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Targeting Allergen to FcγRI Reveals a Novel Th2 Regulatory Pathway Linked to TSLP Receptor

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Abstract

Background—The molecule H22-Fel d 1, which targets cat allergen to FcγRI on dendritic cells, has the potential to treat cat allergy owing to its T-cell modulatory properties.

Objective—To investigate whether the T-cell response induced by H22-Fel d 1 is altered in the presence of the Th2-promoting cytokine, TSLP.

Methods—Studies were performed in cat-allergic subjects with and without atopic dermatitis. Monocyte-derived dendritic cells were primed with H22-Fel d 1 in the presence or absence of TSLP and the resulting T-cell cytokine repertoire was analyzed by flow cytometry. The capacity for H22-Fel d 1 to modulate TSLP receptor expression on dendritic cells was examined by flow cytometry in the presence or absence of inhibitors of Fc receptor signaling molecules.

Results—Surprisingly, TSLP alone was a weak inducer of Th2 responses irrespective of atopic status; however, dendritic cells co-primed with TSLP and H22-Fel d 1 selectively and synergistically amplified Th2 responses in highly atopic subjects. This effect was OX40 ligand-independent pointing to an unconventional TSLP-mediated pathway. Expression of TSLP receptor was upregulated on atopic dendritic cells primed with H22-Fel d 1 through a pathway regulated by FcγRI-associated signaling components, including src related tyrosine kinases and Syk, as well as the downstream molecule, PI3-kinase. Inhibition of TSLP receptor upregulation triggered by H22-Fel d 1 blocked TSLP-mediated Th2 responses.

Conclusion—Discovery of a novel Th2 regulatory pathway linking FcγRI signaling to TSLP receptor upregulation and consequent TSLP-mediated effects questions the validity of receptor-targeted allergen vaccines.

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Clinical Implications—This study establishes a pivotal role for Fc receptor ligation in promoting TSLP-mediated Th2 responses associated with allergic disease.

Capsule Summary—Atopic dendritic cells are equipped to efficiently upregulate TSLP receptor upon Fc receptor ligation by allergen. These findings suggest that dendritic cell-based vaccines that target Fc receptors could amplify Th2-driven inflammatory responses by potentiating the effects of TSLP.

Keywords

H22-Fel d 1; TSLP; TSLP receptor; atopic dermatitis; blood dendritic cells; monocyte-derived dendritic cells; Th2 cells; FcγRI

Introduction

Allergic diseases such as asthma and atopic dermatitis (AD) are a manifestation of inflammatory processes driven by Th2 lymphocytes. Recently, thymic stromal lymphopoietin (TSLP) has been proposed to act as a major “switch” factor in the allergic response based on its capacity to differentiate pro-inflammatory Th2 cells from naïve CD4⁺ T cell precursors in humans. Such cells secrete high levels of IL-4, IL-5, and IL-13, in conjunction with TNF-α, but only low levels of IFN-γ and IL-10^{1, 2}. This process was reported to be mediated by dendritic cells (DCs) through an OX40 ligand pathway activated by TSLP^{1, 3}. In an extension of these studies, TSLP was shown to maintain and polarize circulating Th2 central memory cells, including allergen-specific T cells, suggesting an important role for this cytokine in driving Th2 responses associated with established allergic disease⁴. Direct evidence of a role for TSLP in the pathogenesis of allergic disease is provided in mice lacking the TSLP gene or its receptor. Such animals fail to develop asthma, or else show attenuated disease^{5, 6}. Conversely, mice expressing an inducible TSLP transgene in the skin develop eczematous lesions⁷. In humans, high expression of TSLP is a feature of keratinocytes in the skin lesions of patients with AD and TSLP is also expressed by bronchial epithelial cells derived from the asthmatic lung^{1, 2, 4, 8, 9}. The interactions of human DCs with TSLP-expressing epithelial cells within the respiratory tract or skin are likely pivotal to the generation and maintenance of Th2 responses that orchestrate allergic disease.

