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Dendritic Cell and T cell Responses in Children with Food Allergy

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Abstract

BACKGROUND—Food allergy (FA) and eosinophilic esophagitis (EE) are increasingly common clinical problems. Dendritic cells (DCs) are key regulators of the sensitization and effector phases of allergic immune responses, but their role in these diseases is largely unknown.

OBJECTIVE—To evaluate for alterations in the phenotype and function of DCs in children with IgE-mediated milk allergy or EE compared to their non-affected siblings.

METHODS—Plasmacytoid (pDCs) and monocytoid (mDCs) DCs were prepared from peripheral blood of children with milk allergy (FA), EE, and nonaffected siblings (CON). Purified pDCs and mDCs were cultured alone or with autologous $CD4^+$ lymphocytes. Cytokine levels in plasma, or culture supernatants following stimulation, were measured using multiplex array immunoassay. Cell-surface molecule expression was determined by flow cytometry.

RESULTS—DCs from FA subjects produced greater levels of pro-inflammatory cytokines (IL-6, TNF-α), GM-CSF, and mDC-derived IL-10 compared to controls following allergen exposure. T_H2 but not T_H1 cytokines were spontaneously produced in DC-$CD4^+$ T cell co-cultures from children with FA and were not significantly increased after stimulation with milk extract, suggesting an ongoing activation *in vivo*. This hypothesis was further supported by evidence for elevated IL-5 and IL-13 protein in the plasma of children with both FA and EE. The only significant DC phenotypic differences were: 1) reduced levels of CD80 in EE subjects and 2) FcεRI expression that correlated with serum IgE levels in both groups of subjects.

CONCLUSION—This study suggests that DCs from children with FA and EE produce more pro-inflammatory cytokines, and that their $CD4^+$ T cells are spontaneously activated to produce T_H2 cytokines in the presence of FcεRI-bearing DCs.

Keywords

food allergy; dendritic cell; Th2 cytokines; IgE receptor

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INTRODUCTION

Food allergy (FA) affects about 6% of young children and 3–4% of adults in Westernized countries. Eosinophilic esophagitis (EE), characterized by infiltration of eosinophils within the esophagus, has also become a more common clinical problem and is often driven by abnormal immune responses to food proteins [1–3]. Multiple studies have suggested a role for T_H2 $CD4^+$ T cells in the pathogenesis of these diseases. Some studies attribute allergy to the absence of a T_H1 response and others to an increased T_H2 response after allergen exposure [4–8]. However, the participation of other cell types important in allergic immune responses, including dendritic cells (DCs), has been largely unexplored.

DCs are professional antigen-presenting cells (APCs) capable of activating naïve T cells and responding to innate immune stimuli. There are two major subtypes of immature DCs in the peripheral blood of humans – Blood Dendritic Cell Antigen (BDCA)2^+, $BDCA4^+$, $CD123^{hi}$, $CD11c^-$ plasmacytoid DCs (pDCs) and $BDCA1^+$ $BDCA3^+$ $CD123^{lo}$ $CD11c^+$ myeloid DCs (mDCs). pDCs and mDCs express the αγ2 variant of the high affinity IgE receptor FcεRI, which reportedly increases the efficiency of antigen presentation by up to 1000-fold through a mechanism known as antigen focusing [9,10]. Both subtypes are important in the sensitization and effector phases of allergic asthma and can efficiently induce allergen-dependent T_H2 responses [11]. APCs, including DCs, are also thought to play a central role in the induction and maintenance of oral tolerance [12–14].

Given these data, we hypothesize that DCs also play an integral role in the development of food allergy in general and EE in specific. In this study, we sought to identify phenotypic and functional biomarkers associated with DC activity in FA and EE by comparing these in children with either condition as well as to their non-affected siblings.

METHODS

Study subjects

Children (n=14; 7 male (M) and 7 female (F)) with IgE-mediated cow's milk allergy (FA), 8 (6 M, 2F) with eosinophilic esophagitis (EE), and 11 (3M, 8F) non-affected siblings (CON) of the children with FA and EE were enrolled in this study. All experiments described were performed on all subjects, except in Supplementary Table 1 where limited cell numbers allowed us to do dose titration of allergen on only a subset of subjects (13 subjects with FA, 7 with EE, and 8 CONs). Patient and parental interviews and review of medical records provided details of the following information: (a) all foods being avoided by each subject at the time of enrollment into the study, (b) sensitization to foods (defined by food-specific IgE>0.35 kU_A/L) that were currently being ingested on a regular basis, (c) current medications, and (d) the presence of other allergic diseases (eczema, allergic rhinitis, asthma). This information, along with other significant laboratory parameters, is summarized in Table 1. This study was approved by the Johns Hopkins Institutional Review Board.

