

Proteases Induce Production of Thymic Stromal Lymphopoietin by Airway Epithelial Cells through Protease-Activated Receptor-2¹

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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.

In addition to being a physical barrier between the airway and the immune system, epithelial cells are now thought to play vital roles in both innate and adaptive immune responses (1). Epithelial cells produce chemokines and cytokines that recruit and enhance survival of dendritic cells (DCs)³ and interact directly with DCs through membrane-associated chemokines (2–4). Epithelial cells also express soluble and cell surface molecules that regulate recruitment, differentiation, proliferation, and function of T cells and B cells (2, 5). In particular, newly discovered epithelial-derived cytokines, such as thymic stromal lymphopoietin (TSLP), IL-33, and B cell-activating factor of the TNF family, may play key roles in shaping the functional differentiation and activation of T cells and B cells in the mucosal organs (6).

TSLP is an IL-7-like cytokine that is produced by epithelial cells in the lungs, gut, and skin (1, 7, 8). Expression of TSLP in airways of patients with asthma correlated with the severity of their disease, suggesting that it is involved in the development of allergic airway inflammation (7). TSLP activates DCs to polarize naive T cells toward the Th2 cells that produce IL-4, IL-5, and IL-13 as well as TNF- α (1, 9, 10). Mice expressing TSLP in the lungs develop a spontaneous airway inflammation with characteristics similar to human asthma (11). Conversely, mice deficient in the TSLP receptor (TSLPR) show decreased airway inflammation when they are challenged with allergens (12). Thus, TSLP appears to be necessary and sufficient for the development of Th2-type airway inflammation. However, little is currently known regarding the factors that control the expression of TSLP. In vitro, airway epithelial cells produce TSLP when they are activated by various ligands for TLRs (13–15).

The Th2-type immune response is typically associated with immunity to multicellular helminths (16). These multicellular organisms secrete proteases (17), and the innate immune system appears to have evolved to recognize both TLR ligands and proteases (18). Certain allergens are also proteases, including Amb a from ragweed pollen, Bla g from cockroach, Asp f 5, f 6, and f 11 from *Aspergillus*, and Der p 1, p 3, and p 9 from house dust mite (HDM) (1), suggesting that proteases may be a key link between Th2-type immune responses in antihelminth immunity and allergic responses. Indeed, when these proteases are administered in vivo, they produce Th2 adjuvant activities. The protease activities produced by HDM, *Aspergillus*, or ragweed promoted a Th2 response (19, 20). Moreover, the cysteine proteinase gene from *Leishmania mexicana* has been implicated in the up-regulation of Th2 immunity and the down-regulation of Th1 immunity to this pathogen in mice (21). Recently, a prototypic cysteine protease, papain, enhanced Th2-type sensitization to bystander Ags after papain was administered s.c. into mice (22). Interestingly, in this model, basophils and TSLP were necessary for the papain-induced Th2

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³ Abbreviations used in this paper: DC, dendritic cell; APMSF, 4-amidinophenyl-methanesulfonyl fluoride; E-64, *trans*-epoxysuccinyl-L-leucylamide(4-guanidino)butane; HDM, house dust mite; NHBE, normal human bronchial epithelial; PAR, protease-activated receptor; poly(I: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. Polyinosinic-polycytidylic acid (poly(I:C)) was from InvivoGen. Trypsin from bovine pancreas, *trans*-epoxysuccinyl-L-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 (30 min at 56°C) FBS (Life Technologies), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies) at 37°C and 5% CO₂. To prepare cells for stimulation, BEAS-2B cells were seeded (5×10^4 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 μ M), *Alternaria* extract (25–75 μ g/ml), or poly(I:C) (10 μ g/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(I:C), were pretreated with a serine protease inhibitor, APMSF (50–100 μ M), a cysteine protease inhibitor, E-64 (25–50 μ M), 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 (5×10^4 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) and following the manufacturer's instructions. The transfected cells were grown for 48 h and then stimulated with trypsin (10 nM), papain (100 μ M), *Alternaria* (50 μ g/ml), SLIGKV-NH₂ peptide (500 μ M), or poly(I:C) (10 μ g/ml) for 6 h. The knockdown of target genes and expression of TSLP mRNA was examined by real-time RT-PCR. Reproducibility of certain observations was examined in normal human bronchial epithelial (NHBE) cells. NHBE cells were obtained from Lonza and maintained in serum-free bronchial epithelial cell growth medium (Lonza). To prepare cells for stimulation, NHBE cells were seeded (3×10^4 cells/well) in a 24-well tissue culture plate and grown until 80% confluence. At this stage, NHBE cells

