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Proteases Induce Production of Thymic Stromal Lymphopoietin by Airway Epithelial Cells through Protease-Activated Receptor-2

Hideaki Kouzaki,*† Scott M. O’Grady,‡§ Christopher B. Lawrence,¶ and Hirohito Kita**

Thymic stromal lymphopoietin (TSLP) is produced by epithelial cells and triggers dendritic cell-mediated Th2-type inflammation. Although TSLP is up-regulated in epithelium of patients with asthma, the factors that control TSLP production have not been studied extensively. Because mouse models suggest roles for protease(s) in Th2-type immune responses, we hypothesized that proteases from airborne allergens may induce TSLP production in a human airway epithelial cell line, BEAS-2B. TSLP mRNA and protein were induced when BEAS-2B cells were exposed to prototypic proteases, namely, trypsin and papain. TSLP induction by trypsin required intact protease activity and also a protease-sensing G protein-coupled receptor, protease-activated receptor (PAR)-2; TSLP induction by papain was partially dependent on PAR-2. In humans, exposure to ubiquitous airborne fungi, such as Alternaria, is implicated in the development and exacerbation of asthma. When BEAS-2B cells or normal human bronchial epithelial cells were exposed to Alternaria extract, TSLP was potently induced. The TSLP-inducing activity of Alternaria was partially blocked by treating the extract with a cysteine protease inhibitor, E-64, or by infecting BEAS-2B cells with small interfering RNA for PAR-2. Protease-induced TSLP production by BEAS-2B cells was enhanced synergistically by IL-4 and abolished by IFN-γ. These findings demonstrate that TSLP expression is induced in airway epithelial cells by exposure to allergen-derived proteases and that PAR-2 is involved in the process. By promoting TSLP production in the airways, proteases associated with airborne allergens may facilitate the development and/or exacerbation of Th2-type airway inflammation, particularly in allergic individuals. The Journal of Immunology, 2009, 183: 1427–1434.

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2 Address correspondence and reprint requests to Dr. Hirohito Kita, Division of Allergic Diseases, Mayo Clinic, Rochester, MN 55905. E-mail address: kita.hirohito@mayo.edu

3 Abbreviations used in this paper: DC, dendritic cell; APMSF, 4-amidinophenyl- methanesulfonyl fluoride; E-64, trans-epoxysuccinyl-1-leucylamide(4-guanidino)butane; HDM, house dust mite; NHBE, normal human bronchial epithelial; PAR, protease-activated receptor; poly(L,C), polyinosinic-polycytidylic acid; TSLP, thymic stromal lymphopoietin; siRNA, small interfering RNA.

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response. However, the mechanisms to explain these protease-mediated Th2 responses are not fully understood.

Herein, we investigated whether prototypic proteases and allergen-derived protease(s) activate airway epithelial cells to produce TSLP. We used a common environmental fungus, Alternaria alternata, as a model allergen because an association between exposure to fungi, such as Alternaria and Cladosporium, and asthma has been recognized clinically and epidemiologically (23). Moreover, severe asthma and life-threatening acute exacerbations of asthma have been associated with Alternaria sensitivity or increased airborne exposure to Alternaria (24–28). We found that TSLP was induced in airway epithelial cells by exposure to prototypic proteases or Alternaria proteases. This TSLP response was mediated by a protease-sensing G protein-coupled receptor, namely, protease-activated receptor (PAR)-2, enhanced by a Th2 cytokine, IL-4, and abolished by a Th1 cytokine, IFN-γ. Thus, environmental exposure to allergen-derived proteases may be pivotal in Th2-type airway inflammation and ultimately in the development and/or exacerbation of asthma, especially in allergic individuals.

