Published in final edited form as:

Clin Exp Allergy. 2015 November ; 45(11): 1663–1672. doi:10.1111/cea.12570.

Naturally Occurring Tolerance Acquisition to Foods in Previously Allergic Children is Characterized by Antigen Specificity and Associated with Increased Subsets of Regulatory T cells

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Abstract

Background—Food allergy affects approximately 6–8% of children, and increasing in prevalence. Some children naturally outgrow their food allergy without intervention but the mechanisms by which this occurs remain poorly understood. We sought to investigate the role of regulatory T cells in the development of naturally acquired tolerance.

Methods—Fifty-eight children (1 to 18 years) with either egg or peanut allergy, recent acquisition of natural tolerance to egg or peanut, or no food allergy were studied. Peripheral blood mononuclear cells (PBMC) from these groups were stimulated with relevant antigen for 48 hours and flow cytometry performed to characterize both surface (CD3, CD4, CD25, CD14, CD19, CD127) and intracellular markers (IL-10, Foxp3, and IL-5).

Results—Resting PBMC from naturally tolerant patients had significantly increased CD3+CD4+CD25+CD127loFoxp3+ cells, when compared to allergic or control patients [mean 6.36 vs 2.37 vs 2.62%, respectively, $p < 0.05$]. Upon stimulation with relevant antigen, naturally tolerant patients also had increased IL-10-expressing CD25+CD127lo cells [6.33 vs 1.65 vs 0.7, $p < 0.01$], Foxp3+ cells [mean 12.6 vs 5.42 vs 3%, $p < 0.01$] and CD4+ cells [mean 4.48 vs 1.59 vs 0.87%, $p < 0.01$]; the increase was not observed in PBMCs from allergic or control patients. Additionally, this upregulation was only seen with relevant antigen stimulation and not upon stimulation with unrelated antigen.

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Conflicts of Interest/Corporate Sponsors: none

Conflicts of Interest: All authors report no affiliations or financial interest in the subject matter or materials discussed in the manuscript.

Conclusion—The increased CD3+CD4+CD25+CD127^{lo} cells at baseline and upon stimulation and increased induction of IL-10-producing cells of several types, including Tr1 cells, from naturally tolerant patients suggests an important role for regulatory T cell subsets in the acquisition of natural tolerance.

Keywords

antigen specificity; food allergy; IL-10; natural tolerance; T regulatory cells

INTRODUCTION

Food allergy prevalence rates in children have nearly doubled and are at a rate of 8% in school-aged children according to recent studies [1]. Many children with food allergy, particularly those children allergic to egg, milk, wheat or soy, outgrow their food allergy naturally, without intervention (hereafter referred to as natural tolerance, or NT). Although some of the mechanisms underlying the development of sensitization in food allergy have begun to be described [2–4], the mechanisms associated with the development of natural tolerance to food are not as well defined. Elucidating mechanisms in the development of natural tolerance may lead to development of novel biomarkers for food allergy as well as therapeutic options for the treatment and prevention of food allergy.

Several immunoregulatory cytokines are thought to mediate tolerance due to their ability to downregulate Th2 cytokine responses, as has been shown in mouse models of asthma [5]. In humans, IL-10 has been proposed as a marker for tolerance in the setting of venom immunotherapy, as an elevation in IL-10 was associated with onset of tolerance to bee venom following high dose exposure in bee keepers [6]. Similarly, in a human study of sublingual immunotherapy (SLIT) for hazelnut allergy, increased IL-10 levels were observed in the group receiving treatment with SLIT when compared to those receiving placebo [7]. We have shown that natural tolerance to food is associated with increased IL-10 production from peripheral blood mononuclear cells (PBMCs) in response to antigen [8]. Although IL-10 is produced by many cell types, it has been suggested that T regulatory cells are an important cellular source in the context of food allergy [9, 10].

