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Peanut oral immunotherapy results in increased antigen-induced Treg function and hypomethylation of *FOXP3*

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Abstract

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 to age-matched allergic controls (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) while those that had an allergic reaction were categorized as non-tolerant (NT, n=13).

Results—Antibody and basophil activation measurements did not statistically differentiate between NT vs. IT. However, T-cell function and demethylation of *FOXP3* CpG sites in antigen-induced Treg were significantly different between IT vs. NT participants. When IT participants

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were withdrawn from peanut therapy for an additional 3 months (total of 6 months); only 3 participants remained "immune tolerant" and 4 participants regained sensitivity along with increased methylation of *FOXP3* CpG sites in antigen-induced Treg.

Conclusion—In summary, modifications at the DNA level of antigen-induced T-cell subsets may be predictive of a state of operationally-defined clinical "immune tolerance" during peanut OIT.

Keywords

Food allergy; allergy; oral immunotherapy; peanut; T regulatory cells; desensitization; tolerance; epigenetics; Foxp3

INTRODUCTION

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 antibodies¹, a shift from a T helper type 2 (Th2) response towards a T helper type 1 (Th1) response with increased IFN- γ production^{2–6}, reduction in specific IgE, reduced recruitment or increased anergy/deletion of T effector cells (Teff)^{7–11} and induction of regulatory T cells (Treg)^{12–19}.

As key immune regulatory cells, Treg have been shown to play a pivotal role in maintaining immune tolerance with Treg deficiencies implicated in the development of allergies^{20–24}. Moreover, several studies have demonstrated that epigenetic modifications in CpG-rich regions within the *FOXP3* locus of Treg are associated with stable Foxp3 expression and Treg suppressive function^{25–27,69}. Treg subsets including natural Treg (nTreg, derived from the thymus) and induced Treg (iTreg, derived from the periphery from Teff cells) have been described^{28–31}. iTreg can be characterized as CD4⁺CD25^{hi} cells that proliferate in response to specific antigens (i.e., CFSE^{lo} or CD40L/CD69⁺)^{32–35}. iTreg can be Foxp3⁺ and/or TGF- β ⁺ and/or IL-10⁺^{32,34,36,37,49,68}. Type 1 regulatory T cells (Tr1) release IL-10^{28,38,39} and express CD4, CD49, and LAG3 on their surface^{28,38,39} which differs from nTreg expressing CD4, CD25^{hi}, CD127^{lo}, and perhaps by Helios on their surface^{25–27,40–43}.

We hypothesized that iTreg (CD4⁺CD25^{hi} cells proliferating in response to specific antigen) play a key role in mediating clinical immune tolerance and that assessing epigenetic modulation of the *FOXP3* locus within antigen-induced Treg might provide insight into mechanisms of clinical immune tolerance at the cellular and molecular level. Therefore, we conducted a study with peanut-allergic participants undergoing oral immunotherapy (OIT) to peanut protein over the course of 24 months (24mo), followed by withdrawal from therapy for 3mo, followed by oral food challenge (OFC) at 27mo. We operationally defined "immune tolerant" (IT) patients as those who were non-reactive to an OFC at 27mo and non-tolerant (NT) as those who reacted to an OFC at 27mo. IT participants were withdrawn from peanut another 3mo and rechallenged at 30mo. This work builds on our previous findings in aeroallergen immunotherapy²⁷, by showing that antigen-induced Treg can modulate Teff 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 Treg (ai-Treg).

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

Out of 81 screened, 43 peanut-allergic participants from the clinics at Stanford University Hospital were consented, passed screening, and enrolled in study (Online Repository (OR) Fig. E1). Double-blinded placebo-controlled food challenges (DBPCFC) occurred at screening (see OR for details on eligibility criteria and challenge dosing). Clinical reactivity is defined as any sign of allergic reaction (i.e., 1 or greater on Bock's criteria⁴⁴). Subject demographics are summarized in OR Tables E1 and E2. The protocol was conducted in a hospital setting with trained staff and was performed similarly to Jones et al.¹². The study outline is diagrammed in Fig. E1.