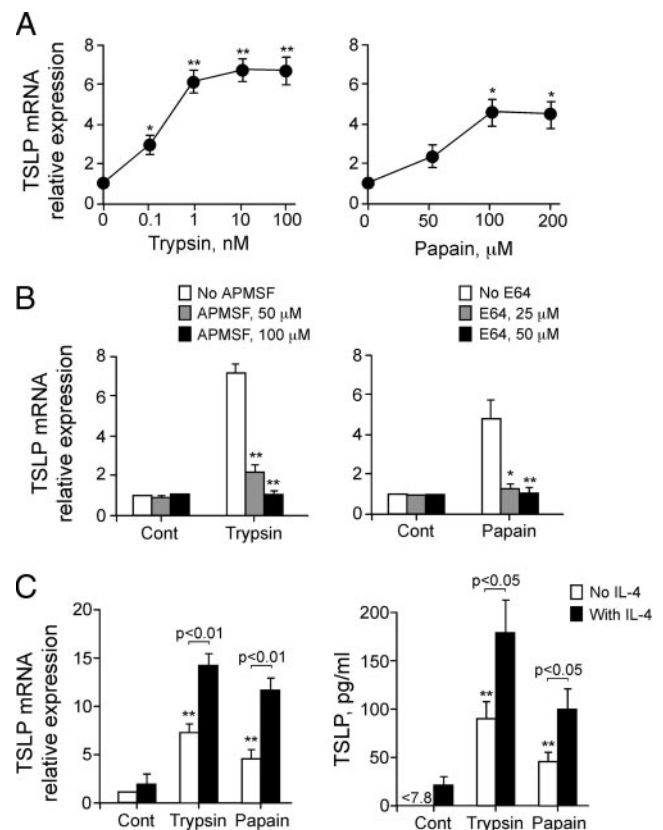


FIGURE 1. Papain and trypsin induce TSLP production in airway epithelial cells. **A**, BEAS-2B cells were incubated with 0.1–100 nM trypsin or 50–200 μ M papain for 6 h. TSLP mRNA was analyzed by real-time RT-PCR. The TSLP transcription was normalized to the 18S rRNA transcription in each sample and 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 μ M 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 μ M 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; $n = 4$.

were stimulated for 6 h with trypsin (10 nM), papain (100 μ M), *Alternaria* extract (50 μ g/ml), or SLIGKV-NH₂ peptide (500 μ M) for 6 h. Cell lysates were collected and used for TSLP mRNA real-time RT-PCR (see below).

RNA isolation and real-time RT-PCR

Total RNA was purified with TRIzol (Invitrogen); DNase digestion utilized DNase I Amplification Grade (Invitrogen). cDNAs were synthesized from 1 μ g of purified RNA samples using a Bio-Rad iScript cDNA synthesis kit. The reaction was incubated at 45°C for 60 min and was stopped by heating to 85°C for 5 min.

