

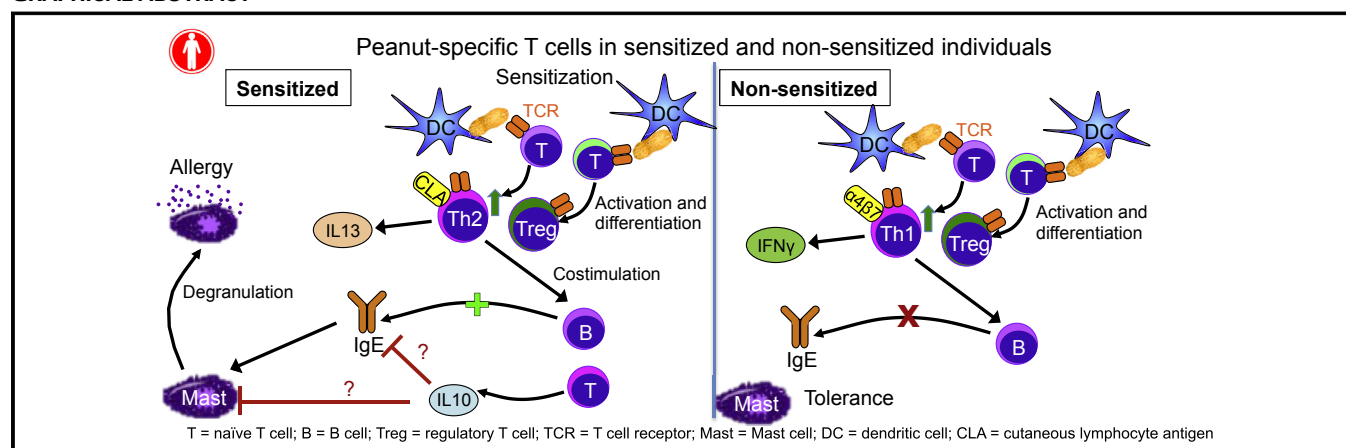
Identification and analysis of peanut-specific effector T and regulatory T cells in children allergic and tolerant to peanut



Katherine A. Weissler, PhD,^a Marjohn Rasooly, MSN,^a Tom DiMaggio, BSN,^a Hyejeong Bolan, MSN,^a Daly Cantave, MSN,^a David Martino, PhD,^{b,c} Melanie R. Neeland, PhD,^b Mimi L. K. Tang, MD, PhD,^{b,c,d} Thanh D. Dang, PhD,^{b,c} Katrina J. Allen, MD, PhD,^{b,c,d} and Pamela A. Frischmeyer-Guerrero, MD, PhD^a
and Melbourne, Australia

Bethesda, Md,

GRAPHICAL ABSTRACT



From ^athe Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda; ^bMurdoch Childrens Research Institute and ^cthe Department of Allergy and Immunology, Royal Children's Hospital, Melbourne; and ^dthe Department of Pediatrics, University of Melbourne.

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Corresponding author: Pamela A. Frischmeyer-Guerrero, MD, PhD, BG 10 RM 11N240B MSC 1889, 10 Center Dr, Bethesda, MD 20892. E-mail: pamela.guerrero@nih.gov.

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Background: Peanut allergy (PA) is potentially life-threatening and generally persists for life. Recent data suggest the skin might be an important route of initial sensitization to peanut, whereas early oral exposure to peanut is protective. In mice regulatory T (Treg) cells are central to the development of food tolerance, but their contribution to the pathogenesis of food allergy in human subjects is less clear.

Objective: We sought to quantify and phenotype CD4⁺ peanut-specific effector T (ps-Teff) cells and peanut-specific regulatory T (ps-Treg) cells in children with and without PA or PS.

Methods: ps-Teff and ps-Treg cells were identified from peripheral blood of children with PA, children with PS, and nonsensitized/nonallergic (NA) school-aged children and 1-year-old infants based on upregulation of CD154 or CD137, respectively, after stimulation with peanut extract. Expression of cytokines and homing receptors was evaluated by using flow cytometry. Methylation at the forkhead box protein 3 (*FOXP3*) locus was measured as a marker of Treg cell stability.

Results: Differential upregulation of CD154 and CD137 efficiently distinguished ps-Teff and ps-Treg cells. A greater percentage of ps-Teff cells from infants with PA and infants with PS expressed the skin-homing molecule cutaneous lymphocyte antigen, suggesting activation after exposure through the skin, compared with NA infants. Although ps-Teff cells in both school-aged and infant children with PA produced primarily Th2 cytokines, a Th1-skewed antipeanut response was seen only in NA school-aged children. The frequency, homing receptor

expression, and stability of ps-Treg cells in infants and school-aged children were similar, regardless of allergic status.

Conclusions: Exposure to peanut through the skin can prime the development of T_H2 ps-Teff cells, which promote sensitization to peanut, despite the presence of normal numbers of ps-Treg cells. (J Allergy Clin Immunol 2018;141:1699-710.)

Key words: Peanut allergy, regulatory T, antigen specificity, CD154, CD137, cutaneous lymphocyte antigen, α 4 β 7, route of sensitization, food allergy

Food allergy is a growing problem around the world, and the associated anaphylactic responses can have fatal consequences.¹ Peanut allergy (PA) is the most common cause of severe reactions in the United States² and is usually lifelong.²⁻⁴ A better understanding of the underlying immunologic events that lead to this disease can facilitate efforts to prevent and treat PA.

Food allergy is postulated to result from a defect in either the establishment or maintenance of oral tolerance, which was defined as a state of unresponsiveness on re-encounter with an antigen after oral exposure. Regulatory T (Treg) cells are an important component of oral tolerance, and several studies have suggested that deficits in the development, function, and/or stability of Treg cells predispose to food allergy. Adoptive transfer of Treg cells can suppress anaphylactic responses to food antigens in murine models,⁵ and mice that cannot produce Treg cells outside the thymus experience spontaneous type 2 inflammation at mucosal sites and generate an antibody response against antigens found in mouse chow.⁶ Loss-of-function mutations in forkhead box protein 3 (*FOXP3*), which encodes the main transcription factor essential for Treg cell development, cause immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, which is associated with profound autoinflammatory disease, as well as food allergy and eczema, in some patients.^{7,8} Additionally, an increase in the frequency and stability of Treg cells was associated with more favorable clinical outcomes in children who underwent oral immunotherapy for PA, and increased numbers of Treg cells were reported in children who naturally outgrew their milk allergy.⁹⁻¹¹

The predominant immune response to peanut in allergic patients is characterized by release of T_H2 effector cytokines (IL-4, IL-5, and IL-13) that induce B cells to produce peanut-specific IgE. Cross-linking of IgE bound to the surfaces of mast cells and basophils results in degranulation and release of inflammatory mediators after peanut exposure, resulting in an allergic reaction.¹² Growing evidence points to the skin as the main site of sensitization to food antigens, particularly peanut. Genetic variants in *FLG*, *SPINK5*, and *DSG1*, which encode epithelial barrier proteins, have all been associated with an increased risk of food allergy.¹³⁻¹⁵ Infants with eczema are 11 times more likely to have PA than those without,¹⁶ and high household consumption of peanut is associated with increased levels of biologically active peanut allergen in household dust and a greater risk of peanut sensitization (PS), particularly if the child has eczema.¹⁷⁻¹⁹

Although recent studies have provided clues regarding the role of T cells in the pathogenesis of PA, these efforts have been hindered by technical difficulties in identifying peanut-specific T cells. Here we have adapted a method used previously to detect T cells that recognize fungal antigens^{20,21} to simultaneously identify peanut-specific effector T (ps-Teff) cells and peanut-

Abbreviations used

CLA:	Cutaneous lymphocyte antigen
CPE:	Crude peanut extract
FOXP3:	Forkhead box protein 3
IQR:	Interquartile range
NA:	Nonallergic
NIH:	National Institutes of Health
OFC:	Oral food challenge
PA:	Peanut allergy
PS:	Peanut sensitization
ps-Teff:	Peanut-specific effector T
ps-Treg:	Peanut-specific regulatory T
SPT:	Skin prick test
Treg:	Regulatory T

specific regulatory T (ps-Treg) cells directly *ex vivo* based on rapid upregulation of CD154 (CD40 ligand) or CD137 (4-1BB), respectively.²² CD154 was originally identified on activated T cells and is crucial for T cell–dependent activation of B-cell responses,^{23,24} whereas CD137 is a known immunoregulatory molecule and a direct target of Foxp3.²⁵ Using this methodology, we sought to explore the frequency and phenotype of ps-Teff and ps-Treg cells in children who are allergic versus tolerant to peanut and thereby illuminate the changes in CD4⁺ T-cell responses that lead to the development of PA.

METHODS

Patient populations

School-aged subjects were recruited to the National Institutes of Health (NIH) under clinical protocol 15-I-0162 (n = 17 patients with PA and n = 19 nonallergic [NA] subjects). Additional demographic and clinical characteristics of this study group are listed in Table E1 and the Methods section in this article's Online Repository at www.jacionline.org.

