Single-cell profiling of peanut-responsive T cells in patients with peanut allergy reveals heterogeneous effector T_H2 subsets

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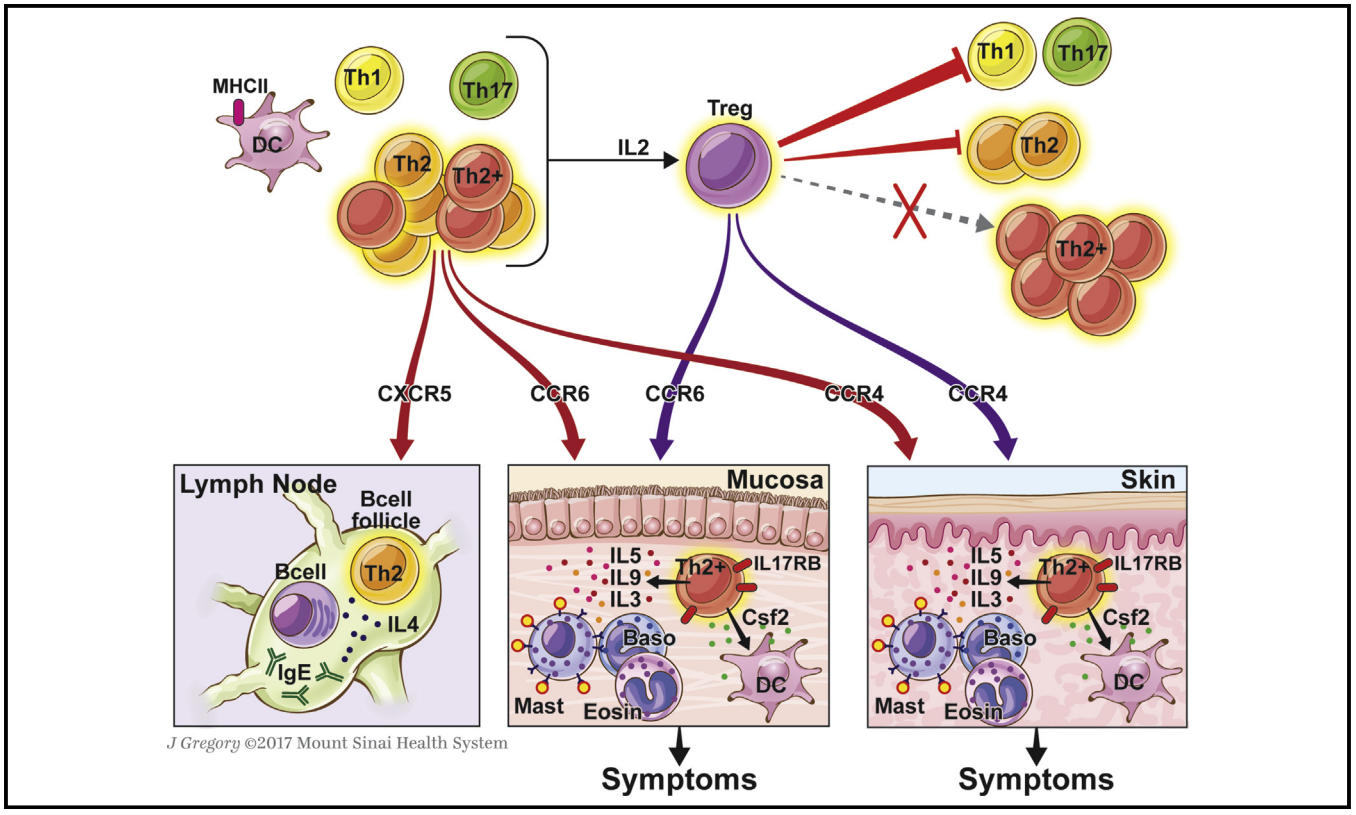
GRAPHICAL ABSTRACT



Background: The contribution of phenotypic variation of peanut-specific T cells to clinical allergy or tolerance to peanut is not well understood.

Objectives: Our objective was to comprehensively phenotype peanut-specific T cells in the peripheral blood of subjects with and without peanut allergy (PA).

Methods: We obtained samples from patients with PA, including a cohort undergoing baseline peanut challenges for an immunotherapy trial (Consortium of Food Allergy Research [CoFAR] 6). Subjects were confirmed as having PA, or if they passed a 1-g peanut challenge, they were termed high-threshold subjects. Healthy control (HC) subjects were also recruited. Peanut-responsive T cells were identified based on CD154 expression after 6 to 18 hours of stimulation with peanut extract. Cells were analyzed by using flow cytometry and single-cell RNA sequencing.

Results: Patients with PA had tissue- and follicle-homing peanut-responsive $CD4^+$ T cells with a heterogeneous pattern of T_H2 differentiation, whereas control subjects had undetectable T-cell responses to peanut. The PA group had a delayed and

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IL-2–dependent upregulation of CD154 on cells expressing regulatory T (Treg) cell markers, which was absent in HC or high-threshold subjects. Depletion of Treg cells enhanced cytokine production in HC subjects and patients with PA *in vitro*, but cytokines associated with highly differentiated T_H2 cells were more resistant to Treg cell suppression in patients with PA. Analysis of gene expression by means of single-cell RNA sequencing identified T cells with highly correlated expression of *IL4*, *IL5*, *IL9*, *IL13*, and the IL-25 receptor *IL17RB*.

Conclusions: These results demonstrate the presence of highly differentiated T_H2 cells producing T_H2-associated cytokines with functions beyond IgE class-switching in patients with PA. A multifunctional T_H2 response was more evident than a Treg cell deficit among peanut-responsive T cells. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: *Food allergy, peanut allergy, T_H2, regulatory T, tolerance*

Abbreviations used

CoFAR:	Consortium of Food Allergy Research
CRTH2:	Chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes
CSF2:	Colony-stimulating factor 2
DEG:	Differentially expressed gene
FoxP3:	Forkhead box P3
HC:	Healthy control
HT:	High threshold
MSSM:	Mount Sinai School of Medicine
PA:	Peanut allergy
peT_H2:	Pathogenic effector T_H2
RNA-seq:	RNA sequencing
TCR:	T-cell receptor
Treg:	Regulatory T

Peanut allergy (PA) is believed to arise from defective oral tolerance pathways or a lack of dietary exposure in early life. In mouse models oral tolerance is an active state of immune regulation mediated by peripherally induced forkhead box p3 $(FoxP3)^+$ regulatory T (Treg) cells that are educated by gastrointestinal dendritic cells through TGF-β– and retinoic acid–dependent mechanisms.[1,2] Support for the clinical importance of oral tolerance comes from the Learning Early About Peanut study, which demonstrated that dietary exposure to peanut early in life (4-11 months of age) could suppress the development of PA in infants with increased risk of PA.[3] The failure or lack of oral tolerance is thought to be a requisite for the development of T_H2-skewed immunity that underlies the pathologic immune responses in patients with PA and other allergic diseases.[4] The T_H2 cytokine IL-4 is necessary for B-cell class-switching to IgE, and a peanut-specific T_H2-skewed $CD4^+$ T-cell profile has been found in patients with PA by using methods from proliferation-based assays to MHC class II tetramers.[5-8]

T_H2-biased immunity can be a benign feature of the young immune system, particularly when partnered with IL-10 production.[9] Therefore features beyond L-4 production might be necessary for pathogenicity, such as homing to B-cell follicles or tissues. Other T_H2-associated cytokines can also contribute to pathogenesis. IL-9 has begun to emerge as an important cytokine associated with food allergy in both mouse models and allergic subjects.[10-12] Treg cells have been reported as a source of IL-4 in mice and human subjects, and these "reprogrammed"

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TABLE I. Demographic characteristics of the study population

	HC subjects	Patients with PA, CoFAR6 cohort	HT subjects, CoFAR6 cohort	Patients with PA, MSSM cohort
No.*	21	75	9	51
Mean age (y), minimum-maximum	30.5 (23-44)	9.5 (4-20)	9.7 (4-16)	10.5 (3-20)
Sex (male/female)	13/7	46/29	7/2	31/19†
Allergy assessment	Self-report	Oral food challenge	Oral food challenge	Convincing clinical history
Median peanut-specific IgE (kU_A/L), minimum-maximum	0 (0-0.29)	78.6 (0.41->100)‡	5.51 (0.25-13.3)	46.2 (0->100)‡
Median peanut-specific IgG_4 (mg_A/L), minimum-maximum	0.13 (0.04-2.58)	0.64 (0.02-7.01)	0.51 (0.13-2.24)	ND

ND, Not measured.
*Numbers are cumulative; numbers for each experiment are presented in the figure legends.
†Sex and age are unreported for 1 subject.
‡Values of greater than 100 kU_A/L were truncated.

T_H2 cells might contribute to food allergy through both T_H2 cytokine production and defective regulatory function.[13] These studies highlight a growing appreciation of the complexity of the food-specific T-cell response in patients with food allergy.

