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Modulation of Dendritic Cell Innate and Adaptive Immune Functions by Oral and Sublingual Immunotherapy

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Abstract

Sublingual (SLIT) and oral immunotherapy (OIT) are promising treatments for food allergy, but underlying mechanisms are poorly understood. Dendritic cells (DC) induce and maintain Th2-type allergen-specific T cells, and also regulate innate immunity through their expression of Toll-like receptors (TLRs). We examined how SLIT and OIT influenced DC innate and adaptive immune responses in children with IgE-mediated cow's milk (CM) allergy. SLIT, but not OIT, decreased TLR-induced IL-6 secretion by myeloid DCs (mDCs). SLIT and OIT altered mDC IL-10 secretion, a potent inhibitor of FcεRI-dependent pro-inflammatory responses. OIT uniquely augmented IFN-α and decreased IL-6 secretion by plasmacytoid DCs (pDCs), which was associated with reduced TLR-induced IL-13 release in pDC-T cell co-cultures. Both SLIT and OIT decreased Th2 cytokine secretion to CM in pDC-T, but not mDC-T, co-cultures. Therefore,

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SLIT and OIT exert unique effects on DC-driven innate and adaptive immune responses, which may inhibit allergic inflammation and promote tolerance.

Keywords

dendritic cell; food allergy; sublingual immunotherapy; oral immunotherapy; innate immunity; adaptive immunity; cow's milk; tolerance

1. Introduction

Food allergy currently affects an estimated 15 million Americans [1, 2]. Although there is no cure, novel forms of immunotherapy (IT) are emerging as viable effective treatments [3-6]. The mechanisms responsible for the clinical benefits of IT are incompletely understood. Prior studies have suggested that IT downregulates Th2 cytokine responses by CD4⁺ T cells and induces antigen-specific T regulatory cells (Tregs) [7-9]. Dendritic cells (DCs) are professional antigen presenting cells (APCs) that take up antigens and prime T cell responses, and therefore likely play an instrumental role in directing these changes in T cell function. Two major classes of DCs have been identified in the peripheral blood of humans: 1) plasmacytoid DCs (pDCs), which are CD123⁺ Blood Dendritic Cell Antigen-2 (BDCA-2)⁺ CD11c⁻ and express TLRs 7 and 9; these cells are a major source of IFN- α following microbial infection and 2) myeloid DCs (mDCs), which are CD123⁻ BDCA1⁺ CD11c⁺ and express TLRs 2, 3, 4, and 8 [10-13]. Both subtypes regulate allergen-driven Th2 cytokine release by CD4⁺ T cells, underscoring a critical role for these cells in the pathogenesis of allergic disease [14, 15].

Recent studies have suggested that DC-derived cytokines released following TLR ligation can regulate T cell tolerance and Th2 differentiation. DC-derived IL-6 limits Th1 responses and promotes commitment to Th2 phenotypes, while IFN- α produced by pDCs potently inhibits IL-4 driven Th2 differentiation of CD4⁺ T cells and destabilizes the Th2 phenotype by suppressing expression of GATA3 [16-18]. Pro-inflammatory cytokines secreted by DCs also have the capacity to break allergen-specific T cell tolerance, and lead to exaggerated Th2 (and Th17) responses [19].

We have previously found that DCs from children allergic to cow's milk produce significantly higher levels of pro-inflammatory cytokines, including IL-6 and TNF- α , following exposure to milk compared to DCs from nonallergic children [14]. Additionally, several studies have revealed global defects in innate immune pathways in individuals with allergic disease, including deficient IFN- α secretion following TLR7 and TLR9 stimulation [20-22]. Whether current treatments for allergic disorders, including immunotherapy, are able to modulate these abnormalities in DC responses is not well understood. Interestingly, subcutaneous immunotherapy (SCIT) was recently shown to restore TLR9-mediated IFN- α responses in DCs from ragweed allergic adults, suggesting IT may have the ability to modulate innate immune function and thereby promote tolerance in allergic individuals [23].

Sublingual (SLIT) and oral (OIT) immunotherapy are promising new treatments for food allergy. In SLIT, an allergen solution is first held under the tongue for a period of time and

then swallowed, whereas in OIT the allergen is delivered in a food vehicle and swallowed immediately. The oral mucosa is relatively devoid of inflammatory cells but rich in tolerogenic APCs (specifically mDCs), suggesting SLIT may have the advantage of eliciting fewer adverse reactions while favoring tolerogenic responses [24]. On the other hand, higher doses of allergen can be delivered with OIT, which may be important for inducing tolerance. We recently reported that OIT was more efficacious for desensitization to cow's milk (CM) than SLIT alone but was accompanied by more systemic side effects [6]. In this study, we sought to examine how SLIT and OIT affect DC innate and adaptive immune functions, given the pivotal role these cells play in tolerance and Th2 differentiation.