Cryopreserved PBMCs were also obtained from a subset of 1-year-old infants enrolled in HealthNuts, a population-based cohort from Melbourne, Australia (n = 14 patients with PA; n = 15 patients with PS; and n = 14 NA subjects). Infants were classified as (1) PA infants, who were defined as having a positive oral food challenge (OFC) result to peanut along with a positive skin prick test (SPT) response (≥ 2 mm) or IgE test result (≥ 0.35 kU/L) for peanut; (2) NA subjects, who were defined as passing an OFC to peanut and having a negative SPT response, IgE test result, or both for peanut; and (3) patients with PS, who were defined as having an SPT wheal response to peanut of 2 mm or greater and/or a peanut-specific IgE level of 0.35 kU/L or greater and a negative OFC result to peanut. Additional details can be found in Table E2 and the Methods section in this article's Online Repository at www.jacionline.org.^{26,27}

The study was approved by local institutional review boards, and informed consent/assent was obtained for all subjects. Approval to conduct the HealthNuts study was obtained from the Victorian State Government Office for Children (reference no. CDF/07/492), the Victorian State Government Department of Human Services (reference no. 10/07), and the Royal Children's Hospital Human Research Ethics Committee (reference no. 27047).

End-point titration SPTs to measure mast cell reactivity

End-point titration SPTs were performed on school-aged children with PA by using serial 10-fold dilutions of peanut extract with the GreerPick system (Greer Laboratories, Lenoir, NC). The starting concentration was the standard peanut extract (1:20 wt/vol) with serial 10-fold dilutions (1:200, 1:2000, 1:20,000, and 1:200,000 wt/vol). Peanut SPTs were not performed on 3

patients with PA because they were unable to discontinue their antihistamines because of eczema.

Crude peanut extract for use in T-cell studies

Peeled fresh peanuts were blended at 2 g per 10 mL of saline, rocked overnight at 4°C, and centrifuged at 5000g for 30 minutes, and the protein layer was collected. Centrifugation and collection of protein layer were repeated twice more, and the resulting solution was passed through a 0.2-μm filter. Endotoxin was reduced by using an endotoxin removal kit (Pierce, Thermo Scientific, Waltham, Mas). The final concentration of endotoxin was approximately 80 EU/mL.

Basophil activation test

Basophil activation was measured by means of upregulation of CD63, as previously described.²⁸ Further details are provided in the [Methods](#) section in this article's Online Repository.

Analysis of peanut-specific T cells

For school-aged subjects recruited at the NIH, blood was collected in sodium heparin, and assays were performed with whole blood. For 1-year-old infants, PBMCs were thawed and rested overnight before being resuspended at 8×10^6 cells/mL in AIM-V medium supplemented with 2.5% human serum, and approximately 5×10^6 cells per well were added to a 48-well plate. Anti-CD40 (clone HB14; Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD28 (clone 15E8; Miltenyi Biotec) were added at a final concentration of 1 μg/mL to whole-blood cultures (6–7 mL) for school-aged children or PBMCs for 1-year-old infants. Crude peanut extract (CPE) was added where indicated at a final concentration of 300 μg/mL, and cultures were incubated at 37°C. Brefeldin A (5 μg/mL) was added after 4.75 hours if intracellular cytokine staining was performed, and cells were harvested after an additional 2 hours. CD4⁺ T cells were enriched from whole blood only by using a magnetic depletion method that resulted in approximately 85% purity (EasySep Direct Human CD4⁺ T Cell Isolation Kit; STEMCELL Technologies, Vancouver, British Columbia, Canada). Live/dead stain (eBioscience, San Diego, Calif) was used to discriminate between live and dead cells. Human immunoglobulin was used as a blocking agent before staining. Cells were surface stained in PBS supplemented with 1% BSA for 25 minutes. When needed, intracellular staining was performed with the eBioscience Foxp3 staining kit, according to the manufacturer's instructions. A BD LSRII (BD, Franklin Lakes, NJ) was used for flow cytometry, and between 8×10^5 and 2×10^6 events were collected. Analysis was performed with FlowJo software (see the [Methods](#) section in this article's Online Repository). Peanut-specific effector T cells and Treg cells were identified by means of upregulation of CD154 or CD137, respectively. The number of cells expressing a given marker were calculated by subtracting the number of cells in that gate per million CD4⁺ T cells in the unstimulated control sample from the number in the peanut-stimulated sample.

Methylation analysis

ps-Teff and ps-Treg cells were sorted from peripheral blood of school-aged children with a BD FACSAria and snap-frozen; methylation status was assessed by means of bisulfite modification and direct pyrosequencing and was performed by EpigenDX (Hopkinton, Mass). The percentage indicated is the average of percentage methylation at 11 different CpG sites in intron 1 (–2236 to –2376 from ATG) of the *FOXP3* locus.

In vitro expansion of ps-Teff and ps-Treg cells, restimulation, and suppression assay

PBMCs were resuspended at 10×10^6 cells/mL and stimulated for 6 hours with 300 μg/mL CPE, 1 μg/mL anti-CD40 (clone HB14; Miltenyi Biotec), and 1 μg/mL anti-CD28 (clone 15E8; Miltenyi Biotec). CD154⁺ and CD137⁺ cells were isolated by using CD154 and CD137 Microbead kits (Miltenyi Biotec) and sequential magnetic columns. CD154⁺ cells were cultured at 5×10^5 cells/mL with CPE-loaded irradiated autologous feeder PBMCs at

5×10^6 cells/mL in X-VIVO 15 medium (Lonza, Basel, Switzerland) supplemented with 2.5% (vol/vol) human AB serum and 4 ng/mL recombinant human IL-2 (PeproTech, Rocky Hills, NJ). CD137⁺ cells were cultured at 2.5×10^4 cells per well in a 96-well U-bottom plate in X-VIVO 15 medium (Lonza) supplemented with 2.5% (vol/vol) human AB serum, 30 nmol/L rapamycin, and 10 ng/mL recombinant human IL-2 (PeproTech). Media were replaced and cultures were split, as necessary. After 17 to 21 days of culture, restimulation and suppression assays were set up using 2×10^5 irradiated autologous CD3-depleted PBMCs alone or loaded with CPE or wheat extract, 1×10^5 expanded CD154⁺ cells labeled with CellTrace Violet, and/or expanded CD137⁺ cells labeled with carboxyfluorescein succinimidyl ester at a 1:1, 1:2, and 1:4 ratio with expanded CD154⁺ cells in a 96-well U-bottom plate in X-VIVO 15 medium supplemented with 2.5% (vol/vol) human AB serum. Cells were harvested after 6 days, dilution of carboxyfluorescein succinimidyl ester and CellTrace Violet was assessed by using flow cytometry, and the division index was identified by using FlowJo software (TreeStar, Ashland, Ore). The percentage suppression was calculated as follows:

$$(100 - [\text{Division index of CD154}^+ \text{ cells in the presence of CD137}^+ \text{ cells} / \text{Division index of CD154}^+ \text{ cells alone}] * 100).$$

Statistical analysis

Statistical analysis was performed with Prism software (GraphPad Software, La Jolla, Calif). The Student *t* test with the Welch correction for unequal variance or a Kruskal-Wallis test was used to determine statistical significance, where appropriate. Comparisons between pairs after a significant result using a Kruskal-Wallis test were performed with the Dunn multiple comparison test. A *P* value of less than .05 was considered significant.

RESULTS

Identification and quantification of peanut-specific CD4⁺ T cells in school-aged children with or without PA

Seventeen patients with PA (median age, 8 years; interquartile range [IQR], 8.5; 71% male) and 19 NA subjects (median age, 12 years; IQR, 5; 53% male) were recruited. Patients with PA had a median total IgE level of 1148 kU/L (IQR, 7617 kU/L), peanut-specific IgE level of 36.1 kU_A/L (IQR, 88.5 kU/L), and wheal size of 5.5 mm (IQR, 4.6 mm) on peanut SPTs. All of the patients with PA were allergic to other foods, 16 of 17 had atopic dermatitis, 12 of 17 had asthma, and 15 of 17 had allergic rhinitis (see [Table E1](#)). All were strictly avoiding peanut in their diet. NA subjects had a median total IgE level of 31.9 kU/L (IQR, 87.5 kU/L), were not sensitized to peanut (peanut-specific IgE levels were less than the level of detection in either a peanut-specific IgE or FX5 food panel, which includes peanut), and were consuming peanut in their diet. One of 19 had atopic dermatitis, 1 of 19 had asthma, and 2 of 19 had allergic rhinitis (see [Table E1](#)).

