Protease Allergens Induce the Expression of IL-25 via Erk and p38 MAPK Pathway

Allergic diseases, including asthma, are characterized by T helper type 2 (Th2) cell-mediated inflammations, coupled with tissue infiltration by eosinophils. In this study, we demonstrate that multiple protease allergens, including papain and DerP1, efficiently induce interleukin (IL)-25 and thymic stromal lymphopoietin (TSLP) gene expression, and this phenomenon is dependent on the protease activities of these allergens. The IL-25 cytokine level in bronchial alveolar lavage (BAL) was also profoundly and significantly increased after treatment with papain. Additionally, the levels of Th2 cytokines were significantly increased, as compared to those in the OVA-only treatment group. The various protease allergens triggered the expression of IL-25 and TSLP mRNA in mouse lung epithelial cells (MLE12) and primary mouse lung epithelial cells; these effects were inhibited by the deactivation of the protease activity of papain. The allergen papain activates the Erk and p38 MAP pathways; the inhibition of these pathways, but not the NF-kB or PI-3 kinase pathways, impairs the induction of IL-25 and TSLP expression by proteases. In this study, we demonstrate that the protease allergens induce IL-25 and TSLP via the MAP kinase signal pathways, and their protease activities are essential to this pathway.

Key Words : IL-25; Thymic Stromal Lymphopoietin; Protease Allergen; Mitogen-Activated Protein Kinases

INTRODUCTION

Asthma and atopic disease have been shown to be induced by environmental allergens, but the molecular mechanisms through which allergens drive the pathogenesis of these diseases remain to be clearly identified. Epithelial cells, the first type of cells to encounter allergens, have been shown to initiate tissue inflammatory responses via the production of a variety of cytokines and chemokines. Moreover, many allergens evidence intrinsic protease activities (1), and some proteases from infectious agents, parasites, and fungi have already been identified as potent allergens (2-7). These different protease allergens have been shown to induce similar Th2 immune responses via the activation of several chemokines and cytokines (1, 6). Thus, protease activities may prove crucial to the initiation of relevant allergic responses. However, the mechanism by which proteases trigger pro-allergic innate immune responses has yet to be clearly assessed.

Two cytokines, interleukin (IL)-25 and thymic stromal lymphopoietin (TSLP), have been recently identified as cytokines that initiate the Th2 allergic response. IL-25 (also known as IL-17E), a member of the IL-17 family, has been implicated in Th2 cell-mediated immunity (8, 9). Recently, IL-25 has been identified as one of the initiators of the Th2 response (10, 11) and has also been shown to be expressed by mast cells (12). The transgenic overexpression of IL-25 by lung epithelial cells results in mucus hyperproduction and airway infiltration of macrophages and eosinophils; conversely, IL-25 blockade reduced airway inflammation and Th2 cytokine production in an allergen-induced asthma model (10, 11, 13). Additionally, the expression of TSLP, an IL-7-like cytokine, is associated with skin or bronchial epithelial cells, although the physiological inducers of TSLP expression have yet to be clearly evaluated. TSLP has been shown to activate dendritic cells (DCs), which in turn prime naïve T cells to express Th2 cytokines, thereby resulting in the initiation of allergic responses (14, 15). Additionally, TSLP can act directly on T cells to promote Th2 differentiation (14, 16, 17). Therefore, IL-25 and TSLP perform pivotal roles in provoking allergic inflammation.
In this report, we have determined that IL-25 and TSLP mRNA were upregulated in lung epithelial cells after a variety of protease allergen treatments in our series of in vivo and in vitro experiments. Moreover, we determined that protease allergens induce IL-25 and TSLP via the intracellular ERK and p38 MAP kinase pathways.

MATERIALS AND METHODS

Allergens

Papain, Aspergillus oryzae protease (Asp; Sigma-Aldrich, St Louis, MO, USA), and rDerP1 (recombinant Dermatophagoides pteronysinus allergen 1; Indoor Biotechnologies, Charlottesville, VA, USA) were reconstituted with sterile PBS to 1 mg/mL and stored at -20°C.

Induction of airway inflammatory reaction

Chicken egg OVA (Sigma-Aldrich) was reconstituted in sterile PBS at 1 mg/mL and stored at -20°C. For intranasal challenge, 10 μL (10 μg) of papain was added to 40 μL (40 μg) of OVA immediately prior to intranasal administration. C57BL/6 mice (Jackson Laboratories, Bar Harbor, MA, USA) were induced with airway inflammation by papain for six total challenges, as described previously (10, 11, 18). One day after the final challenge, the mice were killed for analysis of bronchial alveolar lavage (BAL) fluid. BAL cells were collected and further subjected to differential cell counts. BAL fluid was analyzed for cytokine production by ELISA. All animal studies were approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Lung epithelial cell and Mouse embryonic fibroblast cell culture

Mouse lung epithelial cells (MLE12) were obtained from The American Type Culture Collection. Primary lung epithelial cells were isolated from C57BL/6 mice, as described previously (10, 11, 19), after depletion with anti-CD32/CD16 and anti-CD45 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were treated with 200 ng/mL of Anti-CD45 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were treated with 200 ng/mL of A. oryzae protease. After 2 hr of stimulation, the cells were collected and lysed, and mRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at -20°C.

