

Peanut oral immunotherapy results in increased antigen-induced regulatory T-cell function and hypomethylation of forkhead box protein 3 (*FOXP3*)

Aleena Syed, BSc,^{a,*} Marco A. Garcia, BSc,^{a,*} Shu-Chen Lyu, MSc,^a Robert Bucayu, BSc,^a Arunima Kohli, BSc,^a Satoru Ishida, PhD,^a Jelena P. Berglund, PhD,^d Mindy Tsai, DMSc,^b Holden Maecker, PhD,^c Gerri O'Riordan, RN,^a Stephen J. Galli, MD,^{b,c} and Kari C. Nadeau, MD, PhD^a *Stanford, Calif, and Durham, NC*

Background: The mechanisms contributing to clinical immune tolerance remain incompletely understood. This study provides evidence for specific immune mechanisms that are associated with a model of operationally defined clinical tolerance.

Objective: Our overall objective was to study laboratory changes associated with clinical immune tolerance in antigen-induced T cells, basophils, and antibodies in subjects undergoing oral immunotherapy (OIT) for peanut allergy.

Methods: In a phase 1 single-site study, we studied participants (n = 23) undergoing peanut OIT and compared them with age-matched allergic control subjects (n = 20) undergoing standard of care (abstaining from peanut) for 24 months. Participants were operationally defined as clinically immune tolerant (IT) if they had no detectable allergic reactions to a peanut oral food challenge after 3 months of therapy withdrawal (IT, n = 7), whereas those who had an allergic reaction were categorized as nontolerant (NT; n = 13).

Results: Antibody and basophil activation measurements did not statistically differentiate between NT versus IT participants.

However, T-cell function and demethylation of forkhead box protein 3 (*FOXP3*) CpG sites in antigen-induced regulatory T cells were significantly different between IT versus NT participants.

When IT participants were withdrawn from peanut therapy for an additional 3 months (total of 6 months), only 3 participants remained classified as IT participants, and 4 participants regained sensitivity along with increased methylation of *FOXP3* CpG sites in antigen-induced regulatory T cells.

Conclusion: In summary, modifications at the DNA level of antigen-induced T-cell subsets might be predictive of a state of operationally defined clinical immune tolerance during peanut OIT. (*J Allergy Clin Immunol* 2014;133:500-10.)

Key words: Food allergy, allergy, oral immunotherapy, peanut, T regulatory cells, desensitization, tolerance, epigenetics, forkhead box protein 3

Abbreviations used

ai-Treg:	Antigen-induced regulatory T
APC:	Antigen-presenting cell
CD40L:	CD40 ligand
CFSE:	Carboxyfluorescein succinimidyl ester
DBPCFC:	Double-blind, placebo-controlled food challenge
DC:	Dendritic cell
<i>FOXP3</i> :	Forkhead box protein 3
IT:	Immune tolerant
iTreg:	Induced regulatory T
LAG3:	Lymphocyte activation gene 3
MFI:	Mean fluorescence intensity
nTreg:	Natural regulatory T
ns-Treg:	Nonspecific regulatory T
NT:	Nontolerant
OFC:	Oral food challenge
OIT:	Oral immunotherapy
SPT:	Skin prick test
Teff:	Effector CD4 ⁺ T
T _R 1:	Type 1 regulatory T
Treg:	Regulatory T

The mechanisms of clinical immune tolerance remain largely unknown. Studies of potential mechanisms of immunotherapy-induced tolerance to allergens have shown increases in allergen-specific blocking IgG antibody levels,¹ a shift from a T_H2 response toward a T_H1 response with increased IFN- γ production,²⁻⁶ reduction in specific IgE levels, reduced recruitment or increased anergy/deletion of effector CD4⁺ T (Teff) cells,⁷⁻¹¹ and induction of regulatory T (Treg) cells.¹²⁻¹⁹

As key immune-regulatory cells, Treg cells have been shown to play a pivotal role in maintaining immune tolerance, with Treg cell deficiencies implicated in the development of allergies.²⁰⁻²⁴

From ^athe Division of Allergy, Immunology and Rheumatology, Department of Pediatrics; ^bthe Department of Pathology; and ^cthe Department of Microbiology & Immunology Stanford University School of Medicine, Stanford, and ^dthe Duke Translational Medicine Institute, Regulatory Affairs, Durham.

*These authors contributed equally to this work.

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Corresponding author: Kari C. Nadeau, MD, PhD, Division of Allergy, Immunology and Rheumatology, Stanford University School of Medicine, 269 Campus Dr, CCSR Building, Rm 3215, Stanford, CA 94305. E-mail: knadeau@stanford.edu. 0091-6749/\$36.00

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Moreover, several studies have demonstrated that epigenetic modifications in CpG-rich regions within the forkhead box protein 3 (*FOXP3*) locus of Treg cells are associated with stable *Foxp3* expression and Treg cell-suppressive function.²⁵⁻²⁸ Treg cell subsets, including natural regulatory T (nTreg) cells (derived from the thymus) and induced regulatory T (iTreg) cells (derived from the periphery from Teff cells) have been described.²⁹⁻³² iTreg cells can be characterized as CD4⁺CD25^{hi} cells that proliferate in response to specific antigens (ie, carboxyfluorescein succinimidyl ester [CFSE]^{lo} or CD40 ligand [CD40L]/CD69⁺).³³⁻³⁶ iTreg cells can be *Foxp3*⁺ and/or TGF- β ⁺ and/or IL-10⁺.^{33,35,37-40} Type 1 regulatory T (T_R1) cells release IL-10^{29,41,42} and express CD4, CD49, and lymphocyte activation gene 3 (LAG3) on their surfaces,^{29,41,42} which differs from nTreg cell expression of CD4, CD25^{hi}, CD127^{lo}, and perhaps Helios on their surfaces.^{25-27,43-46}

We hypothesized that iTreg cells (CD4⁺CD25^{hi} cells proliferating in response to specific antigen) play a key role in mediating clinical immune tolerance and that assessment of epigenetic modulation of the *FOXP3* locus within antigen-induced regulatory T (Treg) cells might provide insight into mechanisms of clinical immune tolerance at the cellular and molecular levels. Therefore we conducted a study with participants with peanut allergy undergoing oral immunotherapy (OIT) to peanut protein over the course of 24 months, followed by withdrawal from therapy for 3 months, followed by oral food challenge (OFC) at 27 months. We operationally defined immune-tolerant (IT) participants as those who were nonreactive to an OFC at 27 months and nontolerant (NT) participants as those who reacted to an OFC at 27 months. IT participants were withdrawn from peanut for another 3 months and rechallenged at 30 months. This work builds on our previous findings in aeroallergen immunotherapy²⁷ by showing that ai-Treg cells can modulate Teff cell proliferation to peanut allergen during the course of OIT. We also show that the clinical phenotype of immune tolerance was associated with hypomethylation of *FOXP3* CpG sites in antigen-induced regulatory T (ai-Treg) cells.

METHODS

The protocol for this study was reviewed and approved by the Institutional Review Board of Stanford University. Written informed consent was obtained for all participants before entering the study.

Study design and participants

Of 81 participants screened, 43 with peanut allergy from the clinics at Stanford University Hospital were consented, passed screening, and were enrolled in the study (see Fig E1 in this article's Online Repository at www.jacionline.org). Double-blind, placebo-controlled food challenges (DBPCFCs) occurred at screening (see the Methods section in this article's Online Repository at www.jacionline.org for details on eligibility criteria and challenge dosing). Clinical reactivity is defined as any sign of allergic reaction (ie, score ≥ 1 on the Bock criteria⁴⁷). Subject demographics are summarized in Tables E1 and E2 in this article's Online Repository at www.jacionline.org. The protocol was conducted in a hospital setting with trained staff and was performed similarly to the method used by Jones et al.¹² The study outline is diagrammed in Fig E1.

Collection and processing of samples

Blood was collected at baseline and 3, 6, 9, 12, 18, 24, 27, and 30 months. Laboratory personnel were blinded to participant treatment status. A complete blood count and differential was performed (Stanford Clinical Laboratories). Basophil activation assays were performed, as previously described.⁴⁸

Specific IgE and IgG₄ levels were measured (Stanford Clinical Laboratories). Treg cell, Teff cell, and dendritic cell (DC) subsets were phenotyped by using flow cytometry (LSR II; BD Biosciences, San Jose, Calif). Methylation site analysis was performed on cell subsets, as previously described.⁴⁹ PBMCs were CFSE labeled and cultured with peanut, egg, or timothy grass protein (see the Methods section in this article's Online Repository) to identify ai-Treg and Teff cell subsets. Ai-Treg cells were defined as Treg cells (CD4⁺CD25^{hi}*Foxp3*⁺) that proliferated in the presence of peanut. Proliferation was measured based on CFSE^{lo} or CD40L/CD69⁺ status. nTreg cells were defined by CD4⁺CD25^{hi}*Foxp3*⁺ status, with no proliferation to peanut. Additional information can be found in the Methods section in this article's Online Repository.

Statistical analysis

Comparisons between pretreatment and posttreatment periods or between therapy (NT, IT, or both) groups and control groups were evaluated with the nonparametric Mann-Whitney test, paired Wilcoxon test, and 1-way and 2-way ANOVA (GraphPad Prism Software 5.0; GraphPad Software, La Jolla, Calif), as appropriate. A *P* value of less than .05 was considered statistically significant.

RESULTS

Participants

Twenty-three patients with peanut allergy underwent peanut OIT, whereas 20 age-matched control subjects with peanut allergy underwent standard of care (ie, abstaining from peanut; see Tables E1 and E2 for demographics and Fig E1 for the study schematic). Doses of peanut protein (Byrd Mill, Ashland, Va) were administered orally, with dose escalation every 2 weeks (as tolerated by the subject) up to 4000 mg of protein by 24 months. Both active and control subjects underwent a graded OFC at 24 months. No subject in the control group successfully passed the OFC (ie, all control subjects had signs of clinical reactivity) at 24 months. Participants with no reaction to an OFC were defined as desensitized at 24 months (*n* = 20) and abstained from therapy and avoided peanut-containing foods for 3 months. At 27 months, desensitized participants underwent another OFC. Patients who reacted (ie, exhibited any signs of allergic response) were classified as NT participants (*n* = 13), and those who did not have any clinical allergic reaction were operationally defined as IT participants (*n* = 7). IT participants abstained from OIT and avoided all peanut-containing food for an additional 3 months (total of 6 months of avoidance) and were reassessed for immune tolerance with an OFC at 30 months (IT; *n* = 3).

Humoral and basophil immune markers in IT versus NT participants

Peripheral blood was collected longitudinally and analyzed for immunoglobulin levels and basophil activation to measure immune-monitoring features. Peanut-specific IgE levels showed no statistically significant differences between NT versus IT participants (Fig 1, A). There was a trend for peanut-specific IgG₄ levels and peanut-specific IgG₄/IgE ratios to increase over time in the active treatment groups undergoing OIT; however, differences among the 3 groups did not reach significance (*P* = .24 and *P* = .27, respectively; Fig 1, B and C).