Materials and Methods

Reagents

Recombinant human IL-4 and IFN-γ were from R&D Systems. Polyo- nosinic-polyribocytidylic acid (poly(IC)) was from InvivoGen. Trypsin from bovine pancreas, trans-epoxysoycylin-1-leucylamide (4- guanidino) butane (E-64), and 4-amidinophenylmethanesulfonyl fluoride (APMSF) were from Sigma-Aldrich. Papain was from Carica papaya from Calbiochem. Small interfering RNA (siRNA) for PAR-1, PAR-2, TLR2, and TLR4 and a control siRNA were obtained from Qiagen. Culture filtrate extracts from A. alternata were purchased from Greer Laboratories. The PAR-2 agonist peptide SLIGKV-NH2 was prepared at the Mayo Proteomics Research Center (Mayo Clinic, Rochester, MN).

Cell culture, treatment, and transfection

The human bronchial epithelial cell line BEAS-2B, derived from human bronchial epithelium transformed by an adenovirus 12-SV40 virus, was purchased from American Type Culture Collection. BEAS-2B cells were cultured in DMEM/F12 medium (Invitrogen), supplemented with 10% heat-inactivated FBS (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) at 37°C and 5% CO2. To prepare cells for stimulation, BEAS-2B cells were seeded (5 × 104 cells/well) in a 24-well tissue culture plate (Costar; Corning) and grown until 80% confluence (~2 days). At this stage, the BEAS-2B cells were incubated for up to 24 h with trypsin (0.1–100 nM), papain (50–200 μM), Alternaria extract (25–75 μg/ml), or poly(IC) (10 μg/ml). In some experiments, IL-4 (100 ng/ml) or IFN-γ (100 ng/ml) was added for the duration of incubation. Cell culture supernatants and cell lysates were collected and used for TSLP protein ELISA and TSLP mRNA real-time RT-PCR (see below). Previous reports had noted that high concentrations of fungal extracts or proteases would produce morphologic changes and desquamate epithelial cells (29). At the relatively low concentrations listed above, we did not observe changes in morphology in the BEAS-2B cells for up to 24 h. In some experiments, the stimuli, including trypsin, papain, Alternaria extract, and poly(IC), were pretreated with a serine protease inhibitor, APMSF (50–100 μM), a cysteine protease inhibitor, E-64 (25–50 μM), or their combination for 30 min at room temperature before addition to the BEAS-2B cells. To transfect the BEAS-2B cells, they were seeded at low density (5 × 104 cells/well) overnight in DMEM/F12 supplemented with 10% heat-inactivated FBS. At 30–50% confluence, cells were transfected with siRNA against PAR-1, PAR-2, TLR2, TLR4, or control siRNA at 5 nM using HiPerFect transfection reagent (Qiagen). The real-time RT-PCR contained 1 μl of cDNA, 12.5 μl of Master Mix (TaqMan Universal PCR Master Mix, No AmpErase UNG), and 1.25 μl of TaqMan gene expression assay (200 nM) each of targeted gene and TATA-box gene, and 18S (Applied Biosystems). Reactions were made up to a final volume of 20 μl with sterile water. Amplification and detection of specific products were performed using the iQ 5 Multicolor Real-Time PCR Detection System (Bio-Rad). The real-time RT-PCR protocol was as follows: denaturation by a hot start at 95°C for 10 min, followed by 40 cycles of

![FIGURE 1. Papain and trypsin induce TSLP production in airway epithelial cells. A, BEAS-2B cells were incubated with 0.1–100 nM trypsin or 50–200 μM papain for 6 h. TSLP mRNA was analyzed by real-time RT-PCR. The TSLP transcription was normalized to the 18S rRNA transcription in each sample and expressed as a ratio to the BEAS-2B cultured in the absence of proteases. *, p < 0.05 and **, p < 0.01, compared with cells with medium alone (i.e., no proteases), n = 5. B, Before incubation with BEAS-2B cells, 10 nM trypsin and 100 μM papain were pretreated with their protease inhibitors (APMSF and E-64, respectively) at 37°C for 30 min. TSLP mRNA was analyzed at 6 h. *, p < 0.05 and **, p < 0.01, compared with no protease inhibitors, n = 5. C, BEAS-2B cells were incubated with 10 nM trypsin or 100 μM papain in the presence or absence of 100 ng/ml IL-4. TSLP mRNA expression and protein production were analyzed by real-time RT-PCR and ELISA at 6 and 24 h, respectively. **, p < 0.01, compared with medium alone (Cont) without IL-4; n = 4.](http://www.jimmunol.org/content/content)
Prototypic proteases induce TSLP production from airway epithelial cells