Cell preparation and cultures

Peripheral blood was collected in EDTA following venipuncture and subjected to double Percoll (Pharmacia Biotech) density centrifugation. Plasma was decanted and stored at −20° C. Cell isolation procedures have been described previously [15]. Briefly, the upper fraction of cells consisted of basophil-depleted mononuclear cells that were used to isolate pDCs using $BDCA4^+$ magnetic bead selection (Miltenyi). 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 DCs removal.

DC subtypes ($2.5X10^4$ cells) were cultured in a final volume of 250μl of conditioned-Iscove Modified Dulbecco Media (C-IMDM; Invitrogen Life Technologies) that was supplemented with 5% fetal calf serum (FCS; Invitrogen Life Technologies), 1X nonessential amino acids (Invitrogen Life Technologies), and 10μg/mL gentamicin (Invitrogen Life Technologies), pH 7.2–7.4. Cultures were stimulated with 50μg/mL of an aqueous crude milk extract (Greer) or 5μg/mL of anti-human IgE antibody (prepared in-house) [16] for 24 hours in 96-well flat-bottom plates. This dose of milk extract was chosen as it was found to induce optimal T cell proliferation in DC-T cell co-cultures (data not shown). For DC-T cell co-cultures, 1×10^4 pDCs or mDCs were cultured with 1×10^5 autologous $CD4^+$ T cells in a final volume of 250μl and stimulated with 50μg/mL or 10μg/mL of crude milk extract for 96 hours in 96-well round-bottom plates.

Cytokine measurements

Cytokines were measured using multiplex bead immunoassay (Bioplex, BioRad) according to the manufacturer's directions. A human x-plex panel (consisting of TNF-α, IL-6, GM-CSF, and IL-10), the human Th1/Th2 panel, and the human 27-plex panel were used to evaluate supernatants from pure DC cultures, DC-T cell co-cultures, and plasma, respectively. Limits of detection for this assay are IL-1β 0.6 pg/mL, IL-1Ra 5.5 pg/mL, IL-2 1.6 pg/mL, IL-4 0.7 pg/mL, IL-5 0.6 pg/mL, IL-6 2.6 pg/mL, IL-7 1.1 pg/mL, IL-8 1.0 pg/mL, IL-9 2.5 pg/mL, IL-10 0.3 pg/mL, IL-12 (p70) 3.5 pg/mL, IL-13 0.7 pg/mL, IL-15 2.4 pg/mL, IL-17 3.3 pg/mL, eotaxin 2.5 pg/mL, FGF basic 1.9 pg/mL, G-CSF 1.7 pg/mL, GM-CSF 2.2 pg/mL, IFN-γ 6.4 pg/mL, IP-10 6.1 pg/mL, MCP-1 1.1 pg/mL, MIP-1α 1.6 pg/mL, MIP-1β 2.4 pg/mL, PDGF-BB 2.9 pg/mL, RANTES 1.8 pg/mL, TNF-α 6.0 pg/mL, and VEGF 3.1 pg/mL.

Flow cytometry

Mononuclear cell suspensions were fixed in buffered 4% paraformaldehyde and frozen below −70°C. Prior to and during staining, cells were blocked with FcR blocking reagent according to the manufacturer's directions (Miltenyi). The following antibodies were used: BDCA2-FITC or -Biotin, BDCA1-FITC or -APC, (Miltenyi); CD80-Biotin, CD86-APC, CD19-Pacific Blue; (BD Pharmingen), and/or FcεRIα-PE (eBioscience). Streptavidin-PerCP (BD Pharmingen) was added following staining with the above biotin-conjugated antibodies. Stained cells were analyzed using an LSRII machine (BD Pharmingen). Percent positives were defined by first gating on pDCs or mDCs and then determining the percent of positively stained cells relative to nonstained cells or isotype controls (similar results were obtained when either control was used).

Serologic measurements

Measurements of total serum IgE, milk-specific IgE, and a multi-allergen screen were performed on plasma by the Johns Hopkins Dermatology Allergy and Clinical Immunology (DACI) Reference Laboratory (Baltimore, MD) using a fluorescent-based enzyme immunoassay (FEIA) performed on the ImmunoCAP 250 (Phadia, Kalamazoo, MI, USA). The Phadiatop is a single measurement that detects IgE antibody specific for any of 15 common aeroallergens.

Statistics

Not all data sets were normally distributed, including after logarithmic transformation; therefore, all comparisons were unpaired Wilcoxon rank-sum tests except for Table II where

observations were paired by individual (KaleidaGraph, Synergy Software, Reading, PA). P values were exact except in the case of ties in which asymptotic statistics were used. Linear and Spearman or Pearson (based on whether the data was normally distributed) correlations were performed using Prism Software (GraphPad Software, San Diego, CA). The box defines the 25% and 75% quartiles, division within the box the median, and whiskers the range. Statistically significant p-values, defined as <0.05, are indicated.