We hypothesized that clinical PA results from a combination of disordered proinflammatory T_H2 immunity and an antigen-specific defect in regulatory activity, and we tested this hypothesis in well-characterized patient cohorts and healthy control (HC) subjects, including 84 primarily pediatric subjects who underwent a double-blind, placebo-controlled food challenge to peanut. We performed single-cell analysis using flow cytometry and RNA sequencing (RNA-seq) to determine the phenotype of the T-cell response underlying allergy or tolerance to peanut. Our data show that clinical tolerance to foods is associated with immunologic ignorance or anergy, and the defining feature of PA is the presence of antigen-specific cells, including highly differentiated, tissue-homing, granulocyte growth factor–producing T_H2 cells that might be less susceptible to regulation by Treg cells. The clinical implication is that strategies to eliminate effector T_H2 cells might be more effective for the treatment of PA than strategies aimed at expanding the antigen-specific regulatory response.

METHODS

Human subjects

Informed consent was obtained from all subjects or parents/guardians, and all procedures were approved by the institutional review boards at each of the 5 clinical sites. Blood samples were obtained at baseline from a clinical trial conducted by the Consortium of Food Allergy Research (CoFAR) investigating epicutaneous immunotherapy for PA (CoFAR6; ClinicalTrials.gov identifier NCT01904604).[14] Subjects who responded to a double-blind, placebo-controlled food challenge with a cumulative dose of up to 1 g of peanut were categorized as having PA, whereas those who tolerated the challenge were termed high-threshold (HT) subjects. Additional patients with PA and HC subjects were recruited from the Mount Sinai School of Medicine (MSSM) cohort under other institutional review board–approved protocols. MSSM patients with PA were recruited based on a convincing clinical history but did not undergo oral food challenge as part of this study. Subjects' demographics, including peanut-specific IgE levels, are detailed in Table I. An experimental overview is shown in Fig E1 in this article's Online Repository at www.jacionline.org.

Blood processing and PBMC isolation

For CoFAR6 samples, blood was shipped overnight from participating sites in temperature-controlled boxes (GreenBox; ThermoSafe, Arlington Heights, Ill) with temperature monitors. PBMCs were isolated by means of density centrifugation with Ficoll-Paque Plus (GE Healthcare, Pittsburgh, Pa) and cultured in AIM V medium (Gibco, Grand Island, NY) with 2.5% autologous plasma.

PBMC stimulation

PBMCs were stimulated with 100 μg/mL crude peanut extract or 5 μL/mL anti-CD3/CD28 beads (Invitrogen, Carlsbad, Calif) for 6 hours, 18 hours, 24 hours, or 5 days. Endotoxin was removed from peanut extract by using Detoxi-Gel columns (Thermo Scientific, Waltham, Mass). Residual endotoxin levels measured with the LAL assay (Thermo Scientific) were 14 pg/mL. GolgiPlug (BD Biosciences, San Jose, Calif) was added 4 hours before harvesting. For surface CD154 staining (sorting), blocking anti-CD40 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the culture to maintain CD154 at the surface. In some experiments cells were stimulated with rhIL-2 (STEMCELL Technologies, Vancouver, British Columbia, Canada) at 5 ng/mL, or neutralizing anti–IL-2 (R&D Systems, Minneapolis, Minn) was added at 500 ng/mL. Phorbol 12-myristate 13-acetate/ionomycin/brefeldin cocktail (BD Biosciences) was applied for 6 hours to stimulate cells in some experiments.

Flow cytometry

Harvested PBMCs were stained for viability (Live/Dead Fixable stain; Invitrogen), washed and stained for surface markers, and washed for fixation and permeabilization. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Services, Hatfield, Pa) and treated with permeabilization buffer (eBioscience, San Diego, Calif) before staining with labeled antibodies to detect intracellular CD154 and cytokines. For FoxP3 staining, cells were processed with the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) before staining with antibodies. Antibody panels used for surface and cytoplasmic staining are shown in Tables E1-E4 in this article's Online Repository at www.jacionline.org. Stained cells were analyzed subsequently on an LSR Fortessa (BD Biosciences). For Treg cell sorting or depletion, harvested PBMCs were stained with CD4–Alexa Fluor 488 (Clone OKT4; BioLegend, San Diego, Calif), CD25–Alexa Fluor 700 (Clone BC96; BioLegend), and CD127–phycoerythrin-Cy7 (Clone eBioRDR5; eBioscience). Cells were sorted on the FACSAria IIu (BD Biosciences).

Peanut-specific IgE and IgG_4

Frozen plasma samples were thawed, and peanut-specific IgE and IgG_4 levels were measured by using ImmunoCAP (Thermo Fisher Scientific).

Secreted cytokine measurement

Cell-culture supernatants were centrifuged and stored at −80°C. Supernatant cytokines were measured by using the ProcartaPlex Human Cytokine

Panel 1B (eBioscience) and the Luminex 200 System (Luminex, Austin, Tex) in Mount Sinai's Human Immune Monitoring Core Facility. In other experiments cytokines were measured with the LEGENDplex Human Th Cytokine Panel, according to the manufacturer's instructions (BioLegend), and acquired on the BD LSRFortessa (BD Biosciences).[15] Sensitivity of the 2 cytokine measurement kits is shown in Tables E5 and E6 in this article's Online Repository at www.jacionline.org.

Enrichment and fluorescence-activated cell sorting of CD154$^+$ T cells

PBMCs stimulated for 18 hours were harvested and enriched for CD154$^+$ cells by using the CD154 MicroBead Kit (Miltenyi Biotec). Enriched cells were stained with CD3–allophycocyanin-Cy7 (Clone SK7; eBioscience), CD4–Alexa Fluor 488 (Clone OKT4; BioLegend), and CD154–streptavidin/phycoerythrin (Clone 24-31; eBioscience). CD3$^+$CD4$^+$CD154$^+$ T cells were sorted on the BD FACSAria IIu (BD Biosciences) in Mount Sinai's Flow Cytometry Core Facility and resuspended according to the manufacturer's protocols (Fluidigm, South San Francisco, Calif).

CD154$^+$ T cells were captured after 18 hours of stimulation and sort purified to obtain CD154$^+$ cells at greater than 99% purity. We used the Fluidigm C1 pipeline to obtain single-cell cDNA, prepared barcoded cDNA libraries, and performed 100 NT paired end-read sequencing on multiplexed cells (48 cells per lane) with the Illumina HiSeq (Illumina, San Diego, Calif).

Fluidigm C1 processing and cDNA library construction of single-cell RNA-seq

Sorted Treg and CD154$^+$ T cells were resuspended and loaded onto a Fluidigm C1 Single-Cell Auto Prep IFC for Fluidigm C1 processing, according to the manufacturer's protocols. Single cells were visualized by means of microscopy, and wells containing more than 1 cell were noted and excluded from further steps. cDNA was generated according to Fluidigm C1 protocols with the SMARTer Ultra Low RNA Kit for the Fluidigm C1 System reagents (Clontech Laboratories, Mountain View, Calif). Resultant cDNA from Fluidigm C1 processing was subsequently assessed for quantity and quality by using a Qubit Fluorometer 3.0 (Thermo Fisher Scientific) and 2100 Bioanalyzer or 2200 TapeStation (Agilent Technologies, Santa Clara, Calif). cDNA that passed quality control was subsequently processed for library construction by using Nextera XT DNA Library Prep and Nextera XT Index Kits (all from Illumina). Amplified single-cell libraries were then pooled and submitted to Mount Sinai's Genomics Core Facility for sequencing on the Illumina HiSeq 2500 (100-bp paired-end reads).

Single-cell analysis

RNA-seq raw fastq data were aligned to a hg38 reference genome (UCSC) by using STAR (2.4.2a). Aligned reads were mapped to hg38 genes by using featureCounts from the Subread package (1.4.4).[16] Cells with reads of less than 5×10^5 were removed. The negative binomial model–based method edgeR (3.10.0)[15] was used for single-cell differential expression analysis. Genes expressed in at least 1 cell with at least 1 read per million mapped were kept. The relative log expression method was used to calculate normalization factors between samples. We included plates as covariates to account for any confounding effect of subjects in the generalized linear model and performed quasilikelihood F tests for hypothesis testing. Genes with false discovery rates of less than 0.05 were defined as differentially expressed genes (DEGs). The data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO)[17] and are accessible through GEO Series accession number GSE98852 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE98852).

Molecular state clustering

The Monocle (1.2.0) toolkit was used to find DEGs between cell types/states and to cluster CD154$^+$ cell subgroups.[18] Genes expressed at low levels (<1 read per million mapped in 1 subject) were filtered out, and read counts were converted into reads per kilobase per million mapped reads. Independent component analysis was carried out for dimensional reduction of gene expression. DEGs generated with edgeR-QL were fed as guide genes to order the cells.

