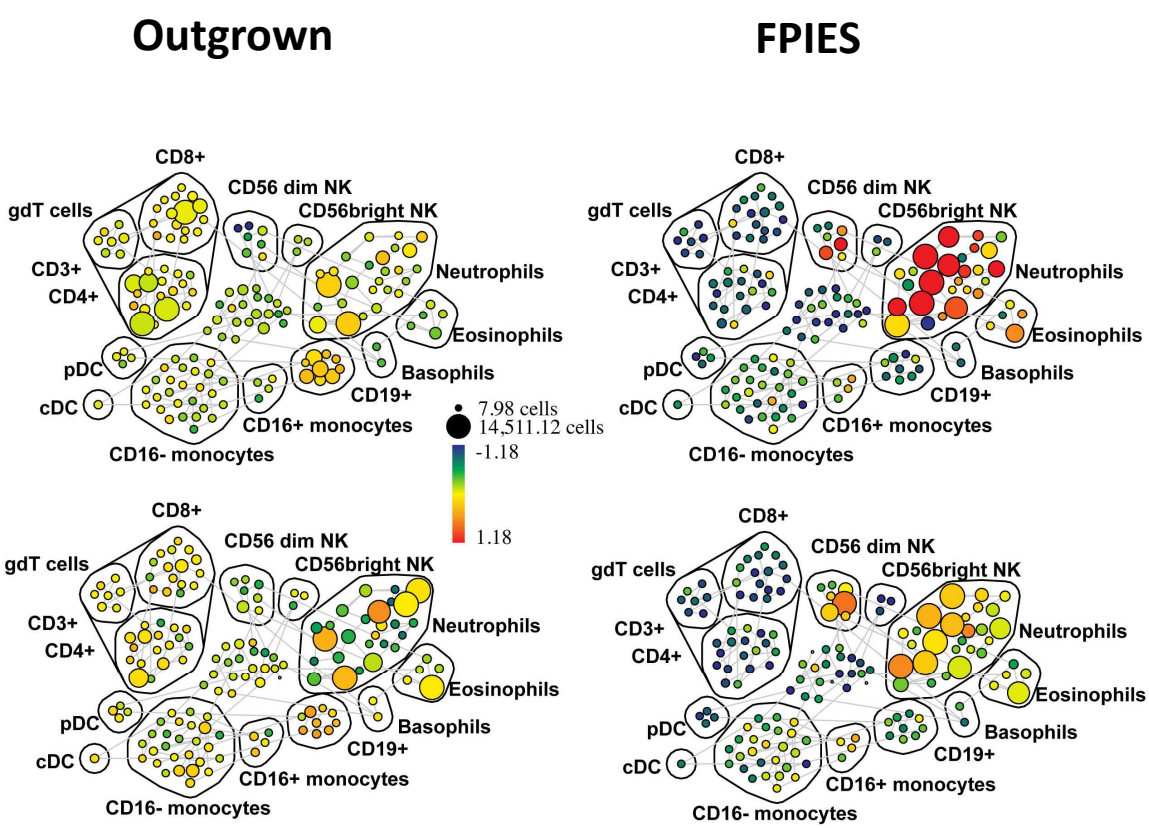
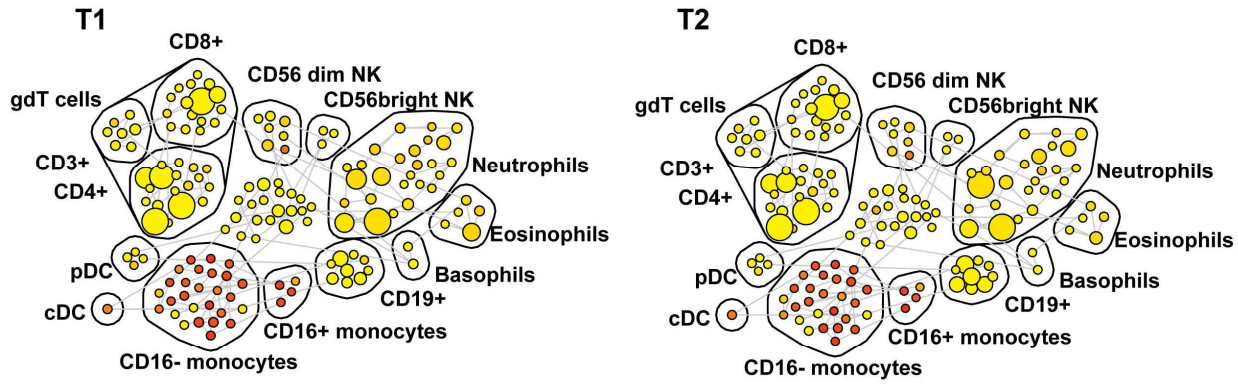