RESULTS

Clinical characteristics of subjects

Thirty-three subjects were enrolled (Table 1). Median total serum IgE levels were 3090 kU/L, 313 kU/L, and 112 kU/L for the FA, EE, and CON subjects, respectively. The corresponding milk-specific IgE levels were 92.5 kU_A/L, 3.34 kU_A/L, and 0.12 kU_A/L. The diagnosis of food allergy was based on a convincing history of reaction following exposure to cow's milk and a milk-specific IgE>0.35 kU_A/L (ImmunoCAP, Phadia, USA). All EE subjects had active disease at the time of enrollment as defined by the presence of chronic gastrointestinal symptoms (including vomiting, dysphagia, abdominal pain, and / or failure to thrive) and >15 eosinophils per high-powered field on recent esophageal biopsies. While many of the subjects with EE experienced acute allergic reactions after ingestion of milk, three of the subjects had gastrointestinal symptoms only (Table 1). All EE subjects had detectable milk-specific IgE and histologic improvement with milk avoidance. All subjects with FA, and most subjects with EE, were actively avoiding multiple foods in addition to cow's milk, and were also regularly ingesting foods to which they were sensitized but not clinically reactive (Table 1). Although several CON subjects had low positive levels of milk-specific IgE, none had a history suggesting allergy to any food nor had they avoided any foods.

DC cytokine responses to allergen stimulation

Previous studies have suggested that DCs are an important source of pro-inflammatory cytokines after treatment with artificial stimuli that crosslink the IgE receptor [16,17]. As shown in Fig. 1, mDCs from children with FA and EE produced significantly greater quantities of TNF-α after stimulation with milk. Both pDCs and mDCs from children with FA also produced more IL-6 in response to milk, with much higher levels produced by pDCs compared to mDCs. No significant difference in IL-6 production was observed between EE subjects and CONs. Higher levels of GM-CSF were produced by mDCs from FA subjects, with a trend in this direction by pDCs after stimulation with allergen [18]. Interestingly, mDCs from children with FA, but not EE, also produced greater quantities of IL-10 in response to milk.

All three groups of subjects produced IL-6, TNF-α, GM-CSF, and IL-10 when stimulated with a goat anti-human IgE antibody [16] that results in cross-linking of the IgE receptor, suggesting that DCs from all subjects were capable of responding to this bivalent stimulus (Fig. 1). Levels of all four cytokines were significantly correlated with expression of FcεRI on pDCs but not mDCs in response to anti-IgE (Supplementary Fig. 1). Levels of IL-10, but not IL-6, TNF-α, or GM-CSF, were significantly higher in FA (pDCs and mDCs) and/or EE (mDCs) subjects compared to CONs following anti-IgE treatment. No significant differences in spontaneous production of IL-6, TNF-α, or GM-CSF by either DC subtype were observed in FA or EE subjects compared to CONs (Fig. 1). mDCs from children with FA produced slight but statistically higher spontaneous levels of IL-10 compared to controls, although the levels were still relatively low compared to those induced with anti-IgE stimulation. These data suggest that DCs from children with FA produce more pro-inflammatory cytokines (IL-6, TNF-α), GM-CSF, and IL-10 (from mDCs) than CONs after

stimulation with allergen. These differences were less obvious for children with EE. For the most part, levels of all cytokines tested were significantly higher than media controls following stimulation with milk or anti-IgE in all three groups of subjects (Fig. 1).

T_H2 cytokine responses in DC-$CD4^+$ T cell co-cultures

To investigate the role of DC subtypes in polarizing $CD4^+$ T-cells toward a T_H2 phenotype, purified pDCs or mDCs were cultured together with autologous $CD4^+$ T cells, and cytokine production was evaluated 96 hours later (Fig. 2 and Supplementary Fig. 2). We observed that T_H2 cytokines (IL-4, IL-5, IL-13, IL-10), IL-2, GM-CSF, and pro-inflammatory TNF-α were all spontaneously produced by both pDC- and mDC-T cell co-cultures from children with FA. To investigate this issue further, an additional 9 subjects with milk allergy were investigated and demonstrated little or no IL-13 production when $CD4^+$ T cells were cultured alone without DCs (data not shown). Spontaneous production of these cytokines by subjects with EE was not statistically different from CONs, except for higher spontaneous release of IL-5 and IL-13. No significant difference in spontaneous production of IFN-γ, a classic T_H1 cytokine, was seen across the three groups of subjects.

Following stimulation with crude milk extract, levels of IL-5, IL-13, IL-4, IL-10, IL-2 (in pDC-T cell co-cultures only), and TNF-α remained significantly higher in the FA group compared to CONs after antigen stimulation (Fig. 2 and Supplementary Fig. 2). In contrast, the EE subjects did not differ significantly from CONs for any cytokines tested after stimulating the cultures with milk extract, even though all EE subjects were clinically reactive to milk. After the addition of milk extract, levels of nearly all cytokines tested increased relative to levels in unstimulated cultures for all three groups of subjects, except for two notable exceptions (Table 2). IL-5 and IL-13 levels in the FA group failed to significantly rise following stimulation with milk extract relative to unstimulated cultures. Similar results were obtained when 10μg/mL of extract was used (Supplementary Table 1). These findings suggest that DCs from FA children can provide *in vitro* accessory cell activity to support the production of IL-5 and IL-13 by CD4 lymphocytes in the absence of exogenous allergen.