2. Materials and Methods

2.1 Study Design and Subject Recruitment

The study was an open-label randomized trial of SLIT and OIT for the treatment of IgE-mediated CM allergy. Subjects were recruited from the Johns Hopkins University pediatric allergy clinic. Details of the study design, enrollment criteria, and clinical outcomes can be found in reference 6. Baseline characteristics of subjects are summarized in Supplementary Table 1. Briefly, 24 subjects 6-17 years of age with documented IgE-mediated CM allergy underwent a double-blind, placebo-controlled food challenge (DBPCFC) to CM followed by initial SLIT escalation (minimum of four weeks) with an aqueous CM extract. Subjects were then randomized to continue SLIT escalation (n=8) to a maximum dose of 7mg of CM protein daily, or to begin OIT with a goal maintenance dose of either 1000mg (n=8) or 2000mg (n=8) of CM protein. These two OIT groups were combined for our analysis to increase power as their clinical courses were similar, and these subjects are herein referred to as "OIT". Key study time points were as follows: T1: baseline screening DBPCFC; T2: end of initial SLIT escalation and randomization to continue SLIT or switch to OIT; T3: end of dose escalation (median 10 weeks (range: 8-14) for SLIT and median 28 weeks (range: 20-41) for OIT); T4: completion of at least 12 weeks of maintenance dosing. At T4, participants underwent a DBPCFC, and subjects in the OIT arm increased their daily maintenance dose to one half the tolerated dose (if greater than the target dose). All subjects then continued daily dosing, and underwent an open oral food challenge (OFC) to 8gm of CM protein at T5 (minimum 60 weeks on maintenance). If the subject passed this challenge without symptoms requiring medication, the subject was considered "desensitized" to CM and therapy was discontinued. A repeat OFC was done one week later at visit T6. If the subject passed this challenge, another OFC was done 5 weeks later at visit T7 (total of six weeks off therapy). All of the subjects in the study demonstrated an increase in their food challenge threshold, with a median fold increase of 7 for the SLIT group and 108 for the OIT group by visit T5. None of the SLIT subjects but 10/16 OIT subjects passed the desensitization challenge at T5. Only 5 of these 10 subjects passed the challenge at T7 after being off therapy for 6 weeks, and were therefore considered clinically tolerant. Two subjects, both in the OIT group, withdrew from the study during the course of therapy due to adverse events. This study was approved by the Johns Hopkins Institutional Review Board (study NA_00014511).

2.2 Cell preparation and cultures

Peripheral blood was collected in EDTA and subjected to double Percoll (Pharmacia Biotech, Inc., Piscataway, NJ) density (1.0751/1.081 g/mL) centrifugation. The upper fraction of cells in the double percoll gradient consisted of basophil-depleted mononuclear cells that were used to isolate pDCs using BDCA4⁺ magnetic bead selection (Miltenyi, Auburn, CA). Cells not retained on this column were then used to isolate mDCs with BDCA1⁺ selection (Miltenyi) after depletion of CD19⁺ B cells. Although the few numbers of DC subtypes isolated did not allow for routine testing of purity, periodic evaluations indicate enrichments achieving up to 95%. CD4⁺ T cells were prepared by positive selection (Miltenyi) from remaining cells after removal of the DC subtypes. DC subtypes (2.0x10⁴ cells) were cultured in duplicate in a final volume of 250µl of conditioned-Iscove Modified Dulbecco Media (C-IMDM) that was supplemented with 5% fetal calf serum (FCS), 1X nonessential amino acids, and 10g/ml gentamicin, pH 7.2-7.4. Cells were cultured for 24 hours with the indicated stimulants in 96-well flat-bottom plates, at which point supernatants were harvested and stored at -80° C for further analysis. For DC-T cell co-cultures, 1x10⁴ pDCs or mDCs were cultured with 1x10⁵ autologous CD4⁺ T cells in a final volume of 250µl and stimulated exactly as described for the pure DC cultures, except supernatants were harvested after 96 hours and cells were cultured in 96-well round-bottom plates. Cultures of pDCs were stimulated with media alone, 10µg/mL of an aqueous crude cow's milk (CM) extract without preservatives (Greer Laboratories), 1.0µg/mL of the TLR7 agonist R837 (InvivoGen, San Diego, CA) or 100nM of the TLR9 agonist CpG ODN-2216 (TriLink, San Diego, CA). Cultures of mDCs were stimulated with media alone, 10µg/mL of an aqueous crude cow's milk (CM) extract without preservatives (Greer Laboratories), 10µg/mL of the TLR2 agonist peptidoglycan from *Staphylococcal aureus* (Fluka Analytical, St. Louis, MO), 25µg/mL of the TLR3 agonist polyinosinic:polycytidylic acid (poly (I:C); Sigma, St. Louis, MO), 10ng/mL of the TLR4 agonist purified lipopolysaccharide from *Escherichia coli* 0111:B4 (Sigma), or 0.5 µg/mL of the TLR7/8 agonist CL-075 (InvivoGen).

2.3 Cytokine measurement

ELISA protocols for the detection of IL-6 (eBioscience, San Diego, CA), IL-10 (eBioscience), and IFN-α (in-house) from pure DC culture supernatants have been described previously [25]. A human x-plex panel (including IL-13, IFN-γ, and IL-10; Biorad, Hercules, CA) was used to evaluate supernatants from DC-T cell co-cultures. Limits of detection for the multiplex assay were IL-13 0.7 pg/ml, IFN-γ 0.6 pg/ml, and IL-10 0.3 pg/mL. Spontaneous cytokine secretion, as measured in cultures stimulated with media alone, was subtracted from the values obtained under each experimental condition for analysis.