Real-time-PCR analysis

Total RNA extracted with TRIzol reagent was used to generate cDNA using oligo-dT, random hexamers, and Super-Script RT II (Invitrogen). For the quantitation of cytokine and transcription factor gene expression, cDNA samples were amplified in iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The primer pairs utilized for Real-time-PCR are shown in Table 1.

Cytokine levels in BAL fluid

The amounts of IL-4, IL-5, IL-13, IL-25, IL-17, and IL-17F in the fluid (BALF) were determined via an enzyme immunoassay, as previously described (10, 11).

Immunoblot analysis

MEF cells were washed with ice-cold PBS and lysed in 0.2 mL of lysis buffer (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM EGTA, 0.05% 2-mercaptoethanol, 1 × protease inhibitors). Cell debris was removed via 15 min of centrifugation at 14,000 × g, and the supernatant was boiled for 5 min in Laemmli sample buffer (Bio-Rad Laboratory) for 5 min. An equal amount of proteins was subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis before blotting onto a PVDF membrane (Amersham and Pharmacia Biotech, Seoul, Korea). The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (pH 7.6) for 1 hr at room temperature, then probed with anti-human β-actin (R&D Systems, Minneapolis, MN, USA), anti-mouse phospho-ERK, anti-mouse phosphoJNK, and anti-mouse p38 MAPK specific antibodies (Cell Signaling Technology Inc., Boston, MA, USA) at 4°C overnight. After washing, the membranes were incubated for 1 hr with secondary rabbit anti-mouse antibody coupled to horseshadish peroxidase (Amersham and Pharmacia Biotech) at room temperature. Antibody-antigen complexes were then detected using an ECL chemiluminescent detection system.
in accordance with the manufacturer’s instructions (Amer- 
sham and Pharmacia Biotech).

Statistics

Data are expressed as mean values±SD and are representa-
tive of at least 2 independent experiments involving at least 4 
mice per group, unless otherwise indicated. Data were ana-
lyzed via Student’s t test (n=2 groups). P values of <0.05 were 
considered significant.

RESULTS

Protease allergens induce IL-25 expression in vivo

Following 6 intranasal administrations of papain, immune 
cell infiltrations, particularly eosinophils, were evident in the 
airway (Figs. 1A, B). The IL-25 cytokine level in BAL was 
also profoundly increased after papain treatment (Fig. 1C). 
Additionally, the levels of Th2 cytokines (IL-4, -5, and -13) 
were significantly increased as compared to those in the OVA-
only treatment group. However, the levels of the Th17 cyto-
kines, IL-17 and IL-17F, in the papain treatment group did 
not differ significantly from those of the control group (Fig. 
1C). We also that the lung cells of the papain-treated mice 
much higher expressed IL-25, IL-5, Gro-alpha, and eotaxin 
genes at much higher levels than those of the control group 
(Fig. 1D). Therefore, the intranasal administration of papain 
specifically induces Th2-type chemokines and cytokines in 
vivo.

Protease allergens triggered IL-25 and TSLP expression 
by epithelial cells and fibroblasts

In order to determine whether the induction of the Th2-
type response is a common feature of protease allergens, we 
subsequently measured the levels of IL-25 and TSLP in the 
MLE12 mouse lung epithelial cell line after stimulation with 
a variety of allergens. Consistent with the data shown in Fig. 1, 
papain treatment triggered the expression of IL-25 and TSLP 
mRNA in the MLE12 cells (Fig. 2A). These effects were inhib-
ted by the deactivation of the protease activity of papain, 
either by boiling or by treatment with protease inhibitors 
(Fig. 2A). It is worth noting that Aspergillus protease (Asp) 
and DerP1, both of which are known as strong allergens with 
protease activity, also induced the expression of IL-25 and 
TSLP (Figs. 2B, C). The induction of IL-25 and TSLP by 
these allergens was also noted in primary lung epithelial (PLE) 
cells and mouse embryonic fibroblast (MEF) cells (Fig. 3A, 
B). The protease allergens also induced the expression of Th2-
associated chemokine genes, particularly the eotaxin gene 
(Fig. 3C).