Prototypic proteases, such as trypsin (a serine protease) and papain (a cysteine protease), are used to model the endogenous proteases naturally found in allergens (e.g., mites, fungi, cockroaches) (30–33). Trypsin and papain are also used to represent endogenous proteases released by inflammatory cells (e.g., mast cell tryptase) at the sites of allergic inflammation (34). Thus, to investigate the effects of endogenous and exogenous proteases on human airway epithelial cells, we exposed BEAS-2B cells to trypsin and papain. After incubation for 6 h with trypsin or papain, mRNA for TSLP in airway epithelial cells stimulated with a TLR3 ligand (37, 38). Therefore, to examine the mechanism of TSLP induction by airway epithelial cells exposed to proteases, we knocked down PAR-2 by transfecting BEAS-2B cells with siRNA for PAR-2 or control RNA and then stimulated them with proteases of these proteases are due to their protease activities, we examined the effects of a serine protease inhibitor, APMSF, and a cysteine protease inhibitor, E-64. When trypsin was pretreated with APMSF, the TSLP mRNA expression was inhibited to baseline level (Fig. 1B). Similarly, when papain was pretreated with E-64, the TSLP mRNA expression was abolished, suggesting that the stimulatory effects of these proteases are mediated by protease activities, but not by unknown contaminants.

In an earlier report, IL-4 synergistically enhanced production of TSLP in airway epithelial cells stimulated with a TLR3 ligand (dsRNA) (13). We also found that IL-4 synergistically enhanced TSLP mRNA expression in BEAS-2B cells stimulated with trypsin or papain, but IL-4 by itself showed minimal effects on TSLP production (Fig. 1B). To confirm these observations at the protein level, we measured TSLP protein by ELISA. Significant amounts of TSLP (control; <7.8, trypsin treated; 80 ± 15, papain treated; 45 ± 5 pg/ml, n = 5) were detected in the supernatants after stimulating BEAS-2B cells with trypsin or papain for 24 h without IL-4 (Fig. 1C). With IL-4, TSLP protein production was enhanced ~2-fold (p < 0.05; n = 5). Thus, the protease activities of trypsin and papain stimulate TSLP mRNA synthesis and protein production from airway epithelial cells.

PAR-2 mediates TSLP production stimulated by proteases

A four-member family of seven transmembrane G protein-coupled receptors, namely, PARs, is activated by proteases, in particular serine proteases (35, 36). In general, PAR-1, PAR-3, and PAR-4 respond to thrombin (a serine protease), and PAR-2 responds to trypsin and trypsin-like serine proteases. The ability of PAR-2 to detect cysteine proteases has been controversial (37, 38). Therefore, to examine the mechanism of TSLP induction by airway epithelial cells exposed to proteases, we knocked down PAR-2 by transfecting BEAS-2B cells with siRNA for PAR-2 or control RNA and then stimulated them with proteases.
for 6 h. PAR-2 mRNA expression was suppressed significantly by PAR-2 siRNA, but not by control siRNA (p < 0.01; n = 5) (Fig. 2A). Induction of TSLP by trypsin was ablated to the baseline level by PAR-2 siRNA (Fig. 2B). TSLP induction by papain was partially (~45%) but significantly reduced by PAR-2 siRNA (p < 0.05; n = 5).