Allergen variants that target DCs could be a useful approach to treat allergic disease based on their ability to modulate APC function, and in turn, alter allergen-specific T-cell responses. Consistent with this view, we recently reported that targeting the major cat allergen, Fel d 1, to the high affinity IgG receptor, FcγRI, on DCs using the allergen variant, H22-Fel d 1, selectively enhanced the frequency of IL-5- and IL-10-expressing T cells *in vitro* in cultures from cat-allergic subjects¹⁰. Interestingly, the Th2 component of the response induced by H22-Fel d 1 was regulated by IL-10, indicating the capacity for this molecule to induce elements of a protective T-cell response.

In order to assess whether DC-based therapies could be efficacious in the clinical setting, it is important to consider the effects of mediators that operate *in vivo* to modulate APCs in allergic individuals. High expression of TSLP at inflamed sites coupled with the presence of TSLP-primed APCs in regional lymph nodes could subvert the induction of a protective T-cell response to H22-Fel d 1 when this molecule is administered via subcutaneous or sublingual routes, or else by instillation into the respiratory tract. Here, we report that Th2 responses triggered by H22-Fel d 1 are amplified in the presence of TSLP in atopic subjects. By dissecting the mechanism involved, we reveal a novel Th2 regulatory pathway linking FcγR signaling to TSLP-driven events. The interaction between allergen and TSLP we describe provides new insight into the regulation of Th2 responses in humans.

Materials and Methods

Human Subjects

Subjects were recruited from the University of Virginia Dermatology Clinic and the University of Virginia Allergic Diseases Clinic, or else by advertisement. Cat-allergic patients with AD reported physician-diagnosed eczema and the presence of itchy rash was confirmed upon physical exam. All AD patients had moderate-to-severe disease based on SCORAD index 11^{12} , high total IgE levels (>250 IU/ml) and high titer IgE ab to cat extract ($CAP \geq 0.7$ IU/ml). Cat-allergic subjects without AD were selected based on high titer IgE ab to cat extract ($CAP > 0.7$ IU/ml). The presence of Fel d 1-specific IgE ab was confirmed in cat-allergic patients with and without AD by Streptavidin CAP assay 13 . All cat-allergic subjects also had high titer IgE ab to dust mite allergen and were generally sensitized to multiple allergens. Control subjects had no measurable serum IgG or IgE antibodies to common allergens, including Fel d 1, and no history of allergic disease. All studies were approved by the University of Virginia Human Investigations Committee.

Cells and Reagents

Recombinant Allergens—Purified recombinant Fel d 1 and Fel d 1 targeted to the high affinity IgG receptor, Fc γ RI, (H22-Fel d 1) were obtained from Indoor Biotechnologies, Inc.. The endotoxin content of Fel d 1 and H22-Fel d 1 was comparable (14.5 and 15.3ng/ml respectively).

Cells—Monocyte-derived DCs (moDCs) were generated from CD14 $^{+}$ monocytes as described previously 10 . These cells were homogeneous based on expression of CD64/Fc γ RI ($>90\%$) and CD11c ($>99\%$). Blood DCs were isolated from PBMCs by magnetic-activated cell sorting (Blood Dendritic Cell Isolation Kit II, Miltenyi Biotec). CD4 $^{+}$ T cells were isolated from fresh PBMCs by negative selection using magnetic-activated cell sorting (Miltenyi Biotec) to $>95\%$ purity. The THP-1 cell line was generously provided by K. Ravichandran (University of Virginia, Charlottesville, VA).

Flow Cytometry Antibodies and Reagents—See online repository materials.

Inhibitors—See online repository materials.

Other Reagents—See online repository materials.

T Cell Cultures

MoDCs were primed with allergen alone (Fel d 1 or H22-Fel d 1, 10 μ g/ml), TSLP alone (15ng/ml), or allergen+TSLP for 48 hours in complete medium containing 10% autologous serum 10 . After washing, moDCs (2×10^5) were co-cultured in 24-well plates with autologous CD4 $^{+}$ T cells (8×10^5). Cultures were supplemented with recombinant human IL-2 (12U/ml) (BioSource International) on day 5, and then restimulated with PMA (50ng/ml) and ionomycin (2 μ g/ml)(Sigma-Aldrich) for 5 hours on day 10. Brefeldin A (BD Biosciences) was added during the final 4 hours of culture. Cells were stained for intracellular and surface markers and analyzed by flow cytometry. Because restimulation with PMA and ionomycin downregulates surface expression of CD4, CD4 $^{+}$ T cells were identified by gating on CD8 neg CD3 $^{+}$ cells. In some experiments, cells were cultured with anti-OX40L monoclonal antibody (20 μ g/ml). For inhibition studies, moDCs were incubated with LY294002 for 1 hour (37°C) prior to priming with various stimuli for 48 hours and co-culturing with T cells (7 days).