Plasma levels of T_H2 cytokines

We next assayed for cytokines in plasma from our three groups of subjects with the intent of supporting the hypothesis that lymphocytes from FA and/or EE subjects exhibit ongoing T_H2 cytokine production *in vivo*. As shown in Fig. 3, levels of IL-5 and IL-13 were significantly elevated in plasma from both FA and EE children compared to CONs. FA subjects also had higher plasma levels of IL-2. However, levels of many other cytokines and chemokines, including IL-1β, IL-1ra, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL12-p70, IL-15, IL-17, eotaxin, basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF, did not differ among the three groups of subjects. Collectively, these data support the conclusion that EE and FA subjects have ongoing production of Th2 cytokines (IL-5 and IL-13) *in vivo* and, as indicated by the *in vitro* data, possess circulating DCs that play a role in this activity.

Comparison of co-stimulatory molecule expression on DCs from children with FA, EE, and CONs

Flow cytometry was performed on mononuclear cell suspensions to investigate whether the ongoing production of T_H2 cytokines by children with FA and EE was associated with altered expression of co-stimulatory molecules on their DCs. (Fig. 4A). First, the frequencies of pDCs and mDCs did not differ significantly across the three groups of subjects. Fig. 4A shows that expression of CD40 and CD86 also did not differ among subjects on either DC subtype. However, children with EE had significantly less staining for

CD80 on their mDCs and there was a similar trend on their pDCs. These data indicate that the greater spontaneous production of cytokines by allergic children was unlikely to be related to altered co-stimulatory molecule expression on their DCs.

Relationship of DC FcεRI expression to serum IgE

Expression of FcεRIα on both subtypes of DCs in the three groups of subjects was highly correlated with serum levels of IgE (Fig. 4B) (pDCs, r^2=0.73, p<0.0001; mDCs, r^2=0.78, p<0.0001). Relative expression of FcεRIα on mDCs was approximately twice as great as that observed on pDCs. Expression of FcεRI on both pDCs and mDCs from children with food allergy (and mDCs only in the EE group) was higher than controls (Supplementary Fig. 3). Using Spearman correlation, spontaneous release of IL-5 and IL-13 from both pDCs and mDCs used as APCs was highly correlated to expression of FcεRI on the respective DC subtype (Fig 4C).

DISCUSSION

FA and EE both appear to be increasing in prevalence in developed countries. The pathogenesis of both FA and most cases of EE is thought to involve a failure of oral tolerance. DCs are known to be essential in both the induction and maintenance of oral tolerance in the intestine, where they play a primary role in presenting dietary antigens to T lymphocytes and thereby directing subsequent immune responses [12–14]. While many EE patients produce food-specific IgE, anaphylaxis to foods is less common. Like FA, EE is associated with a T_H2 dominated response, and recent work has demonstrated a critical role for IL-13 in inducing an EE-specific transcriptome [19]. To our knowledge, the study herein is the first to examine how differences in DC phenotype and/or function may contribute to the pathogenesis of these diseases.

We have shown that DC-$CD4^+$ T cell co-cultures from children with FA spontaneously produced relatively large quantities of T_H2 cytokines in the absence of allergen exposure. EE patients similarly demonstrated ongoing production of IL-5 and IL-13. Of the phenotypic markers investigated, only FcεRI expression on the surface of both pDCs and mDCs correlated with the amount of spontaneously produced IL-5 and IL-13. This suggests an important role for the IgE receptor on the surface of DCs in inducing ongoing cytokine release from $CD4^+$ T cells. Importantly, $CD4^+$ T cells from children with FA, when cultured alone, spontaneously produced no or much lower amounts of T_H2 cytokines, suggesting that DCs were required for this phenomenon. No further increase in IL-5 or IL-13 occurred in DC-T cell co-cultures from FA or EE children after stimulation with milk allergen. We conclude from this observation that T cells from these subjects are already activated *in vivo* in a response dependent on circulating DCs, and that DC/T cell co-cultures are unresponsive to additional allergen stimulation *in vitro*. Evidence that best supports this hypothesis was additionally seen with elevated IL-5 and IL-13 levels in the plasma of subjects with FA and EE –the same two cytokines produced *in vitro* in the DC/T cell co-cultures. Certainly, the role of DCs and their expression of FcεRI/IgE in helping to drive T cell cytokine responses have long been proposed. Moreover, this concept is further supported by our recent findings that reductions in IL-5 and IL-13 produced in response to allergen track with IgE neutralization and FcεRI reduction on DCs following *in vivo* treatment with omalizumab [20].