To detect peanut-specific CD4⁺ T cells, whole blood was cultured with CPE, and upregulation of CD154 and CD137 was used to identify ps-Teff (CD154⁺CD137[–]) and ps-Treg (CD154[–]CD137⁺) cells, respectively, by using the gating strategy depicted in [Fig 1, A and B](#), and quantified in [Fig 1, C](#). Absolute numbers of ps-Teff and ps-Treg cells per 1×10^6 total CD4⁺ T cells, which were calculated by subtracting the number of cells in each gate per 1×10^6 total CD4⁺ T cells in the unstimulated condition from the number in the CPE-stimulated condition, were not different between children with PA and NA children nor was there a significant difference in the ratio of ps-Treg/ps-Teff cells between groups ([Fig 1, D and F](#)). Importantly, the

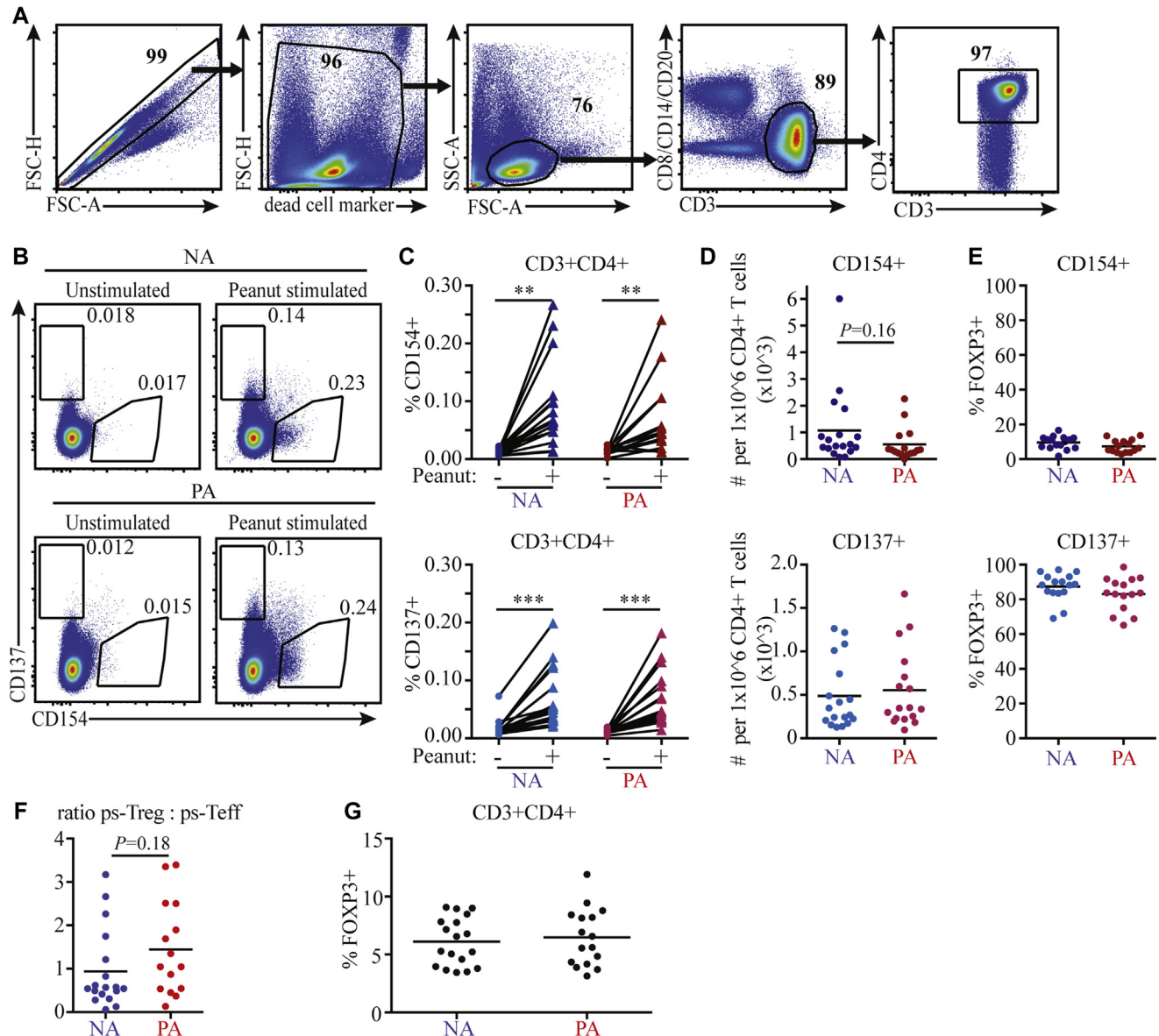


FIG 1. Quantification of ps-Teff and ps-Treg cells by means of rapid upregulation of CD154 and CD137, respectively, in blood from school-aged children. **A**, Gating strategy used to identify CD3⁺CD4⁺ T cells after stimulation of whole blood with CPE for 6.75 hours and subsequent CD4⁺ T-cell enrichment by means of negative selection. FSC, Forward scatter. **B** and **C**, Percentage of CD3⁺CD4⁺ T cells that expressed CD154 or CD137 after culture with medium alone (no peanut) or CPE in NA children or children with PA. Each point in Fig 1, C, represents 1 subject. Statistics were performed by using a paired Student *t* test: ***P* < .01 and ****P* ≤ .001. **D**, Number of CD154⁺ ps-Teff cells and CD137⁺ ps-Treg cells per 1 × 10⁶ CD3⁺CD4⁺ cells was calculated by subtracting the number of CD154⁺ or CD137⁺ cells in the unstimulated condition from the CPE-stimulated condition for each subject. **E**, Percentage of CD154⁺ and CD137⁺ T cells expressing FOXP3 after CPE stimulation. **F**, Ratio of ps-Treg cells/ps-Teff cells in each subject. **G**, Percentage of total CD4⁺ T cells expressing FOXP3 in NA subjects and patients with PA. Statistics were performed by using an unpaired *t* test with Welch correction for unequal variation.

majority of CD154⁺ ps-Teff cells in both groups did not express FOXP3, whereas approximately 90% of CD137⁺ ps-Treg cells were FOXP3⁺ (Fig 1, E).

We next measured the percentage of CD4⁺ T cells expressing FOXP3 in the patients with PA and NA subjects and found no evidence for a global deficiency in Treg cells in children with PA (Fig 1, G).

Examination of ps-Treg cells

Although expression of FOXP3 by the CD137⁺ population supports the identification of these cells as Treg cells, human effector T cells can also upregulate FOXP3 after stimulation.²⁹ To confirm that the CD137⁺ cells were *bona fide* Treg cells, we sorted both CD154⁺ (ps-Teff) and CD137⁺ (ps-Treg) cells after stimulation with CPE and assessed the methylation status