Many types of T regulatory cells exist including thymically-derived Tregs (tTregs) and peripherally derived Tregs (pTregs), which include Tr1 cells, Th3 cells, and Foxp3⁺pTregs. tTregs are known to express CD25 and Foxp3. However, neither CD25 nor Foxp3 upregulation is specific to tTregs, as these markers can also be expressed on activated T cells. Peripherally inducible Tregs such as Tr1 cells and Th3 cells do not constitutively express Foxp3 and are known to mediate their suppressive activity through IL-10 and TGFβ respectively [11, 12]. Tr1 cells can transiently express Foxp3 upon activation, although this expression is not maintained after activation and does not reach the high levels typical of Foxp3⁺ tTreg cells [13]. Low expression of CD127 (CD127^{lo}) has been utilized as a marker to more precisely identify tTregs [14]. Adding to this complexity is the plasticity of Tregs in terms of their ability to upregulate or downregulate Foxp3 in the periphery [15].

In mouse models, it has been shown that adoptive transfer of allergen specific CD25⁺Foxp3⁺ T cells can induce tolerance [16]. In human studies of cow's milk allergy,

the role of regulatory T cells has been explored [17–19]. One study associated CD25+CD27+Foxp3+ cells with natural tolerance to heated milk[18]. Similarly, in another human study of cow's milk allergy, children who outgrew cow's milk allergy (tolerant children), had higher frequencies of CD4+CD25+ cells when compared to cow's milk allergic children [19]. However, the role of more specific markers, such as CD127, and the possible role of other types of Tregs and the immunosuppressive cytokine IL-10 in the context of oral tolerance has not been extensively explored. Building on our previous work demonstrating an increase in antigen induced IL-10 production in PBMCs from NT children [8] we sought to characterize the cellular source of increased IL-10 in patients who developed natural tolerance to egg or peanut, and to determine the antigen specificity of this response.

METHODS

Patient Recruitment

Children ages 1–18 years, with and without egg or peanut allergy or those who had acquired natural tolerance to egg or peanut were recruited via a convenience sample from the Allergy & Immunology Clinics and General Academic Pediatric Clinics at Ann & Robert H. Lurie Children's Hospital of Chicago. IRB approval was obtained for patient recruitment, study protocols used, and informed consent. Assent was also obtained when appropriate.

Food allergy was defined by an allergist/immunologist as history of typical signs and symptoms of food allergy (urticaria, angioedema, respiratory symptoms, or gastrointestinal symptoms within 2 hours of food ingestion) and evidence of specific IgE as determined by skin prick test or ImmunoCAP with high likelihood of reaction (all but 1 with >95% PPV for either skin prick or specific IgE). Children without food allergy or control patients were defined as those without a history of clinical reactivity to egg, peanut, or any other food, and were eating those foods *ad lib*. Patients with natural tolerance to either egg or peanut were defined as those with prior history of food allergy as defined above, but passing an oral food challenge (to 1 whole egg containing a total of 6 grams of protein or 2 tablespoons of peanut butter containing a total of 7 grams of peanut protein) within 6 months of blood draw. Time of recruitment was defined as when PBMCs were obtained and underwent processing.

Exclusion criteria included use of oral steroids, food protein-induced enterocolitis and other non-IgE mediated food allergies, or delayed-type reactions to foods (such as atopic dermatitis).

Specific IgE to egg white and peanut was determined using ImmunoCAP (Phadia, Uppsala, Sweden). Skin prick testing was performed at time of diagnosis using a bifurcated needle with egg white or peanut extract, phenol-saline solution and 1mg/mL histamine solution (Greer Laboratories, Lenoir, NC) using standard clinic protocols.

Peripheral Blood Mononuclear Cell (PBMC) isolation and Ovalbumin and Whole Peanut Extract (WPE) stimulation