APC Phenotyping

MoDCs or blood DCs were primed for 24 hours with allergen alone (10µg/ml), TSLP alone (15ng/ml), or both. In some experiments, moDCs were primed with LPS (0.5ng/ml or 1µg/ml) and THP-1 cells were primed with H22-Fel d 1 alone. Cells were then washed, stained for surface markers, and analyzed by flow cytometry. In time-course experiments for OX40L and TSLPr expression, allergen and TSLP were removed at 48 hours by washing. In some experiments, APCs were incubated for 1 hour (37°C) with kinase inhibitors prior to stimulation with allergen (24 hours) and analysis of TSLPr expression.

Flow Cytometry Analysis

See online repository materials.

Cytokine Assays

See online repository materials.

Calcium Release Assays

See online repository materials.

Statistical Analysis

See online repository materials.

Results

The Th2-Promoting Effect of TSLP Alone is Weak But Increased in Atopic Subjects

Despite reports that TSLP-activated CD11c⁺ DCs can induce a robust Th2 response 1^{-3} , the effects of TSLP have not been examined using cells isolated from humans with well characterized allergic status. Experiments were performed in the following groups: (1) cat-allergic subjects with AD [highly atopic]; (2) cat-allergic subjects without AD [atopic]; and (3) healthy non-atopic controls (see Table I in the Online Repository). For T cell studies, a rigorous statistical approach was used to analyze cytokine-positive CD4⁺ T cells in order to account for low frequency events and within-group variability (see online materials)¹⁰. Stimulation of T cells with TSLP-primed moDCs preferentially enhanced IL-4⁺ T cells and this effect was most marked in cat-allergic patients with AD (normalized index (NI)=3.0 [95% CI: 1.1–8.3] versus 1.9 [0.7–5] for cat-allergic non-AD subjects and 1.7 [0.6–4.5] for controls) (Figures 1A & 1B). However, increases in the frequency of IL-4⁺ T cells were not significant within group or between groups after adjusting for multiple comparisons. TSLP preferentially induced the secretion of Th2 cytokines with significantly higher levels in cat-allergic subjects with and without AD versus controls ($p<0.05$)(Figure 1C). Thus, the Th2-promoting effect of TSLP was modest, but increased in atopic subjects.

MoDCs Co-Primed with H22-Fel d 1 and TSLP Provide a Potent Th2 Stimulus in Atopic Subjects

We previously reported that H22-Fel d 1 induced IL-5-expressing CD4⁺ T cells in parallel with IL-10-expressing cells in cultures from cat-allergic subjects without AD¹⁰. Consistent with those findings, H22-Fel d 1-primed moDCs selectively increased IL-4⁺ and IL-10⁺ CD4⁺ T cells in cultures from cat-allergic non-AD subjects as compared with non-receptor-targeted allergen (Fel d 1)(Figure 2A). Co-priming moDCs from these individuals with H22-Fel d 1 and TSLP enhanced IL-4⁺ T cells as compared with either stimulus alone, and this effect was significant when compared with TSLP alone (NI=4.5 [1.7–12.5])(Figures 2A & B). Surprisingly, moDCs from cat-allergic AD patients primed with H22-Fel d 1 only weakly