Trypsin cleaves the extracellular N terminus of PAR-2 molecules between the R36 and S37 residues to expose a tethered neo-ligand (i.e., S37LIGKV-) that, in turn, binds intramolecularly to PAR-2 and triggers receptor activation (37, 38). A synthetic peptide, SLIGKV-NH₂, that corresponds to the sequence of the tethered neo-ligand can also bind and activate uncleaved PAR-2. We found that, similarly to the enzymatic agonist trypsin, the PAR-2-activating peptide SLIGKV-NH₂ (denoted PAR-2 AP) stimulates TSLP expression by BEAS-2B cells (Fig. 2B) and that the response is ablated by PAR-2 siRNA. These results demonstrate that the stimulation of PAR-2 can induce TSLP secretion and that trypsin-induced TSLP production is mediated by PAR-2. Papain-induced TSLP production appears to involve both PAR-2-dependent and -independent mechanisms.

A. alternata extract induces TSLP production from airway epithelial cells

To examine the relevance of these observations for human asthma, we investigated whether an environmental fungus implicated in asthma (23, 24), namely, *Alternaria*, induces airway epithelial cells to produce TSLP. We used a TLR3 ligand, poly(I:C), as a control because it was previously shown to induce TSLP (13). After a 6-h incubation, TSLP mRNA expression was significantly up-regulated in BEAS-2B cells by *Alternaria* extract in a concentration-dependent manner (Fig. 3A). The TSLP expression in BEAS-2B cells stimulated with poly(I:C) reached a peak at 3 h (Fig. 3A), consistent with a previous report (13), but the TSLP expression induced by *Alternaria* was delayed, reaching a maximum at 6 h. After a 24-h incubation, TSLP protein levels in culture supernatants from BEAS-2B cells incubated with *Alternaria* extracts were increased in a concentration-dependent manner (Fig. 3B). Culture supernatants of BEAS-2B cells incubated with 50 µg/ml *Alternaria* extract or with 10 µg/ml poly(I:C) contained comparable levels of TSLP protein that continued to increase up to 24 h.

As described above (Fig. 1C), IL-4 synergistically enhanced TSLP production from BEAS-2B cells stimulated with trypsin and papain. Thus, we examined whether Th1 (IFN-γ) or Th2 (IL-4) cytokines affect *Alternaria*-induced TSLP expression and production. After 6 h, IL-4 synergistically enhanced both poly(I:C)- and *Alternaria*-induced TSLP mRNA expression (Fig. 4A). IL-4 did not affect the kinetics of TSLP expression induced by poly(I:C) or *Alternaria* (data not shown). Interestingly, IFN-γ strongly inhibited both poly(I:C)- and *Alternaria*-induced TSLP mRNA expression (Fig. 4A; p < 0.05). In addition, IL-4 synergistically enhanced both poly(I:C)- and *Alternaria*-induced TSLP protein production.

**FIGURE 4.** IL-4 enhances and IFN-γ inhibits *Alternaria*-induced TSLP production from airway epithelial cells. BEAS-2B cells were incubated with medium, 10 µg/ml poly(I:C), or 50 µg/ml *Alternaria* extract with or without 100 ng/ml IL-4 or 100 ng/ml IFN-γ. A, TSLP mRNA expression was analyzed at 6 h. B, TSLP protein production was analyzed at 24 h; n = 5.

**FIGURE 5.** Blocking cysteine protease activity inhibits *Alternaria*-induced TSLP protein and mRNA expression. A, *Alternaria* extracts were heated at 37 or 56°C for 30 min. BEAS-2B cells were incubated with preheated *Alternaria* extracts for 6 h. TSLP mRNA expression was expressed as a ratio to cells incubated without *Alternaria*. *, p < 0.05 and **, p < 0.01, compared with *Alternaria* extract heated at 37°C. n = 5. B, *Alternaria* extract (50 µg/ml) or poly(I:C) (10 µg/ml) was pretreated without inhibitors, with 100 µM APMSF, with 50 µM E-64, or with the combination of APMSF and E-64 at 37°C for 30 min. BEAS-2B cells were incubated with medium (control), pretreated *Alternaria* extract (with or without 100 ng/ml IL-4), or pretreated poly(I:C) for 6 h. TSLP mRNA expression was expressed as a ratio to cells incubated with medium alone and no inhibitors; n = 5.
Both TSLP mRNA and TSLP protein from BEAS-2B cells when extract (30 min at 56°C) induced significantly smaller amounts of siRNA as 100%.