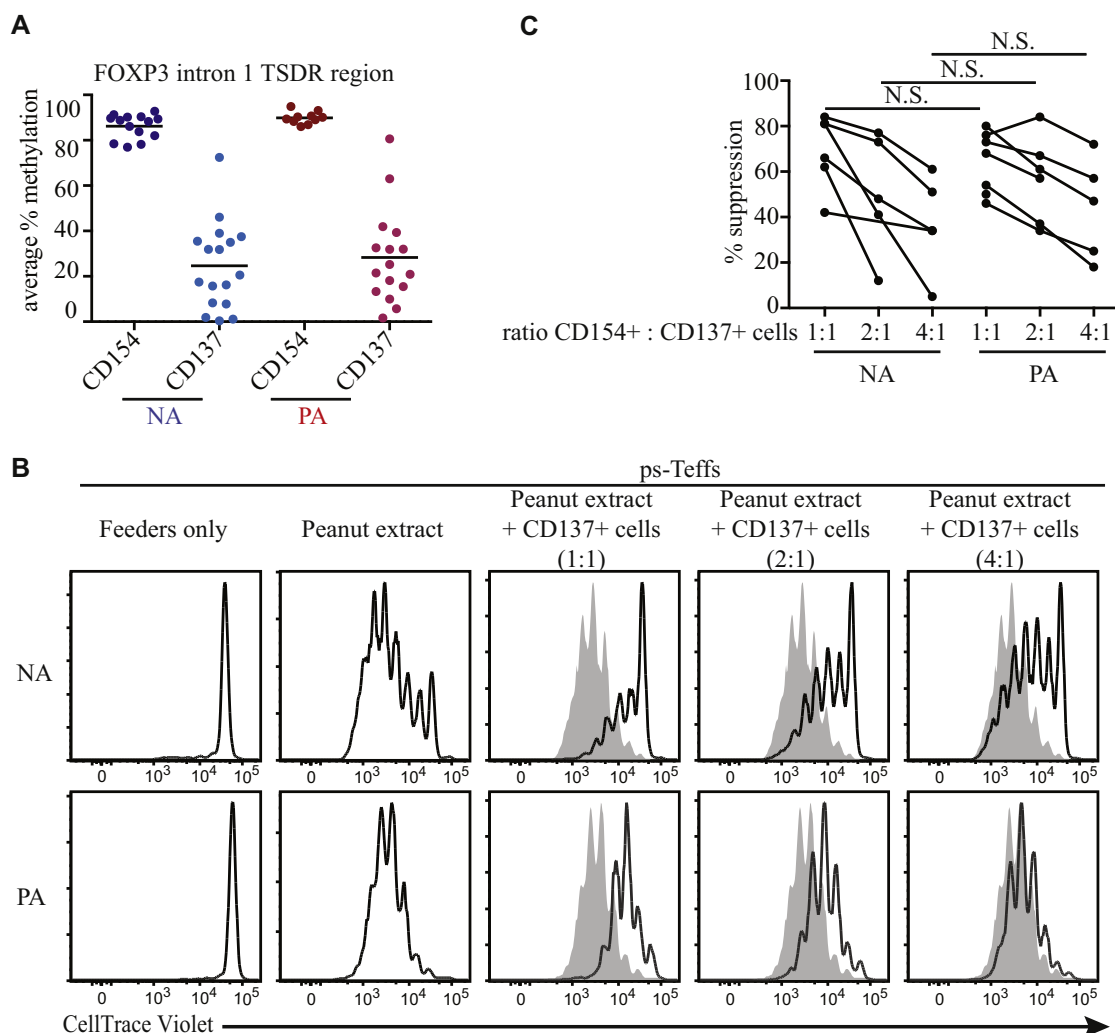


FIG 2. Analysis of Treg cells in school-aged subjects with PA and NA school-aged subjects. **A**, Average percentage methylation across 11 CpG locations in the Treg cell–specific demethylated region (TSDR) of intron 1 of *FOXP3* in fluorescence-activated cell sorting–isolated ps-Teff cells (CD154⁺) and ps-Treg cells (CD137⁺) from school-aged subjects with PA and NA school-aged subjects. **B**, Representative CellTrace Violet dilution by expanded CD154⁺ T cells isolated from NA donors and donors with PA after restimulation with autologous feeders left unloaded or loaded with peanut extract. Autologous CD137⁺ T cells were added to cultures at ratios of 1:1, 1:2, and 1:4 to CD154⁺ cells. Shaded gray histograms show division in response to peanut extract in the absence of CD137⁺ cells. **C**, Percentage suppression of proliferation of CD154⁺ cells stimulated with CPE in the presence of the indicated ratio of CD137⁺ cells relative to cultures that did not contain CD137⁺ cells. Statistics were performed by using an unpaired *t* test with the Welch correction for unequal variation. N.S., Not significant.

across 11 CpG sites in the Treg cell–specific demethylated region of intron 1 of the *FOXP3* locus. Demethylation of this region is important in maintaining a suppressive Treg cell phenotype in both mice and human subjects.^{30–34} We found that the CD137⁺ cell population averaged 0% to 40% methylation across this region of *FOXP3*, which is consistent with what has been reported previously in human Treg cells.³⁴ In contrast, the sorted CD154⁺ cells consistently displayed a methylation rate of close to 90% (Fig 2, A). No differences in *FOXP3* methylation in either ps-Treg or ps-Teff cells were observed between NA subjects and patients with PA (Fig 2, A).

To assess the suppressive capacity of the CD137⁺ cells, CD154⁺ ps-Teff cells and CD137⁺ ps-Treg cells from NA

subjects and patients with PA were isolated by using a magnetic column after stimulation with CPE and expanded *in vitro*, as previously described,^{20,35} to produce sufficient cell numbers for a suppression assay. CD137⁺ cells from NA subjects and patients with PA were equally capable of suppressing the proliferation of autologous CD154⁺ cells in response to CPE when added at ratios of 1:1, 1:2, and 1:4 to cultures (Fig 2, B and C). We set up parallel cultures of CD154⁺ and CD137⁺ cells alone with autologous PBMCs that were left unloaded or loaded with wheat or CPE. Both CD154⁺ and CD137⁺ cells divided more in response to CPE than to autologous feeders alone or loaded with wheat, thus confirming their reactivity to peanut antigen (see Fig E1 in this article's Online Repository at www.jacionline.org).

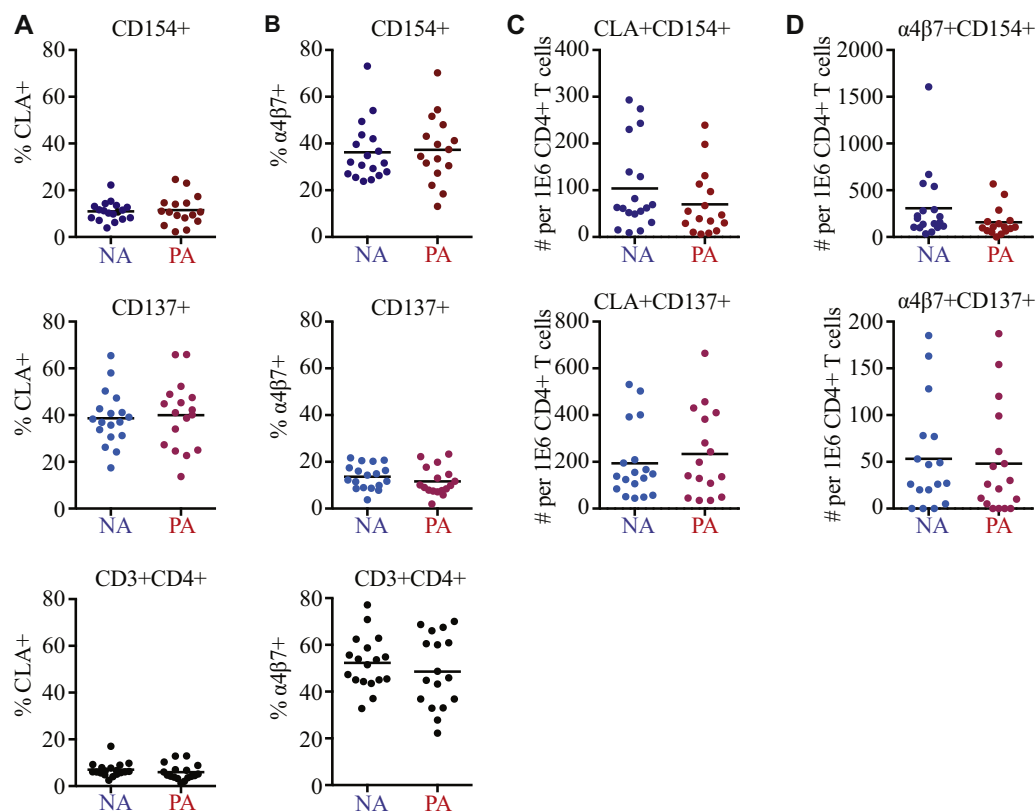


FIG 4. Expression of the homing molecules CLA and $\alpha 4 \beta 7$ on ps-Teff and ps-Treg cells from school-aged children. After stimulation of whole blood from NA school-aged children or school-aged children with PA with CPE, the percentage and number of CD154⁺, CD137⁺, and total CD3⁺CD4⁺ T cells expressing CLA (A and C) and $\alpha 4 \beta 7$ (B and D) was determined by using flow cytometry. Statistics were calculated by using an unpaired *t* test with the Welch correction.

with PA or NA subjects (see Fig E2 in this article's Online Repository at www.jacionline.org).

We next assessed peanut-specific T cells for expression of the skin-homing molecule cutaneous lymphocyte antigen (CLA) and the gut-homing molecule $\alpha 4 \beta 7$, which are strongly correlated with the site of initial antigen encounter for a naive T cell.^{36,37} No differences in expression of CLA or $\alpha 4 \beta 7$ on ps-Teff cells, ps-Treg cells, or total CD4⁺ T cells were observed between children with PA and NA children (Fig 4). Consistent with prior reports of total CD4⁺ Teff and Treg cells, ps-Treg cells expressed relatively more CLA and less $\alpha 4 \beta 7$ than ps-Teff cells.³⁸

Although Treg cells have been shown to potently suppress mast cell degranulation and allergic responses through direct interactions between the 2 cell types,³⁹ we found no significant correlation between the number of total ps-Treg cells or CLA⁺ ps-Treg cells and mast cell reactivity to peanut, as measured by using endpoint titration SPTs to peanut (see Fig E3 in this article's Online Repository at www.jacionline.org).

Characterization of peanut-specific T cells at 1 year of age

The immune system undergoes rapid development early in life as many antigens are encountered for the first time. Because many children experience PA during the first year of life, we sought to evaluate how the T-cell response to peanut develops during this critical window. PBMCs were obtained from a cohort of infants at

1 year of age enrolled in the HealthNuts study.²⁶ Three groups of children were studied: (1) NA subjects (*n* = 14), who were defined as having a negative peanut SPT response and negative OFC result to peanut; (2) patients with PS (*n* = 15), who were defined as having a positive peanut SPT response and positive peanut-specific IgE level but negative OFC result to peanut; and (3) patients with PA (*n* = 14), who were defined as having a positive SPT response, positive peanut-specific IgE level, and positive OFC result to peanut (additional details in Table E2 in this article's Online Repository at www.jacionline.org). Eczema was common in all 3 groups (57% of NA subjects, 73% of patients with PS, and 79% of patients with PA). All of the allergic/sensitized infants were single sensitized, and none of the children had introduced peanut into their diet, although 3 of the children with PS and 1 of the children with PA had documented minimal exposures to peanut at some point during the first year of life (see Table E2).