Collection and processing of samples

Blood was collected at baseline, 3, 6, 9, 12, 18, 24, 27, and 30mo. Lab 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 described⁴⁵. Specific IgE and IgG4 were measured (Stanford Clinical Laboratories). Treg, Teff, and DC subsets were phenotyped using flow cytometry (LSR II, BD Biosciences). Methylation site analysis was performed on cell subsets as previously described⁴⁶. PBMCs were CFSE-labeled and cultured with peanut, egg, or timothy grass protein (see OR) to identify ai-Treg and Teff subsets. Ai-Treg were defined as Treg (CD4+CD25^{hi}Foxp3⁺) that proliferated in the presence of peanut. Proliferation was measured by CFSE^{lo} or CD40L/CD69+. nTreg (natural Treg) were defined by CD4+CD25^{hi}Foxp3⁺ with no proliferation to peanut. Additional information can be found in the OR.

Statistical analysis

Comparisons between pre- and post-treatment or between therapy (NT and/or IT) groups and control groups were evaluated with the nonparametric Mann-Whitney test, paired Wilcoxon test, and one-way and two-way ANOVA (GraphPad Prism Software 5.0), as appropriate. A P value of < 0.05 was considered statistically significant.

RESULTS

Participants

23 peanut-allergic patients underwent peanut OIT while 20 peanut-allergic, age-matched controls underwent standard of care (abstaining from peanut) (OR Table E1 and E2 for Demographics; Fig. E1 for schematic of study). 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 protein by 24mo. Both active and control subjects underwent a graded oral food challenge (OFC) at 24mo. No subject in the control group successfully passed the OFC (i.e., all control subjects had signs of clinical reactivity) at 24mo. Participants with no reaction to OFC were defined as desensitized at 24mo (n=20) and abstained from therapy and avoided peanut-containing foods for 3mo. At 27mo, desensitized participants underwent another OFC. Patients who reacted (i.e., exhibited any signs of allergic response) were classified as non-tolerant (NT, n=13) and those who did not have any clinical allergic reaction were operationally defined as “immune tolerant” (IT, n=7). IT participants abstained from OIT and avoided all peanut-containing food for an additional 3mo (total of 6mo of avoidance) and were reassessed for “immune tolerance” with an OFC at 30mo (IT, n=3).

Humoral and basophil immune markers in IT vs. NT participants

To measure immune monitoring features, peripheral blood was collected longitudinally and analyzed for immunoglobulin levels and basophil activation. Levels of peanut-specific IgE showed no statistically significant differences between NT vs. IT (Fig. 1A). There was a trend for levels of peanut-specific IgG4 and peanut-specific IgG4:Ig E to increase over time in the active treatment groups undergoing OIT; however, differences among the 3 groups did not reach significance (p=0.24 and p=0.27, respectively) (Fig. 1B and Fig. 1C).

IgE binding can mediate basophil activation and several studies suggest basophils could serve as a possible tool to diagnose and monitor allergies^{45,47,48}. To determine the effect of OIT on peripheral basophil activation, surface CD203c was after ex vivo stimulation with peanut allergen as per published techniques^{45,47,48}. Basophil activation decreased at 3mo into OIT (Fig. 1D, *P<0.001) and at 9mo in both IT (mean MFI, 103 [± 46 SD]) and NT (mean MFI, 117 [± 41 SD]) groups, but levels in control participants remained constant (mean MFI at 9mo, 1837 [±140 SD]). Peanut-induced basophil response was most reduced in the IT group, although differences between IT and NT did not achieve statistical significance. Notably, basophils from each group retained high and statistically indistinguishable (P>0.99) levels of responsiveness to activation by anti-IgE (Fig. 1E).

Skin prick test (SPT) wheal diameter was decreased at 12mo for IT and NT participants compared to control (*P<0.05). No difference was observed in SPT results in NT vs. IT (NS, P=0.66) (Fig. 1F).