IgE binding can mediate basophil activation, and several studies suggest basophils could serve as a possible tool to diagnose and monitor allergies.^{48,50,51} Surface CD203c levels were measured after *ex vivo* stimulation with peanut allergen, as per published techniques,^{48,50,51} to determine the effect of OIT

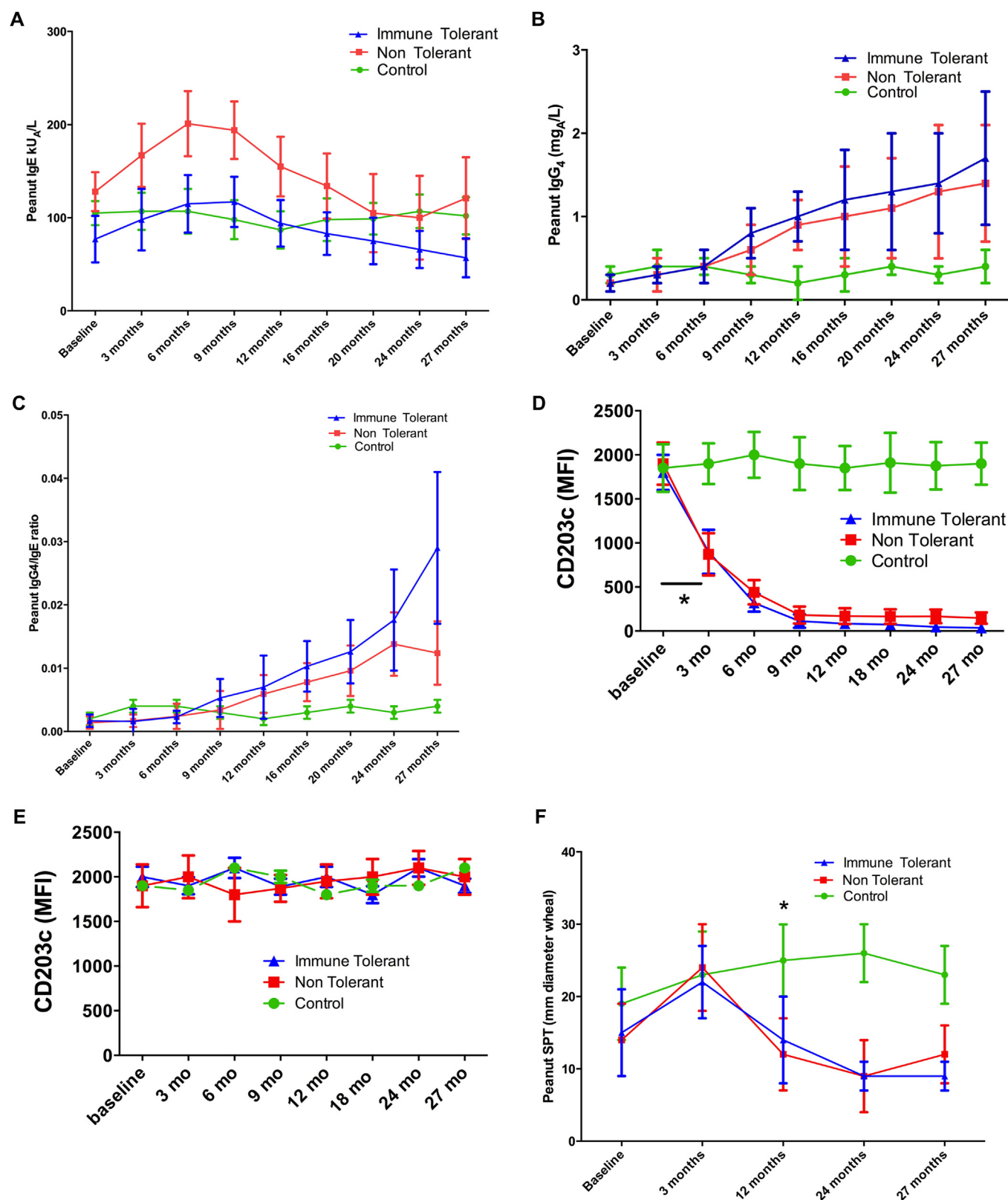


FIG 1. OIT-treated participants (NT participants: *squares*, $n = 13$; IT participants: *triangles*, $n = 7$) or control participants with peanut allergy (*circles*, $n = 20$). **A**, Changes in mean $\pm$ SEM peanut-specific IgE levels (in kilounits of antigen-specific antibody per liter) among IT ($n = 7$), NT ($n = 13$) and control ($n = 20$) participants (not significant, $P = .17$). **B**, Change from baseline in mean $\pm$ SD antibody levels for peanut-specific IgG₄ (in milligrams of antigen-specific antibody per liter). There was a slight increase in IT and NT participants compared with control participants (not significant, $P = .24$). **C**, Mean ($\pm$ SD) peanut-specific IgG₄/IgE

on peripheral basophil activation, Basophil activation decreased at 3 months into OIT ($P < .001$; Fig 1, D) and at 9 months in both the IT (mean $\pm$ SD fluorescence intensity [MFI], 103 ± 46) and NT (mean $\pm$ SD MFI, 117 ± 41 SD) groups, but levels in control participants remained constant (mean $\pm$ SD MFI at 9 months, 1837 ± 140 SD). The peanut-induced basophil response was most reduced in the IT group, although differences between IT and NT participants did not reach statistical significance. Notably, basophils from each group retained high and statistically indistinguishable ($P > .99$) levels of responsiveness to activation by anti-IgE (Fig 1, E).

Skin prick test (SPT) wheal diameters were decreased at 12 months for IT and NT participants compared with control subjects ($P < .05$). No difference was observed in SPT responses in NT versus IT participants (not significant, $P = .66$; Fig 1, F).

ai-Treg cells are induced during OIT and are functionally suppressive

We next investigated whether OIT induced allergen-specific Treg cells. Antigen-induced cells were identified by means of CFSE proliferation assays as $CD4^+CD25^{hi}Foxp3^+CFSE^{lo}$, and T cells and nonspecific regulatory T (ns-Treg) cells were identified as $CD4^+CD25^{hi}Foxp3^+CFSE^{hi}$ T cells, as previously described (for gating, see Fig 2, A and B, and the Methods section in this article's Online Repository at www.jacionline.org).^{33-35,39} Initial studies characterizing T-cell populations identified an increase in ai-Treg cell numbers between baseline and 12 months after starting peanut OIT (Table I). The enhancement of ai-Treg cell function appeared to be specific to peanut because there were no changes in Treg cells specific for other offending allergens (specifically either egg or timothy grass allergen) not used in the OIT protocol (Table I). In addition, there was an increase in intracellular IL-10 levels in the ai-Treg cell population after therapy (Table I). We saw no significant differences at baseline in the IT versus NT versus control groups (see Fig E2 in this article's Online Repository at www.jacionline.org) and grouped the baseline data together ($n = 43$) in Fig 2, D. There were no differences in absolute counts of ns-Treg cells between the control, IT, and NT groups at any time point (Fig 2, C). Absolute counts of ai-Treg cells were significantly increased in IT participants versus NT or control participants beginning 6 months after the start of therapy ($P < .002$; Fig 2, C) and remained so at 24 months ($P < .0001$; Fig 2, D).

The ai-Treg cell population identified in this study could be comprised of iTreg cells^{39,40}; in contrast, ns-Treg cells might represent a population of thymically derived nTreg cells. Although the phenotypic differences between iTreg and nTreg cells are subtle and highly debated,^{40,52-54} functionally, nTreg cells are thought to be nonspecific and control systemic autoimmunity. iTreg cells or "activated" Treg cells were recently discussed by Sakaguchi et al,⁴⁰ activated Treg cells can be identified by $CD4^+CD25^{hi}CD45RA^{lo}Foxp3^+$ status, and iTreg

cells are hypothesized to be specific and are implicated in allergic inflammation.⁵⁵ To further phenotype Treg cell populations, we performed flow cytometry with Helios. Our preliminary data suggest that ai-Treg cells express lower levels of Helios compared with ns-Treg cells (see Fig E3 in this article's Online Repository at www.jacionline.org), possibly indicating that the ai-Treg cell population is comprised of iTreg cells^{25-27,43-45}; however, the ability of levels of Helios expression to discriminate between different subsets of Treg cells is still under active investigation.⁴⁶ Given the increases in IL-10 levels in peanut-induced $CD4^+$ T cells seen in our initial phenotyping studies (Table I), we investigated whether ai-Treg cells could represent $Foxp3^+T_R1$ cells. Our data suggest that the percentage of T_R1 cells is increased in patients receiving therapy compared with those without therapy (see Fig E4 in this article's Online Repository at www.jacionline.org).

Treg cells were sorted by means of flow cytometry from all participants to study function. The suppressive function of ai-Treg cells (defined as $CD4^+CD25^{hi}Foxp3^+$ Treg cells proliferating to peanut) on ai-Teff cell responses was compared at baseline versus 27 months. The data show no significant difference in the suppressive function at baseline for the IT, NT, and control groups (see Fig E2). In Fig 2, E, a Treg cell subpopulation ($CD4^+CD25^{hi}Foxp3^+CD45RO^+$) sorted by means of flow cytometry (FACSaria; BD, San Jose, Calif) was found to suppress Teff cells ($CD4^+CD25^{lo/-}$, $Foxp3^-$) proliferation to peanut but not to other offending allergens or tetanus ($P < .0001$). When naive ($CD45RA^+$) $CD4^+CD25^{hi}Foxp3^+$ Treg cells were sorted out of the total Treg cell population used in the suppression assays, there was no loss in the ability to suppress Teff cell proliferation to peanut allergen (see Fig E5, A, in this article's Online Repository at www.jacionline.org) nor did purified naive Treg cells exhibit any differences in Treg cell function before or after OIT (see Fig E5, B), suggesting that enhanced suppressive function to peanut antigen during OIT was associated with allergen-specific $CD45RO^+$ Treg cells. Of note, there was greater suppression by ai-Treg cells isolated from IT ($95\% \pm 5\%$) than NT ($77\% \pm 21\%$) participants ($P < .0001$; Fig 2, E). Suppressive function of ai-Treg cells from the control group at 27 months was similar to baseline values (Fig 2, E). The increase in ai-Treg cell function did not seem to be due to alterations in the antigen-induced Teff cell function because antigen-induced Teff cell proliferation alone was unchanged between baseline and 27 months (ie, 3 months after stopping OIT) for all treatment groups (see Fig E5, A).