As shown in Fig 5, no significant differences were identified in numbers of ps-Teff or ps-Treg cells among NA children, children with PS, and children with PA. As expected, the majority of CD137⁺ ps-Treg cells and the minority of CD154⁺ ps-Teff cells expressed FOXP3 in all 3 groups (Fig 5, C). Additionally, the level of expression of CD154, as determined by using median fluorescence intensity, was not different among ps-Teff cells from the 3 groups (data not shown). The ratio of ps-Treg/ps-Teff cells was also not different among the 3 groups (Fig 5, D).

We next investigated cytokine production by peanut-specific T cells in response to stimulation with CPE. Both the PS and PA

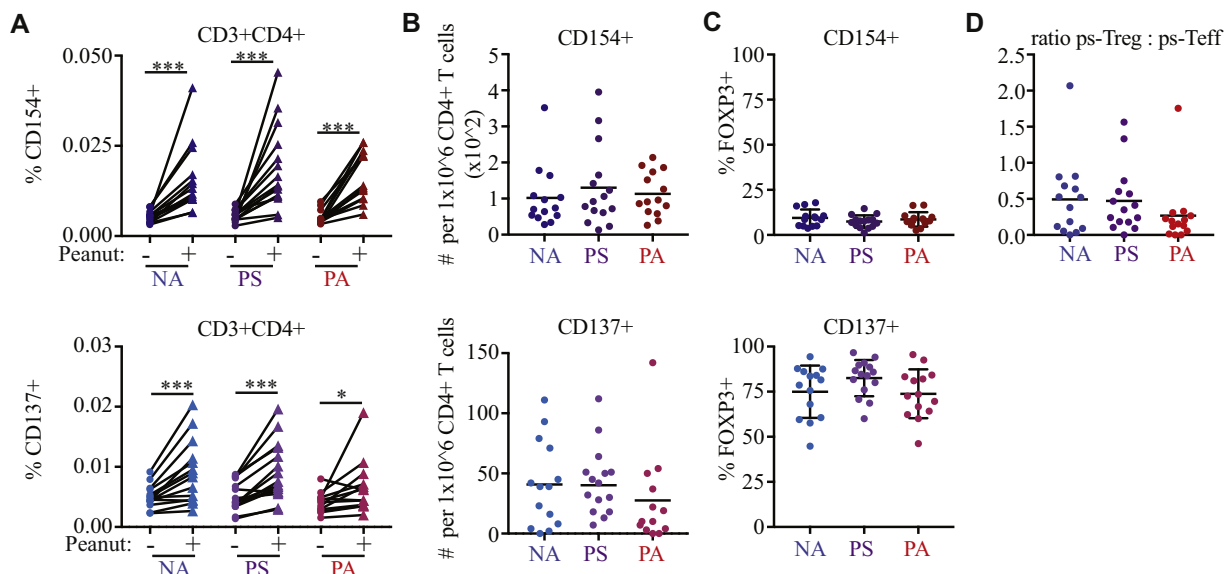


FIG 5. Quantification of ps-Teff and ps-Treg cells by rapid upregulation of CD154 and CD137, respectively, in PBMCs from 1-year-old infants. **A**, Percentage of CD3⁺CD4⁺ T cells that expressed CD154 (top) and CD137 (bottom) after culture of PBMCs in media alone or with CPE from 1-year-old infants who were NA subjects, patients with PS, or patients with PA. Statistics were calculated by using a paired Student *t* test: **P* < .05 and ****P* ≤ .001. **B**, Number of CD154⁺ ps-Teff cells and CD137⁺ ps-Treg cells per 1 × 10⁶ CD3⁺CD4⁺ T cells was calculated by subtracting the number of CD154⁺ or CD137⁺ cells in unstimulated conditions from CPE-stimulated conditions for each subject. Statistics were calculated by using a Kruskal-Wallis test followed by Dunn's multiple comparisons test. **C**, Percentage of CD154⁺ or CD137⁺ cells expressing FOXP3 after stimulation with CPE. **D**, Ratio of the number of CD137⁺ ps-Treg cells to CD154⁺ ps-Teff cells.

groups had a significantly higher percentage of IL-13⁺ ps-Teff cells compared with the NA cohort, and there was a trend toward greater numbers of ps-Teff cells expressing IL-13 in these groups as well (Fig 6, A and B). As in the older children, IL-13 production by ps-Teff cells correlated positively with peanut-specific IgE levels (Fig 6, C). Neither IFN- γ nor IL-17 were significantly induced in ps-Teff cells after treatment with CPE in any of the 3 categories of subjects, and percentages and numbers of IFN- γ ⁺ or IL-17⁺ ps-Teff cells were not different across NA children, children with PS, and children with PA (Fig 6, A and B). Both the percentages and numbers of IL-10⁺ ps-Teff cells tended to be greatest in the children with PS, although this did not reach statistical significance (Fig 6, A and B).

As we had seen in the older cohort of children, ps-Treg cells in NA children, children with PS, and children with PA did not generally produce significant amounts of IL-13, IFN- γ , IL-17, or IL-10 after CPE stimulation at 1 year of age (data not shown).

Next, we assessed expression of the homing molecules CLA and α 4 β 7 on peanut-specific T cells from these infants. Expression of the skin-homing molecule CLA was significantly greater on ps-Teff cells from both infants with PA and infants with PS compared with the NA cohort (Fig 7, A and C). Conversely, α 4 β 7 expression was lower on ps-Teff cells from these 2 groups (Fig 7, B and C). Importantly, the difference in expression of homing molecules was exclusive to ps-Teff cells; neither CLA nor α 4 β 7 expression was different on ps-Treg cells or total CD4⁺FOXP3[−] or CD4⁺FOXP3⁺ T cells among NA children, children with PS, and children with PA (Fig 7). Effector cytokines were primarily produced by CLA⁺ ps-Teff cells after both peanut-specific and nonspecific (phorbol 12-myristate 13-acetate/ionomycin) stimulation in all infants regardless of allergic

status. Percentages of IL-13⁺CLA⁺ ps-Teff cells were greater in those sensitized or allergic to peanut compared with nonallergic control values after peanut stimulation (see Fig E4 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Food allergy stems from a failure to develop tolerance to food antigens, resulting in development of T_H2 effector immune responses and generation of allergen-specific IgE. Emergence of pathogenic responses to food allergens is hypothesized to result in part from a deficiency in the development, function, or both of Treg cells specific for the food. However, technical difficulties in identifying these cells have hampered efforts to investigate a role for Treg cells in food allergy pathogenesis in human subjects. Previous efforts have largely relied on proliferation or tetramer staining, which have inherent limitations. Expansion cultures can select for more proliferative clones and be associated with changes in cellular phenotype, as well as specificity caused by bystander proliferation.⁴⁰ Tetramer staining allows characterization of T cells specific for only 1 epitope at a time and requires knowledge of the patient's HLA status. Furthermore, neither of these methods has allowed for the profiling of the peanut-specific Treg cell population. We have adapted a different strategy to simultaneously identify and characterize ps-Teff and ps-Treg cells directly *ex vivo* that overcomes many of these limitations. Our results suggest that both patients with PS and patients with PA are associated with a T_H2-skewed effector T-cell response to peanut and increased homing of ps-Teff cells to the skin early in life; however, we found no evidence that either PS or PA is