2.4 Statistics

All outcomes were evaluated by using linear regression models with generalized estimating equations (GEE) to account for repeated measures over time. Cytokine concentrations were log transformed for analysis. The difference in cytokine concentrations between a study time point (T3-T7) and baseline (T1) were considered significant only if the overall p-value for

the GEE taking into account multiple comparisons was significant, and the p-value comparing values within the GEE model at one of the time points compared to baseline was also significant. Statistically significant p-values, defined as <0.05 , are indicated. To evaluate the relationship between laboratory outcomes and clinical outcomes, subjects in the OIT group who passed the T5 challenge were divided into those who passed the OFC at visit T7 (Tolerant) and those who failed one of the challenges at T6 or T7 (Desensitized but Not Tolerant). No SLIT subjects passed the challenge at T5; therefore, these subjects were not considered for analysis of tolerance outcomes. In the graphs, the box defines the 25th and 75th percentile; center line the median; whiskers the adjacent values; individual points the outliers.

3. Results

3.1 Pro-inflammatory cytokine secretion by DCs

Subjects with IgE-mediated CM allergy were randomized to receive SLIT or OIT as previously described [6]. To determine the impact of these therapies on pro-inflammatory innate immune responses by circulating DC subtypes, pDCs and mDCs were isolated from the peripheral blood of subjects at baseline and at various time points during IT. Plasmacytoid DCs from subjects undergoing OIT showed modest but significant reductions in spontaneous IL-6 production, as well as in response to both TLR7 and TLR9 stimulation during treatment (Fig. 1A, Supplementary Table 2 and 3). The reduction in IL-6 secretion to TLR7 stimulation was transient and most pronounced after approximately 12 weeks of maintenance dosing (T4), while the decreases in spontaneous and TLR9-induced IL-6 levels were significant only at the tolerance challenge at the end of the study (T7). No significant changes were observed in the subjects that received SLIT (Fig. 1A, Supplementary Table 2).

On the other hand, OIT had no significant effect on mDC IL-6 release to a panel of TLR ligands (Fig. 1B, Supplementary Table 2), while mDCs from SLIT subjects demonstrated a transient increase after 12 weeks of maintenance dosing (T4) that was followed by a decrease in IL-6 production to TLR 2, 3, 4, and 7/8 agonists after 60 weeks of maintenance dosing at visit T5 (Fig. 1B, Supplementary Table 2 and 3). No change in spontaneous IL-6 production was evident (Fig. 1B, Supplementary Table 2). These data suggest that OIT primarily impacts TLR-induced pro-inflammatory cytokine secretion by pDCs, while SLIT affects mDCs.

Treatment of pDCs and mDCs with cow's milk extract elicited very low levels of IL-6 compared to TLR-induced values, and release of CM-induced IL-6 did not change significantly during the course of OIT or SLIT (Supplementary Fig. 1).

3.2 IFN- α production by pDCs in response to TLR stimulation

Plasmacytoid DCs are the primary source of IFN- α following stimulation with either TLR7 or TLR9 agonists [10]. IFN- α may play an important role in inducing tolerance, both by supporting the development of Tregs as well as by suppressing the activity of Th2 cells [16-18, 26-28]. OIT was associated with a significant increase in IFN- α responses to the TLR7 agonist R837 after 60 weeks of maintenance dosing (T5), although not to the TLR9

agonist CpG-2216 (Fig. 2A, Supplementary Table 2 and 3). No significant change in IFN- α production to either TLR ligand was evident at any time point in the SLIT group (Fig. 2A, Supplementary Table 2). These data further suggest that OIT, but not SLIT, significantly impacts pDC innate immune function. Treatment of pDCs with cow's milk extract did not induce significant IFN- α secretion at any timepoint in either SLIT or OIT (Supplementary Fig. 1A).

3.3 mDC IL-10 secretion in response to TLR agonists

Autocrine secretion of IL-10 by mDCs is a key negative regulator of Fc ϵ RI-dependent proinflammatory responses by mDCs [29]. Peptidoglycan, a TLR2 agonist, and poly (I:C), a TLR3 ligand, both elicited significantly increased IL-10 production by mDCs from OIT subjects during treatment (Fig. 2B, Supplementary Table 2 and 3). IL-10 secretion appeared to increase gradually the longer subjects continued on treatment, and only reached significance at the tolerance challenge at T7. While these TLR2 and 3 responses from mDCs isolated from SLIT subjects were not significantly changed during the course of the study, an increase in mDC-derived IL-10 was observed with the TLR7/8 agonist, CL-075, at the end of dose escalation (T3) and after 12 weeks of maintenance dosing (T4), which was not seen in the OIT arm (Fig. 2B, Supplementary Table 2 and 3). However, this increase was transient as levels were below baseline after 60 weeks of maintenance dosing (T5) (Fig. 2B, Supplementary Table 2 and 3). Neither SLIT nor OIT subjects showed any significant change in mDC IL-10 responses to the TLR4 agonist, lipopolysaccharide (Fig. 2B, Supplementary Table 2). Spontaneous IL-10 secretion increased in both arms late in the study, although levels were much lower than those induced following TLR stimulation (Fig. 2B, Supplementary Table 2 and 3). Stimulation with cow's milk extract generally did not induce detectable IL-10 release from mDCs (Supplementary Fig. 1B).