OIT enhances the migratory activity of ai-Treg cells

The functional migratory potential of Treg cells toward an intestinal epithelial cell line was analyzed *in vitro* by using a chemotaxis assay. As shown in Fig 3, A, starting at 12 months, ai-Treg cells from IT or NT participants receiving OIT showed

ratios. There was a slight increase in IT and NT participants compared with control participants (not significant, $P = .27$). D, Expression of CD203c on basophils stimulated with peanut allergen ($1 \mu\text{g/mL}$). Data are presented as means $\pm$ SEMs ($*P < .001$; CI, -1222 to -777.8 ; IT or NT participants versus control participants). IT versus NT participants, $P > .99$ (not significant). E, Expression of CD203c levels on basophils stimulated with anti-IgE ($P > .999$, not significant). F, Significant decrease in SPT response diameters starting at 12 months for IT or NT participants compared with control participants ($*P < .001$; CI, -16.06 to -5.942). No difference in NT versus IT participants was seen ($P = .656$, not significant).

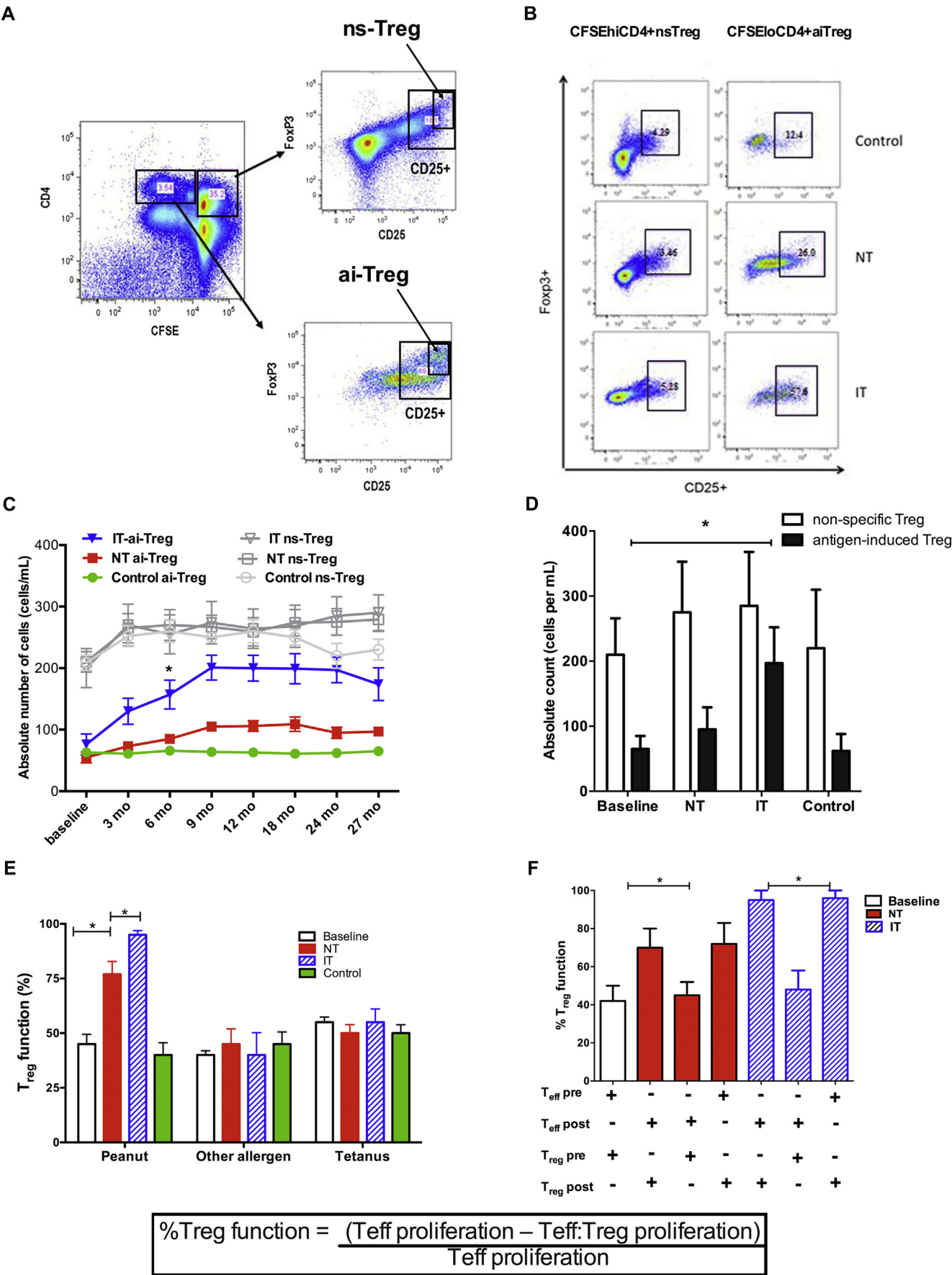


TABLE I. Enhancement of allergen-specific T-cell populations during OIT

Allergen specific T-cell populations*	Treg cells (%), mean $\pm$ SD	IL-10 ⁺ cells (%)	Teff cells (%)	T _H 2 cells (%)	T _H 1 cells (%)
Peanut + memory					
Pre	3.1 $\pm$ 1.3 [‡]	18.9 $\pm$ 4.0 [‡]	24.6 $\pm$ 3.5	57.0 $\pm$ 6.8 [‡]	12.3 $\pm$ 5.7 [†]
Post	16.4 $\pm$ 3.5	52.9 $\pm$ 7.8	23.7 $\pm$ 3.9	19.4 $\pm$ 7.9	22.6 $\pm$ 1.7
Peanut + naive					
Pre	0.2 $\pm$ 0.4 [‡]	11.7 $\pm$ 3.1	2.6 $\pm$ 1.9	17.0 $\pm$ 3.7 [†]	8.2 $\pm$ 1.8 [†]
Post	1.9 $\pm$ 0.9	12.3 $\pm$ 4.1	3.9 $\pm$ 1.3	10.9 $\pm$ 2.8	12.6 $\pm$ 2.8
OOA + memory					
Pre	4.3 $\pm$ 1.5	16.6 $\pm$ 3.2	27.0 $\pm$ 6.3	60.6 $\pm$ 3.5	10.8 $\pm$ 2.1
Post	4.6 $\pm$ 1.3	17.9 $\pm$ 4.8	30.0 $\pm$ 5.4	60.7 $\pm$ 4.7	14.9 $\pm$ 3.4
OOA + naive					
Pre	0.0	18.3 $\pm$ 2.9	3.9 $\pm$ 1.6	13.0 $\pm$ 4.0	7.9 $\pm$ 2.0
Post	1.4 $\pm$ 0.5	18.7 $\pm$ 1.8	3.6 $\pm$ 1.5	11.7 $\pm$ 3.6	8.6 $\pm$ 1.3
Peanut + memory (control)					
Pre	3.1 $\pm$ 1.6	14.7 $\pm$ 1.7	27.4 $\pm$ 4.7	60.3 $\pm$ 5.2	11.3 $\pm$ 2.6
Post	5.0 $\pm$ 1.3	19.4 $\pm$ 2.9	26.1 $\pm$ 3.8	61.1 $\pm$ 3.9	11.4 $\pm$ 2.6

OOA, Other offending allergen; Pre, baseline values before starting OIT; Post, 12 months after starting OIT.

*Percentages shown are percentages of CD4⁺ T cells (mean $\pm$ SD).

Significant differences: [†] $P < .025$ (CI, 4.993 to 15.61) and [‡] $P < .0001$ (CI, 23.72 to 30.28) between the cell populations before (n = 7) and after (n = 7) therapy.

increased migratory activity compared with that seen in control subjects ($P < .001$). Moreover, the migratory activity of ai-Treg cells from IT participants was significantly enhanced starting at 12 months compared with that seen in NT participants.

We next tested whether increased chemokine receptor expression was associated with the increase in migration activity. As shown in Fig 3, B and C, the expression levels of CCR8 increased at 24 and 27 months (ie, at the end of OIT or 3 months after stopping OIT) compared with CCR8 levels at baseline ($P < .001$) in both IT and NT participants. Soler et al⁵⁶ demonstrated that CCR8 is expressed by T_H2 and Treg cells; it is interesting that there is an increase in CCR8 levels in NT participants, which could be due to expression in Treg cells, T_H2 cells, or both. By contrast, CCR4 and CCR7 expression levels did not change significantly over the course of therapy. An example of chemokine receptor staining is shown in Fig 3, D.

Foxp3 is modified in ai-Treg cells during OIT

Because Foxp3 is one of the indicators of Treg cell suppressive function and has been associated with maintenance of iTreg cells and because Foxp3 is a transcription factor for CCR8,⁵⁷⁻⁶² we measured expression of Foxp3 protein in ai-Treg cells using flow cytometry. There was significantly increased Foxp3 protein during OIT in ai-Treg cells from IT participants but not in ai-Treg cells from NT or control participants ($P < .001$; Fig 4, A). Similarly, Foxp3 transcript levels were also increased at 24 and 27 months compared with baseline values in ai-Treg cells from

IT participants but not in ai-Treg cells from NT or control participants (Fig 4, B).

We tested whether DNA methylation of the *FOXP3* gene in Treg cells decreased throughout the course of OIT. ai-Treg cells were identified by double-positive CD69/CD40L expression (in addition to CD4⁺CD25^{hi}Foxp3⁺) on peanut antigen incubation and were purified by means of flow cytometry. Compared with baseline, there was significantly decreased methylation of CpG sites in ai-Treg cells for all OIT participants at 24 and 27 months, with the most pronounced hypomethylation in IT participants ($P < .001$; Fig 4, C). Participants who shifted from the IT group at 27 months to the NT group at 30 months were marked by an increase in methylation of CpG sites (Fig 4, C). ai-Treg cells from control participants (Fig 4, C) and ns-Treg cells from all groups (Fig 4, A) did not change.

DCs induced during OIT can influence epigenetic modifications in T cells

Because iTreg cells can be induced through interactions with DCs, we examined the effects of DCs studied before therapy (baseline) and at 27 months (3 months after stopping OIT) on modulation of *FOXP3* epigenetics in CD4⁺ Teff cells from IT participants versus Teff cells from NT participants, and autologous DCs were purified by means of flow cytometry and cultured together (see Fig E6 in this article's Online Repository at www.jacionline.org) in the presence of peanut protein for 3 days. Autologous cultures did not affect Teff cell viability. Fig E7 in this article's Online

FIG 2. OIT. **A**, Representative staining of ai-Treg and ns-Treg cells. **B**, Representative staining for ns-Treg and ai-Treg cells in control, NT, and IT participants at 30 months. **C**, ns-Treg (open symbols) and ai-Treg (solid symbols) cell absolute counts ($*P < .002$; CI, 2.636 to 141.1; NT participants, n = 7; IT participants, n = 13; control participants, n = 20). **D**, ns-Treg (white bars) and ai-Treg (black bars) cell numbers at baseline and 24 months of treatment ($*P < .0001$; CI, 41.1 to 162.6; NT participants, n = 7; IT participants, n = 13; control participants, n = 20). Changes in ns-Treg cell numbers were not significant ($P = .84$). **E**, Treg cell suppressive activity on conventional responder CD4⁺ T cells (Teff) measured before therapy (baseline) and at 27 months (ie, after 3 months off treatment; filled bars). Suppressed proliferation of Teff cells in response to peanut stimulation in NT and IT participants compared with baseline values ($*P = .0001$; CI, 30.18 to 69.82) but not other allergens or tetanus ($P > .999$). **F**, Suppressive function of Treg cells from before and after OIT. Suppressive function of Treg cells collected before therapy (pre) and at month 27 (post) was assessed toward ai-Teff cells collected before and after therapy (data represent means $\pm$ SEMs; $*P < .001$; CI, -32.03 to -17.97).