stimulated T cells (Figure 2A). By contrast, co-primed moDCs induced a selective and synergistic enhancement in IL-4⁺ T cells as compared with moDCs primed with either H22-Fel d 1 alone (NI=6.7 [2.4–16.7]) or TSLP alone (NI=8.3 [2.9–20]) (Figures 2A, B & C). Co-primed moDCs also induced IL-10⁺ T cells in these patients; however, these cells constituted only a small fraction of cells (Figures 2A & B). Moreover, total cytokine-positive T cells were markedly amplified by co-primed moDCs in the AD group (Figure 2A). A subset of IL-4⁺ T cells induced by co-primed moDCs in both cat-allergic groups also expressed IFN- γ (mean=24.9% $\pm$ 9.5% of total IL-4⁺ T cells, $n=10$) (Figure 2C and data not shown). Notably, the increase in IFN- γ ⁺IL-4⁺ T cells was significant as compared with either H22-Fel d 1-primed (NI=7.7 [2.6–25]) or TSLP-primed (NI=10 [3.2–33.3]) moDCs in the cat-allergic AD group. The Th2-promoting properties of co-primed moDCs from atopic subjects were borne out in the secreted cytokine profile, which showed enhanced IL-5 and IL-13 levels as compared with H22-Fel d 1 or TSLP alone (See Figure E1 in the Online Repository). Co-primed moDCs also induced increased production of IFN- γ as compared with TSLP alone in the AD group; however, mean levels of secreted IFN- γ were more than 7 fold less than Th2 cytokine levels indicating a Th2-dominated cytokine profile (Figure E1). Importantly, moDCs co-primed with non-receptor-targeted Fel d 1 and TSLP did not significantly amplify IL-4⁺ T cells as compared with TSLP-primed moDCs (data not shown). Thus, targeting of allergen to Fc γ RI was required for Th2 amplification. Collectively, these results suggest that moDCs isolated from cat-allergic subjects with and without AD (hereafter referred to as “atopic moDCs”) are equipped to promote a robust Th2 response upon co-priming with H22-Fel d 1 and TSLP, despite the relatively weak Th2-inducing properties of TSLP alone.

The Th2-Promoting Effect of Co-Primed MoDCs is OX40 Ligand-Independent

It was previously reported by Ito and colleagues that priming of CD11c⁺ DCs with TSLP enhanced expression of OX40L and that TSLP-induced Th2 responses were OX40L-dependent¹. Thus, we next investigated whether the Th2-promoting effect of co-primed moDCs was mediated through the OX40/OX40L pathway. Surprisingly, priming with TSLP alone did not increase expression of OX40L on moDCs irrespective of allergic status, though there was a trend towards increased expression of OX40L on co-primed moDCs from atopic subjects (Figure 3A). The lack of an effect of TSLP on OX40L expression was confirmed in time course studies performed up to 96 hours after priming (data not shown). Moreover, blocking OX40L did not inhibit the induction of IL-4⁺ T cells by atopic moDCs primed with any stimulus including those that had been co-primed (Figures 3B & Figure E2). To confirm the biologic activity of anti-OX40L, we tested its capacity to inhibit Th2 responses induced by TSLP-primed blood DCs as opposed to moDCs. Similar to a previous report, the frequency of IL-4⁺ T cells was modestly decreased (by ~35%) in the presence of anti-OX40L antibody, while an isotype control had no effect (Figure E3A and data not shown)¹. Furthermore, OX40L was upregulated on TSLP-primed blood DCs and its ligand (OX40) was readily detected on IL-4⁺ T cells (Figures E3A&E3B). In order to exclude a role for other factors in the Th2-promoting effect of co-primed moDCs, expression of co-stimulatory molecules (CD40, CD80 and CD86) and a broad array of secreted cytokines (IL-1 β , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, (p70), IL-13, IL-15, IL-17, eotaxin, FGF basic, GM-CSF, IFN- γ , IP-10, MCP-1, MIP-1 α , MIP-1 β , PDGFbb, RANTES, TNF- α , VEGF) were analyzed in moDCs. None of these factors were amplified in cultures containing co-primed moDCs from atopic subjects (data not shown).

TSLP-primed DCs have been reported to produce high levels of the Th2-attracting chemokine, CCL17/TARC^{1, 2}. In the present study, levels of CCL17 were not increased above background levels for either TSLP-primed or co-primed moDCs (Figure 3C). Collectively, these findings suggest that the Th2-promoting effect of co-primed moDCs from atopic individuals is mediated

through an unconventional pathway that is OX40L-independent and not related to TARC production.