T-cell receptor clonal inference

T-cell receptor (TCR) clonal analysis was based on TraCeR (0.1).[19] TCR sequences were reconstructed by means of alignment of reads to synthetic human TCR genomes (all possible V-J combinations provided by TraCeR) by using bowtie2 (2.1.0)[20] and assembled reads into TCR contigs by using Trinity (2.2.0).[21] The assembled contigs were then aligned against the IMGT human database by using IgBlast (1.5.0)[22] and quantified as transcripts per million by using kallisto (0.43.0).[23] Only the 2 most highly expressed TCRs from each locus were retained. We searched for cells sharing exact TCR sequences for each cell type.

RESULTS

Peanut-specific T-cell responses and clinical phenotype

Peripheral blood was obtained from a cohort of primarily pediatric patients with PA who reacted to an oral challenge dose of less than 1 g of peanut (CoFAR cohort), subjects with a history of PA and detectable peanut-specific IgE who tolerated a cumulative dose of 1 g of peanut (HT subjects), and healthy adult control subjects who self-reported tolerance to peanut (HC subjects). The PA and HT groups were obtained from subjects undergoing baseline peanut challenges as part of enrollment screening for CoFAR6, a trial of epicutaneous peanut immunotherapy.[14] Demographic information is shown in Table I.

Freshly isolated PBMCs were cultured with peanut extract for 6 hours, harvested, and stained for surface markers and intracellular cytokines. Peanut-responsive T cells were identified by means of expression of CD154, also known as CD40 ligand, which is upregulated on T cells in response to TCR engagement and is essential for providing T-cell help to B cells. Coexpression of the cytokines IL-4, IL-13, IFN-γ, and IL-10 was determined (representative plots of CD154 vs IL-4 and IFN-γ are shown in Fig 1, *A*). The 6-hour stimulation time point was chosen as optimal for intracellular cytokine detection (see Fig E2 in this article's Online Repository at www.jacionline.org).

We observed a significant increase in numbers of CD154$^+$CD4$^+$ T cells after peanut stimulation in patients with PA but not in HT or HC subjects (Fig 1, *B*). Stimulation with egg white protein induced CD154 expression in patients with PA who had egg allergy but not in those who were egg tolerant (data not shown), demonstrating specificity for clinical reactivity. Cytokine expression by CD154$^+$ T cells in patients with PA was dominated by IL-4 and IL-13, with a low but statistically significant increase in IL-10 levels and no significant IFN-γ response (Fig 1, *C*). No increase in numbers of CD154$^+$ cells of any cytokine phenotype was elicited from CD4$^+$ T cells from HT or HC subjects. The frequency of peanut-responsive CD154$^+$IL-4$^+$ or CD154$^+$IL-13$^+$ CD4$^+$ T cells correlated significantly with the level of peanut-specific IgE in patients with PA (Spearman $r = 0.68$ for both IL-4 and IL-13, $P < .0001$). There was no correlation of peanut-responsive T_H2 cell numbers with age (Spearman $r = 0.093$, $P = .46$ and Spearman $r = 0.089$, $P = .48$ for IL-13 and IL-4, respectively; see Fig E3 in this article's Online Repository at www.jacionline.org).

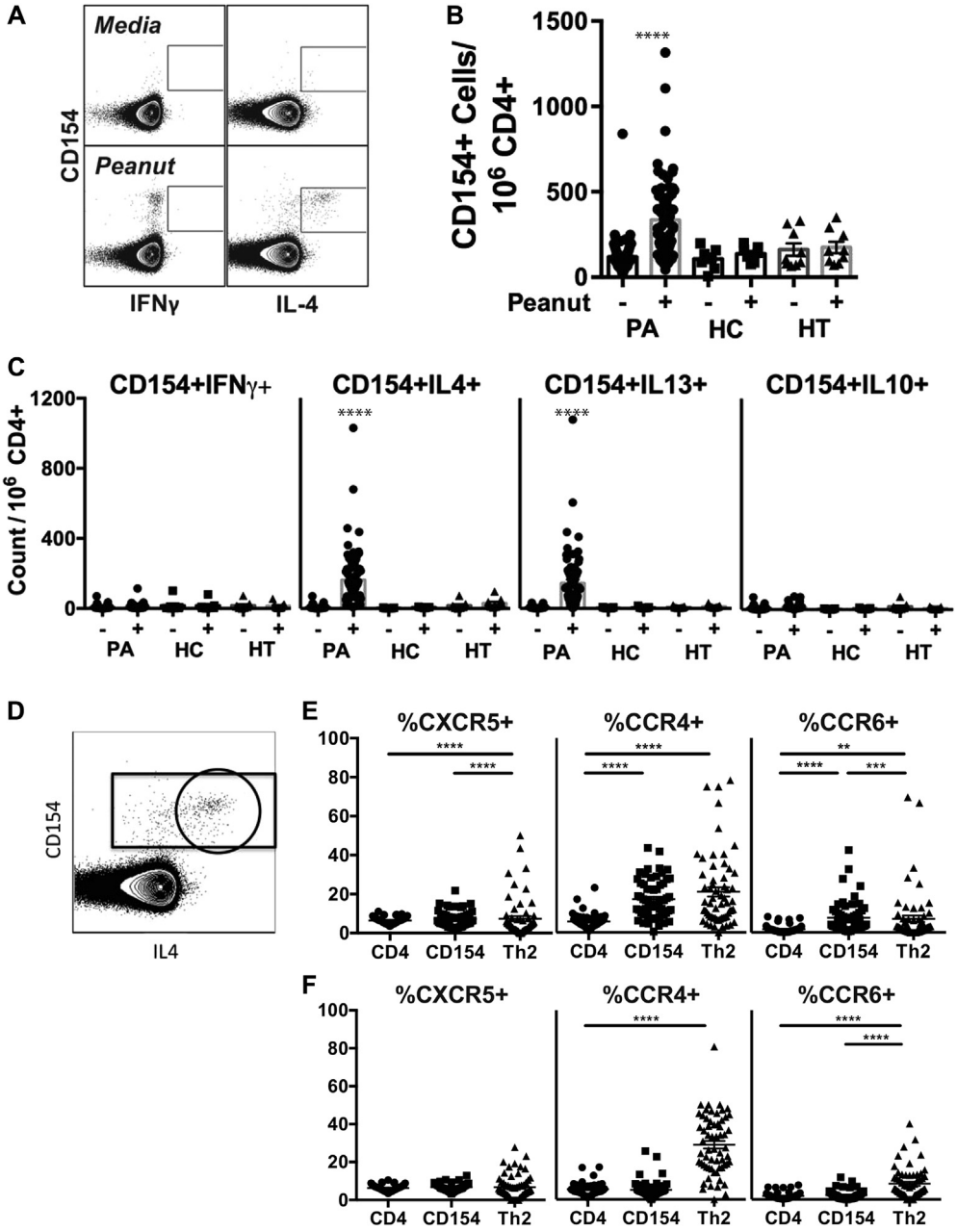

FIG 1. Immunologic responses to peanut stimulation in allergic patients and control subjects. **A,** Representative dot plot of CD154 response to peanut in $CD3^+CD4^+$ cells. **B,** Quantification of $CD154^+$ T cells in patients with PA (n = 69, CoFAR cohort), HC subjects (n = 7), and HT subjects (n = 9) after culture with peanut *(+)* or media *(−)*. **C,** Quantification of total cytokine-positive $CD154^+$ T cells in patients with PA, HC subjects, and HT subjects for IFN-γ, IL-10, IL-4, and IL-13 in response to peanut stimulation. Statistics were calculated by using the Kruskal-Wallis test with the Dunn multiple comparison test. **D-F**, Total $CD3^+CD4^+$ T cells, $CD154^+$ T cells *(rectangle)*, and T_H2 cells ($IL\text{-}4^+CD154^+$ T cells; *gated circle*) were evaluated for CXCR5 (n = 60), CCR4 (n = 61), and CCR6 (n = 61) expression (calculated as percentage positive within indicated populations; Fig 1, *D*) after stimulation for 6 hours with peanut extract (Fig 1, *E*) or anti-CD3/CD28 (Fig 1, *F*). Statistics were calculated by using the Friedman test with the Dunn posttest. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

We examined chemokine receptor expression on peanut-responsive T cells from patients with PA to assess homing capacity (Fig 1, *D-F*). We examined the marker CCR4, which supports homing to the skin and lung; the mucosal homing marker CCR6; and the B-cell follicle homing marker CXCR5. A significantly greater frequency of peanut-responsive $CD4^+$ T cells expressed CCR4 and CCR6 compared with the total $CD4^+$ T-cell population (Fig 1, *E*). This was not due to activation because polyclonally activated $CD4^+$ T cells did not show a similar enrichment in chemokine receptor frequency (Fig 1, *F*). CCR4 and CCR6 were both enriched on activated T_H2 cells (defined as $CD154^+IL\text{-}4^+$ cells; circled in Fig 1, *D*). CXCR5 was significantly enriched on peanut-responsive T_H2 cells but not polyclonally activated T_H2 cells. These data reveal that