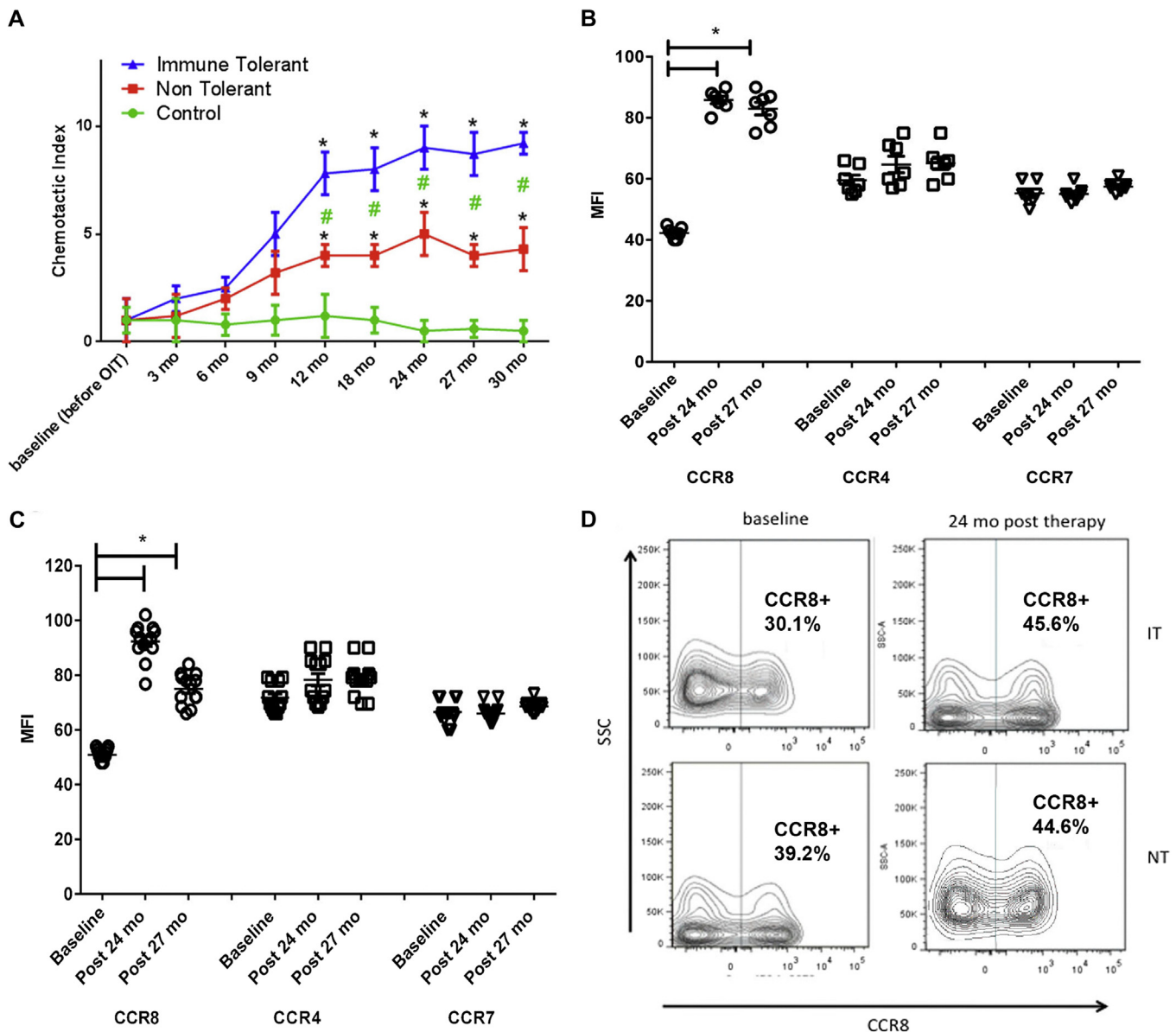


FIG 3. A, Chemotactic indices of ai-Treg cells toward normal intestinal epithelial cells. Indices for cells from IT ($n = 7$) or NT ($n = 13$) participants were significantly higher than those for control participants ($n = 20$) starting at 12 months ($*P < .001$; CI, 5.854 to 7.346). Values for IT participants were significantly higher than those for NT participants starting at 12 months ($\#P < .0001$; CI, 3.004 to 4.596). B and C, Expression levels of chemokine receptors (circles, CCR8; squares, CCR4; triangles CCR7) on ai-Treg cell populations were identified by means of flow cytometry and are presented as MFIs ($\times 10$) at baseline and at 24 months of treatment and at 27 months (ie, 3 months after cessation of treatment) in IT participants (Fig 3, B; $*P < .001$; CI, 38.64 to 48.50) and NT participants (Fig 3, C; $*P < .001$; CI, 34.69 to 48.01). D, An example of chemokine receptor staining.

Repository at www.jacionline.org shows that DCs obtained from either IT or NT participants at baseline did not alter *FOXP3* CpG methylation in Teff cells. However, DCs isolated after therapy significantly decreased the percentage of *FOXP3* CpG methylation in Teff cells ($P < .001$). The percentage of *FOXP3* methylation in Teff cells alone did not change over the course of culture.

DISCUSSION

In this study 20 of 23 participants successfully completed 24 months of OIT, tolerating up to 4 g of peanut protein after

maintenance therapy. After 3 months of peanut avoidance, only 7 of 20 participants were defined as IT participants; these 7 avoided peanut for an additional 3 months (6 months total of avoidance), and only 3 of 7 remained clinically nonreactive (IT). We found that the IT participants had numbers of higher ai-Treg cells with greater suppressive function and with higher levels of *FOXP3* hypomethylation compared with NT and control participants. Thus this study demonstrates a possible mechanism of immune tolerance in subjects receiving OIT involving (1) an increase in numbers of ai-Treg cells with enhanced chemotactic and suppressive behavior and (2) epigenetic modifications within the *FOXP3*

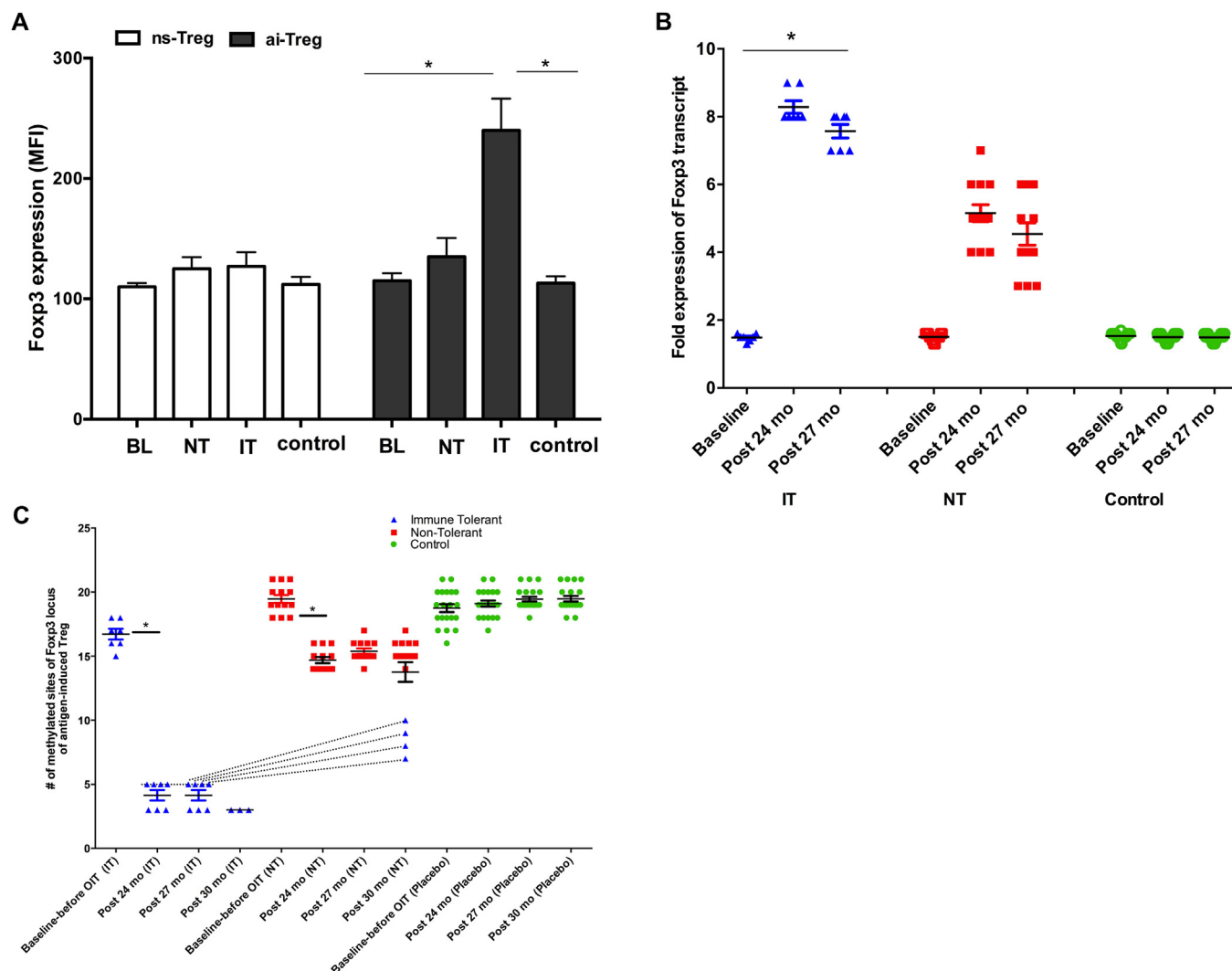


FIG 4. **A**, Intracellular Foxp3 protein expression levels in ns-Treg (white bars) and ai-Treg (black bars) cells. Values are MFIs. There were significant differences in ai-Treg cell counts for IT participants versus NT or control participants ($P < .001$; CI, 90.34 to 159.7); ns-Treg cells were not significant ($P = .1698$). BL, Baseline. **B**, *FOXP3* mRNA in Treg cells isolated from IT participants receiving OIT (triangles), NT participants receiving OIT (squares), and control participants with peanut allergy (circles). Significant differences were found in IT participants at 24 or 27 months compared with baseline values ($P < .001$; CI, 2.871 to 4.437). Differences for NT participants were nonsignificant ($P = .18$). **C**, ai-Treg cells from participants undergoing OIT (IT participants: $n = 7$, blue triangles; NT participants: $n = 13$, red squares) or untreated control participants ($n = 20$, green circles). Four of 7 IT participants (connected by broken lines) were no longer tolerant at 30 months. Data represent mean $\pm$ SEM numbers of methylated sites (IT participants: $P < .001$ vs baseline [CI, 12.13 to 14.16]; NT participants: $P < .001$ vs baseline [CI, 3.919 to 5.620]). Results were not significant for control participants ($P = .15$).

locus of such ai-Treg cells. None of the demographic or clinical features assessed at baseline affected the outcome of IT versus NT participants. All subjects receiving OIT were undergoing maintenance therapy for at least 3 months (ie, from 21 to 24 months); the number of months on maintenance therapy did not distinguish IT versus NT participants.