Approximately 5–20 mL of peripheral blood was collected from each patient in a heparinized tube by a trained phlebotomist. Peripheral blood was processed and samples were prepared within 24 hours of blood draw. PBMCs were isolated with Ficoll gradient, washed with PBS (sterile Dulbeccos' Phosphate buffered saline) and separated into equal aliquots to undergo stimulations, using validated methods [20, 21]. A time course (48, 72, and 120 hours) and concentration response (1, 10, and 100 µg/mL) study was performed to determine optimal time and concentration of antigen required for stimulation. This was found to be a dose of 100µg/mL for both ovalbumin and WPE and a 48-hour incubation time. Thereafter, 2×10^6 cells were stained for flow cytometry immediately and culture of $2\text{--}4 \times 10^6$ PBMC per well in a 12 well plate was used with or without relevant antigen, i.e. either ovalbumin 100 µg/mL (Sigma, Poole, UK) or whole peanut extract 100 mcg/mL. Relevant antigen was defined as the allergen to which the patient was allergic or previously allergic but now naturally tolerant to (either ovalbumin for egg allergic and egg NT patients or WPE for peanut allergic and peanut NT patients). Whereas unrelated antigen (either ovalbumin or peanut) was defined as one that a patient had no prior history of food allergy to and was able to ingest *ad lib*, (either WPE for egg allergic and egg NT patients or ovalbumin for peanut allergic and peanut NT patients). Whole peanut extract was prepared using a validated protocol [22]. Samples were incubated for 48 hours at 37°C in AIM-V media with human serum albumin (Gibco, Grand Island, NY).

Flow Cytometry

To investigate the role of T regulatory cells in natural tolerance to foods, cell surface and intracellular cytokine staining was performed using validated methods [23]. Unstimulated and PBMCs stimulated with ovalbumin and peanut extract were surface stained with markers from the indicated sources (CD3 APC-CY7 (SK7), Biolegend; CD4 V-500 (RPA-T4), BD; CD14 Alexa Fluor-700 (HCD14), Biolegend; CD19 PE (HIB19), BD; RORγT PE (AFKJS-9), eBiosciences; CD25 APC (BC96), Biolegend; and CD127 Pacific Blue (A019D5), Biolegend) and then washed, fixed, and permeabilized for intracellular staining (Foxp3 Alexa Fluor 488 (206D), Biolegend; IL-10 PE-CY7 (JES3-9D7), Biolegend; IL-5 APC (TRFK5), Biolegend) according to manufacturer's instructions (Caltag, Carlsbad, CA). Acquisition was performed using an LSR-II (BD, San Jose, CA), and analyzed using FlowJo software (Treestar, San Carlos, CA). Data are expressed as the mean percentage of cells within the gated CD3CD4 T cells unless otherwise indicated.

Statistical Analysis

Patient demographic and clinical characteristics were compared among non-allergic controls, allergic, and tolerant patients using a chi square test for nominal variables and a Kruskal-Wallis test for continuous variables. The Mann-Whitney U test was used to compare allergic and tolerant patients when comparisons to control patients were not applicable. Analysis of flow cytometry data was performed using a Kruskal-Wallis test when comparing changes among all three groups before and after antigen stimulation. For pairwise comparisons and within-group changes before and after stimulation, the Mann Whitney U test was utilized. For subgroup analyses within the tolerant group (egg NT versus peanut NT) and allergic group (egg allergic ingesting baked egg versus egg allergic strictly avoiding egg), the Mann Whitney U test was utilized. Data analysis was performed using

SAS 9.3 (SAS Institute, Cary, NC) and SPSS version 22 (SPSS, Inc., Chicago, IL). *A priori* power calculation determined n=9 patients in each group to detect a significant difference ($p=0.05$ and power of 80%) in IL-10+Foxp3+ cells between groups. All analyses were conducted with a two-sided type I error rate of 5%.

RESULTS

Patient Characteristics

Fifty-eight patients ranging from 1 to 18 years old (25 non-allergic control, 22 with food allergy, 11 with natural tolerance) participated (Table 1). Of those patients with food allergy, 4 patients had food allergy confirmed by an oral challenge in the clinic and 18 failed an open challenge at home (Supplementary table 1.1). Allergic patients differed significantly from controls in terms of having another atopic condition such as asthma or atopic dermatitis ($p<0.0001$). Naturally tolerant patients also differed significantly from control patients in terms of having another atopic condition ($p=0.0003$). Notably, allergic patients did not differ from naturally tolerant patients in regards to having another atopic condition ($p=0.81$ asthma, $p=0.62$ atopic dermatitis). There were no differences between allergic and tolerant groups in terms of median skin test size at diagnosis or recruitment for egg or peanut, median egg white specific IgE at diagnosis, or median specific IgE for peanut at diagnosis. As expected, due to clinic protocols and treatment guidelines dictating which patients should undergo food challenge, the median egg white specific IgE at recruitment and median peanut specific IgE at recruitment differed significantly between allergic and naturally tolerant patients (8.8 kU/L vs 0.48 kU/L, $p=0.006$ for egg, 12.1 kU/L vs 1.3 kU/L, $p=0.03$ for peanut), with naturally tolerant patients having significantly lower specific IgE at recruitment. As stated previously, there was no difference in specific IgE or skin prick test size at food allergy diagnosis between allergic and tolerant patients. Typically, patients with $sIgE < 2$ kU/L were offered oral food challenge. Supplementary tables 1.1 and 1.2 show further characterization of patients in terms of specific reactions.