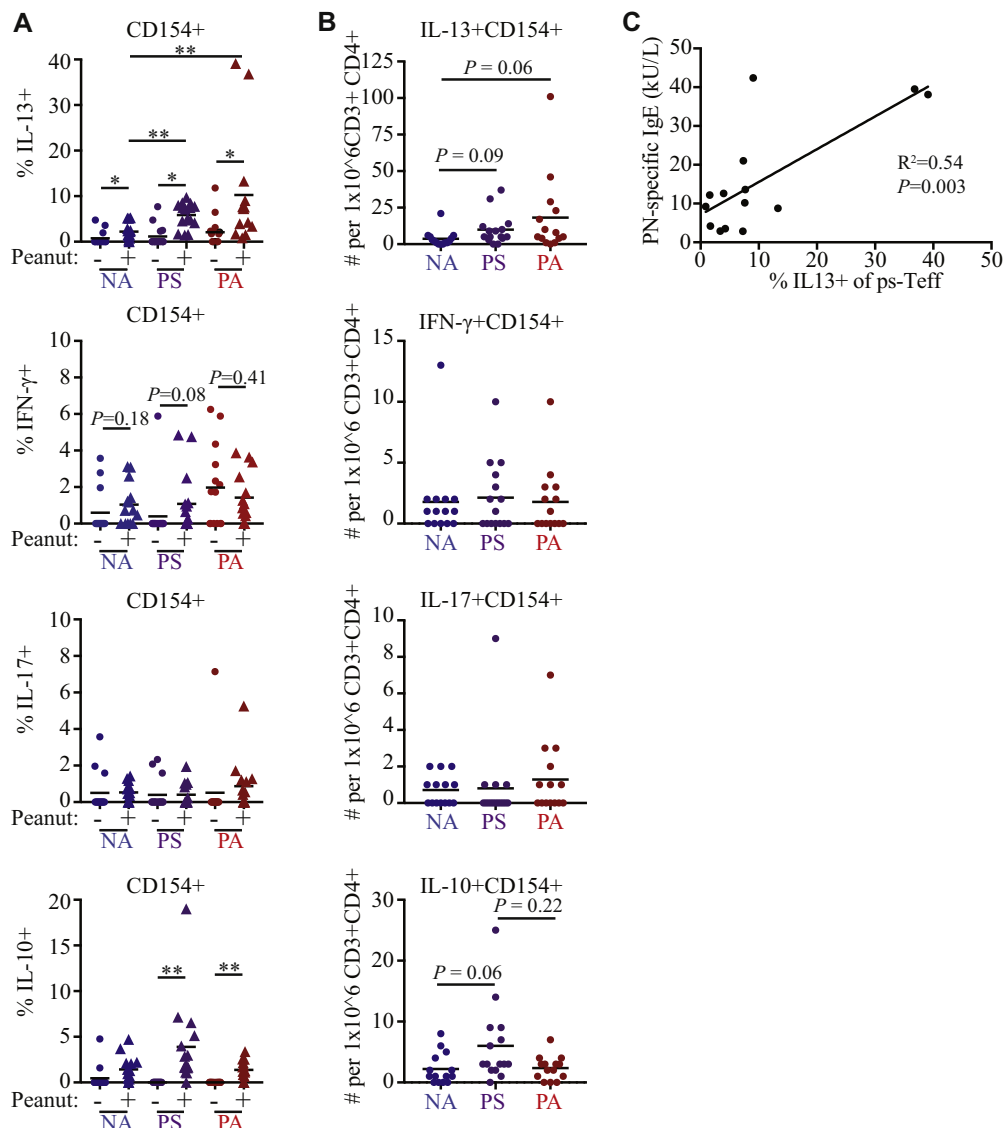


FIG 6. Cytokine production by CD154⁺ ps-Teff cells from 1-year-old infants. **A**, Percentage of CD154⁺ T cells producing each indicated cytokine was measured by using flow cytometry when PBMCs from 1-year-old infants who were NA subjects, patients with PS, or patients with PA were left unstimulated or cultured with CPE. Statistics were calculated by using a paired *t* test between unstimulated and CPE-stimulated samples from the same subject and a Kruskal-Wallis test among the NA, PS, and PA groups: **P* < .05 and ***P* < .01. **B**, Number of CD154⁺ cells per 1 × 10⁶ CD3⁺CD4⁺ T cells expressing the indicated cytokine was calculated by subtracting the number of CD154⁺ cytokine-positive cells in the unstimulated condition from the number in the CPE-stimulated condition. Statistics were calculated by using a Kruskal-Wallis test, followed by the Dunn multiple comparisons test. **C**, Correlation of percentage of ps-Teff cells producing IL-13 and peanut-specific IgE for infants with PA. Linear regression analysis was performed.

associated with a deficiency in the frequency, homing, suppressive function, or stability of ps-Treg cells.

Treg cells are essential for the development of oral tolerance, and Treg cell function has been found to be impaired in a number of monogenic diseases associated with a high risk for food allergy.^{7,41-43} These data suggest that disruption in Treg cell development or function is one mechanism that can predispose to food allergy, but whether defects in Treg cells contribute to the epidemic of food allergy in the general population remains a matter of debate. Although some groups have found an increase in FOXP3⁺ Treg cell counts after successful immunotherapy for PA,^{11,44} others have seen no increase⁴⁵ or only a transient

increase.⁴⁶ These studies all analyzed FOXP3⁺ cells after several days of culturing PBMCs with CPE or used tetramer staining for limited epitopes. Using CD137 upregulation to identify ps-Treg cells directly *ex vivo*, we found these cells were nearly all FOXP3⁺ and did not produce effector cytokines. Methylation analysis of the *FOXP3* locus confirmed Treg cell identity and stability of the CD137⁺ cells and revealed no differences between patients with PA and NA subjects. Furthermore, ps-Treg cells from NA subjects and patients with PA suppressed the proliferation of autologous ps-Teff cells to a similar extent. Collectively, these data suggest that alterations in ps-Treg cells might not be able to explain the development of food allergy.

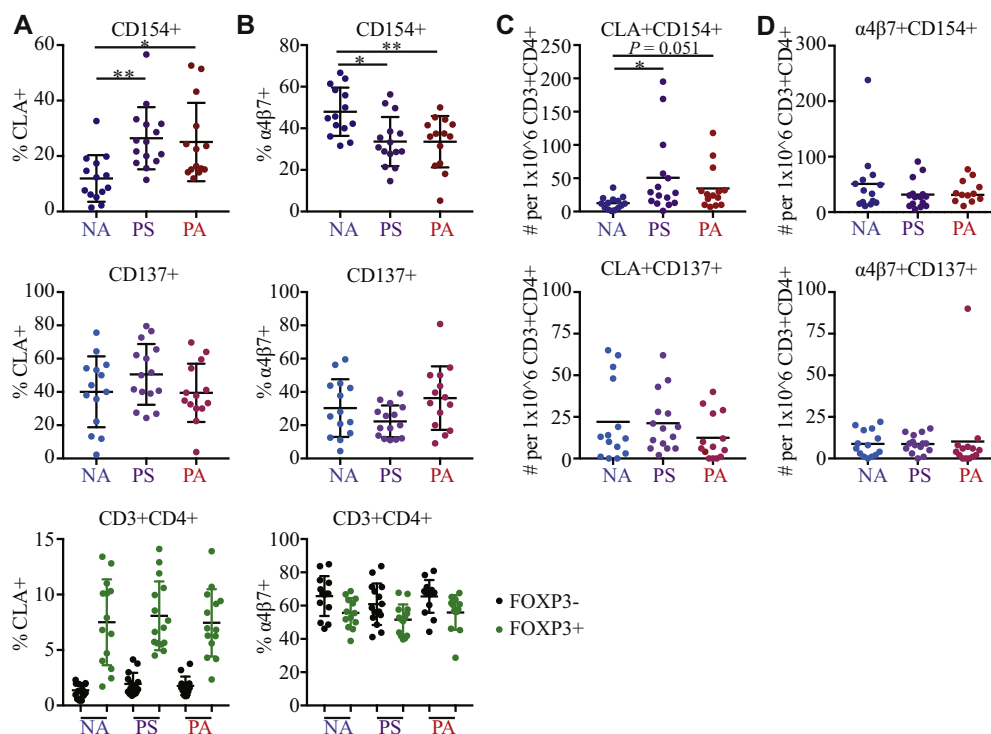


FIG 7. Expression of CLA and $\alpha 4\beta 7$ by peanut-specific T cells from 1-year-old infants. After CPE stimulation of PBMCs from NA infants, infants with PS, or infants with PA, the percentage of CD154⁺, CD137⁺, and total CD3⁺CD4⁺FOXP3⁻ (black) and CD3⁺CD4⁺FOXP3⁺ (green) T cells expressing CLA (**A**) and $\alpha 4\beta 7$ (**B**) was determined by using flow cytometry, and numbers of CLA⁺ (**C**) and $\alpha 4\beta 7$ ⁺ (**D**) CD154⁺ and CD137⁺ cells were calculated. Means and SDs are shown. Statistics were calculated by using the Kruskal-Wallis test, followed by the Dunn multiple comparison test: * $P < .05$ and ** $P < .01$.