In this study we focused on the possible role of Treg cells and whether ai-Treg cells were associated with immune tolerance in OIT. The method of identification of iTreg cells was consistent with other publications^{33-37,39,40} in which iTreg cells were defined as proliferating CD4⁺CD25^{hi}Foxp3⁺ cells (ie, either defined through CFSE¹⁰ or CD40L/CD69⁺ status in response to specific antigen stimulation). Furthermore, our data show that the iTreg

cell population was CD45RO⁺ (and CD45RA⁻), Helios^{10/-}, CD49b⁺/LAG3⁺, Foxp3⁺, and IL-10⁺; therefore the iTreg cells we identified with these markers are consistent with other studies³³⁻³⁷ and are possibly Foxp3⁺ T_R1 cells.^{29,35,41,42} Moreover, we demonstrated that the iTreg cell population induced through peanut OIT was specific in suppression of autologous Teff cell proliferation in response to peanut and not other antigens. The effector T-cell population we used in functional assays was purified as CD4⁺CD25^{10/-} and found to be Foxp3⁻ to prevent cross-contamination.

Previous studies have demonstrated the presence of increased Treg cell numbers in patients with immune tolerance.^{12,14,15,63} ai-Treg cells, despite being in relatively small numbers compared

with other immune cell subsets, have been shown to be associated with natural loss of food allergy.⁶⁴ In contrast to other OIT studies in which high doses of antigen are administered with concomitant omalizumab use,⁶⁵ the present OIT protocol, like that developed by Burks, Jones et al,¹² and others^{12,66-68} started with a low initial dose of peanut followed by slow dose escalations.

Most previous immunotherapy studies have not proved whether Treg cells are functionally suppressive, whether they migrate toward the intestines, or whether they are antigen induced. The population of ai-Treg cells we identified during OIT had a marked increase in Foxp3 expression and associated increases in both chemotaxis toward intestinal epithelial cells and suppressive function toward antigen-induced Teff cells (Figs 2-4) in IT participants. Therefore our data suggest that one possible mechanism of OIT involves modifications of ai-Treg cells to enhance their suppressor function, possibly in the intestinal tissues. Demethylation of *FOXP3* plays a crucial role in Treg cell plasticity and suppressive function^{28,40,69,70} and could play a role in immune tolerance. Importantly, we have documented hypomethylation within the CpG locus of *FOXP3* in ai-Treg cells in IT versus NT participants (Fig 4). We followed participants up to 30 months (ie, until 6 months after stopping OIT) and saw that 4 of 7 IT participants (broken lines in Fig 4, C) were no longer "tolerant" at 30 months. Such "resensitization" was associated with increased methylation of CpG sites in the *FOXP3* locus (Fig 4, C).

Our results suggest possible Treg cell biomarkers that might be useful in predicting a state of immune tolerance; however, many cells in addition to Treg cells are thought to contribute to immune tolerance. Our recent aeroallergen immunotherapy study by Swamy et al²⁷ reported epigenetic changes in the *FOXP3* locus after therapy. The present study performed novel detailed mechanistic studies on sorted antigen-induced T cells and identified Foxp3⁺ T_R1 cells as the major iTreg cell population associated with success of therapy. Although the current study focuses on epigenetic studies of the *FOXP3* locus, our data suggest a potential role for DCs in modulating *FOXP3* epigenetics in Teff cells (see Fig E7). Data from our laboratory demonstrate that DCs expressing indoleamine 2,3-dioxygenase might help promote the conversion of naive CD4⁺ T cells to Treg cells and that this conversion might be mediated through epigenetic changes at the *FOXP3* locus.⁷¹ Janson et al²⁸ have shown that *FOXP3* promoter demethylation was associated with the appearance of a committed Treg cell population, and increased expression of indoleamine 2,3-dioxygenase by DCs has been linked to enhanced tolerogenicity and the suppression of Teff cell proliferation.⁷²

In a recent clinical study by Burks et al,⁷³ participants underwent egg OIT for 22 months. The clinical data in that study and ours are similar, in that the percentage of subjects with loss of clinical reactivity after a period of food allergen avoidance after OIT in our study was 30% (7/23 after 3 months of peanut avoidance) and in the Burks et al⁷³ study was 28% (11/40 after 4-6 weeks egg avoidance). One of the differences between these studies was that the OFC was performed with up to 4.0 g of peanut protein (the same dose as the maintenance dose) in our study, whereas Burks et al used 10 g of egg protein (a much higher dose than the maintenance dose of 2 g of egg protein). The role of specific IgG₄ is under investigation in OIT^{67-69,73}; our data demonstrated a trend but not statistical significance. Although many studies have begun to investigate sustained therapeutic responses after OIT, questions remain regarding the long-term

sustainability and safety of desensitization versus such operationally defined examples of immune tolerance and whether specific IgG₄ levels, IgG₄/IgE ratios, basophil reactivity, and T-cell or other cellular markers could be used to predict an outcome of IT.^{73,74}

Although our data suggest that larger phase 2 trials in OIT are justified and feasible, the limitations of the present study should be mentioned. First, we realize that this was a small cohort study performed at a single site. Second, our mechanistic conclusions are limited to changes detectable by sampling the peripheral circulation. It will be of interest to examine mechanisms at the local organ level as well, as has been done with tonsillar tissue.⁷⁴

In summary, our study investigated the durability of immune tolerance in subjects undergoing food allergy OIT after a minimum of 3 months off therapy. Food allergy OIT is under investigation and is still in the experimental stages.^{67-69,74,75} It is important to note that NT participants, after stopping OIT, were still able to ingest a relatively large amount of peanut protein (compared with their threshold dose at baseline food challenge) before experiencing allergic symptoms (see Table E2). Our data suggest that ai-Treg cells, rather than ns-Treg cells, are a key regulatory cell type modulating the immune response during OIT and that epigenetic regulation of these T cells might contribute to the induction of such immune tolerance. However, none of our data in this small study demonstrate a complete dichotomy between IT versus NT participants; instead, there were incremental differences (some statistically significant and others not) in the extent of mechanistic changes. An interesting goal for future studies will be to determine whether a composite set of values for immune indicators (involving T cells, B cells, basophils, or other cells or plasma markers) might be strongly associated with clinical immune tolerance and its durability. Moreover, large studies will be needed to determine whether such immune indicators can be identified and, if so, whether they will be useful in refining peanut OIT so that it can provide safe, sustained, and effective therapy for patients with peanut allergy.

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Key messages

- Mechanisms of immune tolerance during OIT potentially involve induction of ai-Treg cells.
- Epigenetic changes in the *FOXP3* locus might enhance Treg cell function in this setting.
- Modifications at the DNA level in specific T-cell subsets might be predictive of unresponsiveness during OIT.

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METHODS

Study design and participants

Eighty-one subjects with peanut allergy were screened from the Adult and Pediatric Allergy and Immunology clinics at Stanford University Hospital and are described in the study diagram (Fig E1). Twenty-three subjects with peanut allergy who underwent peanut OIT and 20 age-matched control subjects with peanut allergy who underwent standard of care (ie, abstaining from peanut) were enrolled.

Participants were excluded for some of the following reasons: severe asthma (defined as per the National Heart, Lung, and Blood Institute's Expert Panel Report 2010), severe reaction to peanut ingestions resulting in an intensive care unit hospital admission, and/or eosinophilic disorders. Participants (eligible age, 4-55 years) were enrolled if a number of inclusion criteria were met, including a documented peanut-specific IgE level of 15 kU_A/L or greater, and a positive SPT response to peanut allergen defined as a wheal diameter of 10 mm or larger than that elicited by the saline control. Moreover, a subject must have had a documented allergic reaction (considered positive for an allergic reaction as per the Bock criteria) during a DBPCFC with peanut protein (as peanut flour; Byrd Mill) conducted by Stanford food allergy clinical trial research study staff in a hospital setting. The placebo control for DBPCFCs was oat flour.

At baseline, subjects receiving active OIT reacted to a median dose of 25 mg of peanut protein (range, 6-100 mg) and a median cumulative dose of 32.7 mg of peanut protein (range, 7.7-182.7 mg). Control subjects also reacted to a median of 25 mg of peanut protein (range, 6-100 mg) and a median cumulative dose level of 32.7 mg of peanut protein (range, 7.7-182.7 mg). Three participants dropped out of the active peanut OIT arm (1 because of relocation of residence, 1 because of anxiety, and another because of noncompliance with reaction medications). The control group was a randomized 1:1 unblinded control group (Fig E2). Baseline demographics of study subjects are provided in Tables E1 and E2. Differences in the incidence of atopic disease (ie, asthma and allergic rhinitis) in the treatment and control groups were not statistically significant ($P = .17$).

Peanut protein preparation and OIT for peanut allergen was performed as in the study by Jones et al^{E1} in a hospital setting with trained personnel. Briefly, initial escalating doses of peanut protein (peanut flour, Byrd Mill) on the first day were administered orally, beginning with 0.1 mg of peanut protein, followed by approximate doubling every 30 minutes, up to 6 mg of peanut protein. Doses were increased 25% every 2 weeks up to 4000 mg of peanut protein by 24 months, as per Jones et al.^{E1} Subjects who tolerated 4.0 g of peanut protein with no allergic reaction on repeat food challenge at 24 months (OFC 2) of OIT and with an SPT response (mean diameter) to peanut (Greer peanut extract undiluted; Greer Laboratories, Lenoir, NC) that was at least 1 mm smaller than that elicited by the simultaneous histamine control (Greer histamine control, Greer Laboratories) were considered desensitized and then abstained from therapy for 3 months (months 24-27). At month 27, subjects underwent a food challenge (OFC 3) to 4.0 g of peanut protein or placebo (oat flour) to test for immune tolerance (defined as a lack of clinical reactivity with OFC 3) versus nontolerance (clinical reactivity with OFC 3). OFC 4 was formed on the 7 IT participants after an additional 3 months off peanut OIT (ie, OFC 4 was performed at 30 months). Blood was collected from subjects at baseline and 3, 6, 9, 12, 18, 24, 27, and 30 months for analysis of immune parameters and cellular studies. The protocol for this study was reviewed and approved by the Institutional Review Board of Stanford University. Written informed consent was obtained from all participants (or from parents for minors) before subjects entered the study. International Conference on Harmonisation and Code of Federal Regulation guidelines were followed.