Flow cytometry

Increased frequency of CD3+CD4+CD25+CD127loFoxp3+ cells in NT patients

—Tolerant patients had significantly increased percentages of Foxp3 expressing CD4+CD25+CD127lo cells (Foxp3+CD25+CD127loCD4+ cells) when compared to control and allergic patients at baseline [mean 6.36 vs 2.62 vs 2.37%, $p<0.05$] (Fig 1a, gating strategy in Fig S1). There was no difference in the amount of Foxp3 expressed on a per cell basis (MFI) between the groups [830.14 vs 529.77 vs 619.64 MFI, $p=0.58$] (Fig 1b). Additionally, NT patients had a trend toward higher percentages of the bulk population of CD25+CD127loCD4+ cells that approached statistical significance when compared to control and allergic patients [mean 6.99 vs 4.88 vs 5.67%, respectively, $p=0.06$] (Fig 1c). A subgroup analysis comparing peanut NT versus egg NT patients did not show any significant difference in Foxp3+CD25+CD127loCD4+ cell percentages, MFI of Foxp3, or bulk CD25+CD127loCD4+ cells at baseline. Additionally, subgroup analysis comparing egg allergic patients ingesting baked egg versus egg allergic patients strictly avoiding all types of egg showed no differences in the above aforementioned populations. Supplemental Figure 1 shows the gating strategy utilized.

NT patients exhibit increased IL-10 producing Treg populations upon specific antigen stimulation—Upon stimulation with relevant antigen, (either ovalbumin for egg allergic and egg NT patients or WPE for peanut allergic and peanut NT patients), naturally tolerant patients had significantly higher percentages of IL-10 producing CD25+CD127lo Treg cells (%IL-10+CD25+CD127loCD4+ cells) when compared to control and allergic patients [mean 6.33 vs 0.7 vs 1.65% respectively, $p<0.01$] (Fig 2a, gating strategy Fig S2). This was also true of IL-10 producing Foxp3+CD4+ cells (%IL-10+Foxp3+CD4+ cells). NT patients had higher percentages compared to control and allergic patients [mean 12.6 vs 3 vs 5.42%, $p<0.01$] (Fig 2b, gating strategy Fig S3). Furthermore, NT patients had higher percentages of IL-10 producing CD4+ cells (IL-10+CD4+ cells) when compared to controls and allergics [mean 4.48 vs 0.87 vs 1.59%, $p<0.01$] (Fig 2c, gating strategy Fig S4). Tolerant patients had the lowest percentage of IL-5 producing Foxp3+ cells [mean 9.3 vs 28.73 for control patients vs 22.3% for allergic patients, $p=0.04$] (Fig 2d). There was no difference in IL-5+CD4+ cells among the groups. A subgroup analysis comparing peanut NT versus egg NT patients did not show any significant differences in IL-10 producing CD25+CD127lo cells, IL-10 producing Foxp3+CD4+ cells, IL-10 producing CD4+ cells, or IL-5 producing Foxp3+ cells. Additionally, subgroup analysis comparing egg allergic patients ingesting baked egg versus egg allergic patients strictly avoiding egg showed no differences in the above aforementioned populations. Although the largest percentage of IL-10+ cells was in the CD14+ cells (monocytes), there was no change in the percent of monocytes producing IL-10 after stimulation in any of the three groups (Table II). CD19+ cells (B cells) were not a significant source of IL-10 positive cells and were not upregulated with ovalbumin or peanut stimulation in any of the patient groups (Table II). NT patients exhibit increased IL-10+CD4+ cells and increased percentage changes in IL-10+CD4+ cells and IL-10+CD25+CD127lo cells upon stimulation with relevant antigen.