Outgrown
FPIES
CD8+
CD56 dim NK
CD56bright NK
gdT cells
CD3+
CD4+
Neutrophils
Eosinophils
pDC
Basophils
CD19+
cDC
CD16+ monocytes
CD16- monocytes
7.98 cells
14,511.12 cells
-1.18
1.18

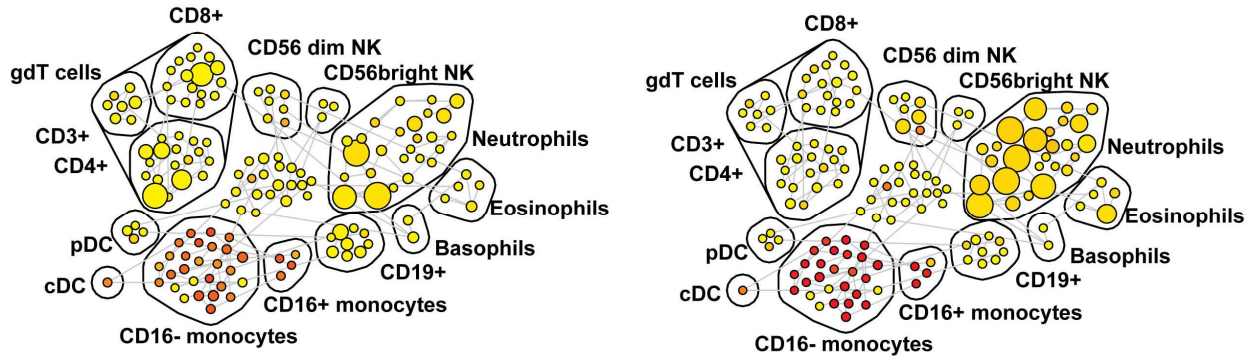
Figure E5

CD163

Outgrown



FPIES



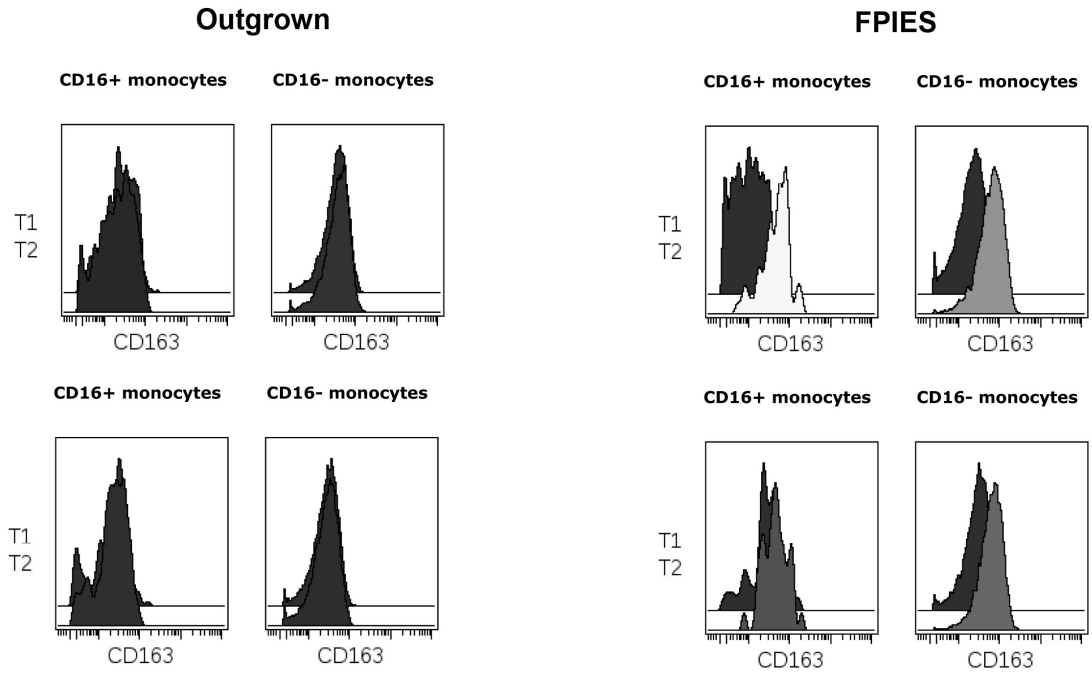
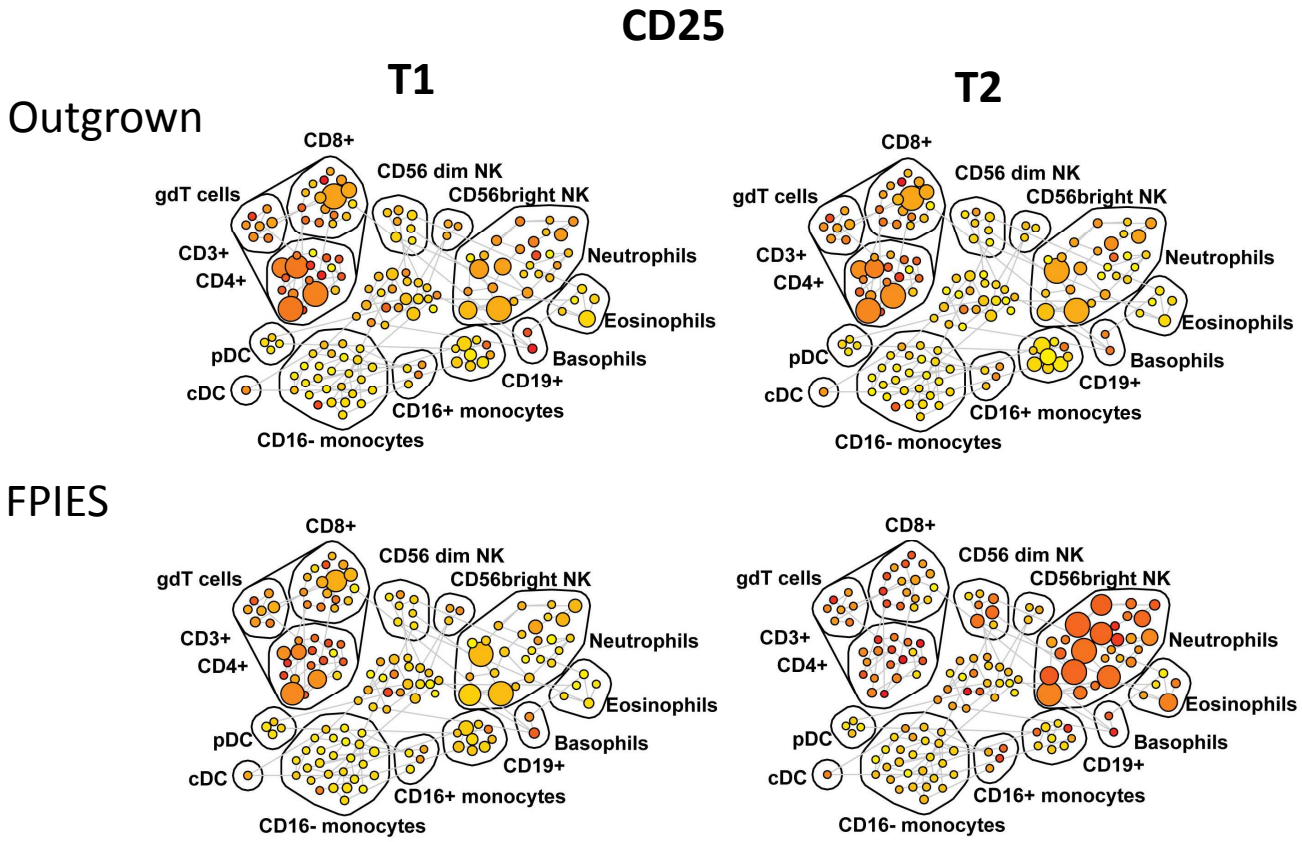