DBPCFCs

Initial DBPCFC for screening. For the first DBPCFC, all subjects underwent spirometry as appropriate per age and had continuous pulse oximetry monitoring and vital signs checked every 15 minutes before being given increasing doses of placebo (oat flour) or allergenic food protein (peanut flour, Byrd Mill) until an objective reaction occurred, as per the Bock criteria. Placebo and peanut challenges were conducted on separate days. The dosages were as follows: 0.1 mg of protein in 15 minutes, 1.6 mg of protein in

30 minutes, 6 mg of protein in 45 minutes, 25 mg of protein in 60 minutes, 50 mg of protein in 60 minutes, and 100 mg of protein in 60 minutes.

Second and subsequent DBPCFCs. Subjects who were eligible for DBPCFCs received up to 5 doses during the challenge (placebo or peanut flour was given on separate days) administered every 30 minutes: 250, 500, 1250, and 2000 mg (4000 mg of protein total).

Measurement of anti-peanut antibody titers

Total and allergen-specific blood IgE and IgG₄ levels were measured in all subjects in the Clinical Laboratories at Stanford Hospital and Clinics using a standard ImmunoCAP assay (Phadia, Uppsala, Sweden).

Basophil activation assay and basophil phenotyping by means of flow cytometry

The basophil stimulation assay was performed, as previously described.^{E2} Three microliters of PBS with or without 1 µg/mL peanut allergen was added to 200 µL of blood, and the mixture was incubated for 20 minutes at 37°C. The incubation was stopped by the addition of ice-cold PBS-EDTA, and the cells were pelleted by means of centrifugation (490g for 5 minutes at 4°C) and stained for 20 minutes on ice with Live/Dead (Invitrogen, Grand Island, NY) and with labeled antibodies to the following surface markers: CD3 (UCHT1), CD11b (VIM12), and CD16 (3G8) from Invitrogen; CD20 (2H7), CD41a (96.2C1), CD56 (B157), CD63 (H5C6), CD66b (G10F5), CD123 (7G3), CCR3 (5E8), CD294 (BM16), and HLA-DR (L243) from BD Biosciences; and CD203c (NP4D6) from BioLegend (San Diego, Calif). After staining, cells were washed and fixed with 2 mL of 1× Lyse/Fix PhosFlow (BD Biosciences) for 30 minutes on ice in the dark and washed a final time by means of centrifugation before analysis on the LSRII flow cytometer (BD Biosciences).

T-cell and DC phenotyping by means of flow cytometry

Cells were fixed with Lyse/Fix PhosFlow buffer (BD Biosciences). For intracellular staining, fixed cells were permeabilized with Perm Buffer III (BD Biosciences) at 4°C for 30 minutes, followed by staining at 4°C for 20 minutes. Flow cytometry was performed with an LSRII flow cytometer (BD Biosciences). Viable cells were identified with a Live/Dead probe (Invitrogen). Phenotypes of T cells were detected with antibodies against surface CD3 (UCHT1), CD4 (SK3), CD25 (4E3), CD127 (SB199), CD45RO (UCHL1), CD45RA (HI100), CD62L (DREG-56), CCR4 (1G1), and CCR8 from BD Biosciences; CCR7, CD69, and CD40L and intracellular IL-10 (JES3-19F1), IL-4 (MP4-25D2), and IL-13 (JES10-52A2) from BD Biosciences; Helios (22F6) from BioLegend; anti-CD49b from BioLegend; anti-LAG3 from R&D Systems (Minneapolis, Minn); and Foxp3 (150D) from BioLegend and stained per the manufacturer-recommended protocol. DC populations were detected with antibodies against HLA-DR (G46-6), Lin1 cocktail (CD3, CD14, CD16, CD19, CD20, CD56), and CD11c (B-ly6) from BD Biosciences and CD123 (6H6) from BioLegend. For T_H1 staining, PBMCs were incubated for 10 minutes at room temperature in the presence of FcR blocking reagent (Miltenyi Biotec, Auburn, Calif) and then stained for CD49b and LAG-3 at 37°C for 15 minutes.

Identification and enumeration of Treg cell subsets

PBMCs from multiple time points for each participant were thawed, labeled with CFSE, and cultured with peanut protein at 100 µg/mL (Byrd Mill) or anti-CD3/CD28 (to test for nonspecific proliferation capacity) for 7 days to identify ai-Treg cells. At day 7, cells were washed and stained for surface CD4, CD25, CD127, CD45RO, CD45RA, CD40L, and CD69 and intracellular Foxp3 and IL-10 along with Live/Dead staining (Invitrogen). ai-Treg cells were defined as the cells that proliferated in response to peanut allergen (CFSE^{lo}) and were CD4⁺CD25^{hi}Foxp3⁺ cells. ns-Treg cells were identified as CD4⁺CD25^{hi}Foxp3⁺CFSE^{hi} cells. Antigen-induced T cells were also identified by isolating CD40L and CD69 double-positive cells after antigen stimulation. A complete blood count with differential was performed on all subjects (Stanford Clinical Laboratories) at each blood draw

(no stimulation with *ex vivo* allergen) to calculate absolute numbers of cell subsets.

T-cell purification

CD4⁺ T cells were enriched from whole blood by means of incubation with the negative selection CD4⁺ isolation kit, according to the manufacturer's methods (StemCell Technologies, Vancouver, British Columbia, Canada). Cells were subsequently stained with Live/Dead (Invitrogen) and CD4 and CD25. Treg (CD4⁺CD25^{hi}) cells were stained and isolated by means of fluorescence-activated cell sorting (FACSARIA, BD) before addition to the suppressor functional assay. Live, autologous non-Treg cells (CD4⁺CD25^{lo}OR[−]Foxp3[−], defined here as Teff cells) were flow sorted simultaneously. Postsort analysis demonstrated that each sorted population was greater than 99% pure and that the sorted Treg cell populations were Foxp3⁺. Sorted T-cell populations were incubated in RPMI-1640 media with 10% FBS for 2 hours to rest the cells before use in further experiments.

Preparation of peanut allergen for *ex vivo* T-cell and basophil stimulation assays

For all *ex vivo* cultures, the same peanut protein used in subjects for OIT (Byrd Mill) was used as the peanut antigen. The peanut protein was dissolved in PBS with no precipitates; the protein concentration was calculated by using the Bio-Rad Assay (Bio-Rad Laboratories, Hercules, Calif), and the stock was diluted to the specified final concentration in cell-culture media.

Suppression assay

Standard thymidine-based suppression assays were performed to analyze Treg cell function. Sorted, unstimulated, autologous Treg and Teff cells were plated in a 96-well culture plates with complete media with autologous, irradiated, CD3-depleted PBMCs (ie, antigen-presenting cells [APCs]) by using a 1 Treg cell/1 Teff cell/10 APC ratio.^{E3} Ratios of 1 Treg cell/4 Teff cells also were performed in a few samples in initial titrations. APCs were incubated overnight with peanut allergen (1 μg/mL) or other allergen (1 μg/mL) at 37°C before suppression assays were performed. Tetanus toxoid antigen (1 μg/mL; Sigma, St Louis, Mo) was used as a positive control antigen. We also tested other allergens to which the subject was found to be allergic (but for which they did not receive OIT), including egg or timothy grass (1 μg/mL, low endotoxin purified allergens; INDOOR Biotechnologies). On day 6, cells were pulsed with 1 μCi of tritiated thymidine per well and harvested on day 7 with a Tomtec cell harvester (Tomtec, Hamden, Conn). Tritiated thymidine incorporation was measured with a 1450 microbeta Wallac Triux liquid scintillation counter. The percentage of Teff cell proliferation was measured by using standard published thymidine incorporation methods, and the percentage of Treg cell function was determined as follows:

(Teff cell proliferation − Teff cell:Treg cell proliferation)/Teff cell proliferation.

Treg cell purification for RT-PCR and epigenetic studies

ai-Treg cell populations were purified by using a CD40L⁺ capture method^{E4} with the coexpression of CD69 and were cell sorted to obtain CD4⁺CD25^{hi} cells (FACSARIA, BD Biosciences) after peanut allergen stimulation at 100 μg/mL for 4 to 6 hours. ns-Treg cells were purified as CD40L[−]/CD69[−] cells and cell sorted for CD4⁺CD25^{hi} cells (FACSARIA, BD Biosciences). RNA was isolated with RNeasy kits (Qiagen, Valencia, Calif), according to the manufacturer's protocols. Similar numbers of cells were used for each subject. For cDNA synthesis, 60 to 100 ng of total RNA was transcribed with cDNA transcription reagents (Applied Biosystems, Carlsbad, Calif) by using random hexamers, according to the manufacturer's protocols. Gene expression was measured in real time by using primers and other reagents purchased from Applied Biosystems and SuperArray (Valencia, Calif). The same number of live cells was used per RT-PCR reaction (100,000). All PCR assays were performed in triplicate. Data were presented

as relative fold expression of the candidate gene to the expression of the house-keeping gene β-glucuronidase.

Methylation site analysis

ai-Treg cell populations, ns-Treg cells, Teff cells, and DCs were purified by using cell sorting alone (FACSARIA, BD Biosciences), as per above. The same number of live cells was used per pyrosequencing reaction. Genomic DNA was isolated with a DNA extraction kit (Qiagen). The DNA was denatured, modified with sodium metabisulfite, purified, and desulfonated by using a CpGenome Fast DNA modification kit (Chemicon International, Billerica, Mass). The disulfite oligonucleotide primers designed with MethPrimer software and used in this analysis were based on our previous methods.^{E5,E6}

Chemotaxis assay

CD4⁺ T cells were labeled with CFSE and stimulated with media containing 100 μg/mL peanut allergen for 7 days. The activated CD4⁺ T cells (5 × 10⁵) were added to the top compartment of a 5-mm Transwell system (Corning Costar, Tewksbury, Mass). Human intestinal epithelial cells (CACO-2; ATCC, Manassas, Va) were added to the bottom well, and the transwells were incubated for 2.5 hours at 37°C in the absence of exogenous chemokines. ai-Treg cells (CD4⁺CD25^{hi}CFSE^{lo}) migrating toward intestinal epithelial cells were identified by means of flow cytometry, and the number of migrating ai-Treg cells was divided by the number of cells that spontaneously migrated toward media alone to calculate chemotactic indices. Each assay was performed in duplicate.