H22-Fel d 1 Enhances TSLP Receptor Expression in Atopic MoDCs

The high affinity IgG receptor comprises an IgG binding α -chain complexed with a γ -chain dimer that contains a signaling motif (immune receptor tyrosine-based activation motif (ITAM)). This motif is critical to an array of Fc γ RI-mediated effector functions. In order to investigate whether targeting allergen to Fc γ RI enhances the TSLP pathway in atopic moDCs, we tested whether H22-Fel d 1 influenced TSLP receptor (TSLPr) expression. TSLPr was constitutively expressed on atopic moDCs and priming with H22-Fel d 1 markedly enhanced TSLPr expression with maximal levels occurring at 24 hours (Figures 4A & 4B and Figure E4). TSLP alone had no effect on TSLPr expression, while co-priming with TSLP reduced TSLPr expression at 24 hours but enhanced TSLPr upregulation at later time points; however, this effect was not observed in all atopic subjects (Figure 4B & data not shown). In contrast to atopic cells, non-atopic moDCs generally lacked constitutive expression of TSLPr and priming with H22-Fel d 1 enhanced receptor expression much less efficiently than in atopic cells (Figures 4A & 4B). Non-receptor-targeted allergen only weakly induced TSLPr expression and this effect was variable and restricted to atopic subjects (Figure 4A and Figure E4).

Since the properties of moDCs may differ from circulating CD11c⁺ DCs, we next examined whether DCs isolated from the blood of atopic subjects also upregulated TSLPr in response to H22-Fel d 1. Similar to atopic moDCs, blood DCs constitutively expressed TSLPr and receptor expression was further enhanced after priming with H22-Fel d 1, while TSLP had no effect (Figure 4C). TSLPr expression was also downregulated in co-primed DCs as compared with H22-Fel d 1-primed cells; however, this was more marked than for moDCs. This could reflect more efficient internalization of upregulated TSLPr upon binding to TSLP.

The H22 sFv domain of H22-Fel d 1 that targets the allergen molecule to Fc γ RI, binds outside the Fc binding pocket and is monovalent. As such, it does not cross-link Fc γ RI. Though cross-linking is not a pre-requisite for Fc γ RI-mediated endocytosis, IgG antibodies are required for internalization of H22 sFv bound to Fc γ RI^{14, 15}. The ability for serum to influence TSLPr expression was examined using THP-1 monocytes which have been used extensively to characterize cellular events triggered by ligation of Fc γ RI. Interestingly, H22-Fel d 1-mediated upregulation of TSLPr was enhanced in culture medium containing 10% human serum as compared with 10% fetal bovine serum, irrespective of atopic status (Figure E5). Thus, while serum antibodies may facilitate endocytosis, the difference in the capacity to upregulate TSLPr expression between atopic and non-atopic moDCs was primarily intrinsic to these cells.

FcR Signaling Components Modulate H22-Fel d 1-Induced TSLP Receptor Expression in Atopic MoDCs

Initial studies demonstrated that priming atopic moDCs with H22-Fel d 1, but not non-receptor-targeted Fel d 1, led to the release of intracellular calcium supporting a role for signaling through Fc γ RI in H22-Fel d 1-mediated TSLPr upregulation (Figure 5A). Thus, we next sought to identify signaling components that positively regulated TSLPr expression induced by H22-Fel d 1 in atopic cells. The src-related tyrosine kinases (SRTKs) and the tyrosine kinase Syk, associate with the Fc γ chain ITAM domain^{16–18}. These kinases can be inhibited by PP2 and piceatannol respectively. In the presence of PP2 there was a paradoxical increase in H22-Fel d 1-induced TSLPr expression in both moDCs and blood DCs from atopic patients, suggesting a negative regulatory role for SRTKs ($p=0.006$)(Figure 5B). By contrast, piceatannol had variable effects ($p>0.05$)(Figure 5B). Inhibition of the downstream signaling component PI3-kinase, by LY294002 generally reduced TSLPr expression suggesting a positive regulatory

role ($p=0.05$)(Figure 5B). These findings implicated FcR signaling components in modulation of TSLPr expression induced by receptor-targeted allergen.

TSLP Receptor is Induced by LPS, But Only at High Dose

Contaminating LPS in allergen preparations can act as a Th2 adjuvant ^{19–22}. The preparation of H22-Fel d 1 used in our system contained low levels of endotoxin (~0.4ng/ml working concentration). Thus, we next examined whether LPS contributed to TSLPr upregulation mediated by H22-Fel d 1. Low dose LPS (0.5ng/ml) did not induce TSLPr expression while at high dose (1μg/ml), TSLPr expression was markedly upregulated (Figure E6). Thus, while high dose LPS can upregulate TSLPr, the capacity for H22-Fel d 1 to induce TSLPr is not explained by contaminating low dose LPS.