Antigen-induced Treg are induced during OIT and are functionally suppressive

We next investigated whether OIT induced allergen-specific Treg. Antigen--induced cells were identified by CFSE proliferation assays as CD4⁺CD25^{hi}Foxp3⁺CFSE^{lo} T cells and non-specific Treg (ns-Treg) were identified as CD4⁺CD25^{hi}Foxp3⁺CFSE^{hi} T cells as previously described^{32–34,49} (for gating, see Fig. 2A and 2B and OR). Initial studies characterizing T cell populations identified an increase in ai-Treg between baseline and 12mo after starting peanut OIT (Table I). The enhancement of antigen-induced Treg function appeared to be specific to peanut, as there were no changes in Treg specific for other offending allergens (OAA, 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 in the ai-Treg population after therapy (Table I). We saw no significant differences at baseline in IT vs. NT vs. Control groups (Fig. E2) and grouped the baseline data together (n=43) in Fig. 2D. There were no differences in absolute counts of ns-Treg between control, IT, and NT subjects at any time point (Fig. 2C). Absolute counts of ai-Treg were significantly increased in IT vs. NT or control participants beginning at 6mo after start of therapy (Fig. 2C, *P<0.002) and remained so at 24mo (Fig. 2D, *P<0.0001).

The ai-Treg population identified in this study could be comprised of iTreg^{49, 68}; in contrast, ns-Treg may represent a population of thymically-derived nTreg. While the phenotypic differences between iTreg and nTreg are subtle and highly debated^{50–52, 68}, functionally, nTreg are thought to be nonspecific, and control systemic autoimmunity. Induced Treg or “activated” Treg were recently discussed by Sakaguchi and colleagues; activated Treg can be identified by CD4⁺CD25^{hi}CD45RA^{lo}Foxp3⁺; iTreg are hypothesized to be specific, and are implicated in allergic inflammation⁵³. In order to further phenotype Treg populations, we performed flow cytometry using Helios. Our preliminary data suggest that ai-Treg express lower levels of Helios compared to ns-Treg (Fig. E3A and B), possibly indicating that the ai-Treg population are comprised of iTreg^{25–27, 40–42}; however the ability of levels of Helios expression to discriminate among different subset of Tregs is still under active investigation⁴³. Given the increases in IL-10 in peanut-induced CD4⁺ T cells seen in our initial phenotyping studies (Table I), we investigated whether ai-Treg could represent Foxp3⁺ Tr1 cells. Our data suggest that the percentage of Tr1 cells is increased in patients on therapy compared to those without therapy (Fig. E4).

To study function, Treg were sorted by flow cytometry from all participants. The suppressive function of ai-Treg (defined as CD4⁺CD25^{hi}Foxp3⁺ Treg proliferating to peanut) on ai-Teff responses was compared at baseline vs. 27mo. The data show no significant difference in the suppressive function at baseline for IT, NT, and Control groups (Fig. E2). In Fig. 2E, a Treg subpopulation (CD4⁺CD25^{hi}Foxp3⁺CD45RO⁺) sorted by flow cytometry (FACS Aria, BD, San Jose, CA) was found to suppress Teff (CD4⁺CD25^{lo/-}, Foxp3⁻) proliferation to peanut (but not to other offending allergens or tetanus) (*P<0.0001). When naïve (CD45RA⁺) CD4⁺CD25^{hi}Foxp3⁺ Treg cells were sorted out of the total Treg population used in the suppression assays, there was no loss in the ability to suppress Teff proliferation to peanut allergen (Fig. E5A) nor did purified naïve Treg cells exhibit any differences in Treg function pre or post-OIT (Fig. E5B), suggesting that enhanced suppressive function to peanut antigen during OIT was associated with allergen-specific CD45RO⁺Treg. Of note, there was greater suppression by ai-Treg isolated from IT (95±5%) than NT (77±21%) participants (P<0.0001) (Fig. 2E). Suppressive function of ai-Treg from the control group at 27mo was similar to baseline (Fig. 2E). The increase in antigen-induced Treg function did not seem to be due to alterations in the antigen-induced Teff function, as antigen-induced Teff proliferation alone was unchanged between baseline and 27mo (i.e., 3mo after stopping OIT) for all treatment groups (Fig. E5A).

OIT enhances the migratory activity of antigen-induced Treg

The functional migratory potential of Treg towards an intestinal epithelium cell line was analyzed *in vitro* by a chemotaxis assay. As shown in Fig. 3A, starting at 12mo, ai-Treg from IT or NT OIT participants showed increased migration activity compared to control (*P<0.001). Moreover, the migratory activity of ai-Treg from IT participants was significantly enhanced starting at 12mo compared to NT participants.