Unlike the FA group, levels of IL-5 and IL-13 produced in co-cultures stimulated with milk allergen from EE subjects did not differ significantly from controls, despite comparable levels of IL-5 and IL-13 in the plasma of FA and EE subjects. Several explanations seem possible. The median milk-specific IgE levels of subjects with EE in our study were almost 30-fold lower than that of the food allergic group. Alternatively, the majority of

lymphocytes responsible for T_H2 cytokine production in EE may be localized to the esophageal tissues and are no longer present in peripheral blood. Indeed, lymphocytes have been found to accumulate in the esophagi of patients diagnosed with EE [8,21]. No difference in IFN-γ, a T_H1 cytokine, was observed between FA or EE subjects and controls in either stimulated or unstimulated cultures. These data support earlier studies that have suggested the magnitude of the T_H2 response, rather than the absence of a T_H1 response, underlies clinical disease.

The spontaneous induction of T_H2 cytokines in DC-$CD4^+$ T cell co-cultures from FA and EE subjects was not associated with increased co-stimulatory molecule expression on DCs from these subjects prior to culture. In fact, EE subjects had significantly less staining for CD80. Alterations in CD80 have previously been implicated in allergic disease. After stimulation with Derp1, Charbonnier et al. [22] showed that CD86 and HLA-DR were upregulated equally on DCs in control and dust mite-allergic subjects, but only mDCs from normal subjects demonstrated increased expression of CD80. CD80 has also been shown to be required for induction of low-dose oral tolerance to peanut in mice [23]. The diminished expression of CD80 on DCs from EE patients may, therefore, contribute to their lack or oral tolerance.

Assuming that IgE is playing a role in the DC-dependent T cell cytokine responses observed among our FA and EE subjects, then it seems relevant to comment here on what has been known for decades regarding basophil function in these subjects. In particular, May *et al.* [24] reported in 1976 that up to 80% of children with food allergy have basophils that spontaneously release histamine. Subsequent studies by Sampson *et al.* [25] suggested a role for histamine releasing factor (HRF) (along with IgE) in this phenomenon. Histamine release reportedly returned to normal after children were placed on appropriate elimination diets [25]. Basophils from a majority of the FA children in our study also demonstrated spontaneous histamine release (unpublished data). All of them were regularly ingesting multiple foods to which they were sensitized but clinically tolerant. We propose that these food antigens may form IgE complexes in the systemic circulation that subsequently bind and activate FcεRI on basophils and DCs leading to their activation. Indeed, circulating basophils from allergic children have previously been shown to be activated, and our data suggests DCs from food allergic children could be as well, which subsequently facilitates T cell activation [26]. Although not tested here, this is certainly one mechanistic hypothesis that requires investigation in future studies.

We found that DCs from children with FA produced much higher quantities of IL-6 (pDCs and mDCs) and TNF-α (mDCs) after stimulation with milk allergen compared to their nonaffected siblings. Assuming an IgE-dependent activation, the lack of a significant difference in TNF-α production by pDCs from FA subjects could reflect differences in the kinetics of IL-6 and TNF-α secretion by pDCs. Schroeder *et al.* [16] have shown that pDC-derived TNF-α peaks much earlier (8 hours) and then declines, while IL-6 continues to increase at 24 hours (the time point chosen in this study) following IgE crosslinking. The higher expression of pro-inflammatory cytokines by DCs following food allergen exposure is likely to be an important mechanism by which DCs promote allergic inflammation in children with food allergy. The milk extract used in this study does contain endotoxin. While this may also contribute to the release of pro-inflammatory cytokines by mDCs after stimulation with allergen, based on our own experience and other published observations, human pDCs do not express TLR4, and therefore would not be expected to respond to endotoxin [27]. Treatment with artificial stimuli known to crosslink the IgE receptor on DCs does lead to release of IL-6 and TNF-α, and the greater expression of FcεRI on DCs from allergic subjects may contribute to the greater amount of pro-inflammatory cytokines released after treatment with allergen. Indeed, we did observe a positive correlation between

expression of FcεRI and production of IL-6, TNFα, IL-10 and GM-CSF by pDCs in response to anti-IgE treatment. However, the three groups of subjects did not differ in the amount of IL-6 or TNF-α produced from DCs after treatment with anti-IgE. This suggests a critical role for other receptors on the DC in mediating the increased pro-inflammatory cytokines produced in response to milk in the children with FA and EE. Of note, although none of the control subjects in our study had food allergy, some might still be classified as allergic. A number of them had sensitivity to environmental allergens, and a few had symptoms of allergic rhinitis. The age and sex distribution of the groups studied also varied in some instances, and it is possible these disparities also contributed to the differences in DC cytokine production that was observed. Finally, although none of our control subjects reported a history of ever reacting to any foods, we cannot completely exclude the possibility that they may have outgrown a food allergy in the past.