When examining the CD4+ cells upon stimulation with relevant antigen, only NT patients increased IL-10+CD4+ cells when comparing unstimulated and stimulated cells [mean 0.66 vs 4.48%, or approximately 7-fold increase with stimulation, $p<0.01$]. Allergic patients, in contrast, showed no change in IL-10+CD4+ cells and instead trended towards a decrease in IL-10+CD4+ cell percentage [mean 4.42 vs 1.59%, $p=0.1$] (Fig 3a). In the Foxp3+CD25+CD127lo cells analyses, tolerant patients had decreased percentages of these cells after stimulation with relevant antigen [mean 11.44 vs 3.97%, $p=0.03$], whereas controls [mean 2.62 vs 2.94%, $p=0.59$] and allergic patients [mean 2.37 vs 3.07%, $p=0.4$] had no change (Fig 3b). Of note, absolute numbers of Foxp3+CD25+CD127lo cells remained the same in tolerant patients after stimulation with relevant antigen (data not shown). When comparing unstimulated and stimulated cells, tolerant patients had a significantly greater absolute change, from baseline, in IL-10 producing CD4+ cells upon stimulation with relevant antigen which was not seen in control or allergic patients [4.16 vs .05 vs -0.62 $p<0.01$] (Fig 3c). This represented an approximately 677% change from baseline in IL-10 producing CD4+ cells for tolerant patients. Similarly when comparing change in IL-10+CD25+CD127lo cells upon stimulation among tolerant, control, and allergic patients, tolerant patients had a significantly greater absolute percentage change in these cells [6.2 vs 0 vs -0.5, $p<0.01$] (Fig 3d). This represented an approximately 48-fold increase from baseline with stimulation.

Cells from a subset of patients underwent stimulation with unrelated antigen (either WPE for egg allergic and egg NT patients or ovalbumin for peanut allergic and peanut NT patients) in addition to relevant antigen (n=4 for NT patients, n=5 for allergic patients) and upon stimulation with relevant antigen versus unrelated antigen, only NT patients had an increased percentage of IL-10+CD4+ cells in response to relevant antigen. Additionally, this IL-10+CD4 cell increase was not observed upon stimulation with unrelated antigen [mean 4.48 vs 1.13%, p=0.03] (Fig 4a), and only occurred in response to the relevant antigen. Similarly, only NT patients had more IL-10+CD25+CD127lo cells in response to relevant antigen [mean 6.33 vs 0.69%, p=0.02] (Fig 4c) and these differences were not seen with unrelated antigen. Additionally, this specificity was not observed in cells from allergic patients when comparing relevant and unrelated antigen stimulation (Figs 4b and 4d).

DISCUSSION

Previous studies by our group have shown an increased allergen-specific IL-10 production in children who naturally developed tolerance (NT) to egg when compared to those children with persistence of egg allergy and control subjects without food allergy [8]. Here we sought to identify the cellular source of this increase in IL-10 seen in NT patients. Our findings demonstrate that NT patients uniquely increase production of IL-10 from CD25+CD127loCD4+, Foxp3+CD4+, and CD4+ cells after stimulation with relevant antigen. These findings were only observed in NT patients, and only after stimulation with relevant antigen and not with unrelated antigen. These observations support the notion that tolerance acquisition is an active, antigen specific process.