The real-time RT-PCR contained 1 μ l of cDNA, 12.5 μ l of Master Mix (TaqMan Universal PCR Master Mix, No AmpErase UNG), and 1.25 μ l of TaqMan gene expression assay of the target genes: TSLP (Hs00263639_m1), PAR-1 (Hs00169258_m1), PAR-2 (Hs00608346_m1), TLR4 (Hs00152939_m1), and 18S (Hs99999901_s1) (18S; as an endogenous control; Applied Biosystems). Reactions were made up to a final volume of 20.0 μ l 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

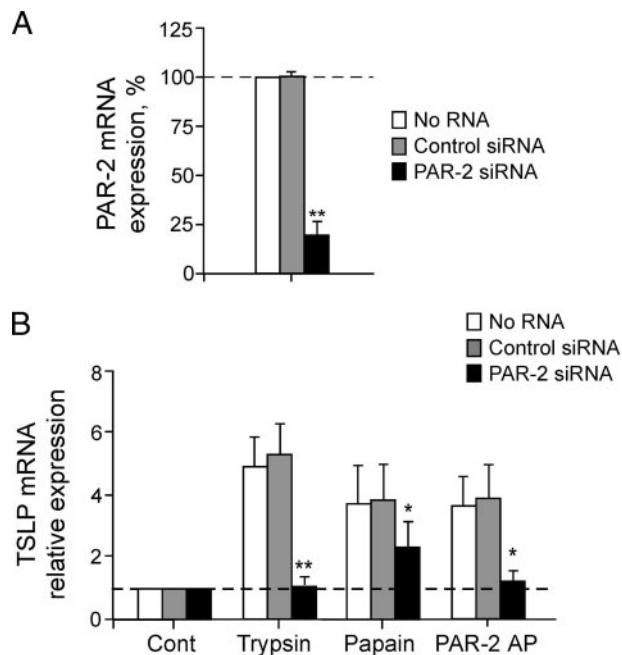


FIGURE 2. Knockdown of PAR-2 suppresses epithelial cell responses to proteases. *A*, BEAS-2B cells were transfected with 5 nM siRNA against PAR-2 or control siRNA for 48 h. Expression of PAR-2 mRNA was examined by real-time RT-PCR. *B*, Transfected BEAS-2B cells were stimulated for 6 h with medium alone (Cont), 10 nM trypsin, 100 μ M papain, or 500 μ M PAR-2-activating SLIGKV-NH₂ peptide (PAR-2 AP). The levels of TSLP mRNA were determined as in Fig. 1. TSLP mRNA expression was expressed as a ratio to the cells cultured with medium alone (Cont) without siRNA. *, $p < 0.05$ and **, $p < 0.01$, compared with the samples with the same stimuli but without siRNA; $n = 5$.

of a two-step program (denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min). Transcription was normalized to the 18S rRNA transcription in each sample and expressed as relative expression compared with the parallel BEAS-2B cells cultured in the absence of stimuli or cytokines.

ELISA analysis of TSLP protein released in cell supernatants

Immunoreactive TSLP in the supernatants from BEAS-2B cells was quantitated using a specific ELISA with matched Abs according to the manufacturer's instructions (R&D Systems). The sensitivity limit of the TSLP assay was 7.8 pg/ml.

Statistical analysis

All data are reported as the mean $\pm$ SEM from the indicated number of samples. Two-sided differences between two samples were analyzed with the Mann-Whitney U test. Values of $p < 0.05$ were considered significant.

Results

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 exogenous 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 was up-regulated in BEAS-2B cells in a concentration-dependent manner (Fig. 1A; $n = 5$). The effects of trypsin appeared to be stronger than those of papain. To verify that the stimulatory effects