3.4 TLR responses in OIT subjects according to clinical outcome

After a minimum of 60 weeks of maintenance dosing (T5), none of the SLIT subjects but 10 of the 16 OIT subjects were able to pass an oral food challenge (OFC) to CM. These 10 subjects were then taken off therapy to try and distinguish between subjects who were truly "tolerant" to CM versus those that were desensitized only and required regular exposure to CM to retain their lack of reactivity. To determine whether any change in DC innate immune function predicted tolerance versus desensitization among these 10 OIT subjects, we compared cytokine responses between those subjects who were tolerant and passed their milk challenge after therapy had been withdrawn for 6 weeks, to those who failed a challenge at visit T6 or T7 (during the period when treatment had been discontinued), and were therefore considered desensitized but not truly tolerant. In both Tolerant and Desensitized OIT subjects, spontaneous and TLR9-induced pDC IL-6 responses decreased, while no change in IL-6 secretion following TLR7 stimulation was observed (Fig. 3A). The decline in TLR9-induced IL6 was significant in the Tolerant subjects after only 12 weeks of maintenance dosing (T4) and remained decreased compared to baseline, while in the Desensitized group IL-6 levels were significantly reduced from baseline only at the time of the tolerance challenge (T7; Fig. 3A). No clear pattern in IFN- α responses by pDCs emerged that distinguished Tolerant and Desensitized subjects (data not shown).

Overall, Tolerant subjects showed a trend toward increased, followed by decreased, mDC IL-6 responses to TLR stimulation, although the decrease reached significance only in the TLR2 and TLR7/8 stimulated cultures after at least 60 weeks of maintenance dosing (T5; Fig. 3B). Spontaneous IL-6 also decreased significantly in mDC cultures in the Tolerant group by the end of the study (T7; Fig. 3B). In contrast, no significant changes in IL-6 production were seen in mDC cultures stimulated with the panel of TLR ligands from Desensitized OIT subjects (Fig. 3B). mDC IL-10 responses did not effectively distinguish Tolerant and Desensitized OIT subjects (data not shown).

3.5 TLR-induced effector cytokine secretion in DC-T cell co-cultures

Given the ability of TLR agonists to induce cytokine secretion by DCs that influence T cell tolerance and effector cytokine expression, we next examined whether the changes in innate immune responses by DCs that we observed during SLIT and OIT influenced cytokine responses in DC-T cell co-cultures [19]. We found that OIT, but not SLIT, decreased release of IL-13 in pDC-T cell co-cultures stimulated with both TLR7 and TLR9 agonists (Fig. 4A). Th2 cytokine expression was significantly reduced from baseline after 60 weeks of maintenance dosing (T5) following TLR7 stimulation and by the end of the study (T7) following TLR7 and TLR9 stimulation (Fig. 4A).

IL-13 responses in mDC-T cell co-cultures from the SLIT cohort stimulated with both TLR2 and TLR3 agonists were decreased at the end of dose escalation (T3), but subsequently increased again in cultures stimulated with the TLR3 agonist (Fig. 4B). OIT subjects manifested reduced IL13 secretion in mDC-T cell co-cultures stimulated with TLR2 and TLR7/8 agonists, but not until after at least 60 weeks of maintenance dosing (T5; Fig. 4B). Neither cohort showed a change in spontaneous or TLR4-stimulated IL-13 release (Fig. 4B).

Both SLIT and OIT were associated with decreases in IL-10 and IFN- γ secretion in TLR ligand-treated pDC-T cell co-cultures (Fig. 5A, Supplementary Fig. 2A). For IL-10, decreased secretion was evident following TLR7 and 9 stimulation early during the course of treatment (by T3, the end of dose escalation) and remained lower compared to baseline for the duration of the study (Fig. 5A). IFN- γ also started to decrease early, and was significantly decreased in OIT after 12 weeks of maintenance dosing (T4) for TLR7 and 9 conditions, and in SLIT after 60 weeks of maintenance (T5) in TLR7 ligand-treated cultures only (Supplementary Fig. 2A). Neither group exhibited a change in IL-10 secretion in mDC-T cell co-cultures stimulated with the entire panel of TLR ligands (Fig. 5B). Interestingly, OIT had no effect on TLR-induced release of IFN- γ at any point in the study, while SLIT mitigated IFN- γ production in response to the entire panel of TLR ligands (Supplementary Fig. 2B). In some cases, this decrease was evident by the end of dose escalation (T3; for TLR7/8 stimulated cultures), but in other cases, no significant decrease was seen until either at least 12 (T4; TLR3 condition) or 60 weeks of maintenance dosing (T5; TLR2 and TLR4 conditions). Spontaneously produced IL10 decreased in mDC-T cell co-cultures from SLIT subjects and IFN- γ in OIT subjects, but in both cases, levels of cytokine were low compared to TLR-induced values (Fig. 5B, Supplementary Fig. 2B).

We further evaluated whether DC-T cell responses following TLR stimulation predicted clinical responses. Among subjects receiving OIT, IL-13 responses in pDC-T cell co-

cultures were significantly reduced in both Desensitized (after 60 weeks of maintenance dosing, T5) and Tolerant (after 12 weeks of maintenance dosing, T4) subjects following TLR7 stimulation, and at the end of the study (T7) in the Tolerant group only (Supplementary Fig. 3A). In the Desensitized subjects, IL-13 levels in mDC-T cell co-cultures were significantly decreased compared to baseline after 60 weeks of maintenance dosing (T5) and/or at the end of the study (T7) following treatment with TLR2, 3, and 7/8 agonists (Supplementary Fig. 3B). In the Tolerant group, an increase in IL-13 was apparent by the end of dose escalation (T3) following treatment with TLR2, TLR3, and TLR4 agonists, but only reached statistical significance in the TLR3 condition. However, levels subsequently decreased from baseline by 60 weeks of maintenance (T5), and again the decrease was only statistically significant in the TLR3-treated cultures (T5; Supplementary Fig. 3B).