Blocking TSLPr Upregulation in Co-Primed MoDCs Abolishes Their Th2-Promoting Effect

Since the PI3-kinase inhibitor, LY294002, inhibited TSLPr upregulation induced by H22-Fel d 1, we postulated that this molecule would block the robust Th2 response induced by co-primed moDCs. Cultures were established using atopic moDCs from a patient that exhibited a strong positive regulatory effect for PI3-kinase on H22-Fel d 1-induced TSLPr expression (grey asterisk, Figure 5). Inhibition of PI3-kinase had no effect on the frequency of IL-4⁺ T cells induced by non-primed moDCs (Figure 6, left panel). By contrast, inhibition in co-primed moDCs resulted in a preferential decrease in IL-4⁺ T cells (12 fold versus <2 fold for IFN-γ⁺ T cells) and IL-4⁺IFN-γ⁺ T cells (35 fold)(Figure 6). IL-4⁺ T cells induced by H22-Fel d 1 alone were also markedly reduced (7 fold) while there was no effect on TSLP-induced IL-4⁺ T cells. These findings support the view that TSLPr upregulation triggered by H22-Fel d 1 promotes TSLP-driven Th2 responses.

Discussion

We have identified a novel immune pathway that amplifies Th2 responses in humans. Specifically, we have shown that targeting cat allergen to FcγRI upregulates expression of TSLPr in atopic moDCs. Upregulation of TSLPr was most pronounced in moDCs from atopic patients and Th2 responses triggered by H22-Fel d 1 were markedly amplified in the presence of TSLP in these individuals. These findings indicated that receptor-targeted allergen potentiates the Th2-promoting effect of TSLP through TSLPr upregulation. In support of this theory, we further showed that TSLPr upregulation was regulated by FcR signaling components, and inhibition of TSLPr upregulation abolished the Th2-promoting effect of TSLP elicited by H22-Fel d 1. This study is the first report of an immune pathway linking Fc receptor-mediated events to TSLPr expression and consequent TSLP-driven Th2 responses.

While TSLP was previously shown to be sufficient to induce asthma-like disease in mouse models and to drive Th2 responses in human-based *in vitro* systems, the role of allergen in those studies was largely ignored ^{1–6}. In contrast to previous reports, TSLP alone was actually a weak inducer of Th2 responses in our system, irrespective of allergic status. By contrast, receptor-targeted allergen and TSLP acted coordinately to exert a potent Th2-promoting effect. This phenomenon was restricted to atopic individuals and was dependent upon the capacity to upregulate TSLPr. These observations are in line with a recent report that both antigen *and* TSLP are required for the development of full airway inflammation in a mouse asthma model ²³.

The Th2-promoting effect of TSLP that was triggered by H22-Fel d 1 was most pronounced in cat-allergic patients with AD as judged by a synergistic induction of IL-4⁺ T cells. Notably, H22-Fel d 1-primed moDCs induced only a weak T cell response in these patients (see Figure 2A). Since T cell anergy can be a feature of AD, we postulate that co-priming moDCs with

H22-Fel d 1 and TSLP surpasses a stimulation threshold required for optimal T-cell activation in these patients.

H22-Fel d 1 has the capacity to rapidly upregulate TSLPr expression in both blood DCs and moDCs and expression of TSLPr appears to be modulated in a similar manner in both cell types. Upregulation of TSLPr in atopic moDCs induced by H22-Fel d 1 was positively regulated by PI3-kinase, which acts downstream of Syk in FcγRI signaling²⁴. Thus, we expected that inhibition of Syk and its upstream activating components (SRTKs) would yield similar results to those for PI3-kinase. However, inhibition of SRTKs actually enhanced expression of TSLPr in atopic cells, suggesting that ITAM-associated molecules provide a “braking mechanism” for TSLPr upregulation. Since PI3-kinase is shared among different signaling pathways, the effects of LY294002 could reflect engagement of this molecule in pathways not related to FcγRI. Arguing against this, LY294002 did not alter TSLPr expression in non-primed moDCs suggesting that its effects were specifically linked to FcγRI-triggered events (data not shown). Another important consideration is that inhibitors may act on other molecules through non-kinase “off-target” effects. Such off-target effects may occur in the concentration range for LY294002 commonly used to inhibit PI3-kinase²⁵. In our study, LY294002 was used at 25μM; however, inhibition of H22-Fel d 1-induced TSLPr expression was evident at concentrations of LY294002 as low as 0.25μM with marked inhibition at 2.5μM suggesting that the effects of LY294002 were specific (data not shown). Moreover, the SRTK inhibitor, PP2, had no effect on constitutive levels of TSLPr on moDCs, providing further evidence that inhibitory molecules acted on the FcγRI pathway in H22-Fel d 1-primed moDCs (data not shown).