Figure E6


CD25
T1
T2
Outgrown
FPIES
CD8+
CD56 dim NK
CD56bright NK
gdT cells
CD3+
CD4+
Neutrophils
Eosinophils
pDC
Basophils
cDC
CD19+
CD16+ monocytes
CD16- monocytes

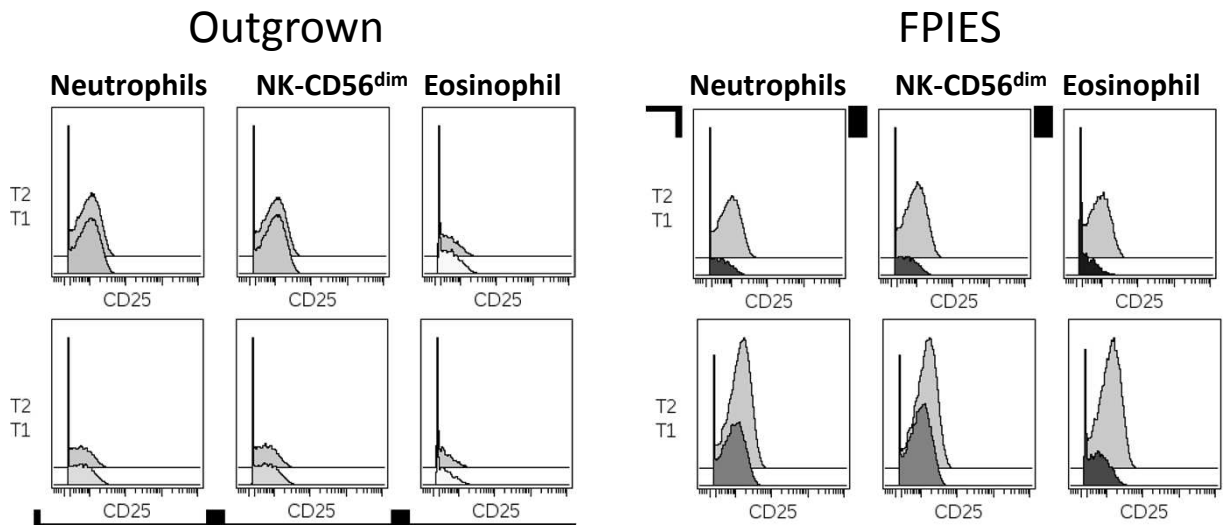

Outgrown
FPIES
Neutrophils
NK-CD56dim
Eosinophil