Although no discrepancy in the frequency of ps-Treg cells was identified in school-aged children with established PA versus age-matched NA subjects, we considered the possibility that a transient deficiency in ps-Treg cell counts early in life, when peanut is first introduced into the diet, can contribute to the development of PA. To address this question, we compared the frequency of ps-Treg cells in 1-year-old infants with OFC-confirmed PA with that in age-matched infants sensitized but clinically tolerant to peanut and NA control subjects. All 3 groups of children showed similar numbers of ps-Treg cells, arguing against a quantitative defect in Treg cell development early in life as an explanation for the development of PA. Our data are consistent with the results of a recent study that concluded that the development of allergic responses to aeroallergens was also not the result of an impaired Treg cell response in allergic patients.³⁵ This group found no difference in aeroantigen-specific Treg cell frequency, phenotype, T-cell receptor avidity, suppressive capacity, or T-cell receptor β repertoire diversity between allergic patients and NA subjects. In both our older cohort of children with PA and our younger cohorts with PA or PS, we also saw a pronounced T_H2-dominated immune response by ps-Teff cells. Similar to what has been reported for both peanut and cat allergy,^{47,48} we found a positive correlation between IL-13 production by ps-Teff cells and peanut-specific IgE levels for both age groups, as well as between IL-13 production and basophil activation test results for the older cohort. Although greater numbers of IFN- γ ⁺ ps-Teff cells were evident in the older group of NA children compared with those with PA, this difference was not seen in

the 1-year-old infants. In fact, T_H1 responses by ps-Teff cells to peanut were low in all children at this age but dominated the effector T-cell response to peanut in older NA children who were ingesting peanut in their diet. Although these data suggest that an initial deficiency in T_H1 responses to peanut might not cause PA, failure of IFN- γ ⁺ ps-Teff cells to expand can contribute to disease persistence. Furthermore, we found that the total number of ps-Teff cells was not greater in patients with PA, and in fact, the NA group tended to have a greater frequency of ps-Teff cells among school-aged children. We hypothesize that this is likely a consequence of continued exposure to peanut by the NA subjects, resulting in expansion of peanut-specific T cells. In contrast, allergic subjects are instructed to avoid peanut, and subsequently, peanut-specific T-cell clones might not have the opportunity to expand.

Both Treg and Teff cells acquire expression of homing receptors that reflect the tissue site of initial activation. T cells activated in the mesenteric lymph nodes or Peyer patches express $\alpha 4\beta 7$, which directs their trafficking to the gut, whereas those activated in skin lymph nodes express CLA, which mediates their localization to the skin.^{36,37,49} Although expression of CLA and $\alpha 4\beta 7$ was similar on both ps-Treg and ps-Teff cells in school-aged children with and without PA, a higher percentage of ps-Teff cells from infants with PA and infants with PS at 1 year of age expressed CLA and correspondingly fewer ps-Teff cells expressed $\alpha 4\beta 7$ compared with their NA counterparts. Importantly, expression of CLA and $\alpha 4\beta 7$ on ps-Treg cells was not different among the 3 groups of infants, suggesting that lack of proper

ps-Treg cell homing is not responsible for the development of T_H2 effector responses in children with PS and those with PA.

Our data are consistent with the dual allergen-exposure hypothesis, which theorizes that initial exposure to a food allergen through the skin results in generation of an allergic T_H2 response and sensitization, whereas exposure through consumption induces tolerance.⁵⁰ A prior study found that sorted CLA⁺ memory cells from patients with PA proliferated more in response to peanut than sorted α4β7⁺ memory cells,⁵¹ although our study is the first to show differential expression of these markers on identified peanut-specific T cells. The increase in CLA expression on ps-Teff cells from infants with PA and infants with PS is unlikely to be explained merely by a higher rate of eczema in these 2 groups because the majority of NA infants received a diagnosis of eczema as well, and no difference in CLA expression was evident on total CD4⁺ T cells (FOXP3⁺ or FOXP3[−]) across the 3 groups of subjects. Furthermore, CLA expression was similar on ps-Teff cells from school-aged children with PA and NA school-aged children, despite the fact that nearly all of the children with PA and only 1 of the NA children had received a diagnosis of eczema in this cohort. The higher frequency of α4β7⁺ ps-Teff cells in the NA infants cannot be explained by greater ingestion of peanut in this group because none of the 1-year-old infants introduced peanut into their diet. However, we cannot exclude the possibility that NA children had ingested small amounts of peanut antigen either through breast milk⁵² or as a minor component of other food items. Additionally, the allergic and sensitized groups might have had more contact with peanut through the skin, thus allowing for the development and expansion of CLA⁺ ps-Teff cells. Nonoral environmental, peanut exposure has been found to correlate positively with development of PA.⁵³ Our observation that CLA⁺ ps-Teff cells were the primary source of effector cytokines in all infants emphasizes the critical role of the skin in determining immune responses to peanut early in life.

ps-Teff cells from both infants with PS and infants with PA showed altered homing and T_H2-dominated effector responses, suggesting that impairment in the skin barrier early in life, along with environmental peanut exposure, promotes sensitization to peanut, whereas other factors must determine clinical reactivity to peanut. Although not statistically different, we found a trend toward greater numbers of IL-10⁺ ps-Teff cells in infants with PS relative to their counterparts with PA and NA subjects. Prior studies have implicated IL-10 as a key tolerogenic cytokine in food allergy; children who outgrew a food allergy or who were predisposed to food allergy but remained tolerant to cow's milk were more likely to have CD4⁺ T cells that secreted IL-10 in response to the tolerated food antigen than allergic children or those with no food allergy.^{54,55} Although further validation is needed, these data support a potential tolerogenic role for IL-10 in suppressing clinical allergic responses to peanut in infants sensitized to the food.

Our study has several limitations. First, the results from this study did not use paired longitudinal samples but rather samples from allergic children at different ages, and thus we cannot be certain that the changes in T-cell responses in our infant and school-aged cohorts were due to age alone. Second, ps-Teff and ps-Treg cells might not recirculate after encountering peanut antigen in the gut, and thus our ability to detect them in peripheral blood and draw conclusions might be limited. However, others have found T cells specific for orally administered antigens in the blood, which suggests recirculation can occur.^{36,56} Finally, there

might be factors that predispose to food allergy in the innate immune system, which are outside the scope of the present study and require further investigation.⁵⁷

In summary, our data suggest that PA does not result from a quantitative deficiency in the development of ps-Treg cells or IFN-γ⁺ ps-Teff cell but rather the emergence of a pathogenic T_H2 effector response as a result of impairment in the skin barrier and environmental peanut exposure.

Key messages

- The frequency, stability (as assessed by methylation status of the *FOXP3* locus), and homing receptor expression of ps-Treg cells in peripheral blood were similar in children with PA and NA children.
- ps-Teff cells from school-aged children with PA were more likely to produce IL-13 and less likely to express IFN-γ compared with NA control subjects; however, although ps-Teff cells from infants with PS or infants with PA were also T_H2 skewed, few IFN-γ⁺ ps-Teff cells were detected in 1-year-old infants, regardless of allergic status.
- ps-Teff cells from infants sensitized or allergic to peanut at 1 year of age are more likely to express the skin-homing receptor CLA compared with NA control subjects, suggesting they were likely primed after exposure to peanut through the skin.

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METHODS

Patient populations

School-aged subjects were recruited to the NIH under clinical protocol 15-I-0162 (n = 17 patients with PA and n = 19 NA subjects). PA was defined as a documented history of an immediate hypersensitivity reaction after peanut exposure along with a positive SPT response (wheal >3 mm larger than that elicited by the negative control), peanut-specific IgE levels (>0.35 kU_A/L; ImmunoCAP; Thermo Scientific, Waltham, Mass), and basophil activation test results to peanut. All NA subjects had a negative peanut-specific IgE or FX5 food panel result (allergen mix of 6 the most common food allergens including peanut; ImmunoCAP) and were consuming peanut in their diet.

Basophil activation test

Whole blood collected in heparin was incubated with 1 μ mol/L N-formyl-L-methionyl-L-leucyl-L-phenylalanine (Sigma, St Louis, Mo) as a nonspecific positive control, anti-IgE antibody (0.01, 0.1, and 1 μ g/mL) as a positive control, and CPE (0.01, 0.1, 1, 10, 100, 250, and 500 ng/mL) for 15 minutes at 37°C. Cold EDTA (20 mmol/L) was added to inhibit further basophil activation, antibodies were added for flow cytometric analysis, and cells

were fixed and erythrocytes were lysed with BD Lyse/Fix buffer, according to the manufacturer's instructions. Flow cytometric data were collected by using a BD LSR II. Basophils were identified as CD123⁺BDCA2⁺HLA-DR⁺ cells, and expression of CD63 was assessed as a marker of degranulation. The area under the curve of CD63 expression for all dilutions of CPE was determined.

Flow cytometric analysis

The following antibodies were used to characterize and sort peanut-specific T cells: anti-CD154 fluorescein isothiocyanate (24-31), anti-CD137 phycoerythrin (PE; 4B4), anti-CD8 Alexa Fluor 700 (RPA-R8), anti-CD14 Alexa Fluor 700 (61D3), anti-CD20 Alexa Fluor 700 (2H7), anti-IL-10 peridinin-chlorophyll-protein complex/ef710, and phycoerythrin-Cy7 (JES3-9D7) (eBioscience). Anti-CD3 BV510 (HIT3a), anti-CD4 BV711 (SK3), anti-IL-13 BV421 (JES10-5A2), anti-IL-17A BV786 (N49-653), anti-FOXP3 Alexa Fluor 647 (259D/C7), anti-CLA BV605 (HECA-452), anti-IFN- γ BV650 (4S.B3), and anti-IFN- γ BV605 (B27; BD Biosciences) were also used. The reagent anti-human α 4 β 7 integrin (Act-1; catalog no. 11718; from Dr A. A. Ansari) was obtained through the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH.