DC-T-cell culture

Plasmacytoid and myeloid combined DC populations were purified by means of flow sorting of (FACSARIA, BD Biosciences) PBMCs to obtain live HLA-DR⁺Lin1[−]CD11c⁺CD123⁺ DCs (Fig E6). Autologous CD4⁺ Teff cell populations were isolated from frozen PBMCs by means of magnetic bead separation per the manufacturer's protocol (Miltenyi Biotech); their phenotype was confirmed as greater than 95% pure CD4⁺CD25^{lo}OR[−] T cells. Autologous DCs and CD4⁺ T cells were cocultured at a ratio of 1:3 for 72 hours at 37°C in RPMI plus 10% FBS supplemented with Pen-Strep antibiotic (Gibco, Grand Island, NY) with and without 5 μg/mL peanut allergen (Byrd Mill). The concentration of allergen used was based on titration results that demonstrated effective stimulation with 5 μg/mL peanut protein.

Peanut allergen

For all *ex vivo* cultures, the same peanut protein used in participants for OIT (Byrd Mill) was used as the peanut antigen. The peanut protein was dissolved in PBS with no precipitates; protein concentration was calculated by using the Bio-Rad Assay, and the stock was diluted to the specified final concentration in cell-culture media.

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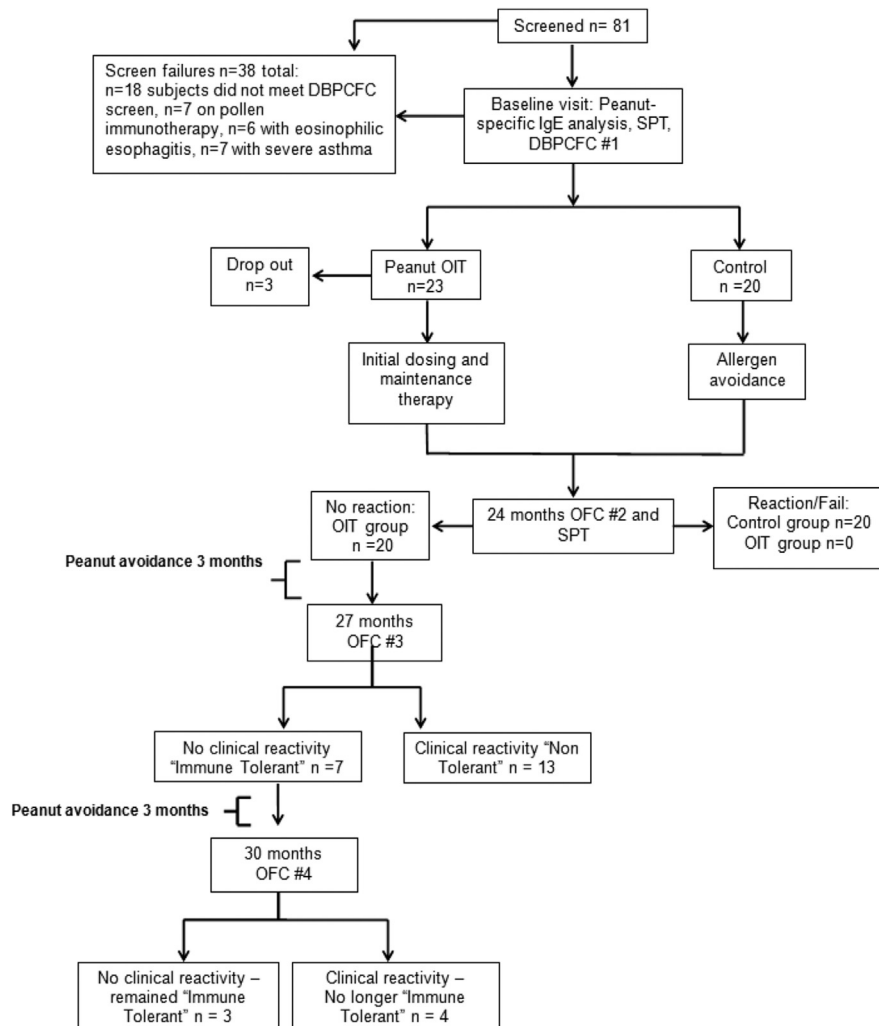


FIG E1. Study flow diagram.

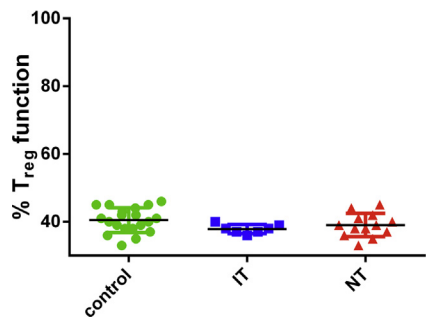


FIG E2. The suppressive function (presented as percentage Treg cell function) of baseline samples from IT, NT, or control participants is statistically indistinguishable. Means (*horizontal long line*) with SEMs (*horizontal short lines*) are graphed (not significant, $P = .178$).

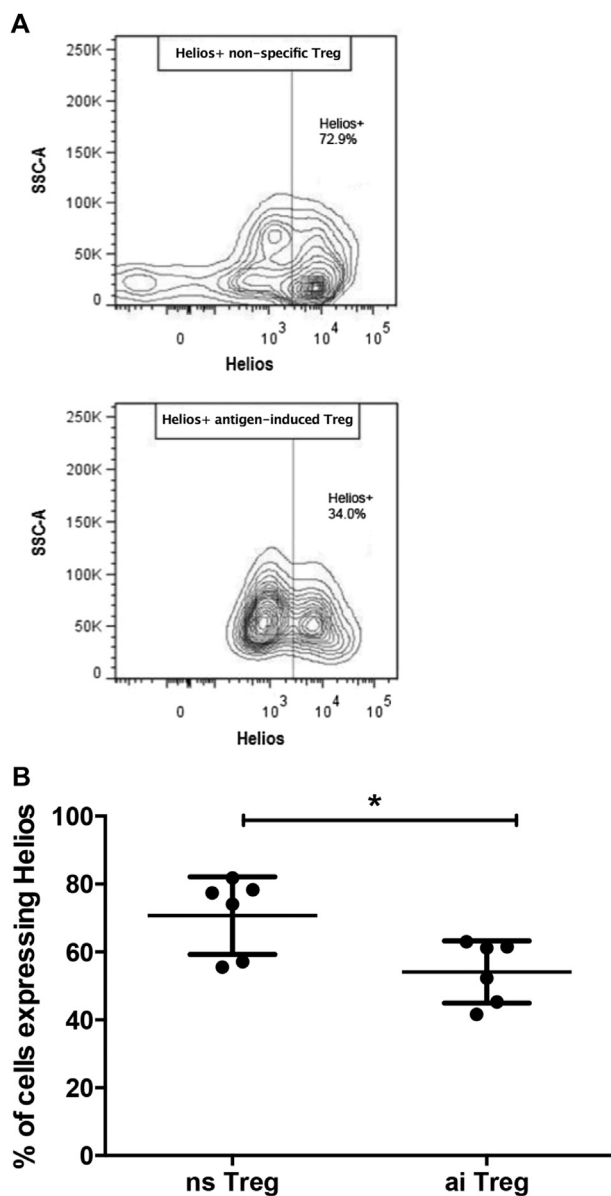


FIG E3. A, Helios expression assessed by using standard flow cytometric methods in ns-Treg cells (CFSE^{hi}CD4⁺CD25^{hi}FoxP3⁺) and ai-Treg cells (CFSE^{lo}CD4⁺CD25^{hi}FoxP3⁺) in a representative sample from 1 subject. SSC, Side scatter. **B,** Composite Helios expression in ns-Treg and ai-Treg cells in untreated patients with peanut allergy (**P* = .009). Data represent means $\pm$ SEMs (*n* = 6 per group).

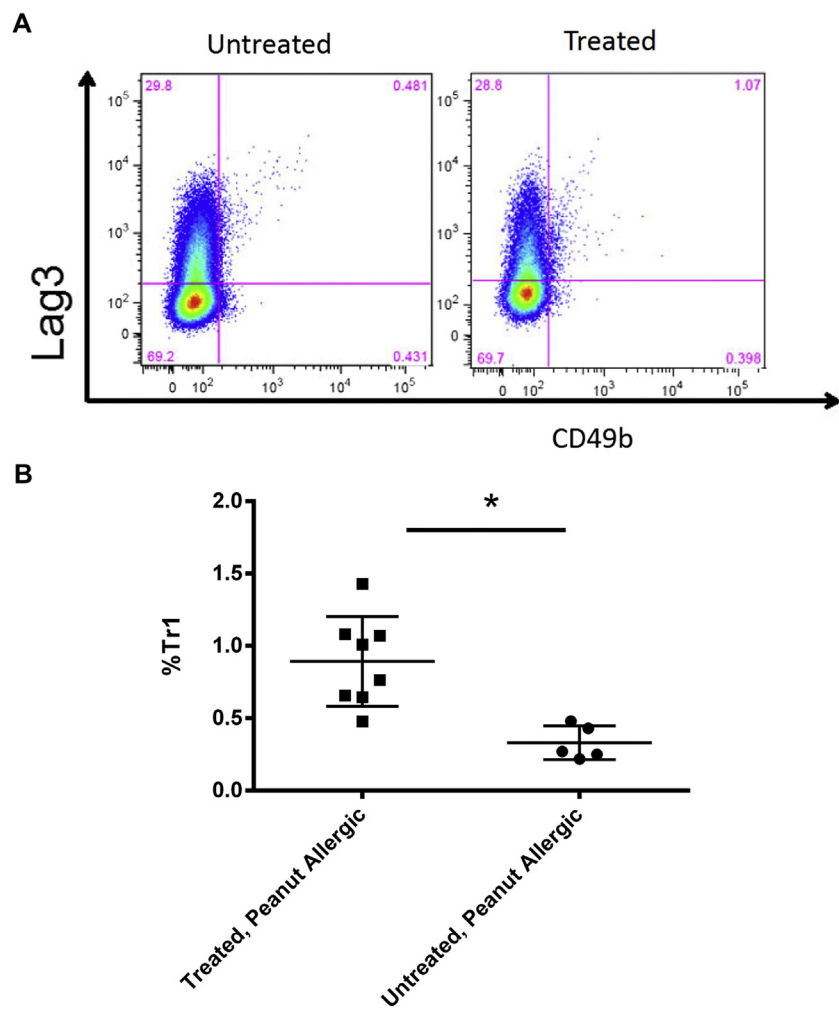


FIG E4. A, Representative staining of T_R1 cells in 1 untreated and 1 treated patient with peanut allergy ($CD4^+CD45RA^-CD49B^+Lag3^+$). **B,** Percentage T_R1 cells of $CD4^+CD45RA^-$ cells in untreated ($n = 5$) and treated (4-36 months after therapy, $n = 8$) subjects ($*P = .001$).