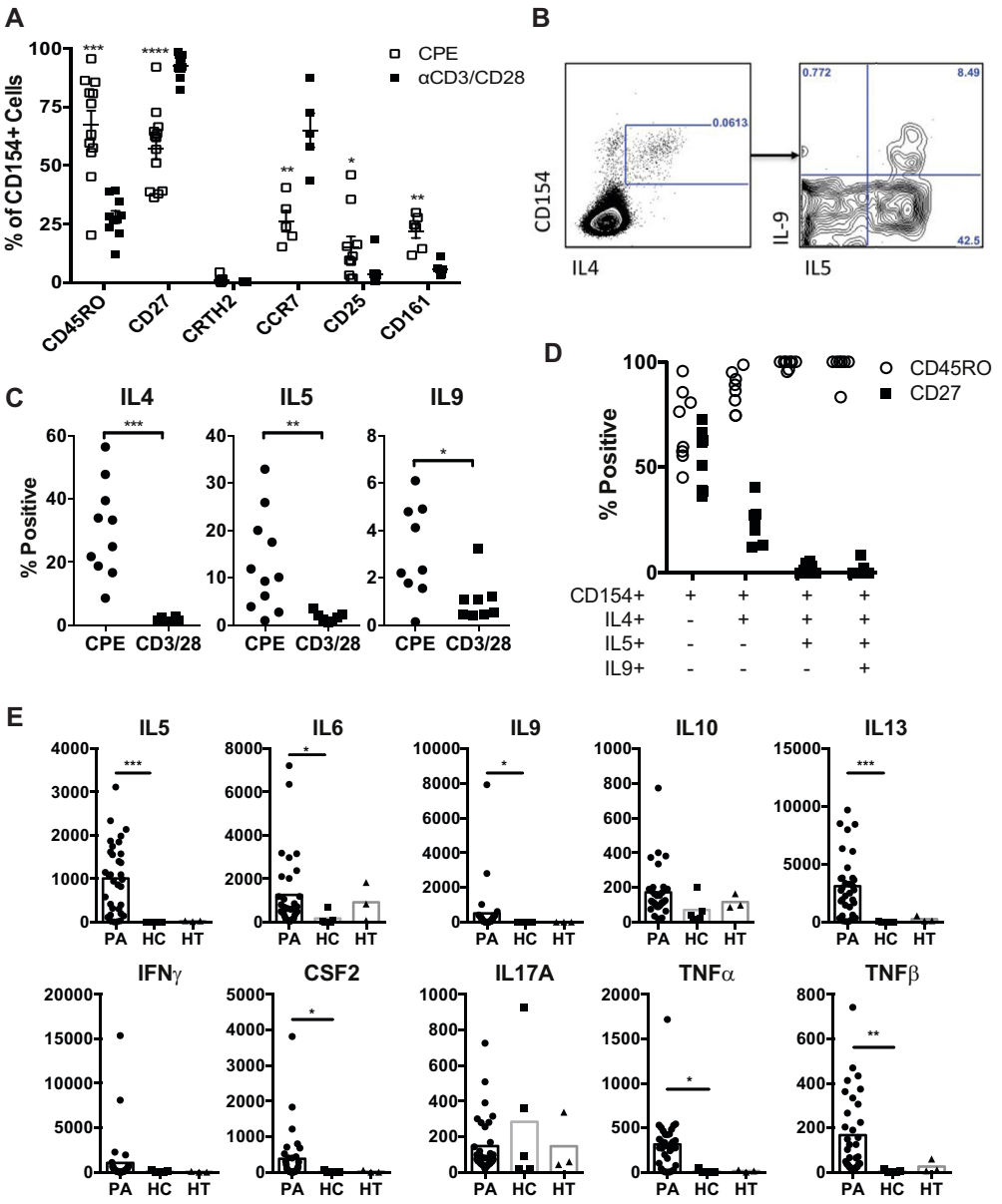

FIG 2. Phenotypic heterogeneity of peanut-responsive T_H2 cells. **A,** Frequency of expression of memory and differentiation markers on $CD154^+$ cells after stimulation with peanut (crude peanut extract *[CPE]*) or anti-CD3/CD28. Each *symbol* represents 1 subject (n = 6-13, MSSM PA cohort). **B,** Representative flow cytometric plot showing coexpression of IL-5 and IL-9 in peanut-responsive T_H2 ($CD154^+IL\text{-}4^+$) cells. **C,** Percentage frequency of $CD154^+$ T cells coexpressing indicated cytokines after stimulation with peanut (CPE) or anti-CD3/CD28. Each *symbol* represents an individual subject (n = 6-11, MSSM PA cohort). **D,** Memory marker expression on each cell subset. Each *bar* represents the mean and SEM of 8 subjects (MSSM PA cohort). **E,** Quantification of secreted cytokines after peanut stimulation of PBMCs from patients with PA (n = 33), HC subjects (n = 5), and HT subjects (n = 3) for 5 days. Statistics were calculated by using the Mann-Whitney *U* test. $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

peanut-responsive T_H2 cells from patients with PA include skin, lung, and intestinal homing populations, as well as follicle-homing T cells with the potential to regulate IgE production.

Phenotypic heterogeneity of peanut-responsive T_H2 cells in patients with PA

We recruited additional patients with PA to perform phenotypic analysis of peanut-responsive T cells (MSSM cohort, demographic information in Table I). Memory and differentiation markers, including CD45RO, CD27, CCR7, CD25, CD161, and chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes (CRTH2), were examined. $CD4^+$ T cells upregulating CD154 after peanut exposure were compared with $CD4^+$ T cells upregulating CD154 after polyclonal stimulation to control for effects of activation (Fig 2, *A*). Peanut-activated cells were enriched for the memory marker CD45RO and expressed less CD27 and CCR7 compared with polyclonally activated T cells. CD25 and CD161 expression was also enriched on peanut-activated T cells, although these markers were expressed