IL-10 was significantly decreased following both TLR7 (from the end of dose escalation T3 through the end of the study T7) and TLR9 (after 12 weeks of maintenance dosing T4 and at the end of the study T7) stimulation in pDC-T cell co-cultures from the Tolerant group, but only after TLR9 treatment (at visits T3, T4, and T7) in the Desensitized subjects (Supplementary Fig. 4A). No differences in TLR-induced IL-10 were apparent in mDC-T cell co-cultures between Desensitized and Tolerant OIT subjects, except for a significant decrease in IL-10 at the end of the study (T7) following TLR4 stimulation in the Tolerant group (Supplementary Fig. 4B).

IFN- γ was decreased in pDC-T cell cultures under TLR7 (after 12 weeks of maintenance dosing T4 and at the end of the study T7) and TLR9 stimulated conditions (at T7) in Desensitized subjects but only at the end of the study (T7) after TLR7 treatment in those that were considered tolerant (Supplementary Fig. 5A). IFN- γ responses did not change significantly in mDC-T cell co-cultures from Desensitized or Tolerant subjects following treatment with TLR agonists, except levels did significantly decrease in the Desensitized group after 60 weeks of maintenance (T5) and at the end of the study (T7) following TLR7/8 stimulation (Supplementary Fig. 5B).

3.5 Allergen-induced effector cytokine secretion in DC-T cell co-cultures

We next asked how SLIT and OIT influenced Th2 adaptive immune responses to allergen. CD4⁺ T cells from both SLIT and OIT subjects demonstrated reduced secretion of Th2 cytokines (IL-5 and IL-13) in response to milk when co-cultured with pDCs (Fig. 6). This decrease occurred earlier in OIT subjects (for IL13, by T3 - the end of dose escalation), but only after 60 weeks of maintenance dosing (T5) in subjects receiving SLIT (Fig. 6). However, no decrease in Th2 cytokines was seen in analogous mDC-T cell co-cultures from SLIT or OIT subjects, and in fact, CM-induced IL-5 levels significantly increased in the OIT group (at T4 and T7) and IL-13 significantly rose in the SLIT group (at T5; Fig. 6). These data suggest that the two DC subtypes regulate adaptive Th2 immune responses to allergen differently following IT.

Secretion of IFN- γ following stimulation with CM did not change significantly in SLIT or OIT during the course of treatment and IL-10 was noted to decrease significantly only in OIT subjects at the end of the study (T7) in pDC-T co-cultures (Supplementary Fig. 6).

Spontaneous release of IFN- γ and IL-10 was significantly reduced in both pDC- and mDC-T cell co-cultures under various conditions, particularly in subjects receiving SLIT (Supplementary Fig. 6).

Tolerant OIT subjects manifested no significant changes in IL-5 or IL-13 secretion in either pDC-T cell or mDC-T cell co-cultures stimulated with CM extract during the course of therapy (Supplementary Fig. 7). However, Desensitized OIT subjects demonstrated significantly decreased CM-induced IL-5 and IL-13 production in pDC-T cell co-cultures, but significantly increased Th2 cytokines in mDC-T cell co-cultures. Secretion of IFN- γ and IL-10 to CM did not predict clinical responses (data not shown).

The majority of subjects in the study (19/24) were also sensitized to peanut, and therefore we were able to compare T cell responses to the allergen used for treatment (milk) compared to a food antigen to which subjects were allergic but had not received IT (peanut). Among peanut (PN)-allergic subjects, no significant change in PN-induced Th2 cytokine release was evident in either pDC-T cell or mDC-T cell co-cultures, with the exception of transiently reduced IL-13 in mDC-T cell co-cultures from SLIT subjects at the end of dose escalation (T3; Fig 6).

4. Discussion

Dendritic cells are considered essential to the development of tolerance, yet how the function of these cells is influenced by allergen immunotherapy is largely unknown. Here, we demonstrate for the first time in humans that both SLIT and OIT are associated with changes in DC innate immune functions that may inhibit allergic inflammation and promote the development of tolerance. These therapies appear to at least partially correct the abnormalities in DC secretion of pro-inflammatory cytokines and IFN- α that have been reported in allergic populations [14, 21, 22]. Furthermore, CM-induced Th2 cytokine secretion by CD4⁺ T cells decreased during SLIT and OIT but only in pDC-T cell co-cultures. Th2 cytokine levels in mDC-T cell co-cultures stimulated with milk failed to change, or even increased, during the course of therapy. These data suggest that IT has disparate effects on the two major DC subtypes in humans, and how these cells influence antigen-specific adaptive immune responses. Finally, SLIT and OIT exerted distinct effects on the function of the two major circulating DC subtypes, suggesting that the route of allergen delivery and/or dose of antigen delivered can result in unique immunologic consequences that may influence clinical efficacy.