Interestingly, the absolute numbers of Foxp3+CD25+CD127loCD4+ cells did not change after stimulation with relevant antigen, but a percentage decrease in these cells was observed upon stimulation with relevant antigen in NT patients (Fig 3b). Based on previous studies, we hypothesize that these cells (Foxp3+CD25+CD127loCD4+ cells) are thymically derived tTregs [14, 24]. This finding along with the increased percentage of IL-10+CD4+ cells and IL-10+Foxp3+CD4+ cells observed after relevant antigen stimulation in NT patients, suggests a relative increase of pTregs upon antigen stimulation. Additionally, we hypothesize, that these IL-10+CD4+ producing cells, induced by relevant antigen only, are inducible Tr1 cells [11]. Tr1 cells are antigen specific adaptive Tregs and are known to be induced in the periphery after exposure to antigen in the presence of IL-10. Although Tr1 cells do not rely on Foxp3 expression for their function, they may transiently express Foxp3 upon activation [13]. These activated Tr1 cells that transiently express Foxp3 may be the IL-10+Foxp3+CD4+ cells that we have shown to increase in NT upon antigen stimulation. These cells are not induced by unrelated antigen suggesting that they are important in exposure to relevant NT allergen only. Importantly, although Tr1 cells may constitutively lack Foxp3 expression, they have comparable regulatory function to tTreg cells [13, 25, 26]. From studies of immunotherapy, a role for this Tr1 subset of regulatory T cells in the development of tolerance has been supported [27, 28]. In the context of cow's milk allergy, an increase in IL-10 secretion from milk-specific T cell clones has been shown in atopic patients with tolerance to milk. Additionally, Nadeau's group have shown in the context of peanut oral immunotherapy that Foxp3+ Tr1 cells were the major pTreg cell population

associated with success of oral immunotherapy [29]. To our knowledge, we are the first to describe a potential role of Tr1 cells in the acquisition of natural tolerance to egg or peanut.

The cytokine expressing CD25+CD127lo cells shown to be increased in NT patients did not also express Foxp3, suggesting that Foxp3 expression may not be a requisite for functionality of these cells. This has also been suggested in previous studies where the suppressive capability CD25+CD127lo cells has been shown despite lack of Foxp3 expression [14]. Further studies would help elucidate the functional capabilities and the role of these cells in oral tolerance acquisition.

Other cell types besides T regulatory cells produce IL-10, such as monocytes and B cells [30]. However, we have shown that with stimulation there is no increase in frequency of IL-10 producing monocytes or B cells, in comparison to the robust increase seen in the T regulatory cell populations. This suggests that these regulatory populations are likely important contributors to the increased IL-10 production observed in tolerant patients.

In our previous study, we demonstrated that egg NT patients had significantly increased IL-10 protein level compared to allergic and control patients after ovalbumin stimulation [8]. This upregulation was not seen after nonspecific stimulation with anti CD3/CD28. Findings from this current study further support the concept of antigen specific responses in the upregulation of IL-10 production as well as an increase in IL-10 positive cells. Reliable markers for identifying antigen-specific T regulatory cells are currently limited [31]. However, we have shown that the increase in production of IL-10 from CD25+CD127loCD4+, Foxp3+CD4+, and CD4+ cells observed in NT patients after antigen stimulation, is only observed after relevant antigen stimulation and not after unrelated antigen stimulation in these same patients, implying antigen-specific T regulatory cell involvement. In addition, these relationships persist among cells proliferating in response to antigen as measured by CFSElow expression (data not shown). Antigen specific T regulatory cells have been described to be functionally far superior to CD25+CD127lo+CD4+ T reg cells *in vitro* in the context of allogeneic graft rejection and therefore have been hypothesized to have high therapeutic potential in the context of allergic immune responses in patients [31].

Findings from oral immunotherapy studies in food allergy support the notion of tolerance acquisition being an active, immunologic process [29]. Our findings support the concept that natural tolerance is also an active immunologic process, is antigen specific and is not simply passively achieved.

While our study does not precisely identify the “moment” of natural oral tolerance acquisition, we attempted to address this by defining those who were tolerant as having passed a food challenge within 6 months at the time of the experiment. However, it is possible that some patients categorized as allergic, may have been in the process of developing tolerance, as even though they had a clear history of reaction as well as elevated sIgE they did not undergo food challenge at the time of recruitment. In this study, we hypothesized that Foxp3+CD25+CD127loCD4+ cells were tTreg cells as the addition of CD127lo to Foxp3 and CD25 positivity has been shown to more precisely identify tTreg

cells [14] [24]. As novel techniques and markers arise, future studies could utilize methods that more precisely identify and distinguish recent thymic emigrant tTregs from pTregs, for example the use of Helios expression (although the specificity of this marker is still debated [32]), or epigenetic modification[33]. As allergic and naturally tolerant patients differed from control patients in terms of having other atopic conditions such as asthma and atopic dermatitis, whether or not this could influence the development of tolerance and IL-10 producing Treg cell generation should be considered. However, allergic patients did not differ significantly from naturally tolerant patients in regards to other atopic disease. As such, presumably, this should not be a contributing factor to the development of natural tolerance and IL-10 producing T reg cell generation. Additionally, this study focused on IL-10 expression, elaborating on our prior study, but it is possible that there are other cytokines and processes involved in natural tolerance.