We next tested whether increased chemokine receptor expression was associated with the increase in migration activity. As shown in Fig. 3B and 3C, the expression levels of CCR8 increased at 24 and 27mo (i.e., at the end of OIT or 3mo after stopping OIT) as compared to CCR8 levels at baseline (*P<0.001) in both IT and NT participants. Soler et al. demonstrated that CCR8 is expressed by Th2 cells and Treg⁸³; it is interesting that there is an increase in CCR8 in NT subjects, which could be due to expression in Treg and/or Th2. 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. 3D.

Foxp3 is modified in antigen-induced Treg during OIT

Since Foxp3 is one of the indicators of Treg suppressive function and has been associated with maintenance of iTreg, and since Foxp3 is a transcription factor for CCR8⁵⁴⁻⁵⁹, we measured expression of Foxp3 protein in ai-Treg using flow cytometry; there was significantly increased Foxp3 protein during OIT in ai-Treg from IT participants, but not in ai-Treg from NT or control participants (Fig. 4A, *P<0.001). Similarly, Foxp3 transcript levels were also increased at 24 and 27mo compared to baseline in ai-Treg from IT, but not in ai-Treg from NT or control participants (Fig. 4B).

We tested whether DNA methylation of the *FOXP3* gene in Treg decreased throughout the course of OIT. AiTreg were identified by double positive CD69/ CD40L expression (in addition to CD4⁺CD25^{hi}Foxp3⁺) upon peanut antigen incubation and were purified by flow cytometry. Compared to baseline, there was significantly decreased methylation of CpG sites in ai-Treg for all OIT participants at 24 and 27mo, with most pronounced hypomethylation in IT participants (Fig. 4C, *P<0.001). Participants that shifted from IT at 27mo to NT at 30mo were marked by an increase in methylation of CpG sites (Fig. 4C). Ai-Treg from control participants (Fig. 4C) and ns-Treg from all groups did not change (Fig. 4A).

Dendritic Cells (DCs) induced during OIT can influence epigenetic modifications in T cells

Since iTreg can be induced via interactions with DCs, we examined the effects of DCs studied before therapy (baseline) and at 27mo (3mo after stopping OIT) on modulation of *FOXP3* epigenetics in CD4⁺Teff IT vs. NT. Teff cells and autologous DCs were purified by flow cytometry and cultured together (Fig. E6) in the presence of peanut protein for 3 days. Autologous cultures did not affect Teff viability. Fig. E7 shows that DCs obtained from either IT or NT participants at baseline did not alter *FOXP3* CpG methylation in Teff. However, DCs isolated post-therapy significantly decreased the percentage of *FOXP3* CpG methylation in Teff (*P<0.001). The percent of *FOXP3* methylation in Teff alone did not change over the course of the culture.

DISCUSSION

In this study, 20/23 participants successfully completed 24mo of oral immunotherapy, tolerating up to 4 g of peanut protein after maintenance therapy. After 3mo of peanut avoidance, only 7/20 participants were defined as “immune tolerant”; these 7 avoided peanut for an additional 3mo (6mo total of avoidance) and only 3/7 remained clinically non-reactive (i.e., “immune tolerant”). We found that the IT participants had higher numbers of ai-Treg with greater suppressive function, and with higher levels of *FOXP3* hypomethylation, compared to NT and control participants. Thus, this study demonstrates a possible mechanism of IT in OIT involving: i) an increase in ai-Treg with enhanced chemotactic and suppressive behavior, and ii) epigenetic modifications within the *FOXP3* locus of such ai-Treg. None of the demographic or clinical features assessed at baseline affected the outcome of IT vs. NT. All subjects on OIT were on maintenance for at least 3mo (i.e., from 21mo to 24mo); number of months on maintenance did not distinguish IT vs. NT.