DCs in the oral mucosa are purported to be the primary target of SLIT [30]. In humans, these cells are comprised mainly of mDCs, while pDCs are undetectable [31]. Interestingly, SLIT had a significant impact on circulating mDC, but not pDC, innate immune function. After an initial increase, SLIT elicited decreases in pro-inflammatory IL-6 secretion by mDCs in response to the entire panel of TLR ligands. Of note, we previously published that a same similar pattern of increased/decreased responsiveness was also observed for basophils from these subjects receiving SLIT [6]. Whether the two responses are linked to one another is not currently known, but their correlative nature help substantiate the notion that neither were due to chance alone. Nonetheless, IL-6 secretion by bone marrow-derived

myeloid DCs is known to promote skewing to Th2, and recently pro-inflammatory cytokine secretion (including IL-6) by mDCs was found to be key in breaking tolerance by allergen-specific CD4⁺ T cells [19, 32]. These data suggest that the changes in mDC function during SLIT may contribute to the clinical desensitization evident in these patients. Reduced secretion of IL-6 might also facilitate *de novo* generation of adaptive Tregs, which may play a key role in the induction of peripheral T cell tolerance during IT [9, 33].

In contrast to SLIT, subjects undergoing OIT as a group did not manifest any significant changes in mDC IL-6 secretion. However, mDCs from the subset of OIT subjects who achieved true tolerance to CM did show decreased IL-6 release following treatment with innate immune stimuli that mirrored the changes in mDC responses seen in the SLIT group, whereas these changes were not apparent in OIT subjects that failed to achieve tolerance. These data suggest that reduced pro-inflammatory cytokine secretion by mDCs may promote the development of clinical tolerance, even though this effect may not be sufficient, based on the lack of tolerance acquisition in subjects undergoing SLIT alone. These findings raise the question of whether the addition of SLIT to OIT would enhance acquisition of clinical tolerance compared to OIT alone. pDCs from tolerant subjects also showed less IL-6 secretion following TLR9 stimulation than those who were desensitized only, although the decrease was significant in both groups. Therefore, reduced pro-inflammatory cytokine secretion by both DC subtypes may contribute to the efficacy of these therapies.

In this study, we were unfortunately not able to examine how SLIT and OIT influenced expression of TLRs. It is our experience that such measures are hampered by a lack of validated reagents (e.g. antibodies) suitable for evaluating protein for some TLRs, particularly TLR7. Thus, studies have generally relied on changes in mRNA expression, which would have required purifying additional DCs from an already limited source (i.e. children). Instead, we elected to investigate functional changes, which are a better indication of how SLIT/OIT affects innate immunity. Prior studies in adult patients undergoing subcutaneous immunotherapy for Hymenoptera venom allergy did find reduced TLR2, but not TLR1 or TLR4, expression on mDCs during the course of treatment. However, mDC cytokine responses to innate immune stimuli were not evaluated in this study [34].

Both SLIT and OIT were associated with significant increases in mDC IL-10 secretion following treatment with innate immune stimuli. IL-10 is often regarded as a key cytokine that drives the development of tolerance in patients undergoing IT [35, 36]. Autocrine secretion of IL-10 by mDCs suppresses FcεRI-dependent pro-inflammatory responses [29]. Therefore, increased IL-10 production by mDCs during SLIT and/or OIT may reduce inflammatory responses to allergen. Intriguingly, IFN-α, a key anti-viral cytokine produced by pDCs, acts on mDCs to augment autocrine secretion of IL-10 by mDCs, suggesting these two cell types may work together to promote anti-allergic responses [29]. We found increased IFN-α secretion from pDCs in subjects undergoing OIT, suggesting OIT may exert beneficial changes in both pDC and mDC innate immune function that synergistically promote tolerance. Reduced IL-6 responses to TLR agonists were also seen in pDC cultures from OIT subjects, while SLIT had no significant effect on either IL-6 or IFN-α production by pDCs. Enhanced IFN-α responses following TLR9 stimulation have also been reported during subcutaneous immunotherapy, but only to higher doses of CpG than those used in our

study [23]. While not addressed in our study, it is possible that the increased IFN- α secretion by pDCs during IT enhances the development of antigen-specific Tregs, since pDC-derived IFN- α is known to promote the generation of adaptive Tregs [26].

Several recent studies have suggested that cytokines released by DCs following TLR stimulation may dictate Th2 differentiation. DC-derived IL-6 was found to potently promote, and IFN- α inhibit, Th2 immune responses [17, 18]. In our study, we found OIT reduced IL-6, and enhanced IFN- α , secretion by pDCs stimulated with TLR7 and/or 9 agonists. These changes were associated with reduced IL-13 release in pDC-T cell co-cultures stimulated with the same TLR agonists. These alterations in DC function were only observed in subjects who underwent OIT; whether this contributes to the increased clinical efficacy of OIT compared to SLIT remains to be seen. Interestingly, SLIT was associated with greater decreases in mDC IL-6 release to TLR ligands compared to OIT, although both forms of therapy were associated with reduced IL-13 secretion in mDC-T cell co-cultures following TLR2 stimulation.

Both SLIT and OIT were associated with significant decreases in IFN- γ and IL-10 secretion in pDC-T cell co-cultures following treatment with innate immune stimuli. SLIT also decreased IFN- γ responses to TLR agonists in mDC-T cell co-cultures, which was not evident in OIT subjects. These results support the possibility that SLIT has a greater effect on certain mDC innate immune functions compared to OIT. Some differences were evident in TLR-induced DC-T cell cytokine responses between OIT subjects who achieved tolerance versus those who were desensitized only, but no clear pattern emerged that predicted clinical responsiveness.