![Fig. 1. Papain induced allergic inflammation with significant induction of IL-25. Mice were intranasally treated with papain and OVA (Papain) 
or OVA only (OVA) for 6 repetitions. (A) Total cell number of bronchoalveolar lavage fluid (BALF). (B) Differential cell counts of BALF. (C) 
Concentration of cytokines in BALF. (D) Chemokine and cytokine mRNA expression of lung cells (*P<0.05; n=5 mice per group; 3 inde-
pendent experiments).]
Protease allergens induce IL-25 gene expression via the ERK and p38 MAP kinase pathways

In an effort to address the intracellular mechanism that regulates IL-25 and TSLP expression upon stimulation with protease allergens, we evaluated the activation of intracellular MAPK and NF-κB. Papain induced the phosphorylation of ERK, JNK, and p38 MAPK in MEF cells within as short a time as 15 min (Fig. 4A). Boiled papain marginally induced p-JNK with delayed kinetics, and failed to induce the phosphorylation (Fig. 4B). On the other hand, the levels of IκBα were reduced 2 hr after papain treatment, which might be the results of a secondary response. In order to evaluate the functions of different signaling pathways in allergen-induced IL-25 and TSLP gene expression, we employed pathway-specific inhibitors. IL-25 and TSLP expression were profoundly inhibited by Erk, JNK, and p38 inhibitors (Fig. 4C, data not shown). Likewise, Asp protease also induced ErK, JNK, and
p38 phosphorylation in MEF cells (data not shown).

Toll-like receptors (TLR) are important pattern recognition receptors for infectious agents, which signal through the adapter proteins MyD88 and/or TRIF. In order to evaluate the relationship between the toll-like receptors and the protease allergens, we utilized papain to treat MyD88-/- TIRAP-/- or TRIF-/- MEF cells. The IL-25 gene was expressed normally from all types of MEF cells in response to papain stimulation (Fig. 4D, data not shown). These results showed that protease-induced allergic responses were not mediated by the toll-like receptors.

**DISCUSSION**

In an effort to determine the mechanism underlying allergen-induced Th2 response, we first analyzed the innate and adaptive factors triggered by papain as a model protease allergen in vivo.

In this study, following 6 intranasal administrations of papain, we readily detected strong Th2 responses, including immune cell (particularly eosinophils) infiltrations, and increased levels of Th2 cytokines (including IL-25) in the airway (Fig. 1). Additionally, various proteases were shown to elicit IL-25 and TSLP expression from mouse lung epithelial cells, and their activities were reduced by boiling or protease inhibitor treatment (Figs. 2, 3). Our group and others recently demonstrated that IL-25 and TSLP perform important functions in the initiation of allergic asthma (10, 11, 20). IL-25 (also known as IL-17E), a member of the IL-17 family, has been previously implicated in Th2 cell-mediated immunity (8, 9). Recently, IL-25 has also been implicated as one of the initiators of the Th2 response (10), and has also been shown to be expressed by mast cells (12). The transgenic overexpression of IL-25 by lung epithelial cells results in mucus production and airway infiltration of macrophages and eosinophils; conversely, the blockage of IL-25 reduces airway inflammation and Th2 cytokine production in an allergen-induced asthma model (10, 13). Additionally, the expression of TSLP, an IL-7-like cytokine, is associated with skin or bronchial epithelial cells, although the physiological inducers of TSLP expression have yet to be clearly identified. TSLP has been previously demonstrated to activate DCs, which then subsequently prime naive T cells to express Th2 cytokines, thereby precipitating allergic responses (14, 15). Additionally, TSLP can operate directly on T cells to promote Th2 differentiation (14, 17).

Kiss et al. (2007) previously reported that the Asp allergen can induce eosinophilic airway inflammation and lung IL-4 production in the absence of adaptive immune cells; these responses required intact protease activity (18). In our study, different protease allergens elicited the expression of IL-25 and TSLP genes, which depended heavily on the protease activity. These results show that the induction of TSLP and IL-25 constitutes an innate immune response to protease allergens in lung epithelial cells.

Recently, IL-25-induced cytokines and chemokines have been shown to be regulated principally by costimulation-activated MAPKs and NF-κB, in addition to the subsequent upregulation of the IL-25 receptor (21). In our study, papain...
induced ERK, JNK, and p38 MAPK phosphorylation in MEF cells, while boiled papain marginally induced p-JNK with delayed kinetics, but failed to induce ERK and p38 phosphorylation. Additionally, IL-25 and TSLP expression were inhibited by ERK, JNK, and p38-specific MAPK inhibitors (Fig. 4). These results showed that allergen-induced IL-25 and TSLP production were regulated by the MAP kinase pathways. Interestingly, in our study, NF-κB was activated 2 hr after papain stimulation; this phenomenon may be induced by IL-25 secretion in the stimulated cells themselves. Collectively, our findings revealed that protease activity in a variety of allergens activates the MAPK pathway to induce IL-25 and TSLP in a TLR-pathway-independent manner. These observations may reflect the existence of non-TLR-related, protease-activated innate immune receptors in mouse respiratory epithelial cells in the initiation of allergic inflammation.

In conclusion, the protease allergens induce IL-25 and TSLP via the MAP kinase signal pathways, and that their protease activities are critically important in this pathway. The identification of innate immune molecules and sensors of protease activity will prove crucial to our further understanding of the initial pathogenesis of allergic reaction. Additionally, targeting the MAPK pathway may provide a novel strategy for preventing allergic inflammation.

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