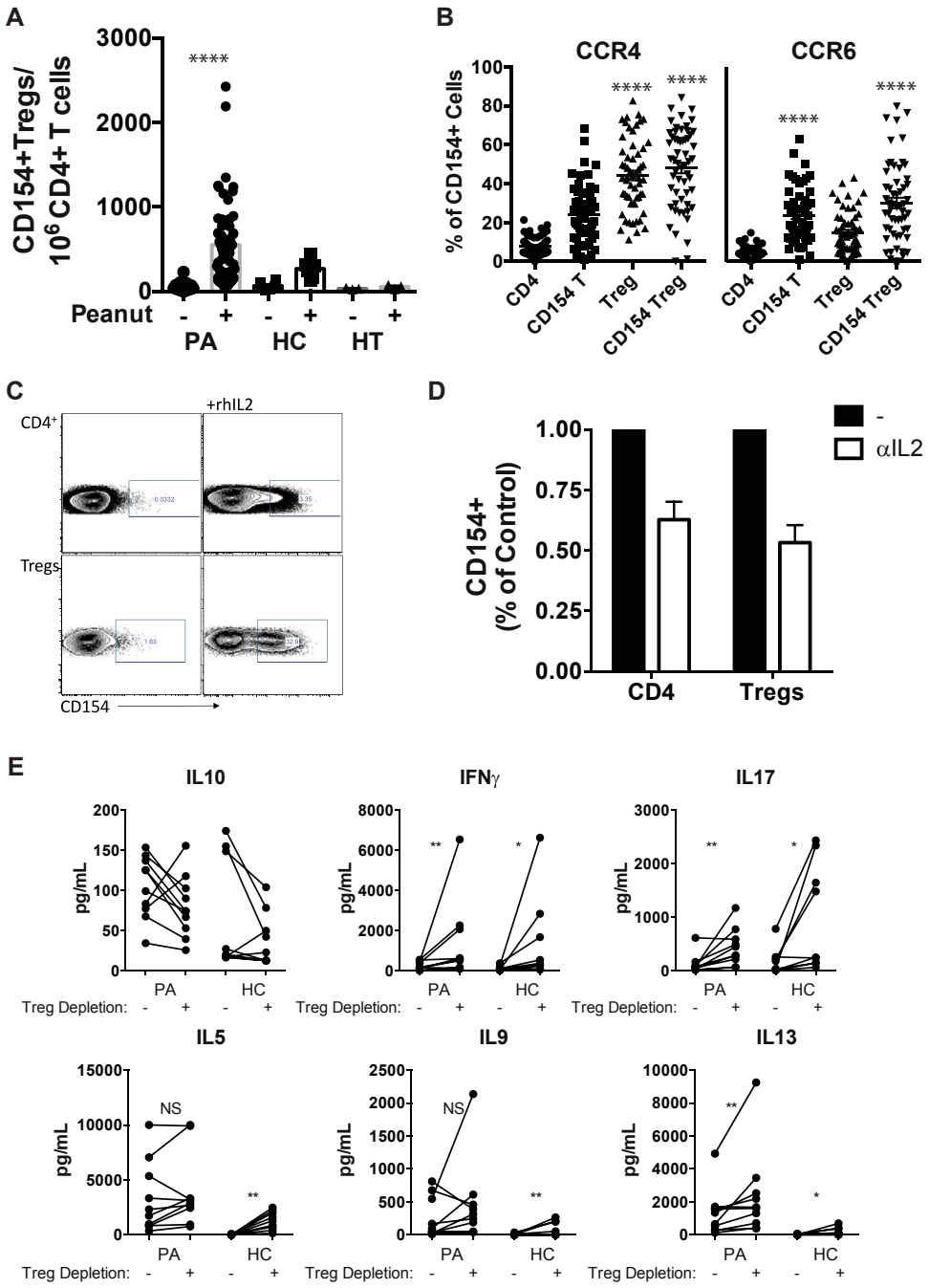

FIG 3. Identification and phenotypic analysis of peanut-responsive Treg cells. **A,** Quantification of $CD154^+FoxP3^+CD25^+CD127^{low}CD4^+$ T cells after stimulation with peanut *(+)* for 18 hours in patients with PA (n = 62, CoFAR cohort), HC subjects (n = 6), and HT subjects (n = 3). **B,** Expression of CCR4 and CCR6 (n = 57, CoFAR PA cohort) on $CD4^+$ T cells, $CD154^+CD4^+$ T cells *(CD154 T)*, $FoxP3^+CD25^+CD127^{low}$ Treg cells *(Treg)*, and $CD154^+FoxP3^+CD25^+CD127^-$ cells *(CD154 Treg)* after peanut stimulation. **C,** Representative dot plots showing the effect of rhIL-2 on CD154 expression in $CD4^+$ T cells or Treg cells after 18 hours. **D,** Effect of IL-2 neutralization on CD154 expression on $CD4^+$ T cells or Treg cells after 18 hours of peanut stimulation (n = 4 patients with PA). **E,** Effect of Treg cell depletion (removal of $CD3^+CD4^+CD25^{high}CD127^{low}$ by using fluorescence-activated cell sorting) on peanut-induced cytokine secretion. Individual values are shown for patients with PA (MSSM cohort, n = 10) or HC subjects (n = 9). $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$. *NS,* Not significant. Statistics were calculated with the Wilcoxon matched pairs signed-rank test (Fig 3, *A* and *E*) or the Friedman test with the Dunn posttest correction (Fig 3, *B*).

by a minority of peanut-responsive T cells. The marker CRTH2, which is constitutively expressed on basophils, eosinophils, and a small subset of $CD4^+$ T cells (see Fig E4 in this article's Online Repository at www.jacionline.org),[24,25] was present on a population of T cells distinct from peanut-responsive T cells (Fig 2, *A*, and see Fig E4). Therefore peanut-responsive $CD154^+$ T cells

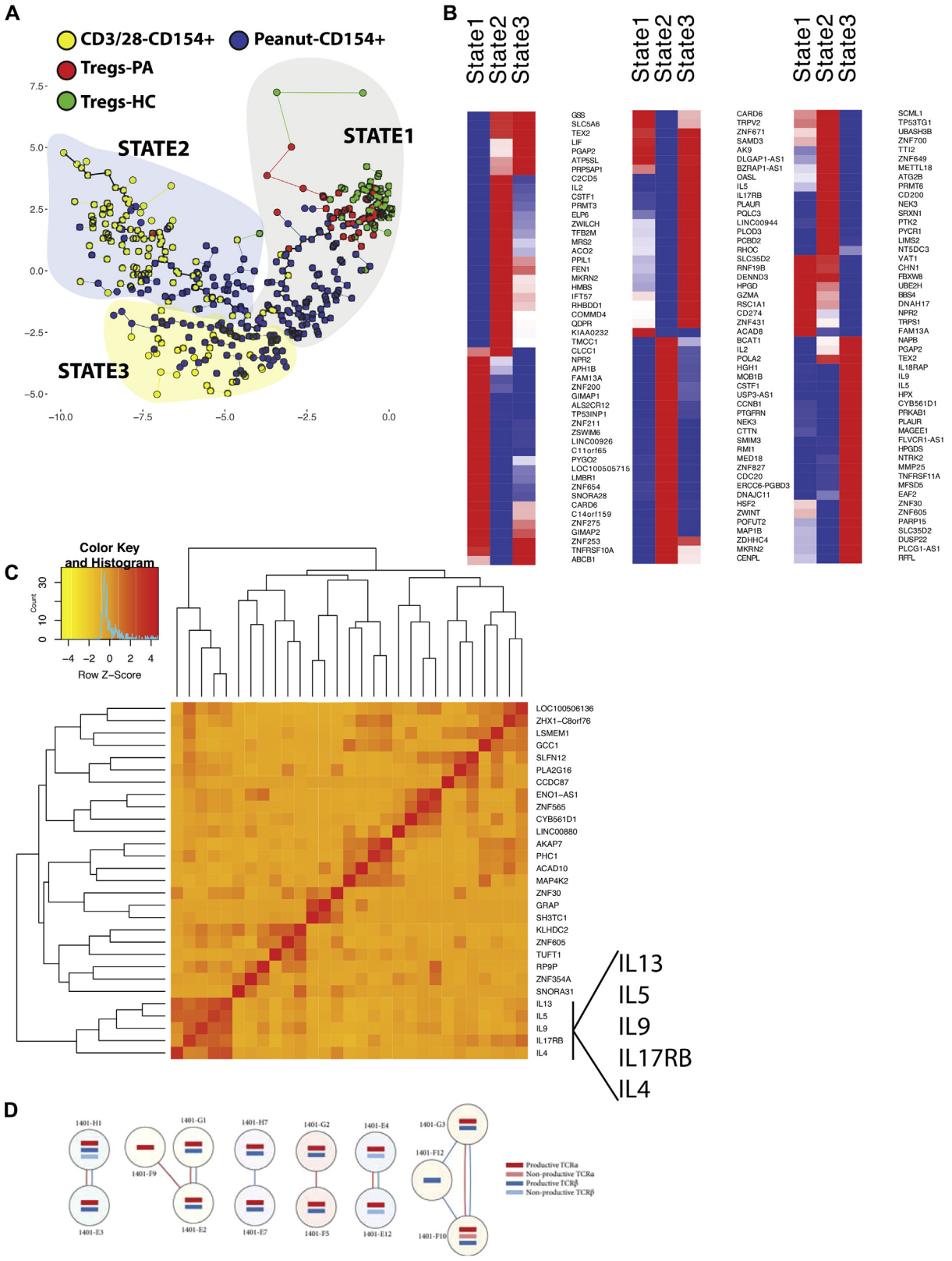

FIG 4. Single-cell sequencing analysis of CD154^{+} T cells and Treg cells. **A,** Minimum spanning tree showing clustering and ordering of peanut-activated T cells, polyclonally activated T cells, and Treg cells from patients with PA or HC subjects into 3 states and the distribution of cells by phenotype within those states. **B,** Heat maps showing genes differentially expressed between peanut-activated T cells in the 3 states. **C,** Pearson correlation of genes significantly upregulated in state 3. The *x- and y-axes* depict a hierarchy of genes that exhibit similar correlation values and are thus coexpressed. Only genes with a correlation of greater than 0.5 with another significantly upregulated gene are shown. **D,** TCR analysis of peanut-activated cells from a donor with PA (Subject ID 1401) indicating shared TCR α and/or β chains. Each *circle* indicates 1 cell, with the *red line* indicating a shared α chain, and the *blue line* indicating a shared β chain.

are selectively enriched for effector memory T cells in patients with PA but are heterogeneous and lack unified surface markers.

We examined coexpression of IL-5 and IL-9 with IL-4 in peanut-responsive T cells (Fig 2, *B-D*). After 6 hours of stimulation with peanut extract, we observed a subset of $CD154^+IL\text{-}4^+$ cells coexpressing IL-5 and, within that subset, a smaller subset of cells coexpressing IL-9 (Fig 2, *B*). We did not observe IL-5 or IL-9 expression in $CD154^+$ cells that did not express IL-4, suggesting that these cytokines were derived from T_H2 cells rather than T_H9 subsets. As cells acquired coexpression of IL-5 and IL-9, they became uniformly positive for the memory marker CD45RO and negative for CD27, which is indicative of terminal differentiation (Fig 2, *D*). We will refer to these T_H2 cells coexpressing IL-5, IL-9, or both as T_H2^+ cells.

For a more comprehensive profile of the effector phenotype of the peanut-specific immune response, we measured cytokines secreted into the culture supernatant using multiplex analysis 5 days after peanut stimulation. A time course of cytokine response demonstrated that T_H2 cytokines were first detectable in supernatants after 4 days of stimulation and were maximal at 5 days (data not shown). PBMCs from patients with PA secreted an array of T_H2-associated (IL-5, IL-9, and IL-13) and proinflammatory (IL-6, GM-CSF, TNF-α, and TNF-β) cytokines (Fig 2, *E*). IL-4 levels were less than levels of detection in most samples. Like IL-2, IL-4 consumption in *ex vivo* cultures has been described[26] and might explain the lack of detectable IL-4. IFN-γ, IL-10, and IL-17 were detectable, but levels were not significantly increased in response to peanut stimulation, even in HT or HC subjects.