PCR. Data are expressed as ratio to the mock-transfected cells without siRNA against PAR-1, PAR-2, or TLR4 or 5 nM control siRNA for 48 h.

/H11569/H11569 expressed as a ratio to cells incubated without siRNA.

/H9262

PAR-2 and protease activity in Alternaria-induced TSLP mRNA expression. A, BEAS-2B cells were transfected with 5 nM siRNA against PAR-1, PAR-2, or TLR4 or 5 nM control siRNA for 48 h. Expression of mRNA for target molecules was examined by real-time RT-PCR. Data are expressed as ratio to the mock-transfected cells without siRNA as 100%.

** p < 0.01, compared with control cells without siRNA; n = 5.

PAR-2 and protease activity in Alternaria extract mediate TSLP production

Next, we characterized the BEAS-2B cell-stimulatory activities in Alternaria extract and investigated the mechanism for cellular activation. First, we examined the effects of heat treatment on the Alternaria-induced TSLP production. The heat-treated Alternaria extract (30 min at 56°C) induced significantly smaller amounts of both TSLP mRNA and TSLP protein from BEAS-2B cells when compared with the control-treated Alternaria extract (30 min at 37°C) (p < 0.01 and <0.05, respectively; Fig. 5A). The response of BEAS-2B to 75 μg/ml heat-treated Alternaria extract was apparently less than the response to 25 μg/ml control-treated Alternaria extract, suggesting that heat treatment removed >67% of stimulatory activity.

Second, we investigated whether protease activity in the Alternaria extract is involved in Alternaria-induced TSLP mRNA expression. The Alternaria extract was preincubated with a serine protease inhibitor, APMSF, or cysteine protease inhibitor, E-64, or their combination before incubation with the BEAS-2B cells. E-64 partially (~50%) but significantly inhibited TSLP mRNA expression induced by Alternaria extract with or without IL-4 (p < 0.01 or p < 0.05, respectively; n = 5); APMSF showed no inhibition (Fig. 5B). A combination of E-64 and APMSF showed inhibition comparable to E-64 alone. In contrast, pretreatment of poly(I:C) with AMPSF, E-64, or their combination showed no effects on the TSLP mRNA expression induced by poly(I:C) plus IL-4, suggesting that the carryover of these protease inhibitors to the BEAS-2B culture does not affect TSLP expression induced by a nonprotease stimulus.

Finally, we examined the receptor involved by using a gene knockdown approach. BEAS-2B cells were transfected with siRNAs specific for PAR-1, PAR-2, or IL-4 or a control siRNA. Transfection with these specific siRNAs, but not the control siRNA, significantly suppressed the target molecule expression (Fig. 6A). These knockdown BEAS-2B cells were then stimulated with Alternaria extract or poly(I:C). The Alternaria-induced TSLP mRNA expression was significantly inhibited (~60%) in the PAR-2 siRNA knockdown cells (p < 0.01, n = 6; Fig. 6B); in contrast, the Alternaria-induced TSLP mRNA expression was not affected in the PAR-1 siRNA or TLR4 siRNA knockdown cells (Fig. 6C). Furthermore, the poly(I:C)-induced TSLP mRNA expression was not affected in the PAR-2 siRNA knockdown cells (Fig. 6B). Thus, both E-64-sensitive, heat-labile protease activity and PAR-2 are likely involved in the TSLP induction when BEAS-2B cells are exposed to Alternaria.

Finally, to examine the physiological significance of these observations, we examined the reproducibility of certain findings in NHBE cells. Incubation of NHBE cells with trypsin, papain, or PAR-2-activating peptide, SLIGKV-NH₂ peptide (PAR-2 AP), or 50 μg/ml Alternaria extract for 6 h.