While cow's milk-induced Th2 cytokine secretion in pDC-T cell cultures decreased during SLIT and OIT, no change or even an increase in Th2 cytokines was seen in analogous mDC-T cell co-cultures. These data support a prominent role for pDCs in driving the decrease in Th2 responses to allergen during IT, which is interesting since pDCs are known to play a critical role in the induction of tolerance in both the lung and gastrointestinal tract.[27, 28] Interestingly, the pattern of decreased Th2 cytokine responses to CM in pDC-T, and increased Th2 cytokines in mDC-T cell co-cultures, was more evident in OIT subjects who were only desensitized to CM compared to those who were considered tolerant. The changes in DC-T cell Th2 cytokine responses during the course of IT were antigen-specific, as no significant changes in Th2 cytokine secretion to a different allergen (peanut) were seen among SLIT or OIT subjects who were peanut allergic. The effect of immunotherapy on DC-T cell function also appeared to be specific for Th2 immune responses to CM, since no change in IFN- γ or IL-10 release after stimulation with CM was observed.

The lack of increase in IFN- γ and IL-10 secretion following CM stimulation, and the decrease in both cytokines following treatment with innate immune stimuli, is at odds with some, but not all, prior studies evaluating immunotherapy for food allergy. Bedoret et al. did find reduced Th2 and increased Th1 responses during the course of milk IT in combination with omalizumab; however, differences in the clinical design of this study, as well as the assays used to measure T cell cytokine responses, may contribute to our different findings [37]. Other studies of IT for food allergy have found results similar to ours including a

decrease in Th2 cytokine responses but no evidence of increased Th1 immunity [38]. Moreover, Suarez-Fueyo et al. demonstrated that the increase in IFN- γ during SLIT for grass pollen allergy was not mediated by T cells; therefore, we cannot exclude the possibility that other cell types did indeed lead to increased Th1 and IL-10 responses in our study that we never detected since we only evaluated the T cell arm [39].

There are several limitations to this study. All subjects were treated briefly (4 weeks) with low doses of SLIT at the beginning of the trial in order to decrease the risk of reactions during dose build-up. However, OIT subjects had received OIT exclusively for a minimum of 14 weeks by visit T3, a minimum of 26 weeks by T4, and a minimum of 74 weeks by T5. The length of time subjects required to reach specific time points also varied, depending in part on the frequency and/or severity of reactions with dosing. This pilot study also had no placebo group, but the consistent and distinct differences in the function of the two DC subtypes observed between subjects receiving SLIT versus OIT suggest that these changes were due to differences in treatment strategies and not chance alone. Finally, limited sample sizes make it impossible to make more definitive statements regarding the relationship between our DC findings and clinical outcomes, and provide the impetus to examine these questions in larger clinical trials.

In summary, this pilot study suggests that both SLIT and OIT for food allergy induce pro-tolerogenic changes in innate immune function by both pDCs and mDCs. These therapies also reduce allergen-induced Th2 cytokine responses by CD4⁺ T cells, but only when co-cultured with pDCs. The mechanisms by which allergen-specific immunotherapy modulates innate immune responses will be the subject of future investigations. Small quantities of dietary antigens reach the systemic circulation, and therefore would have the potential to influence the function of circulating cells, including basophils and DCs. The changes in innate immune responses that we observed might also be related to the changes in allergen-specific IgE and IgG that accompany immunotherapy. Studies have demonstrated that engagement of the high affinity IgE receptor Fc ϵ RI on pDCs inhibits TLR9-induced secretion of IFN- α by these cells, and IgG immune complexes are known to have immunomodulatory activities in the innate immune system [25, 40-42]. Other studies have suggested that certain allergens can directly or indirectly trigger TLRs on DCs [43, 44]. Since CM-specific IgE increased early in this study and then declined, and CM-IgG4 increased after the end of dose escalation, if some TLR responses are indeed influenced by immunoglobulin levels, this may partly explain why certain changes in TLR activity were only evident at some timepoints in the study [6]. While larger clinical studies are needed to verify our findings, a greater understanding of how innate immunity contributes to the effectiveness of SLIT and OIT and influences adaptive immune responses may inform the development of pro-tolerogenic adjuvants that will enhance the efficacy of these therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DC	Dendritic cell
TLR	Toll like receptor
IT	Immunotherapy
SLIT	Sublingual immunotherapy
OIT	Oral immunotherapy
SCIT	Subcutaneous immunotherapy
CM	Cow's milk
pDC	Plasmacytoid dendritic cell
mDC	Myeloid dendritic cell
BDCA	Blood Dendritic Cell Antigen
Treg	T regulatory cell
APC	Antigen presenting cell
DBPCFC	Double blind placebo-controlled food challenge
OFC	Oral food challenge
GEE	Generalized estimating equation
poly (I:C)	polyinosinic:polycytidylic acid

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- SLIT and OIT for CM allergy significantly alter DC innate and adaptive immune functions
- SLIT exerts a greater effect on mDC function and OIT on pDC responses
- Changes in TLR-driven DC cytokine responses during OIT/SLIT may promote tolerance

Figure 1.

IL-6 secretion by DCs from SLIT (n=8) and OIT (n=16) subjects in response to TLR stimulation. pDCs or mDCs were cultured with the indicated panels of TLR ligands for 24 hours. (A) IL-6 levels in culture supernatants from pDCs stimulated with media alone, TLR7 or 9 agonists and (B) mDCs stimulated with media alone, TLR2, 3, 4, or 7/8 agonists. Spontaneous cytokine secretion, as measured in media alone, was subtracted to obtain TLR-induced values. Outcomes were evaluated by using linear regression models with generalized estimating equations (GEE) to account for repeated measures over time. * p<0.05; ** p<0.01; T1: baseline; T3: end of dose escalation; T4: completion of at least 12 weeks of maintenance dosing; T5: completion of at least 60 weeks of maintenance dosing; T7: six weeks off therapy. The box defines the 25th and 75th percentile; center line the median; whiskers the adjacent values; individual points the outliers.