Identification of peanut-responsive $CD4^+$ T cells bearing regulatory markers

CD154 expression has been reported to be expressed on Treg cells with slower kinetics than effector cells.[27] We did not observe expression of CD154 on $CD4^+CD25^{hi}CD127^{low}$ cells at 6 hours of stimulation, but at 18 hours of peanut or polyclonal stimulation, we observed upregulation of CD154 on these cells (see Fig E5 in this article's Online Repository at www.jacionline.org). Importantly, depletion of $CD25^+$ cells before stimulation abolished the population of $CD154^+CD3^+CD4^+CD25^{hi}CD127^{low}FoxP3^+$ cells, indicating that CD25 was present on the cells before stimulation (see Fig E5).

We examined the frequency of peanut-responsive cells with Treg cell markers in patients with PA, HT subjects, and HC subjects. We observed a significant increase in CD154 expression after 18 hours of peanut stimulation on $CD3^+CD4^+CD25^{hi}CD127^{low}FoxP3^+$ cells from patients with PA, which was lower or absent in HC and HT subjects (Fig 3, *A*). Assessment of chemokine receptor expression on $CD154^+$ cells with regulatory markers showed high expression of CCR4, which was similar to the total population of Treg cells, and levels of CCR6 that were enriched compared with those of either total $CD4^+$ T cells or total Treg cells (Fig 3, *B*). Peanut-responsive cells with Treg cell markers expressed high levels of the memory marker CD45RO, intermediate levels of CD27, and low levels of CCR7, which is consistent with a tissue-homing memory T-cell phenotype (see Fig E6 in this article's Online Repository at www.jacionline.org). Similar to self-reactive Treg cells identified with tetramers,[28] these peanut-responsive Treg cells expressed neither IL-10 nor IFN-γ (data not shown).

It has been reported that CD154 can be regulated by IL-2.[29] Because of the slow kinetics of the Treg cell response to peanut, relatively high frequency of cells as a percentage of total Treg cells, and activation of Treg cells only in patients with PA, we investigated the link between IL-2 and CD154 expression on Treg cells. Treatment of PBMCs with rhIL-2 for 18 hours increased CD154 expression on $CD4^+$ T cells and, more strikingly, on Treg cells (Fig 3, *C*). Neutralization of IL-2 suppressed CD154 expression on $CD4^+$ T cells after anti-CD3/CD28 stimulation at 18 but not 6 hours (data not shown) and reduced by approximately 50% the frequency of peanut-responsive Treg cells identified after 18 hours of stimulation with peanut extract (Fig 3, *D*). These results indicate that Treg cells upregulate CD154 as a secondary response to release of IL-2, likely from effector cells observed to be activated at 6 hours.

The low frequency of antigen-responsive cells (500-600 cells per million $CD4^+$ T cells) and limited blood volumes available precluded the performance of suppression assays with purified Treg cells responding to peanut stimulation. As an alternative approach to assess Treg cell function, we depleted PBMCs from patients with PA and HC subjects of $CD4^+CD25^{hi}CD127^{low}$ cells using fluorescence-activated cell sorting before stimulation with peanut extract and determined the effect on cytokine secretion (Fig 3, *E*). Peanut-induced IFN-γ and IL-17 production was significantly increased in cultures of Treg cell–depleted PBMCs from patients with PA and HC subjects, whereas IL-10 production was slightly but not significantly decreased. Levels of IL-13, which was produced at higher levels in patients with PA, were also increased significantly by Treg cell depletion in both patients with PA and HC subjects. In contrast, secretion of IL-5 and IL-9 was significantly enhanced by Treg cell removal in HC subjects but not in patients with PA.

Single-cell transcriptional profile of peanut-responsive $CD4^+$ T cells

We observed substantial heterogeneity in the phenotype of peanut-responsive cells as identified by CD154 expression after stimulation with peanut. To exhaustively phenotype these cells at molecular resolution, we performed single-cell RNA-seq on $CD154^+$ T cells captured after 18 hours of stimulation. After applying quality control measures, single-cell sequencing data were available for 212 peanut-responsive $CD154^+$ cells from 5 patients with PA and 122 αCD3/CD28-activated $CD154^+$ cells from 3 patients with PA. As an additional reference population, we sorted $CD4^+CD25^{hi}CD127^{low}$ Treg cells from freshly isolated PBMCs from 4 patients with PA or 4 HC subjects (97 resting Treg cells), for a total of 431 cells (sequencing information can be found in Table E7 in this article's Online Repository at www.jacionline.org).

Next, by using Monocle,[18] a notion of expression-state clustering and evolution was generated with guide genes identified as differentially expressed between resting Treg cells from HC subjects and polyclonally activated T cells from patients with PA (Fig 4, *A*, and see Fig E7, *A*). This comparison was chosen to examine how peanut-responsive T cells would cluster between 2 biologically distinct cell populations, and indeed, 3 states or clusters of cells based on this notion of transcriptional similarity were identified. Cells from different subjects were distributed across all 3 states (see Fig E7, *B*). State 1 contained 98% of the resting Treg cell populations (Fig 4, *A*), only 4% of the

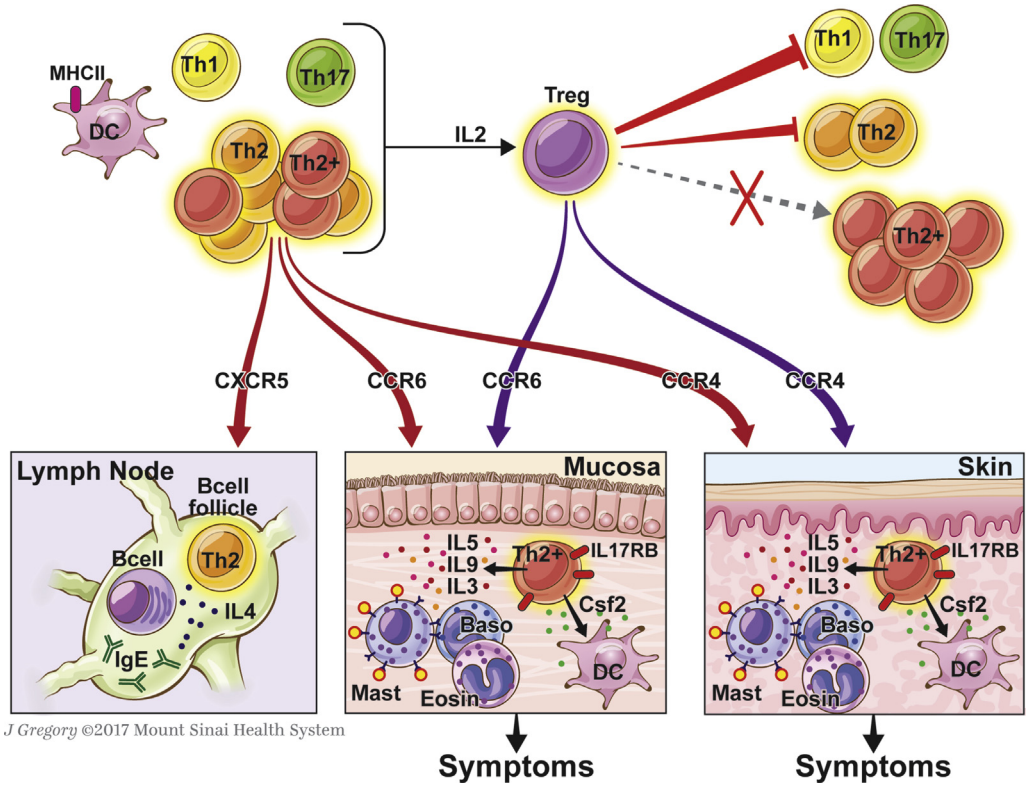

FIG 5. Proposed model of the contribution of peanut-responsive T cells to PA. Stimulation of PBMCs with peanut leads to early (6 hours) activation of memory T_H2 cells expressing IL-4 and IL-13 and memory T_H2^+ cells coexpressing IL-4 and IL-13 with IL-5 ± IL-9. T_H1 and T_H17 cells remain quiescent. T_H2 cells express molecules to facilitate homing to the skin, lung, intestine, or B-cell follicles, where we hypothesize that they can promote allergic effector cell expansion (skin and mucosa) or IgE class-switching (follicle). The heterogeneous T-cell effector T-cell response to peanut also includes IL-3 and CSF2, which can also contribute to tissue inflammation through actions on allergic effector cells and dendritic cells *(DCs)*, respectively. These distal effects on effector cells and IgE production would be expected to contribute to symptoms on peanut re-exposure. Release of IL-2 promotes subsequent activation of Treg cells. Treg cells completely suppress *(red arrows)* T_H1 and T_H17 responses and partially suppress T_H2 responses but do not significantly suppress T_H2^+ responses. We speculate that targeting the heterogeneous T_H2 response, including T_H2^+ cells, through molecules, such as *IL17RB*, might be effective in the treatment of PA.

polyclonally activated CD154$^+$ cells, and 45% of the peanut-activated CD154$^+$ cells. State 2 contained 2% of the resting Treg cell population, 75% of the polyclonally activated CD154$^+$ cells, and 25% of the peanut-activated CD154$^+$ cells. State 3 contained no resting Treg cells, 25% of the polyclonally activated CD154$^+$ cells, and 30% of the peanut-activated CD154$^+$ cells. Therefore Treg cells were associated primarily with state 1, most of the polyclonally activated cells were associated with state 2, and peanut-activated cells were distributed across the 3 states. Other analytic methods, such as Seurat,[30] primarily identified the 3 input cell types but were not able to effectively subcluster peanut-responsive T cells (data not shown).