The levels of TSLP mRNA were determined by real-time RT-PCR. The TSLP transcription was normalized to the 18S rRNA transcription in each sample and expressed as a ratio to the NHBE cells cultured with medium alone. ** p < 0.01, compared with cells with medium alone; n = 5.

Discussion

TSLP triggers DC-mediated activation of a Th2-type airway inflammation response (39). However, the environmental factors that control the expression of TSLP are largely unknown. Several TLR ligands induce TSLP production (13–15). Our study provides the first evidence that exposure to protease(s) induces TSLP in airway epithelial cells. This conclusion is based on several observations: 1) protease activities of trypsin and papain induce TSLP mRNA and protein in BEAS-2B cells; 2) Alternaria extract also induces TSLP mRNA and protein; 3) Alternaria-mediated TSLP induction is highly heat labile and partially inhibited by a cysteine protease.
inhibitor, E-64; 4) the induction of TSLP by proteases and Alternaria extract depends on a protease-sensing receptor, PAR-2; and 5) trypsin, papain, and Alternaria extract also induce TSLP mRNA in NHBE cells. Many allergens relevant to human diseases, such as fungi, mite, cockroach, and pollens, have protease activities (34); these protease activities of allergens potently induce Th2-type immune responses in several experimental animal models (19, 20, 22, 40). Our results provide a mechanistic understanding for these protease-related observations and suggest that airway epithelial cells may play pivotal roles by recognizing allergen-derived protease activities, producing TSLP, and inducing and/or exacerbating Th2-type inflammatory responses in the airways.

We found that PAR-2 exerts a critical role when BEAS-2B cells respond to trypsin or papain and induce TSLP (Fig. 2). PAR-2, but not PAR-1 or TLR4, was also partially involved in the TSLP induced in response to Alternaria extract (Fig. 6). Previously, Kauffman et al. (29) showed that fungal proteases activate epithelial cells, generating several cytokines and chemokines. Recently, chitinase from Streptomyces griseus stimulated the intracellular calcium response and IL-8 production in human bronchial epithelial cells through PAR-2 (41). Thus, mucosal surfaces, human PAR-2 may monitor the activities of certain exogenous proteases and perhaps nonprotease enzymes and may play a gatekeeper’s role to regulate subsequent immune responses. In mice, in vivo airway administration of a PAR-2 agonist peptide enhanced the Th2-type sensitization to an innocuous Ag, OVA (42). Similarly, both Th2-type airway inflammation and airway hyperreactivity were attenuated in mice deficient in PAR-2 and enhanced in mice overexpressing PAR-2 (43), suggesting roles for PAR-2 in regulating Th2-type immune responses. In humans, patients with asthma show increased expression of PAR-2 on their airway epithelial cells (44, 45). Therefore, further studies on the roles of PAR-2 in response to both environmental proteases and glycosidases as well as to endogenous enzymes (e.g., mammalian chitinases) and in the production of epithelial-derived immunoregulatory factors, such as TSLP, IL-33, and B cell-activating factor of the TNF family, may provide important information to understand better the interactions between the airway immune system and environmental factors.

Although the roles for the PAR-2 molecule to recognize trypsin or trypsin-like protease activity are well established, the ability of PAR-2 to recognize cysteine protease activity has been controversial. For example, PAR-2 mediated the intracellular calcium response in A549 cells stimulated with a cysteine protease from the allergen Der p 1 (46). In another study, Der p 1 stimulated IL-8 expression independently from PAR-2 while a serine protease, Der p 3, stimulated IL-8 expression that depended on PAR-2 (38). In our study, papain-induced TSLP expression was partially inhibited by PAR-2 siRNA transfection while the same treatment totally inhibited TSLP response (Fig. 4). Thus, the receptor mechanisms to recognize trypsin and papain or serine proteases and cysteine proteases may not be the same. Moreover, the cellular responses to papain and other cysteine proteases may involve additional PARs, such as PAR-3 and PAR-4, or PAR-independent mechanisms.