Both DC subtypes from FA subjects also produced larger quantities of GM-CSF following stimulation with milk. GM-CSF is an important growth factor for eosinophils that can also activate APCs [28,29]. This may represent another mechanism by which DCs promote T_H2 responses in this disease. With the exception of greater TNF-α production by mDCs, no differences in secretion of pro-inflammatory cytokines, GM-CSF, or IL-10 by either pDCs or mDCs from subjects with EE were observed compared to nonaffected controls following milk stimulation. This is in spite of documented clinical reactivity to milk by all of the subjects with EE in this study. These findings may also suggest that the relevant pathogenic cells in subjects with EE are localized to the gastrointestinal tract, and therefore the function of peripheral DCs may be relatively spared.

Interestingly, mDCs from FA children produced greater quantities of IL-10. IL-10 may promote T_H2 responses by decreasing secretion of IL-12 and thereby indirectly inhibit the differentiation of T_H1 cells [30]. Alternatively, IL-10 has also been shown to have immunosuppressive effects on both T_H1 and T_H2 cells, and may indirectly interfere with IgE synthesis and eosinophil survival [31]. Recent work has suggested that autocrine secretion of IL-10 following FcεRI crosslinking on mDCs may be a mechanism to inhibit TNF-α secretion by these cells, and thereby diminish their pro-inflammatory activities following allergen exposure [17,32].

In summary, we found that DCs from children with FA produced greater quantities of pro-inflammatory cytokines and GM-CSF after allergen stimulation that may promote allergic inflammation and T_H2 responses in these diseases. Evidence for ongoing DC-dependent T cell production of T_H2, but not T_H1, cytokines was also evident in co-cultures from children with FA. Surprisingly, the IL-5 and IL-13 levels produced in these cultures were not increased after *in vitro* stimulation with milk. This observation was also associated with elevated IL-5/IL-13 protein in the plasma of FA (and EE) subjects compared to controls.

Of the phenotypic markers investigated, only FcεRI on the surface of both pDCs and mDCs correlated with the amount of “spontaneously” produced IL-5 and IL-13. Coupled with previous reports, these findings collectively suggest an important role for IgE (and its receptor, FcεRI) in promoting DC-dependent secretion of these cytokines by $CD4^+$ T cells in children with multiple food allergies and/or EE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FA	food allergy
EE	eosinophilic esophagitis
DC	dendritic cell
APC	antigen-presenting cell
BDCA	Blood Dendritic Cell Antigen
pDC	plasmacytoid dendritic cell
mDC	monocytoid dendritic cell
CON	control
HSHR	high spontaneous histamine release
HRF	histamine releasing factor
MFI	mean fluorescence intensity

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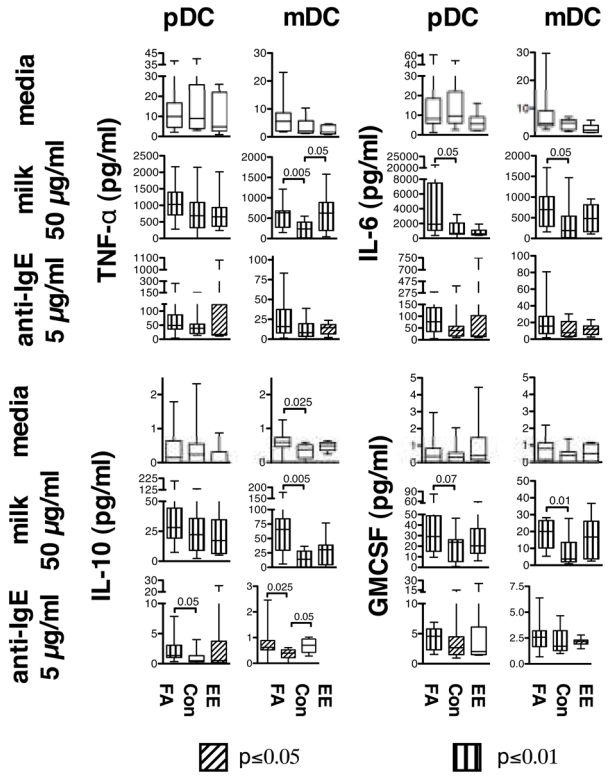

Fig. 1.

Cytokine secretion by purified DCs. pDCs or mDCs isolated from peripheral blood of FA, CON, and EE children were cultured in the presence of media alone, 50μg/mL of crude milk extract, or 5μg/mL of goat polyclonal anti-human IgE. TNF-α, IL-6, IL-10, and GM-CSF were measured 24 hours after stimulation. Levels of each cytokine under each condition tested were compared across groups and statistically significant differences are indicated. Levels of each cytokine following treatment with milk or anti-IgE to levels produced by cells cultured in media alone were also compared within each group of subjects, and significant differences are indicated by markings within the boxes according to the legend.

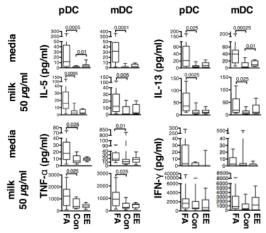

Fig. 2.

Cytokine secretion by DC-CD4$^+$ T cell co-cultures. pDCs or mDCs from FA, CON, and EE subjects were cultured together with autologous CD4$^+$ T cells in media alone or 50 μg/mL of crude milk extract. Indicated cytokines (pg/mL) were measured 96 hours after stimulation.