Interestingly, TSLPr expression was also upregulated by non-receptor-targeted allergen; however, this process was highly variable, less efficient than for H22-Fel d 1, and restricted to atopic cells. This effect is likely mediated through ligation of Fc receptors expressed at high density on atopic moDCs, by IgE- or IgG-bound allergen. It was previously reported that moDCs from atopic patients express increased levels of FcεRI, and this is driven by enhanced expression of the γ chains of the receptor²⁶. Notably, these γ chains can be shared among FcεRI and FcγRI. Moreover, high expression of the high affinity IgE receptor, FcεRI, on DCs from atopic subjects is pivotal to allergen uptake and T cell activation²⁷. Thus, DCs from atopic individuals possess the molecular machinery necessary to upregulate TSLPr through Fc receptor ligation upon allergen encounter. With this in mind, the present study provides insight into how allergens may induce potent Th2 responses in humans, despite their relatively weak Th2-inducing properties *ex vivo*. In line with this view, we have recently confirmed that moDCs primed with dust mite allergen (Der p 1) in the presence of TSLP, can drive a robust Th2 response in a subset of highly atopic subjects (Hulse and Woodfolk, unpublished data).

A surprising feature of our study was the relatively weak Th2-promoting capacity of TSLP. In addition, the TSLP-mediated amplification of Th2 responses triggered through FcγRI did not appear to involve OX40L or increased production of the Th2-attracting chemokine, TARC/CCL17. This is in contrast to previous reports on the effects of TSLP mediated through CD11c⁺ blood DCs^{1, 2}. One possible explanation is that TSLP-primed moDCs, unlike TSLP-primed blood DCs, secrete IL-12 which may counter-regulate the Th2-promoting effects of TSLP¹; however, no IL-12 was detected in our system. It should be pointed out that while no increase in TARC was noted for primed versus non-primed moDCs in our system, background levels were high. This could reflect exhaustion of TARC/CCL17 production during generation of moDCs *in vitro*. Nevertheless, our findings are in line with recent findings in a TSLP-driven mouse model of AD showing that Th2 polarization mediated by Langerhans cells did not involve secretion of TARC/CCL17 by these cells and was OX40L-independent.²⁸ Consistent with activation of an alternate TSLP-mediated pathway triggered through FcγRI, cells expressing IFN-γ constituted up to 37% of IL-4⁺ T cells induced by co-primed atopic moDCs.

From a clinical standpoint, our findings question whether DC-targeted allergens will be clinically efficacious in atopic individuals. The route of vaccine administration will no doubt be a key factor to consider if TSLP-mediated effects are to be avoided. On the other hand, our results suggest that targeting Fc receptor signaling components may provide a useful strategy for modulating Th2 responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Atopic dermatitis
DC	dendritic cells
ITAM	immune receptor tyrosine-based activation motif
moDCs	monocyte-derived dendritic cells
NI	normalized index
SCORAD	SCORing atopic dermatitis
SRTK	src-related tyrosine kinase
TARC	thymus and activation-regulated chemokine
TSLP	thymic stromal lymphopoietin
TSLPr	TSLP receptor

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Figure 1. The Th2-Promoting Effect of TSLP is Weak in Atopic Subjects

CD4 $^+$ T cells were co-cultured with moDCs primed for 48 hours with TSLP. (A) Cells were stained for intracellular cytokines (day 10) and analyzed by flow cytometry, gating on CD4 $^+$ T cells. Relative change for each T cell type as compared with non-stimulated cells, expressed as normalized index (n=5 subjects per group). Error bars represent 95% confidence intervals. (B) Representative dot plots showing unstimulated (NS) and TSLP-stimulated CD4 $^+$ T cells stained for IL-4 and IFN- γ . Percentages of cells in each quadrant are shown. (C) Secreted cytokines in non-stimulated (NS) and TSLP-stimulated cultures (n=5 subjects per group). Bars represent the mean $\pm$ SEM. *Significant versus control group.