Figure 2.

TLR agonist-induced IFN- α and IL-10 production by pDCs and mDCs, respectively, from SLIT (n=8) and OIT (n=16) subjects. pDCs or mDCs were cultured with the indicated panels of TLR ligands for 24 hours. (A) IFN- α levels in culture supernatants from pDCs stimulated with media alone and TLR7 or 9 agonists. (B) IL-10 levels secreted by mDCs stimulated with media alone and TLR2, 3, 4, or 7/8 agonists. Spontaneous cytokine secretion, as measured in media alone, was subtracted to obtain TLR-induced values. Outcomes were evaluated by using linear regression models with generalized estimating equations (GEE) to account for repeated measures over time. * $p < 0.05$; ** $p < 0.01$; T1: baseline; T3: end of dose escalation; T4: completion of at least 12 weeks of maintenance dosing; T5: completion of at least 60 weeks of maintenance dosing; T7: six weeks off therapy. The box defines the 25th and 75th percentile; center line the median; whiskers the adjacent values; individual points the outliers.

Figure 3.

IL-6 secretion by DCs from OIT subjects who did (Tolerant; n=5) or did not (Desensitized; n=5) achieve clinical tolerance following stimulation with TLR ligands. pDCs or mDCs were cultured with the indicated panels of TLR ligands for 24 hours. (A) IL-6 levels in culture supernatants from pDCs stimulated with media alone, TLR7 or 9 agonists and (B) mDCs stimulated with media alone, TLR2, 3, 4, or 7/8 agonists. Spontaneous cytokine secretion, as measured in media alone, was subtracted to obtain TLR-induced values. Outcomes were evaluated by using linear regression models with generalized estimating equations (GEE) to account for repeated measures over time. * p<0.05; ** p<0.01; T1: baseline; T3: end of dose escalation; T4: completion of at least 12 weeks of maintenance dosing; T5: completion of at least 60 weeks of maintenance dosing; T7: six weeks off therapy. The box defines the 25th and 75th percentile; center line the median; whiskers the adjacent values; individual points the outliers.

Figure 4.

IL-13 production in DC-T cell co-culture supernatants from SLIT (n=8) and OIT subjects (n=16) following stimulation with TLR ligands. pDCs or mDCs were co-cultured with autologous CD4⁺ T cells for 96 hours and stimulated with the indicated panels of TLR agonists. (A) pDC-CD4⁺ T cell co-cultures stimulated with media alone, TLR7 or 9 agonists. (B) mDC-CD4⁺ T cell co-cultures stimulated with media alone, TLR2, 3, 4, or 7/8 agonists. Spontaneous cytokine secretion, as measured in media alone, was subtracted to obtain TLR-induced values. Outcomes were evaluated by using linear regression models with generalized estimating equations (GEE) to account for repeated measures over time. * p<0.05; ** p<0.01; T1: baseline; T3: end of dose escalation; T4: completion of at least 12 weeks of maintenance dosing; T5: completion of at least 60 weeks of maintenance dosing; T7: six weeks off therapy. The box defines the 25th and 75th percentile; center line the median; whiskers the adjacent values; individual points the outliers.

Figure 5.

IL-10 production in DC-T cell co-culture supernatants from SLIT (n=8) and OIT (n=16) subjects following stimulation with TLR ligands. pDCs or mDCs were co-cultured with autologous CD4⁺ T cells for 96 hours and stimulated with the indicated panels of TLR agonists. (A) pDC-CD4⁺ T cell co-cultures stimulated with media alone, TLR7 or 9 agonists. (B) mDC-CD4⁺ T cell co-cultures stimulated with media alone, TLR2, 3, 4, or 7/8 agonists. Spontaneous cytokine secretion, as measured in media alone, was subtracted to obtain TLR-induced values. Outcomes were evaluated by using linear regression models with generalized estimating equations (GEE) to account for repeated measures over time. * p<0.05; ** p<0.01; T1: baseline; T3: end of dose escalation; T4: completion of at least 12 weeks of maintenance dosing; T5: completion of at least 60 weeks of maintenance dosing; T7: six weeks off therapy. The box defines the 25th and 75th percentile; center line the median; whiskers the adjacent values; individual points the outliers.

Figure 6.

Th2 cytokine responses to allergen during SLIT (n=8) and OIT (n=16). IL-5 and IL-13 levels were measured in culture supernatants from CD4⁺ T cells co-cultured with pDCs or mDCs stimulated with media alone, cow's milk extract (CM) or peanut extract (PN) at the indicated time points (spontaneous cytokine secretion, as measured in media alone, was subtracted to obtain allergen-induced values). Outcomes were evaluated by using linear regression models with generalized estimating equations (GEE) to account for repeated measures over time. * p<0.05; ** p<0.01; T1: baseline; T3: end of dose escalation; T4: completion of at least 12 weeks of maintenance dosing; T5: completion of at least 60 weeks of maintenance dosing; T7: six weeks off therapy. The box defines the 25th and 75th

percentile; center line the median; whiskers the adjacent values; individual points the outliers.