T2
T1
CD25

Figure E7

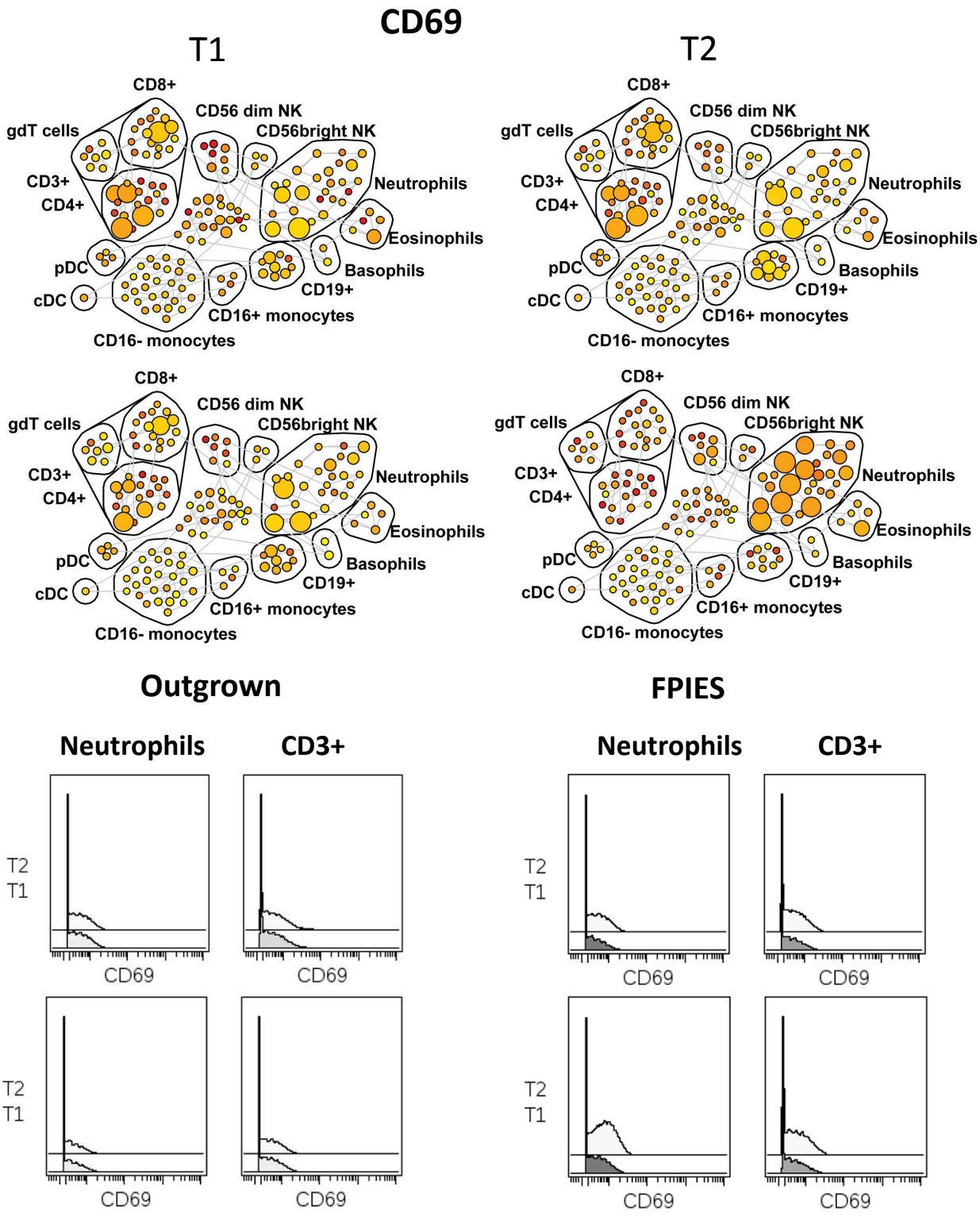

CD69
T1
T2
CD8+
CD56 dim NK
CD56bright NK
gdT cells
CD3+
CD4+
Neutrophils
Eosinophils
pDC
Basophils
CD19+
cDC
CD16+ monocytes
CD16- monocytes
Outgrown
FPIES
Neutrophils
CD3+
T2
T1
CD69