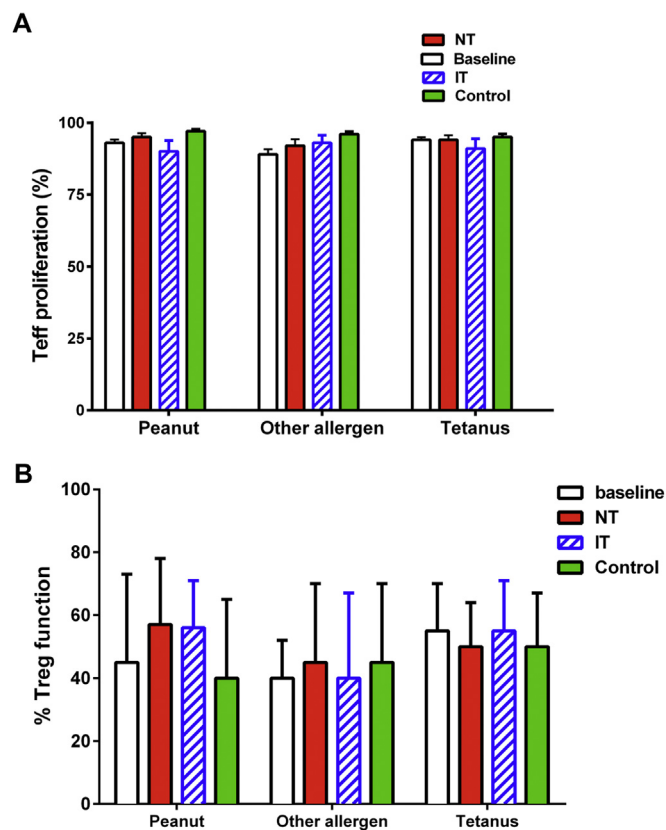


FIG E5. A, Teff cell proliferation in response to peanut, other allergen, or tetanus antigen in the absence of Treg cells from patients undergoing OIT and control patients at baseline and 27 months after therapy. **B,** Suppressive function of purified naive Treg cells obtained before and after OIT. Percentage of Teff cell proliferation was measured by using standard published thymidine incorporation methods, and percentage Treg cell function was defined as follows: (Teff cell proliferation – Teff cell:Treg cell proliferation)/Teff cell proliferation (please see the Methods section for determining proliferation to antigens used in each autologous assay: peanut, other allergen, and tetanus). No significant differences were found ($P = .76$).

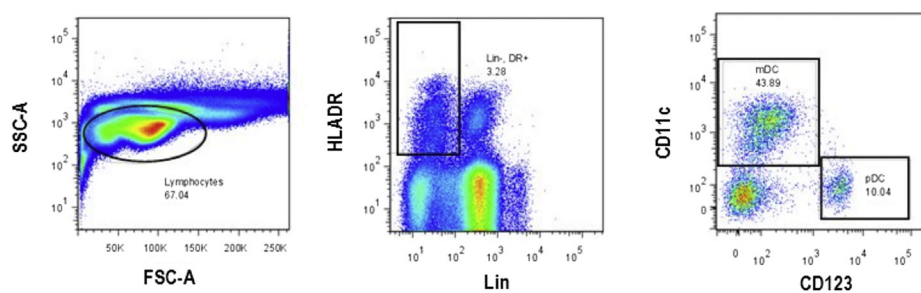


FIG E6. Gating strategy for identifying myeloid DC (*mDC*) and plasmacytoid DC (*pDC*) populations in PBMCs. From lymphocytes gated for DCs ($\text{Lin}^- \text{HLADR}^+$), *mDC*s were gated as $\text{CD11c}^+ \text{CD123}^-$, and *pDC*s were gated as $\text{CD11c}^- \text{CD123}^+$. *FSC*, Forward scatter; *SSC*, side scatter.

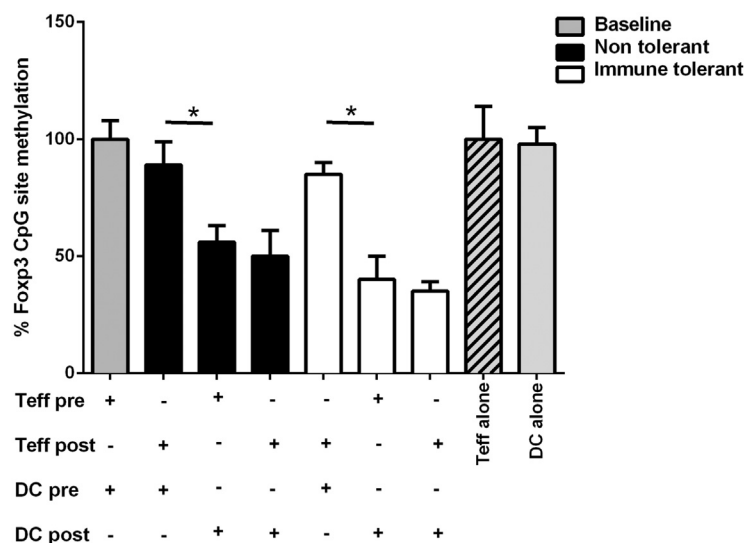


FIG E7. DC-dependent modification of Foxp3 CpG methylation in DC-T-cell cultures in the presence of peanut allergen. Autologous Teff cells (purified by using flow sorting; FACSARIA, BD Biosciences) and DCs purified from subjects receiving OIT by using flow cytometry (FACSARIA, BD Biosciences; *black bars*, NT; *white bars*, IT) before and after therapy were cocultured, and CpG methylation within the *FOXP3* locus was assessed after 3 days of culture. *Gray bar*, Baseline Foxp3 CpG methylation from cultures of DCs and Teff cells from the pretherapy time point. For control subjects, methylation levels in Teff cells and DCs cultured alone with antigen are shown. Data represent means $\pm$ SEMs. * $P < .001$ (CI, 36.21 to 53.79).

TABLE E1. Summary of demographics of participants

	Patients receiving OIT		Control subjects	
	No.	Percent	No.	Percent
No. of participants	23	NA	20	NA
Age (y), median	10.4	NA	12	NA
Age (y), range	5-45	NA	6-20	NA
Male sex	12	60	8	40
Asthma	14	70	7	35
Allergic rhinitis	14	70	6	30
Other food allergies	8	40	7	35
Other atopic conditions*	11	55	3	15
No other atopic conditions	3	15	0	0
SPT (mm), median (range)	14.7 (7-30)	NA	19.0 (12-34)	NA
Peanut-specific IgE (kU _A /L), median (range)	100 (19-318)	NA	84 (18-309)	NA
Dose level OFC (mg protein), median (range)	25 (6-100)	NA	25 (6-100)	NA
Cumulative dose Level OFC (mg protein [range])	32.7 (7.7-182.7)	NA	32.7 (7.7-182.7)	NA
Peanut-specific IgG ₄ (mg _A /L), median (range)	0.2 (0.1-0.6)	NA	0.3 (0.2-0.8)	NA

NA, Not applicable.

*Please refer to [Table E2](#) for data on individual participants. Differences in the incidence of atopic disease in patients receiving OIT versus control participants were nonsignificant ($P = .17$).

TABLE E2. Detailed demographics of participants

Patient ID	Age (y)	Sex	Peanut-specific IgE (kU _A /L)	SPT wheal (average diameter [mm])	Concomitant atopic disease	Dose level of peanut protein on screening DBPCFC with positive clinical reactivity* (mg)	Dose level of peanut protein on 3rd oral food challenge with positive clinical reactivity* (mg ‡)
A	26	M	20.9	17	Asthma, atopic dermatitis, other food allergies	6	NT, 500
B	8	M	138	11	Asthma, allergic rhinitis, atopic dermatitis, other food allergies	25	IT
C	7	M	101	9	None	6	NT, 1000
D	6	F	124	12	Allergic rhinitis	6	IT
E	5	M	103	30	Allergic rhinitis	50	Drop out, 2000
F	5	M	252	7	Allergic rhinitis	6	NT, 500
G	11	F	317	25	Asthma, allergic rhinitis, other food allergies	100	NT, 2000
H	10	F	69	8.5	None	6	IT
I	6	F	248	19.5	Asthma, allergic rhinitis, other food allergies	25	NT, 500
J	7	F	116	21.5	Asthma, atopic dermatitis, other food allergies	25	IT
K	7	M	170	11	Asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis	50	NT, 1000
L	7	M	54.9	25	Asthma, allergic rhinitis, atopic dermatitis, other food allergies	25	Drop out, 100
M	11	M	163	7	Allergic rhinitis, atopic dermatitis	6	NT, 1000
N	45	M	20.6	10	Asthma, allergic rhinitis	25	IT
O	12	F	19	18	Asthma	25	IT
P	9	F	48.7	20	Asthma, allergic rhinitis, atopic dermatitis, other food allergies	100	NT, 2000
Q	6	F	168	8	Asthma, atopic dermatitis	25	NT, 100
R	7	F	32.7	14	Asthma, allergic conjunctivitis	100	NT, 500
S	5	M	33	7.8	Allergic rhinitis	50	Drop out, 1000
T	13	M	53.1	8.5	Asthma, allergic rhinitis	6	IT
U	8	M	27.4	16.5	Asthma, allergic rhinitis	6	NT, 500
V	10	M	62.4	17	Asthma, allergic rhinitis, atopic dermatitis, other food allergies	50	NT, 2000
W	6	F	58	15	Asthma	6	NT, 1000
P1	10	F	103	25	Asthma	100	100
P2	11	F	51	28	Atopic dermatitis, other food allergies	6	6
P3	15	F	195	16.5	Allergic rhinitis	6	6
P4	11	M	178	12	Allergic conjunctivitis	25	6
P5	6	F	239	17	Other food allergies	100	25
P6	14	M	174	12	Asthma	50	25
P7	9	M	115	20	Asthma, allergic rhinitis	25	50
P8	10	M	65	16	Other food allergies	6	6
P9	15	F	42	13	Allergic rhinitis	6	25
P10	12	M	18	21	Other food allergies	25	100
P11	7	F	48	16	Asthma	50	6
P12	20	F	264	34	Asthma	100	25
P13	17	F	72	25	Atopic dermatitis, other food allergies	6	6
P14	6	F	309	16	Asthma	25	25
P15	18	M	149	15	Allergic rhinitis	50	6
P16	11	F	33	17	Other food allergies	100	25
P17	14	M	70	23	Asthma	6	25
P18	7	F	31	22	Allergic rhinitis	6	6
P19	13	M	84	23	Other food allergies	6	25
P20	15	F	42	13	Allergic rhinitis	6	100

*Based on the Bock criteria from Sampson H. Anaphylaxis and emergency treatment. Pediatrics 2003;111:1601.

‡Note: IT participants had no clinical reactivity at 4000 mg of peanut protein at 27 months.