Among various environmental factors, the association between exposure to Alternaria and human asthma, particularly severe and life-threatening asthma, has long been recognized (23, 24). Our use of Alternaria extract may have both advantages and disadvantages. Alternaria extract contains many molecules produced by the fungus, including proteases, other proteins and peptides, and carbohydrates (data not shown). Thus, it likely reflects real-life exposure in humans, but dissecting the specific receptors and molecules involved is complex. Nonetheless, our data suggest that protease(s) produced by Alternaria plays a substantial role in inducing TSLP from airway epithelial cells. Previously, Alternaria extracts stimulated chemokine production from primary nasal epithelial cells that was highly dependent on protease activity (29). We also found that heat-labile, E-64-sensitive cysteine protease activity, but not serine protease activity, is likely involved (Fig. 5). Our observations are consistent with a recent study where papain induced TSLP in mouse basophils (22). With BEAS-2B cells, we found that a cysteine protease inhibitor, E-64, was highly effective in abolishing TSLP expression by the authentic cysteine protease papain (Fig. 1). However, because E-64 only partially inhibited TSLP expression induced by Alternaria (Fig. 5), other classes of proteases, such as aspartate proteases, or glycosidases might also be involved in Alternaria’s activity.

Other innate immune-stimulatory molecules, such as TLR ligands, may also be involved in the Alternaria-induced TSLP expression. TLRs, such as TLR2 and TLR4, play important roles in both innate and adaptive immunity to fungi (47). Furthermore, ligands for TLR2, TLR3, TLR8, and TLR9 effectively stimulated TSLP production from airway epithelial cells (13–15). Interestingly, a nonenzymatic mite allergen, Der p 2, likely stimulates an innate immune response through TLR4 by molecular mimicry of a lipid-recognition protein, MD-2 (48). Furthermore, β-glucan moieties, but not proteases, in HDM extract, mediated CCL20 production by airway epithelial cells (49). Therefore, several receptors, including PARs, TLRs, and lectin-type receptors, may be involved in both the recognition of and the initiation of immune responses to environmental allergens. Interestingly, analyses of PAR-2 and TLR4 signal transduction suggest that these receptors may physically interact and cooperate in their inflammatory responses (50). Furthermore, in Drosophila, fungal infection is recognized by both pathogen-associated molecular patterns and fungal protease activities (18). Thus, innate immune receptors, such as TLRs and PARs, may not work in isolation, but instead cooperate to fine-tune their specificity and to regulate the magnitude of cellular responses.

The dichotomy of the effects of Th1 and Th2 cytokines on TSLP expression needs to be noted. IL-4 synergistically enhanced the TSLP response to Alternaria, but IFN-γ abolished the TSLP response (Fig. 4). IL-4 also enhanced TSLP mRNA expression and protein production induced by prototypic proteases (Fig. 1), suggesting that the local cytokine milieu influences the epithelial TSLP response. In allergic individuals, airway exposure to Alternaria or other allergen proteases may result in an elevated TSLP response compared with normal individuals, leading to profound Th2-type airway inflammation. In contrast, individuals with stronger Th1 responses (51) may be spared from the effects of environmental proteases. Although a detailed analysis of the relevant signal transduction mechanisms are beyond the scope of this manuscript, our observations can be explained by a mechanism centered on NF-κB. IL-1β and TNF-α regulate TSLP expression in a NF-κB-dependent manner (15). PAR stimulation also induces cytokine and transcription responses through MAPK and NF-κB (38, 52–54). Importantly, synergy between NF-κB and STAT6 has been shown in TSLP induction in response to dsRNA and IL-4 (13), and IFN-γ-mediated acetylation of STAT1 suppresses NF-κB activation (55). Furthermore, dsRNA-induced TSLP production by human keratinocytes was enhanced by IL-4, IL-13, and TNF-α and inhibited by IFN-γ, TGF-β, or IL-17 (56), consistent with our observations. Alternatively, cytokines may modulate expression of PARs. In mice infected with helminths, PAR-1 expression was enhanced in an IL-13- or STAT6-dependent mechanism.
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