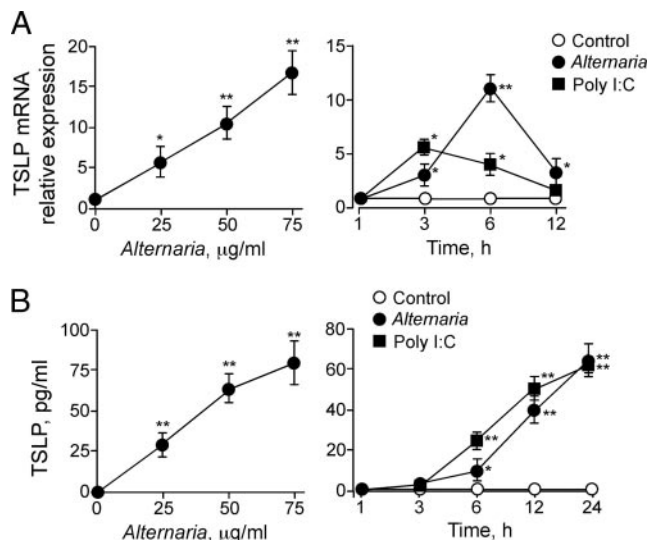


FIGURE 3. *Alternaria* extract induces TSLP production from airway epithelial cells. *A*, left panel, BEAS-2B cells were incubated with *Alternaria* extract for 6 h. *A*, right panel, BEAS-2B cells were incubated with medium alone (control), 10 μ g/ml poly(I:C), or 50 μ g/ml *Alternaria* extract for up to 12 h. TSLP mRNA expression was analyzed as in Fig. 1. *B*, left panel, BEAS-2B cells were incubated with *Alternaria* extract for 24 h. *B*, right panel, BEAS-2B cells were incubated with medium alone (control), 10 μ g/ml poly(I:C) or 50 μ g/ml *Alternaria* extract for up to 24 h. TSLP protein levels in the supernatants were analyzed by ELISA. *, $p < 0.05$ and **, $p < 0.01$, as compared with medium alone; $n = 5$.

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 expression (Fig. 1C). 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

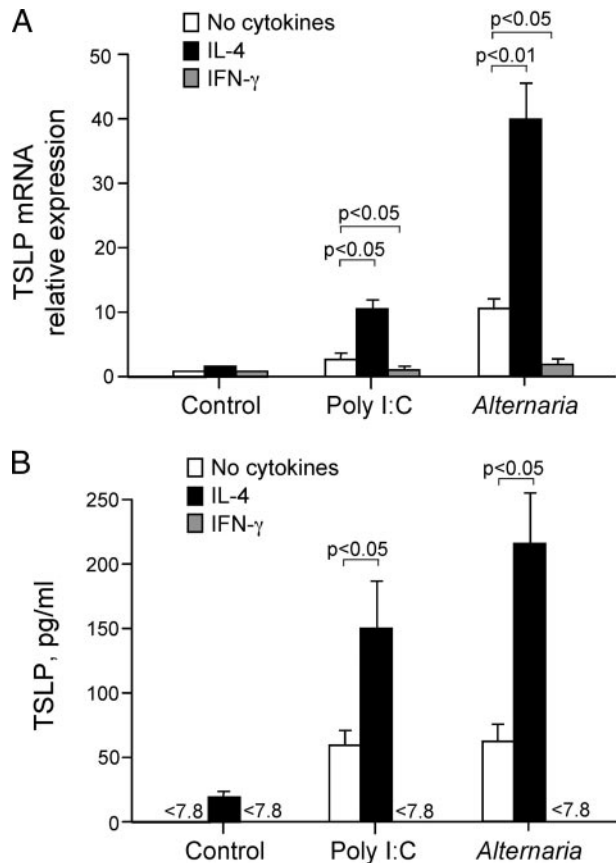


FIGURE 4. IL-4 enhances and IFN- γ inhibits *Alternaria*-induced TSLP production from airway epithelial cells. BEAS-2B cells were incubated with medium, 10 μ g/ml poly(I:C), or 50 μ g/ml *Alternaria* extract with or without 100 ng/ml IL-4 or 100 ng/ml IFN- γ . A, TSLP mRNA expression was analyzed at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

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 abolished 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 abolished 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