In this study, we focused on the possible role of Treg and whether antigen-induced Treg were associated with “immune tolerance” in OIT. The method of identification of iTreg was consistent with other publications^{32–36,49,68} in which iTreg were defined as proliferating CD4⁺CD25^{hi}Foxp3⁺ cells (i.e. either defined through CFSE^{lo} or CD40L/CD69⁺ in response to specific antigen stimulation). Furthermore, our data show that the iTreg population was CD45RO⁺ (and CD45RA^{neg}), Helios^{lo/neg}, CD49b⁺/LAG3⁺, Foxp3⁺, and IL-10⁺; therefore, the iTreg we identified with these markers are consistent with other studies^{32–36} and are possibly Foxp3⁺ Tr1 cells^{28,34,38,39}. Moreover, we demonstrated that the iTreg population induced through peanut OIT was specific in suppression of autologous Teff proliferation in response to peanut, not other antigens. So as to prevent cross contamination, the effector T cell population we used in functional assays was purified as CD4⁺CD25^{lo/neg} and found to be Foxp3[–].

Previous studies have demonstrated the presence of increased Treg in patients who develop “immune tolerance”^{12,14,15,60}. Antigen-induced Treg, despite being in relatively small numbers compared to other immune cell subsets, have been shown to be associated with natural loss of food allergy⁶¹. In contrast to other OIT studies in which high doses of antigen are administered with concomitant omalizumab use⁶², the present OIT protocol, like that developed by Burks, Jones, and others^{12,63–65}, started with a low initial dose of peanut followed by slow dose escalations.

Most previous immunotherapy studies have not proven whether Treg are functionally suppressive, whether they migrate towards intestines, or whether they are antigen-induced. The population of ai-Treg that 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 (Fig. 2–4) in IT participants. Our data therefore suggest that one possible mechanism of OIT involves modifications of ai-Treg to enhance their suppressor function, possibly in the intestinal tissues. Demethylation of *FOXP3* plays a crucial role in Treg plasticity and suppressive function^{66–69} and could play a role in immune tolerance. Importantly, we have documented hypomethylation within the CpG locus of *FOXP3* in ai-Treg in IT vs. NT subjects (Fig. 4). We followed participants up

to 30mo (i.e., until 6mo after stopping OIT) and saw that 4/7 IT participants (broken lines in Fig. 4C) were no longer “tolerant” at 30mo. Such “resensitization” was associated with increased methylation of CpG sites in the *FOXP3* locus (Fig. 4C).

Our results suggest possible Treg biomarkers that might be useful in predicting a state of “immune tolerance”; however, many cells in addition to Treg are thought to contribute to IT. Our recent aeroallergen immunotherapy study by Swamy et al.²⁷ reported epigenetic changes in the *FOXP3* locus following therapy. The present study performed novel detailed mechanistic studies on sorted antigen-induced T cells and identified Foxp3+ Tr1 cells as the major iTreg population associated with success of therapy. While 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 (Fig. E7). Data from our laboratory demonstrate that DCs expressing indoleamine 2,3 dioxygenase (IDO) may help promote the conversion of naïve CD4+ T cells to Treg and that this conversion may be mediated via epigenetic changes at the *FOXP3* locus⁸⁴. Janson, et al. have shown that *FOXP3* promoter demethylation was associated with the appearance of a committed Treg population⁶⁹, and increased expression of IDO by DCs has been linked to enhanced tolerogenicity and the suppression of Teff proliferation⁷⁰.

In a recent clinical study by Burks et al.⁷¹, participants underwent egg OIT for 22mo. The clinical data in that study and ours are similar, in that the % of subjects with loss of clinical reactivity after a period of food allergen avoidance after OIT in our study was 30% (7/23 after 3mo peanut avoidance) and, in the Burks, et al.⁶⁰ 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 4.0g of peanut protein (the same dose as the maintenance dose) in our study whereas Burks et al. used 10g of egg protein (a much higher dose than the maintenance dose of 2g egg protein). The role of specific IgG4 is under investigation in OIT^{64–66,72}; 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 vs. such operationally defined examples of “immune tolerance” and whether specific IgG4, IgG4:IgE ratio, basophil reactivity, T cell or other cellular markers could be used to predict an outcome of IT^{72,74}.

While our data suggest that larger phase 2 trials in OIT are justified and feasible, limitations of the present study should be mentioned. First, we realize that this was a small cohort study performed at a single site. Secondly, 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 using tonsillar tissue⁷⁴.