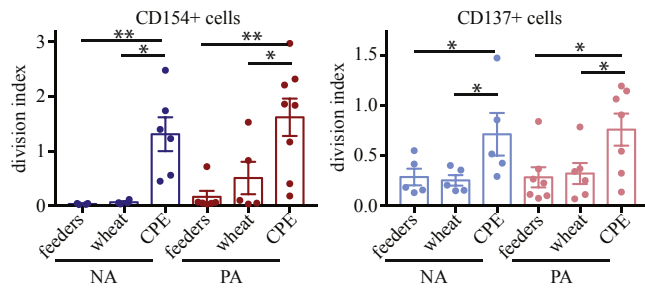


FIG E1. Assay validation. CD154⁺ and CD137⁺ T cells were isolated from PBMCs after 6 hours of stimulation with CPE and expanded separately *in vitro* for 17 to 21 days. After expansion, 1×10^5 expanded CD154⁺ cells labeled with CellTrace Violet or 1×10^5 expanded CD137⁺ cells labeled with carboxyfluorescein succinimidyl ester (CFSE) were restimulated with 2×10^5 irradiated autologous CD3-depleted PBMCs alone or loaded with CPE or wheat extract for 6 days. Dilution of CFSE (CD154⁺ cells) or CellTrace Violet (CD137⁺ cells) was assessed by using flow cytometry, and the division index was calculated. Statistics were calculated by using a ratio paired *t* test: **P* < .05 and ***P* < .01.

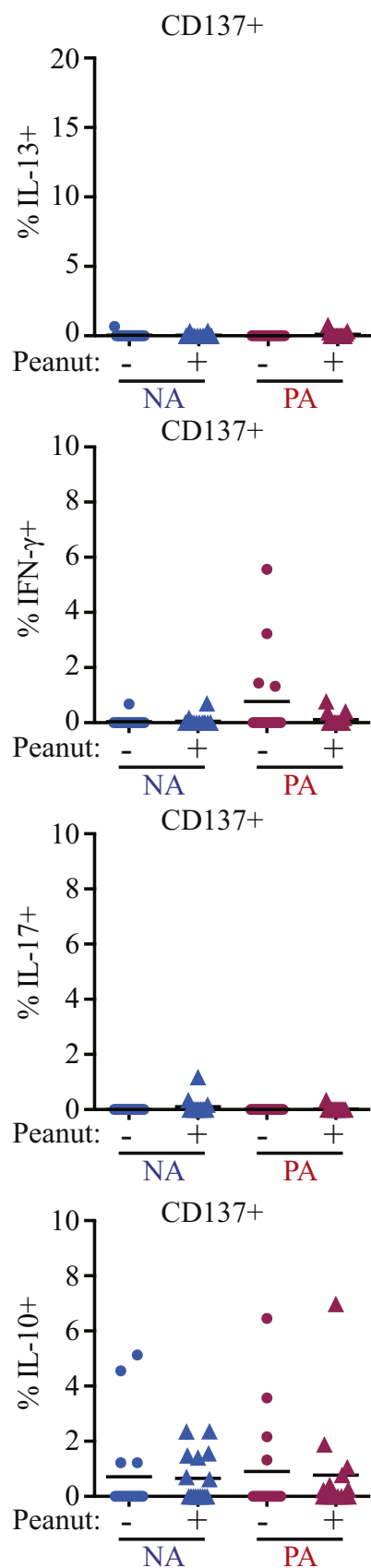


FIG E2. Cytokine production by CD137⁺ ps-Treg cells from school-aged children. Percentage of CD137⁺ T cells producing each indicated cytokine was measured by using flow cytometry when whole blood from NA school-aged children or school-aged children with PA was left unstimulated or cultured with CPE.

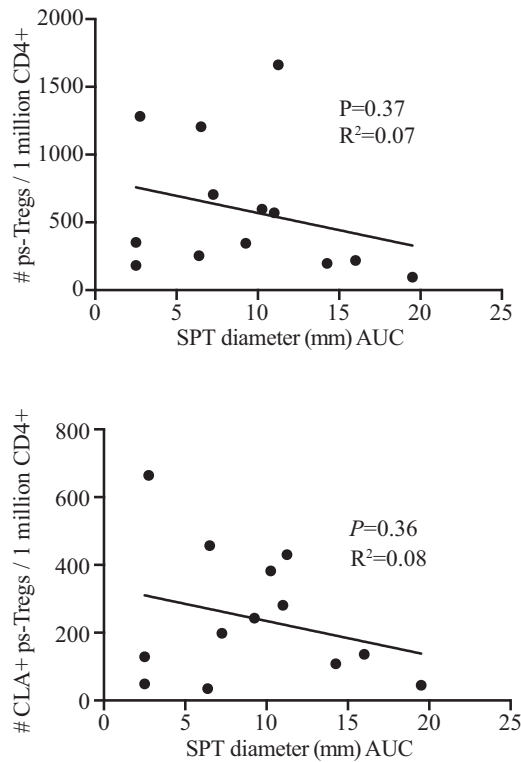


FIG E3. Correlation between peanut SPT response and ps-Treg cell numbers in school-aged children with PA. Diameter of the wheal in response to a peanut SPT at several dilutions of peanut extract (see the [Methods](#) section) was used to determine the area under the curve (*AUC*). Correlation between wheal size and number of total (*top*) and CLA⁺ (*bottom*) ps-Treg cells per million CD4⁺ cells was determined by using linear regression analysis.

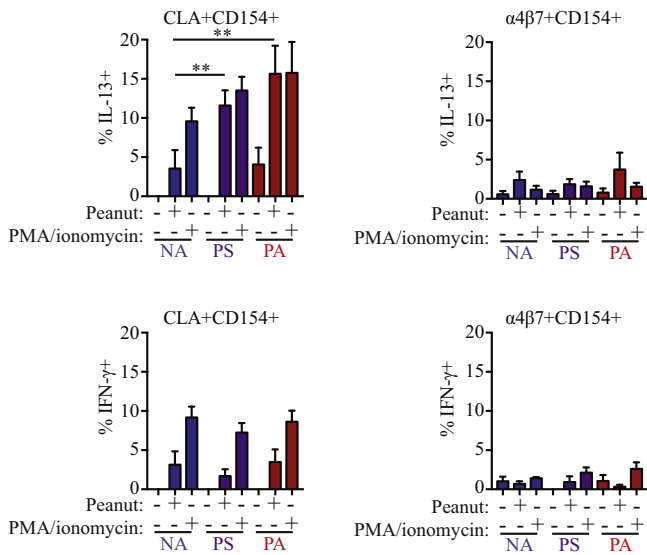


FIG E4. Cytokine production by skin- and gut-homing Teff cells. PBMCs from 1-year-old infants were cultured in medium alone or stimulated with CPE or phorbol 12-myristate 13-acetate (*PMA*)/ionomycin for 6.45 hours. Brefeldin A was added for the final 2 hours of culture. Percentages of CLA⁺CD154⁺ or α4β7⁺CD154⁺ cells expressing IL-13 or IFN-γ were then determined by using flow cytometry. ***P* < .01.

TABLE E1. Characteristics of school-aged study groups at the NIH (n = 36)

	NA subjects	Patients with PA
No.	19	17
Male sex, no. (%)	10 (53)	12 (71)
Age (y), median (lower-upper quartiles)	12 (10-15)	8 (5-13.5)
Peanut SPT wheal size (mm), median (lower-upper quartiles)	Not done	5.5 (4.9-9.5)
Peanut IgE (kU _A /L), median (lower-upper quartiles)	<0.5	36.1 (11.5-100)
Total IgE (kU/L), median (lower-upper quartiles)	31.9 (9.4-96.9)	1,148 (394-8,011)
Other food allergies, no. (%)	0 (0)	17 (100)
Atopic dermatitis, no. (%)	1 (5)	16 (94)
Asthma, no. (%)	1 (5)	12 (71)
Allergic rhinitis, no. (%)	2 (11)	15 (88)
Positive peanut SPT response at indicated dilution, no. (%)	Not done	Not done on 3
1:20		14 (100)
1:200		10 (71)
1:2,000		7 (50)
1:20,000		1 (7)

TABLE E2. Characteristics of 1-year-old infant study groups (n = 43)

	NA subjects	Patients with PS	Patients with PA
No.	14	15	14
Male sex, no. (%)	8 (57)	9 (73)	11 (79)
Peanut SPT wheal size (mm), median (lower-upper quartiles)	0 (0)	4 (3-5)	8 (6-12.5)
Peanut IgE (kU _A /L), median (lower-upper quartiles)	Not done	1.7 (0.6-6.1)	11.2 (4.0-25.3)
Eczema, no. (%)	8 (57)	11 (73)	11 (79)
Accidental oral peanut exposure, no. (%)	0 (0)	3 (20)	1 (7.1)