We selected only the peanut-activated T cells to identify DEGs between states (Fig 4, *B*). The complete DEG list is provided in Supplemental Excel File 1 in this article's Online Repository at www.jacionline.org, and a DEG list comparing all peanut-activated T cells with all polyclonally activated T cells is provided in Supplemental Excel File 2 in this article's Online Repository at www.jacionline.org. State 3, which was comprised mostly but not exclusively of peanut-activated T cells, was a clearly proinflammatory T_H2-associated state. DEGs (false discovery rate < 5%) included *IL5*, *IL9*, *IL13*, *IL4*, colony-stimulating factor 2 *(CSF2)*, and *IL3*, as well as the IL-25 receptor *(IL17RB)*. Enrichment analysis identified pathways, including "regulation of immunoglobulin production" and "regulation of JAK-STAT" cascade (see Table E8 in this article's Online Repository at www.jacionline.org). Fig E8 in this article's Online Repository at www.jacionline.org shows gene expression of *IL2*, *IL5*, and *IL9* by individual cells across states. The correlation of gene expression in peanut-activated T cells in state 3 is shown in Fig 4, *C*. A cluster of 5 genes, including *IL4*, *IL5*, *IL9*, *IL13*, and *IL17RB*, comprised the dominant cluster of correlated genes.

Pathway analysis of DEGs in state 2 identified cell cycle or cell division (see Table E9 in this article's Online Repository at www.jacionline.org), and DEGs included *IL2* and chemokines and cytokines, such as *CCL22* and *IL17F*. Peanut-activated T cells in state 1, which clustered together with resting Treg cells, were not defined by a regulatory gene signature. The top pathways associated with DEGs in peanut-activated cells in state 1 were mitochondrion organization, metabolic process, and DNA strand elongation (see Table E10 in this article's Online Repository at www.jacionline.org), suggesting an early activation state. Identification of 5 clusters rather than 3 did not separate the peanut-responsive T cells from Treg cells in state 1 (data not shown).

Genes differentially expressed between peanut-activated T cells and Treg cells within state 1 are listed in Supplemental Excel File 3 in this article's Online Repository at www.jacionline.org. DEGs upregulated in peanut-responsive cells in state 1 compared with peanut-responsive cells in the other 2 states include several surface receptors or channels (*NPR2*, *GIMAP2*, and *CLCC1*). Although genes upregulated in the 3 different states suggest association with cell cycle, a formal analysis of cell-cycle genes did not indicate significant contribution to variance. Application of a machine learning classifier for cell-cycle allocation to predict the cell cycle[31] demonstrated that cells were predominantly in G1 and that clustering was independent of cell cycle (see Fig E9 in this article's Online Repository at www.jacionline.org).

We reconstructed the full complementarity-determining region 3 directly from the single-cell RNA-seq data to characterize the TCR sequence of the α and β chains (see Fig E10 and Table E11 in this article's Online Repository at www.jacionline.org). Using TraCeR (https://www.github.com/teichlab/tracer),[19] we identified 4 clonal expansions, each comprised of a pair of cells with identical α and β TCR sequences from a single patient with PA (Fig 4, *D*, and see Fig E11 in this article's Online Repository at www.jacionline.org). Clones were found in all 3 states, and interestingly, pairs of clones were found in different states (see Fig E11). Clonality was not uniquely associated with T_H2-polarized cells, and instead, the most highly upregulated gene was *PLA2G15* (see Fig E11), a phospholipase that results in a lymphoproliferative and autoimmune disorder when deleted.[32]

DISCUSSION

We studied the T-cell response to peanut at the cellular and molecular levels in a total of 156 human subjects, including 84 who underwent a double-blind, placebo-controlled food challenge to determine their threshold of clinical reactivity to peanut, and 21 HC subjects. In patients with PA, we identified a peanut-responsive population of highly differentiated T_H2 cells and a delayed IL-2–dependent activation of cells expressing regulatory markers, whereas nonallergic control subjects had a marked absence of peanut-reactive immune response. A summary model that places our findings in the pathogenesis of PA is shown in Fig 5.

By using a multifaceted approach to immune profiling, the effector response to peanut, including detection of intracellular and secreted cytokines, as well as single-cell profiling, we identified a highly heterogeneous T_H2 response to peanut in patients with PA, including cells coexpressing IL-5 ± IL-9, which we refer to as T_H2^+ cells (Fig 5). To our knowledge, this is the first report of single-cell RNA-seq of food allergen–responsive T cells. Previously, transcriptional profiling of peanut-responsive $CD4^+$ T cells was done on the bulk level by using microarray analysis[12] and on the single cell level by using a panel of allergy- and immune tolerance–focused genes.[11] The identification of peanut-responsive T-cell clones in this exploratory analysis is remarkable because we sequenced a relatively small number of cells (40-50 per patient), cells were activated with a crude peanut extract containing many peanut allergens, and stimulation time did not allow for expansion of allergen-responsive T cells. Although preliminary, the positive identification of peanut-responsive clones prompts additional investigation of more patients with higher-throughput single-cell capture approaches, such as DropSeq,[30] to comprehensively study association of phenotype with TCR specificity.

We found that peanut-responsive cells clustered into 3 states, one of which was enriched for highly differentiated T_H2 cells (state 3). Correlation analysis showed that *IL4*, *IL5*, *IL9*, *IL13*, and *IL17RB* were highly coexpressed in peanut-responsive T cells. *IL3* and *CSF2* (GM-CSF) were also differentially expressed in this T_H2 state but did not correlate with the cluster of other T_H2-related cytokines and might be distinct effector cell phenotypes. Using bulk analysis methods, we recently identified a highly differentiated T_H2 (IL-5 and IL-9) and proinflammatory (TNF-α and CSF2) immune profile in cohorts of patients with egg allergy.[10,33] IL-9 is differentially expressed by peanut-responsive T cells from patients with PA, as identified through bulk analysis by using a microarray.[34] Wisniewski et al[6] also identified a multifunctional T_H2 response to peanut in patients with PA by using a proliferation-based flow cytometry approach. The T_H2-associated cytokines we identified as upregulated using single-cell RNA-seq (*IL3*, *IL5*, *IL9*, and *CSF2*) are critical for the differentiation of granulocytes, including mast cells, eosinophils, and basophils, and we hypothesize that these T cell–derived granulocyte growth factors control the allergic milieu of the skin or mucosa (Fig 5). IL-9 drives intestinal mastocytosis, is increased in the intestines of patients with food allergy, and is critical for food-induced allergic symptoms.[10,33] OX40 ligand–expressing dendritic cells promote production of IL-3 from naive $CD4^+$ T cells, which then recruit basophils and promote T_H2 priming of $CD4^+$ T cells.[35] The role of T cell–derived IL-5, IL-3, CSF2, and TNF-α in food allergy remains to be identified. The unique association of the IL-25 receptor *IL17RB* on these highly differentiated T_H2 cells suggests a role for tissue-derived IL-25 in PA pathogenesis, which is supported by work in animal models of food allergy.[36,37] *IL17RB* was also previously reported as highly differentially expressed in peanut-responsive T cells analyzed by using a bulk microarray.[11]

The peanut-responsive T_H2 cells described here share several features of cells described as T_H2A and pathogenic effector T_H2 (peT_H2) cells.[7,38-40] Common features include multicytokine potential, including IL-5 and IL-9, lack of expression of CD27, and expression of hematopoietic prostaglandin D synthase and *IL17RB*. Both T_H2A and peT_H2 cells are $CRTH2^+CD161^{high}$,[38,40] which is distinct from the peanut-responsive T_H2^+ cells described here. peT_H2 cells correlate with eosinophilia and were proposed as a T_H2 phenotype that distinguished T_H2 diseases characterized by allergic inflammation of tissues (eosinophilic gastroenteritis or atopic dermatitis) from an IgE-mediated T_H2 disease (PA).[7,40] T_H2A cells have been described across distinct allergies, including aeroallergens and peanut.[38] The reason for this discrepancy in expression of CD161 and CRTH2 between peT_H2 cells, T_H2A cells, and the T_H2^+ cells we describe here is not clear. We used an activation-based detection approach, whereas T_H2A cells were identified with a tetramer-based approach, and it is possible that activation downregulates CRTH2 expression. However, we observed CRTH2 expression on phorbol 12-myristate 13-acetate/ionomycin-stimulated $CD154^+$ cells, and peT_H2 cells have been described to emerge *in vitro* through multiple rounds of stimulation.[40] Despite the discrepancy in expression of CRTH2 and CD161, which might be related to methods of antigen-specific cell detection or culture conditions, expression of $CD45RO^+/CD45RA^-$, $CD27^-$, hematopoietic prostaglandin D synthase–positive, and $IL17RB^+$ are consistent markers of

these allergen-specific T_H2 cells with multicytokine production across multiple studies (including peT_H2, T_H2A, and T_H2^+ cells).