In summary, our study investigated the durability of “immune tolerance” in food allergy OIT after a minimum of 3mo off therapy. Food allergy OIT is under investigation and is still in experimental stages^{64–66,73,74}. It is important to note that NT participants, after stopping OIT, were still able to ingest a relatively large amount of peanut protein (as compared to their threshold dose at baseline food challenge) prior to developing allergic symptoms (Table E2). Our data suggest that ai-Treg, rather than ns-Treg, 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 vs. NT; instead, there were incremental differences (some statistically significant, 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 peanut allergy patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BL	Baseline
mo	Months
DBPCFC	Double Blind Placebo Controlled Food Challenge
DC	Dendritic Cell
Foxp3	Forkhead Box Protein 3
ns-Treg	Non-specific regulatory T cell
OFC	Oral Food Challenge
OIT	Oral Immunotherapy
ai-Treg	antigen-induced regulatory T cell
SPT	Skin Prick Test
Teff	Effector CD4+T cell
Treg	Regulatory T cell

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KEY MESSAGES

- Mechanisms of “immune tolerance” during OIT potentially involve induction of antigen-induced Treg.
- Epigenetic changes in the Foxp3 locus may enhance Treg function in this setting.
- Modifications at the DNA level in specific T-cell subsets may be predictive of unresponsiveness during OIT.

Fig. 1.

OIT-treated participants (squares, n=13, NT or triangles, n=7, IT) or peanut-allergic control participants (circles, n=20). A) Changes in mean peanut-specific IgE levels (kU_A/L $\pm$ SD) among IT (N=7), NT (N=13) and Control (N=20) subjects (NS, P=0.17) B) Change from baseline in mean antibody levels ($\pm$ SD) for peanut-specific IgG₄ (mg_A/L). Slight increase in IT and NT compared to Control (NS, P=0.24). C) Mean ($\pm$ SD) of peanut-specific IgG₄:IgE ratios. Slight increase in IT and NT compared to Control (NS, P=0.27) D) Expression of CD203c levels on basophils stimulated with peanut allergen (1 μ g/ml). Data are

presented as mean $\pm$ SEM (*P < 0.001, CI: -1222 to -777.8) IT or NT vs. Control. IT vs. NT (NS, P > 0.99) Mg = milligrams of antigen-specific antibody; MFI=mean fluorescence intensity. E) Expression of CD203c levels on basophils stimulated with anti-IgE (NS, P > 0.999). F) Significant decrease in SPT diameter starting at 12mo for IT or NT compared to Control (*P < 0.001, CI: -16.06 to -5.942). No difference in NT vs. IT (NS, P = 0.656).

Fig. 2.

A) Representative staining of ai-Treg and ns-Treg. B) Representative staining for ns-Treg and ai-Treg in Control, NT and IT participants at 30mo C) ns-Treg (open shapes) and ai-Treg (closed shapes) absolute counts (* $P < 0.002$, CI: 2.636 to 141.1, NTN=7, ITN=13, ControlN=20). D) ns-Treg (white bars) and ai-Treg (black bars) at baseline and 24mo of treatment (* $P < 0.0001$, CI: 41.1 to 162.6, NT N=7, ITN=13, Control N=20). Changes in ns-Treg not significant ($P=0.84$). E) Treg suppressive activity on conventional responder CD4+ T cells (Teff) measured pre-therapy (baseline) and at 27mo—i.e., after 3mo off treatment

(filled bars). Suppressed proliferation of Teff in response to peanut stimulation in NT and IT compared to baseline (* $P=0.0001$, CI: 30.18 to 69.82) but not other allergens or tetanus ($P>0.999$). F) Suppressive function of Treg from pre and post-OIT. Suppressive function of Treg collected pre-therapy (pre) and at month 27 (post) was assessed towards ai-Teff collected pre- and post-therapy (data represent mean + SEM; * $P < 0.001$, CI: -32.03 to -17.97).

Fig. 3.