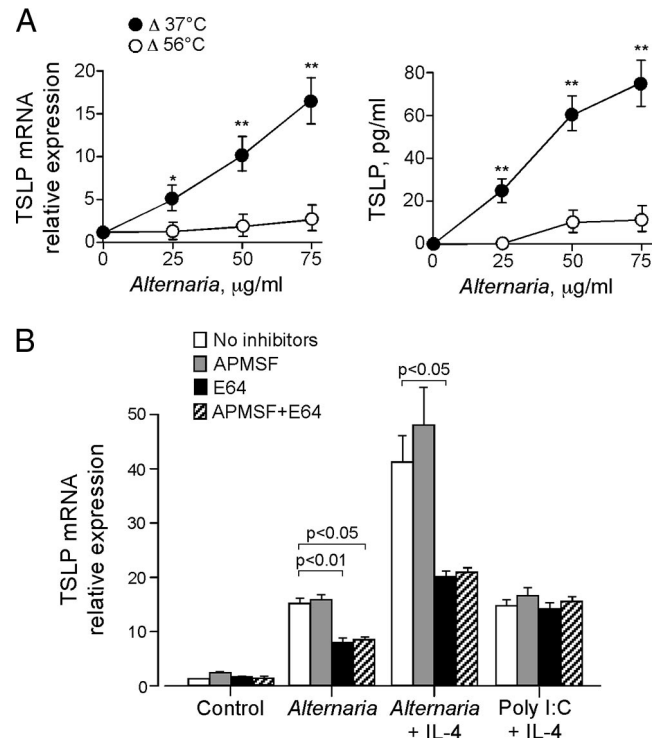


FIGURE 5. Blocking cysteine protease activity inhibits *Alternaria*-induced TSLP protein and mRNA expression. A, *Alternaria* extracts were heated at 37 or 56°C for 30 min. BEAS-2B cells were incubated with preheated *Alternaria* extracts for 6 h. TSLP mRNA expression was expressed as a ratio to cells incubated without *Alternaria*. *, $p < 0.05$ and **, $p < 0.01$, compared with *Alternaria* extract heated at 37°C. $n = 5$. B, *Alternaria* extract (50 μ g/ml) or poly(I:C) (10 μ g/ml) was pretreated without inhibitors, with 100 μ M APMSF, with 50 μ M 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; $n = 5$.

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* extract increased in a concentration-dependent manner (Fig. 3B). Culture supernatants of BEAS-2B cells incubated with 50 μ g/ml *Alternaria* extract or with 10 μ g/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

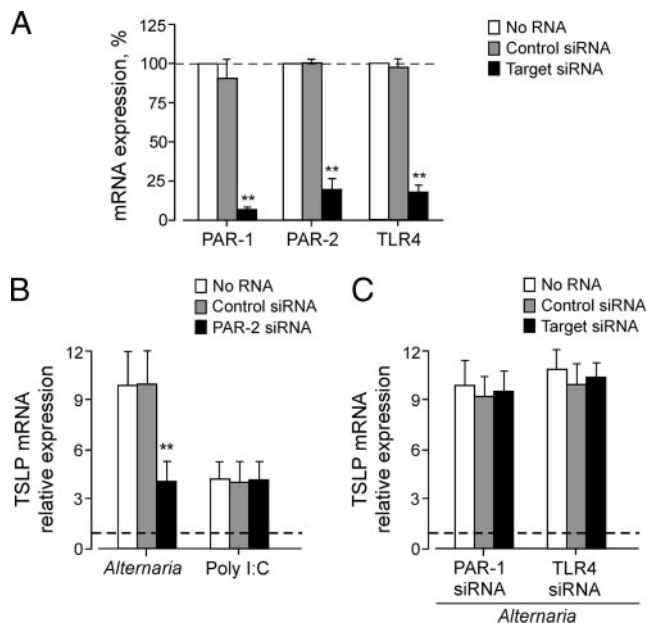


FIGURE 6. PAR-2 knockdown partially inhibits *Alternaria*-induced TSLP mRNA expression. **A**, BEAS-2B cells were transfected with 5 nM siRNA against PAR-1, PAR-2, or TLR4 or 5 nM control siRNA for 48 h. Expression of mRNA for target molecules was examined by real-time RT-PCR. Data are expressed as ratio to the mock-transfected cells without siRNA as 100%. **, $p < 0.01$, compared with control cells without siRNA; $n = 5$. **B** and **C**, Transfected BEAS-2B cells were stimulated with 50 $\mu\text{g}/\text{ml}$ *Alternaria* or 10 $\mu\text{g}/\text{ml}$ poly(I:C) for 6 h. TSLP mRNA expression was expressed as a ratio to cells incubated without siRNA. **, $p < 0.01$, compared with no siRNA; $n = 5$.