Homing plays a key role in T-cell function. We found that peanut-responsive T_H2 cells were enriched for the homing markers CCR4, CCR6, and CXCR5. Although CCR6 is often used as a surface marker of T_H17 cells, previously, we showed that CCR6 is required for homing of pathogenic T_H2 cells to the small intestine and development of food-induced allergic symptoms in mice.[41] CCR4 is expressed on both T_H2 and Treg cells and facilitates homing to the skin[42] and lungs.[43] The skin, lungs, and gastrointestinal tract are common sites of manifestations of food allergy, and we speculate that the homing of T cells producing an array of T_H2-related cytokines contributes to allergic inflammation in the tissues, which contributes to acute responses to food allergen (Fig 5). Provision of B-cell help by T cells is dependent on homing to B-cell follicles in a CXCR5-dependent manner. IL-4 production by T_H2 cells contributes to allergy in large part through support of IgE class-switching, and T cells require CXCR5 expression to interact with B cells in the follicles of the lymph node.[42] We observed significantly enriched expression of CXCR5 on peanut-responsive T_H2 cells in the blood of patients with PA, although the frequency of these cells was low compared with the total population of peanut-responsive cells. Lymph nodes, such as the tonsils, would likely be more informative than blood on the frequency and phenotype of peanut-specific follicular helper T cells. It would be of interest to compare this $CXCR5^+$ T_H2 population in those with IgE-mediated versus non–IgE-mediated allergic diseases to foods (ie, milk allergy with immediate hypersensitivity reactions vs milk allergy contributing to eosinophilic esophagitis). We hypothesize that this $CXCR5^+$ T_H2 population would be uniquely associated with IgE-mediated disease.

Treg cells are thought to be deficient or "reprogrammed" in patients with food allergy.[13] We identified a population of cells bearing regulatory markers that upregulated CD154 in a delayed manner compared with T_H2 cells (detectable at 18 vs 6 hours). Deletion of $CD25^+$ cells before stimulation completely abolished this response, indicating that they expressed Treg cell markers before stimulation. These cells expressed markers consistent with peripherally induced Treg cells.

The delayed kinetics of activation, frequency (approximately 5% of the Treg cell population), and IL-2 dependence suggest that these cells are the result of bystander activation rather than antigen-specific Treg cells. There is conflicting evidence on the antigen specificity of Treg cells. Antigen-specific Treg cells (identified as Treg cells based on regulatory marker expression and lack of cytokine production on stimulation) were identified in human subjects by using an array of MHC class II tetramers specific for self-antigens or microbial antigens.[28] Tetramer-positive Treg cells were identifiable but very rare cells (<1% of tetramer-positive cells for any given antigen). Bacher et al[44] used CD137 (4-1BB) as an activation marker of antigen-specific Treg cells and examined the frequency of Treg cells specific for a variety of antigens and allergens. Treg cells specific for aeroallergens or microbial antigens could be readily found in peripheral blood of healthy subjects, but food-specific Treg cells were markedly few to absent. In contrast to these findings in HC subjects, food allergen–responsive cells with a Treg cell phenotype and regulatory function in suppression assays have been identified by using proliferation assays (carboxyfluorescein isothiocyanate dilution) in allergic patients.[45,46] In addition, single-cell transcriptional profiling using tetramer-selected cells and a curated panel of genes identified a population of peanut-specific cells with expression of regulatory genes (FoxP3, CD25, IL-10, and IFN-γ) in patients with PA.[47] Although our data suggest that Treg cells can be bystander activated, Treg cells as a population are functional and dampen the immune response to peanut.

The potential suppressive role of CD154 on Treg cells remains to be clarified. The fact that cytokines commonly produced by highly differentiated T_H2 cells (IL-5 and IL-9) were less affected by removal of Treg cells in patients with PA suggests that T_H2^+ cells might be more resistant to regulation by Treg cells than other T-cell subsets. Memory T cells have been described to be less susceptible to suppression than naive T cells,[48] and we speculate that as T_H2 cells differentiate to become T_H2^+ cells, they become less susceptible to the regulatory effects of Treg cells. Bacher et al[44] have proposed, based on their findings in the context of birch allergy, that T_H2 cells escape Treg cell control because of differing antigen specificities.

There is little information available on the immune basis of tolerance to foods under homeostatic conditions in healthy human subjects, although a lack of food-specific Treg cells has been documented in healthy subjects.[44] In contrast to patients with PA, we observed a marked absence in T-cell reactivity to peanut in HC subjects, as well as those we termed HT subjects, who were sensitized to peanut and had a clinical history of PA but passed a food challenge with a cumulative dose of 1 g of peanut protein. The HT group was not rechallenged with a higher dose of peanut protein, and therefore we do not know what proportion of this group would react at a higher dose of peanut versus being fully tolerant. Importantly, the HT cohort was consistent in age and diet with the PA cohort, whereas the HC cohort was adult and not avoiding peanut in the diet. Despite the diversity of these 2 control groups in age and diet, they exhibited a consistent absence of T-cell response, as measured based on CD154-based detection or cytokine production. Although the HC group was not matched for age, we did not observe any trend of an association between peanut-specific T-cell response and age within the PA group. Furthermore, the age range of the PA group was 4 to 20 years, with 14 patients older than 13 years. Therefore it is unlikely that the absence of peanut-responsive T cells in the HC group, which was also observed in the HT group, was due to age. This lack of T-cell reactivity was accompanied by relatively low levels (HT) or no (HC) peanut-specific IgE and a low peanut-specific IgG_4 levels. The relative absence of allergen-specific T-cell responses in HC subjects is consistent with 2 previous reports that used MHC class II tetramers or CD154 expression to detect peanut-reactive T cells in HC subjects.[7,8] Our data suggest that naturally occurring clinical tolerance, whether complete tolerance in adult HC subjects not restricting peanut in the diet or a higher threshold of reactivity to peanut in HT subjects, is characterized by immunologic ignorance or anergy rather than an antigen-specific counterregulatory T_H1 or Treg cell response. This does not rule out a role for Treg cells in the healthy response to foods because our data show that removal of the entire Treg cell compartment enhances the immune response to peanut in HC subjects. Indeed, patients with a variant of immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, who lack functional Treg cells, have been reported to have allergies to multiple foods, clearly showing a role for Treg cells in immune tolerance to foods.[49] However, our data

suggest that this regulatory activity might not be limited to antigen-specific Treg cells.

In summary, we have identified a heterogeneous population of T_H2, T_H2^+, and other growth factor–producing peanut-responsive cells the with capacity for tissue and lymph node homing that we postulate play a central role in PA pathogenesis. We did not find evidence for a lack of peanut-responsive Treg cells or disrupted Treg cell response in patients with PA. This is consistent with the findings of Bacher et al,[44] who identified the presence of T_H2 effector cells with different antigen specificity than Treg cells in patients with birch allergy, with no deficiency in numbers or suppressive capacity of Treg cells.

These results have several implications for therapy. The relative resistance of IL-5 and IL-9 to regulation by Treg cells in patients with PA, as well as the lack of detectable antigen-responsive Treg cells in HC and HT subjects, suggests that eliminating or targeting peanut-specific T_H2 cells would be more effective for the treatment of PA than boosting the pool of peanut-specific Treg cells. Tight correlation between the IL-25 receptor and cytokine expression in highly differentiated T_H2 cells, as well as the common finding of IL-25 receptor expression by allergen-specific T cells across multiple studies, suggests *IL17RB* as a target for modifying the phenotype or eliminating these cells.

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Key messages

- **PA was associated with a multifunctional T-cell response, including highly differentiated T_H2 cells producing IL-5 and IL-9, cells expressing *CSF2* and *IL3*, and a delayed IL-2–dependent activation of Treg cells.**
- **Subjects with a higher threshold of reactivity to peanut, as well as HC subjects, exhibited a lack of peanut-specific T_H2 or Treg cell response, suggesting anergy or deletion as a basis of tolerance.**
- **The contribution of highly differentiated multicytokine-producing T_H2 cells to the pathogenesis of PA needs further investigation.**

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