Although our study did not directly examine the proliferative and suppressive capacity of these regulatory T cell subsets directly, we used IL-10 expression to assess functional activation for these cells. As none of these subsets expressed measureable IL-10 at baseline, but only upon activation with relevant antigen, we hypothesize that these cells are responding in an immunoregulatory capacity to that antigen which then leads to decreased Th2 responses. However, further study regarding the functional suppressive capacity of these cells as well as the effect of blocking IL-10 (or IL-10R) would be important and desirable future work to further mechanistic understanding of NT.

In summary, this study supports the concept of T regulatory cell involvement in the development of natural tolerance to foods. This study provides novel evidence of the specificity of T regulatory cell responses in natural tolerance to foods. Furthermore, our data suggest that natural tolerance acquisition is an “active” process that is antigen specific rather than passively achieved. Specifically, IL-10 producing regulatory subsets, such as Tr1 cells may be a T regulatory subset that is critical to the development of natural tolerance to foods. Understanding the mechanisms of natural tolerance to foods may help identify targets for therapeutic development. Additionally, identifying similarities and differences between the mechanistic processes and cell types involved in tolerance achieved via oral immunotherapy versus natural tolerance may help evaluate and modify current therapeutic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Project Support: Northwestern University Feinberg School of Medicine, Thrasher Research Fund, Gerber Foundation, Bunning Food Allergy Initiative, National Institutes of Health NIAID K23 A1-100995; R37HL068546, R01HL078860 and P01AI106683 from the NIH and by the Ernest S. Bazley Foundation

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Fig. 1. Naturally Tolerant Patients had Increased CD4+CD25+CD127loFoxp3+ T Regulatory Cells at Baseline

A. Tolerant patients had increased thymically derived T regulatory cells as compared to allergic and control patients. **B.** There was no difference in Foxp3 MFI in these cells among tolerant patients as compared to allergic and control patients. **C.** At baseline, there was no difference in CD25+CD127lo cells among control, allergic, and tolerant patients. Data are expressed as the mean percentage cells within gated CD3+CD4+ T cells. Box plots with median as dark horizontal line in wide bars representing lower and upper quartiles. Whiskers are minimum and maximum. * represents p-value <0.05 calculated with Kruskal-Wallis test and Mann Whitney for pairwise comparisons.

Fig. 2. Naturally Tolerant Patients had Increased IL-10 Producing T Regulatory Cell Subsets and Decreased IL-5+Foxp3+ Cells After Stimulation with Relevant Antigen Compared to Control and Allergic Patients

Upon stimulation with relevant antigen: **A.** tolerant patients had increased IL-10 producing CD25+CD127lo cells when compared to control and allergic patients. **B.** Tolerant patients had increased IL-10 producing Foxp3+ cells compared to control and allergic patients. **C.** Tolerant patients had increased IL-10 producing CD4+ cells compared to control and allergic patients. **D.** Tolerant patients had decreased IL-5 producing Foxp3+ cells compared to control and allergic patients. Scatterplots with dark horizontal line representing mean and whiskers representing standard deviation. * represents p-value < 0.05 calculated with Kruskal-Wallis test and Mann Whitney for pairwise comparisons.

Fig. 3. Tolerant Patients Increased IL-10 Producing CD4+ Cells and CD25+CD127lo Cells Upon Stimulation with Relevant Antigen

A. The percentage of IL-10 producing CD4+ cells increased in tolerant patients upon stimulation with relevant antigen. **B.** The percentage of CD25+CD127loFoxp3+ cells decreased in tolerant patients and did not change in allergic or control patients. **C.** Percent change in IL-10 producing CD4+ cells in tolerant patients increased and not in allergic and control patients. **D.** Percent change in IL-10 producing CD25+CD127lo cells increased in tolerant patients compared to allergic and control patients. Data expressed in A, B represent the mean percentage cells within gated CD3+CD4+ T cells. Data in C, D represent the absolute difference between unstimulated and stimulated conditions. Bar graph with whiskers representing standard deviation. Box plots with median as dark horizontal line in wide bars representing lower and upper quartiles. Whiskers are minimum and maximum. * represents p-value <0.05 calculated with Kruskal-Wallis test and Mann Whitney for pairwise comparisons. ** represents p-value <0.005 calculated with Kruskal-Wallis test and Mann Whitney for pairwise comparisons.