Figure 2. Dendritic Cells Co-Primed with H22-Fel d 1 and TSLP Provide a Potent Th2 Stimulus in Atopic Subjects

CD4⁺ T cells were co-cultured with moDCs which had been primed for 48 hours with Fel d 1 (Fel), H22-Fel d 1 (H-Fel), TSLP, or H22-Fel d 1+TSLP. Cytokine-positive T cells were analyzed on day 10. (A) Summary data showing the percentage of each T cell type induced by different stimuli (5 subjects per group). Values are geometric means. Black and grey asterisks denote significant differences for IL-10⁺ and IL-4⁺ cell types respectively, after adjusting for multiple comparisons. (B) Relative change in each T cell type for H22-Fel d 1+TSLP versus H22-Fel d 1 alone (left panel), and H22-Fel d 1+TSLP versus TSLP alone (right panel) (n=5 per group). Error bars represent 95% confidence intervals. *Significant after adjusting for multiple comparisons. (C) Representative dot plot from an AD patient showing the phenotype of gated CD4⁺ T cells induced by different stimuli.

Figure 3. The Th2-Promoting Effect of Co-Primed MoDCs is OX40 Ligand-Independent

(A) Expression of OX40 ligand on moDCs after priming with different stimuli for 48 hours. Bars represent the mean fluorescence intensity (MFI) $\pm$ SEM (n=5 subjects per group). *p<0.05. (B) Effect of anti-OX40L mAb on the percentage of IL-4⁺ T cells induced (day 10) by atopic moDCs primed with different stimuli for 48 hours. Bars represent the mean $\pm$ SEM (4 subjects). (C) CCL17/TARC levels in supernatants from cultures containing moDCs primed with different stimuli for 48 hours. Bars represent the mean $\pm$ SEM (n=5 subjects per group).

Figure 4. H22-Fel d 1 Enhances TSLPr Expression in Atopic MoDCs

(A) Comparison of TSLPr expression on moDCs from atopic and non-atopic subjects. MoDCs were primed with different stimuli and expression of TSLPr analyzed at 24 hours. Data is representative of 4 atopic and 4 non-atopic subjects. FSC, forward scatter. (B) Time course of TSLPr expression on moDCs (representative of 6 subjects). Solid red histogram corresponds to the fluorescence-minus-one control (FMO). (C) TSLPr expression on blood DCs. DCs were isolated, primed with different stimuli and analyzed for TSLPr expression at 24 hours. Data is representative of 3 experiments.

Figure 5. FcR Signaling Components Modulate H22-Fel d 1-Induced TSLP Receptor Expression in Atopic MoDCs

(A) Calcium mobilization in atopic moDCs primed with different stimuli. The y axis represents the ratio of free to bound calcium. Data is representative of 4 atopic subjects. (B) Effect of kinase inhibitors on H22-Fel d 1-induced TSLPr expression in atopic DCs. Cells were incubated with inhibitors for 1 hour before priming with H22-Fel d 1 and analyzing for TSLPr expression (24 hours). Inhibitors tested were PP2 (src-related tyrosine kinase inhibitor); piceatannol (Syk kinase inhibitor); and LY294002 (PI3-kinase inhibitor). Data is shown for 8 different atopic patients. * $p \leq 0.05$.

Figure 6. Blocking TSLPr Upregulation in Co-Primed MoDCs Abolishes Their Th2-Promoting Effect

Atopic or non-atopic moDCs were incubated with no inhibitor, or with LY294002 prior to priming with different stimuli. Cells were then washed and cultured with CD4⁺ T cells for 7 days. Intracellular cytokine expression was analyzed by flow cytometry. Dot plots show the phenotype of gated CD4⁺ T cells. No inh.: no inhibitor.