Figure E8

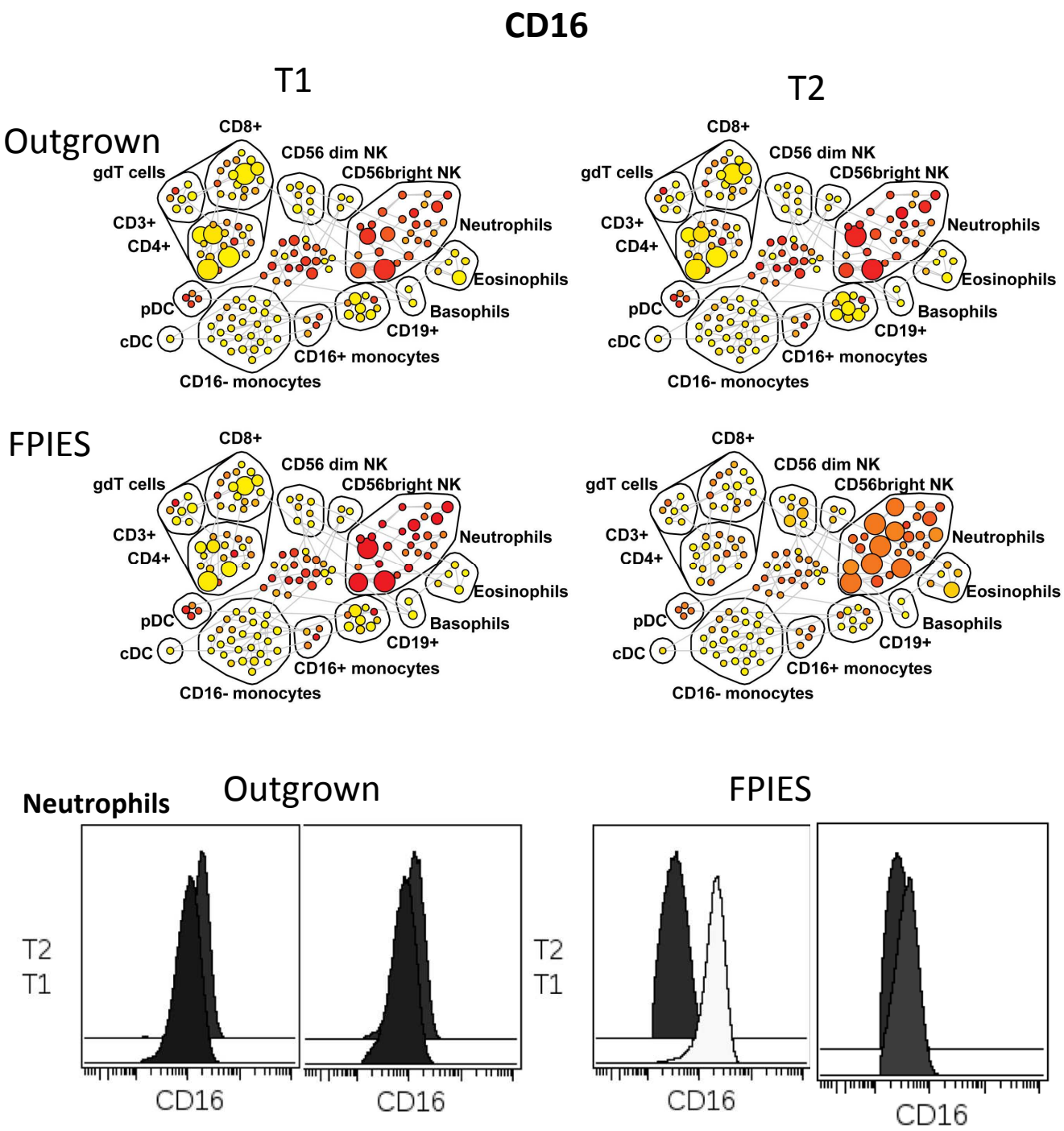

CD16
T1
T2
Outgrown
FPIES
CD8+
CD56 dim NK
CD56bright NK
gdT cells
CD3+
CD4+
Neutrophils
Eosinophils
pDC
Basophils
cDC
CD19+
CD16+ monocytes
CD16- monocytes
Neutrophils
Outgrown
FPIES
T2
T1
CD16
CD16
CD16
CD16

Figure E9