Fig. 4. Tolerant Patients Increased IL-10 Producing Cells in Response to Relevant Antigen Only Upon stimulation with relevant antigen versus unrelated antigen: **A.** Tolerant patients increased IL-10 producing CD4+ cells in response to relevant antigen only and not unrelated antigen. **B.** There was no increase in IL-10+CD4+ cells in allergic patients in response to unrelated or relevant antigen. **C.** Tolerant patients increased IL-10 producing CD25+CD127lo cells in response to relevant antigen only and not irrelevant antigen. **D.** There was no increase in IL-10+CD25+CD127lo cells in allergic patients in response to unrelated or relevant antigen. Data are expressed as the mean percentage cells within gated CD3+CD4+ T cells (A,B) and within gated CD25+CD127loCD4+ cells (C,D). Scatter plots with mean as dark horizontal line. * represents p-value < 0.05 calculated with Kruskal-Wallis test and Mann Whitney for pairwise comparisons.

Table 1

Patient Characteristics

	Control	Allergic	Tolerant	p values [*]
Number	25	22	11	
Age in years, median (range)	8.6 (1.4, 15.3)	6 (0.9, 17.6)	6.3 (2.7, 11.8)	0.271
Male Sex, n (%)	17 (68%)	15 (68%)	9 (82%)	0.666
Race:				0.115
White	8 (32%)	13 (59%)	8 (72%)	
Hispanic	11 (44%)	5 (22%)	3 (27%)	
Other	6 (24%)	4 (18%)	0	
Asthma	0	11 (50%)	5 (45%)	0.001
Atopic dermatitis	0	12(55%)	5 (45%)	0.001
Reaction:				0.919
Skin (urticaria, angioedema)		8 (36%)	4 (36%)	
Anaphylaxis		4 (18%)	2 (18%)	
GI symptoms (profuse emesis)		1 (5%)	0	
Median skin test size egg white at initial diagnosis in mm (range)	--	7.5 (5, 12)	7 (2, 14)	0.892
Median egg white specific IgE kU/L at initial diagnosis (range)	--	11 (3.2, 35.3)	3.6 (0.36, 25.2)	0.113
Median egg white specific IgE kU/L at recruitment (range)	--	8.8 (0.2, 26.4)	0.48 (0.1, 1.69)	0.006
Median skin test size peanut at initial diagnosis in mm (range)	--	9 (2, 14)	6.5 (5, 20)	.968
Median peanut specific IgE kU/L at initial diagnosis (range)	--	3.69 (.33, >100)	3.38 (1.7, >100)	.699
Median peanut specific IgE kU/L at recruitment (range)	--	12.1 (.26, >100)	1.3 (0.1, 34.5)	.034
Tolerating egg baked goods, n (%)	--	8 (36%)	--	

* p-values from Kruskal Wallis, Chi-Square, and Mann Whitney tests comparing control, allergic, and tolerant groups or allergic and tolerant groups when not applicable to controls.

Table II

Phenotype of IL-10 producing cells with ovalbumin or peanut stimulation

	Allergic	Tolerant	p value [*]
No.	20	9	
%IL-10+/CD3+CD4+, mean (confidence interval)	1.59 (0.61–2.58)	4.48 (3.04–5.93)	<0.01
%IL-10+/CD3+CD4+Foxp3+	5.42 (3.3–7.54)	12.6 (5.61–19.58)	0.04
%IL-10/CD25+CD127lo	1.65 (0.26–3.04)	6.33 (–1.31–13.97)	0.11
%IL-10+/CD14+	4.48 (–37.14–46.09)	17.91 (2.25–33.6)	0.37
%IL-10+/CD19+	0.57 (–2.86–4)	1.62 (–2.16–5.41)	0.85

*
p-values from Mann Whitney U test