(Fig. 4B; $p < 0.05$). No TSLP protein was detectable in the BEAS-2B supernatants incubated with poly(I:C) or *Alternaria* extract in the presence of IFN- γ (Fig. 4B).

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 $\mu\text{g}/\text{ml}$ heat-treated *Alternaria* extract was apparently less than the response to 25 $\mu\text{g}/\text{ml}$ 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 ($\sim 50\%$) 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 APMSF, 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

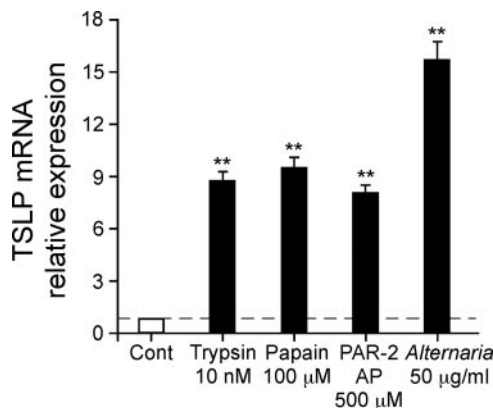


FIGURE 7. Proteases and *Alternaria* extract induce TSLP expression in NHBE cells. NHBE cells were stimulated for 6 h with medium alone (Cont), 10 nM trypsin, 100 μM papain, 500 μM PAR-2-activating SLIGKV-NH $_2$ peptide (PAR-2 AP), or 50 $\mu\text{g}/\text{ml}$ *Alternaria* extract for 6 h. The levels 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; $n = 5$.

culture does not affect TSLP expression induced by a nonprotease stimulus.

Third, 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 TLR4 or a control siRNA. Transfection with these specific siRNAs, but not the control siRNA, significantly suppressed the target molecule expression $> 80\%$ (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 ($\sim 60\%$) 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-NH $_2$, significantly increased expression of TSLP mRNA ($p < 0.01$, $n = 5$; Fig. 7). The magnitudes of TSLP mRNA expression in NHBE cells were roughly comparable to those induced in BEAS-2B cells (Figs. 1 and 2). Furthermore, *Alternaria* extract also significantly enhanced TSLP mRNA expression in NHBE cells (~ 15.5 -fold over medium control; Fig. 7).

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, at 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 blocked trypsin-induced TSLP expression (Fig. 2). 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- κ B. IL-1 β and TNF- α regulate TSLP expression in a NF- κ B-dependent manner (15). PAR stimulation also induces cytokine and transcription responses through MAPK and NF- κ B (38, 52–54). Importantly, synergy between NF- κ B 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- κ B 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

(57). Thus, the potential relationships among allergic status, environmental exposure (e.g., proteases and TLR ligands), and TSLP expression deserve future clinical studies.

In conclusion, our data support the novel finding that the protease activities of the fungus *Alternaria* are recognized by airway epithelial cells, resulting in TSLP production. We propose a model in which perturbation of PAR-2 on airway epithelial cells by proteases from environmental fungi and perhaps other allergens results in the production and secretion of TSLP. This TSLP can then stimulate airway mucosal DCs, leading to the subsequent development and/or exacerbation of Th2-type inflammatory responses. These airway responses to proteases may be pronounced in individuals with allergic phenotypes. Thus, PARs may be non-TLRs that form an interface between innate and adaptive immune responses. A better understanding of the protease/PAR pathways may allow us to develop novel therapeutic and preventive strategies for asthma and allergic diseases.

Disclosures

The authors have no financial conflict of interest.

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