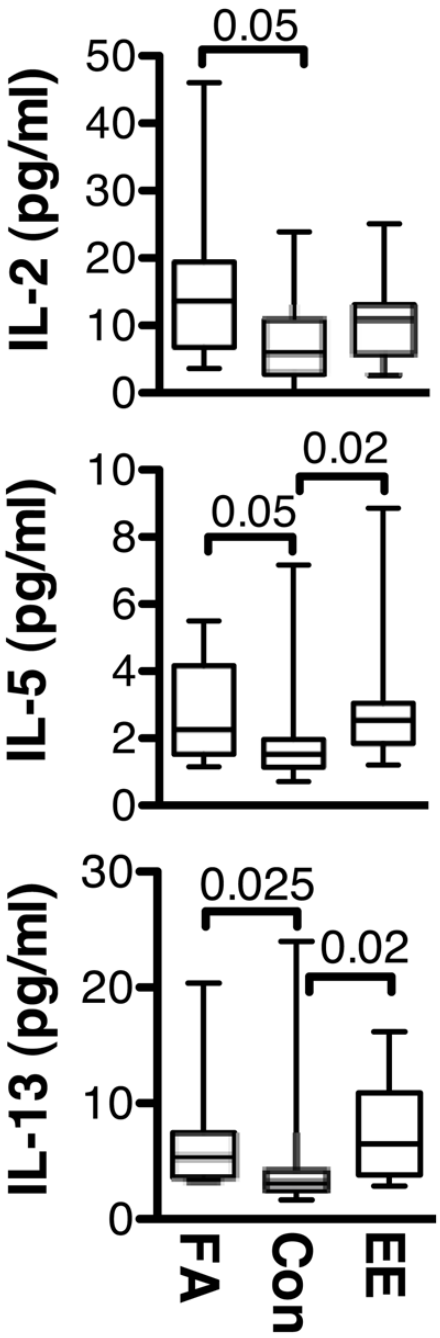

Fig. 3.
Plasma cytokine levels in children with FA, EE, and CONs. Plasma levels of IL-2, IL-5, and IL-13 (pg/mL) are indicated.

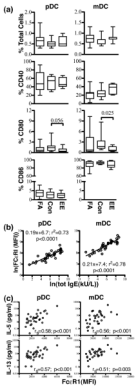

Fig. 4.

Flow cytometric analysis of DCs from children with FA, EE, and CONs. (a) The relative frequency of pDCs and mDCs as a percentage of total mononuclear cells (% total cells), as well as the percent of pDCs and mDCs expressing CD40, CD80, and CD86 in FA, CON, and EE subjects, are represented. (b) Expression (mean fluorescence intensity; MFI) of FcεRIα on pDCs and mDCs. All subjects are represented (FA, EE, CON). Levels of FcεRIα expression on both pDCs and mDCs are highly correlated to total serum IgE levels (KU/L). (c) Spearman correlation was used to compare the level of IL-5 and IL-13 spontaneously produced in pDC- or mDC-CD4^{+} T cell co-cultures to the level of expression of FcεRI (expressed in MFI).

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Table 1

Patient Characteristics

	Age	Milk IgE	Total IgE	Phad	Avoiding	Sens	Acute Rxn	Meds	Other
FA1	9.3	1108.0	4318	9.12	M, E, P, Se, T, O(3)	Y	Y	IC	A, AR
FA2	6.5	394.0	3634	5.24	M, E, P, Se, T, O(3)	Y	Y	IC	A
FA3	6.9	5.4	335	2.63	M, E, P,T	Y	Y	LRA	E, A, AR
FA4	8.4	94.5	4006	51.70	M, E, P, Se, T, O(5)	Y	Y	LRA	E, A, AR
FA5	9.8	32.0	1171	77.00	M, E, P, T, O(1)	Y	Y	IC	E, A, AR
FA6	10.8	904.0	4603	68.70	M, E, P, Se, T, W, O(9)	Y	Y	NC	E, AR
FA7	8.3	77.4	998	8.84	M, E, Se, W, O(9)	Y	Y	NC	E, A, AR
FA8	6.1	810.0	5960	82.30	M, E, P,S, Se, T, W, O(*)	Y	Y	IC	E, A, AR
FA9	5.9	51.5	4469	614.00	M, E, P, Se, T, O(1)	Y	Y	LRA	E, A
FA10	6.3	90.4	1423	41.00	M, E, P, Se, T, W, O(1)	Y	Y		E, A, AR
FA11	4.2	20.8	238	25.80	M, T	Y	Y		E
FA12	4.8	88.7	2545	49.20	M, P,S, Se, T, W, O(2)	Y	Y		E, A, AR
FA13	8.6	652.0	3998	91.10	M, E, P, Se, T, O(*)	Y	Y	IC, NC	E, A, AR
FA14	5.8	133.2	1361	50.80	M, P, Se, T, O(1)	Y	Y	LRA	E, A
Med	**6.7**	**92.5**	**3090**	**50.00**					
IQR	**2.6**	**529.5**	**3022**	**61.64**					
EE1	21.6	2.7	350	23.10	M, E, P	Y	Y	SC	AR
EE2	2.2	5.3	548	23.30	M, P,S, Se, T, W, O(§)	Y	Y	IC, NC, AH, LRA	E, A, AR
EE3	13.1	0.18	276	35.40	M	Y	Y	SC	AR
EE4	5.9	3.99	1007	22.60	M, P, T	Y	N‡		AR
EE5	18.0	0.92	67	2.21	N†	Y	N‡	SC	AR
EE6	10.5	6.78	553	49.60	M, E, P,S, Se, T, O(2)	Y	Y	AH	E, A, AR
EE7	7.5	28.40	157	0.45	M, E, P, T, W, O(3)	Y	Y		E, A, AR
EE8	15.1	0.54	204	28.50	M, E	Y	N‡	SC	AR
Med	**11.8**	**3.34**	**313**	**23.20**					
IQR	**8.7**	**4.85**	**357**	**12.72**					
Con1	11.3	0.15	187	1.37	N	NT	N		
Con2	10.3	0.12	129	17.60	N	NT	N		AR