A) Chemotactic Indices of ai-Treg toward normal IECs. Indices for cells from immune tolerant (n=7) or non-tolerant (n=13) subjects were significantly higher than those for control (n=20) participants starting at 12mo (*P< 0.001, CI: 5.854 to 7.346). Values for IT subjects were significantly higher than those for NT subjects starting at 12mo (#P<0.0001, CI: 3.004 to 4.596). B and C) Expression levels of chemokine receptors (circles, CCR8; squares, CCR4; triangles CCR7) on ai-Treg populations were identified by flow cytometry and are presented as MFI (x10) at baseline and at 24mo of treatment and at 27mo (i.e., 3mo post cessation of treatment) in IT patients (Fig. 3B) (*P < 0.001, CI: 38.64 to 48.50) and NT patients (Fig. 3C) (*P<0.001, CI: 34.69 to 48.01). An example of chemokine receptor staining is shown in Fig. 3D.

Fig. 4.

(A) Intracellular Foxp3 protein expression levels in ns-Treg (white bars) and ai-Treg (black bars). Values are mean fluorescence intensity (MFI). Significant differences in ai-Treg for IT vs. NT or control (* $P < 0.001$, CI: 90.34 to 159.7), not significant for ns-Treg ($P = 0.1698$). (B) *FOXP3* mRNA in Treg isolated from IT OIT participants (triangles), NT OIT participants (squares) and peanut-allergic control participants (circles). Significant differences in IT participants at 24 or 27mo compared to baseline (* $P < 0.001$, CI: 2.871 to 4.437). Difference for NT participants non-significant ($P = 0.18$). (C) ai-Treg from participants undergoing OIT (IT, $n = 7$, blue triangles; NT, $n = 13$, red squares) or untreated controls ($n = 20$, green circles). 4/7 IT participants (connected via broken lines) were no longer “tolerant” at 30mo. Data represent mean number of methylated sites + SEM; (IT: * $P < 0.001$ vs. baseline CI: 12.13 to 14.16, NT: * $P < 0.001$ vs. baseline CI: 3.919 to 5.620). Not significant for control ($P = 0.15$). MFI= mean fluorescence intensity.

Table 1

Enhancement of allergen-specific T cell populations during OIT.

Allergen-specific T cell populations ^A		% Treg Mean ±SD	%IL-10+	%Teff	%Th2	%Th1
Peanut+ memory	Pre	3.1 ± 1.3 ^{**}	18.9 ± 4.0 ^{**}	24.6 ± 3.5	57.0 ± 6.8 ^{**}	12.3 ± 5.7 [*]
	Post	16.4 ± 3.5	52.9 ± 7.8	23.7 ± 3.9	19.4 ± 7.9	22.6 ± 1.7
Peanut+ naïve	Pre	0.2 ± 0.4 ^{**}	11.7 ± 3.1	2.6 ± 1.9	17.0 ± 3.7 [*]	8.2 ± 1.8 [*]
	Post	1.9 ± 0.9	12.3 ± 4.1	3.9 ± 1.3	10.9 ± 2.8	12.6 ± 2.8
OOA+ memory	Pre	4.3 ± 1.5	16.6 ± 3.2	27.0 ± 6.3	60.6 ± 3.5	10.8 ± 2.1
	Post	4.6 ± 1.3	17.9 ± 4.8	30.0 ± 5.4	60.7 ± 4.7	14.9 ± 3.4
OOA+ naïve	Pre	0.0	18.3 ± 2.9	3.9 ± 1.6	13.0 ± 4.0	7.9 ± 2.0
	Post	1.4 ± 0.5	18.7 ± 1.8	3.6 ± 1.5	11.7 ± 3.6	8.6 ± 1.3
Peanut+ memory (control)	Pre	3.1 ± 1.6	14.7 ± 1.7	27.4 ± 4.7	60.3 ± 5.2	11.3 ± 2.6
	Post	5.0 ± 1.3	19.4 ± 2.9	26.1 ± 3.8	61.1 ± 3.9	11.4 ± 2.6

^A Percentages shown are % of CD4+ T cells, mean ±SD.

Significant differences (*P < 0.025, CI: 4.993 to 15.61, ** P < 0.0001 CI: 23.72 to 30.28) between the cell populations pre (n=7) and post (n=7) therapy.

Abbreviations: SD; standard deviation, OOA; other offending allergen, Pre; baseline values prior to starting OIT, Post; 12 months after starting OIT.