	Age	Milk IgE	Total IgE	Phad	Avoiding	Sens	Acute Rxn	Meds	Other
Con3	10.2	0.38	144	2.94	N	NT	N		
Con4	6.0	0.25	112	0.10	N	NT	N		
Con5	7.5	1.52	18.80	<0.35	N	NT	N		
Con6	12.5	0.23	1056	19.10	N	NT	N		
Con7	13.4	<0.10	16	0.15	N	NT	N		
Con8	2.2	<0.10	161	0.15	N	NT	N		
Con9	17.2	<0.10	9	0.48	N	NT	N		AR
Con10	14.0	<0.10	27	0.16	N	NT	N	AH	AR
Con11	16.9	<0.10	4	0.27	N	NT	N		AR
Med	**11.3**	**0.12**	**112**	**0.27**					
IQR	**4.8**	**0.24**	**135**	**2.01**					

†Subject was not following a food avoidance diet at time of enrollment into the study; now exclusively on elemental formula

‡These subjects experienced abdominal pain and/or vomiting after ingestion of concentrated or large quantities of milk

§Diet limited to elemental formula exclusively

*Diet limited to elemental formula and few foods (amarenth, tapicoa, rice, fruit, vegetables, chicken, turkey, pork)

Age: Age in years (y) at time of enrollment

Milk IgE: Milk-specific IgE (kUA/L) **Total IgE**: Total serum IgE (kU/L) **Phad**: Phadiatop for most common aeroallergens (kU/L)

Avoiding: Foods being avoided in the diet at the time of enrollment into the study M, milk; E, egg; P, peanut; S, soy; Se, sesame; T, tree nuts; W, wheat; O, other (number of other foods); N, none

Sens: Subject was (Y) or was not (N) regularly ingesting food(s) in their diet to which they were sensitized but clinically tolerant; NT, not tested

Acute Rxn: Subject did (Y, yes) or did not (N, no) experience acute hypersensitivity reaction after ingestion of milk

Meds: Current medications actively used at time of enrollment into the study: SC, swallowed corticosteroid; IC, inhaled corticosteroid; NC, intranasal corticosteroid; LRA, leukotriene receptor antagonist; AH, antihistamine

Other: Other atopic diseases diagnosed or reported: E, eczema; A, asthma; AR, allergic rhinitis

Table 2

Changes in cytokine secretion with milk stimulation. P values comparing difference in cytokine levels produced by pDCs or mDCs co-cultured with $CD4^+$ T cells in the presence or absence of milk extract (50 μg/ml milk extract) using Wilcoxon Matched-Pairs Signed-Ranks Test.

	pDC			mDC		
	FA	**Con**	**EE**	**FA**	**Con**	**EE**
IL-5	**0.6257**	0.0320	**0.9453**	0.0166*	0.0420	**0.5469**
IL-13	0.0419	0.0010	**0.3828**	**1.0000**	0.0322	**0.6400**
IL-4	0.0001	0.0059	0.0142	0.0002	**0.1025**	0.0156
IL-10	0.0001	0.0010	0.0078	0.0001	0.0010	0.0078
GM-CSF	0.0001	0.0010	0.0078	0.0001	0.0010	0.0078
IL-2	0.0001	0.0078	0.0010	0.0134	0.0010	0.0078
IL-12	0.0001	0.0010	0.0078	0.0001	0.0010	0.0078
TNF-α	0.0001	0.0010	0.0078	0.0001	0.0010	0.0078
IFN-γ	0.0001	0.0010	0.0078	0.0017	0.0010	0.0078

*levels statistically higher in media alone than after stimulation with milk extract